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Ceftazidime-Resistant *Klebsiella pneumoniae* and *Escherichia coli* Isolates Producing TEM-10 and TEM-43 β-Lactamases from St. Louis, Missouri

YOUJUN YANG,[1]* NIRAJA BHACHECH,[1] PATRICIA A. BRADFORD,[1] BRADLEY D. JETT,[2] DANIEL F. SAHM,[2]† AND KAREN BUSH[1]‡

Wyeth-Ayerst Research, Pearl River, New York,[1] and Barnes-Jewish Hospital, Washington University School of Medicine, St. Louis, Missouri[2]

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Ceftazidime-resistant *Escherichia coli* and *Klebsiella pneumoniae* (49 and 102 isolates, respectively) were collected from Barnes-Jewish Hospital, St. Louis, Mo., from 1992 to 1996. They were uniformly resistant to ceftazidime, generally resistant to aztreonam, and variably susceptible to cefotaxime. Four representative *E. coli* strains and 15 *Klebsiella* strains were examined. From one to four β-lactamases were produced per strain, with three possible enzymes related to ceftazidime resistance: enzymes with pI values of 5.6, 6.1, or 7.6. By pulsed-field gel electrophoresis there were at least 13 different *Klebsiella* strain types and 3 different *E. coli* strain types, indicating that the outbreak was not clonal. After cloning and sequencing of the β-lactamase-encoding genes, the enzyme with a pI of 5.6 was identified as TEM-10. The enzyme with a pI of 6.1 was a novel TEM variant (TEM-43) with Lys at 104, His at 164, and Thr at 182. TEM-43 showed broad-spectrum hydrolytic activity against all penicillins, with the highest hydrolysis rate for ceftazidime compared to those for the other expanded-spectrum cephalosporins. Aztreonam was also a good substrate for TEM-43, with hydrolytic activity similar to that of ceftazidime and affinity higher than that of ceftazidime. The TEM-43 β-lactamase was well inhibited by clavulanic acid and tazobactam at concentrations of <10 nM. Sulbactam was less effective than the other inhibitors. The Thr182 mutation previously reported in an inhibitor-resistant β-lactamase did not cause the TEM-43 enzyme to become resistant to any of the inhibitors.

Extended spectrum β-lactamases (ESBLs) exhibit an enhanced ability to hydrolyze the expanded-spectrum β-lactams such as cefotaxime and ceftazidime. These enzymes are usually encoded by multidrug resistance plasmids which are readily transferable and which may carry genes encoding resistance to other antibiotics such as aminoglycosides (6, 22). Many ESBLs are related to TEM- and SHV-type enzymes with one or more amino acid alterations by single- or multiple-step mutations (10). When first reported in the literature, ESBL-producing organisms were found in nosocomial isolates from large metropolitan hospitals. Today, ESBLs have been identified worldwide, not only from the major teaching hospitals but also from community hospitals and nursing homes (4, 13, 27). The rapid spread of ESBLs has caused significant threats to the therapy for infections and usage of the expanded-spectrum β-lactams.

Since 1992 the Clinical Microbiology Laboratory of Barnes-Jewish Hospital in St. Louis, Mo., has used the antimicrobial susceptibility profiles obtained with members of the family *Enterobacteriaceae* to identify potential ESBL-producing isolates. Strains for which the β-lactamase inhibitors sulbactam or clavulanic acid enhanced expanded-spectrum cephalosporin activity were classified as presumptive ESBL producers. From 1992 to 1996, 49 *Escherichia coli* isolates and 102 *Klebsiella pneumoniae* isolates putatively producing ESBLs were encountered. These represented approximately 1 and 4% of all clinical *E. coli* and *K. pneumoniae* isolates from this center, respectively. Prior to this study the only ESBL reported from St. Louis was TEM-10, produced in a *Proteus mirabilis* isolate in a different St. Louis hospital, although one presumed ESBL-producing *K. pneumoniae* isolate was suspected in that same hospital (20).

In this study 15 representative *K. pneumoniae* isolates and 4 *E. coli* isolates from the Barnes-Jewish Hospital were examined for β-lactamase production. Several presumptive ESBL-producing isolates were identified on the basis of their resistance profiles. Genes encoding commonly produced enzymes from this group of isolates were cloned and sequenced; one of these enzymes was identified as a novel ESBL, TEM-43, containing mutations previously identified in both ESBLs and inhibitor-resistant TEMs. The biochemical and genetic properties of TEM-43 are presented in this report.

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MATERIALS AND METHODS

Bacterial strains. Clinical strains of *K. pneumoniae* (n = 49) and *E. coli* (n = 102) were isolated from patients at Barnes-Jewish Hospital. Isolates 125, 156, 166, and 179, which produced a single β-lactamase with a pI of 5.6 or a pI of 6.1, were selected for further study (see Table 1).

