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A Novel Extended-Spectrum TEM-Type β-Lactamase (TEM-52) Associated with Decreased Susceptibility to Moxalactam in *Klebsiella pneumoniae*

CLAIRE POYART,[1]* PIERRE MUGNIER,[2]† GILLES QUESNE,[1] PATRICK BERCHE,[1] AND PATRICK TRIEU-CUOT[1]

Laboratoire de Microbiologie, Faculté de Médecine Necker-Enfants Malades, 75730 Paris Cedex 15,[1] and Laboratoire de Recherche Moléculaire sur les Antibiotiques, Université Paris VI, 75 270 Paris Cedex 06,[2] France

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Klebsiella pneumoniae **NEM865 was isolated from the culture of a stool sample from a patient previously treated with ceftazidime (CAZ). Analysis of this strain by the disk diffusion test revealed synergies between amoxicillin-clavulanate (AMX-CA) and CAZ, AMX-CA and cefotaxime (CTX), AMX-CA and aztreonam (ATM), and more surprisingly, AMX-CA and moxalactam (MOX). Clavulanic acid (CA) decreased the MICs of CAZ, CTX, and MOX, which suggested that NEM865 produced a novel extended-spectrum β-lactamase. Genetic, restriction endonuclease, and Southern blot analyses revealed that the resistance phenotype was due to the presence in NEM865 of a 13.5-kb mobilizable plasmid, designated pNEC865, harboring a Tn*3*-like element. Sequence analysis revealed that the *blaT* gene of pNEC865 differed from bla_{TEM-1} by three mutations leading to the following amino acid substitutions: Glu_{104}→Lys, Met_{182}→Thr, and Gly_{238}→Ser (Ambler numbering). The association of these three mutations has thus far never been described, and the *blaT* gene carried by pNEC865 was therefore designated bla_{TEM-52}. The enzymatic parameters of TEM-52 and TEM-3 were found to be very similar except for those for MOX, for which the affinity of TEM-52 (K_i, 0.16 μM) was 10-fold higher than that of TEM-3 (K_i, 1.9 μM). Allelic replacement analysis revealed that the combination of Lys_{104}, Thr_{182}, and Ser_{238} was responsible for the increase in the MICs of MOX for the TEM-52 producers.**

Klebsiella pneumoniae is an important pathogen that is usually susceptible to extended-spectrum cephalosporins. However, strains producing extended-spectrum β-lactamases (ESBLs) were described in the early 1980s, and, since that time, there has been an increase in the incidence of ceftazidime (CAZ)-resistant *Klebsiella* strains responsible for nosocomial outbreaks (8, 16, 27). In most cases, ESBLs are plasmid-encoded enzymes providing resistance to oxyiminocephalosporins (CAZ, cefotaxime [CTX], ceftriaxone, cefpirome, and cefepime [FEP]) and to aztreonam (ATM) (16, 24). Cephamycins (cefoxitin [FOX] and cefotetan [CTT]), moxalactam (MOX), and carbapenems are stable toward most ESBLs, and strains producing such enzymes remained susceptible to these molecules (7, 24). The molecular basis of the extended spectrum often involves point mutations within plasmid-mediated β-lactamase genes resulting in either single or multiple amino acid substitutions in the corresponding enzymes (7, 16, 24). We describe here a novel TEM-type ESBL able to hydrolyze MOX. The enzyme is produced by a clinical isolate of *K. pneumoniae*.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *K. pneumoniae* NEM865 was isolated from the culture of a stool sample from a 2-year-old girl previously treated with CAZ for 2 weeks. *K. pneumoniae* NEM533, a nalidixic acid-resistant mutant of *K. pneumoniae* 5214-K (28; this study) and *Escherichia coli* K802N (33) resistant to nalidixic acid were used as recipients in mating experiments. *E. coli* TG1 (11) and plasmid pBGS18 (31) were used in the cloning experiments as the host strain and vector, respectively. Plasmids pBR322 (32) and pCFF04 (30) were used as templates for the amplification of bla_{TEM-1} and bla_{TEM-3}, respectively. Bacteria were grown in Mueller-Hinton (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) media at 37°C.

