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TEM-121, a Novel Complex Mutant of TEM-Type β-Lactamase from *Enterobacter aerogenes*

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Enterobacter aerogenes **clinical isolate LOR was resistant to penicillins and ceftazidime but susceptible to cefuroxime, cephalothin, cefoxitin, cefotaxime, ceftriaxone, and cefepime. PCR and cloning experiments from this strain identified a novel TEM-type β-lactamase (TEM-121) differing by five amino acid substitutions from β-lactamase TEM-2 (Glu104Lys, Arg164Ser, Ala237Thr, Glu240Lys, and Arg244Ser) and by only one amino acid change from the extended-spectrum β-lactamase (ESBL) TEM-24 (Arg244Ser), with the last substitution also being identified in the inhibitor-resistant β-lactamase IRT-2. Kinetic parameters indicated that TEM-121 hydrolyzed ceftazidime and aztreonam (like TEM-24) and was inhibited weakly by clavulanic acid and strongly by tazobactam. Thus, TEM-121 is a novel complex mutant TEM β-lactamase (CMT-4) combining the kinetic properties of an ESBL and an inhibitor-resistant TEM enzyme.**

TEM-type β-lactamases are widespread among enterobacterial clinical isolates. These Ambler class A enzymes behave mostly as broad-spectrum penicillinases. They are usually susceptible to clavulanic acid and confer resistance to amino-, carboxy-, and ureido-penicillins. Under selective pressure, bla_{TEM} genes have evolved, giving rise to point mutant derivatives that confer resistance to expanded-spectrum cephalosporins or to β-lactam–clavulanic acid combinations. The extended-spectrum β-lactamases (ESBLs) have a few amino acid substitutions at Ambler positions 104, 164, 238, and 240 (2, 14, 15), whereas substitutions of residues at positions 69, 130, 244, 275, and 276 are able to confer an inhibitor-resistant TEM (IRT) profile (2, 8). During the past decade, TEM-type enzymes combining amino acid substitutions specific to both IRT-type and ESBL-type β-lactamases have been reported and designated complex mutant TEM (CMT) β-lactamases (12, 18, 22). This novel denomination takes into account the amino acid sequence of the variant enzyme and does not account for its enzymatic properties. Three CMT β-lactamases are known: TEM-50/CMT-1 from *Escherichia coli* GR102 (22), TEM-68/CMT-2 from *Klebsiella pneumoniae* 3151 (12), and TEM-89/CMT-3 from *Proteus mirabilis* Pm 631 (18). TEM-50/CMT-1 and TEM-68/CMT-2 are susceptible to clavulanic acid and hydrolyze extended-spectrum cephalosporins like ESBL enzymes, whereas TEM-89/CMT-3 confers the same pattern of resistance found in IRT β-lactamases.

We describe here a novel CMT enzyme, TEM-121/CMT-4, from an *Enterobacter aerogenes* isolate. This is the first CMT enzyme that confers a high level of resistance to ceftazidime combined with a reduced susceptibility to clavulanic acid.

MATERIALS AND METHODS

Bacterial strains and plasmids. Identification of *E. aerogenes* clinical isolates was performed using API 20E and API 32GN systems (Biomerieux, Marcy l'Etoile, France). *E. aerogenes* CF24.03, which produces TEM-24, was a gift from C. De Champs (11). *E. coli* DH10B was used for cloning experiments (2).

Susceptibility testing. Antibiotic-containing disks were used for antibiotic susceptibility testing by the disk diffusion assay (Sanofi-Diagnostics Pasteur, Marnes-la-Coquette, France), as described previously (4). The double-disk synergy test was performed with ceftazidime- and amoxicillin-clavulanic acid-containing disks on Mueller-Hinton agar plates, and the results were interpreted as described previously (13). MICs were determined by an agar dilution technique on Mueller-Hinton agar (Sanofi-Diagnostics Pasteur, Paris, France) with an inoculum of 10^4 CFU per spot and were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (17).

