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A TEM-Derived Extended-Spectrum β-Lactamase in *Pseudomonas aeruginosa*

P. MUGNIER,[1]* P. DUBROUS,[2] I. CASIN,[1,3] G. ARLET,[3] AND E. COLLATZ[1]

Laboratoire de Recherche Moléculaire sur les Antibiotiques, Université Paris VI, 75270 Paris Cedex 06,[1]
Biologie Médicale, Hôpital d'Instruction des Armées Percy, 92140 Clamart,[2] *and*
Laboratorie de Bactériologie, Hôpital Saint-Louis, 75010 Paris,[3] *France*

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A clinical strain of *Pseudomonas aeruginosa*, PAe1100, was found to be resistant to all antipseudomonal β-lactam antibiotics and to aminoglycosides, including gentamicin, amikacin, and isepamicin. PAe1100 produced two β-lactamases, TEM-2 (pI 5.6) and a novel, TEM-derived extended-spectrum β-lactamase called TEM-42 (pI 5.8), susceptible to inhibition by clavulanate, sulbactam, and tazobactam. Both enzymes, as well as the aminoglycoside resistance which resulted from AAC(3)-IIa and AAC(6′)-I production, were encoded by an 18-kb nonconjugative plasmid, pLRM1, that could be transferred to *Escherichia coli* by transformation. The gene coding for TEM-42 had four mutations that led to as many amino acid substitutions with respect to TEM-2: Val for Ala at position 42 (Ala42), Ser for Gly238, Lys for Glu240, and Met for Thr265 (Ambler numbering). The double mutation Ser for Gly238 and Lys for Glu240, which has so far only been described in SHV-type but not TEM-type enzymes, conferred concomitant high-level resistance to cefotaxime and ceftazidime. The novel, TEM-derived extended-spectrum β-lactamase appears to be the first of its class to be described in *P. aeruginosa*.

The first TEM-type β-lactamase, produced by a clinical *Escherichia coli* strain, was reported in 1965 (19) and was later called TEM-1 (34). A variant of this enzyme, TEM-2, originally observed in *Pseudomonas aeruginosa* (7, 33), differs from TEM-1 only in one amino acid, but not in its substrate profile (15, 29). Until now, TEM-derived extended-spectrum β-lactamases (ESBLs) (15, 24, 29) have only been described in members of the family *Enterobacteriaceae*, beginning in 1987 with TEM-3 produced by *Klebsiella pneumoniae* (13) and with about 30 variants reported to date (3, 11). The production of ESBLs confers resistance at various levels to expanded-spectrum cephalosporins, such as cefotaxime and ceftazidime, and to aztreonam, but not normally to the cephamycins and carbapenems (25). The reasons why TEM-derived ESBLs have never been reported in *P. aeruginosa* is not clear. One reason might be that, at least in France (16), only 10% of the ticarcillin-resistant *P. aeruginosa* strains produce a TEM-type enzyme, while the OXA-type and the PSE- and CARB-type enzymes are more frequent. In addition, *P. aeruginosa* has the potential to derepress the production of its chromosomal cephalosporinase efficiently and thereby become resistant to expanded-spectrum cephalosporins (32). The acquisition of an ESBL-encoding gene might then be of a lesser advantage for this species than for the klebsiellae, which do not have this potential, and the same might apply to explain the low frequency of ESBL-producing strains in the genera *Enterobacter* and *Citrobacter*, which do have this potential. Nevertheless, the production of some non-TEM-type ESBLs has been reported in *P. aeruginosa*, such as PER-1, which is a class A enzyme only remotely related to the TEM-type β-lactamases (36, 37), and OXA-10 (formerly called PSE-2) or OXA-2 derivatives, which confer high-level resistance to ceftazidime, cefsulodin, and aztreonam (17, 18, 35).

* Corresponding author. Mailing address: L.R.M.A., Université Paris VI, 15, rue de l'Ecole de Médecine, 75270 Paris Cedex 06, France. Phone: 33-1-43.29.28.65. Fax: 33-1-43.25.68.12.

