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TEM-80, a Novel Inhibitor-Resistant β-Lactamase in a Clinical Isolate of *Enterobacter cloacae*

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Enterobacter cloacae **Ecl261 was isolated with** ***Escherichia coli*** **Ec257 from the urine of a patient living in a nursing home. Both isolates were resistant to ticarcillin (MICs, 1,024 μg/ml), without significant potentiation of its activity by 2 μg of clavulanate per ml (MICs, 512 μg/ml), and susceptible to naturally active cephalosporins. This inhibitor-resistant phenotype was conferred in both strains by similar conjugative plasmids of 40 kb (Ecl261) and 30 kb (Ec257), which also conveyed resistance to sulfonamides and trimethoprim. Clinical and transconjugant strains produced a β-lactamase with a pI of 5.2 which belonged to the TEM family, as indicated by specific PCR amplification. Compared with TEM-1, this enzyme exhibited lower catalytic efficiencies (14- and 120-fold less for amoxicillin and ticarcillin, respectively), and higher concentrations of β-lactamase inhibitors were required to yield a 50% reduction in benzylpenicillin hydrolysis (750-, 82-, and 50-fold higher concentrations for clavulanate, sulbactam, and tazobactam, respectively). Gene sequencing revealed four nucleotide differences with the nucleotide sequence of** $bla_{\text{TEM-1A}}$**. The first replacement (T32C), located in the promoter region, was described as being responsible for the increase in the level of β-lactamase production. The three other changes led to amino acid substitutions that define a new inhibitor-resistant TEM (IRT) β-lactamase, TEM-80 (alternate name, IRT-24). Two of them, Met69Leu and Asn276Asp, have previously been related to inhibitor resistance. The additional mutation, Ile127Val, was demonstrated by site-directed mutagenesis to have a very weak effect, at least alone, on the IRT phenotype. This is the first description of an IRT β-lactamase in** ***E. cloacae*****. The horizontal transfer of** $bla_{\text{TEM-80}}$ **may have occurred either from Ec257 to Ecl261 or in the reverse order.**

Enterobacter cloacae is a member of the commensal digestive flora of humans. In recent years, this species has emerged as an important opportunistic pathogen, especially in debilitated patients, for example, those who have implanted foreign devices and those who are hospitalized in intensive care units (36). Unfortunately, the problem appears to have escaped the confines of the hospital setting, and community-acquired infections due to *E. cloacae* have also been described (13, 31). The emergence of *E. cloacae* as a cause of nosocomial infections can be related to its intrinsic resistance to broad-spectrum β-lactam antibiotics, used in prophylactic therapy (36). Indeed, *E. cloacae* strains are naturally resistant to amoxicillin, amoxicillin-clavulanate, narrow-spectrum, cephalosporins, and cefoxitin due to the production of an inducible species-specific Bush group 1 cephalosporinase (8). However, carboxypenicillins and ureidopenicillins are active against the wild-type strains. Epidemiological studies showed that one-half or more of the strains tested remain susceptible to these molecules (36).

Several reports have demonstrated the ease with which *E. cloacae* strains can acquire β-lactam resistance, especially when they are subjected to antibiotic pressure. Although overproduction of the AmpC β-lactamase is the most common mechanism involved in β-lactam resistance in *E. cloacae*, other β-lactamases can also be found (36). In particular, wild-type strains of *E. cloacae* may become resistant to broad-spectrum penicillins, like ticarcillin and piperacillin, via the acquisition of plasmids encoding TEM-1, TEM-2, and SHV-1 β-lactamases (26, 35). Recently, *E. cloacae* strains with resistance to extended-spectrum cephalosporins conferred by TEM- and SHV-type extended-spectrum β-lactamases have been detected at high frequencies in Greece (41). β-Lactamase inhibitors usually restore the activities of carboxy- and ureidopenicillins against the Bush group 2b and 2be β-lactamases. Various extended-spectrum β-lactamases not derived from TEM or SHV enzymes have been described in rare isolates of *E. cloacae*, such as the SFO-1 (28), IBC-1 (16), CTX-M-3 (14), CTX-M-8 (5), and VEB-1 (17) β-lactamases. In addition, carbapenem-resistant strains of *E. cloacae* with the Bush group 2f carbapenemases Nmc-A (30) and IMI-1 (33) have also been reported. All these β-lactamases are inhibited by clavulanate. However, despite the great diversity of acquired β-lactamases encountered in this species, no plasmid-encoded inhibitor-resistant TEM (IRT) β-lactamase has been identified at present.

In 1998, a patient who was living in a nursing home and who carried an indwelling urinary catheter, developed a urinary tract infection simultaneously due to a strain of *E. cloacae* (Ecl261) and a strain of *Escherichia coli* (Ec257). For both isolates there was limited potentiation of ticarcillin and piperacillin activities by clavulanate and tazobactam, respectively, despite the susceptibilities of the isolates to extended-spectrum cephalosporins such as cefotaxime, suggesting the presence of

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TABLE 1. Oligonucleotides used in this study

Primer use and primer	Sequence (5′ → 3′)	Nucleotide position[a]	Reference
Amplification and sequencing			
TEM-7	ATAAAAATTCTTGAAGAC	−7	38
TEMB	TCTAAAGTATATATGAGAGTAAACTTGGTCTG	1103	4
Sequencing			
TEMC	GGGCAAGAGCAACTCGG	461	4
TEMD	CAGCAATGGCAACAACGTTG	753	4
TEMF	CAACGTTGTTGCCATTGCTGCAG	772	4
TEMG	ACCGAGTTGCTCTTGCCC	478	4
Mutagenesis experiment[b]			
Val127F	GAATTATGCAGTGCTGCC**GTA**ACCATGATGGATAACAC	563	This study
Val127R	GTGTTATCACTCATGGT**TAC**GGCAGCACTGCATAATTC	600	This study

[a] Nucleotide positions are numbered as described by Sutcliffe (39).
[b] Mutagenic primers in which Ile (ATA) was replaced by Val (GTA in the forward orientation and TAC in the reverse orientation). Boldface characters denote the mismatched bases.

an IRT enzyme. In order to confirm the presence of such an enzyme in *E. cloacae* strains, biochemical and genetic β-lactamase characterizations were undertaken.

