ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1993, p. 984–990
0066-4804/93/050984-07$02.00/0

Vol. 37, No. 5

Plasmid-Mediated AmpC-Type β-Lactamase Isolated from *Klebsiella pneumoniae* Confers Resistance to Broad-Spectrum β-Lactams, Including Moxalactam

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Received 14 May 1992/Accepted 2 March 1993

Klebsiella pneumoniae **NU2936 was isolated from a patient and was found to produce a plasmid-encoded β-lactamase (MOX-1) which conferred resistance to broad spectrum β-lactams, including moxalactam, flomoxef, ceftizoxime, cefotaxime, and ceftazidime. Resistance could be transferred from *K. pneumoniae* NU2936 to *Escherichia coli* CSH2 by conjugation with a transfer frequency of 5×10^{-7}. The structural gene of MOX-1 (bla_{MOX-1}) was cloned and expressed in *E. coli* HB101. The MIC of moxalactam for *E. coli* HB101 producing MOX-1 was >512 μg/ml. The apparent molecular mass and pI of this enzyme were calculated to be 38 kDa and 8.9, respectively. Hg^{2+} and Cu^{2+} failed to block enzyme activity, and the presence of EDTA in the reaction buffer did not reduce the enzyme activity. However, clavulanate and cloxacillin, serine β-lactamase inhibitors, inhibited the enzyme activity competitively (K_is = 5.60 and 0.35 μM, respectively). The kinetic study of MOX-1 suggested that it effectively hydrolyzed broad-spectrum β-lactams. A hybridization study confirmed that bla_{MOX-1} is encoded on a large resident plasmid (pRMOX1; 180 kb) of strain NU2936. By deletion analysis, the functional region was localized within a 1.2-kb region of the plasmid. By amino acid sequencing, 18 of 33 amino acid residues at the N terminus of MOX-1 were found to be identical to those of *Pseudomonas aeruginosa* AmpC. These findings suggest that MOX-1 is a plasmid-mediated AmpC-type β-lactamase that provides enteric bacteria resistance to broad-spectrum β-lactams, including moxalactam.**

Extended-spectrum β-lactamases that confer resistance to broad-spectrum β-lactam antibiotics have been found worldwide as a result of the increasing use of these drugs (10, 15, 16, 29, 31). They are mostly encoded by the R plasmids (5, 6, 19, 25, 44). Most of these plasmid-mediated β-lactamases belong to class A (1), and they have been grouped as TEM- or SHV-related β-lactamases on the basis of their substrate profiles, deduced amino acid sequences, isoelectric points, and DNA hybridization. However, only a few AmpC-related β-lactamases mediated by R plasmid have been reported. AmpC β-lactamases encoded by the bacterial chromosome belong to class C and are usually inducible in many gram-negative bacteria, such as *Enterobacter cloacae* (14), *Citrobacter freundii* (21), *Pseudomonas maltophilia* (*Xanthomonas maltophilia*) (32), and *Serratia marcescens* (27), and production of these enzymes results in increased resistance to broad-spectrum β-lactams (34, 36). The plasmid-encoded β-lactamase MIR-1 found in a clinical *Klebsiella pneumoniae* isolate showed homology at the nucleotide sequence level with that of *E. cloacae* AmpC, and it gave resistance to oxyimino- and α-methoxy β-lactams (28). Recently, the plasmid-mediated CMY-2 β-lactamase was found to show a high degree of homology with the chromosomal AmpC of *C. freundii* (7). Other extended-spectrum β-lactamases such as CMY-1 (8), BIL-1 (29), and FEC-1 (25) encoded by R plasmids were considered to be derivatives of AmpC-type β-lactamase. However, no amino acid or nucleotide sequences were shown in those reports. Some of these β-lactamases may belong to a TEM- or SHV-related enzyme. On the other hand, a group of chromosomal metallo β-lactamases (12), which also give resistance to broad-spectrum β-lactam antibiotics, have been found in *Bacteroides* spp. (48) and *Serratia* spp. (46), and they require Zn^{2+} for the enzyme activities. Recently, a plasmid-encoded metallo β-lactamase has also been found (43).