PCR, cloning experiments, recombinant plasmid analysis, and DNA sequencing. Whole-cell DNAs of *E. aerogenes* LOR and *E. aerogenes* CF24.03 were extracted as described previously (4). Whole-cell DNA of *E. aerogenes* strain LOR was used as the template in standard PCR experiments (4) with primers preAmpC-EA1 (5′-GAGGAAGATAGCTGCGTGTC-3′) and preAmpC-EA2 (5′-TCTACCAGCTAGTGCCAACC-3′) to amplify the bla_{AmpC} gene and with laboratory-designed primers to detect the class A β-lactamase genes bla_{TEM}, bla_{SHV}, bla_{PER-1}, bla_{VEB-1}, and bla_{GES-1} (19). Amplification products obtained with primers preTEM-1 (5′-GTATCCGCTCATGAGACAATA-3′) and preTEM-2 (5′-TCTAAAGTATATATGAGTAAACTTGGTCTG-3′) and whole-cell DNA of *E. aerogenes* strains LOR and CF24.03 as templates were ligated into pBK-CMV phagemid (Stratagene, Amsterdam, The Netherlands) that had been previously digested with restriction enzyme ScaI (Amersham Pharmacia Biotech, Orsay, France). Recombinant phagemids were transformed into *E. coli* strain DH10B by electroporation with a Gene Pulser II apparatus (Bio-Rad, Ivry-sur-Seine, France). Transformants were selected on Trypticase soy agar containing amoxicillin (100 μg/ml) and kanamycin (30 μg/ml). The PCR products of the bla_{AmpC} and bla_{TEM} genes were sequenced on both strands with an Applied Biosystems sequencer (ABI 377). The nucleotide and deduced protein sequences were analyzed with software available at the website of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The ClustalW program (www.infobiogen.fr) was used for the alignment of multiple protein sequences.

Plasmid content and transformation. Plasmid DNA content of recombinant strains was extracted using a plasmid Midi kit (QIAGEN, Courtaboeuf, France). Transformation experiments were performed as previously described (4). Transformant strains were selected on amoxicillin (100 μg/ml)-containing plates.

β-Lactamase extracts and β-lactamase purification. Cultures of recombinant *E. coli* DH10B(pBK-TEM-121) and *E. coli* DH10B(pBK-TEM-24) were grown overnight at 37°C in 4 liters of Trypticase soy broth containing amoxicillin (100 μg/ml) and kanamycin (30 μg/ml), resuspended in 40 ml of 100 mM sodium phosphate buffer (pH 7), disrupted by sonication, and centrifuged at 20,000 × *g*

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for 1 h at 4°C as previously described (4). β-Lactamase extracts were filtered through a 0.45-μm-pore-size filter (Millipore, Saint-Quentin-en-Yvelines, France) and dialyzed overnight against 20 mM bis-Tris (pH 6.8) at 4°C. The enzyme extracts were loaded onto a Q-Sepharose column preequilibrated with the same buffer, and the resulting enzyme extracts were recovered in the flowthrough. The extracts were then dialyzed against 20 mM Tris-HCl buffer (pH 9) overnight at 4°C and loaded onto a preequilibrated Q-Sepharose column. The β-lactamase activity was retained, and the proteins were subsequently eluted with a linear NaCl gradient (0 to 1 M). The β-lactamase-containing fractions were tested using a nitrocefin test. The fractions with the highest β-lactamase activities were pooled and dialyzed against 50 mM phosphate buffer (pH 7). The purified β-lactamase extracts were used for determination of enzyme activities. Their purity was estimated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4).

IEF analysis. The β-lactamase extracts from cultures of clinical isolates and purified enzymes were subjected to analytical isoelectric focusing (IEF) as previously described (4), using an ampholine polyacrylamide gel with a pH range of 3.5 to 9.5 (ampholine polyacrylamide gel plate; Amersham Pharmacia Biotech) for 90 min at 1,500 V, 50 mA, and 30 W. The focused β-lactamases were detected by overlaying the gel with a 1 mM nitrocefin solution (Calbiochem, Merck Eurolab SAS, Fontenay-sous-bois, France).

Kinetic parameters. Purified β-lactamase extracts were used for kinetic measurements performed at 30°C in 100 mM sodium phosphate (pH 7.0). The initial rates of hydrolysis were determined with an ULTROSPEC 2000 UV spectrophotometer (Amersham Pharmacia Biotech) and were analyzed with Swift II software (Amersham Pharmacia Biotech). One unit of enzyme activity was defined as the activity that hydrolyzed 1 μmol of benzylpenicillin per min. The k_{cat} and K_m values were determined by analyzing β-lactam hydrolysis under initial rate conditions by using the Eadie-Hofstee linearization of the Michaelis-Menten equation as described previously (10). The 50% inhibitory concentrations (IC_{50}s) were determined for clavulanic acid, tazobactam, and sulbactam. Various concentrations of these inhibitors were preincubated with the purified enzyme for 3 min at 30°C to determine the concentrations that reduced the rate of hydrolysis of 100 μM benzylpenicillin by 50%. The protein content was measured by the Bio-Rad DC protein assay (Bio-Rad S.A.).

Nucleotide sequence accession number. The nucleotide sequence of the $bla_{TEM-121}$ gene has been submitted to the GenBank nucleotide sequence database and has been assigned accession number AY307374.