Antibiotics and susceptibilities. The antibiotics used in this study were obtained from their respective manufacturers, as indicated: benzylpenicillin, ampicillin, aztreonam, and cephaloridine, from E. R. Squibb & Sons, Princeton, N.J.; piperacillin and tazobactam, from Lederle Laboratories, Pearl River, N.Y.; ticarcillin and clavulanic acid, from SmithKline Beecham, Worthing, England; cefoperazone and sulbactam, from Pfizer, Groton, Conn.; cefoxitin and imipenem, from Merck, Rahway, N.J.; cefuroxime and ceftazidime, from Glaxo, Inc., Greenford, United Kingdom; cefotaxime, from Hoechst-Roussel, Somerville, N.J.; ceftriaxone, from Roche Laboratories, Nutley, N.J.; cefazolin, cephalothin, cefamandole, and moxalactam, from Eli Lilly, Indianapolis, Ind.; cephalothin, carbenicillin, oxacillin, and cloxacillin, from Sigma Chemicals Co., St.

* Corresponding author. Mailing address: 205/227, Infectious Disease Research, Wyeth-Ayerst, 401, North Middletown Rd., Pearl River, NY 10965. Phone: (914) 732-4466. Fax: (914) 732-2480. E-mail: Yangy@war.wyeth.com.

† Present address: MRL Pharmaceutical Services Inc., Reston, Va.

‡ Present address: R. W. Johnson Pharmaceutical Research Institute, Raritan, N.J.

Louis, Mo.; and nitrocefin, from BBL, Cockeysville, Md. All antibiotic solutions were prepared fresh before use. Antibiotic susceptibility was determined by serial twofold dilution in Mueller-Hinton II agar with an inoculum of 10^{-4} CFU/spot according to the criteria established by National Committee for Clinical Laboratory Standards (18). The synergy tests were performed with a 2-to-1 ratio of drug to inhibitor for amoxicillin-clavulanate and ampicillin-sulbactam and with constant concentrations of 2 and 4 μg/ml for clavulanate and tazobactam, respectively, in the ticarcillin-clavulanate and piperacillin-tazobactam tests.

Initial identification of the clinical isolates. Disc diffusion tests for each ceftazidime-resistant isolate were performed by a standard protocol (18). Pulsed-field gel electrophoresis (PFGE) was performed on a CHEF Mapper (Bio-Rad) by a standard procedure (15).

Crude β-lactamase extraction and IEF. The β-lactamases of the clinical isolates were extracted from 100 ml of a Trypticase soy broth culture by five cycles of freezing and thawing followed by centrifugation. The β-lactamase activity and pIs were determined by isolectric focusing (IEF) electrophoresis at 10°C on a Multiphor apparatus (LKB-Pharmacia) with prepared PAG plates (pH range, 3.5 to 9.5; Pharmacia). TEM-1, TEM-2, K1, SHV-1, and P99 were used as standards. TEM-10 and TEM-28 were used for comparison. Enzymes were detected after the gel was overlaid with nitrocefin (16).

Nucleic acid techniques. All standard recombinant DNA techniques were performed as described by Sambrook et al. (28). Restriction enzymes, calf intestinal phosphate, T4 DNA ligase, and materials for PCR were obtained from New England Biolabs (Beverly, Mass.) and Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and were used as directed by the manufacturers. To clone the enzyme with a pI of 6.1, plasmid DNA was prepared from clinical isolate 156 by the Qiagen column method according to the manufacturer's directions (Qiagen Inc., Chatsworth, Calif.). This purified DNA was used as a template for PCR amplification with *Taq* polymerase. One cycle of denaturation was followed by 30 cycles of annealing, extension, and denaturation and a final cycle of annealing and prolonged extension. The primers used for PCR were kindly provided by B. Rasmussen and were designed to amplify the TEM-1 mature protein-coding sequence with an *Eco*RI site incorporated into the amino terminus of the primer and a *Bam*HI site incorporated into the carboxy terminus of the primer to facilitate cloning. The PCR fragment was cloned into pCLL2300, a kanamycin resistance-conferring cloning vector (25), and the resultant plasmid (pCLL3416) was transformed into *E. coli* DH5α (25). Double-stranded DNA analysis was done with a Sequenase kit (United States Biochemicals) with [^{35}S]dATP. A set of nested primers complementary to the TEM-coding sequence (26) was selected so as to avoid overlap with known mutations in the TEM gene. For the enzyme with a pI of 5.6, plasmid was prepared from clinical isolate 166 by alkaline lysis (1), digested with *Bgl*II, and cloned into pCLL2300. The resultant plasmid (pCLL3421) was transformed into *E. coli* DH5α. Sequencing was performed as described previously (30).