Here we report a plasmid-mediated AmpC β-lactamase, MOX-1, which shows significant homology in its N-terminal amino acid sequence with AmpC of *Pseudomonas aeruginosa* (23), and that the production of MOX-1 in *Escherichia coli* confers a high level of resistance to various kinds of broad-spectrum β-lactams, including moxalactam.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used for the analysis of MOX-1 are listed in Table 1. *K. pneumoniae* NU2936, which shows a high level of resistance to various broad-spectrum β-lactam antibiotics, was isolated from a patient with bladder cancer and urinary tract infection at Nagoya University Hospital in 1991. Bacteria were grown in LB broth or 2× YT medium (33) supplemented with appropriate antibiotics.

Antibiotics. Antibiotics were obtained from the following sources: ampicillin and cefminox, Meiji-seika Co., Tokyo, Japan; cephaloridine, flomoxef, and moxalactam, Shionogi Pharmaceutical Co., Osaka, Japan; chloramphenicol and cloxacillin, Sigma Chemical Co., St. Louis, Mo.; cefotaxime, Farbwerke Hoechst AG, Frankfurt, Germany; cefazolin and ceftizoxime, Fujisawa Pharmaceutical Co., Osaka, Japan; ceftazidime, Japan Glaxo Co., Tokyo, Japan; cefmetazole, Sankyo Co., Tokyo, Japan; aztreonam, Eizai Co., Ltd., Tokyo, Japan; imipenem, Banyu Pharmaceutical Co., Tokyo, Japan; rifampin, Nippon CIBA-GEIGY Co.,

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TABLE 1. Bacterial strains and plasmids used in the present study[a]

Strain or plasmid	Relevant properties	Source or reference
Strains		
K. pneumoniae NU2936	Clinical isolate; moxalactam-resistant strain, Tc^r	This study
E. coli CSH2	*metB* F$^-$ NA^r, Rif^r	T. Sawai, Chiba University
E. coli HB101	F$^-$ *hsdS20* (r$^-$ m$^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ$^-$	A. Ohta, Tokyo University
E. coli JM109	*recA1 supE44 endA1 hsdR gyrA96 relA1 thi-1* Δ(*lac pro*) F′ [*traD36 proA*$^+$ *B*$^+$ *lacI*^{q}*ZΔM15*]	4, 47
P. aeruginosa PAO1	Laboratory strain	K. Hirai, Kyorin Pharmaceutical Co., Ltd.
Plasmids		
pRMOX1	A large plasmid encoding β-lactamase MOX-1 found in *K. pneumoniae* NU2936 $bla_{MOX-1}$$^+$ Tc^r	This study
pREMNU21	A subclone carrying the bla_{MOX-1} of pRMOX1; vector = pHSG398, Cm^r	This study
pKOB5-3	A recombinant plasmid encoding a β-lactamase gene of *K. oxytoca* E23004	3
pDAB22	A recombinant plasmid encoding the *ampC* gene of *E. coli* K-12 strain D21	3
pHSG398	A cloning vector; Cm^r	41
pBR322	Ap^r (TEM-1) Tc^r	40
pKK232-8	Δ(*cat-pro*), Ap^r	9
pBluescript II SK$^+$	A cloning vector, Ap^r	Stratagene

[a] Cm, chloramphenicol; Tc, tetracycline; Ap, ampicillin; NA, nalidixic acid; Rif, rifampicin; bla_{MOX-1}, the structural gene of MOX-1.

Hyogo, Japan; clavulanate, Beecham Pharmaceuticals, Brentford, United Kingdom; and cefotetan, Yamanouchi Pharmaceutical Co., Tokyo, Japan.

