ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2011, p. 1262–1265 Vol. 55, No. 3
0066-4804/11/$12.00 doi:10.1128/AAC.01359-10

Characterization of the Novel CMT Enzyme TEM-154▽

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Received 5 October 2010/Returned for modification 5 December 2010/Accepted 8 December 2010

TEM-154, identified in Portugal in 2004, associated the substitutions observed in the extended-spectrum β-lactamase (ESBL) TEM-12 and in the inhibitor-resistant penicillinase (IRT) TEM-33. This enzyme exhibited hydrolytic activity against ceftazidime and a low level of resistance to clavulanic acid. Surprisingly, the substitution Met69Leu enhanced the catalytic efficiency of oxyimino β-lactams conferred by the substitution Arg164Ser. Its discovery confirms the dissemination of the complex mutant group of TEM enzymes in European countries.

TEM-type β-lactamases are the most prevalent mechanism of resistance to β-lactam antibiotics in *Enterobacteriaceae*. Among these β-lactamases, extended spectrum β-lactamases (ESBL) and inhibitor-resistant penicillinases (IRT) have emerged from the parental penicillinases TEM-1 and TEM-2 (2). Since the 1990s, another subgroup of enzymes that combine the substitutions observed in the ESBL and in the IRT appeared; its members were designated complex mutant TEM (CMT) (21). Nine different CMT, mainly in *E. coli* strains, have been described: TEM-50, TEM-68, TEM-89, TEM-121, TEM-109, TEM-125, TEM-151, TEM-152, and TEM-158, most of which have been identified in France (7, 14, 16–21).

In 2004, during a 2-year study of ESBL-producing *Enterobacteriaceae* from different Portuguese clinical settings (12), Machado et al. identified a novel TEM enzyme that associated the Arg164Ser substitution observed in the ESBL TEM-12 (4) with the Met69Leu substitution observed in TEM-33 (IRT-5) (3). This β-lactamase, designated TEM-154, was produced by an *Escherichia coli* strain, H258, which is resistant to penicillins (alone or associated with clavulanic acid), ceftazidime, all tested aminoglycosides except amikacin, all tested quinolones, sulfamethoxazole, tetracycline, and chloramphenicol.

In this study, we characterized the genetic support and the enzymatic activity of TEM-154. *E. coli* H258 produces only one β-lactamase, which has a pI of 5.3. The French double-disk synergy test and CLSI MIC testing confirmed the production of an ESBL by this strain (5, 6). The TEM-154-harboring plasmid did not transfer in mating experiments (19). However, an *E. coli* DH5α transformant harboring the parental phenotype of resistance to β-lactams was obtained throughout electroporation of plasmid DNA. Plasmid DNAs were extracted from the clinical strain and transformant by the method of Kado and Liu (8). Plasmid size was determined by comparison with those of plasmids Rsa (39 kb), TP114 (61 kb), pCFF04 (85 kb), and pCFF14 (180 kb), as previously described (15). Plasmid content analysis revealed one plasmid of about 180 kb. TEM-specific PCR and sequencing experiments were performed on the clinical strain H258 and on the transformant as previously described (17) and confirmed the presence of $bla_{\text{TEM-154}}$. This gene harbored a pattern of silent mutations identical to those of $bla_{\text{TEM-1a}}$ and a promoting sequence, P_a/P_b (11).

E. coli DH5α clones producing TEM-154, TEM-12, TEM-33, and TEM-1 were obtained using the vector pBK-CMV, as previously described (17). The correct orientation of the different inserts was checked by PCR and sequencing using the primers pBK-CMV1′ (5′CTAGTGGATCCAAGAATTCAAAAAGC3′) and pBK-CMV2′ (5′AATTGGGTACACTTACCTGGTACCC3′). Direct sequencing of both strands was performed on two independent PCR products, which were obtained from the clinical strain *E. coli* H258 and from the different *E. coli* DH5α clones. The MICs for the clinical strain and the different *E. coli* DH5α clones are presented in Table 1. *E. coli* DH5α clones, which produced the enzyme TEM-154 and its parental enzymes (TEM-12, TEM-33, and TEM-1) from the same genetic background, were used to compare microbiologically these related TEM-encoding genes, as previously described (17). *E. coli* H258 and the corresponding clone had a high level of resistance to penicillins similar to that of the clones producing TEM-12, TEM-33, and TEM-1 (512 to >2,048 μg/ml). The MICs of cephalothin, cefuroxime, cefoxitin, cefotaxime, and imipenem were similar for all *E. coli* DH5α clones. Only the TEM-154 and TEM-12 enzymes, which harbored ESBL-type mutations, conferred a high level of resistance to ceftazidime (32 to 512 μg/ml). However, ceftazidime MICs were 2- to 4-fold higher for the TEM-154-producing *E. coli* clone than for the TEM-12-producing clone. Likewise, the MICs of cefepime and aztreonam for TEM-154-producing

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▽ Published ahead of print on 20 December 2010.

