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Novel Plasmid-Mediated β-Lactamase (MIR-1) Conferring Resistance to Oxyimino- and α-Methoxy β-Lactams in Clinical Isolates of *Klebsiella pneumoniae*

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Klebsiella pneumoniae **isolates from 11 patients at the Miriam Hospital were identified as resistant to cefoxitin and ceftibuten as well as to aztreonam, cefotaxime, and ceftazidime. Resistance could be transferred by conjugation or transformation with plasmid DNA into *Escherichia coli* and was due to the production of a β-lactamase with an isoelectric point of 8.4 named MIR-1. In *E. coli*, MIR-1 conferred resistance to aztreonam, cefotaxime, ceftazidime, ceftibuten, ceftriaxone, and such α-methoxy beta-lactams as cefmetazole, cefotetan, cefoxitin, and moxalactam. In vitro, MIR-1 hydrolyzed cephalothin and cephaloridine much more rapidly than it did penicillin G, ampicillin, or carbenicillin. Cefotaxime was hydrolyzed at 10% the rate of cephaloridine. Cefoxitin inactivation could only be detected by a microbiological test. The inhibition profile of MIR-1 was similar to that of chromosomally mediated class I β-lactamases. Potassium clavulanate had little effect on cefoxitin or ceftibuten resistance and was a poor inhibitor of MIR-1 activity. Cefoxitin or imipenem did not induce MIR-1. The gene determining MIR-1 was cloned on a 1.4-kb *AccI-PstI* fragment. Under stringent conditions, probes for TEM-1 and SHV-1 genes and the *E. coli ampC* gene failed to hybridize with the MIR-1 gene. However, a provisional sequence of 150 bp of the MIR-1 gene proved to be 90% identical to the sequence of *ampC* from *Enterobacter cloacae* but only 71% identical to that of *E. coli*, thus explaining the lack of hybridization to the *E. coli ampC* probe. Plasmid profiles of the 11 *K. pneumoniae* clinical isolates were not identical, but each contained a plasmid from 40 to 60 kb that hybridized with the cloned MIR-1 gene. Both transfer-proficient and transfer-deficient MIR-1 plasmids belonged to the N incompatibility group. Thus, the resistance of these *K. pneumoniae* strains was the result of plasmid acquisition of a class I β-lactamase, a new resistance determinant that expands the kinds of beta-lactam resistance capable of spread by plasmid dissemination among clinical isolates.**

Plasmid-mediated extended-spectrum β-lactamases conferring resistance to broad-spectrum cephalosporins but not to cephamycins were first detected in gram-negative bacilli in Europe in 1983 (19, 20) and have since become more common. Some primarily hydrolyze cefotaxime (45), and others primarily hydrolyze ceftazidime (40). Beta-lactam antibiotics with an α-methoxy substituent in the C7 position such as cefoxitin, cefotetan, or moxalactam have been resistant to hydrolysis by plasmid-mediated β-lactamases (13). However, chromosomal β-lactamases produced by bacilli such as enterobacters, serratiae, citrobacters, and morganellae are able to hydrolyze cephamycins and, if produced in sufficient amount, can confer resistance to these antibiotics (43). Recently, the plasmid-mediated β-lactamase CMY-1 produced by a wound isolate of *Klebsiella pneumoniae* from a patient in South Korea has been reported to confer resistance to cefoxitin (2).

In this report we describe a novel plasmid-mediated β-lactamase, MIR-1, with an isoelectric point (pI) of 8.4 which was found in 11 clinical isolates of *K. pneumoniae* and subsequently cloned into *Escherichia coli*. MIR-1 conferred resistance to penicillins and broad-spectrum cephalosporins, including cefoxitin and ceftibuten, but not to cefepime, cefpirome, meropenem, or imipenem. This pattern of resistance was similar to that determined by chromosomally-encoded β-lactamases. The substrate and inhibition profiles of MIR-1 were compared with those of class A TEM-1 and TEM-6 plasmid-mediated β-lactamases and with those of class C chromosomal β-lactamases from *E. coli* (pI 8.5) and from *Enterobacter cloacae* P99 (pI 7.66). MIR-1 resembled the chromosomal β-lactamases in biochemical properties, and the cloned gene resembled the *ampC* gene of *E. cloacae* in sequence.

This is the first documented example of plasmid-mediated cephalosporinase production by multiple nosocomial isolates. The presence of such a resistance determinant on a plasmid raises concern for rapid dissemination among gram-negative bacilli and possible loss of effectiveness of α-methoxy beta-lactams.

