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TEM-71, a Novel Plasmid-Encoded, Extended-Spectrum β-Lactamase Produced by a Clinical Isolate of *Klebsiella pneumoniae*

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TEM-71, a novel extended-spectrum β-lactamase from a *Klebsiella pneumoniae* clinical isolate, had an isoelectric point of 6.0 and a substrate profile showing preferential hydrolysis of cefotaxime over ceftazidime. It differed from TEM-1 by two substitutions, Gly238Ser and Glu240Lys, and was under the control of the strong *P4* promoter.

Extended-spectrum β-lactamases (ESBLs) are enzymes that confer resistance to oxyimino-β-lactams such as cefotaxime, ceftazidime, ceftriaxone, and the monobactam aztreonam but not to cephamycins or carbapenems (5). ESBLs, which are predominantly derivatives of plasmid-mediated TEM or SHV β-lactamases, arise through mutations that introduce one or more amino acid substitutions that alter the configuration or binding properties of the active site, resulting in an expansion of the substrate range of the enzymes (G. A. Jacoby and K. Bush, website [http://www.lahey.org/studies/webt.htm]) (3, 5). ESBL-producing clinical isolates are frequently associated with nosocomial outbreaks (9, 21), with production detected most commonly in *Klebsiella pneumoniae* (8, 17) in addition to other members of the *Enterobacteriaceae* family (8) and *Pseudomonas aeruginosa* (19). Novel ESBLs continue to be reported at an alarming rate (Jacoby and Bush, website) (3). Here, we describe another novel ESBL, designated TEM-71, produced by a clinical isolate of *K. pneumoniae* obtained from the blood of a patient in San Antonio, Tex., in 1994.

The susceptibility of *K. pneumoniae* 3635 to selected antimicrobial agents was determined by broth microdilution with cation-adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) according to National Committee for Clinical Laboratory Standards (NCCLS) methods (20).

The antimicrobial agents used for susceptibility testing or kinetic studies were in some cases obtained from different sources. Amoxicillin, ampicillin, cefotaxime, ceftriaxone, cephaloridine, penicillin G, and piperacillin were from Sigma Chemical Co. (St. Louis, Mo.); aztreonam was from Bristol-Myers Squibb (Princeton, N.J.) and ICN Biomedicals, Inc. (Aurora, Ohio); cefepime was from Bristol-Myers Squibb; clavulanic acid was from GlaxoSmithKline (Collegeville, Pa.) and U.S. Pharmacopeia (Rockville, Md.); cefpodoxime was from Pharmacia & Upjohn (Kalamazoo, Mich.); ceftazidime was from Eli Lilly (Indianapolis, Ind.) and U.S. Pharmacopeia; cefoxitin and imipenem were from Merck (Rahway, N.J.); and tazobactam was from Wyeth-Ayerst Research (Pearl River, N.Y.).

Isoelectric focusing was performed as previously described (22), and the isoelectric point (pI) of the enzyme was determined in reference to TEM-1 (pI, 5.4), TEM-2 (pI, 5.6), TEM-3 (pI, 6.3), and SHV-3 (pI, 7.0).

Plasmid DNA was prepared by using a Qiagen plasmid midi kit (Qiagen, Chatsworth, Calif.) as previously described (22). The plasmids were separated vertically in a 0.9% agarose gel prepared with 0.5× Tris-borate-EDTA buffer and electrophoresed first at 40 V for 50 min and then at 120 V for 4 h.

Conjugal transfer of β-lactam resistance plasmids was performed as previously described (13). A spontaneous mutant of *Escherichia coli* J53 (F^- *met pro*) resistant to 250 μg of sodium azide per ml was used as a recipient in resistance transfer studies (13).

Hybridization studies were performed as described previously (22). DNA transferred from agarose gels to positively charged nylon membranes (Zeta-Probe; Bio-Rad Laboratories, Hercules, Calif.) was hybridized with a 351-bp digoxigenin-labeled bla_{TEM} DNA probe (22).

