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Characterization of TEM-56, a Novel β-Lactamase Produced by a *Klebsiella pneumoniae* Clinical Isolate

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TEM-56 produced by a *Klebsiella pneumoniae* clinical isolate is a novel β-lactamase of isoelectric point 6.4 that confers a moderate resistance level to expanded-spectrum cephalosporins. The amino acid sequence deduced from the corresponding *bla* gene showed two amino acid replacements with respect to the TEM-2 sequence: Glu-104 to Lys and His-153 to Arg. This enzyme showed catalytic properties close to those of TEM-18. Thus, TEM-56 appears as a new TEM mutant, an intermediary between TEM-18 and the extended-spectrum β-lactamase TEM-21.

In Dijon University Hospital, Dijon, France, *Klebsiella pneumoniae* isolates with decreased susceptibility to cephalosporins or aztreonam are submitted to epidemiological studies performed by analytical isoelectric focusing and pulsed-field gel electrophoresis (8, 14, 17). Over a 2-year period (1995 to 1996), 22 strains producing a β-lactamase of pI 6.4 were isolated. Analysis of chromosomal DNA by pulsed-field gel electrophoresis revealed that the 22 strains were closely related according to Tenover's criteria (24). For 21 of them, a double-disk synergy test (11) was positive between clavulanic acid and cefotaxime, whereas it was considered negative for strain Kp 395. The MICs for β-lactams of strains of the first group were similar, whereas those for the last strain differed. Kp 395 showed high MICs for penicillins and cephalothin, but the MICs were low for expanded-spectrum cephalosporins and aztreonam and similar to those seen for *Escherichia coli* strains producing TEM-18 (Table 1). Conversely, MICs for these agents against Kp 377 (one representative strain of the first group) were comparable to those for the TEM-3-producing strain. Clavulanic acid potentiated expanded-spectrum cephalosporins and aztreonam, but the MICs of penicillins remained high.

For Kp 395 and Kp 377, a plasmid of 120 kb was extracted by the method of Birnboim and Doly (4), but all conjugation experiments carried to transfer resistance failed. Transformation experiments using *E. coli* HB 101 or *E. coli* DH_{5a} competent cells failed, too. PCR analysis was performed as described previously (16) on plasmid DNA extracted from both strains with primers J (forward, 5′-CTTATTCCCTTTTTTGCGGC-3′) and E (reverse, 5′-GGTCTGACAGTTACCAATGC-3′) (6) at positions 236 and 1079 of the TEM family gene β-lactamase, respectively, with numbering according to Sutcliffe (23). The promoter region was amplified with the following primers: GOV1 (forward, 5′-ATAAAATTCTTGAAGACGAAA-3′) (16) and SIE2 (reverse, 5′-AAAACTCTCAAGGATCTTAC C-3′) (this study) at positions −5 and 380, respectively. PCR products were sequenced with an Applied Biosystems 373A sequencer according to the manufacturer's instructions. Analysis of the sequences (Table 2) revealed that the two bla_{TEM} genes were derived from bla_{TEM-2}. Moreover, their promoter regions are identical to that of bla_{TEM-2}.

The deduced amino acid sequences of the enzymes produced by Kp 377 and Kp 395 are reported in Table 3. The en-

TABLE 1. MICs for *K. pneumoniae* clinical isolates Kp 395 and Kp 377 and *E. coli* strains producing TEM-18, TEM-2, or TEM-3

