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A New TEM-Derived Extended-Spectrum β-Lactamase (TEM-91) with an R164C Substitution at the Ω-Loop Confers Ceftazidime Resistance

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A new plasmid-mediated TEM-derived extended-spectrum β-lactamase, TEM-91, was identified in a ceftazidime-resistant (MIC, >128 μg per ml) *Escherichia coli* strain isolated in 1996 in Japan. TEM-91 has three amino acid substitutions, R164C, M184T, and E240K, compared with TEM-1 penicillinase. The isoelectric point (pI), K_m, and k_{cat} of TEM-91 for ceftazidime were 5.7, 179 μM, and 29.0 s^{-1}, respectively. The K_i of clavulanic acid for ceftazidime hydrolysis was 30.3 nM.

Since broad-spectrum β-lactams, including expanded-spectrum cephalosporins, cephamycins, and carbapenems, are efficacious agents for the control of infectious diseases caused by gram-negative bacilli, the emergence and proliferation of such microorganisms that have acquired consistent resistance to the above-mentioned antimicrobial agents are becoming actual impediments in clinical settings (2, 3, 11). For instance, the worldwide proliferation of extended-spectrum-β-lactamase (ESBL)-producing clinical isolates belonging to the family *Enterobacteriaceae*, such as *Escherichia coli* and *Klebsiella pneumoniae*, is creating real problems in the provision of high-grade medical treatment, including organ transplantations, surgical operations, and chemotherapy for patients with malignancies (6, 7). More than 119 TEM-related β-lactamases, including inhibitor-resistant enzymes and TEM-derived ESBLs that hydrolyze oxyimino-cephalosporins and monobactams, have been recorded to date (12; Jacoby, G. A., and K. Bush, Amino acid sequences from TEM, SHV, and OXA extended-spectrum and inhibitor resistant β-lactamases [http://www.lahey.org/studies/webt.htm]). According to the report by the National Nosocomial Infections Surveillance system conducted by the Centers for Disease Control and Prevention in the United States, *K. pneumoniae* resistant to oxyimino-cephalosporins increased to >10% in intensive care units in 2001 (5), and most of these strains are speculated to be ESBL producers. In Japan, however, only a few TEM-derived ESBLs have been reported, although several *E. coli* and *K. pneumoniae* strains producing CTX-M-type enzymes, including Toho-1, or SHV-derived ESBLs, such as SHV-12, have been isolated in geographically separate medical institutions (9, 23, 24). In this study, a new TEM-derived ESBL, TEM-91, possessing three amino acid substitutions, R164C, M182T, and E240K, was characterized.

The ceftazidime (CAZ)-resistant *E. coli* strain HKY322 was isolated from a urine sample of a patient in 1996, and the MIC of CAZ for this strain was >128 μg per ml. The strain, however, was susceptible to other oxyimino-β-lactams, such as cefotaxime, as shown in Table 1. The CAZ resistance was transferred to *E. coli* CSH2 (F^- *metB* Na^r Rif^r) by conjugation analysis (10) concurrently with the transmission of a resident large plasmid. The inhibition tests with clavulanic acid and PCR analyses suggested that this strain produced a TEM-type enzyme. An *Eco*RI-digested DNA fragment carrying the gene for CAZ resistance in strain HKY322 was ligated with a cloning vector, pBCSK$^+$, and CAZ resistance was expressed in *E. coli* XL1-Blue cells by a transformation with the recombinant plasmids. The antibiotic susceptibility profiles of the parental strain HKY322 and the clone *E. coli* XL1-Blue(pBCTEM91) are shown in Table 1. Strain HKY322 showed resistance to CAZ, as well as to ampicillin and piperacillin, but did not show resistance to other oxyimino-cephalosporins, such as cefotaxime (MIC, 4 μg/ml) and ceftriaxone (MIC, 1 μg/ml) (Table 1).

Nucleotide sequencing analyses of both strands of the cloned DNA fragment were performed with an ABI Prism 377 DNA sequencer using the M13 universal primer and truncated mutants made with a deletion kit (Takara Co. Ltd., Kyoto, Japan). From cloning and sequencing analyses of the genetic determinant for CAZ resistance, strain HKY322 was found to produce a new TEM-derived ESBL, TEM-91. Four point mutations were found in the coding region of the bla_{TEM-91} gene compared with the bla_{TEM-1A} gene (EMBL accession number X54604), and these mutations caused three amino acid substitutions, R164C, M182T, and E240K, numbered according to the residue numbering scheme for class A β-lactamases by Ambler et al. (1) and Leflon-Guibout et al. (16) (Table 2).

