ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2001, p. 3651–3653 Vol. 45, No. 12
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.12.3651–3653.2001
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Evolution of TEM-Related Extended-Spectrum β-Lactamases in Korea

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Received 23 February 2001/Returned for modification 23 June 2001/Accepted 21 August 2001

TEM-52, differing from TEM-1 by having the substitutions Glu-104→Lys, Met-182→Thr, and Gly-238→Ser, has previously been described as the most prevalent extended-spectrum β-lactamase (ESBL) in Korea. In a further survey, we discovered the ESBLs TEM-15, which is like TEM-52 but lacks the substitution at residue 182, and TEM-88, which is like TEM-52 with an additional Gly-196→Asp substitution. TEM-88 retained the activity of TEM-52 against moxalactam. Otherwise, the kinetic properties of the three ESBLs failed to show an advantage to this evolution.

Blood culture isolates of *Escherichia coli* and *Klebsiella pneumoniae* were collected from Seoul National University Children's Hospital between 1994 and 1999. Among 16 isolates of extended-spectrum β-lactamase (ESBL)-producing *E. coli* and 36 isolates of ESBL-producing *K. pneumoniae*, 12 and 18 isolates, respectively, produced TEM-derived enzymes. Ten *E. coli* and 15 *K. pneumoniae* isolates produced TEM-52 β-lactamase, two *K. pneumoniae* strains produced TEM-15 β-lactamase, and two *E. coli* isolates and one *K. pneumoniae* isolate produced a novel TEM-related ESBL (unpublished data). In this study, we characterized the novel TEM-type ESBL TEM-88 and compared its biochemical characteristics to those of the TEM-15 and TEM-52 β-lactamases.

K. pneumoniae strain K28 was isolated from the blood of a patient in a pediatric oncology unit in 1998. Analytical isoelectric focusing (7) demonstrated that strain K28 produced β-lactamases with isoelectric point (pI) values of 5.6 and 7.6. The gene for ceftazidime resistance, along with that for a pI 5.6 enzyme and a plasmid of about 150 kb termed pMG272, was transferred by conjugation to *E. coli* J53Azi^r (9). For nucleotide sequencing, the bla_{TEM} gene was amplified with pMG272 as the template and primers T1 (5′-ATA AAA TTC TTG AAG ACG AAA-3′) and T2 (5′-GAC AGT TAC CAA TGC TTA ATC A-3′) (6). The amplified PCR product was purified using a QIAEX gel extraction kit (Qiagen, Chatsworth, Calif.). Both strands were sequenced using published TEM primers (6) and a dideoxy termination cycle sequencing kit (Perkin-Elmer Cetus, Norwalk, Conn.). The deduced amino acid sequence of TEM-88 had four amino acid substitutions from TEM-1: Glu-104→Lys, Met-182→Thr, Gly-196→Asp, and Gly-238→Ser (numbered according to the proposal of Ambler et al. [1]) (Table 1). The amino acid replacement at position 196 has not been observed in other TEM-related ESBL genes (http://www.lahey.org/studies/webt.htm). TEM-88 differed from TEM-52 by 1 amino acid at position 196, and TEM-52 differed from TEM-15 by 1 amino acid at position 182 (11) (Table 1). TEM-15, TEM-52, and TEM-88 are the only TEM-type ESBLs identified in Korea to date. With these findings, we speculated that TEM-15 developed into TEM-52 and that TEM-52 evolved into TEM-88 (Table 1). In order to find out whether there was a functional advantage in such changes, we analyzed and compared the biochemical characteristics of TEM-15, TEM-52, and TEM-88.

The bla_{TEM-88} gene was cloned from plasmid pMG272 with *Eco*RI as an 18-kb insert into the vector plasmid pBC SK (Stratagene, La Jolla, Calif.) to produce plasmid pMG273. Plasmid pMG273 was introduced by electroporation into *E. coli* XL1-Blue (Stratagene), which was used for the kinetic assays. To represent TEM-52 β-lactamase, a clinical isolate (9) and *E. coli* transconjugant J53 Azi^r(pMG276) were used. The bla_{TEM-15} gene was cloned by PCR into the pPCR-Script Cam vector (Stratagene) from *K. pneumoniae* strain 23 (unpublished), and the resulting plasmid, pMG275, was transformed by electroporation into *E. coli* XL1-Blue. The identity of bla_{TEM-15} was reconfirmed by sequencing.

Antimicrobial susceptibility testing was performed using

TABLE 1. Amino acid substitutions in TEM-type β-lactamases

β-Lactamase	pI	Residue (coding triplet) at amino acid:			
		104	182	196	238
TEM-1	5.4	Glu (GAG)	Met (ATG)	Gly (GGC)	Gly (GGT)
TEM-15	5.9	Lys (AAG)			Ser (AGT)
TEM-52	5.9	Lys (AAG)	Thr (ACG)		Ser (AGT)
TEM-88	5.6	Lys (AAG)	Thr (ACG)	Asp (GAC)	Ser (AGT)

[a] Abbreviations: Asp, aspartic Acid; Glu, glutamic acid; Gly, glycine; Lys, lysine; Met, methionine; Ser, serine; Thr, threonine.