Antibiotic susceptibility testing. Disk diffusion tests were performed, and the results were interpreted according to the guidelines of the Comité Français de l'Antibiogramme (1). The MICs were determined on Mueller-Hinton agar by a dilution method with an inoculum of 10^4 CFU per spot. The following antimicrobial agents were kindly provided by the indicated manufacturers: amoxicillin (AMX), ticarcillin, and clavulanic acid (CA), Smith Kline Beecham; cefalothin and CAZ, Glaxo Group Research Ltd., Greenford, England; cefotetan, ICI Pharmaceuticals Inc.; FOX and imipenem, Merck-Sharp & Dohme-Chibret); MOX, Eli Lilly & Co., Indianapolis, Ind.; sulbactam (SUL), Pfizer Inc., New York, N.Y.; CTX, Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.; tazobactam (TZ), Taiho Laboratories, Tokushima, Japan; and ATM and benzylpenicillin, Bristol-Myers-Squibb, Princeton, N.J.

Genetic techniques. Mating on filters was performed as described previously (33). Transfer frequencies were expressed as the number of transconjugants per donor CFU after the mating period. The antibiotic concentrations for the selection of transconjugants were 20 μg/ml for CAZ and 50 μg/ml for nalidixic acid.

Isoelectric focusing of β-lactamases. Analytical isoelectric focusing was done with CAZ-resistant *E. coli* transconjugants and transformants. Supernatants of sonicates were subjected to isoelectric focusing for 2 h by using a mini IEF cell 111 (Bio-Rad) and a gradient made up of two-thirds of polyampholytes with a pH range of 4 to 6 and one-third with a pH range of 3 to 10 (Serva). Extracts from TEM-1, TEM-2, and TEM-3-producing strains were used as standards for pIs of 5.4, 5.6, and 6.3, respectively. β-Lactamases were revealed by overlaying the gel with nitrocefin (1 mg/ml) in phosphate buffer (50 mM, pH 7).

Recombinant DNA techniques. Isolation of plasmid DNA, transformation, restriction endonuclease digestion, ligation, agarose gel electrophoresis, and other standard recombinant techniques were performed as described by Sambrook et al. (29). DNA-DNA hybridization was performed as follows. DNA was transferred onto nylon membranes (Boehringer Mannheim, Mannheim, Germany) as described previously (29). Purified plasmid ColE1::Tn*3* DNA was used as a probe and was labeled with [α-^{32}P]dCTP (Amersham, les Ulis, France) by nick translation. Prehybridization and hybridization were carried out for 4 and

* Corresponding author. Mailing address: Laboratoire de Microbiologie, Faculté de Médecine Necker-Enfants Malades, 75730 Paris Cedex 15, France. Phone: (33) (1) 40 61 56 79. Fax: (33) (1) 40 61 55 92. E-mail: cpoyart@pasteur.fr.

† Present address: Department of Biosciences, University of Kent, Canterbury, Kent CTZ NJ, England.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characters[a]	Source or reference
K. pneumoniae		
NEM865	Tic Caz Mox Cm Sm Gm Su Tmp Tc	Clinical isolate
NEM533	Nal	Spontaneous mutant of *K. pneumoniae* 5214-K (28) and this work
NEM835	Nal Tic Caz Mox	Conjugation NEM865 × NEM533
E. coli		
K802N	Nal; *hsdR*$^+$ *hsdM*$^+$ *gal met supE*	33
TG1	F′ *traD36 lacI*^{q} Δ(*lacz*)*M15proA*$^+$ *B*$^-$/*supE* Δ(*hsdM-mcrB*)5($r_k^-m_k^-$ McrB$^-$)*thi* Δ(*lac-proAB*)	11
NEM837	Nal Tic Caz Mox	Conjugation NEM865 × K802N
Plasmids		
pNEC865	Tic Caz Mox; Mob (13.5 kb)	This work
pBGS18	Km (cloning vector)	31
pBGS18Ω*bla*$_{TEM-52}$	Km Tic Caz Mox; recombinant derivative of pBGS18 carrying a 1-kb *Eco*RI-*Bam*HI fragment amplified from pNEC865	This work
pBGS18Ω*bla*$_{TEM-3}$	Km Tic Caz; recombinant derivative of pBGS18 carrying a 1-kb *Eco*RI-*Bam*HI fragment amplified from pCFF04	This work
pBGS18Ω*bla*$_{TEM-1}$	Km Tic; recombinant derivative of pBGS18 carrying a 1-kb *Eco*RI-*Bam*HI fragment amplified from pBR322	This work
pBGS18Ω*bla*$_{TEM-52-H}$	Km Tic; recombinant derivative of pBGS18 carrying the 660-bp *Eco*RI-*Pst*I fragment of pBGS18Ω*bla*$_{TEM-52}$ and the 340-bp *Pst*I fragment of pBGS18Ω*bla*$_{TEM-1}$	This work

[a] Resistance to antibiotics is denoted as follows: Tic, ticarcillin; Caz, ceftazidime; Mox, moxalactam; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Nal, nalidix acid; Su, sulfamide; Tmp, trimethoprim; and Tc, tetracycline. Mob, mobilizable.

