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A Novel Plasmid-Mediated β-lactamase that Hydrolyzes Broad-Spectrum Cephalosporins in a Clinical Isolate of *Klebsiella pneumoniae*

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A new extended-spectrum β-lactamase with an isoelectric point (pI) of 6.2 was detected in *Klebsiella pneumoniae* F161 that was isolated from a patient with infection. This strain was highly resistant to the third or fourth generation cephalosporins such as ceftazidime, ceftriaxone, cefoperazone, and cefpirome. Analysis of this strain by the double disk diffusion test showed synergies between amoxicillin-clavulanate (AMX-CA) and cefotaxime, and AMX-CA and aztreonam, which suggested that this strain produced a extended-spectrum β-lactamase (ESBL). Genetic analysis revealed that the resistance was due to the presence of a 9.4-kb plasmid, designated as pKP161, encoding for new β-lactamase gene (*bla*). Sequence analysis showed that a new *bla* gene of pKP161 differed from bla_{TEM-1} by three mutations leading to the following amino acid substitutions: $Val_{84} \rightarrow Ile$, $Ala_{184} \rightarrow Val$, and $Gly_{238} \rightarrow Ser$. These mutations have not been reported previously in the TEM type β-lactamases produced by clinical strains. The novel β-lactamase was overexpressed in *E. coli* and purified by ion exchange chromatography on Q-Sepharose and CM-Sepharose, and then further purified by gel filtration on Sehadex G-200. The catalytic activity of the purified β-lactamase was confirmed by the nitrocefin disk.

Key words: Extended-spectrum β-lactamase (ESBL), *bla*, TEM-type β-lactamase, *Klebsiella pneumoniae*, Double disk synergy test, Isoelectric point (pI) value, Cephalosporin

INTRODUCTION

Klebsiella pneumoniae is an important pathogen that is usually susceptible to extended-spectrum cephalosporins. However, clinical isolates producing extended-spectrum β-lactamases (ESBLs) that are usually encoded by plasmid-mediated genes were described in the early 1980s. Since that time, there has been an increase in incidence of cephalosporin-resistant *Klebsiella* strains responsible for nosocomial outbreaks (Essack *et al.*, 2001; Neuwirth *et al.*, 2000). Recently, strains for which the β-lactamases inhibitors clavulanate or sulbactam enhanced expanded spectrum cephalosporins activity were classified as presumptive ESBL producers (Blahova *et al.*, 1997; Jarlier *et al.*, 1988). Most ESBLs are variants of the classical TEM and SHV β-lactamase, but with one or more amino acid substitutions. Extensive use of newer generation cephalosporins has been a strong factor selecting for ESBL variants formed in a given environment, promoting their further evolution and spread in bacterial populations by means of plasmid transmission. Today, ESBLs have been identified worldwide, and transferred between different wards, as well as between hospitals, with the case of international travel, between different countries (Gouby *et al.*, 1994). The rapid spread of ESBLs has caused significant threats to the therapy for infections and the usage of the expanded spectrum β-lactams. But most studies on ESBLs have focused on the resistant strains collected in Europe and North America. To redress this situation, we investigated ESBLs in nosocomial *K. pneumoniae* isolates collected at

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a major hospital in Korea.

In this paper, we describe a novel TEM-type β-lactamase that can hydrolyze cefpirome, ceftazidime, ceftriaxone, and cefoperazone. This plasmid-mediated β-lactamase is produced by a clinical isolate of *K. pneumoniae* F161.

MATERIALS AND METHODS

Bacterial strains

The strain of *Klebsiella pneumoniae* F161 with unusual pattern of resistance to extended broad-spectrum cephalosporins was isolated at Kang-Nam Seong-Shim Hospital, Seoul, Korea. *Escherichia coli* 1193E (TEM-1), *E. coli* 3455E (TEM-3), *E. coli* 3457E (TEM-7), *E. coli* 2639E (TEM-9) were gift from Glaxo-Wellcome Co. All strains were grown at 37°C in Mueller-Hinton broth (MHB) or on Mueller-Hinton agar (MHA) plates.