Strain or β-lactamase produced	MIC (μg/ml)[a]							
	AMX	TIC	PIP	CFT	FOX	ATM	CTX	CAZ
K. pneumoniae								
Kp 395 (TEM-56)	>8,192 (1,024)	>8,192 (1,024)	>2,048 (16)	512	4 (4)	1 (0.25)	0.25 (≤0.06)	4 (2)
Kp 377 (TEM-21)	>8,192 (32)	>8,192 (16)	1,024 (4)	256	4 (4)	16 (0.12)	128 (≤0.06)	32 (1)
E. coli								
TEM-18	8,192 (512)	1,024 (64)	512 (16)	16	8 (8)	2 (1)	0.25 (0.12)	4 (2)
TEM-3	8,192 (16)	8,192 (32)	256 (2)	128	4 (4)	8 (0.5)	64 (0.12)	32 (0.5)
TEM-2	4,096 (16)	4,096 (32)	512 (2)	32	2 (2)	≤0.06 (≤0.06)	≤0.06 (≤0.06)	0.5 (0.25)

[a] AMX, amoxicillin; TIC, ticarcillin; PIP, piperacillin; CFT, cephalothin; FOX, cefoxitin; ATM, aztreonam; CTX, cefotaxime; CAZ, ceftazidime. The values in parentheses are MICs in the presence of 2 μg/ml of clavulanic acid.

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TABLE 2. Nucleotide substitutions in bla_{TEM-2}, bla_{TEM-21}, and bla_{TEM-56} genes

Region and nucleotide position[a]	Nucleotide in:		
	bla_{TEM-2}	bla_{TEM-21}	bla_{TEM-56}
Promoter region			
32*[b]	T	T	T
175*	A	A	A
Coding region			
226*	C	C	C
317*	A (Lys-39[c])	A	A
346*	G	G	G
436*	T	T	T
512	G (Glu-104)	A (Lys[d])	A (Lys)
604*	G	G	G
660	A (His-153)	G (Arg)	G (Arg)
682*	C	C	C
914	G (Gly-238)	A (Ser)	G
925*	A	A	A

[a] Nucleotide numbering is according to that given by Sutcliffe (23) and amino acid numbering is according to Ambler et al. (1).
[b] Nucleotides marked with an asterisk are involved in the differences between bla_{TEM-2}, bla_{TEM-1A}, and bla_{TEM-1B} genes (9, 10, 23).
[c] Gln for TEM-1.
[d] For TEM-21 and TEM-56, there are indicated amino acids differing from TEM-2.

zyme produced by Kp 377 was TEM-21 of pI 6.4 (Lys-39, Lys-104, Arg-153, and Ser-238) (2, 3, 25) and that produced by Kp 395 was a new enzyme: TEM-56 of pI 6.4 also (Lys-39, Lys-104, and Arg-153). It appears that TEM-18 (Lys-39 and Lys-104) (R. Labia and D. Sirot, personal communication), TEM-56, and TEM-3 (Lys-39, Lys-104, and Ser-238) are intermediate mutants between TEM-2 and TEM-21. Within the TEM family, Arg-153 has been found only in TEM-21 and TEM-56.

β-Lactamases TEM-2, TEM-18, TEM-56, TEM-3, and TEM-21 were purified to homogeneity according to previously described methods using ammonium sulfate precipitation, ion-exchange chromatography, and size exclusion chromatography (7, 15). Kinetic constants (Table 3) were determined by computerized microacidimetry (13), at pH 7 and 37°C, in distilled water containing 85 mM NaCl. TEM-56 hydrolyzed ceftriaxone and cefotaxime moderately, but the action on cefuroxime and ceftazidime was hardly detectable, a situation very similar to that of TEM-18. Concerning penicillins, the kinetics of TEM-56 and TEM-18 were comparable to those of TEM-2. TEM-21 and TEM-3 had considerable activity for expanded-spectrum cephalosporins and low K_m values for penicillins. TEM-56, TEM-21, TEM-18, and TEM-3 enzymes are susceptible to β-lactamase inhibitors and, in some instances, more so than native TEM-2 (Table 4). Aztreonam was a poor substrate for all tested β-lactamases (data not shown).