Susceptibility testing. MICs were determined by the agar dilution method with Mueller-Hinton II agar (BBL Microbiology Systems, Cockeysville, Md.) containing graded concentrations of antibiotics. A 5-μl portion of an inoculum (approximately 10^6 CFU/ml) cultured to the logarithmic phase of growth in Mueller-Hinton broth (BBL) was applied. Plates were incubated overnight at 37°C.

Transfer of resistance. Tests for the transfer of the resistance were carried out by using *E. coli* CSH2 as the recipient. After conjugation for 120 min at 37°C, transconjugants were selected on LB agar plates supplemented with rifampin (100 μg/ml), nalidixic acid (50 μg/ml), and moxalactam (30 μg/ml). The frequency of transfer was expressed relative to the number of donor cells.

Purification of MOX-1 and AmpC of *P. aeruginosa* PAO1. Bacteria were grown overnight in 500 ml of LB broth with shaking at 37°C. The cells were harvested by centrifugation and were suspended in 10 ml of 50 mM sodium phosphate buffer (pH 7.0). For preparation of cell extracts, the cells were ruptured by ultrasonication at 4°C. Cell debris was removed by centrifugation at 40,000 × *g* for 2 h. The clear supernatant was used to study the effects of metal ions and EDTA on the β-lactamase activity. The crude enzyme extracts were chromatographed through a Sephadex G-75 (Pharmacia, Uppsala, Sweden) column preequilibrated with 50 mM phosphate buffer (pH 7.0). The eluent was chromatographed again through a DEAE-Sephacel (Pharmacia) column preequilibrated with 50 mM phosphate buffer (pH 7.0). The same buffer was used for elution. Fractions which showed enzyme activity were pooled and concentrated by ultrafiltration with Centricut 10 (Kurabo Co., Osaka, Japan). The purity of the preparation was confirmed by high-pressure liquid chromatography gel filtration as reported previously (3).

Kinetic study of MOX-1 and *P. aeruginosa* AmpC. Purified MOX-1 and AmpC of *P. aeruginosa* were assayed against various β-lactam substrates at 30°C in 50 mM phosphate buffer (pH 7.0) by using an autospectrophotometer (UV2200, Shimadzu, Kyoto, Japan). The absorption maxima of the substrates used were as follows: ampicillin, 235 nm; aztreonam, 250 nm; cefminox, 275 nm; cefmetazole, 285 nm; cefotaxime, 264 nm; cefotetan, 295 nm; ceftazidime, 272 nm; ceftizoxime, 250 nm; cephaloridine, 295 nm; and moxalactam, 274 nm. The molar extinction coefficients were calculated by the method of Seeberg et al. (36). K_m and V_{max} values were obtained by a least-squares fit to plots of the initial steady-state velocities at different substrate concentrations. Relative V_{max} and relative V_{max}/K_m values were calculated for comparison of enzyme activities, as recommended by Bush and Sykes (11).

Molecular mass determination and isoelectric focusing. Molecular mass was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the purified enzyme preparation. Isoelectric focusing of the enzyme was done with Multiphor II (Pharmacia-LKB) by using Ampholine-gel (Pharmacia-LKB), with a pH range of 3.5 to 9.5. The pH gradient was determined with an isoelectric focusing calibration kit (pH 3 to 10; Pharmacia).

Inhibition test of MOX-1 by Hg^{2+}, Cu^{2+}, EDTA, clavulanate, and cloxacillin. The inhibitory effect of Hg^{2+}, Cu^{2+}, EDTA, clavulanate, or cloxacillin for MOX-1 was tested by the method of Watanabe et al. (43). A solution of enzyme was preincubated with an inhibitor in 50 mM phosphate buffer (pH 7.0) at 30°C for 5 min, and the remaining activity was assayed spectrophotometrically with 100 μM cephaloridine as the substrate.