Antimicrobial agents

Ceftriaxone and cefoperazone were purchased from Sigma. Cefpirome was synthesized at LG Chem Research Park, LG Chemical Ltd. Ceftazidime (Glaxo-Wellcome) was kindly provided by its manufacturer. Antibiotic standard disks (amoxicillin/clavulanate, aztreonam, ceftriaxone, cefotaxim, moxalactam) were purchased from Becton Dickinson Microbiology Systems.

Susceptibility test

MICs (Minimal Inhibitory Concentration) of various cephalosporins against β-lactamase harboring strains were determined by the agar dilution method as described by NCCLS M7-A5 (National Committee for Clinical Laboratory Standards M7-A5, 2000). Mueller-Hinton medium (Difco Laboratories) was used for testing aerobic and facultative organisms. Test strains were grown for 18 h in MHB. These overnight cultures were diluted with the same fresh medium to a density of approximately 10^7 CFU/ml, and applied to MHA plates, which have serially diluted antimicrobial agent, by use of an inoculator to yield 10^4 CFU per spot. MIC was considered to be the lowest concentration that completely inhibited growth on agar plates, disregarding a single colony or a faint haze caused by the inoculum.

Double disk synergy test

To confirm the presence of ESBL from *K. pneumoniae* F161, the double disk penetration test, which was originally developed for the detection of an extended-spectrum β-lactamase (ESBL) by Jarlier was performed on *K. pneumoniae* F161 as described previously (Jarlier *et al.*, 1988). Disks containing aztreonam, moxalactam, cefotaxim, and ceftriaxone were located around an amoxicillin/clavulanate. Enhancement of the zone of inhibition between the amoxicillin/clavulanate disk and the other drug containing disks was indication of the presence of a clavulanate sensitive extended-spectrum β-lactamase (Blahova *et al.*, 1997; Jarlier *et al.*, 1988).

Plasmid isolation

Plasmid DNA was prepared from the clinical strain by a cleared-lysate procedure with a purification step involving cesium chloride-ethidium bromide density gradient ultracentrifugation (Sambrook and Russell, 1989). The minimal size of the plasmid DNA was assessed as follows: Plasmid DNA digested with *Pst*I, *Hind*III and *BamH*I (Boehringer Mannheim) was electrophoresed on a 0.8% agarose gel. The fragment sizes were determined by comparison with standard size markers, and the minimal size of the whole plasmid was deduced by the addition of the sizes of these fragments.

DNA amplification using the polymerase chain reaction

The PCR primers used in this study are listed in Table I. An 861-bp DNA fragment of the *bla* gene from pKP161 of *K. pneumoniae* F161 and *E. coli* 2639E (TEM-9) was amplified, respectively, using the polymerase chain reaction (PCR) with primer 1 & 2 designed for the detection

Table I. PCR primers and plasmids used in this study

	Sequence or phenotype	Source of reference
Primers		
Primer 1	5'-ATGAGTATTCAACATTTCCGT-3'	Kim *et al.*, 1995
Primer 2	5'-TTACCAATGCTTAATCAGTGA-3'	Kim *et al.*, 1995
Primer 3	5'-TAATACGACTCACTATA-3'	Chanal *et al.*, 1992
Primer 4	5'-CCGCACTAGTGATATCCC-3'	Chanal *et al.*, 1992
Plasmids		
pDK101	*Xcm*I cloning site, ampicillin resistant, vector for DNA sequencing	Kovalic *et al.*, 1991
pLB513	*Xcm*I cloning site, kanamycin resistant, vector for protein expression	Kwak and Kim, 1995

of TEM-type β-lactamase (Kim *et al.*, 1995). Thirty cycles of amplification (30 sec at 94°C, 30 sec at 57°C, and 1 min at 72°C, with a final elongation step of 10 min at 72°C) were performed by Power-block system (Ericomp). PCR product was ligated with *Xcm*I digested pDK101 for DNA sequencing (Kovalic *et al.*, 1991), and *Xcm*I digested pLB513 for protein expression (Kwak and Kim, 1995), respectively. The recombinant plasmids were then transformed into *E. coli* JM109 for DNA sequencing and *E. coli* BL21 (DE3) for protein expression by electroporation.