TABLE 1. MICs of β-lactam antibiotics for *E. coli* H258 and the *E. coli* recombinants DH5α(pBK-TEM-154), DH5α(pBK-TEM-12), DH5α(pBK-TEM-33), DH5α(pBK-TEM-1), and DH5α(pBK-CMV)

β-Lactam(s)[a]	MIC (μg/ml) for *E. coli* strain:						
	H258 (pH258-TEM-154)	DH5α (pBK-TEM-154)	DH5α (pBK-TEM-12)	DH5α (pBK-TEM-33)	DH5α (pBK-TEM-1)	DH5α (pBK-CMV)	DH5α
Amoxicillin	>2,048	>2,048	>2,048	>2,048	>2,048	4	4
Amoxicillin + CLA	>1,024	>1,024	64	512	16	4	4
Ticarcillin	>2,048	>2,048	>2,048	>2,048	>2,048	2	2
Ticarcillin + CLA	1,024	>1,024	32	128	32	2	2
Piperacillin	1,024	>2,048	>2,048	512	512	2	2
Piperacillin + TZB	16	8	2	4	2	2	2
Cephalothin	8	8	8	4	4	4	4
Cefuroxime	8	8	8	4	4	4	4
Cefoxitin	2	4	4	4	4	4	4
Cefotaxime	0.06	0.25	0.12	0.06	0.06	0.06	0.06
Cefotaxime + CLA	0.06	0.03	0.06	0.06	0.06	0.06	0.06
Ceftazidime	64	512	32	0.25	0.12	0.12	0.12
Ceftazidime + CLA	1	8	0.5	0.12	0.12	0.12	0.12
Aztreonam	2	1	4	0.06	0.12	0.12	0.12
Aztreonam + CLA	0.25	0.12	0.12	0.06	0.12	0.06	0.06
Cefepime	1	2	1	0.06	0.06	0.06	0.06
Cefepime + CLA	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Imipenem	0.12	0.12	0.25	0.12	0.25	0.25	0.25

[a] CLA, clavulanic acid at 2 μg/ml; TZB, tazobactam at 4 μg/ml.

E. coli were close to those of the TEM-12-producing clone (1 to 2 μg/ml) and higher than those for the TEM-1- and TEM-33-producing *E. coli* DH5α clones (0.06 to 0.12 μg/ml).

Clavulanic acid did not restore the susceptibility of TEM-154-producing *E. coli* to penicillins, and the MICs of penicillin-clavulanic acid combinations were even higher than those observed for the TEM-33-producing clone (1,024 to >1,024 versus 128 to 512 μg/ml). Clavulanic acid decreased the MICs of oxyimino β-lactams for both TEM-154- and TEM-12-producing strains, but the ceftazidime-clavulanic acid MIC remained in the intermediate range for the TEM-154-producing clone, probably because of its high level of resistance to ceftazidime (512 versus 32 to 64 μg/ml). Tazobactam restored the susceptibility to piperacillin of the TEM-154-producing strains, as was observed for the other TEM-producing clones.

TEM-33, TEM-12, and TEM-154 were overproduced in *E. coli* BL21(DE3), as previously described (17). The different TEM enzymes were purified to homogeneity, and the level of purity was estimated to be >95% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1, 10). Matrix-assisted laser desorption–time of flight mass spectra (MALDI-TOF MS) were acquired on a Voyager DE-PRO mass spectrometer (ABSciex, les Ulis, France) equipped with a delayed-extraction MALDI source and a pulsed nitrogen laser (337 nm). The purified proteins were resuspended in 5 mM ammonium hydrogen carbonate. Sinapinic acid was used as a matrix, and mass was measured using a peptide mixture (trypsinogen, enolase, bovine serum albumin; LaserBio Labs, Sophia-Antipolis, France) as an external standard as previously described (13). MALDI-TOF MS values (28,882.0 Da, 28,830.6 Da, and 28,815.6 Da) were in excellent agreement with the calculated molecular masses of the mature proteins (28,888.9 Da, 28,837.9 Da, and 28,819.9 Da, respectively). Their kinetic parameters (Table 2) were determined by computerized microacidimetry as previously described (9) and compared with those previously determined for TEM-1 (21). The 50% inhibitory concentrations (IC_{50}s) were determined, as previously described (21), with 100 μM benzylpenicillin as the reporter substrate, 1 nM enzyme, and 10 min of incubation (complete inactivation).

TEM-154 had 1.8- to 72-fold-higher k_{cat} values against penicillins than TEM-12 and TEM-33. However, its activity against all these substrates except ticarcillin was lower than that of the parental penicillinase, TEM-1. TEM-154 K_m values against benzylpenicillin and amoxicillin were closer to those of TEM-33 and TEM-1 than to those of TEM-12, whereas its K_m values against piperacillin and ticarcillin were the highest of the four enzymes.