A 1,082-bp PCR product that included both the bla_{TEM-71} structural gene and its upstream regulatory region was amplified from both *K. pneumoniae* 3635 and its transconjugant with oligonucleotide primers (18) and concentrations of PCR reactants (22) as previously described. Cycling parameters for the amplification of the bla_{TEM} gene included a 5-min denaturation at 96°C followed by 35 cycles of denaturation (96°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 1 min) and ending with a final extension period of 72°C for 10 min. A 351-bp digoxigenin-labeled bla_{TEM} probe was prepared under the same conditions, but an alternate deoxynucleoside triphosphate mixture containing substituted nucleosides was used (22).

The DNA sequences of both strands of the bla_{TEM-71} gene in *K. pneumoniae* 3635 and its *E. coli* transconjugant J53 (pMG259) were determined from purified PCR products

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TABLE 1. MICs of selected antimicrobial agents for various strains[a]

Antimicrobial agent	MIC (μg/ml) for:		
	K. pneumoniae 3635	*E. coli* J53	*E. coli* J53 (pMG259)
Amoxicillin-clavulanate[b]	8	8	8
Ampicillin	>64	8	>64
Aztreonam	16	0.12	>64
Aztreonam-clavulanate[c]	≤0.03	0.06	0.12
Cefepime	1	0.06	2
Cefotaxime	8	0.25	16
Cefotaxime-clavulanate[c]	≤0.03	0.06	0.06
Cefoxitin	2	2	4
Cefpodoxime	32	2	128
Cefpodoxime-clavulanate[c]	0.12	1	0.25
Ceftazidime	32	0.25	128
Ceftazidime-clavulanate[c]	0.12	0.12	0.25
Ceftriaxone	8	0.25	32
Ceftriaxone-clavulanate[c]	≤0.06	≤0.06	≤0.06
Imipenem	≤1	≤1	≤1
Piperacillin	>128	≤2	>128
Piperacillin-tazobactam[d]	4	2	≤1

[a] Quality control organisms for MIC determinations were *Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *K. pneumoniae* ATCC 700603, *P. aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213.
[b] 2:1 concentration ratio.
[c] Clavulanic acid was used at a final concentration of 4 μg/ml.
[d] Tazobactam was used at a final concentration of 4 μg/ml.

(QIAquick spin columns; Qiagen) by using previously described oligonucleotides (18, 23). Purified pMG259 was used as a template to confirm and complete the $bla_{\text{TEM-71}}$ sequence, particularly outside of the coding region of the gene.

The cycle sequencing reactions, purification of products, and analysis of data were performed as previously described (22). The DNA sequences of the leading and lagging strands were determined by using products of independent PCRs. DNA sequencing data were analyzed with DNASIS for Windows (Hitachi Software Genetic Systems, San Francisco, Calif.).

Transconjugant *E. coli* J53 (pMG259), the source of the TEM-71 enzyme, was grown overnight at 37°C in 2 liters of Trypticase soy broth supplemented with 200 μg of ampicillin/ml. The enzyme was purified for kinetic analysis as previously described (22). The enzyme for k_{cat} determination was further purified by anion-exchange chromatography through a HiTrap Q column (Amersham Pharmacia, Piscataway, N.J.) in 20 mM Tris (pH 7.5). This preparation was 35% pure and unstable except in the presence of 10% glycerol. The purified enzyme focused as a single β-lactamase with a pI of 6.0. The protein concentration was determined by BCA protein assay (Pierce, Rockford, Ill.).

Kinetic studies to determine K_m and $V_{\max}$ values were performed as previously described (22). The inhibition of hydrolysis was measured after the addition of 985 μl of cephaloridine (100 μM) to 5 μl of enzyme preincubated for 5 min with the inhibitor in 10 μl of phosphate buffer (pH 7.0) at 25°C (4). The 50% inhibitory concentrations were determined graphically (4).

Based on NCCLS interpretive criteria for non-ESBL-producing *Enterobacteriaceae* (20), *K. pneumoniae* 3635 was resistant to ampicillin, cefpodoxime, ceftazidime, and piperacillin, intermediate to aztreonam, and susceptible to amoxicillin-clavulanate, cefepime, cefotaxime, cefoxitin, ceftriaxone, imipenem, and piperacillin-tazobactam (Table 1). Reductions in the MICs of aztreonam, cefotaxime, cefpodoxime, ceftazidime, and ceftriaxone by three or more twofold dilutions in the presence of clavulanic acid were indicative of ESBL production (Table 1).