MATERIALS AND METHODS

Bacterial strains. Strains and plasmids used are listed in Table 1. Between 6 September 1988 and 30 May 1989, clinical isolates of *K. pneumoniae* at the Miriam Hospital that were resistant to cefoxitin and ceftibuten and intermediate in resistance or resistant to cefotaxime and ceftazidime were saved for further study. Strains were identified by the API 20E system (Analytab Products, Plainview, N.Y.). Susceptibility testing was performed by Kirby-Bauer diffusion assay on cation-supplemented Mueller-Hinton agar by using standard commercial disks with or without 10 μg of supplemental potassium clavulanate.

Antibiotics. Antibiotics used were as follows: cefsulodin (Abbott Laboratories, Chicago, Ill.); nitrocefin (BBL Microbiology Systems, Cockeysville, Md.); amoxicillin, potas-

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties	Reference or source
E. coli		
C600	F⁻ *lacY leu thr thi*	25
HB101	F⁻ *lacY leu pro thi recA rpsL20*	25
J53-2	F⁻ *met pro* Rif^r	7
J53(R6K)	TEM-1 producer	M. Matthew
J53(R1010)	SHV-1 producer	32
87120702	AmpC hyperproducer	G. Miller
A15R$^+$(HB114-5748)	TEM-6 producer	A. Bauernfeind
E. cloacae		
P99	Prototype strain	Glaxo
55M	P99 hyperproducer	9
75043003	Inducible P99 producer	G. Miller
K. pneumoniae 45C, 96D, 244C, 258B, 265D, 280B, 561E, 636C, 758B, 834C, 844C, 928B, 1162D, 1293D, 1429D	Cefoxitin-resistant clinical isolates	This study
Plasmids		
R1	Ap (TEM-1) Cm Km Sm Su	12
R269N-1	Ap (TEM-1) Km Sm Tc IncN	12
pBGS131	Km M13 mcr[a]	35
pBR322	Ap (TEM-1) Tc	25
pMG226	Ap (TEM-6) Cm Km Sm	13
pMLC28	Cm M13 mcr[b]	B. Seed
pMON38	Ap (SHV-1) Cm	11
pNU81	Ap (AmpC) Km	16

[a] Multiple cloning region from M13tg131.
[b] Multiple cloning region from pUC12 and pUC13 (26).

sium clavulanate, and temocillin (Beecham Laboratories, Bristol, Tenn.); cefepime (Bristol-Myers Co., Wallingford, Conn.); ceftazidime and cefuroxime (Glaxo, Greenford, England); cefotaxime and cefpirome (Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J.); carumonam and ceftriaxone (Hoffmann-La Roche, Inc., Nutley, N.J.); cefotetan and meropenem (ICI Pharmaceuticals, Wilmington, Del.); tazobactam (Lederle Laboratories, Pearl River, N.Y.); moxalactam (Eli Lilly, Indianapolis, Ind.); cefoxitin and imipenem (Merck Sharp & Dohme, West Point, Pa.); carbenicillin, cefoperazone, and sulbactam (Pfizer, Inc., New York, N.Y.); ceftibuten (Schering Corporation, Bloomfield, N.J.); cephaloridine, cephalothin, cloxacillin, and penicillin G (Sigma Chemical Company, St. Louis, Mo.); ceftizoxime (Smith Kline & French Laboratories, Philadelphia, Pa.); ampicillin trihydrate and aztreonam (E.R. Squibb & Sons, Princeton, N.J.); cefotiam (Takeda Chemical Industries, Ltd., Osaka, Japan); and cefmetazole (The Upjohn Co., Kalamazoo, Mich.).

Disks containing antibiotics were obtained from BBL except for those containing cefpirome (Hoechst-Roussel), ceftibuten (Schering), and moxalactam (Eli Lilly).

Susceptibility testing. MICs were determined by agar dilution by using Trypticase soy agar plates (BBL Microbiology Systems) containing graded concentrations of antibiotics and an inoculum of 10^4 to 10^5 organisms per spot applied by a replica-plating device (13). Plates were incubated overnight at 37°C.

Analytical isoelectric focusing. Crude sonic extracts were applied to a polyacrylamide gel with pH range 3.5 to 10 as described by Matthew et al. (31). Differential isoelectric focusing was performed with an overlay of filter paper soaked in a solution containing 0.75 mg of nitrocefin per ml plus 5 mg of cefoxitin per ml.

β-Lactamase assays. Alkalimetric assays were conducted by the method of Labia et al. (22) by using sonic extracts without further purification, as described previously (50). The titrant was 0.005 N NaOH, and the reaction mixture contained 500 μM of substrate dissolved in 85 mM NaCl buffer. NaOH consumption with time was monitored at 37°C with a pH stat radiometer comprising a pHM82 pH meter, a TTT 80 titrator, and an ABU 80 autoburette (Radiometer, Copenhagen, Denmark). Spontaneous hydrolysis of beta-lactams was controlled by using reaction mixtures without enzyme.