Purification of TEM-43 β-lactamase. *E. coli* DH5α(pCLL3416), which contained the gene encoding TEM-43, was used to isolate enzyme for further biochemical characterization. Crude enzyme extracts were obtained by five cycles of freezing-thawing from 4 liters of the logarithmic-phase cells. β-Lactamase extracts were recovered in the supernatant after centrifugation. The enzyme was first chromatographed to Sephadex G-75 equilibrated in 50 mM phosphate buffer (pH 7.0). The eluate containing β-lactamase activity was dialyzed against 20 mM phosphate buffer at pH 5.8 and was then chromatographed on a CM-C50 cation-exchange column (25 by 1.5 cm) equilibrated with the dialyzing buffer. The enzyme was eluted from the column by stepwise elution (200 ml) at pH 6.5. The purity of TEM-43 β-lactamase was examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 12% acrylamide and Bio-Rad low-molecular-weight markers (molecular weights, 10,000 to 100,000) as standards. Proteins were stained with Coomassie brilliant blue. IEF was also performed to confirm the presence of only TEM-43 by using nitrocefin as the staining reagent. The bicinchoninic acid assay (Pierce Biochemicals, Rockford, Ill.) was used to determine the protein concentrations of the enzyme preparations.

The pI 5.6 enzyme was partially purified from the transformant *E. coli* DH5α(pCLL3421) by single-step Sephadex G75 chromatography. The relative maximum hydrolysis rates and K_m values of this enzyme for selective β-lactams were determined spectrophotometrically.

Enzyme kinetics and inhibition studies. The kinetic parameters of the purified TEM-43 enzyme were determined with a Beckman DH7400 or a Gilford 250 spectrophotometer. Values of K_m and V_{max} were determined as described previously (31). At least two independent kinetic evaluations were performed for each substrate, with the initial velocities obtained for six to eight substrate concentrations. Calculations were performed with the computer program ENZPACK (Biosoft; Elsevier). The average values of the kinetic parameters for each substrate were reported, with the standard deviation for each value being less than 25%. Inhibition studies were performed as follows. Enzyme and inhibitor were preincubated in a volume of 50 μl for 10 min at 25°C before the addition of 100 μM nitrocefin in 50 mM phosphate buffer (final volume, 1,000 μl). The concentration of inhibitor that inhibits 50% of the enzyme activity (IC_{50}) was determined graphically.

Nucleotide sequence accession number. The nucleotide sequence of bla_{TEM-43} is registered in GenBank as accession no. U95363.

RESULTS

Epidemiology. The Clinical Microbiology Laboratory of Barnes-Jewish Hospital in St. Louis used ceftazidime, ceftriaxone, aztreonam, and cefotetan susceptibility profiles to identify 49 *E. coli* and 102 *K. pneumoniae* strains producing presumptive ESBLs from 1992 to 1996. Any *E. coli* or *K. pneumoniae* isolates failing to exhibit susceptibility by disk diffusion (i.e., resistant or intermediate) to either ceftazidime, ceftriaxone, or aztreonam were tested by a double-disk method to determine the effect of sulbactam or clavulanic acid on the resistance profile. Strains for which β-lactamase inhibitors enhanced cephalosporin activity were classified as presumptive ESBL producers. The profiles obtained with the non-β-lactams tested against a collection of these isolates exhibited substantial heterogeneity and indicated that multiple strains of ESBL producers were being encountered (11). Approximately 1 and 4% of all clinical *E. coli* and *K. pneumoniae* isolates, respectively, were identified as ESBL producers.

Susceptibility tests and initial strain identification. Representative isolates of ceftazidime-resistant *E. coli* and *K. pneumoniae* were examined by PFGE, disc diffusion susceptibility testing, and IEF. The PFGE analysis indicated that among the 15 isolates of *K. pneumoniae*, 3 strains were identical, 2 strains gave a second pattern, and 10 strains had unique patterns (data not shown). For the three *E. coli* isolates tested, two strains were related but not identical and one was unique. These data indicated that the ceftazidime outbreak described above was not clonal.

The disc susceptibility tests indicated that all isolates were uniformly resistant to the penicillins tested and to ceftazidime (Table 1). All isolates were susceptible to imipenem. Susceptibilities to cefotaxime, cefoxitin, and aztreonam varied among the isolates. Tazobactam was able to restore the piperacillin susceptibilities of 14 of 19 isolates and change a resistant designation to intermediate for the other 5 isolates. Clavulanic acid restored the ampicillin susceptibilities of 5 of the 19 isolates, with an intermediate category for the other 14 isolates. Six of the isolates remained resistant to the ampicillin-sulbactam combination.