TEM-18 and TEM-56 gave similar and moderate decreases in susceptibility levels of cefotaxime, ceftazidime, and aztreonam. On the contrary, TEM-21 and TEM-3 conferred a high level of resistance to expanded-spectrum cephalosporins. This indicates that the Glu-104 to Lys amino acid substitution enables the mutant to hydrolyze these cephalosporins but not enough to confer a true resistance, as already noticed in the variants obtained by site-directed mutagenesis (18, 20, 22, 27). Conversely, the substitution of Gly-238 for Ser has a key role, as already demonstrated (5, 21, 26). Concerning the His-153 for Arg amino acid substitution, comparison of TEM-56 and TEM-18, on one hand, and TEM-21 and TEM-3, on the other hand, shows that this substitution does not significantly modify the hydrolytic properties of the enzyme, as also shown by enzyme kinetics.

Comparison of a large set of class A β-lactamase sequences (12, 19) shows that residue 153 is not conserved, but the most frequently encountered is arginine. The role of the His-153 for Arg amino acid substitution remains unclear. Often, extended-spectrum or inhibitor-resistant TEM-derived β-lactamases may present one or a few secondary amino acid substitutions whose roles are not always elucidated. Within presently reported TEM mutant β-lactamases, about 10 positions have been described (http://www.lahey.org/studies/webt.htm).

It is noteworthy that the first of the 22 strains producing an enzyme of pI 6.4 was Kp 395, producing TEM-56. This strain was isolated from a patient who had received multiple β-lactam therapies, and TEM-56 was detected only once. Then Kp 377, producing TEM-21, was isolated from a patient in the same ward. This suggests that bla_{TEM-21}, which differs from bla_{TEM-56} by a single base at position 914, was selected under antibiotic pressure in vivo. Then the strain spread in the ward and in other departments subsequent to patients' transfers.

TABLE 3. Comparative kinetic parameters of β-lactamase TEM-2 and its mutants

Drug	Kinetic parameters of[a]:														
	TEM-2			TEM-18			TEM-56			TEM-3			TEM-21		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
Benzylpenicillin	1,500	18	83	1,400	24	58	1,400	22	63	45	6	7.5	50	9	5.5
Amoxicillin	1,200	20	60	1,300	41	31.7	1,330	40	33	31	5	6.2	43	20	2.15
Ticarcillin	195	12	16.2	280	6	47	300	7	43	9	2	4.5	7	3	2.3
Piperacillin	1,275	40	31.8	1,360	40	34	1,350	38	35	72	12	6	46	12	3.8
Cephalothin	300	350	0.86	225	135	1.7	250	140	1.8	48	23	2.1	18	55	0.33
Cephaloridine	825	800	1.0	1,360	1,200	1.1	1,350	1,200	1.1	43	30	1.4	120	120	1.0
Cefoperazone	700	230	3.0	550	170	3.2	550	180	3.0	28	20	1.4	19	30	0.63
Cefuroxime	3	1,000	<0.01	10	480	0.02	8	470	0.02	28	90	0.31	18	85	0.21
Ceftriaxone	<1	ND	<0.01	21	800	0.03	16	840	0.02	81	80	1	53	210	0.25
Cefotaxime	1.5	ND	<0.01	35	1,660	0.02	28	1,700	0.02	202	100	2	120	400	0.3
Ceftazidime	<1	ND	<0.01	<1	ND	<0.01	<1	ND	<0.01	18	200	0.1	4	ND	<0.01

[a] k_{cat} values are given in per second units, K_m values are given in micromolar units, and k_{cat}/K_m values are given in per micromole per second units. The values given for k_{cat} and K_m were computed on the basis of at least three independent experiments. ND, not detected (high K_m or K_i values).

TABLE 4. 50% inhibitory concentrations (IC_{50}s) of β-lactamase inhibitors for β-lactamase TEM-2 and its mutants

β-Lactamase	IC_{50} (μM) of:		
	Clavulanic acid	Sulbactam	Tazobactam
TEM-2	0.16	8	0.1
TEM-18	0.007	4.2	0.1
TEM-56	0.008	4.3	0.1
TEM-3	0.01	0.4	0.01
TEM-21	0.01	2	0.07

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