The purification of TEM-91 from bacterial culture of *E. coli* XL1-Blue(pBCTEM91) was performed according to the methods described previously (14) with a HiLoad 16/60 Superdex 200 prepgrade column (Pharmacia Biotech, Uppsala, Sweden) preequilibrated with 50 mM Tris-HCl buffer (pH 8.0). Anionic-exchange chromatography was performed on a HiTrap Q HP column (Pharmacia Biotech) preequilibrated with the same buffer by using a high-performance liquid chromatography system (Pharmacia Biotech). Proteins were eluted with a linear gradient of 0 to 500 mM NaCl in the same buffer. Fractions with activity were pooled and concentrated with an Ultra-

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TABLE 1. Susceptibilities of *E. coli* HKY322 producing TEM-91 and transconjugant to β-lactams and β-lactam–β-lactamase inhibitor combinations

E. coli strain	MIC (μg/ml)[a]										
	AMP	CER	CAZ	CAZ-CLA 4	CTX	CTX-CLA 4	CPM	MOX	CMNX	ATM	IPM
HKY322	>128	128	>128	0.5	4	<0.5	16	2	<0.5	32	<0.5
XL1-Blue-(pBCTEM91)	128	64	64	<0.5	1	<0.5	2	1	1	8	<0.5
XL1-Blue	4	2	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

[a] Abbreviations: AMP, ampicillin; CER, cephaloridine; CAZ, ceftazidime; CLA, clavulanic acid; CTX, cefotaxime; CPM, cefpirome; MOX, moxalactam; CMNX, cefminox; ATM, aztreonam; IPM, imipenem. The number after "CLA" indicates concentration (micrograms per milliliter).

free-15 centrifugal filter device (Millipore Corporation, Bedford, Mass.). This elution process was repeated four times. Fractions with activity were then passed through the size exclusion and anion-exchange columns once again. The purified enzymes were used for subsequent β-lactamase assays. To determine the isoelectric point (pI), 10 μl of purified enzyme solution was loaded onto an Immobiline DryStrip (pHs 3 to 10; Pharmacia Biotech) with an IPGphor electrophoresis system (Pharmacia Biotech). The pI of TEM-91 was 5.7.

TEM-91 was assayed against various β-lactam substrates at 30°C in 50 mM phosphate buffer (pH 7.0) with an autospectrophotometer (model V-550; Nihon Bunko Ltd., Tokyo, Japan). The absorption maxima of the substrates used were as follows: for ampicillin, 235 nm; for aztreonam, 315 nm; for cefotaxime, 264 nm; for CAZ, 272 nm; and for cephaloridine, 295 nm. The molar extinction coefficients were calculated by the method of Seeberg et al. (20). K_m and k_{cat} values were obtained by a Michaelis-Menten plot of the initial steady-state velocities at different substrate concentrations. Inhibition studies were done at 30°C in 50 mM phosphate buffer (pH 7.0) with ampicillin or CAZ as substrates and clavulanate and sulbactam as inhibitors. Kinetic parameters of TEM-91 are shown in Table 3. CAZ was expected to have a relatively low affinity to the TEM-91 compared to those of cephaloridine and cefotaxime, but the relatively high hydrolytic velocity of TEM-91 for CAZ resulted in high hydrolysis of CAZ. However, TEM-91 hardly hydrolyzes cefotaxime, in contrast to TEM-72 (18), which has both the M182T and E240K substitutions. Moreover, TEM-91 showed low hydrolytic capacity (k_{cat}/K_m = 1.32×10^2) for aztreonam compared to that for CAZ, an infrequent occurrence in ESBLs, where aztreonam and CAZ hydrolysis are often closely associated. This enzyme was blocked effectively by clavulanic acid (K_i for CAZ, 30.3 nM).

R164H and R164S substitutions have been reported for several TEM-enzymes, such as TEM-5 and TEM-46. However, the substitution R164C found in TEM-91 is not common (http://www.lahey.org/studies/webt.htm). Only TEM-87 was reported to possess the R164C substitution, and this enzyme also hydrolyzes CAZ (17). Class A β-lactamases have a conserved structural domain Ω-loop consisting of amino acid residues R164 through D179, and this portion is fastened by a salt bridge across the side chains of R164 and D179 (12, 15, 21, 22, 23). It has also been suggested that steric hindrance of the tight Ω-loop structure toward the bulky 7β side chains of oxyiminocephalosporins results in the poor hydrolytic activity of TEM-1 penicillinase against these substrates (reference 23 and http://www.lahey.org/studies/webt.htm). From this perspective, it is reasonable to speculate that the hydrolytic activity of TEM-91 for CAZ may well depend on the enlargement of the active-site cavity through distortion of the Ω-loop structure as a result of the R164C substitution, which disrupts the salt bridge between R164 and D179 as was suggested for SHV-24 (14). The E240K substitution just near the active-site pocket of the enzyme would also reduce steric hindrance for bulky 7β functionality of CAZ as well as stabilization of the enzyme (19), and this might well result in the acceleration of the CAZ hydrolysis cycle of TEM-91. Actually, a high level of CAZ-hydrolyzing activity has been reported to occur in TEM-5 (CAZ-1), TEM-24 (CAZ-6), TEM-46 (CAZ-9), and TEM-61, which have E240K as well as the R164S or R164H substitution (http://www.lahey.org/studies/webt.htm).