RESULTS AND DISCUSSION

Isolation and preliminary antibiotic susceptibility testing. *E. aerogenes* strain LOR was isolated from the urine of a 54-year-old woman in December 2002. She had been hospitalized at the Bicêtre hospital (Le Kremlin-Bicêtre, France) for kidney and pancreas transplantation. During the surgery follow-up, she developed a urinary tract infection due to *E. aerogenes* LOR. The patient was treated three times with amoxicillin (1 g) combined with clavulanic acid but developed, 7 days later, a pyelonephritis due to *E. aerogenes* LOR, for which she received imipenem.

E. aerogenes LOR was selected for further study on the basis of its uncommon pattern of resistance to β-lactam antibiotics, including ceftazidime and aztreonam, and susceptibility to cephalothin, cefuroxime, cefotaxime, and ceftriaxone. No synergy was detectable between extended-spectrum cephalosporins and clavulanic acid on the disk diffusion antibiogram when the test was performed under standard conditions (13). The isolate was also resistant to cotrimoxazole, fluoroquinolones, and all aminoglycosides except gentamicin (data not shown).

PCR experiments and cloning of β-lactamase genes. Preliminary PCR experiments using whole-cell DNA of *E. aerogenes* strain LOR and primers designed to amplify several internal fragments of ESBL genes gave positive results only for the bla_{TEM} gene. External primers for bla_{TEM} and whole-cell DNAs of *E. aerogenes* strains LOR and CF24.03 producing TEM-24 as templates were used to amplify by PCR a 996-bp fragment that did not include the promoter sequences. The corresponding PCR products were cloned into pBK-CMV, giving rise to recombinant plasmids pBK-TEM-121 and pBK-TEM-24, respectively. A PCR experiment using primers preAmpC-EA1 and preAmpC-EA2 with whole-cell DNA of *E. aerogenes* LOR yielded a 1,250-bp PCR product containing the entire bla_{AmpC} gene with its own promoter, which was also sequenced.

DNA sequencing. Analysis of the inserted nucleotide sequence from a recombinant plasmid (pBK-TEM-121) revealed a 286-amino-acid protein differing by five amino acid substitutions from β-lactamase TEM-2 (Glu104Lys, Arg164Ser, Ala237Thr, Glu240Lys, and Arg244Ser) and by only one amino acid change from TEM-24 (Arg244Ser) (2, 9). This enzyme was designated TEM-121/CMT-4 in accordance with TEM and IRT nomenclature (http://www.lahey.org/studies/webt.html).

Herein, we describe a β-lactamase CMT that has likely evolved from TEM-24 by an Arg-to-Ser replacement at position 244. This substitution has been reported alone and in combination with a substitution of Met to Leu at position 69 in β-lactamases TEM-30 (IRT-2) (8) and TEM-77 (IRT-21) (15). Three CMTs have been identified so far in clinical isolates. (i) CMT-1/TEM-50 combines amino acid substitutions of the ESBL TEM-15 (Glu to Lys at position 104 and Gly to Ser at position 238) with two IRT-type substitutions (Met to Leu at position 69 and Asn to Asp at position 276). (ii) CMT-2/TEM-68 associates amino acid substitutions encountered in the ESBL TEM-47 (Gly to Ser at position 238 and Glu to Lys at position 240) (12) with one amino acid substitution found in IRT-9/TEM-38, Arg to Leu at position 275 (1). (iii) CMT-3/TEM-89 contains the Glu104Lys and Gly238Ser ESBL-type substitutions, encountered in the ESBL TEM-3, and the Ser130Gly substitution identified in β-lactamase IRT-17/TEM-59 (5).

DNA sequence analysis of the PCR product of the bla_{ampC} gene from *E. aerogenes* LOR showed that the cephalosporinase shared 100% identity with that reported for the previously identified AmpC enzyme of another *E. aerogenes* strain (20). Analysis of the promoter sequences upstream of the coding sequence revealed that the −35 and −10 promoter sequences were identical to those defining a wild-type promoter (20) but were separated by 19 instead of 17 bp (20). This uncommon distance may explain a weak expression of the cephalosporinase produced by *E. aerogenes* LOR (3), which was consistent with the pattern of resistance and the results of the isoelectric focusing experiments (see below).

IEF analysis. IEF results showed that *E. aerogenes* LOR and recombinant *E. coli* DH10B(pBK-TEM-121) expressed only one β-lactamase with a pI value of 6.3, corresponding to β-lactamase TEM-121. *E. aerogenes* CF24.03 produced β-lactamases with pI values of 8.5 and 6.5, likely corresponding to the chromosomally encoded AmpC-type β-lactamase and TEM-24, respectively.