DNA techniques. Plasmid DNA was prepared by the rapid alkaline extraction method and was purified by low-melting-temperature agarose (agarose-L; Nippon Gene Co., Ltd., Toyama, Japan) gel electrophoresis and then electroelution. *K. pneumoniae* NU2936 and *E. coli* CSH2 harboring pRMOX1 were examined for large plasmids by the methods of Kado and Liu (18). Restriction endonucleases, exonucle-

TABLE 2. Antibiotic susceptibilities of *K. pnumoniae* NU2936, transconjugant, and *E. coli* HB101 (pREMNU21)

Antibiotic	MIC (μg/ml) for:			
	K. pneumoniae NU2936	*E. coli* CSH2(pRMOX1)	*E. coli* HB101	*E. coli* HB101(pREMNU21)
Cefazolin	>512	>512	2	>512
Cephaloridine	128	128	4	128
Cefotaxime	512	512	<0.1	>512
Ceftazidime	64	32	0.5	16
Ceftizoxime	>512	256	<0.1	512
Cefmetazole	>512	256	0.5	512
Cefminox	>512	512	0.5	>512
Cefotetan	>512	512	<0.1	>512
Moxalactam	>512	512	<0.1	>512
Flomoxef	256	128	<0.1	256
Ampicillin	>512	>512	4	>512
Aztreonam	32	16	0.5	16
Imipenem	0.5	0.5	0.5	0.5

ase III, mung bean nuclease, Klenow fragment, and T4 DNA ligase were obtained from Nippon Gene Co., Ltd. Deletion analysis was done according to the manufacturer's (Nippon Gene Co., Ltd.) recommendation. Recombinant plasmids were transformed into *E. coli* JM109. As a hybridization probe, the 3.0-kb *Pst*I DNA fragment of pREMNU21 was excised from a low-melting-temperature agarose gel after electrophoresis and was labeled with [α-^{32}P]dCTP by using a multi-prime-labeling kit (Nippon Gene Co., Ltd.). Plasmid DNA was blotted onto nylon membranes (Hybond-N; Amersham, Buckinghamshire, United Kingdom) by the method of Southern (38). DNA hybridization was achieved as described elsewhere (3).

Determination of amino acid sequence at the N terminus of MOX-1. Thirty-three amino acid residues in the N terminus of MOX-1 were sequenced by a peptide sequencer model 473A system (ABI) by using the purified enzyme preparation.

RESULTS

Plasmid pRMOX1 harbored by a clinical isolate of *K. pneumoniae* NU2936. *K. pneumoniae* NU2936 was isolated from a patient with bladder cancer who suffered from urinary tract infection and who had been treated with various β-lactam antibiotics (moxalactam, cefmetazole, and piperacillin). This strain showed resistance to these antibiotics (Table 2). Two large plasmids were found in this strain. By conjugation experiments, the larger plasmid, pRMOX1 (180 kb), was transferred into *E. coli* CSH2 at a low frequency (5×10^{-7}), with the simultaneous transfer of resistance to tetracycline (Fig. 1; Table 2). Transconjugant *E. coli* CSH2(pRMOX1) was highly resistant to a variety of broad-spectrum β-lactams, including moxalactam, cefotaxime, ceftazidime, and ceftizoxime. However, the transconjugant as well as *K. pneumoniae* NU2936 were susceptible to imipenem.

Cloning of the bla_{MOX-1} gene. *Pst*I-digested fragments of pRMOX-1 were ligated into the polylinker site of pHSG398. These recombinants were introduced into *E. coli* HB101, and transformants showing resistance to both moxalactam and chloramphenicol were isolated. A 5.2-kb derivative containing a 3.0-kb insert was termed pREMNU21 and was mapped with various restriction enzymes (Fig. 2). *E. coli* HB101 harboring pREMNU21 showed resistance to β-lactams similar to that observed in *K. pneumoniae* NU2936. The transcriptional direction of the bla_{MOX-1} gene was determined by using the pKK232-8 promoter selection vector, which is a pBR322 derivative containing a promoterless chloramphenicol acetyltransferase (CAT) gene. Only a recombinant of pKK232-8 carrying the 0.9-kb *Pst*I-*Bam*HI fragment of pREMNU21 showed CAT activity, so the tran-