18 h, respectively, at 65°C in 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 sodium citrate), 0.5% sodium dodecyl sulfate (SDS), and 0.05% nonfat dry milk, followed by two washings in 2× SSC–0.1% SDS at room temperature for 30 min and two washings in 0.2× SSC–0.1% SDS at 65°C for 45 min.

PCR amplification, cloning, and sequencing. The pairs of primers C1 (5′-ggGAATTCTCGGGGAAATGTGCGCGGAAC-3′) and C2 (5′-gggatccGAGTAAACTTGGTCTGACAG-3′), which delineate *bla*$_{TEM-1}$ of pBR322 (32), were used in the PCR experiments, which were carried out as described previously (25). These primers were designed to add *Eco*RI and *Bam*HI sites (bases in lowercase letters) at the 5′ and 3′ ends of the *blaT* gene, respectively. The amplicons were purified, digested with *Eco*RI and *Bam*HI, and cloned into pBGS18 digested with the same enzymes, and recombinant DNAs were introduced by transformation into *E. coli* TG1. The entire nucleotide sequences of both strands of three cloned amplicons obtained from independent PCRs were determined by using the dideoxy chain termination method of Sanger with the DYE ABI-PRISM sequencing kit on a Genetic ABI-PRISM 310 Sequencer Analyzer (Perkin-Elmer, Applied Biosystem Division, Roissy, France).

β-Lactamase preparation. Crude enzyme extracts were prepared for kinetics assays as described previously (22). The kinetic parameters were determined spectrophotometrically in sodium phosphate buffer (50 mM, pH 7.0) at 30°C with a model 550 double-beam spectrophotometer (Perkin-Elmer Corp.). One unit of β-lactamase was defined as the amount of enzyme able to hydrolyze 1.0 μmol of cephaloridine per min. Wavelengths of 233 nm for benzylpenicillin, 235 nm for ampicillin, 260 nm for cephaloridine and CAZ, and 267 nm for CTX were used. V_{max} and K_m values were calculated by computerized linear regression analysis of Woolf-Augustinsson-Hofstee plots (velocity versus velocity divided by substrate concentration). For the determination of the K_i values of MOX and the concentrations of clavulanic acid required to inhibit 50% of the β-lactamase activity (IC_{50}), the inhibitor was preincubated with the enzyme for 5 min at 30°C before the addition of the substrate. The K_i values of MOX were deduced from Dixon plots. The protein concentrations were measured by the technique of Bradford (6).

Nucleotide sequence accession number. The nucleotide sequence of *bla*$_{TEM-52}$ has been assigned EMBL accession number Y13612.

RESULTS AND DISCUSSION

Properties of *K. pneumoniae* NEM865. NEM865 was isolated in March 1996 at the Hospital Necker-Enfants Malades from the culture of a stool sample from a 2-year-old girl originating from Athens, Greece. The review of the records revealed that the patient was previously treated with CAZ for 2 weeks. A stool swab collected from this patient on admission as part of a surveillance study for multiply resistant enterobacteria grew *K. pneumoniae* NEM865. Analysis of this strain by the conventional disk diffusion antibiotic susceptibility test suggested that β-lactam resistance was due to the presence of an ESBL combined with another mechanism of resistance. No zones of inhibition were detected for penicillins, tested alone or in combination with a β-lactamase inhibitor, or for FOX or CAZ. Synergies were observed between AMX-CA and cephalosporins such as CAZ, CTX, and FEP, between AMX-CA and ATM, and more surprisingly, between AMX-CA and MOX (Fig. 1A presents some of these results). NEM865 was also resistant to chloramphenicol, gentamicin, streptomycin, sulfamethoxazole, and tetracycline (data not shown). The MICs of β-lactams for *K. pneumoniae* NEM865 indicated that this strain was resistant to all antibiotics tested except imipenem (Table 2). In order to evaluate the effects of β-lactamase inhibitors on MOX activity, CA, SUL, and TZ were combined with MOX at a final concentration of 4 μg/ml. Eightfold de-