K. pneumoniae 3635 contained a β-lactamase with a pI of 6.0 and a second β-lactamase with a pI of 7.6 (data not shown). The latter was identified by DNA sequence analysis as SHV-1. Strain 3635 carried two plasmids with molecular sizes of approximately 101 and 71 kb. Resistance to ceftazidime was associated with the 71-kb plasmid, which was transferable to *E. coli* J53 via conjugation. The bla_{TEM} gene was localized to the 71-kb plasmid with a bla_{TEM}-specific digoxigenin-labeled probe (data not shown). The transconjugant *E. coli* J53 (pMG259) expressed a single β-lactamase with a pI of 6.0 and was resistant to ampicillin, aztreonam, cefpodoxime, ceftazidime, and piperacillin (Table 1).

The MICs of aztreonam and several of the extended-spectrum cephalosporins were consistently higher for the transconjugant *E. coli* J53 (pMG259) than for *K. pneumoniae* 3635 (Table 1). Quantitative studies showed that the higher MICs for the transconjugant were due to an increase in β-lactamase activity, possibly suggesting better expression in *E. coli* (data not shown).

A comparison of nucleotide mutations and inferred amino acid substitutions at key sites within the regulatory and coding regions of $bla_{\text{TEM-71}}$, $bla_{\text{TEM-1a}}$ (25), $bla_{\text{TEM-1b}}$ (10), $bla_{\text{TEM-1c}}$ (11), $bla_{\text{TEM-1d}}$ (15, 16), $bla_{\text{TEM-1e}}$ (15, 16), $bla_{\text{TEM-1f}}$ (15, 16), and $bla_{\text{TEM-2}}$ (10) is shown in Table 2. The DNA sequence of $bla_{\text{TEM-71}}$ revealed that it could be a derivative of the recently

TABLE 2. Nucleotide mutations and amino acid substitutions among selected bla_{TEM} genes and corresponding enzymes[a]

Gene (promoter)	Nucleotide (amino acid) at indicated position[b]													
	32	162	175	226 (6)	317 (39)	346 (48)	436 (78)	604 (134)	682 (160)	913 (237)	914 (238)	917 (240)	925 (242)	Reference
$bla_{\text{TEM-1a}}$ (*P3*)	C	G	A	C	C (Gln)	A	C	G	T	C	G (Gly)	G (Glu)	G	25
$bla_{\text{TEM-1b}}$ (*P3*)			G	T			T	T						10
$bla_{\text{TEM-1c}}$ (*P3*)							T							11
$bla_{\text{TEM-1d}}$ (*P3*)							T			T				15, 16
$bla_{\text{TEM-1e}}$ (*P3*)			G	T		G	T	T						15, 16
$bla_{\text{TEM-1f}}$ (*P4*)		T				G	T		C				A	15, 16
$bla_{\text{TEM-71}}$ (*P4*)		T				G	T		C		**A (Ser)**	**A (Lys)**	A	This study
$bla_{\text{TEM-2}}$ (*Pa*/*Pb*)	T				**A (Lys)**	G	T		C				A	10

[a] Nucleotide changes that result in amino acid differences from TEM-1 (25) are shown in boldface.
[b] Nucleotide numbering according to Sutcliffe (25); amino acid numbering (in parentheses) according to Ambler et al. (1).