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TABLE 2. MICs of β-lactams for strains producing TEM-related extended-spectrum β-lactamases

Enzyme	Strain	MIC (μg/ml)[a]							
		AMX	AMX-CLA	ATM	CAZ	CEF	CTX	FOX	MOX
TEM-15	*K. pneumoniae* K23	>256	12	4	32	>256	>256	6	1
	E. coli J53(pMG274)	>256	12	12	32	>256	>256	6	0.25
	E. coli XL1-Blue(pMG275)	>256	8	1	4	>256	24	6	0.5
TEM-52	*K. pneumoniae* KpS15	>256	6	16	192	>256	>256	8	4
	E. coli J53(pMG276)	>256	8	6	24	>256	>256	6	2
TEM-88	*K. pneumoniae* K28	>256	16	16	16	>256	>256	8	2
	E. coli J53(pMG272)	>256	16	8	32	>256	>256	8	2
	E. coli XL1-Blue(pMG273)	>256	8	1	4	32	96	6	ND

[a] Abbreviations: AMX, amoxicillin; ATM, aztreonam; CAZ, ceftazidime; CEF, cephalothin; CLA, clavulanic acid; CTX, cefotaxime; FOX, cefoxitin; MOX, moxalactam; ND, not done.

Etest strips (AB Biodisk, Dalvägen, Sweden). The MICs of amoxicillin, amoxicillin-clavulanic acid, cephalothin, cefotaxime, ceftazidime, and aztreonam were similar for transformant or transconjugant *E. coli* strains producing TEM-15, TEM-52, or TEM-88 (Table 2). TEM-52 and TEM-88, but not TEM-15, augmented resistance to moxalactam.

Kinetic assays for β-lactam hydrolysis were performed with *E. coli* XL1-Blue(pMG275), *E. coli* J53(pMG276), and *E. coli* XL1-Blue(pMG273). β-Lactamase extracts were prepared by three freeze-thaw cycles followed by Sephadex G-75 chromatography with 0.1 M phosphate buffer, pH 7.0 (Pharmacia Biotech Inc., Piscataway, N.J.) (3). Antimicrobials used for hydrolysis assays were benzylpenicillin, cephaloridine, cefotaxime, moxalactam (Sigma, St. Louis, Mo.), ceftazidime (Glaxo Group Research, Ltd., Greenford, England), and aztreonam (Bristol-Myers Squibb, Princeton, N.J.). Initial hydrolysis rates were determined spectrophotometrically at 37°C with 0.1 M phosphate buffer, pH 7.0. The computer program GraFit (Erithacus Software Ltd., Staines, United Kingdom) and linear regression using a Hanes plot (10) were used for calculating kinetic parameters. For benzylpenicillin, half-time analysis with a single-process curve was used (12). Although moxalactam is stable in the presence of most ESBLs, it was included as a substrate because TEM-52 is known to have a higher affinity for moxalactam than TEM-3 or TEM-1 (11). For moxalactam, a 50% inhibitory concentration (IC_{50}) was determined using cephaloridine as the substrate at five times the K_m for each enzyme, because hydrolysis rates were too small to determine. The relative values for maximum rate of hydrolysis (V_{max}) and K_m were determined as the means of two or three determinations.

All 3 enzymes showed similar biochemical characteristics such as similar relative V_{max} and K_m values for cefotaxime, ceftazidime, cephaloridine, and benzylpenicillin; more effective hydrolysis of cefotaxime than ceftazidime; and very weak hydrolysis of aztreonam (Table 3). The IC_{50} of TEM-52 or TEM-88 for moxalactam was three- or fourfold lower than that of TEM-15 (Table 3), indicating that TEM-52 had a higher affinity for moxalactam than TEM-15 and that TEM-88 with a further Gly-196→Asp substitution retained this property.

In molecular modeling, TEM residue 196 is quite far from the binding site of the enzyme and positioned on the surface of an α-helix behind the B3 sheet (5). Mutagenesis studies have also indicated that residue 196 is tolerant of substitutions that have no effect on activity (4). The Gly-196→Asp change in TEM-88 compared to TEM-52 is thus functionally silent, similar to substitutions observed in TEM-57 and TEM-90 (2, 8). Evolution from TEM-15 to TEM-52 to TEM-88 in Korea does not seem to be based on improved ability to hydrolyze oxyimino-β-lactams.

Nucleotide sequence accession number. The nucleotide sequence of the bla_{TEM-88} gene has been submitted to GenBank under accession no. AY027590.

H.P. and G.A.J. were supported in part by a Merit Review award from the VA/DoD Collaborative Research Program on Mechanisms of Emerging Pathogens.

TABLE 3. Kinetic parameters of TEM-15, TEM-52, and TEM-88 β-lactamases

Substrate or inhibitor	TEM-15			TEM-52			TEM-88		
	K_m (μM)	Relative V_{max} (%)	IC_{50}[a] (μM)	K_m (μM)	Relative V_{max} (%)	IC_{50} (μM)	K_m (μM)	Relative V_{max} (%)	IC_{50} (μM)
Benzylpenicillin	6	100		6	100		7	100	
Cephaloridine	32	189		28	130		22	161	
Cefotaxime	59	292		43	249		46	274	
Ceftazidime	257	19		199	20		213	24	
Aztreonam	IND[b]	<0.5		IND	<0.5		IND	<0.5	
Moxalactam			0.32			0.09			0.07

[a] Measured with cephaloridine as the substrate.
[b] IND, indeterminate (activity too low to measure K_m accurately).

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