Here we describe the unusual occurrence of a TEM-derived ESBL in a clinical isolate of *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the study are listed in Table 1. *P. aeruginosa* PAe1100 was isolated from the skin of a severely burned patient 1 month after his admission to the Percy Hospital, Clamart, France, in 1994. The patient had received no antimicrobial treatment before the date of isolation of the strain except for perioperative antibiotic prophylaxis twice, with piperacillin, amikacin, and teicoplanin. No other gram-negative strain with a similar phenotype was found during the same period. PAe1100 belonged to serogroup O:6, as determined with antisera from Sanofi Diagnostics Pasteur (Marnes-La-Coquette, France). It was resistant to all clinically used anti-*Pseudomonas* β-lactam antibiotics, such as ticarcillin, piperacillin, ceftazidime, cefsulodine, aztreonam, and imipenem, and was also resistant to gentamicin, netilmicin, tobramycin, amikacin, isepamicin, sulfonamides, and mercuric ions. Bacterial strains were grown in Mueller-Hinton (MH) or brain heart infusion (BHI) medium at 37°C.

MIC determinations and antibiotics. MICs were determined on MH agar containing serially twofold diluted antibiotics. Plates inoculated with a Steers-type inoculator and ca. 10^4 CFU per spot were incubated at 37°C for 18 h. The MICs of the β-lactams were determined alone or in combination with clavulanic acid, sulbactam, or tazobactam, at 2, 8, and 4 μg/ml, respectively.

The antimicrobial agents were provided as follows: ampicillin and aztreonam, Bristol Myers Squibb; moxalactam, Eli Lilly Laboratories; ceftazidime and cephaloridine, Glaxo Group Research, Ltd.; cefotaxime, Hoechst Roussel Pharmaceuticals Ltd.; piperacillin and tazobactam, Lederle Laboratories; sulbactam, Pfizer Laboratories; clavulanic acid and ticarcillin, SmithKline Beecham; benzylpenicillin, Spécia Rhône-Poulenc-Rorer Laboratories; and cefsulodin, Takeda Laboratories.

β-Lactamase preparation. Crude enzyme extracts were prepared from 20-ml cultures grown at 37°C under vigorous agitation to an optical density at 650 nm of ca. 0.8. The cells were harvested by centrifugation and were disrupted by sonication in a volume of 2 ml of phosphate buffer (10 mM; pH 7.0). The lysates were centrifuged at 100,000 × *g* for 30 min. The supernatants were stored in aliquots at −20°C and were used for the determination of isoelectric points and kinetic parameters. The protein concentrations were measured by the technique of Bradford (10), and the hydrolysis rates were determined with cephaloridine and ceftazidime as substrates.

Isoelectric focusing. The supernatants of the sonicates were subjected to isoelectric focusing for 2 h, using a mini IEF cell 111 (Bio-Rad) and a gradient made up of two-thirds of polyampholytes with a pH range of from 4 to 6 and one-third with a pH range of from 3 to 10 (Serva). Extracts from TEM-1-, TEM-2-, and TEM-3-producing strains were used as standards for pIs of 5.4, 5.6, and 6.3, respectively. β-Lactamases were revealed by overlay with nitrocefin (1 mg/ml) in phosphate buffer (50 mM; pH 7).

TABLE 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Genotype or description	Resistance phenotype[a]	Reference or source
Strain			
PAe1100	*P. aeruginosa*, clinical strain	Ti Ca Im Gm Ak Su Hg	This study
HB101	*E. coli*, F$^-$ *D*(*gpt-proA*)*62 leuB6 supE44 ara-14 galK2 lacY1 D*(*mcrC-mrr*) *rpsL20*(StrR) *xyl-5 mtl-1 recA13*	Sm	New England Biolabs
PAO38-Rif	High-level rifampin-resistant PAO38 derivative obtained in vitro	Rif	This study
C1aNal(RP4CAZ)	In vitro-derived mutant of *E. coli* C1aNal(RP4) selected on ceftazidime	Ca	3a
Plasmid			
pLRM1	Nonconjugative, ca. 18-kb plasmid from PAe1100	Ti Ca Gm Ak	This study
pUC19-13	1.7-kb central *Pvu*II fragment of Tn*903* containing *aph(3′)-Ia* cloned into the *Sca*I site of pUC19	Km	This study
pAZ331	4.4-kb *Eco*RI-*Sal*I fragment of pLRM1 cloned into the *Eco*RI-*Sal*I site of pUC19-13	Ti Ca Gm Km	This study
pBG66 (derivative)	Derived from pBR322 and pBR325 and carrying the triple (G238S, E240K, T265M[b]) TEM mutation constructed in vitro	Ti Ca Cm	23