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MATERIALS AND METHODS

Bacterial strains. *E. cloacae* Ecl261 and *E. coli* Ec257 were collected in February 1998 in a private laboratory from a urine sample of a 89-year-old male patient living in a nursing home near Bordeaux, France. This patient presented with a background of debilitation that included diabetes, alcoholism, nicotine addiction, cardiac insufficiency, and emphysema. In addition, he had a nonoperable prostatic adenoma causing urine retention and had carried an indwelling urinary catheter for 6 months. Due to repeated episodes of exacerbation of chronic bronchitis, the patient had received several courses of amoxicillin-clavulanate prior to the isolation of these strains.

The *E. cloacae* and *E. coli* clinical strains were identified with the API 20E system (BioMérieux SA). Several other strains of *E. coli* were used in this study: a rifampin-resistant (Rif^r) mutant of *E. coli* K-12 was used as the recipient in conjugation transfer experiments, and an *E. coli* strain, strain CF0042, producing TEM-35 (44) was used as a control for MIC and pI determinations and was also used in the kinetic studies. *E. coli* JM109 carrying plasmid pBR322 served as the source of the bla_{TEM-1} β-lactamase gene in site-directed mutagenesis experiments.

Antimicrobial susceptibility testing. Antibiotic susceptibility patterns were determined by the disk diffusion method in Mueller-Hinton agar by using 22 disks (http://www.sfm.asso.fr). The MICs of various β-lactams, alone or in combination with 2 μg of clavulanic acid per ml, 4 μg of tazobactam per ml, and 8 μg of sulbactam per ml, were determined by a twofold dilution method in Mueller-Hinton agar by using a final inoculum of 10^4 to 10^5 CFU per spot (http://www.sfm.asso.fr). The antibiotics tested in this study were kindly provided as standard powders by the indicated suppliers: ampicillin, Bristol-Myers Squibb; amoxicillin, ticarcillin, and clavulanic acid, SmithKline Beecham; cefotaxime, Aventis Pharma-Hoechst Marion Roussel; cefoxitin, Merck Sharp & Dohme Chibret; cephalothin, Eli Lilly; piperacillin and tazobactam, Wyeth-Léderlé; and sulbactam, Pfizer Laboratories.

Isoelectric focusing. Crude β-lactamase extracts were obtained by sonication and were analyzed by isoelectric focusing in polyacrylamide gels containing ampholines (Pharmacia LKB) with a pH range of 3.5 to 10.0, as described by Matthew et al. (29). β-Lactamase activities were detected by an iodine-starch procedure in agar gel with benzylpenicillin (75 μg/ml) as the substrate (2). The pIs of the β-lactamases studied were determined by comparison with the pIs of reference β-lactamases: TEM-1 (pI 5.4), TEM-2 (pI 5.6), and TEM-35 (pI 5.2).

Transfer experiments. Conjugation assays between donor (Ec257 or Ecl261) and recipient (*E. coli* K-12 Rif^r) cells were carried out by a broth mating procedure in brain heart infusion medium, as described previously (4). Transconjugants were selected on Mueller-Hinton agar containing rifampin (50 μg/ml) and ampicillin (100 μg/ml).

Kinetic studies. The β-lactamases produced by transconjugants Tc257 and Tc261 and by strains producing the reference enzyme (TEM-1) and TEM-35 and the mutant enzyme (TEM-127V) were purified to homogeneity from 4 liters of broth cultures (brain heart infusion broth; Difco) by previously described methods (7, 43). After precipitation with ammonium sulfate the enzyme was submitted to gel filtration chromatography on Toyopearl HW-50 resin (fractionation range, 500 to 80,000 Da) (Sigma). Then the active fractions were pooled, dialyzed, concentrated, and purified by ion-exchange chromatography with resin MonoQ HR 5/5 and with fast-performance liquid chromatography (Amersham Biosciences) (7, 9). The purified enzymes were homogeneous, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Kinetic constants were determined by computerized microacidimetry at pH 7 and 37°C in a 0.1 M NaCl solution (22). The inhibition constant (K_i) was determined by microacidimetry as a competition procedure with benzylpenicillin as the substrate. The concentrations of inhibitors that gave a 50% reduction of hydrolysis of benzylpenicillin at 1 mM (IC_{50}s) were measured after 10 min of preincubation of the enzymes with the inhibitors.

Plasmid DNA analysis. Plasmid DNA was extracted and purified by the protocol and with the reagents provided with a commercial kit (Qiagen Plasmid Midi kit; Qiagen) and was then analyzed by electrophoresis on a 0.9% (wt/vol) agarose gel and visualized by staining with ethidium bromide under UV light. The sizes of the plasmids were estimated, after *Eco*RI and *Eco*RV enzymatic restriction, by comparison with the sizes of the fragments of phage lambda DNA digested with *Pst*I or *Hin*dIII.

PCR amplification of bla_{TEM} genes. The amplification of bla_{TEM} genes was performed with 5 ng of purified plasmid DNA mixed with 200 μM each deoxynucleoside triphosphate, 0.5 μM each primer, and 1.25 U of *Taq* polymerase (Perkin-Elmer Applied Biosystems Division) in the appropriate buffer. After a denaturation step at 94°C for 5 min, 35 subsequent cycles of amplification were performed, with each one consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final step at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1.5% (wt/vol) agarose gel, and the amplicon size was evaluated by comparison with the sizes of the fragments of phage lambda DNA digested with *Pst*I. The PCR products were purified with the microcolumns of the MicrospinTM Sephacryl S-400 purification system (Pharmacia LKB).

Sequence analysis. Both strands of all bla_{TEM} genes reported here were sequenced with sets of custom-made bla_{TEM}-specific primers (Table 1) by an automated fluorescent method by use of dye terminator chemistry (AmpliTaq DNA polymerase FS Dye Terminator Cycle Sequencing Ready Reaction kit; Perkin-Elmer Applied Biosystems Division) and an ABI Prism 377 sequencer (Perkin-Elmer Applied Biosystems Division).