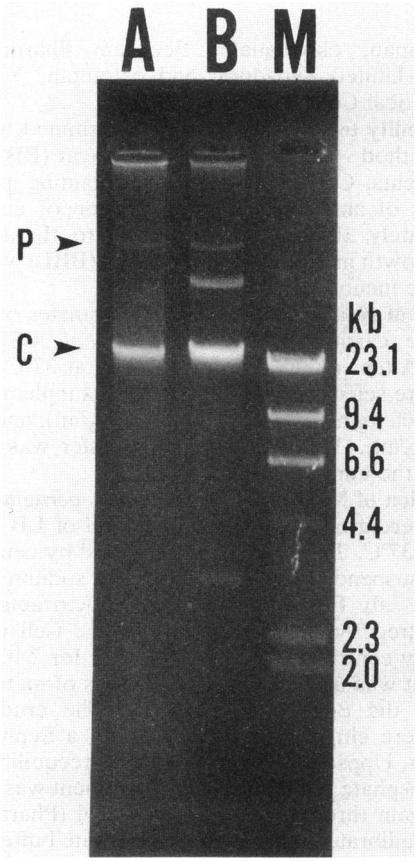

FIG. 1. Agarose gel electrophoresis of large plasmids of *K. pneumoniae* NU2936 and its transconjugant *E. coli* CSH2(pRMOX1). Lane A, plasmid pRMOX1 transferred from *K. pneumoniae* NU2936 to *E. coli* CSH2 (the pRMOX1 and chromosomal DNAs are indicated by P and C, respectively); lane B, plasmids of *K. pneumoniae* NU2936, including pRMOX1, which are indicated by the arrowheads; lane M, *Hin*dIII-digested bacteriophage lambda DNA.

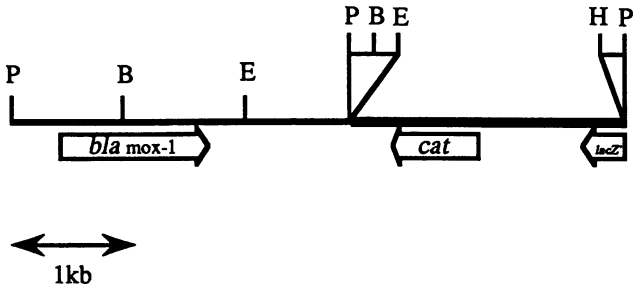

FIG. 2. Restriction map of recombinant plasmid pREMNU21. The direction and length of the β-lactamase gene bla_{MOX-1} were determined. Arrows indicate the extent and transcriptional direction of bla_{MOX-1}, *cat*, and *lacZ'*. The thick line represents the vector pHSG398, and the thin line represents the insert. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; bla_{MOX-1}, structural gene of β-lactamase MOX-1; *cat*, chloramphenicol acetyltransferase; *lacZ'*, 3'-truncated *lacZ* (β-galactosidase gene).

scriptional direction was determined as shown in Fig. 2. This shows that the transcription of bla_{MOX-1} is not under the control of the *cat* promoter of pHSG398. To determine the coding region of MOX-1 on pREMNU21, the 3.0-kb *Pst*I fragment of pREMNU21 was transferred into the polylinker site of pBluescript II SK$^+$. The position of bla_{MOX-1} on pREMNU21 was localized within a 1.2-kb region by deletion analysis with exonuclease III, mung bean nuclease, and the Klenow fragment. The 3.0-kb *Pst*I fragment of pREMNU21 was used as a DNA probe for confirming the origin of the bla_{MOX-1} gene. We achieved the hybridization under moderate stringency conditions, by which 60 to 100% homology could be detected. The bla_{MOX-1} gene probe hybridized with a resident large plasmid, pRMOX1, of *K. pneumoniae* NU2936. However, no significant hybridization was observed with the chromosomal DNA of the parental strain (data not shown). Moreover, the probe did not hybridize with either the plasmid encoding the *bla* gene of *K. pneumoniae* LEN-1 or the plasmid carrying *ampC* of *E. coli* K-12. Additionally, the probe did not hybridize with the chromosomal DNAs of *P. aeruginosa* PAO1, *Proteus vulgaris*, *S. marcescens*, and *K. pneumoniae*, nor did the bla_{MOX-1} probe hybridize with pBR322 (40) carrying the TEM-1 β-lactamase gene (data not shown).

