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MECHANISMS OF RESISTANCE

TEM-184, a Novel TEM-Derived Extended-Spectrum β-Lactamase with Enhanced Activity against Aztreonam

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ABSTRACT TEM-184, a novel TEM-derived extended-spectrum β-lactamase (ESBL), was isolated from an *Escherichia coli* ST354 clinical strain. Compared to TEM-1, TEM-184 contains the mutations Q6K, E104K, I127V, R164S, and M182T. Kinetic analysis of this enzyme revealed extended-spectrum activity against aztreonam in particular. TEM-184 was also susceptible to inhibitors, including clavulanic acid, tazobactam, and avibactam.

KEYWORDS ESBL, avibactam, inhibitors

TEM-type enzymes are likely the most prevalent acquired β-lactamases among *Escherichia coli* and other *Enterobacterales* in the clinical setting and were the first broad-spectrum enzymes showing the ability to evolve extended-spectrum β-lactamase (ESBL) activity or resistance to mechanism-based inhibitors following specific amino acid replacements (1, 2). Indeed, a very large array of TEM variants with ESBL activity and a lower number of variants resistant to inhibitors (IRT) or with a combined phenotype (CMT) have been described (ftp://ftp.ncbi.nlm.nih.gov/pathogen/betalactamases/allele.tab), underscoring the outstanding evolutionary plasticity of these enzymes, and characterization of these variants has provided an invaluable amount of information on the structure-function relationships of these enzymes and, more generally, of serine β-lactamases.

In this study, we identified and characterized, from a kinetic point of view, a novel natural TEM-type variant with ESBL activity, named TEM-184, isolated from an *E. coli* clinical isolate.

The $bla_{TEM-184}$ gene was detected in *E. coli* isolated from abdominal drainage of an elderly patient who had undergone abdominal surgery for a relapsing colonic adenocarcinoma. The patient had received amoxicillin-clavulanate for 24 h as surgical prophylaxis. After 5 days, the patient developed a surgical-site infection, yielding an *E. coli* isolate resistant to fluoroquinolones and cefotaxime but susceptible to ceftazidime, aztreonam, and carbapenems (isolate CT-Eco1, not stored), and was treated with ceftazidime (2 g t.i.d.). After initial improvement, fever and signs of infection relapsed, and an *E. coli* isolate that was also resistant to ceftazidime and aztreonam (isolate CT-Eco2) was isolated from the drainage discharge (Table 1). CT-Eco2 was positive for ESBL production by combo-disk test with cefotaxime and clavulanate (3). Genotyping by multilocus sequence typing (MLST) analysis using the Warwick schema (http://mlst.warwick.ac.uk) showed that the isolate belonged to sequence type 354 (ST354), a lineage that has been sporadically reported as responsible for invasive human infections and in association with β-lactamase production (NDM-5 and CMY-2 enzymes) (4,

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TABLE 1 Antimicrobial susceptibility of CT-Eco2 and *E. coli* BL21(DE3) with and without $bla_{TEM-184}$ gene

Species	Isolate name	Genotype	MIC (mg/liter)[a]													
			CTX	CAZ	FEP	AZT	CAZ-AVI	AZT-AVI	PIP	IMI	MEM	ERT	AK	GM	CIP	PTZ
E. coli	CT-Eco2	CTX-M-14+TEM-184	64	>128	>32	>64	≤1	[b]	>128	0.5	0.25	0.12	1	0.5	>2	>256
E. coli	BL21(DE3)	pLB-II-TEM-184	1	128	4	64	0.25	0.12	>128	0.12	0.25	0.12	1	0.5	0.12	0.5
E. coli	BL21(DE3)	pLB-II	≤0.06	≤0.06	≤0.06	0.5	≤0.06	0.5	0.5	0.12	0.12	0.12	1	0.12	0.12	0.5

[a]MICs were determined using reference broth microdilution (17). Category classification was based on EUCAST clinical breakpoints (http://www.eucast.org/clinical_breakpoints). CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; CAZ-AVI, ceftazidime-avibactam (avibactam at fixed concentration of 4 mg/liter); AZT-AVI, aztreonam-avibactam (avibactam at fixed concentration of 4 mg/liter); PIP, piperacillin; IMI, imipenem; MEM, meropenem; ERT, ertapenem; AK, amikacin; GM, gentamicin; CIP, ciprofloxacin; PTZ, piperacillin-tazobactam (tazobactam at fixed concentration of 4 mg/liter).
[b]Not tested.