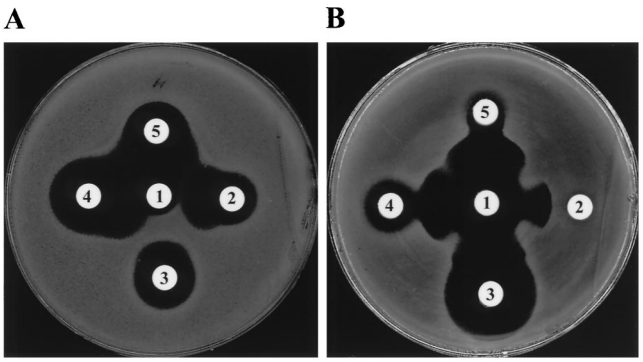

FIG. 1. Detection of the production of an ESBL by the double-disk synergy test. The bacterial strains were wild-type strain *K. pneumoniae* NEM865 (A) and *E. coli* TG1 containing pBGS18Ω*bla*$_{TEM-52}$ (B). Disks: 1, AMX-CA (AMX at 20 μg and CA at 10 μg); 2, CTX (30 μg); 3, MOX (40 μg); 4, ATM (30 μg); 5, CAZ (30 μg). Note the potentiation of cephalosporins, ATM, and MOX by CA.

TABLE 2. MICs for the strains tested

Strain	MIC (μg/ml)[a]												
	AMX	AMX + CA	TIC	TIC + CA	CF	FOX	CTX	CAZ	MOX	MOX + CA	MOX + SUL	MOX + TZ	CTT	ATM	IMP
K. pneumoniae															
NEM865	>1,024	>1,024	>1,024	512	512	64	128	256	16	2	2	2	8	32	0.5
NEM533	8	2	4	2	4	4	<0.06	0.25	0.25	0.25	0.25	0.25	0.125	0.06	<0.5
NEM835 (NEM835/pNEC865)	>1,024	16	>1,024	16	128	4	32	64	4	0.25	0.25	0.25	2	16	<0.5
E. coli															
K802N	1	1	0.5	0.5	4	4	<0.06	0.25	0.25	0.25	0.25	0.25	0.25	0.25	<0.5
NEM837 (K802N/pNEC865)	>1,024	16	>1,024	16	128	4	32	64	4	0.25	0.25	0.25	2	8	<0.5
TG1/pBGS18Ω*bla*$_{TEM-52}$	>1,024	32	>1,024	32	256	4	32	128	4	0.25	0.25	0.25	2	8	<0.5
TG1/pBGS18Ω*bla*$_{TEM-3}$	>1,024	16	>1,024	16	64	4	16	32	0.25	0.25	0.25	0.25	0.25	8	<0.5
TG1/pBGS18Ω*bla*$_{TEM-1}$	>1,024	8	>1,024	8	32	4	<0.06	0.25	0.25	0.25	0.25	0.25	0.25	0.125	<0.5
TG1/pBGS18Ω*bla*$_{TEM-52H}$	>1,024	128	>1,024	128	256	4	0.25	8	0.5	0.5	0.5	0.5	0.5	0.5	<0.5
TG1/pBGS18	2	2	1	1	4	4	<0.06	0.25	0.25	0.25	0.25	0.25	0.25	0.25	<0.5

[a] TIC, ticarcillin; CF, cephalothin; IMP, imipenem. The other abbreviations are defined in the text. CA, SUL, and TZ were each used at a final concentration of 4 μg/ml.

FIG. 2. Restriction and Southern analyses of pNEC865. Lanes 1 and 1′ and lanes 2 and 2′, *Pst*I digestion of ColE1::Tn*3* and pNEC865, respectively; lanes 3 and 3′ and lanes 4 and 4′, *Bam*HI-*Pst*I digestion of ColE1::Tn*3* and pNEC865, respectively. Plasmid ColE1::Tn*3* was used as a probe. DNA fragments internal to Tn*3* are indicated by white arrows.

creases in the MICs of MOX were obtained with the three β-lactamase inhibitors (Table 2). These results suggested that *K. pneumoniae* NEM865 produces of a novel type of ESBL which is able to hydrolyze MOX.