Site-directed mutagenesis experiments. Ile127Val site-directed mutagenesis of the TEM-1 β-lactamase was performed with the QuickChange site-directed mutagenesis kit (Stratagene). A pair of complementary mutagenic oligonucleotide primers, primers TEMValF and TEMValR (Table 1), was designed. Mu-

TABLE 2. MICs of β-lactam antibiotics alone or in combination with suicide inhibitors

Strain	Plasmid-mediated enzyme	>MIC (μg/ml)[a]									
		AMP	SAM	AMX	AMC	TIC	TIM	PIP	TZP	CEF	CTX
E. coli Ec257	TEM-80	1,024	512	2,048	1,024	1,024	512	128	64	8	4
E. cloacae Ec1261	TEM-80	1,024	1,024	2,048	2,048	1,024	512	64	64	>512	1
E. coli Tc257	TEM-80	1,024	512	2,048	1,024	1,024	512	128	64	4	8
E. coli Tc261	TEM-80	1,024	512	2,048	1,024	1,024	512	128	64	4	2
E. coli CF004	TEM-35	512	512	1,024	1,024	512	512	128	16	8	2
E. coli JM109	TEM-127V	2,048	2,048	4,096	128	>4,096	256	32	8	32	8
E. coli JM109	TEM-1	4,096	2,048	4,096	128	>4,096	256	32	16	32	8
E. coli K-12	None	2	1	4	2	2	2	1	1	4	2
E. coli JM109	None	1	1	2	1	2	1	1	1	4	4

[a] AMP, ampicillin; SAM, ampicillin-sulbactam; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; TIC, ticarcillin; TIM, ticarcillin-clavulanic acid; PIP, piperacillin; TZP, piperacillin-tazobactam; CEF, cephalothin; CTX, cefotaxime.

tagenesis was carried out with the *Pfu* Turbo DNA polymerase provided with the kit. Plasmid pBR322 was used as the DNA template for the mutagenic PCR; and the cycling parameters were 95°C for 30 s, 55°C for 1 min, and 68°C for 10 min for a total of 16 cycles. After amplification, the *Dpn*I restriction enzyme was added to the digested methylated (parental) DNA. Competent JM109 cells were transformed by electroporation with mutagenic DNA by heat pulsing for 45 s at 42°C. After 1 h of incubation at 37°C, the transformed cells were plated onto Luria-Bertani agar medium containing 15 μg of tetracycline per ml.

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to the GenBank/EMBL database and has been assigned accession number AF347054.

RESULTS

Antibiotic susceptibility pattern. By the disk diffusion method, *E. coli* strain Ec257 exhibited an IRT phenotype; i.e., it was resistant to amoxicillin and amoxicillin-clavulanate but was susceptible to cephalothin, cefoxitin, and cefotaxime, according to French national susceptibility breakpoints (http://www.sfm.asso.fr). In addition, this strain was resistant to various non-β-lactam antibiotics including chloramphenicol, tetracycline, sulfonamides, and trimethoprim. The antibiogram of *E. cloacae* strain Ecl261 showed that it was resistant to ticarcillin and clavulanate-ticarcillin and fully susceptible to extended-spectrum cephalosporins such as cefotaxime, also suggesting an IRT phenotype. Strain Ecl261 was additionally resistant to sulfonamides and trimethoprim. *E. coli* transconjugants Tc257 (Ec257 donor) and Tc261 (Ecl261 donor) displayed the same pattern of antibiotic resistance: an IRT phenotype associated with resistance to sulfonamides and trimethoprim. The MICs of the β-lactams are listed in Table 2. Ampicillin, amoxicillin, and ticarcillin MICs for the clinical and transconjugant strains were 1,024, 2,048, and 1,024 μg/ml, respectively. The isolates were more susceptible to piperacillin (MICs, 64 to 128 μg/ml). At the fixed concentrations chosen, the β-lactamase inhibitors lowered the MICs of penicillins by at most 2 dilutions or less, even for the piperacillin-tazobactam combination. The *E. coli* strains remained susceptible to cephalothin, with MICs ranging from 4 to 8 μg/ml.

Biochemical β-lactamase characterization. Analytical isoelectric focusing of crude β-lactamase extracts of strains Ec257 and Ecl261 and their respective transconjugants gave an identical band for each strain which comigrated with the reference TEM-35 enzyme at pI 5.2 (data not shown).

The catalytic efficiencies (k_{cat}/K_m) of the enzyme produced by the transconjugants were drastically reduced compared with those for the wild-type TEM-1 enzyme (23-, 14-, 120-, and 104-fold lower for benzylpenicillin, amoxicillin, ticarcillin, and cephalothin, respectively) (Table 3). The k_{cat}/K_m ratios for the enzymes produced by the transconjugants were close to those obtained for the reference IRT, TEM-35. However, the transconjugant enzymes had a lower affinity for penicillins compared with that of TEM-35 (2.75-, 2.25-, and 1.9-fold higher values of K_m for benzylpenicillin, ticarcillin, and amoxicillin, respectively). The inhibition parameters measured for clavulanic acid, sulbactam, and tazobactam (Table 4) showed that the K_i values of the enzyme produced by transconjugants were increased by factors of 680, 152, and 180, respectively, compared with those of TEM-1, and higher concentrations of suicide inhibitors were required to give a 50% reduction in benzylpenicillin hydrolysis (750-, 82- and 50-fold higher for clavulanate, sulbactam, and tazobactam, respectively). The K_i values and IC_{50}s of the enzyme produced by the transconjugants were similar to those of the reference enzyme, TEM-35, but they were within twofold of those of the reference enzyme. Tazobactam retained a significant inhibitory potency (IC_{50}, 5

TABLE 3. Kinetic parameters for TEM-80 and TEM-127V compared with those for TEM-35 and TEM-1[a]

Enzyme	Benzylpenicillin			Ticarcillin			Amoxixillin			Cephalothin		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	$_{cat}$ (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
TEM-80	1,000	440	2.3	150	1,500	0.10	1,100	420	2.6	10	1,800	0.005
TEM-35	1,000	160	6.3	120	670	0.18	910	220	4.1	19	1,300	0.014
TEM-1	1,200	22	54	120	10	12	920	25	37	120	230	0.52
TEM-127V	1,400	57	24	160	24	6.8	1,100	69	16	170	560	0.30

[a] k_{cat} standard deviation, 10%; K_m standard deviation, 15%.