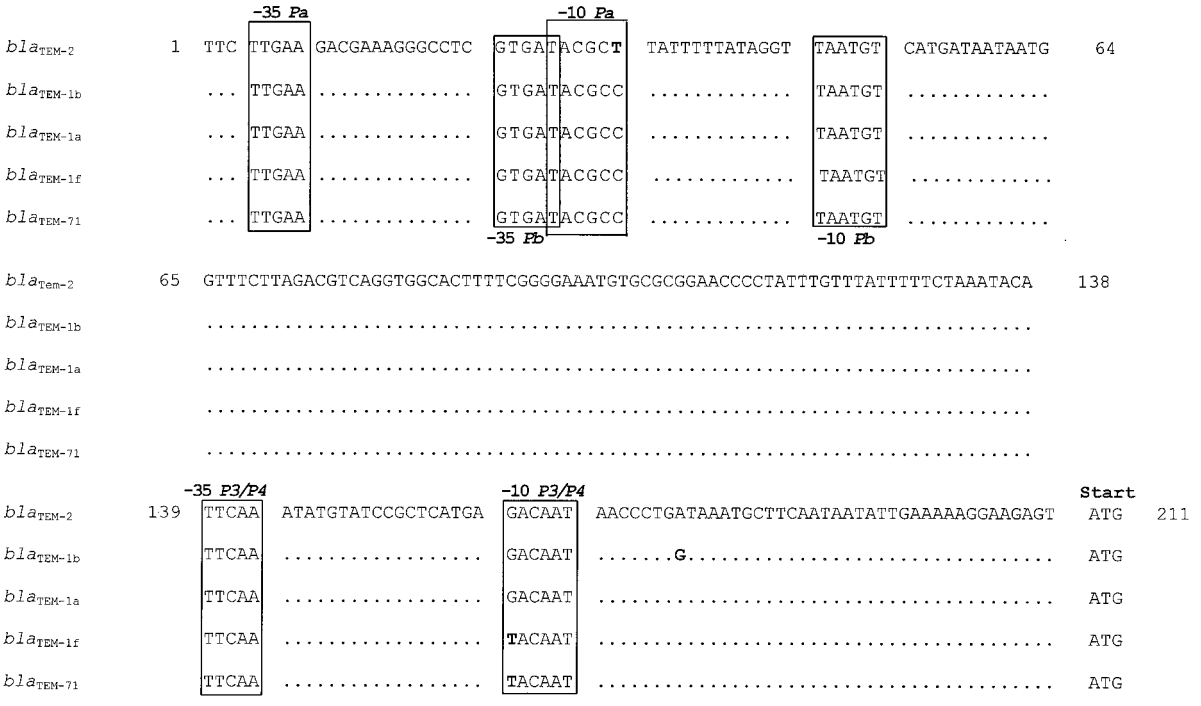

FIG. 1. Comparison of promoter sequences of the β-lactamase genes bla_{TEM-2} (10), bla_{TEM-1b} (10), bla_{TEM-1a} (25), bla_{TEM-1f} (15, 16), and bla_{TEM-71}. The differences are shown in boldface. The −35 and −10 regions of *Pa*, *Pb*, and *P3/P4* are boxed. The start codon is indicated. The nucleotide positions are numbered according to Sutcliffe (25).

described bla_{TEM-1f} gene (15, 16). Although both bla_{TEM-71} and bla_{TEM-1f} possess all of the silent mutations found in bla_{TEM-2} (10) compared to the bla_{TEM-1a} gene (25), neither gene encodes the Gln39Lys substitution characteristic of the TEM-2 enzyme. Furthermore, the *P4* promoter (containing a $G_{162}\rightarrow T$ transversion which is located at position 1 of the −10 consensus Pribnow box sequence of the *P3* promoter) (11, 24) (Fig. 1) was found upstream of both bla_{TEM-71} and bla_{TEM-1f} (15, 16) instead of the overlapping *Pa* and *Pb* promoters located upstream from the bla_{TEM-2} gene (7, 10) (Table 2 and Fig. 1). The *P4* promoter has been shown to result in hyperproduction of TEM-1 (24).

The amino acid sequence inferred from the DNA sequence of bla_{TEM-71} revealed two substitutions compared to the TEM-1 enzyme (25): Gly238Ser and Glu240Lys, both thought to play key roles in the hydrolysis of oxyimino-β-lactams and the monobactam aztreonam (6, 14, 26). TEM-71 is the first natural variant of TEM β-lactamase that contains both a serine at position 238 and a lysine at position 240 unaccompanied by additional substitutions (Jacoby and Bush, website).