[a] Abbreviations: Ti, ticarcillin; Ca, ceftazidime; Im, imipenem; Km, kanamycin; Gm, gentamicin; Ak, amikacin; Sm, streptomycin; Cm, chloramphenicol; Rif, rifampin; Su, sulfonamide; Tc, tetracycline; Hg, mercuric ions.

[b] The designations represent, for example, a Ser for a Gly at position 238 (G238S).

Plasmid isolation and conjugation experiments. Plasmid DNA of PAe1100 was extracted by an alkaline lysis method modified from the method described by Birnboim and Doly (5). The only plasmid detected was called pLRM1. The plasmid size was estimated after digestion with various endonucleases (Boehringer Mannheim) and agarose gel electrophoresis, with the Raoul (Appligene) fragments used as molecular size markers.

Attempts to transfer the β-lactam resistance marker into HB101 and PAO38-Rif (Table 1) were made, using mating procedures in solid and liquid media (44). *E. coli* C1aNal(RP4CAZ) was used as a donor control strain. Transconjugants were selected on MH agar plates containing streptomycin (500 μg/ml) and ceftazidime (10 μg/ml) or rifampin (500 μg/ml) and ceftazidime (10 μg/ml), respectively.

Transformation and cloning experiments. *E. coli* HB101 was transformed with pLRM1 by electroporation (Bio-Rad gene pulser). Transformants were selected on BHI agar containing ceftazidime (10 μg/ml). Fragments of pLRM1, digested with various restriction endonucleases, were ligated into the similarly digested vector pUC19-13 with T4 DNA ligase at 16°C for 16 h. The restriction enzymes and DNA ligase were used as suggested by the manufacturer (Boehringer Mannheim). Transformants were selected on MH agar plates containing kanamycin (100 μg/ml) and ceftazidime (10 μg/ml).

DNA amplification by PCR, PCR mapping, and DNA-DNA hybridization. The amplification procedure comprised denaturation at 94°C for 2 min; this was followed by 40 cycles including denaturation for 1 min at 92°C, annealing for 1 min at 55°C, and polymerization for 1 to 3 min at 72°C. One step of polymerization at 72°C for 7 min was added after the last cycle. The reactions were performed in a final volume of 100 μl with 1.25 U of *Taq* DNA polymerase (Boehringer Mannheim). The oligonucleotides used in the study are listed in Table 2. The DNA templates were crude lysates of TEM-producing *P. aeruginosa* and *E. coli* strains for the amplification of the bla_{TEM} genes and purified plasmid preparations of pAZ331 and pLRM1 for the PCR mapping experiments. For hybridization, DNA was transferred to Hybond N$^+$ membranes (Amersham). The probe used was the fragment amplified with primers AN6 and PM3 and labelled with the ECL random prime labelling system (Amersham). The labelling reaction, hybridization under stringent conditions, and detection were carried out according to the recommendations of the manufacturer.

Restriction fragment length polymorphism. PCR-generated bla_{TEM} gene fragments were digested with various restriction endonucleases to identify fragment length polymorphisms indicative of mutations occurring in ESBLs (3). The fragments from ca. 1 μg of PCR product digested with 5 U of endonuclease were separated on low-melting agarose (3%) gels, with fragments of bla_{TEM-2}, bla_{TEM-3}, and bla_{TEM-5} used as controls.

DNA sequencing. The PCR-generated fragments obtained with total DNA from HB101(pLRM1) and primers A and B (Table 2) were digested with *Bam*HI and *Hin*dIII and were cloned into the similarly digested vector M13mp18 (Boehringer Mannheim). DNA sequencing by the dideoxy chain termination method was carried out with the T7 sequencing kit (Pharmacia).