TABLE 4. Inhibition parameters for TEM-80 and TEM-127V compared with those for TEM-35 and TEM-1

Enzyme	Clavulanic acid		Sulbactam		Tazobactam	
	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)
TEM-80	68	60	137	500	1.8	5.0
TEM-35	30	41	100	440	1.2	3.2
TEM-1	0.1	0.08	0.9	6.1	0.01	0.1
TEM-127V	0.15	0.1	1.4	7.0	0.015	0.15

μM) 12 to 100 times higher than that of clavulanate (IC_{50}, 60 μM) or sulbactam (IC_{50}, 500 μM).

Plasmid analysis. Upon mating of *E. cloacae* Ecl261 or *E. coli* Ec257 with an *E. coli* K-12 Rif^r recipient strain, ampicillin-resistant transconjugants Tc261 or Tc257, respectively, were selected in both cases with a high frequency of 10^{-3} per donor cell. The results of plasmid analysis after agarose gel electrophoresis of clinical strains and their respective transconjugants is shown in Fig. 1. The plasmid content from Ecl261 yielded a single band (lane 1), in contrast to the result for Ec257, for which at least four additional bands of smaller sizes and in lower quantities were visible (lane 2). Transconjugant plasmids pTc261 from Tc261 (lane 3) and pTc257 from Tc257 (lane 4) harbored a single plasmid, with the two bands observed probably corresponding to the different supercoiled plasmid forms (open and closed circular forms). The distinct migration of the open circular forms showed that these two undigested plasmids were of different sizes, with pTc257 being smaller than pTc261. Restriction of the transconjugant plasmids with *Eco*RI or *Eco*RV gave fragments with calculated sizes of ca. 40 kb for pTc261 and ca. 30 kb for pTc257. Indeed, after *Eco*RI digestion, the profile obtained with pTc261 consisted of eight restriction fragments of 10.7, 9.0, 8.3, 4.5, 3.5, 2.2, 2.0, and 1.3 kb (lane 6). Plasmid pTc257 was composed of five *Eco*RI bands of 9.0, 8.3, 6.3, 4.5, and 2.2 kb, four of which were identical in size to those given by pTc261 (lane 7), with the 6.3-kb fragment possibly being generated by a single 10-kb deletion in pTc261 involving the adjacent 10.7-, 3.5-, 2.0-, and 1.3-kb fragments. Similarly, restriction of pTc261 with *Eco*RV (lane 8) displayed 11 fragments (9.0, 8.0, 5.5, 5.0, 4.5, 3.5, 2.5, 1.8, 1.1, 0.9, and 0.4 kb), while that of pTc257 (lane 9) gave 6 fragments (9.0, 8.0, 7.5, 3.5, 2.5, and 0.4 kb), including 5 common bands.

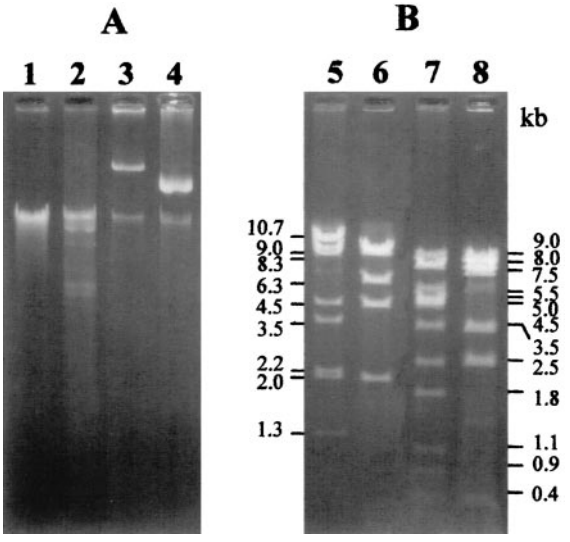

FIG. 1. Agarose gel electrophoresis of plasmid DNA from clinical isolates and their transconjugants. (A and B) Undigested and digested plasmid DNA, respectively. Lanes 1 to 4, plasmid content from Ecl261, Ec257, Tc261, and Tc257, respectively; lanes 5 and 6, *Eco*RI restriction fragments from pTc261 and pTc257, respectively; lanes 7 and 8, *Eco*RV restriction fragments from pTc261 and pTc257, respectively. The sizes are indicated in kilobases.

Gene sequence analysis. PCR amplification with primers specific for the bla_{TEM} genes gave positive results. The TEM-7 and TEMB primer pair (Table 1) yielded a fragment of 1,110 bp which covered the entire *bla* gene, including the promoter and the coding regions. Double-strand sequencing led to identical results with PCR products from both Ecl261 and Ec257. The analysis showed that they were derived from the bla_{TEM-1A} gene sequence by four mutations: A32T, A407C, A581G, and A1022G (according to the numbering of Sutcliffe [39]) (Table 5). The first replacement was located in the promoter region (12), and the three other ones were situated in the coding region, leading to the amino acid changes Met69Leu, Ile127Val, and Asn276Asp (according to the ABL numbering system of Ambler et al. [1]). The combination of these three