5). Analysis of β-lactamase genes by PCR and sequencing revealed that CT-Eco2 carried a $bla_{CTX-M-14}$ gene and a bla_{TEM} gene that encoded a new TEM variant, i.e., TEM-184 (6). Compared with TEM-1, TEM-184 carried five amino acid modifications, including Q6K, E104K, I127V, R164S, and M182T, which were previously detected in other TEM variants but never in this combination.

The $bla_{TEM-184}$ gene was amplified by PCR from CT-Eco2 using the specific primers TEM-for 5′-GGGGGCATATGATGAGTATTCAACATTTCCGT-3′ and TEM-rev 5′-GGGGGGGATCCTTACCAATGCTTAATCAGTGA-3′. The amplicon, after digestion with NdeI and BamHI (restriction sites underlined), was cloned in the pET-9(a) (Agilent Technologies, Santa Clara, CA) and pLB-II (7) vectors to yield recombinant plasmids pET-TEM-184 and pLB-TEM-184, respectively.

The TEM-184 enzyme was purified from a stationary-phase culture of *E. coli* BL21(DE3)/pET-TEM-184 grown aerobically at 37°C in 1.2 liters of tryptic soy broth containing kanamycin (50 μg/ml) as follows. Cells were collected by centrifugation (8,900 × *g* for 10 min), resuspended in 30 ml of 20 mM Tris-HCl, pH 7.4, and disrupted by sonication (5 times for 60 s each time at 60 W, on ice). After ultracentrifugation at 105,000 × *g* for 1 h, the cleared supernatant was loaded onto a Q-Sepharose FF column (bed volume, 100 ml) equilibrated with 20 mM Tris-HCl buffer (pH 7.4) and eluted with a linear NaCl gradient (0 to 1 M) in 20 mM Tris-HCl buffer (pH 7.4). The β-lactamase-containing fractions were pooled, concentrated by Centricon (cutoff, 10 kDa), and loaded on a Superdex 200 gel filtration column (XK 16/40; GE Healthcare, Milan, Italy) equilibrated with sodium-phosphate buffer 25 mM, pH 7.0 (buffer A). The eluted β-lactamase-containing fractions (5 ml) were collected, and the enzyme preparation was estimated to be >95% pure by SDS-PAGE (data not shown). Kinetic parameters were determined at 25°C in buffer A using a Lambda 25 spectrophotometer (Perkin-Elmer, Monza, Italy) as described previously (8). The K_m, k_{cat}, and K_i values were calculated using the equations reported by De Meester et al. (9).

In SDS-PAGE, the purified TEM-184 enzyme showed a molecular mass of 28.7 kDa. The isoelectric point, determined by isoelectric focusing, was 5.8.

TEM-184 was able to efficiently hydrolyze penicillins, ceftazidime, and aztreonam, which was one of the best substrates. Lower catalytic efficiencies, due to lower k_{cat} and in some cases higher K_m values, were detected for cefotaxime, cefazolin, and cefepime (Table 2). Clavulanic acid, tazobactam, and avibactam were overall good inhibitors of TEM-184, with K_i values of 0.24, 0.38, and 0.59 μM, respectively. Table 2 compares the K_m, k_{cat}, and k_{cat}/K_m values of TEM-184 with those of TEM-87 (E104K, R164C, M182T), TEM-107 (E104K, R164H, M182T, G238S), and TEM-149 (E104K, R164S, M182T, E240V) ESBLs (10–12), with which TEM-184 showed some amino acid similarities (Fig. 1). Compared with TEM-87 and TEM-149, TEM-184 showed higher K_m values for ceftazidime, cefepime, and piperacillin. TEM-184 showed higher k_{cat} and k_{cat}/K_m values for aztreonam than those observed for TEM variants reported in Table 2. Other TEM variants (TEM-63, TEM-131, TEM-177, TEM-205, TEM-211) have similar amino acid substitutions, but kinetic constants are not available.