Purification of MOX-1 and kinetic study. The pREMNU21-encoded MOX-1 was purified from the cell extracts as described in Materials and Methods. Sixty milligrams (dry weight) of purified MOX-1 was harvested from 1,000 ml of bacterial culture. The purified enzyme protein appeared as a single band in SDS-PAGE by Coomassie blue staining (Fig. 3). The final purity of enzyme was considered to be greater than 95%. The apparent molecular mass and pI of MOX-1 were 38 kDa and 8.9, respectively. The kinetic parameters (K_m and relative V_{max}) and hydrolytic efficiency (V_{max}/K_m) of MOX-1 are given in Table 3. Hydrolytic efficiency was greater for moxalactam, although the V_{max} value for moxalactam was lower than those for the other substrates tested. This may be because of the higher affinity (lower K_m) of MOX-1 to moxalactam. Cefotaxime was a poorer substrate than cephaloridine, although its MICs were greater. The kinetic parameters and hydrolytic efficiency of *P. aeruginosa* AmpC are also shown in Table 3. The substrate profiles of MOX-1 were similar to those of *P. aeruginosa* AmpC for the five substrates tested.

Inhibition analysis of MOX-1 activity. The presence of

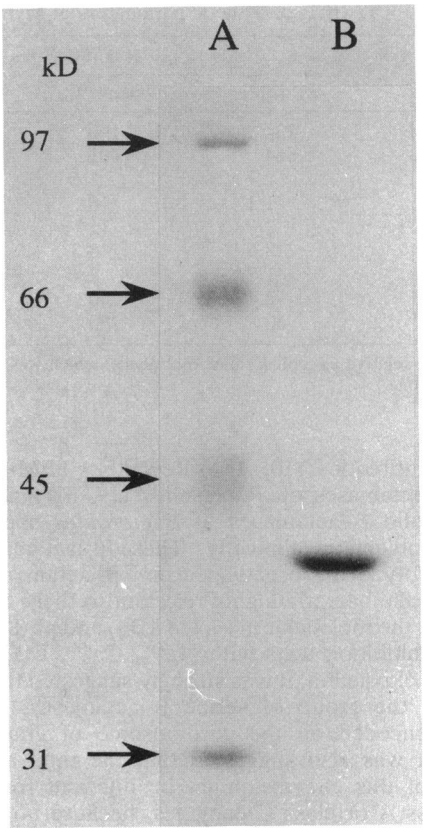

FIG. 3. SDS-PAGE of purified MOX-1. Lanes: A, protein molecular mass standards; B, purified β-lactamase MOX-1. The purified MOX-1 was stained as a single band by Coomassie blue staining.

Hg^{2+} or Cu^{2+} ion in the reaction buffer did not reduce the MOX-1 activity, and the addition of 50 mM EDTA did not result in a reduction in enzyme activity. On the other hand, clavulanate and cloxacillin, which are serine β-lactamase inhibitors, inhibited MOX-1 activity. The K_i values of clavulanate and cloxacillin to cephaloridine were calculated to be 5.60 and 0.35 μM, respectively.

Amino acid sequence of MOX-1. The sequence of 33 amino acid residues at the N terminus of MOX-1 was determined and compared with those of the other AmpC enzymes (Fig. 4). It showed significant homology with the chromosomal AmpC β-lactamases of gram-negative rods. Among them, the greatest homology was observed with the AmpC of *P. aeruginosa* (23). Eighteen of 33 amino acid residues of MOX-1 were identical to those of AmpC of *P. aeruginosa*, and at least 5 residues were substituted conservatively (Fig. 4).