TABLE 5. Nucleotide differences between bla_{TEM-80} and other related bla_{TEM} sequences

bla gene (promoter)[c]	Nucleotide[a] (amino acid[b]) position in:						Reference
	Promoter region		Coding region				
	32	162	317 (39)	407 (69)	581 (127)	1022 (276)	
bla_{TEM-1A} (*P3*)	C	G	C (Gln)	A (Met)	A (Ile)	A (Asn)	39
bla_{TEM-80} (*Pa*/*Pb*)	T			C (Leu)	G (Val)	G (Asp)	This study
$bla_{TEM-127V}$ (*P3*)					G (Val)		This study
bla_{TEM-33} (*P3*)				C (Leu)			38
bla_{TEM-35} (*P3*)[d]			A (Lys)	C (Leu)		G (Asp)	44
bla_{TEM-81} (*Pa*/*Pb*)	T			C (Leu)	G (Val)		24
bla_{TEM-84} (*P4*)		T				G (Asp)	24

[a] Nucleotide positions are numbered as described by Sutcliffe (39) in comparison with the sequence of bla_{TEM-1A}. Only nucleotide positions located in the coding region which lead to an amino acid substitution are indicated.
[b] Amino acid numbering is according to the ABL numbering system of Ambler et al. (1).
[c] Promoters are numbered as described by Leflon-Guibout et al. (23).
[d] The promoter sequence of the bla_{TEM-35} gene used in this study was controlled by sequencing.

substitutions has never been described before, and thus, the novel enzyme found in both Ecl261 and Ec257 was called TEM-80 (alternative name, IRT-24) (http://www.lahey.org/studies/webt.htm).

Ile127Val site-directed mutagenesis. In order to determine whether the single replacement of Ile127 by a valine might cause inhibitor resistance, site-directed mutagenesis was performed at this position of the TEM-1 β-lactamase. The introduction of a guanine base at position 581 instead of an adenine resulted in the change of an ATA (Ile) to a GTA (Val) codon in the $bla_{\text{TEM-1A}}$ β-lactamase gene located on the pBR322 plasmid of *E. coli* JM109. This substitution led to the formation of a mutant enzyme designated TEM-127V.

After checking of the bla_{TEM} sequence, the MICs of different β-lactam antibiotics alone or in combination with β-lactamase inhibitors were determined for the two strains harboring either the wild-type or the mutant bla_{TEM} gene. The differences in the MICs for the two isogenic strains were at most twofold (Table 2). Furthermore, the catalytic efficiencies (k_{cat}/K_m) of the TEM-127V-producing mutant were not significantly altered, but the K_m values of the enzyme were about two to three times higher than those of the native enzyme. Moreover, the presence of Val127 in the TEM-1 β-lactamase did not affect the inhibitory parameters (K_i and IC_{50}) (Tables 3 and 4).

DISCUSSION

Clinical use of suicide inactivators such as clavulanic acid has inexorably led to the selection of mutants mainly derived from parental TEM-1 enzymes. Four amino acid changes at position 69, 130, 244, and 276 can account for the IRT phenotype (42). At present, at least 27 IRT β-lactamases produced by clinical isolates have been reported (http://www.lahey.org/studies/webt.htm). In this study, we characterized a novel combination of mutations in an IRT-type enzyme isolated from two strains, *E. cloacae* and *E. coli*. The IRT β-lactamases occur almost exclusively in *E. coli*, in which they are present in up to 2 to 5% of European clinical strains, including hospital-acquired as well as community-acquired isolates (18, 24). Indeed, the isolation of *E. coli* Ec257, which was resistant to the combination of amoxicillin-clavulanate but susceptible to cephalothin, strongly suggested the presence of an IRT enzyme in this strain. In a previous French study, about 87% of the *E. coli* strains exhibiting such a phenotype were demonstrated to produce an IRT-type β-lactamase, with the remaining strains probably expressing an OXA-1 β-lactamase (11, 34). Nevertheless, the presence of plasmid-mediated IRT enzymes has also been found in members of the family *Enterobacteriacae* other than *E. coli*, i.e., *Citrobacter freundii* (20), *Klebsiella pneumoniae* (3, 25), *Proteus mirabilis* (6), and *Klebsiella oxytoca* (4). The isolation from the same urine sample of *E. cloacae* Ecl261 presenting with full susceptibility to cefuroxime and extended-spectrum cephalosporins but resistance to ticarcillin in which susceptibility was not restored by the addition of the clavulanate inhibitor suggested the presence of an IRT enzyme in this strain as well.

For the two clinical strains and their transconjugants, the MICs of penicillins were high, even for piperacillin (MICs, 64 to 128 μg/ml), and the addition of inhibitors reduced these MICs by only 2 dilutions or less. It is noteworthy that the transconjugants had higher levels of resistance to the piperacillin-tazobactam combination (MICs, 64 μg/ml) than those observed for strains carrying most other IRT-type enzymes reported in the literature (MICs, ≤8 μg/ml) (11). The transferable β-lactamases produced by Ecl261, Ec257, and their respective transconjugants had identical pIs (pI 5.2). Of the about 20 IRT enzymes for which pIs have been reported (http://www.lahey.org/studies/webt.htm), the pI is 5.2 for one-half of them because these enzymes are more often derived from TEM-1 (pI 5.4) than from TEM-2 (pI 5.6). The chromosomal cephalosporinase was not hyperproduced in *E. cloacae* Ecl261, as indicated by the low MIC of cefotaxime and by isoelectric focusing (no detectable enzyme at pI > 8). The IRT phenotype conferred by this pI 5.2 enzyme was consistent with the substrate profile. The kinetic studies (Tables 3 and 4) demonstrated that this enzyme possesses a highly reduced catalytic efficiency (k_{cat}/K_m), particularly against ticarcillin and cephalothin, and that higher concentrations of inhibitors are required for inhibition of this enzyme compared with the concentrations required for TEM-1, with tazobactam remaining the most efficient compound.