Susceptibility testing. MICs of β-lactams for *E. aerogenes* isolates LOR and CF24.03 and *E. coli* DH10B harboring recombinant plasmids are reported in Table 1. Susceptibility data showed that *E. aerogenes* LOR was resistant to amino-, carboxy-, and ureido-penicillins. Clavulanic acid did not restore susceptibility to amoxicillin, whereas tazobactam restored susceptibility to piperacillin. *E. aerogenes* LOR was resistant to

TABLE 1. MICs of β-lactams for *E. aerogenes* LOR and CF23.04 isolates and for recombinant *E. coli* DH10B(pBK-TEM-121), DH10B(pBK-TEM-24), and DH10B

β-Lactam[a]	MIC (μg/ml) for the following isolate[b]:				
	E. aerogenes LOR	*E. coli* DH10B(pBK-TEM-121)	*E. aerogenes* CF24.03	*E. coli* DH10B(pBK-TEM-24)	*E. coli* DH10B
Amoxicillin	>512	>512	>512	>512	2
Amoxicillin-CLA	16	32	512	4	2
Ticarcillin	>512	>512	>512	512	1
Ticarcillin-CLA	128	256	128	32	1
Piperacillin	32	64	128	128	1
Piperacillin-TZB	4	8	16	8	1
Cephalothin	8	16	>512	>512	4
Cefoxitin	4	2	128	2	2
Cefuroxime	8	8	>512	256	0.5
Ceftriaxone	<0.06	<0.06	32	8	<0.06
Cefotaxime	<0.06	<0.06	32	8	<0.06
Ceftazidime	128	256	512	256	<0.06
Aztreonam	8	16	512	256	0.06
Cefepime	2	4	4	4	0.06
Cefpirome	2	4	4	4	0.06
Imipenem	0.06	0.06	0.06	0.06	0.06

[a] CLA, clavulanic acid at 2 μg/ml; TZB, tazobactam at 4 μg/ml.
[b] *E. aerogenes* isolate LOR produced β-lactamase TEM-121, whereas *E. aerogenes* CF24.03 produced TEM-24 and AmpC-type β-lactamases. Recombinant *E. coli* DH10B(pBK-TEM-121) and *E. coli* DH10B(pBK-TEM-24) produced β-lactamases TEM-121 and TEM-24, respectively.

ceftazidime but was paradoxically susceptible to narrow-spectrum cephalosporins (cephalothin, cephaloridine, cefuroxime, and cefoxitin) and also to cefotaxime, ceftriaxone, cefepime, aztreonam, and imipenem. The resistance profile of recombinant *E. coli* strain DH10B(pBK-TEM-121) mirrored that of the parental strain, *E. aerogenes* LOR. *E. aerogenes* CF24.03, which produces TEM-24 and AmpC-type β-lactamases, had reduced susceptibility to cefepime and cefpirome, was susceptible to imipenem, and was resistant to the other β-lactams. MICs for *E. coli* DH10B(pBK-TEM-24) showed that β-lactamase TEM-24 conferred resistance to penicillins, to narrow-spectrum cephalosporins except cefoxitin, and to ceftazidime, aztreonam, cefotaxime, and ceftriaxone and a reduced susceptibility to cefepime and cefpirome. Addition of clavulanic acid and tazobactam inhibitors restored the activities of amoxicillin and piperacillin, respectively (Table 1).

Kinetic parameters. After purification from culture extracts of recombinant *E. coli* DH10B(pBK-TEM-121) and *E. coli* DH10B(pBK-TEM-24), the specific activities of β-lactamases TEM-121 and TEM-24 against benzylpenicillin were determined to be 3.4 and 3.1 U/mg of protein, respectively. β-Lactamases were purified to near homogeneity as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

The kinetic parameters of the purified β-lactamases TEM-121 and TEM-24 are presented in Table 2. The catalytic efficiencies of both enzymes for benzylpenicillin, ticarcillin, ceftazidime, cefepime, and cefpirome were similar, whereas TEM-121 hydrolyzed amoxicillin, cephalothin, cephaloridine, cefuroxime, aztreonam, cefotaxime, and ceftriaxone at much lower levels than TEM-24 (Table 2). This difference was due mostly to a decrease of affinity (shown by a higher K_m value), whereas the k_{cat} values remained similar. No catalytic activity of either enzyme against cefoxitin, moxalactam, or imipenem was detectable.