Enzyme assays. β-Lactamase activity was determined spectrophotometrically in sodium phosphate buffer (50 mM; pH 7.0) at 30°C with a model 550S double-beam spectrophotometer (Perkin-Elmer Corp.). One unit of β-lactamase was defined as the amount of enzyme able to hydrolyze 1 μmol of cephaloridine per min. We used 3 mU of β-lactamase in each reaction mixture except those containing ceftazidime and aztreonam, in which we used 12 mU. The hydrolysis rates of penicillin G were measured with both amounts of enzyme, allowing the determination of relative hydrolysis rates (V_{rel}) for all β-lactam antibiotics tested.

V_{rel} and K_m values were calculated by computerized linear regression analysis of Eadie-Hofstee (V_0 versus V_0/S, where V_0 is the initial velocity and S is the substrate concentration) plots. The concentrations of β-lactamase inhibitors required to inhibit 50% of the β-lactamase activity (IC_{50}) were measured after 10 min of preincubation of the enzyme with the inhibitor and with cephaloridine (100 μM) as the substrate. The wavelengths used were 233 nm for penicillin G, 260 nm for cephaloridine and ceftazidime, 263 nm for cefsulodin, 267 nm for cefotaxime, and 318 nm for aztreonam.

RESULTS

Transfer and cloning of the ESBL gene bla_{TEM-42}. *P. aeruginosa* PAe1100 contained a plasmid, pLRM1, of ca. 18 kb (data not shown). It appeared to be nonconjugative since it failed, despite the use of two techniques and repeated attempts, to transfer to *E. coli* HB101 or *P. aeruginosa* PAO38-Rif (Table 1)

TABLE 2. Oligonucleotides used for amplification and PCR mapping

Primer (gene)	Nucleotide sequence (5′-3′)[a]	Reference or origin
A (bla_{TEM-1})	G(207)TATGGATCCTCAACATTTCCGTGTCG(233)	43, 47
502 (bla_{TEM-1})	A(498)GAATGACTTGGTTGAGTACTCACGA(523)	43, this study
503 (bla_{TEM-1})	G(707)CTCCGGTTCCCAACGATCAAGGC(684)	43, this study
B (bla_{TEM-1})	A(1067)CCAAAGCTTAATCAGTGAGGCA(1045)	43, 47
AN6 [*aac(6′)-Ib*]	C(429)GCGCGGATCCCACACTGCGCCTCATGA(445)	45, 9
PM3 [*aac(6′)-Ib*]	G(798)ACGGGTCGTTTGAATTCTGGTG(821)	45, this study
PM9 [*aac(3)-IIa*]	A(186)TGCATACGCAGAAGGCAAT(205)	1, this study
PM10 [*aac(3)-IIa*]	C(1046)TAACCTGAAGGCTCGCAAG(1027)	1, this study

[a] The underlined nucleotides represent restriction sites.

TABLE 3. MICs of β-lactam antibiotics for PAe1100 and *E. coli* transformants

Antibiotic + inhibitor[a]	MIC (μg/ml) for strain (plasmid):			
	PAe1100	HB101 (pLRM1)	HB101 (pAZ331)	HB101 (pUC19-13)
Ampicillin	>512	>512	>512	4
Ampicillin + Cla	>512	8	4	4
Ampicillin + Sul	>512	32	2	2
Ticarcillin	>512	>512	>512	4
Ticarcillin + Cla	16	64	64	4
Ticarcillin + Sul	256	256	16	2
Ticarcillin + Taz	32	32	32	4
Piperacillin	128	256	512	1
Piperacillin + Cla	4	1	1	1
Piperacillin + Sul	8	1	1	1
Piperacillin + Taz	8	1	1	1
Cefotaxime	128	8	16	0.06
Cefotaxime + Cla	16	0.06	0.06	0.06
Cefotaxime + Sul	8	0.06	0.06	0.03
Ceftazidime	64	64	64	0.12
Ceftazidime + Cla	1	0.25	0.25	0.12
Ceftazidime + Sul	1	0.12	0.12	0.12
Ceftazidime + Taz	2	0.25	0.25	0.25
Aztreonam	128	64	128	0.06
Aztreonam + Cla	2	0.12	0.12	0.12
Aztreonam + Sul	4	0.12	0.06	0.06
Aztreonam + Taz	4	0.12	0.12	0.06
Cefsulodin	512	—[b]	—	—
Cefsulodin + Cla	2	—	—	—
Imipenem	8	0.12	0.12	0.12
Moxalactam	8	—	—	—