DNA sequencing and protein analysis

Double-stranded DNA was isolated with the QIAprep spin miniprep kit (Qiagene), and DNA sequencing was performed with the sequenase, version 2.0, DNA sequencing kit (United States Biochemicals) and a set of primer 3 & 4 (Table I)

Overexpression and purification of new TEM protein

The plasmids used in this study are listed in Table I. A new *bla* gene was cloned in plasmid pLB513, and overexpressed in *E. coli* BL21(DE3). A transformant strain, *E. coli* BL21(DE3) carrying plasmid pLB513-*bla*, grown in M9ZB medium (NH_4Cl 1 g, KH_2PO_4 3 g, Na_2HPO_4 6 g, glucose 4 g, 1 M $MgSO_4$ 1 ml, N-Z-amine A 10 g, NaCl 5 g in 1 liter of water) containing kanamycin (40 μg/ml), was induced with IPTG (isopropyl-β-D-thiogalactopyranoside; 0.4 mM) for 10 min before rifampin (200 μg/ml) was added. The culture was incubated for an additional 3 h and harvested by centrifugation. New TEM-type β-lactamase was purified with Q-Sepharose, CM-Sepharose, and Sehadex G-200 as described (Yang *et al.*, 1998). The concentration of protein was measured by using Micro BCA (bicinchoninic acid) protein assay reagent (Pierce, USA) with bovine serum albumin (Pierce, USA) as a standard (Smith *et al.*, 1985). The purified TEM-type β-lactamase was divided into aliquots and stored at 70°C until used.

M_r and isoelectric focusing analysis

The relative β-lactamase molecular mass was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with 15% acrylamide. Proteins were boiled for 5 min at 100°C in a solution containing 1% SDS and 4% mercaptoethanol before being loaded on the gel. The low-range marker proteins (Bio-Rad, USA) were subjected to the same treatment and used as the standard reference.

Isoelectric focusing (IEF) was performed by the method of Matthew (Matthew *et al.*, 1975), using a NOVEX Xcell mini-cell with precast polyacrylamide gel (5% wt/vol) containing Ampholines (pH range, pH 3-10). β-lactamase bands were visualized by staining with a paper strip soaked with 250 μM nitrocefin. When β-lactam ring of nitrocefin was hydrolyzed by β-lactamase, a distinctive color change from yellow to red was detected.

RESULTS

Antibacterial susceptibility

The MICs of cephalosporins against *K. pneumoniae* F161, *E. coli* 1193E (TEM-1), *E. coli* 3455E (TEM-3), *E. coli* 3457E (TEM-7), *E. coli* 2639E (TEM-9), and several *E. coli* JM109 strains transformed with each plasmid harboring *bla* gene, were determined by the agar dilution method. Table II showed that *E. coli* transformant with new TEM-type β-lactamase originated from pKP161 was most

Table II. Antibacterial activities of cephalosporins against various TEM-type β-lactamase harboring strains

Strains		β-lactamase	MIC (μg/ml)			
			Cefpirome	Ceftazidime	Ceftriaxone	Cefoperazone
K. pneumoniae	F161	Novel TEM	16	64	32	128
E. coli	1193E	TEM-1	0.063	0.25	0.063	8
E. coli	3455E	TEM-3	2	8	4	16
E. coli	3457E	TEM-7	4	16	0.25	ND[c]
E. coli	2639E	TEM-9	4	>128	4	32
E. coli	JM109[a]	Novel TEM	4	16	16	64
E. coli	JM109[a]	TEM-1	0.063	0.13	0.031	2
E. coli	JM109[a]	TEM-3	1	4	2	8
E. coli	JM109[a]	TEM-7	0.25	4	0.063	2
E. coli	JM109[a]	TEM-9	0.063	0.13	0.063	2
E. coli	JM109[b]	(–)	0.031	0.13	0.031	0.063