IEF with crude extracts indicated that these isolates produced one to four types of β-lactamases per strain. On the basis of the susceptibility and IEF results, the possible identities of these isolates were predicted (Table 1). Among these strains, four produced a single enzyme. Isolates 125 and 166 produced β-lactamases with a pI of 5.6, identical to that produced by TEM-10 or TEM-26. Isolates 156 and 179 produced a β-lactamase that focused at pH 6.1, which aligned with TEM-28. These four isolates were further studied by β-lactamase gene sequencing and biochemical characterization of purified enzymes.

Genetic analysis of TEM-43 β-lactamase gene. *E. coli* 156, containing a novel β-lactamase with a pI of 6.1, was selected for further molecular and biochemical characterization. This enzyme was cloned and expressed in *E. coli* DH5α(pCLL3416) by PCR amplification. DNA analysis of the gene cloned from strain 156 showed this β-lactamase with a pI of 6.1 to be a derivative of TEM-1 encoded on transposon Tn*2*. The amino acid sequences derived from the nucleic acid data indicated that this enzyme was novel and differed from TEM-1 by three amino acids: lysine for glutamate at position 104, histidine for arginine at position 164, and threonine for methionine at position 182. Table 2 indicates the amino acid substitutions of several TEM-type β-lactamases. The predicted molecular size from the sequence was 28,860, consistent with the size observed by SDS-PAGE.

TABLE 1. Profiles of St. Louis isolates

Strain	Organism	Kirby-Bauer susceptibility zone diameters (mm)[a]										Presence of β-lactamases with the following pIs:						Possible identity of β-lactamase(s)
		AMP	AMC	SAM	PIP	PTZ	CAZ	CTX	ATM	FOX	IPM	5.4	5.6	6.1	7.6	8.7	9.0	
114	*K. pneumoniae*	6	18	14	6	22	8	17	6	23	27	X			X		X	TEM-1 or TEM-7; SHV-1, SHV-2, SHV-4, or SHV-7; chromosomal
117	*K. pneumoniae*	6	14	8	6	18	6	22	6	14	26	X	X		X	X		TEM-1 or TEM-7; TEM-2, TEM-10, or TEM-26; SHV-1, SHV-2, SHV-4, SHV-6, or SHV-7; AmpC type
119	*K. pneumoniae*	6	18	10	6	23	8	28	13	23	30	X						TEM-7 (?)
122	*K. pneumoniae*	6	16	17	6	25	10	23	13	13	26		X		X	X		TEM-2, TEM-10, or TEM-26; SHV-1, SHV-2, SHV-4, SHV-6, or SHV-7; chromosomal
123	*K. pneumoniae*	6	15	6	6	18	6	23	10	13	28	X	X		X	X		TEM-1 or TEM-7; TEM-2, TEM-10, or TEM-26; SHV-1; AmpC type
125	*K. pneumoniae*	6	16	13	6	18	6	23	9	17	27		X					TEM-10 or TEM-26
149	*E. coli*	6	14	19	6	25	8	23	14	24	21			X				TEM-6, TEM-28, or TEM-43
154	*E. coli*	14	20	19	15	25	26	29	32	12	32						X	AmpC type
156	*E. coli*	6	15	15	6	25	6	14	6	17	30			X				TEM-43
162	*Klebsiella oxytoca*	6	16	14	6	22	6	23	10	23	27			X		X		TEM-6, TEM-28, or TEM-43; chromosomal
164	*K. pneumoniae*	6	17	10	6	19	8	16	7	15	27	X						TEM-7 (?)
166	*E. coli*	6	17	18	6	25	13	28	20	21	25		X					TEM-10
171	*K. pneumoniae*	6	16	17	6	23	9	23	16	23	24			X		X		TEM-6, TEM-28, or TEM-43; chromosomal
179	*K. pneumoniae*	6	15	16	6	22	6	24	11	22	26			X				TEM-6, TEM-28, or TEM-43
181	*K. pneumoniae*	6	17	13	6	21	10	6	14	18	26	X			X	X		TEM-1 or TEM-7; SHV-1, SHV-2, SHV-4, or SHV-6; chromosomal
185	*K. pneumoniae*	6	16	9	6	18	11	14	11	24	26	X			X	X		TEM-1 or TEM-7; SHV-1, SHV-2, SHV-4, SHV-6, or SHV-7; chromosomal
193	*K. pneumoniae*	6	20	18	6	24	10	25	17	25	25			X	X			TEM-6, TEM-28, or TEM-43; SHV-1
196	*K. pneumoniae*	6	15	17	6	22	6	23	13	24	27			X	X			TEM-6, TEM-28, or TEM-43; SHV-1
201	*K. pneumoniae*	6	16	15	6	22	6	21	15	19	28			X	X			TEM-6, TEM-28, or TEM-43; SHV-1, SHV-2, SHV-4, SHV-6, or SHV-7