This β-lactamase inhibitor resistance of strains Ec257 and Ecl261, together with resistance to sulfonamides and trimethoprim, was easily transferred by conjugation to *E. coli* K-12, as is usually described for IRT β-lactamase-encoding genes (4, 9, 25, 44). Endonuclease digestion of the conjugative plasmids from the transconjugants demonstrated that pTc257 (30-kb plasmid) and pTc261 (40-kb plasmid) might be derived from each other by a single deletion or insertion event involving a 10-kb fragment, consistent with the horizontal transfer of the plasmid carrying the $bla_{\text{TEM-80}}$ gene between Ec257 and Ecl261. Considering the high frequency of IRT enzymes in *E. coli*, such a transfer would be expected to occur from *E. coli* to *E. cloacae*. However, deletions due to an interrupted transfer are common during conjugation events, and since the *E. coli* plasmid is smaller than that of *E. cloacae*, this transfer might also have occurred from *E. cloacae* to *E. coli*.

As suggested by the pI value, PCR amplifications showed that strains Ec257 and Ecl261 and their transconjugants carry a bla_{TEM} gene. Nucleotide sequence analysis revealed the presence of a new IRT enzyme, TEM-80, identical in both Ec257 and Ecl261. The gene encoding TEM-80 differed from $bla_{\text{TEM-1A}}$ (39) by four mutations. One of them corresponded to the presence at position 32 of a thymine instead of the cytosine that occurs in the wild-type $bla_{\text{TEM-1A}}$ sequence. Such a mutation has previously been described to convert the weak promoter *P3* into the two overlapping *Pa* and *Pb* promoters and to result in a large increase in the level of β-lactamase production (12). This change, first characterized in TEM-2 promoters (12), has also been reported in IRT β-lactamases (23).

The three remaining mutations led to amino acid substitutions in the coding region. Two of them, Met69Leu and Asn276Asp, have already been described in other IRTs produced by clinical strains, either alone for Met69Leu in the TEM-33 enzyme (44) and Asn276Asp in the TEM-84 enzyme (24) or in combination in the TEM-35 β-lactamase (7, 44). In vitro amino acid replacements at these two positions have been recognized as major contributors to clinically significant levels of resistance to β-lactamase inactivators (10, 11, 21, 37, 42).

Furthermore, studies showed that a double amino acid substitution (Leu69 plus Asp276) gave rise to enzymes that were more resistant to clavulanic acid than β-lactamases with a single Leu-69 or Asp276 mutation (11, 40).

The third substitution, found in TEM-80, is the change Ile127Val. Recently, another IRT enzyme, TEM-81, with the substitutions Met69Leu and Ile127Val has been reported (24), but the role of the latter mutation in the IRT phenotype has not been investigated. The amino acid at position 127 is located at the end of α helix H4 (residues 119 to 128). Helices H4 and H2 are linked by a disulfide bridge between Cys77 and Cys123, and residue 127 is very close to residue Lys73, located at the beginning of the H2 helix, known to have an important role in β-lactam hydrolysis (27). The lack of a methyl group at this position (in valine but not in isoleucine) might induce a conformational alteration of the active site. On the other hand, this change is unlikely to be crucial for β-lactam activity, since a valine at position 127 is often present in class A β-lactamases (e.g., in PSE-3) (1, 32), or another hydrophobic amino acid is present in the remaining enzymes of this class (e.g., Met in PC1 or Leu in CTX-M) (5).

First, in order to study the role of this substitution at position 127 in the IRT phenotype, TEM-80 (Met69Leu, Ile127Val, and Asn276Asp) and TEM-35 (Met69Leu and Asn276Asp) were compared. These two enzymes differ only by the substitution Ile127Val; Ile and Val are amino acids with uncharged groups, which explains the same pI value (pI 5.2) for TEM-80 and TEM-35. The MICs of piperacillin-tazobactam for *E. coli* TEM-80-producing strains (MICs, 64 μg/ml) were higher than those for TEM-35-producing strains (MICs, 16 μg/ml). However, this increase could be explained by the presence of strong promoters upstream of the $bla_{\text{TEM-80}}$ gene (Table 5). Nevertheless, the decrease in the k_{cat}/K_m ratio and the increase in the values for the inhibition parameters (k_{cat} and IC_{50}) were always higher for this enzyme than for TEM-35. These differences are mostly related to higher K_m values for TEM-80 than for TEM-35.

In a second step, in order to confirm this result, an in vitro mutant was constructed by site-directed mutagenesis. The MICs of β-lactam–β-lactamase inhibitor combinations were not significantly different for strains producing TEM-1 and TEM-127V. Nevertheless, the MICs were very high due to the large quantity of TEM β-lactamase expressed as a result of the high copy number of plasmid pBR322 carrying the bla_{TEM} gene. Such a high level of β-lactamase expression might have masked small differences in MICs. However, the presence of Val127 in the TEM-1 β-lactamase did not affect the inhibitory parameters (K_i and IC_{50}). Furthermore, the catalytic efficiencies (k_{cat}/K_m) of the TEM-127V enzyme were not significantly altered, but the K_m values were about two times higher than those of the native enzyme, demonstrating a lower affinity of this enzyme for its substrates. Thus, the Ile127Val substitution seems to have a very weak effect, at least alone, on the IRT phenotype. Nevertheless, this mutation might play a role, even a minor one, in cooperation with other changes, as described for the substitution at position 182 in the TEM-32 and IRT-3 enzymes (Met69Ile and Met182Thr) (15, 19).

In conclusion, we report here on a novel IRT enzyme, TEM-80, found in a clinical isolate of *E. cloacae* associated with an *E. coli* isolate in the same urine sample. The difficulty in identifying an IRT phenotype in *Enterobacter* spp. and particularly in strains hyperproducing their chromosomal cephalosporinase might lead to underestimation of the frequency of IRT in this species. In our case, the coexistence with an IRT-producing *E. coli* isolate was a useful clue for the detection of this type of enzyme in *E. cloacae*.