TEM-154 hydrolytic activity against cephalothin was close to that of the ESBL TEM-12 and was lower than that of TEM-1. The K_m value for this substrate was 3- to 4-fold lower than that of the three other enzymes. However, its K_m decrease was probably too low to increase the cephalothin MIC for the TEM-154-producing clone.

Surprisingly, TEM-154 displayed hydrolytic activity close to that of TEM-12 against cefotaxime and aztreonam and even greater activity against ceftazidime and had lower K_m values for these substrates. Overall, TEM-154 had higher catalytic efficiency than the ESBL TEM-12 against cefuroxime, cefotaxime, ceftazidime, and aztreonam, suggesting that the IRT-type substitution Met69Leu enhanced the activity against oxyimino β-lactams conferred by the substitution Arg164Ser. These enhanced catalytic efficiencies probably explained the MIC discrepancies of cefotaxime and ceftazidime observed for the clones which produced TEM-154 and TEM-12. However, the enhancement observed for cefuroxime was probably too low to increase the MIC for the TEM-154-producing strain.

Finally, TEM-154 exhibited higher susceptibility to clavulanic acid than TEM-33 and was less susceptible than TEM-12 and TEM-1 (IC_{50}, 0.67 versus 0.02, 0.08, and 1.8 μM). TEM-154 was as susceptible to tazobactam as TEM-12 and TEM-1 (IC_{50}, 0.14 versus 0.13 and 0.13 μM).

TEM-154 was the 10th member of the complex mutant sub-

TABLE 2. Kinetic parameters of β-lactamases TEM-154, TEM-12, TEM-33, and TEM-1[a]

β-Lactam	TEM-154			TEM-12			TEM-33			TEM-1[b]		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Benzylpenicillin	357	25	14.3	80	7	11	150	15	10	1200	25	48
Amoxicillin	196	21	9.3	60	7.5	8	111	23	5	920	26	35.4
Ticarcillin	143	90	1.6	19	12	1.6	2	7	0.2	115	10	11.5
Piperacillin	465	64	7.3	89	15	6	83	15	6	987	45	21.9
Cephalothin	42	79	0.5	46	327	0.1	5	317	0.01	122	250	0.5
Cefuroxime	1.2	16	0.08	1.6	81	0.02	<0.1	ND	ND	<0.1	ND	ND
Ceftazidime	39	166	0.2	11	254	0.04	<0.1	ND	ND	<0.1	ND	ND
Cefotaxime	4.9	83	0.06	11	320	0.03	<0.1	ND	ND	1.2	ND	ND
Aztreonam	2.5	56	0.04	2	247	0.008	<0.1	ND	ND	<0.1	ND	ND

[a] ND, not determined. The standard deviations for the results of the analysis were ≤15%.
[b] Data are from the work of Sirot et al. (21)

group of the TEM β-lactamases. It combined hydrolytic activity against oxyimino β-lactams and decreased susceptibility to clavulanic acid. Compared with the other CMT enzymes, TEM-154 had a catalytic efficiency against ceftazidime close to that of TEM-121 (CMT-4) and TEM-109 (CMT-5) and higher than that of the other CMT-type enzymes (k_{cat}/K_m, 0.2 versus 0.2, 0.27, and 0.009 to 0.07 $\mu M^{-1} \cdot s^{-1}$) (13, 15–20). Such efficiency was similar to those of ESBL ceftazidimases such as TEM-6 and TEM-28 (k_{cat}/K_m, 0.4 and 0.27 $\mu M^{-1} \cdot s^{-1}$) (18, 19).

TEM-154 was slightly less resistant to clavulanate than IRT enzymes and conferred a high level of resistance to penicillin-clavulanate acid combinations. However, this resistance level did not make it difficult to identify it as an ESBL, unlike with the CMT-type enzymes TEM-121, TEM-125, TEM-152, and TEM-158 (16–18, 20). After the previous reports of CMT enzymes in France and in Poland (7, 14, 16–21), the discovery of this novel CMT in a Portuguese clinical setting confirms the emergence of this subgroup of TEM β-lactamases in Europe.

Nucleotide sequence accession number. The GenBank accession number for $bla_{TEM-154}$ is FJ807656.

We thank Marlene Jan and Rolande Perroux for their technical assistance and Sophie Quevillon-Cheruel for providing the modified pET9a plasmid.

This work was supported in a part by grants from the Ministère de l'Enseignement Supérieur et de la Recherche (JE2526), the INRA (USC2018), the Centre Hospitalier Régional Universitaire de Clermont-Ferrand, France, and the Ministère de la Santé, de la Jeunesse et des Sports.

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