Microbiological assay. To determine beta-lactam inactivation by crude sonic extracts, the microbiological assay described by Masuda et al. (29) was used. The indicator strain was *Micrococcus luteus* ATCC 9341 or *Escherichia coli* ATCC 25922 (for testing aztreonam). Inhibitory zones to commercial cefoxitin or cefotaxime disks were determined for *M. luteus* by Kirby-Bauer agar diffusion on Mueller-Hinton agar. Blank disks were impregnated with 15 μl of β-lactamase standardized for approximately the same activity against nitrocefin. The preparations were applied undiluted or diluted 1:2, 1:4, or 1:8. Disks were placed on seeded Mueller-Hinton agar plates at a distance from the cefoxitin disk such that they would be at the inner periphery of the expected zone. Plates were read after overnight incubation at 37°C. Inactivation of cefoxitin by β-lactamase was detected as growth of the indicator strain within the expected cefotaxime or cefoxitin zone of inhibition.

Induction of β-lactamase. Cefoxitin (10 μg/ml) or imipenem (0.5 μg/ml) was added to log-phase broth cultures of *K. pneumoniae* 96D and *E. coli* C600(pMG231) to test induction. After 3 h of growth at 37°C with shaking, sonic extracts were prepared. Cells grown without inducer served as controls. β-Lactamase activity was determined from the slope of nitrocefin hydrolysis measured spectrophotometrically. *E. cloacae* P99, a known inducible strain, was tested similarly as a control.

Inhibition studies. Spectrophotometric studies for determination of the concentrations required to inhibit 50% of the β-lactamase activity (I_{50}s) were performed on a Shimadzu UV260 spectrophotometer. Assays were carried out by a modification of the procedure of O'Callaghan et al. (37). The reaction mixture contained 100 μM nitrocefin dissolved in 0.1 M phosphate buffer, pH 7.0, at 37°C. Enzyme preparations were standardized to achieve similar rates of hydrolysis of nitrocefin. Enzyme and inhibitor were preincubated in equal volumes for 5 min before being added to the cuvette. Activity was calculated from initial linear hydrolysis rates measured at 482 nm. The concentration required to inhibit 50% of the β-lactamase activity was derived from linear regression analysis.

Nitrocefin competition assay. Inhibition profiles to 18 competing substrates or inhibitors were determined by a nitrocefin competition assay, as recently described (39).

Genetic studies. *Klebsiella* isolates were mated with *E. coli* J53-2 in L broth overnight at both 30 and 37°C with selection of transconjugants on plates containing 50 μg of ampicillin or cefoxitin per ml by using 100 μg of rifampin per ml for

counterselection (34). Plasmid DNA, prepared by the procedure of Takahashi and Nagano (49), was used to transform *E. coli* C600 to cefoxitin resistance by the technique of Lederberg and Cohen (23). Transconjugants or transformants were characterized by spot testing on plates containing antibiotics or $HgCl_2$ (42). Incompatibility matings were performed as previously described (34). Plasmid molecular weights were estimated by agarose gel electrophoresis in comparison with standard plasmids of known size (49). Tests with bacteriophage IKe, which propagates on bacteria carrying a plasmid of the N incompatibility group (IncN) (18), were performed on R medium agar with R-top agar overlays (36).

DNA techniques. Restriction endonucleases and T4 DNA ligase were obtained from New England BioLabs, Inc. (Beverly, Mass.), and were used as recommended by the manufacturer. Cloning and subcloning were done as previously described (25).

Plasmid DNA was transferred to nitrocellulose or nylon filters by using the method of Southern (47). β-Lactamase probes were labeled with digoxigenin as described by the manufacturer (Boehringer-Mannheim, Indianapolis, Ind.). Test fragments included a 424-bp *Bgl*I-*Hin*cII fragment of pBR322 internal to the TEM-1 β-lactamase gene, a 150-bp *Pvu*II fragment of pMON38 internal to the SHV-1 β-lactamase gene, and a 685-bp *Pst*I-*Xho*I fragment of pNU81 internal to the *E. coli ampC* gene (11, 15). Hybridization was performed under stringent conditions by using solutions containing various concentrations of SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7.0]) with or without sodium dodecyl sulfate. Hybridization with labeled probe took place in 5× SSC at 68°C for at least 6 h followed by washing for 5 min twice in 2× SSC–0.1% (wt/vol) sodium dodecyl sulfate at room temperature and by washing for 15 min twice in 0.1× SSC–0.1% sodium dodecyl sulfate at 68°C.