DISCUSSION

The most prominent mechanism of resistance to β-lactam antibiotics in clinical gram-negative bacterial isolates is the production of β-lactamases. Many kinds of improved β-lactam antibiotics which tolerate the β-lactamases of gram-negative bacteria have been developed for clinical uses. However, many extended-spectrum β-lactamases also have appeared to enable bacteria to survive under conditions resulting from the use of a large amount of broad-spectrum

TABLE 3. Kinetic parameters of MOX-1 and AmpC of *P. aeruginosa* PAO1 for each β-lactam antibiotic

Substrate	MOX-1			AmpC of *P. aeruginosa* PAO1		
	K_m (μM)	Relative V_{max}[a]	Relative V_{max}/K_m[a]	K_m (μM)	Relative V_{max}[a]	Relative V_{max}/K_m[a]
Cephaloridine	134	100	100	85	100	100
Cefotaxime	1,064	201	25	ND[b]	ND	ND
Ceftazidime	2.7	1.5	75	4.5	0.45	8.5
Cefmetazole	1.1	10	793	1.1	1.4	108
Cefminox	3.8	11	234	ND	ND	ND
Cefotetan	8.5	8.3	83	ND	ND	ND
Moxalactam	1.7	2.4	187	3.5	2.1	51
Ampicillin	2.4	40	2,180	2.1	70	2,833
Aztreonam	40	80	267	ND	ND	ND

[a] Values are relative to cephaloridine hydrolysis, which was equal to 100.
[b] ND, not done.

β-lactam antibiotics (10, 15). Recently, TEM- or SHV-related β-lactamases on R plasmids (19, 31) and chromosomal metallo β-lactamases of *Bacteroides* spp. (12, 48) became problematic clinically. These β-lactamases effectively hydrolyze the broad-spectrum β-lactam antibiotics, and they make bacteria highly resistant to these agents. On the basis of the molecular mass (38 kDa) and pI (8.9) and the results of inhibition tests with Hg^{2+}, Cu^{2+}, EDTA, clavulanate, and cloxacillin, it was strongly suggested that MOX-1 belongs to the group of serine β-lactamases, which are generally encoded on the chromosome of gram-negative bacteria. It was also suggested that the total amino acid sequence of this enzyme might be different from that of known class A or class C enzymes, because no significant hybridizations with a TEM-related β-lactamase gene or *E. coli ampC* (17) were detected by Southern hybridization with the bla_{MOX-1} probe. Although the plasmid-mediated MIR-1 β-lactamase found in *K. pneumoniae* showed nucleotide sequence homology with the chromosomal *ampC* of *E. cloacae* (13, 28), MOX-1 showed a rather closer relationship with the chromosomal AmpC of *P. aeruginosa* (23) by amino acid sequence analysis at the N terminus. Recently, CMY-2 was found to show a high degree of homology with the AmpC-type β-lactamases of *C. freundii* (7). However, as far as we know, this is the first report of a plasmid-mediated AmpC-type β-lactamase, suggesting a close evolutionary relationship with the chromosomal AmpC of *P. aeruginosa*, which confers a high level of resistance to various broad-spectrum β-lactams, including moxalactam.

		10	20	30	
K. pneumoniae	MOX-1	GEASPVDPLR	PVVDASIQPL	LKEHRIPGMA	VAV
		*** *.*.*.	..***..**.	.*.. ***.*	**.
P. aeruginosa	AmpC	GEA-PADRLK	ALVDAAVQPV	MKANDIPGLA	VAI
		* ..*	**...* **	.*....****	***
E. cloacae	AmpC	TPVSE-KQLA	EVVANTITPL	MKAQSVPGMA	VAV
		. ....	.*. .* **	.....*****	***
E. coli	AmpC	AP——QQIN	DIVHRTITPL	IEQQKIPGMA	VAV
		. ..	. ** ****	.*.. .****	.**
S. marcescens	AmpC	A——QQ-Q	DI-DAVIQPL	MKKYGVPGMA	IAV
		.. . ...	.*. .* **	..*. *****	**.
C. freundii	AmpC	AAKTE-QQIQ	DIVNRTITPL	MQEQAIPGMA	VAI