Expression of TEM-184 in *E. coli* BL21(DE3), transformed with recombinant plasmid pLB-TEM-184, conferred resistance to piperacillin, ceftazidime, and aztreonam. On the

TABLE 2 Kinetic parameters of TEM-184 compared with TEM-1, TEM-10, TEM-87, TEM-107, and TEM-149 ESBLs

	TEM-184 (Q6K, E104K, I127V, R164S, M182T)[a]			TEM-1[b]			TEM-10[b] (R164S, E240K)			TEM-87[c] (E104K, R164C, M182T)			TEM-107[d] (E104K, R164H, M182T, G238S)			TEM-149[e] (E104K, R164S, M182T, E240V)		
Substrate	K_m (μM)[f]	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
Cefazolin	98 ± 8	4	0.04	513	200	0.39	417	12.5	0.03	54 ± 2	13	0.24	NA	NA	NA	68 ± 5	2.2	0.03
Cefotaxime	44 ± 3	2	0.04	6,000	9.0	1.5×10^{-3}	30	0.3	0.01	53 ± 3	2.2	0.04	22	3	0.14	43 ± 2	0.07	1.6×10^{-3}
Ceftazidime	333 ± 18	53	0.16	4,280	0.3	0.7×10^{-4}	607	17	0.03	94 ± 5	23	0.24	86	15	0.17	19 ± 1	8.3	0.44
Cefepime	400 ± 25	4	0.01	NA[f]	NA	NA	NA	NA	NA	23 ± 1	4.4	0.19	26	3	0.11	16 ± 1	2.4	0.15
Aztreonam	21 ± 1.5	13	0.62	1,430	1.0	6.9×10^{-4}	28	2	0.07	50 ± 1	5.3	0.11	17	2	0.12	51 ± 3	4.7	0.09
Piperacillin	192 ± 15	27	0.14	NA	NA	NA	NA	NA	NA	14 ± 1	70	5.0	3	11	3.67	1.4[g] ± 0.3	2.7	1.92
Benzylpenicillin	68 ± 7	24	0.35	25	1,600	64.0	2	51	25.50	6.2 ± 0.5	39	6.29	NA	NA	NA	0.6[g] ± 0.05	3.5	5.80
Carbenicillin	29 ± 2	22	0.76	NA	NA	NA	NA	NA	NA	1.5 ± 0.5	6.7	2.24	NA	NA	NA	NA	NA	NA
Nitrocefin	190 ± 10	1,470	7.74	55	930	16.9	47	59	1.25	16 ± 2	31	1.94	5	72	14.4	20 ± 2	7.4	0.37

[a]Each kinetic value is the mean of three different measurements; the error was below 10%.
[b]Data from Raquet et al. (18).
[c]Data from Perilli et al. (10).
[d]Data from Lee et al. (11).
[e]Data from Perilli et al. (12).
[f]NA, not available.
[g]K_m was calculated as K_i using nitrocefin as reporter substrate (9).