Genetic support of the ESBL β-lactamase gene in *K. pneumoniae* NEM865. *K. pneumoniae* NEM865 was mated with *K. pneumoniae* NEM533 and *E. coli* K802N. Transconjugants of NEM533 and K802N resistant to nalidixic acid (50 μg/ml) and CAZ (10 μg/ml) were obtained at frequencies of 3×10^{-6} and 5×10^{-5}, respectively. Resistance to chloramphenicol, gentamicin, streptomycin, sulfamethoxazole, and tetracycline were not cotransferred with CAZ resistance. The plasmid contents of NEM865 and randomly selected NEM533 and K802N transconjugants (six of each) were analyzed by agarose gel electrophoresis following digestion with *Hin*dIII (data not shown). A large plasmid (>50 kb) and a small plasmid (13.5 kb) were detected in the wild-type clinical isolate NEM865, whereas only the small replicon, designated pNEC865, was present in all the transconjugants studied (data not shown). A transconjugant of *K. pneumoniae* NEM533 and *E. coli* K802N were selected for further studies and were designated NEM835 and NEM837, respectively (Table 1). Neither of the selected *K. pneumoniae* NEM835 or *E. coli* NEM837 transconjugants was able to retransfer ($<10^{-8}$ per donor) CAZ resistance to appropriate *K. pneumoniae* or *E. coli* recipients (data not shown). These results suggest that the gene conferring CAZ resistance is carried by pNEC865 and that this plasmid could be transferred by mobilization in its original host, NEM865. Plasmid pNEC865 and ColE1::Tn*3* (12) were compared by restriction endonuclease and Southern blot analyses (Fig. 2). Tn*3* contains one *Pst*I (658 bp) and two *Pst*I-*Bam*HI (1919 and 920 bp) internal DNA fragments (13). DNA fragments of similar sizes and carrying sequences homologous to ColE1::Tn*3* were detected in pNEC865 (Fig. 2). Furthermore, partial sequencing (200 bp) of the 920-bp *Bam*HI-*Pst*I fragment of pNEC865 revealed that the segment sequenced was almost identical to the corresponding region of the Tn*3* *tnpR* gene (data not shown). These data indicate that pNEC865 harbors a Tn*3*-like element. Plasmid pNEC865 has an approximate size of 13.5 kb and carries only the β-lactamase resistance gene. These features are unusual for a plasmid encoding an ESBL, because such plasmids are usually large (≥80 kb) and carry multiple antibiotic resistance genes (17).

TABLE 3. Amino acid substitutions of TEM-type variants at critical positions

β-Lactamase	pI	Amino acid at the following position[a]:						Reference or source
		39	69	104	164	182	238	
TEM-1	5.4	Gln	Met	Glu	Arg	Met	Gly	32
TEM-2	5.6	**Lys**	Met	Glu	Arg	Met	Gly	3
TEM-3	6.3	**Lys**	Met	**Lys**	Arg	Met	**Ser**	30
TEM-15	6.0	Gln	Met	**Lys**	Arg	Met	**Ser**	20
TEM-20	5.4	Gln	Met	Glu	Arg	**Thr**	**Ser**	4
TEM-32	5.4	Gln	**Ile**	Glu	Arg	**Thr**	Gly	10, 14
TEM-43	6.1	Gln	Met	**Lys**	**His**	**Thr**	Gly	35
TEM-52	6.0	Gln	Met	**Lys**	Arg	**Thr**	**Ser**	This work
TEM-52H	—[b]	Gln	Met	**Lys**	Arg	**Thr**	Gly	This work

[a] Amino acid residues are numbered as described previously (2). Amino acids shown in boldface type represent changes from the amino acids in TEM-1 (32).
[b] —, not determined.