[a] Disk diffusion tests were performed by a standard method, and the results were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (17, 18). Abbreviations: AMP, ampicillin, AMC, amoxicillin clavulanic acid; SAM, ampicillin plus sulbactam; TIC, ticarcillin, TIM, ticarcillin plus clavulanic acid; PIP, piperacillin, PTZ, piperacillin plus tazobactam; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam, IMP, imipenem; FOX, cefoxitin.

TABLE 2. Antimicrobial susceptibilities of representative isolates producing a single β-lactamase

Strain	Organism	pI of enzyme	MIC (μg/ml)[a]											
			AMP	AMC	SAM	TIC	TIM	PIP	PTZ	CAZ	CTX	ATM	IPM	FOX
125	*K. pneumoniae*	5.6	>128	16	32	>128	128	>128	16	>128	1	64	0.125	16
166	*E. coli*	5.6	>128	16	16	>128	32	32	2	32	0.25	16	0.125	16
156	*E. coli*	6.1	>128	16	32	>128	>128	>128	4	>128	32	>128	0.5	4
179	*K. pneumoniae*	6.1	>128	16	16	>128	128	>128	4	128	1	32	0.125	2
DH5α(pCLL2300)	*E. coli*	None	4	4	4	4	4	2	1	0.25	≤0.12	≤0.12	0.25	4
DH5α(pCLL3416)	*E. coli*	6.1	>128	16	8	>128	8	128	0.5	32	0.25	4	0.5	8

[a] Abbreviations: AMP, ampicillin; AMC, amoxicillin plus clavulanic acid; SAM, ampicillin plus sulbactam, TIC, ticarcillin; TIM, ticarcillin plus clavulanic acid; PIP, piperacillin; PTZ, piperacillin plus tazobactam; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; IPM, imipenem; FOX, cefoxitin.

The nucleotide sequence indicated that the β-lactamase with a pI of 5.6 produced by isolate 166 was identical to TEM-10 (3). The relative hydrolysis rates of the partially purified pI 5.6 β-lactamase showed the same pattern as that for TEM-10 (data not shown).

The antimicrobial susceptibilities of the TEM-43-producing clinical isolate 156 and the DH5α(pCLL3416) clone are compared in Table 2. The introduction of the TEM-43 gene into *E. coli* DH5α(pCLL3416) resulted in a greater than twofold increase in the MICs of penicillins, ceftazidime, and aztreonam. The MICs for the other three isolates producing a single β-lactamase are also listed in Table 2 for comparison. Strain 156 was more resistant to cefotaxime and aztreonam than the other clinical isolates. All strains were susceptible to imipenem (MICs, <0.5 μg/ml). In combination with the β-lactamase inhibitors, the activities of ampicillin and piperacillin against the clinical isolates were improved at least fourfold, as were their activities against the TEM-43 clone DH5α(pCLL3416).

Biochemical characterization of TEM-43. SDS-PAGE demonstrated that the TEM-43 β-lactamase was purified to 93% homogeneity by two steps of chromatography. IEF demonstrated that the purified TEM-43 enzyme focused at pH 6.1 on the IEF gel stained with nitrocefin. No other contaminating β-lactamase band was observed. TEM-43 was a broad-spectrum β-lactamase with strong hydrolytic activity against all penicillins and cephalosporins tested (Table 3). Benzylpenicillin, piperacillin, cephaloridine, and nitrocefin were good substrates, with strong hydrolytic activities shown by the enzyme. The k_{cat} value for piperacillin was close to that for benzylpenicillin. Among the expanded-spectrum cephalosporins tested, ceftazidime was a better substrate for TEM-43 than the other expanded-spectrum cephalosporins. However, TEM-43 had a lower affinity for ceftazidime than for the other expanded-spectrum cephalosporins. Although the hydrolysis rates for cefuroxime, ceftriaxone, and cefoperazone were lower, TEM-43 had a higher affinity for these drugs (lower K_m values; Table 3). Therefore, the catalytic efficiencies for all of these expanded-spectrum β-lactamases were similar. Aztreonam was also a good substrate for TEM-43, with a k_{cat} similar to that for ceftazidime and a higher affinity than that for ceftazidime. Imipenem and cefoxitin were poor substrates for TEM-43, with relative hydrolytic rates for imipenem and cefoxitin being less than 0.001% of that for benzylpenicillin. The TEM-43 β-lactamase was inhibited by all three β-lactamase inhibitors tested. Clavulanic acid and tazobactam were much better inhibitors (IC_{50}s, 3.4 and 6.7 nM respectively) than sulbactam (IC_{50}, 106 nM), consistent with the observations for other TEM-derived ESBLs (3, 4).