[a]Transformed strains by plasmid harboring bla_{TEM} gene
[b]Wild type JM109
[c]Not Determined

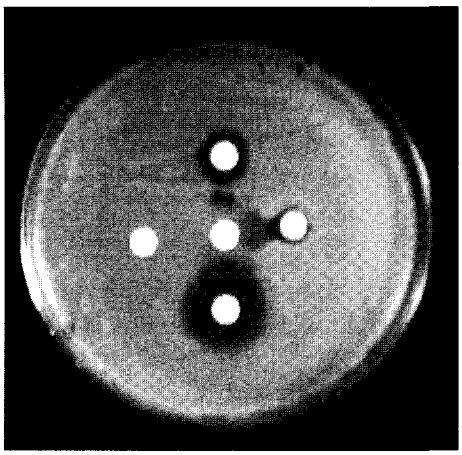

Fig. 1. Double disk synergy obtained with *K. pneumoniae* F161, producing extended broad-spectrum β-lactamase (ESBL). Four 30 μg antibiotic disk (top: aztreonam, bottom: moxalactam, right: cefotaxam, left: ceftriaxone) were placed around a disk of 20 μg of amoxicillin plus 10 μg of clavulanate.

resistant to several cephalosporins among all transformants with each TEM β-lactamase. This result indicated that a new TEM-type β-lactamase from *K. pneumoniae* F161 had more potent β-lactamase activity against several cephalosporins than the other TEM-type β-lactamases reported previously.

Double disk synergy test

Disks containing aztreonam, moxalactam, cefotaxim, and ceftriaxone were located around an amoxicillin/clavulanate. Fig. 1 showed that the positioning of an amoxicillin/clavulanate disk near disks containing aztreonam, cefotazime, ceftriaxone, and moxalactam induced synergistic antibacterial activities of azteronam, cefotaxime, and moxalactam when the agents were tested against lawn culture of *K. pneumoniae* F161. Enhancement of the zone of inhibition between the amoxicillin/clavulanate disk and the other drug containing disks was indication of the presence of a clavulanate sensitive ESBL in *K. pneumoniae* F161.

Plasmid characterization

Restriction enzyme digests of purified pKP161 plasmid from *K. pneumoniae* F161 were obtained by using *Bam HI*, *Hind*III, and *Pst*I. The fragment sizes were determined by comparing with standard size markers, and the size of the whole plasmid was deduced to be 9.4-kb by the addition of the sizes of these fragments.

Nucleotide sequence analysis of the new bla_{TEM} gene

An 861-bp bla_{TEM} fragment was amplified from pKP161 plasmid with primer 1 & 2. The PCR product was cloned into pDK101 and its nucleotide sequence was determined by dideoxynucleotide chain termination procedure. The new *bla* gene of pKP161 was characterized by three amino acid residue substitution, G452 →A (Val84Ile), C753 →T (Ala184Val), and G914 →A (Gly238Ser), and two silent mutations, T436 →C and G604 →T, when compared to the sequence of the $bla_{TEM\text{-}1a}$ gene (Sutcliffe, 1978). This mutation has not been reported previously in the TEM-type β-lactamases produced by clinical strains (Table III).