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REFERENCES

1. **Ambler, R. P., A. F. Coulson, J. M. Frère, J. M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem. J. **276:**269–270.
2. **Barthelemy, M., M. Guionie, and R. Labia.** 1978. Beta-lactamases: determination of their isoelectric points. Antimicrob. Agents Chemother. **13:**695–698.
3. **Bermudes, H., F. Jude, C. Arpin, C. Quentin, A. Morand, and R. Labia.** 1997. Characterization of an inhibitor-resistant TEM (IRT) β-lactamase in a novel strain of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **41:** 222.
4. **Bermudes, H., F. Jude, E. B. Chaibi, C. Arpin, C. Bebear, R. Labia, and C. Quentin.** 1999. Molecular characterization of TEM-59 (IRT-17), a novel inhibitor-resistant TEM-derived β-lactamase in a clinical isolate of *Klebsiella oxytoca*. Antimicrob. Agents Chemother. **43:**1657–1661.
5. **Bonnet, R., J. L. Sampaio, R. Labia, C. De Champs, D. Sirot, C. Chanal, and J. Sirot.** 2000. A novel CTX-M β-lactamase (CTX-M-8) in cefotaxime-resistant *Enterobacteriaceae* isolated in Brazil. Antimicrob. Agents Chemother. **44:**1936–1942.
6. **Bret, L., C. Chanal, D. Sirot, R. Labia, and J. Sirot.** 1996. Characterization of an inhibitor-resistant enzyme IRT-2 derived from TEM-2 β-lactamase produced by *Proteus mirabilis* strains. J. Antimicrob. Chemother. **38:**183–191.
7. **Brun, T., J. Péduzzi, M. M. Caniça, G. Paul, P. Névot, M. Barthélémy, and R. Labia.** 1994. Characterization and amino acid sequence of IRT-4, a novel TEM-type enzyme with a decreased susceptibility to β-lactamase inhibitors. FEMS Microbiol. Lett. **120:**111–117.
8. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
9. **Caniça, M. M., M. Barthélémy, L. Gilly, R. Labia, R. Krishnamoorthy, and G. Paul.** 1997. Properties of IRT-14 (TEM-45), a newly characterized mutant of TEM-type β-lactamases. Antimicrob. Agents Chemother. **41:**374–378.
10. **Chaibi, E. B., J. Péduzzi, S. Farzaneh, M. Barthélémy, D. Sirot, and R. Labia.** 1998. Clinical inhibitor-resistant mutants of the β-lactamase TEM-1 at amino-acid position 69. Kinetic analysis and molecular modelling. Biochim. Biophys. Acta **1382:**38–46.
11. **Chaïbi, E. B., D. Sirot, G. Paul, and R. Labia.** 1999. Inhibitor-resistant TEM β-lactamases: phenotypic, genetic and biochemical characteristics. J. Antimicrob. Chemother. **43:**447–458.
12. **Chen, S. T., and R. C. Clowes.** 1984. Two improved promoter sequences for the β-lactamase expression arising from a single base-pair substitution. Nucleic Acids Res. **12:**3219–3234.
13. **Cullmann, W.** 1996. Comparative evaluation of orally active antibiotics against community-acquired pathogens: results of eight European countries. Chemotherapy (Basel) **42:**11–20.
14. **Doucet-Populaire, F., J. C. Ghnassia, R. Bonnet, and J. Sirot.** 2000. First isolation of a CTX-M-3-producing *Enterobacter cloacae* in France. Antimicrob. Agents Chemother. **44:**3239–3240.
15. **Farzaneh, S., E. B. Chaibi, J. Peduzzi, M. Barthelemy, R. Labia, J. Blazquez, and F. Baquero.** 1996. Implication of Ile-69 and Thr-182 residues in kinetic characteristics of IRT-3 (TEM-32) β-lactamase. Antimicrob. Agents Chemother. **40:**2434–2436.
16. **Giakkoupi, P., L. S. Tzouvelekis, A. Tsakris, V. Loukova, D. Sofianou, and E. Tzelepi.** 2000. IBC-1, a novel integron-associated class A β-lactamase with extended-spectrum properties produced by an *Enterobacter cloacae* clinical strain. Antimicrob. Agents Chemother. **44:**2247–2253.
17. **Girlich, D., L. Poirel, A. Leelaporn, A. Karim, C. Tribuddharat, M. Fennewald, and P. Nordmann.** 2001. Molecular epidemiology of the integron-located VEB-1 extended-spectrum β-lactamase in nosocomial enterobacterial isolates in Bangkok, Thailand. J. Clin. Microbiol. **39:**175–182.
18. **Henquell, C., D. Sirot, C. Chanal, C. De Champs, P. Chatron, B. Lafeuille,**