DISCUSSION

Ceftazidime-resistant *E. coli* and *K. pneumoniae* strains were isolated from Barnes-Jewish Hospital in St. Louis. The number of presumed ESBL-producing isolates has been large, compared to data recorded from other single centers in the United States (3, 24), but the proportion of ESBL-producing isolates among all isolates remains <5%.

TEM-43 showed a hydrolytic profile similar to those of other TEM variants originating from the United States, such as TEM-10, TEM-12, and TEM-26 (3, 19). All these enzymes exhibit hydrolytic activity against ceftazidime as well as aztreonam. Like TEM-10, TEM-26, and TEM-28, cefotaxime was hydrolyzed by TEM-43 but at a lower level. TEM-43 had a significantly better affinity (lower K_m) for cephaloridine, cefotaxime, and aztreonam than the TEM-1 parent enzyme. However, the K_m of TEM-43 for cephaloridine was increased compared to those of the other related ESBLs. Imipenem and cefoxitin were poor substrates (Table 3) for TEM-43, as expected for the TEM-derived ESBLs.

The substitution profile of TEM-43 is similar to that of TEM-6; both of these ESBLs have the same amino acid changes at positions 104 and 164 (14, 29). Both of these en-

TABLE 3. Kinetic parameters for TEM-43 β-lactamase

Antibiotic	k_{cat} (s^{-1})	Relative k_{cat}	K_m (μM)	k_{cat}/K_m mM^{-1} s^{-1}	Relative k_{cat}/K_m
Benzylpenicillin	240	100	26	9,200	100
Ampicillin	49	20	13	3,800	41
Carbenicillin	26	11	39	670	7.3
Piperacillin	190	79	120	1,600	17
Oxacillin	18	7.5	21	860	9.3
Cloxacillin	6.4	2.7	16	400	4.3
Cephalothin	26	11	55	470	5.1
Cephaloridine	170	71	120	1,400	15
Nitrocefin	120	50	44	2,700	29
Cefuroxime	7.5	3.1	16	470	5.1
Cefotaxime	6.9	2.8	33	210	2.3
Ceftriaxone	2.8	1.2	10	280	3.0
Ceftazidime	43	18	160	270	2.9
Cefoperazone	3.0	1.3	7.6	390	4.2
Cefoxitin	0.02	<0.001	ND[a]	ND	ND
Imipenem	0.02	<0.001	ND	ND	ND
Aztreonam	42	18	57	740	8.0

[a] ND, not determined. The hydrolysis rate was too slow to obtain an accurate K_m.

zymes are members of the His164-substituted TEM variants, a growing family of ESBLs that is less numerous than extended-spectrum TEMs with Ser164 substitutions. When TEM-6 was evaluated for hydrolysis characteristics in our laboratory (5), the hydrolytic profile was similar to that for TEM-43. However, aztreonam was hydrolyzed, with TEM-6 having a relative V_{max} that was approximately 15% of the observed hydrolysis rate for ceftazidime. This is in contrast to TEM-43, which hydrolyzes both aztreonam and ceftazidime with identical k_{cat} values.

The unique ESBL substitution in TEM-43 is the threonine-for-methionine substitution at position 182. This change at position 182 has been reported for an inhibitor-resistant TEM-type β-lactamase, TEM-32, (7) and for two ESBLs, TEM-20 and TEM-52 (23). In a study with laboratory-derived TEM mutants (7), substitution of only Thr for Met at position 182 gave a TEM enzyme with increased hydrolytic properties for penicillins and the early cephalosporins; no data on the hydrolysis of expanded-spectrum β-lactams were provided.

TEM-32 additionally has an isoleucine-for-methionine substitution at position 69 (this substitution has been shown to be the dominant factor for inhibitor resistance [12]) but no changes from TEM-1 at positions 104 and 164. Assuming that the Ile69 substitution rather than the Thr182 substitution is fully responsible for inhibitor resistance, as shown by Farzaneh et al. (7), it was expected that the TEM-43 β-lactamase would be susceptible to inhibition by the classic β-lactamase inhibitors. It was also reported that the hydrogen bond between the hydroxyl of Thr182 and the carbonyl of Glu64 was expected to be responsible for the increase in catalytic activity of TEM-32 (21). Although the Met182Thr substitution at position 182 was identified for TEM-43, as it was in the inhibitor resistant TEM-32 β-lactamase, resistance to the inhibitors was not observed.