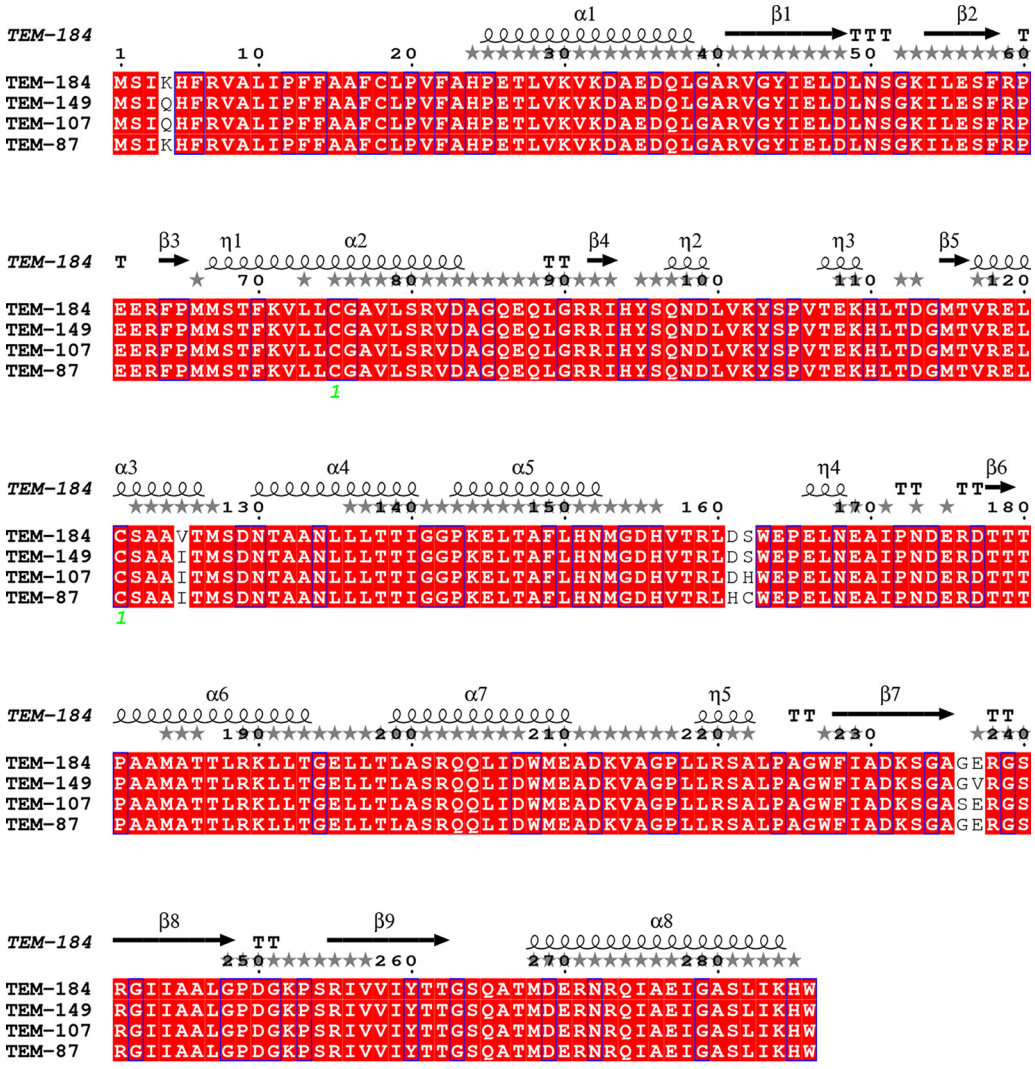

FIG 1 Sequence alignment of TEM-184 with sequences of TEM-87 (accession no. AF250872), TEM-107 (accession no. AY101764), and TEM-149 (accession no. DQ369751). Top, secondary structure annotation of TEM-1 (PDB-ID 1M40). Arrows, β-strands; spirals, α-helices; red boxes, conserved position; red letters, similar residues. Aligned sequence was made using EsPrit (v.3.0).

other hand, the strain was intermediate to cefepime and remained susceptible to cefotaxime and β-lactam/β-lactamase inhibitor combinations, including piperacillin-tazobactam, ceftazidime-avibactam, and aztreonam-avibactam. MIC values of TEM-184 were similar to those of TEM-87 and TEM-149, with high values for piperacillin, ceftazidime, and aztreonam and low values for cefepime.

With the exception of I127V, all substitutions in TEM-184 were well described in TEM-type ESBLs. Residue Q6K in the signal peptide may have a role in efficient protein secretion across the membrane (13). Residue K104 seems to improve the catalytic activities of ESBLs against ceftazidime and aztreonam (14). Residue 164 is located in the omega loop and usually makes a salt bond and a hydrogen bond with D179; when the arginine is replaced by serine, the omega loop becomes more flexible because of the elimination of the electrostatic attraction between residues 164 and D179, allowing for better accommodation of bulky β-lactam substituents (14). The M182T mutation acts as a global suppressor of β-lactamase substitution that stabilizes the enzyme (15). This substitution has been identified in both ESBLs and inhibitor-resistant enzymes, and, when it is present in combination with R164S, it exerts a positive effect on TEM variants. Residue I127V was found in TEM-80 (also called IRT-24), which is resistant to

inhibitors but also carries the M69L and N276D substitutions (16). In fact, as demonstrated by Arpin et al. (16), the substitution of I127V alone does not affect the inhibitor-resistant profile of the enzyme. Amino acid 127 is located at the end of the α3 helix (Fig. 1), and it is close to lysine 73. The lack of a methyl group at this position in valine 127 might result in a conformational alteration of the active site, which, in TEM-184, may increase the ability to hydrolyze aztreonam but is deleterious toward cefepime, cefotaxime, and cefazolin. It would be interesting to further test this hypothesis by site-directed mutagenesis experiments.

Accession number(s). The sequence for TEM-184 has been deposited in the GenBank database under accession number FR848831.

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We have no conflicts of interest to declare.

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