[a] Cla, clavulanic acid (2 μg/ml); Sul, sulbactam (8 μg/ml); Taz, tazobactam (4 μg/ml).
[b] —, not determined (naturally resistant to cefsulodin).

under conditions in which the transfer of a conjugative plasmid, RP4CAZ, to both strains was obtained. *E. coli* HB101 was, however, readily transformed by electroporation with pLRM1, which conferred resistance to all the β-lactams except imipenem and to the aminoglycosides (Tables 1 and 3), but not to the sulfonamides and mercuric ions. Isoelectric focusing revealed two β-lactamases, one with a pI of 5.6 and one with a pI of 5.8, called TEM-42, both in PAe1100 and in HB101 (pLRM1).

Plasmid pLRM1 was digested with several restriction enzymes in order to clone the β-lactamase genes separately. A 4.4-kb *Eco*RI-*Sal*I fragment, cloned into pUC19-13, coded for TEM-42 only. The recombinant plasmid, pAZ331, conferred the same β-lactam resistance profile to HB101 as pLRM1. The enzyme with a pI of 5.6 was not cloned, but sequence analysis (see below) revealed that it was a typical TEM-2 enzyme. Plasmid pAZ331 also encoded resistance to gentamicin, but not amikacin. Amplification with primers specific for the 5′ and 3′ extremities of the *aac(3)-IIa* gene (1, 41) yielded a fragment of the expected size of 860 bp. Partial PCR mapping and restriction analysis of pLRM1 allowed us to draw the map shown in Fig. 1, with the bla_{TEM-42}, *aac(3)-IIa*, and bla_{TEM-2} genes in the same orientation. On the other hand, no fragment was amplified with the *aac(6′)-Ib*-specific primers (Table 2) and pLRM1 as the template, and no hybridization between pLRM1 and an *aac(6′)-Ib* internal fragment was observed (data not shown). This makes it unlikely that the AAC(6′)-I phenotype of PAe1100 and HB101(pLRM1), and particularly the resistance to amikacin and isepamicin, is conferred by a typical *aac(6′)-Ib* gene.

β-Lactam resistance phenotypes of PAe1100 and the *E. coli* transformants. By a disk diffusion test, a moderate effect of synergy between clavulanic acid and ceftazidime, cefotaxime, or aztreonam was observed against PAe1100, an effect which was much more pronounced against HB101(pLRM1) (data not shown). The MICs of the β-lactam antibiotics for PAe1100, HB101(pLRM1), and HB101(pAZ331) are presented in Table 3 and indicate that all three strains were highly resistant to the β-lactams tested except moxalactam.

Three points concerning the association of β-lactams with β-lactamase inhibitors seem worth mentioning. (i) Sulbactam in association with ampicillin or ticarcillin was somewhat more effective than clavulanic acid when TEM-42 was produced alone in HB101(pAZ331), but it failed to restore the activities of these antibiotics against HB101(pLRM1), which concomitantly produced TEM-2. (ii) Although clavulanic acid failed to restore the activity of ticarcillin against HB101(pLRM1) completely, it did so against PAe1100. In agreement with this observation were the specific activities of the β-lactamases encoded by pLRM1, which were found to be 3.5 times higher in HB101 than in PAe1100. (iii) Tazobactam appeared to be as efficient as clavulanic acid, when combined with ticarcillin, against the three strains.

The ESBL produced by PAe1100 conferred resistance to cefotaxime, ceftazidime, and aztreonam, with MICs of 128, 64, and 128 μg/ml, respectively. The activities of these β-lactams were restored when the β-lactams were combined with any one of the three inhibitors. As for the resistance to cefsulodin, TEM-2 is known to cause only a slight increase in the MIC of this drug (26). The restoration of its activity against PAe1100 when the drug was combined with clavulanate suggests strongly that the resistance to cefsulodin is conferred by TEM-42. The resistance of PAe1100 to imipenem was not transferred to *E. coli* HB101 with plasmid pLRM1 (Table 3).