Substitution of the threonine at position 182 has recently been identified in the TEM β-lactamases as a global suppressor (9). This substitution was shown to restore enzymatic activity to TEM variants with multiple mutations and provide additional protein stability. The Thr182 substitution alone may not convey a selective enzymatic advantage to the wild-type TEM-1 enzyme with respect either to the ability to be inhibited or to the ability to hydrolyze poor substrates. However, good substrates may be hydrolyzed somewhat more efficiently (7). It is possible that the differences between the relative hydrolysis profiles for ceftazidime and aztreonam with TEM-6 and TEM-43 may be related to the Thr182 mutation. Huang and Palzkill (9) hypothesized that the Thr182 mutation, considered to be a relatively "neutral" mutation, may allow more destabilizing mutations to occur to respond to antibiotic pressure without compromising the ability of the β-lactamase to function. It is quite likely that similar amino acid substitutions that appear to have no noticeable effect on enzymatic function will be identified. Because some of these neutral substitutions may not alter the pI or the phenotypic response to standard antibiotics, they will remain covert until a second mutation occurs. It is expected that these kinds of mutants already exist among the normal distribution of TEM-producing isolates. These may be identified only when additional ESBLs or inhibitor-resistant TEM variants are sequenced.

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REFERENCES

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
2. **Blazquez, J., M. Baquero, R. Caton, I. Alos, and F. Baquero.** 1993. Characterization of a new TEM-type β-lactamase resistant to clavulanate, sulbactam, and tazobactam in a clinical isolate of *Escherichia coli*. Antimicrob. Agents Chemother. **37:**2059–2063.
3. **Bradford, P. A., C. Cherubin, V. Idemyor, B. Rasmussen, and K. Bush.** 1994. Multiply resistant *Klebsiella pneumoniae* strains from two Chicago hospitals: identification of the extended-spectrum TEM-12 and TEM-10 ceftazidime-hydrolyzing β-lactamases in a single isolate. Antimicrob. Agents Chemother. **38:**761–766.
4. **Bradford, P. A., C. Urban, A. Jaiswal, N. Mariano, B. Rasmussen, S. Projan, J. J. Rahal, and K. Bush.** 1995. SHV-7, a novel cefotaxime-hydrolyzing β-lactamase, identified in *Escherichia coli* isolates from hospitalized nursing home patients. Antimicrob. Agents Chemother. **39:**899–905.
5. **Bradford, P. A., N. Jacobus, N. Bhachech, and K. Bush.** 1996. TEM-28 from an *Escherichia coli* clinical isolate is a member of the His-164 family of TEM-1 extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **40:**260–262.
6. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
7. **Farzaneh, S., E. B. Chaibe, J. Peduzzi, M. Barthelemy, R. Lambia, J. Blazquez, and F. Baquero.** 1996. Implication of Ile-69 and Thr-182 residues in kinetic characteristics of IRT-3 (TEM-32) β-lactamase. Antimicrob. Agents Chemother. **40:**2434–2436.
8. **Henquell, C., C. Chanal, D. Sirot, R. Labia, and J. Sirot.** 1995. Molecular characterization of nine different types of mutants among 107 inhibitor-resistant TEM β-lactamases from clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **39:**427–430.
9. **Huang, W., and T. Palzkill.** 1997. A natural polymorphism in β-lactamase is a global suppressor. Proc. Natl. Acad. Sci. USA **94:**8801–8806.
10. **Jacoby, G. A., and A. A. Medeiros.** 1991. More extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **35:**1697–1704.
11. **Jett, B. D., D. J. Ritchie, R. Reichley, T. C. Bailey, and D. F. Sahm.** 1995. In vitro activity of various β-lactam antimicrobial agents against clinical isolates of *Escherichia coli* and *Klebsiella* spp. resistant to oxyimino cephalosporins. Antimicrob. Agents Chemother. **39:**1187–1190.
12. **Knox, J. R.** 1995. Extended-spectrum and inhibitor resistant TEM-type β-lactamases: mutations, specificities, and three-dimensional structure. Antimicrob. Agents Chemother. **39:**2593–2601.
13. **Livermore, D. M., and M. Yuan.** 1996. Antibiotic resistance and production of extended-spectrum β-lactamases amongst *Klebsiella* spp. from intensive care units in Europe. J. Antimicrob. Chemother. **38:**409–424.
14. **Mabilat, C., and P. Courvalin.** 1990. Development of "oligotyping" for characterization and molecular epidemiology of TEM β-lactamases in members of the family *Enterobacteriaceae*. Antimicrob. Agents Chemother. **34:**2210–2216.
15. **Maslow, J. N., A. M. Slutshy, and R. D. Arbeit.** 1993. Application of pulsed-field gel electrophoresis to molecular epidemiology, p. 563–572. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic molecular microbiology principles and applications. American Society for Microbiology, Washington, D.C.
16. **Matthew, M., A. M. Harris, M. Marshall, and G. W. Ross.** 1975. The use of analytical isoelectric focusing for detection and identification of β-lactamases. J. Gen. Microbiol. **88:**169–178.
17. **National Committee for Clinical Laboratory Standards.** 1997. Performance standards for antimicrobial disk susceptibility tests. Approved standard M2-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.
18. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility test for bacterial that grow aerobically. Approved standard M7-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
19. **Naumovski, L., J. P. Quinn, D. Miyashiro, M. Patel, K. Bush, S. B. Singer, D. Graves, T. Palzkill, and A. M. Arvin.** 1992. Outbreak of ceftazidime resistance due to a novel extended-spectrum β-lactamase in isolates from cancer patients. Antimicrob. Agents Chemother. **36:**1991–1996.
20. **Palzkill, T., K. S. Thomason, C. S. Sanders, E. S. Moland, W. Huang, and T. W. Milligan.** 1995. New variant TEM-10 β-lactamase gene produced by clinical isolate of *Proteus mirabalis*. Antimicrob. Agents Chemother. **39:**1199–1200.
21. **Papanicolaou, G. A., A. Mederios, and G. A. Jacoby.** 1990. Novel plasmid-mediated β-lactamase (MIR-1) conferring resistance to oxymino- and α-methoxy β-lactams in clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **34:**2200–2209.
22. **Philippon, A., R. Labia, and G. Jacoby.** 1989. Extended spectrum β-lactamases. Antimicrob. Agents Chemother. **33:**1131–1136.
23. **Poyart, C., P. Mugnier, G. Quesne, P. Berche, and P. Trieu-Cuot.** 1998. A novel extended-spectrum TEM-type β-lactamase (TEM-52) associated with decreased susceptibility to moxalactam in *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **42:**108–113.
24. **Quinn, J. P., D. Miyashiro, D. Sahm, R. Flamm, and K. Bush.** 1989. Novel plasmid-mediated β-lactamase (TEM-10) conferring selective resistance to ceftazidime and aztreonam in clinical isolates of *Klebsiella pneumoniae*. An-