Overexpression and purification of new TEM-type β-lactamase

New TEM-type β-lactamase was overexpressed in *E. coli* and purified to apparent homogeneity (Fig. 2). Crude preparation of β-lactamase from *E. coli* BL21 (DE3) harboring pLB513-bla were obtained from sonic extracts prepared in 0.05 M phosphate buffer, pH 7.0 (TEM-28). β-lactamase was initially purified by ion exchange chromatography on Q-Sepharose in buffer A (10 mM Tis (pH 8.0), 1 mM EDTA, 10% glycerol, 1 mM DTT). The enzyme was eluted with gradient buffer A solution (0 to 150 mM KCl). β-lactamase in column fractions was detected by nitrocefin disk and SDS-PAGE. β-lactamase was further purified by ion exchange chromatography on CM-Sepharose in buffer B (10 mM potassium acetate (pH 5.0), 1 mM EDTA, 1 mM DTT). The enzyme was eluted with gradient buffer B solution (400 to 750 mM KCl). β-lactamase was finally purified to apparent homogeneity by Sephadex G-200 chromatography in 0.05 mM phosphate buffer (pH 7.0).

M_r and isoelectric focusing analysis

The SDS-polyacrylamide gel electrophoresis analysis showed the purified new β-lactamase to have a relative mass of 28 kD in Fig. 2. Analytical isoelectric focusing was also performed with enzyme extracts of IPTG induced *E. coli* BL21(DE3) harboring pLB513-bla. Following IEF, β-lactamase bands were visualized by staining with nitrocefin. As shown in Fig. 3, an isoelectric point value of 6.2 was determined.

DISCUSSION

The emergence and wide spread of ESBLs by target bacterial strains, particularly caused by the dissemination of plasmid-mediated β-lactamase, have complicated chemotherapy of gram-negative bacteria infections. Since the plasmid-mediated β-lactamase conferring resistance to the newer cephalosporins was first detected in gram-negative bacteria in 1983, the contribution of TEM-derived ESBLs in the resistance mechanism to β-lactam-stable antibiotics has been reported worldwide. ESBLs are found most frequently in *K. pneumoniae* and occasionally

Table III. Amino acid substitutions of TEM-type variants at critical positions

β-Lacatamase	pI	Amino acid at position[a]																												
		6	21	39	42	51	69	84	92	104	115	127	130	153	164	165	182	184	196	204	218	237	238	240	244	262	265	268	275	276
TEM-1	5.4	Q	L	Q	A	L	M	V	G	E	D	I	S	H	R	W	M	A	G	Q	G	A	G	E	R	V	T	S	R	N
TEM-9	5.5		F							K					S												M			
TEM-11	5.6			K											H															
TEM-19	5.4																						S							
TEM-22	6.3			K						K												G	S							
TEM-30	5.2																								S					
TEM-39	5.4						L									R														D
TEM-49	6.0		F																				S	K			M	G		
TEM-52	6.0									K							T						S							
TEM-61	6.5			K											H									K						
TEM-66	6.0			K					D	K													S							
TEM-74	5.2		F																						S		M			
TEM-77							L																		S					
TEM-84																													D	
TEM-88	5.6									K							T		D				S							
TEM-93											G						T						S	K						
New TEM	6.2							I										V					S							

[a]Nucleotide numbering is according to the system of Sutcliffe.

*Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isolucine; L, leucine; M, methionine, N, asparagine; P, proline; Q, qlutamine; R, arginine; S, serine; T, threonine; V,valine; W, tryptophan.

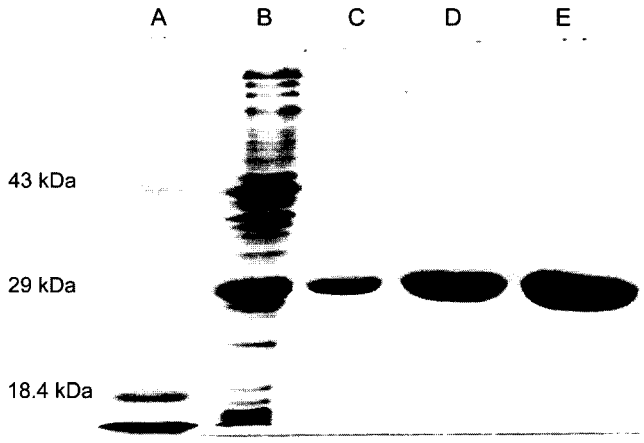

Fig. 2. Purification of new TEM-type β-lactamase by chromatography. (A) Markers for Molecular Weight, (B) Cell lysate, (C) Q-Sepharose (0~150 mM KCl), (D) CM-Sepharose (400~750 mM KCl), (E) Sephadex G-200.