By using the disk diffusion test, the synergies between AMC-CA and CTX, AMX-CA and CAZ, AMX-CA and ATM, and AMX-CA and MOX observed with the wild-type strain NEM865 were also observed with NEM835 and NEM837 (data not shown). In comparison with NEM865, *K. pneumoniae* NEM835 was resistant to CTX and CAZ, with fourfold decreases in the MICs of these antibiotics. The MICs of cephamycins (FOX and CTT) and MOX were also lower, confirming that high-level resistance to cephamycins in wild-type strain NEM865 was due to the production of an ESBL combined with another mechanism of β-lactam resistance. Other enzymes (MIR-1, CMY-1, FOX-1, and LAT-1, etc.) that degrade α-methoxycephalosporins and MOX have been described previously (7). However, the β-lactam resistance conferred by these enzymes is usually not reversed by inhibitor combinations, a feature enabling the distinction between such enzymes and TEM-derived ESBLs. Therefore, the reduced susceptibility to cephamycins in the wild-type strain NEM865 was probably related to the decreased permeability of the strain for β-lactams, as described previously (21, 23). The MICs of MOX for *K. pneumoniae* NEM835 and *E. coli* NEM837 transconjugants fell into the susceptible range but remained higher than those for the recipient strains (4 versus 0.25 μg/ml). The combination of MOX with a β-lactamase inhibitor (CA, SUL, or TZ) restored the susceptibility to this antibiotic (Table 2). Interestingly, the production of this novel ESBL by *K. pneumoniae* or *E. coli* transconjugants also increased the MIC of CTT but not that of FOX. Isoelectric focusing revealed the production of a β-lactamase with a pI of 6.0 by the wild-type strain NEM865 and the transconjugants *K. pneumoniae* NEM835 and *E. coli* NEM837 (data not shown).

Cloning and sequencing of the *blaT* gene of pNEC865. A 1-kb DNA fragment amplified from pNEC865 and carrying the *blaT* gene was cloned into pBGS18, and the resulting plasmid was introduced into *E. coli* TG1. The β-lactam resistance phenotypes of the *E. coli* transformants were similar to those of the *E. coli* transconjugants (Fig. 1B and Table 2). Sequence analysis revealed that the *blaT* gene of pNEC865 differed from bla_{TEM-1} by three mutations, leading to three amino acid substitutions: Lys for Glu at position 104, Thr for Met at position 182, and Ser for Gly at position 238 (Table 3). Since the combination of these three substitutions has not been described previously, the ESBL produced by pNEC865 was designated TEM-52. TEM-52 is closely related to TEM-3, one of the first ESBLs to be described (30), and TEM-15 (20). The same critical substitutions involved in the extension of the β-lactamase spectrum were present in these enzymes at positions 104 and 238, but TEM-52 differed from TEM-3 by a Glu-to-Lys change and a Thr-to-Met change at positions 39 and 182, respectively, and from TEM-15 by a Thr-to-Met change at position 182 (Table 3).

Enzyme assays and analysis of the amino acid substitutions in TEM-52. In order to compare the kinetic constants of TEM-52 with those of TEM-1 and TEM-3, bla_{TEM-1} and bla_{TEM-3} genes were amplified, cloned into pBGS18, and introduced into *E. coli* TG1 as described above (Table 1). The cloned bla_{TEM-1} and bla_{TEM-3} genes were sequenced to verify that no misincorporation of nucleotides occurred during the PCRs. Comparison of the MICs of cephamycins and MOX for TG1 strains producing TEM-3 or TEM-52 confirmed that this novel ESBL confers resistance to CTT and MOX but not to FOX (Table 2). The K_m value and the relative rates of hydrolysis (V_{rel}) of TEM-52 were compared to those of TEM-3, which revealed that the biochemical characteristics of these two β-lactamases were similar (Table 4). It is worth noting that the K_m values of these enzymes were typical of those of ESBLs belonging to the Ser_{238} family (26). In addition, the IC_{50} of CA for TEM-52 (14 nM) and TEM-3 (10 nM) were also nearly identical. Therefore, the mutation Met_{182}→Thr does not result in K_m or IC_{50} modifications. The specific activity of TEM-52 was 3.5-fold higher that of TEM-3 when ampicillin was used as the substrate (data not shown). These differences in specific activity might be responsible for the higher MICs of β-lactams for strain TG1 producing TEM-52 compared to those for strain TG1 producing TEM-3 (Table 2). Because MOX hydrolysis was not measurable in vitro under our conditions, K_i values were determined to compare the respective affinities of TEM-52 and TEM-3 for this antibiotic. The affinity of TEM-52 for MOX was 10-fold higher than that of TEM-3 (Table 3). This increased affinity might explain the differences in the MICs of MOX for TG1 strains producing TEM-52 or TEM-3 (Table 2).