TEM-48 and TEM-49 are two enzymes that contain both the Gly238Ser and Glu240Lys substitutions in addition to other mutations (9). A comparison of the DNA sequences of the bla_{TEM-48} and bla_{TEM-49} genes with the sequence of bla_{TEM-71} suggests the possibility of a close relationship. The three genes differ within the coding region by only three mutations: C→T transitions at nucleotide positions 263 (Leu21Phe substitution in TEM-48 and TEM-49) and 990 (Thr265Met substitution in TEM-48 and TEM-49) and an A→G transition at position 998 (Ser268Gly substitution in TEM-49) (9). The bla_{TEM-48}, bla_{TEM-49}, and bla_{TEM-71} genes share G→A transitions at nucleotide positions 914 (Gly238Ser substitution in TEM-48, TEM-49, and TEM-71) and 917 (Glu240Lys substitution in TEM-48, TEM-49, and TEM-71) as well as four silent mutations ($A_{346}\rightarrow G$, $C_{436}\rightarrow T$, $T_{682}\rightarrow C$, and $G_{925}\rightarrow A$) (9) (Table 2) in their sequences compared to the DNA sequence of bla_{TEM-1a} (25). Whether these genes also share similar promoters is not known, since the nucleotide sequences reported for bla_{TEM-48} and bla_{TEM-49} did not include their upstream regulatory regions (9).

The simultaneous presence of the Gly238Ser and Glu240Lys substitutions is more typically observed within the SHV family of enzymes than in variants of TEM β-lactamase (Jacoby and Bush, website) (3). Included among these is SHV-5, which, like TEM-71, contains the mutations at positions 238 and 240 without additional substitutions (2).

The kinetic parameters for TEM-71 are consistent with those of an ESBL (Table 3). The highest V_{max} values were for

TABLE 3. Kinetic properties of purified TEM-71 β-lactamase

Substrate	Mean V_{max}[a] ± SD	Relative V_{max} (%)	Mean K_m ± SD (μM)	Relative V_{max}/K_m (%)
Penicillin G	0.49 ± 0.004	100	2.1 ± 0.14	100
Cephaloridine[b]	0.87 ± 0.06	180	8.3 ± 1.2	43
Ceftazidime	0.11 ± 0.005	22	100 ± 20	0.4
Cefotaxime	1.1 ± 0.05	220	73 ± 5.0	6.0
Aztreonam[c]	<0.03	<6	ND[d]	ND

[a] Nanomoles of substrate hydrolyzed per minute per milligram of protein.
[b] k_{cat} for cephaloridine was 13 s^{-1}.
[c] Values based on estimated V_{max}.
[d] ND, not determined. The rate of hydrolysis of aztreonam was too low to obtain an accurate K_m value.

cefotaxime and cephaloridine and were approximately twice the rate for penicillin and 10-fold greater than that for ceftazidime. Both extended-spectrum cephalosporins were hydrolyzed at relative rates at least 3 orders of magnitude higher than by TEM-1 (5). The lowest hydrolysis rate of the five substrates tested was for aztreonam.

The TEM-71 enzyme had strong binding for both penicillin and cephaloridine, based on K_m values of 2.1 and 8.3 μM, respectively (Table 3). The relative hydrolytic efficiencies, measured by V_{max}/K_m, revealed that penicillin was hydrolyzed at least twice as efficiently as cephaloridine and approximately 17 and 250 times better than cefotaxime and ceftazidime, respectively. Cefotaxime was hydrolyzed 15 times as efficiently as ceftazidime. Although the rates of hydrolysis of ceftazidime and aztreonam compared to that of cefotaxime were lower than expected given their MICs, this relationship has been reported in the characterization of other β-lactamases as well (22, 28).

Although the substitutions in SHV-5 and TEM-71 are analogous, their kinetic properties appear to differ (12). Cefotaxime is hydrolyzed approximately 2 times more efficiently than ceftazidime by SHV-5 as opposed to 15 times more efficiently by TEM-71. However, both enzymes exhibited a preference for cefotaxime hydrolysis over ceftazidime. Notably, neither enzyme hydrolyzed aztreonam at a measurable rate, in contrast to most TEM- and SHV-derived ESBLs.

Clavulanic acid and tazobactam both inhibited the TEM-71 β-lactamase, with 50% inhibitory concentrations of 6.1 and 7.8 nM, respectively (data not shown). As expected for serine-based β-lactamases, no inhibition was observed when the enzyme was assayed with 10 mM EDTA at pH 7.0.

In summary, bla_{TEM-71}, reported here from *K. pneumoniae* but recently also discovered in an *E. coli* blood isolate (27), is an example of the ongoing evolution of ESBL genes linking mutations that expand the substrate-binding and hydrolytic properties of the enzymes with strong promoters that enhance gene expression.

Nucleotide sequence accession number. The nucleotide sequence of bla_{TEM-71} reported in this study will appear under the GenBank accession number AF203816.

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