Analysis of the amino acid substitutions in TEM-42 and their correlation with MICs. An 860-bp bla_{TEM} fragment was amplified from total DNA of HB101(pLRM1) with primers A and B and was digested with restriction enzymes which cut the

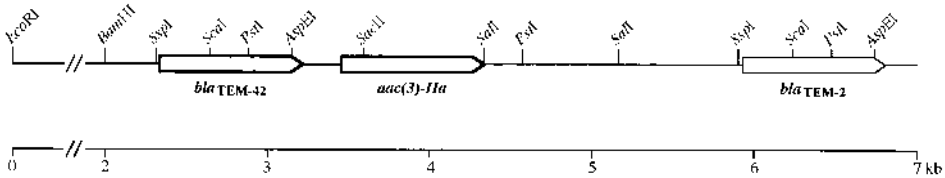

FIG. 1. Restriction map of the bla_{TEM} and *aac(3)-II* cluster of pLRM1. The fragment of 4.4 kb cloned into pAZ331 is indicated in boldface.

TABLE 4. Nucleotide and amino acid substitutions in bla_{TEM-42} and TEM-42, respectively

Position (nucleotide or amino acid[a])	Substitution[b]	
	TEM-2	TEM-42
327	G**C**A	G**T**A
42	Ala	**Val**
915	**G**GT	**A**GT
238	Gly	**Ser**
918	**G**AG	**A**AG
240	Glu	**Lys**
990	A**C**G	A**T**G
265	Thr	**Met**

[a] Nucleotide numbering is according to Sutcliffe (43); amino acid numbering is according to Ambler et al. (2).
[b] Boldface indicates the nucleotide or amino acid change.

bla_{TEM} genes at positions where mutational, ESBL-related changes are known to occur, which can be revealed by restriction fragment length polymorphism analysis (3). A modification of the patterns resulting from digestion with *Hpa*II and *Hph*I (data not shown) was observed, suggesting mutations at positions 914 and 917 and substitutions of Gly at position 238 (Gly238) and Glu240, respectively. The PCR products generated from HB101(pLRM1) were cloned into M13mp18. Sequence analysis of multiple clones showed the presence of two distinct bla_{TEM} genes, one bla_{TEM-2} and the other a bla_{TEM-2} variant called bla_{TEM-42}.

Four nucleotide substitutions were found in the variant, each leading to an amino acid substitution (Table 4). These were Val for Ala42, Ser for Gly238, Lys for Glu240, and Met for Thr265.

The coincidence of Ser238 and Lys240 has not been described previously in a TEM-derived enzyme. It is reminiscent of what has been observed in the SHV-4, SHV-5, and SHV-7 enzymes (4, 12, 39). Production of these double mutant enzymes confers high-level resistance to cefotaxime and ceftazidime (Table 5). The high-level resistance to cefotaxime and ceftazidime conferred by the recombinant plasmid pAZ331 was eight times greater than those reported for an *E. coli* strain producing a TEM-1-derived, triple mutant enzyme (23) lacking the Val for Ala42 change of TEM-42 (Table 5). To assess whether this particular change contributed to the high MICs observed for the TEM-42 producer or whether merely an abundant level of enzyme production was the cause, the specific activities of TEM-42 and the triple mutant enzyme were determined in their respective producers. As indicated in Table 5, the specific β-lactamase activity in the TEM-42 producer was about eight times higher, whether it was determined with ceftazidime or cephaloridine.

TABLE 5. Amino acid variations in TEM-2, TEM-42, SHV-5, and the TEM triple mutant obtained in vitro[a] and correlation with selected MIC changes for *E. coli*

Enzyme	Amino acid at position[b]				Sp act (mU/mg)[c]		Fold MICs[d]	
	42	238	240	265	Cri	Caz	Ctx	Caz
TEM-2	Ala	Gly	Glu	Thr	ND[e]	ND	1	1
TEM-42	Val	Ser	Lys	Met	330	25	256	512
G238S, E240K, T265M (pBG66 derivative)[a]	—[f]	Ser	Lys	Met	39	3.5	32	64
SHV-5	Gly	Ser	Lys	—	ND	ND	128[g]	512[g]

[a] This mutant was constructed in vitro (23) (see footnote *b* of Table 1 for description of mutant designations).
[b] Numbering according to Ambler et al. (2).
[c] Cri, cephaloridine; Caz, ceftazidime.
[d] Values determined with respect to those for the corresponding β-lactamase-free susceptible *E. coli* strains. Ctx, cefotaxime; Caz, ceftazidime.
[e] ND, not determined.
[f] No amino acid change with respect to TEM-2.
[g] Data from Gutmann et al. (20).