FIG. 4. Comparison of the amino acid sequences at the N termini of the MOX-1 and chromosomal AmpC β-lactamases of gram-negative rods. The sequence of 33 amino acid residues at the N terminus of MOX-1 of *K. pneumoniae* NU2936 was compared with that of each chromosomal AmpC β-lactamase by using the GENETYX system (SDC Co., Ltd., Tokyo, Japan). Amino acid residues identical to those of MOX-1 are marked in each chromosomal AmpC with asterisks just above corresponding residue, and conservative substitutions are marked with periods. The sequences of amino acid residues of each AmpC were from the indicated references: *P. aeruginosa* AmpC (23), *E. cloacae* AmpC (13), *E. coli* AmpC (17), *S. marcescens* AmpC (24), and *C. freundii* AmpC (22).

Although MOX-1 showed a closer relationship to the chromosomal AmpC of *P. aeruginosa* PAO1 than to those of enteric bacteria (17, 22, 27, 45), the bla_{MOX-1} probe did not hybridize with the chromosomal *ampC* gene of *P. aeruginosa* PAO1. This might be because of the stringency of DNA hybridization by which homology only greater than 60% can be detected. Taking the amino acid sequence at the N terminus into consideration, it is speculated that the total DNA homology between bla_{MOX-1} and *ampC* of *P. aeruginosa* PAO1 may be less than 60%. Presumably, this is why no significant hybridization was detected in this case.

It has been shown by DNA analysis that many plasmid-mediated extended-spectrum β-lactamases belong to the TEM- or SHV-related β-lactamases (15). Clinical *K. pneumoniae* isolates show moderate resistance to ampicillin because of the constitutive production of the chromosomal penicillinase (24, 35). Arakawa et al. (2) demonstrated a close evolutionary relationship between plasmid-encoded penicillinases (TEM type) and the chromosomal β-lactamase of *K. pneumoniae* LEN-1. It was later found that SHV-1 also shows a high degree of homology with the chromosomal β-lactamase of *K. pneumoniae* LEN-1 (26). Arakawa et al. (3) also reported that a clinical isolate of *K. oxytoca* E23004 has a chromosomal class A β-lactamase which confers bacterial resistance to a broad-spectrum β-lactam, cefoperazone. Recently, a plasmid-mediated MEN-1 was found to show a close relationship to the chromosomal β-lactamase of *Klebsiella oxytoca* (5). It was therefore suggested that the chromosomal β-lactamase gene of *K. pneumoniae* might be the origin of the plasmid-mediated extended-spectrum β-lactamases. Many plasmid-mediated TEM- or SHV-related β-lactamases have been found in *K. pneumoniae*. However, it remains unknown why only a few plasmid-mediated AmpC-type β-lactamases have been found. It may be because of differences between the class A and class C β-lactamases in regulating gene expression. Although expression of the chromosomal *bla* gene in *K. pneumoniae* is constitutive, those of chromosomal *ampC* genes of *E. cloacae*, *C. freundii*, *S. marcescens*, and *P. aeruginosa* were inducible under the regulation of AmpD, AmpR, and AmpG (20, 21). In the present study, we found that bla_{MOX-1} encodes AmpC-type β-lactamase and is expressed constitutively in *E. coli* HB101. Deletion analysis showed that the

region upstream of the structural gene bla_{MOX-1} on pREMNU21 was about 0.6 kb, so *ampR* cannot be encoded in this region. Therefore, the nucleotide sequence in the promoter region of bla_{MOX-1} may well be quite different from that of the inducible chromosomal *ampC* genes of gram-negative bacteria.

ACKNOWLEDGMENTS

This work was supported by a grant for scientific research from Yault Co., Ltd., Tokyo, Japan.

We thank K. Hirai for providing *P. aeruginosa* PAO1.

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