TABLE 4. Kinetic parameters of TEM-52, TEM-3, TEM-52H, and TEM-1

Substrate	TEM-52			TEM-3			TEM-52H		TEM-1	
	K_m (μM)	K_i (μM)[a]	Relative V_{max} (%)	K_m (μM)	K_i (μM)	Relative V_{max} (%)	K_m (μM)	Relative V_{max} (%)	K_m (μM)	Relative V_{max} (%)
Benzylpenicillin	5.3	—[b]	100	3.8	—	100	29	100	26	100
Ampicillin	4.1	—	66	8.2	—	126	40	113	40	122
Cephaloridine	13	—	130	19	—	120	532	95	244	25
CAZ	239	—	50	350	—	65	982	0.2	—	—
CTX	30	—	229	35	—	252	1,170	2.3	—	—
MOX	—	0.16	—	—	1.9	—	—	—	—	—

[a] K_i constants were measured with cephaloridine used as the substrate.
[b] —, not determined.

The mutation Met_{182}→Thr has been described previously in three different variants of TEM-1 (32): TEM-20, TEM-32 (IRT-3), and TEM-43 (4, 10, 14, 35). Amino acid residues Thr_{182} and Ser_{238} are found with TEM-20, residues Ile_{69} and Thr_{182} are found with TEM-32, and residues Lys_{104}, His_{164}, and Thr_{182} are found with TEM-43 (Table 3). None of these enzymes were shown to confer the resistance phenotype observed with TEM-52. We have shown that TEM-52 differed from TEM-3 and TEM-15 by two substitutions and one substitution, respectively (Table 3). The mutation at position 39, which also differentiates TEM-1 from TEM-2, has been associated only with minor changes in the substrate profile (5, 7, 9). Therefore, it is likely that the amino acid change responsible for the difference between the enzymatic profiles of TEM-52 and TEM-3 is the Met_{182}→Thr replacement.

The association of Lys_{104}, Thr_{182}, and Ser_{238} in TEM-52 is responsible for MOX hydrolysis. To investigate the role of the three mutations (Lys_{104}, Thr_{182}, and Ser_{238}) in TEM-52 involved with MOX hydrolysis, a *bla*$_{TEM-52}$–*bla*$_{TEM-1}$ hybrid gene was constructed by replacing the 340-bp *Pst*I fragment of pBGS18Ω*bla*$_{TEM-52}$ with that of pBGS18Ω*bla*$_{TEM-1}$ (Table 1). The resulting plasmid, pBGS18Ω*bla*$_{TEM-52H}$, encoded a hybrid TEM β-lactamase, designated TEM-52H, which differed from TEM-52 by a Ser_{238}→Gly change (Table 3). This enzyme, like other TEM variants containing only the Lys_{104} substitution, was unable to confer resistance to 7-oxyiminocephalosporins (Table 2) (5). The kinetic constants of this hybrid protein were similar to those of TEM-18, an ESBL containing the Lys_{104} substitution (Table 4) (26). These results suggest that the combination of Thr_{182} with Lys_{104} does not account for the extended spectrum of TEM-52. On the other hand, MOX hydrolysis by TEM-3 or TEM-15, which contains Lys_{104} and Ser_{238}, or by TEM-20, which contains Thr_{182} and Ser_{238}, has never been studied. Taken together, these results suggest that the combination of Lys_{104}, Thr_{182}, and Ser_{238} is responsible for the extended spectrum of TEM-52 and is required for MOX hydrolysis.

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}→Ser, might enlarge the active site to give enzymes with higher affinities for 7-oxyiminocephalosporins (15, 34). The Glu_{104}→Lys change, which has been observed for many ESBLs, may perturb the seryl-aspartyl-asparagyl loop and its interaction with the substrate (15). Molecular modeling studies have led to the suggestion that the presence of a threonine at position 182 probably strengthens a hydrogen bond which stabilizes the active site and, consequently, increases the catalytic activity of the enzyme (10, 18). A cooperative effect of these three mutations in TEM-52 might account for the ability of this enzyme to hydrolyze MOX.

In conclusion, these results highlight the remarkable adaptability of *K. pneumoniae* to selective antibiotic pressure. Until this report, cephamycins and MOX were considered to be stable to ESBLs and cephamycins remained an option for the treatment of infections due to organisms producing such enzymes (16, 19, 27). Further work is required to determine whether the level of resistance to MOX observed in vitro has clinical significance.

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