TABLE 6. Kinetic parameters for TEM-2, TEM-42, and SHV-5

Substrate	TEM-2[a]		TEM-42		SHV-5[b]	
	Km (μM)	Relative V_{max} (%)	K_m (μM)	Relative V_{max} (%)	Km (μM)	Relative V_{max} (%)
Penicillin G	15	100	1.4	100	ND[c]	100
Cephaloridine	610	72	54	160	31	140
Cefotaxime	510[d]/>3,000	<0.1	124	110	7	25
Cefsulodin	ND	0.6[e,f]	189	7	ND	ND
Ceftazidime	480[d]/>3,000	<0.1	98	3	23	11
Aztreonam	2,900[d]	0.4[d,f]	7.1	2	ND	1

[a] Data from Gutmann et al. (21).
[b] Data from Gutmann et al. (20).
[c] ND, not determined.
[d] Data from Bush et al. (14).
[e] Data from King et al. (27).
[f] Determined with respect to cephaloridine.

Enzyme assays. The catalytic activity of TEM-42 was measured against benzylpenicillin, cephaloridine, cefotaxime, cefsulodin, ceftazidime, and aztreonam (Table 6). The K_m values and the V_{rel} values, with the V_{rel} of benzylpenicillin set at 100, are presented in Table 6, along with those available for TEM-2 and SHV-5. The V_{rel}s of cefotaxime, ceftazidime, and aztreonam were greatly increased compared with those determined for TEM-2. The K_m values of cefotaxime and ceftazidime were appreciably lower than those for TEM-2 (14, 21, 30), but not quite as low as those for SHV-5. However, ceftazidime was clearly less hydrolyzed than penicillin G and cephaloridine by both enzymes. The particularly low K_m value of aztreonam was likely to result from the simultaneous amino acid change at positions 238 and 240 in TEM-42, since higher K_m values of aztreonam were previously found for TEM derivatives in which only one or the other of these two changes occurred (24).

TEM-2 is considered to have only modest (26) or no (31) activity against cefsulodin in vitro. The V_{rel} and K_m values measured with cefsulodin for TEM-42 were found to be very close to those measured with ceftazidime. Thus, it seems safe to say that the amino acid changes in TEM-42 contribute to the kinetic values observed with cefsulodin.

The IC_{50}s of clavulanic acid, sulbactam, and tazobactam for TEM-42 were 15, 8, and 4 nM, respectively (Table 7). The decrease in the IC_{50} of clavulanic acid was close to those found for other TEM-type ESBLs, such as TEM-3. Especially notable was the low IC_{50} of sulbactam, which decreased from close to 2 μM for TEM-2 to approximately 10 nM for TEM-42. This

TABLE 7. Inhibition profiles of TEM-42, TEM-2, and TEM-3

Inhibitor	IC_{50} (nM)		
	TEM-2[a]	TEM-42	TEM-3[b]
Clavulanic acid	70	15	26
Sulbactam	1,700	8	50
Tazobactam	82	4	20

[a] Data from Gutmann et al. (21).
[b] Data from Kitzis et al. (28).

explains its inhibitory efficiency in association with ampicillin or ticarcillin in the absence of TEM-2 (Table 3).