Inhibition studies determining IC_{50}s confirmed that TEM-24 was inhibited by clavulanic acid (0.02 μM), tazobactam (1 μM), and sulbactam (2 μM). On the other hand, TEM-121 was weakly inhibited by clavulanic acid (1 μM) and sulbactam (10 μM), whereas tazobactam remained a better inhibitor (0.3 μM) of TEM-121 than of TEM-24.

β-Lactamase TEM-121 exhibited a peculiar spectrum of hydrolysis compared to that of other CMT β-lactamases, since it hydrolyzed expanded-spectrum cephalosporins but was resistant to clavulanic acid. CMT-1/TEM-50 and CMT-2/TEM-68 also displayed significant ESBL activity but were still susceptible to clavulanic acid, with the IC_{50}s of clavulanic acid being 0.15 and 0.25 μM for CMT-1 and CMT-2, respectively (12, 22). On the other hand, the resulting catalytic activity of CMT-3/TEM-89 was similar to that of classical IRT β-lactamases, conferring high resistance to penicillins alone or in combination with clavulanic acid, as was observed for TEM-121, but

TABLE 2. Kinetic parameters of β-lactamases TEM-121 and TEM-24[a]

β-Lactam	TEM-121			TEM-24		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Benzylpenicillin	25	15	1,700	20	9	2,200
Amoxicillin	10	500	20	6	55	110
Ticarcillin	10	75	140	10	60	160
Piperacillin	7	15	140	6	6	1,000
Cephaloridine	70	270	260	90	90	1,000
Cephalothin	20	420	50	30	30	1,000
Cefuroxime	>5	>700	1	45	330	140
Ceftriaxone	>2	>500	4	10	10	1,000
Cefotaxime	0.5	430	10	8.5	25	340
Ceftazidime	40	150	270	120	180	670
Cefepime	6	40	150	9	90	100
Cefpirome	30	360	80	45	310	145
Aztreonam	6.5	80	80	16	55	290

[a] Values are the means of results from at least three independent experiments.

sparing extended-spectrum cephalosporins, whereas ceftazidime was highly hydrolyzed by TEM-121. The IC_{50} of CMT-3 for clavulanic acid was much higher than that of TEM-3 (90 and 0.01 μM, respectively) (18). A similar situation was observed for β-lactamase SHV-10, which is related to the ESBL SHV-9 but has an additional Ser-to-Gly change at position 130. SHV-10 has kept its ability to hydrolyze penicillins but has lost nearly all activity against cephalosporins (21).

Conclusions. We report a novel CMT enzyme, TEM-121/CMT-4, that has likely evolved from TEM-24, one of the most prevalent ESBLs isolated in France (7, 11, 16). β-Lactamase TEM-121 has hydrolysis activity against several expanded-spectrum cephalosporins, such as ceftazidime, cefepime, and cefpirome, and also against aztreonam, but has lost hydrolysis activity against cefotaxime and ceftriaxone compared to β-lactamase TEM-24. This discrepancy was due mainly to a loss of affinity of TEM-121 for these substrates. As observed previously (23), the extension of the substrate profile observed with TEM-121 is mostly due to the presence of Lys104 and Lys240. Both lysine residues play a role by interacting with the carboxylate of the aminothiazole oxime side chain of aztreonam, thus increasing the affinity for this substrate. In addition, the hydrogen-bonding interactions involving the oxime and N7 of Lys104 play a major role in hydrolysis of most expanded-spectrum cephalosporins (23).

Moreover, the Arg-to-Ser change at position 244 in the TEM-121/CMT-4 mutant enzyme was responsible for its high level of resistance to clavulanic acid (IC_{50}, 2 μM) compared to the IC_{50}s of β-lactamases TEM-24 and TEM-1 for this inhibitor (IC_{50}s, 0.05 and 0.08 μM, respectively). However, as is the case for IRT β-lactamases (4, 6), TEM-121/CMT-4 has a good susceptibility to tazobactam inhibition. It is interesting that TEM-121, compared to TEM-2, possesses two additional lysine residues and lacks an arginine residue. The two extra lysines likely explain the good activity of tazobactam against this CMT and maintain its important hydrolytic activities against ceftazidime and aztreonam (23). This is the first example of a TEM-type enzyme that retains activity against several expanded-spectrum cephalosporin molecules and that was not well inhibited by serine-based β-lactamase inhibitors.

In conclusion, this is the first report of an Arg-to-Ser substitution at Ambler position 244 in a TEM-type ESBL. Detection of such an ESBL-producing strain that does not display visible synergy between extended-spectrum cephalosporins and clavulanic acid is important, since an isolate under examination may otherwise be wrongly reported as being susceptible to ceftriaxone, cefepime, aztreonam, and cefpirome.

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