P. Texier, J. Sirot, and R. Cluzel. 1994. Frequency of inhibitor-resistant TEM β-lactamases in *Escherichia coli* isolates from urinary tract infections in France. J. Antimicrob. Chemother. **34:**707–714.
19. **Huang, W., and T. Palzkill.** 1997. A natural polymorphism in β-lactamase is a global suppressor. Proc. Natl. Acad. Sci. USA **94:**8801–8806.
20. **Hunter, J. E., J. E. Corkill, A. G. McLennan, J. N. Fletcher, and C. A. Hart.** 1993. Plasmid encoded β-lactamases resistant to inhibition by clavulanic acid produced by calf faecal coliforms. Res. Vet. Sci. **55:**367–370.
21. **Imtiaz, U., E. Billings, J. R. Knox, E. K. Manavathu, S. A. Lerner, and S. Mobashery.** 1993. Inactivation of class A β-lactamases by clavulanic acid: the role of arginine-244 in a proposed nonconcerted sequence of events. J. Am. Chem. Soc. **115:**4435–4442.
22. **Labia, R., J. Andrillon, and F. Le Goffic.** 1973. Computerized microacidimetric determination of β-lactamase Michaelis-Menten constants. FEBS Lett. **33:**42–44.
23. **Leflon-Guibout, V., B. Heym, and M. H. Nicolas-Chanoine.** 2000. Updated sequence information and proposed nomenclature for bla_{TEM} genes and their promoters. Antimicrob. Agents Chemother. **44:**3232–3234.
24. **Leflon-Guibout, V., V. Speldooren, B. Heym, and M. H. Nicolas-Chanoine.** 2000. Epidemiological survey of amoxicillin-clavulanate resistance and corresponding molecular mechanisms in *Escherichia coli* isolates in France: new genetic features of bla_{TEM} genes. Antimicrob. Agents Chemother. **44:**2709–2714.
25. **Lemozy, J., D. Sirot, C. Chanal, C. Huc, R. Labia, H. Dabernat, and J. Sirot.** 1995. First characterization of inhibitor-resistant TEM (IRT) β-lactamases in *Klebsiella pneumoniae* strains. Antimicrob. Agents Chemother. **39:**2580–2582.
26. **Liu, P. Y., D. Gur, L. M. Hall, and D. M. Livermore.** 1992. Survey of the prevalence of β-lactamases amongst 1000 gram-negative bacilli isolated consecutively at the Royal London Hospital. J. Antimicrob. Chemother. **30:**429–447.
27. **Matagne, A., and J. M. Frère.** 1995. Contribution of mutant analysis to the understanding of enzyme catalysis: the case of class A β-lactamases. Biochim. Biophys. Acta **1246:**109–127.
28. **Matsumoto, Y., and M. Inoue.** 1999. Characterization of SFO-1, a plasmid-mediated inducible class A β-lactamase from *Enterobacter cloacae*. Antimicrob. Agents Chemother. **43:**307–313.
29. **Matthew, A., A. M. Harris, M. J. Marshall, and G. W. Ross.** 1975. The use of analytical isoelectric focusing for detection and identification of β-lactamases. J. Gen. Microbiol. **88:**169–178.
30. **Nordmann, P., S. Mariotte, T. Naas, R. Labia, and M. H. Nicolas.** 1993. Biochemical properties of a carbapenem-hydrolyzing β-lactamase from *Enterobacter cloacae* and cloning of the gene into *Escherichia coli*. Antimicrob. Agents Chemother. **37:**939–946.
31. **Pareja, A., C. Bernal, A. Leyva, G. Piedrola, and M. C. Maroto.** 1992. Etiologic study of patients with community-acquired pneumonia. Chest **101:**1207–1210.
32. **Péduzzi, J., A. Reynaud, P. Baron, M. Barthélémy, and R. Labia.** 1994. Chromosomally encoded cephalosporin-hydrolyzing β-lactamase of *Proteus vulgaris* RO104 belongs to Ambler's class A. Biochim. Biophys. Acta **1207:**31–39.
33. **Rasmussen, B. A., K. Bush, D. Keeney, Y. Yang, R. Hare, C. O'Gara, and A. A. Medeiros.** 1996. Characterization of IMI-1 β-lactamase, a class A carbapenem-hydrolyzing enzyme from *Enterobacter cloacae*. Antimicrob. Agents Chemother. **40:**2080–2086.
34. **Romaszko, J. P., L. Bret, C. Henquell, D. Sirot, C. Chanal, and J. Sirot.** 1995. Detection of β-lactamases resistant to inhibitors (IRT) by the disk diffusion method. Pathol. Biol. (Paris) **43:**306–309.
35. **Rubio, M. C., J. Gil, J. Castillo, I. Otal, M. L. Gómez-Lus, E. Rubio, C. Sarraseca, A. Torrellas, and R. Gómez-Lus.** 1989. The susceptibility to amoxycillin/clavulanate of *Enterobacteriaceae* with plasmid-mediated ampicillin resistance: a twelve-year study of strains in one Spanish hospital. J. Antimicrob. Chemother. **24**(Suppl. B)**:**35–40.
36. **Sanders, W. E., and C. C. Sanders.** 1997. *Enterobacter* spp.: pathogens poised to flourish at the turn of the century. Clin. Microbiol. Rev. **10:**220–241.
37. **Saves, I., O. Burlet-Schiltz, P. Swarén, F. Lefèvre, J. M. Masson, J. C. Promé, and J. P. Samama.** 1995. The asparagine to aspartic acid substitution at position 276 of TEM-35 and TEM-36 is involved in the β-lactamase resistance to clavulanic acid. J. Biol. Chem. **270:**18240–18245.
38. **Speldooren, V., B. Heym, R. Labia, and M. H. Nicolas-Chanoine.** 1998. Discriminatory detection of inhibitor-resistant β-lactamases in *Escherichia coli* by single-strand conformation polymorphism-PCR. Antimicrob. Agents Chemother. **42:**879–884.
39. **Sutcliffe, J. G.** 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. USA **75:**3737–3741.
40. **Swarén, P., D. Golemi, S. Cabantous, A. Bulychev, L. Maveyraud, S. Mobashery, and J. P. Samama.** 1999. X-ray structure of the Asn276Asp variant of the *Escherichia coli* TEM-1 β-lactamase: direct observation of electrostatic modulation in resistance to inactivation by clavulanic acid. Biochemistry **38:**9570–9576.
41. **Tzelepi, E., P. Giakkoupi, D. Sofianou, V. Loukova, A. Kemeroglou, and A. Tsakris.** 2000. Detection of extended-spectrum β-lactamases in clinical isolates of *Enterobacter cloacae* and *Enterobacter aerogenes*. J. Clin. Microbiol. **38:**542–546.
42. **Vakulenko, S. B., B. Geryk, L. P. Kotra, S. Mobashery, and S. A. Lerner.** 1998. Selection and characterization of β-lactam–β-lactamase inactivator-resistant mutants following PCR mutagenesis of the TEM-1 β-lactamase gene. Antimicrob. Agents Chemother. **42:**1542–1548.
43. **Vedel, G., A. Belaaouaj, L. Gilly, R. Labia, A. Philippon, P. Névot, and G. Paul.** 1992. Clinical isolates of *Escherichia coli* producing TRI β-lactamases: novel TEM-enzymes conferring resistance to β-lactamase inhibitors. J. Antimicrob. Chemother. **30:**449–462.
44. **Zhou, X. Y., F. Bordon, D. Sirot, M. D. Kitzis, and L. Gutmann.** 1994. Emergence of clinical isolates of *Escherichia coli* producing TEM-1 derivatives or an OXA-1 β-lactamase conferring resistance to β-lactamase inhibitors. Antimicrob. Agents Chemother. **38:**1085–1089.