The substitutions found at R164 are usually R164S and R164H and are caused by point mutations at nucleotide position 692 (CGT→AGT) and at nucleotide position 693 (CGT→CAT), respectively. The R164C substitution found in bla_{TEM-91}, however, is caused by a point mutation at nucleotide position 692 (CGT→TGT). Theoretically, the probability of occurrence of each point mutation should be the same among these three mutation types. The codon usage of UGU is not rare in *E. coli*, and molecular sizes of simple side chains of Ser (—CH_2 OH) and Cys (—CH_2 SH) are similar, while a long side chain [—$(CH_2)_3$ $C(NH_2)$—N^+H_2] of arginine is projected into the active-site pocket in wild-type class A β-lactamases. One might wonder, therefore, why only two enzymes, TEM-87 and TEM-91, have an R164C substitution among the TEM-derived enzymes reported, despite the fact that R164S and

TABLE 2. Silent mutations and amino acid substitutions found in TEM-91 compared with TEM-1A and TEM-1B

β-Lactamase	Codon (amino acid substitution, position) containing a mutation at position[a]:						
	209	226	436	604	692	747	917
TEM-91	**A**TG (M, 3)	TT**C** (F, 8)	GG**T** (G, 78)	GC**G** (A, 134)	**T**GT (C, 164)	A**C**G (T, 182)	**A**AG (K, 240)
TEM-1A	**A**TG (M, 3)	TT**C** (F, 8)	GG**C** (G, 78)	GC**G** (A, 134)	**C**GT (R, 164)	A**T**G (M, 182)	**G**AG (E, 240)
TEM-1B	**A**TG (M, 3)	TT**T** (F, 8)	GG**T** (G, 78)	GC**T** (A, 134)	**C**GT (R, 164)	A**T**G (M, 182)	**G**AG (E, 240)

[a] Standard numbering schemes for class A β-lactamases by Ambler et al. (1) and Leflon-Guibout et al. (16). Boldface nucleic acids are the mutation.

TABLE 3. Kinetic parameters for TEM-91 β-lactamase[a]

Agent	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_i (nM)[b]	
				CLA	SUL
Ampicillin	15.5	94.1	6.07×10^6	34.6	33.8
Cephaloridine	31.6	36.3	1.15×10^6	ND	ND
Ceftazidime	179	29.0	1.62×10^5	30.3	57.9
Cefotaxime	32.1	2.96	9.22×10^4	ND	ND
Aztreonam	94.8	0.0125	1.32×10^2	ND	ND

[a] Data are mean values from three measurements; each standard deviation was lower than 10%.
[b] Abbreviations: CLA, clavulanic acid; SUL, sulbactam; ND, not done.

R164H substitutions have thus far been found in at least 14 and 8 TEM-derived enzymes, respectively (http://www.lahey.org/studies/webt.htm). The R164C substitution observed in TEM-91 may thus have negative effects on the retention of the tertiary structure or function of this enzyme due to its susceptibility to oxidation or alkylating substances. The R164C substitution may also offset the folding and stability defects that occur with the M182T substitution (8), since substitutions at R164 are often associated with the M182T substitution, as has been observed for TEM-43, TEM-63, TEM-87, and TEM-107 (http://www.lahey.org/studies/webt.htm).

Strains producing TEM-derived ESBLs are still rare in Japan compared with their presence in Western countries, although some CTX-M-related class A β-lactamases, including Toho-1 and CAZ-hydrolyzing SHV-12 (9, 25), and several cephamycin-hydrolyzing class C β-lactamases, such as MOX-1 and CMY-9 (4), have been identified in Japan, as well as carbapenem-hydrolyzing metallo-β-lactamases such as IMP-1 (13). Thus, there is a need for more prudent use of broad-spectrum β-lactams and intensive surveillance of gram-negative bacilli that have acquired consistent resistance to oxyimino-β-lactams.

Nucleotide sequence accession number. The nucleotide sequence of bla_{TEM-91} and the deduced amino acid sequence of TEM-91 have been deposited in the EMBL and GenBank nucleotide sequence data banks through the DNA Data Bank of Japan with the assigned accession number AB049569.

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