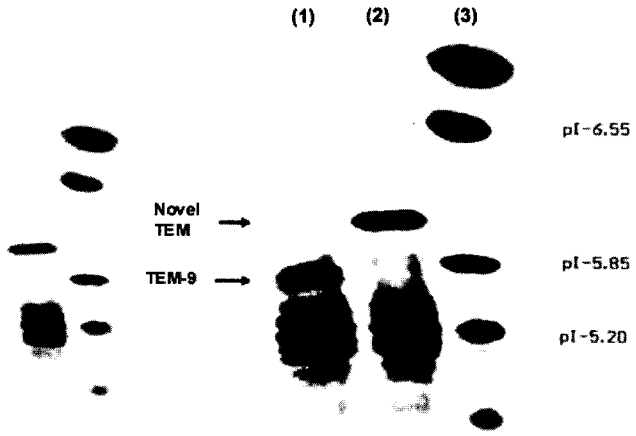

Fig. 3. Isoelectric-focusing gel showing the band of β-lactamase from *E. coli* BL21(DE3). lane (1): TEM-9 β-lactamase, lane (2): novel TEM-type β-lactamase, lane (3): Control marker.

in *E. coli*; other species of the family *Enterobacteriaceae* are rarely producers of ESBLs (Sirot, 1995).

In this study, we isolated *K. pneumoniae* F161 strain for which the MICs of cefpirome, ceftazidime, ceftriaxone, and ceftriaxone were 16, 64, 32, and 128 μg/ml, respectively. This isolate produced a novel TEM-type β-lactamase characterized by an isoelectric point of 6.2. This enzyme is encoded by 9.4-kb plasmid, designated as pKP161, which can be transferred to *E. coli* JM109. The phenotype of resistance of *E. coli* JM109 transformed by pKP161 was very similar to that of *K. pneumoniae* F161 to cephalosporins.

Analysis of the nucleotide sequences revealed that the new *bla* gene of pKP161 differed from bla_{TEM-1a} by three mutations (nucleotides 452, 753, and 914) leading to the following amino acid substitutions: $Val_{84} \rightarrow$Ile, $Ala_{184} \rightarrow$ Val, and $Gly_{238} \rightarrow$Ser. The combination of amino acid replacements found in the novel TEM-type β-lactamase was unique and unknown to date, but the change in Gly_{238} of β-lactamase have been previously documented in the other ESBLs (Fiett *et al.*, 2000; Poyart *et al.*, 1998). Kinetic and molecular modeling analyses of ESBLs have provided the following explanations for the influences of certain mutations on the substrate profiles of these enzymes. The residue at position 238 is situated at the end of the β-3 sheet, and mutations at this residue, such as $Gly_{238} \rightarrow$Ser, might enlarge the active site to give enzymes with the highest affinities for 7-oxyiminocephalosporins (Huletsky *et al.*, 1993; Venkatachalam *et al.*, 1994). However, the variations in Val_{84} and Ala_{184} of the novel β-lactamase have never been reported in TEM-type ESLBs. It would be interesting to study how these new variations in Val_{84} and Ala_{184} affect the enzymatic activity.

In summary, a new TEM-type extended spectrum β-lactamase was found in a strain of *K. pneumoniae* isolated in Korea. In view of its novelty in amino acid sequence of the enzyme, further studies would be necessary to establish the kinetic parameters of the enzyme.

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