DISCUSSION

The multidrug-resistant strain *P. aeruginosa* PAe1100 was a unique clinical isolate from a patient who had not undergone any long-term antibiotic therapy. It contained a nonconjugative plasmid, pLRM1, of ca. 18 kb which codes for two β-lactamases, TEM-2 and a novel, TEM-2-derived ESBL called TEM-42. To our knowledge, the production of a penicillinase and its extended-spectrum derivative, which are both encoded by the same plasmid, has not been described previously. However, as in PAe1100, the concomitant production of a penicillinase and an ESBL leading to resistance to the combination of penicillins that are active against gram-negative bacteria with a β-lactamase inhibitor has been observed previously (12, 40). Plasmid pLRM1 also coded for two aminoglycoside-modifying enzymes, an AAC(3)-II (1, 41) and, as judged from the aminoglycoside resistance pattern (41), most likely an AAC(6′)-I, but apparently not a typical AAC(6′)-Ib. The fact that both β-lactamase genes and both aminoglycoside resistance genes were found on the same plasmid raised the possibility that they are part of an integron-like structure (6, 22). However, no resistance to sulfonamides or mercuric ions typically associated with these structures was conferred by pLRM1.

TEM-42 appears to be the first TEM-derived ESBL described in *P. aeruginosa*. This enzyme has cefotaxime-hydrolyzing as well as ceftazidime-hydrolyzing activities and also confers resistance to aztreonam and cefsulodin. Nucleotide sequence analysis of several cloned copies of what appeared to be one PCR-generated fragment confirmed that pLRM1 carried a typical $bla_{\text{TEM-2}}$ gene, in addition to a variant of this gene. The $bla_{\text{TEM-42}}$ gene had four nucleotide substitutions that were novel inasmuch as their combination has not been described previously and all led to amino acid changes (Table 4).

The likely contribution of the amino acid changes to the observed alterations in the cefotaxime and ceftazidime susceptibilities of TEM-42-producing *E. coli* can be comparatively inferred from the data in the literature. In the bla_{TEM} mutants constructed in vitro, substitution of Ser for Gly238 resulted in a small increase in the MICs of cefotaxime and ceftazidime (8, 46), while substitution of Lys for Glu240 entailed a 4- to 16-fold increase in the MIC of ceftazidime, but not that of cefotaxime (8, 46). High-level resistance to both cefotaxime and ceftazidime was observed when residues Ser238 and Lys240 were simultaneously present (46). The MIC of neither cefotaxime nor ceftazidime was noticeably altered when Met instead of Thr was present at position 265 (8, 23), a mutation also observed in TEM-42. TEM-42 conferred higher-level resistance to cefotaxime and ceftazidime than the triple mutant (Ser for Gly238, Lys for Glu240, and Met for Thr265) obtained in vitro (23) (Table 5), with the only structural difference between the two enzymes being a Val instead of an Ala residue at position 42, apart from the Lys39 of TEM-42, typical for TEM-2 and its derivatives. However, from the results reported in the literature (38, 42), the significance of this particular change is not evident. In the present case, the difference between the specific activities (Table 5) of TEM-42 and the triple mutant (23) appears to be sufficient to explain the difference between the MICs of cefotaxime and ceftazidime for the respective producers.

As for the enzyme characteristics, the K_m values of cephaloridine and cefotaxime for TEM-42 are very close to those for the double and the triple mutants altered at positions 238 and 240 and at positions 238, 240, and 265, respectively (23) (Table 6). The mutations at positions 238 and 240 are also present in SHV-5, but its kinetic parameters are quite different from those of TEM-42, especially as far as the K_m values are concerned. These differences are more likely to be linked to the only moderate percentage (68%) of identical amino acids in TEM and SHV and to different β-lactam–β-lactamase interactions overall. Besides, simple comparison of deduced amino acid functions in homologous positions may not be legitimate since, as suggested by Venkatachalam et al. (46), identical mutations could result in distinct modifications of the functional features of TEM- and SHV-type enzymes.

TEM-42 is susceptible to inhibition by clavulanic acid, sulbactam, and tazobactam. As such, it behaves very much like TEM-3, especially with its high affinity for sulbactam. Both enzymes have only one amino acid in common among those typically altered in ESBLs, i.e., Ser238. Whether this is critical for interaction with sulbactam remains subject to speculation.

On the practical level, TEM-42 production causes high-level resistance to several clinically useful anti-*Pseudomonas* β-lactam antibiotics, such as aztreonam, cefsulodin, and ceftazidime, but is, in return, accompanied by the susceptibility of the producing bacteria to the protective effects of these compounds with all three currently commercially available β-lactamase inhibitors.

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