timicrob. Agents Chemother. **33:**1451–1456.
25. **Rasmussen, B. A., Y. Gluzman, and F. P. Tally.** 1990. Cloning and sequencing of the class B β-lactamase gene (*ccrA*) from *Bacteroides fragilis* TAL3636. Antimicrob. Agents Chemother. **34:**1590–1592.
26. **Rasmussen, B. A., P. A. Bradford, J. P. Quinn, J. Wiener, R. A. Weinstein, and K. Bush.** 1993. Genetically diverse ceftazidime-resistant isolates from a single center: biochemical and genetic characterization of TEM-10 β-lactamases encoded by different nucleotide sequences. Antimicrob. Agents Chemother. **37:**1989–1992.
27. **Rice, L. B., E. C. Eckstein, J. DeVente, and D. M. Shlaes.** 1996. Ceftazidime-resistant *Klebsiella pneumoniae* isolates recovered at the Cleveland Department of Veterans Affairs Medical Center. Clin. Infect. Dis. **23:**118–124.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sowek, J. A., S. B. Singer, S. Ohringer, M. F. Malley, T. J. Dougherty, J. Z. Gougoutas, and K. Bush.** 1991. Substitution of lysine at position 104 or 240 of TEM-1_{pTZ18R} β-lactamase enhances the effect of serine-164 substitution on hydrolysis or affinity for cephalosporins and the monobactam aztreonam. Biochemistry **30:**3179–3188.
30. **Sutcliffe, J. G.** 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. USA **75:**3737–3741.
31. **Yang, Y., and K. Bush.** 1996. Biochemical characterization of the carbapenem-hydrolyzing β-lactamase AsbM1 from *Aeromonas sobria* AER 14M: a member of a novel subgroup of metallo-β-lactamases. FEMS Microbiol. Lett. **137:**193–200.
32. **Yang, Y., N. Bhachech, P. A. Bradford, B. D. Jett, D. F. Sahm, and K. Bush.** 1996. Ceftazidime-resistant *Klebsiella pneumoniae* and *E. coli* isolates producing TEM-10 and TEM-43 β-lactamases from St. Louis, abstr. C-25, p. 38. *In* Program and abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.