TABLE 2. Susceptibility of *E. coli* C600 derivatives to broad-spectrum beta-lactams by agar dilution assay

Antibiotic	MIC (μg/ml) for *E. coli* C600 carrying plasmid encoding indicated β-lactamase			
	R⁻[a]	R1 (TEM-1)	pMG226 (TEM-6)	pMG231 (MIR-1)
Ampicillin	4	1,000	2,000	1,000
+Clavulanate[b]	8	8	16	≥256
+Sulbactam[b]	4	64	64	≥256
+Tazobactam[b]	8	8	32	≥256
Aztreonam	0.125	0.125	64	128
Carumonam	0.25	0.25	2	64
Cefepime	0.125	0.125	4	1
Cefmetazole	4	4	2	≥64
Cefotaxime	0.125	0.125	1	64
Cefotetan	0.5	0.5	1	≥64
Cefotiam	0.25	0.25	4	≥256
Cefoxitin	16	16	16	≥256
Cefpirome	0.125	0.125	2	1
Ceftazidime	0.5	0.25	128	128
Ceftibuten	1	1	2	≥256
Ceftizoxime	0.125	0.125	0.5	≥64
Ceftriaxone	0.125	0.125	8	128
Cefuroxime	8	8	16	≥64
Imipenem	0.5	0.25	0.25	1
Meropenem	0.03	0.03	≤0.015	0.125
Moxalactam	0.25	0.25	2	64
Temocillin	16	16	32	64

[a] R⁻, Plasmid-free.
[b] β-Lactamase inhibitors were used at concentrations of 8 μg/ml.

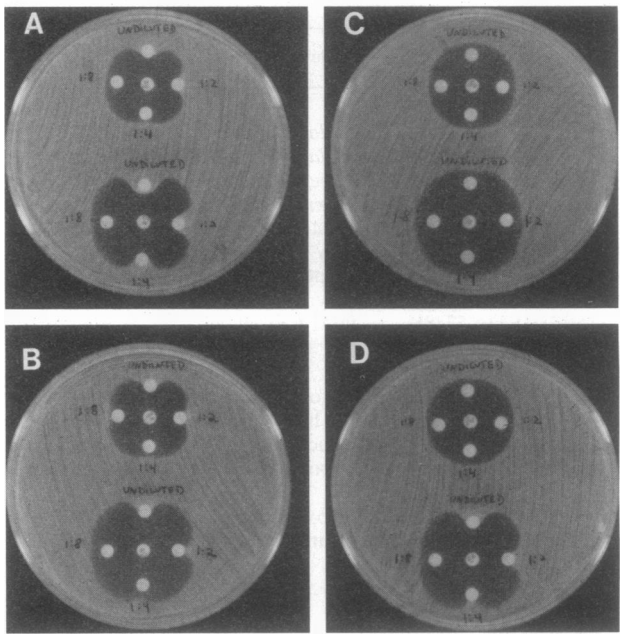

FIG. 1. Masuda bioassay. Inactivation of cefoxitin (upper half of plate) and cefotaxime (lower half) by crude sonic extracts of β-lactamases undiluted and in 1:2, 1:4, and 1:8 dilutions. (A) MIR-1; (B) AmpC; (C) TEM-1; (D) TEM-6.

Bound label was detected by enzyme-linked immunoassay, as described by Boehringer-Mannheim.

The cloned MIR-1 gene was used as a probe to detect plasmids carrying the MIR-1 gene. The 1.4-kb *Acc*I-*Pst*I insert in plasmid pMG232 was labeled with digoxigenin. Hybridizing DNA was visualized via chemiluminescence (Southern-Light; Tropix, Inc., Bedford, Mass.).

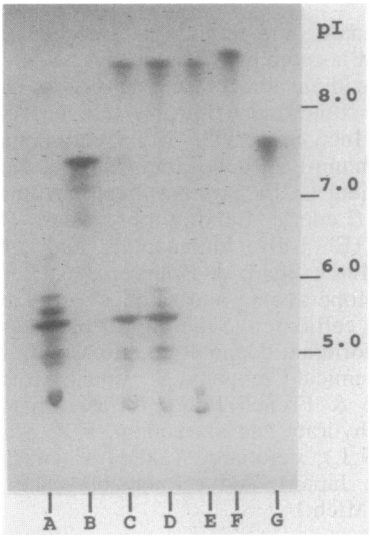

FIG. 2. Isoelectric focusing gel showing β-lactamase band pattern of strains containing MIR-1 β-lactamase or standard β-lactamases or both. Lane A, TEM-1 (pI 5.4); lane B, SHV-1 (pI 7.6); lane C, *K. pneumoniae* 96D; lane D, *E. coli* C600(pMG230); lane E, *E. coli* C600(pMG231); lane F, *E. coli* AmpC (pI 8.5); lane G, *E. cloacae* P99 (pI 7.66). The band with a pI of 7.6, weakly visualized in lane C, was more evident on other gels.

TABLE 3. Substrate profile of crude sonic extracts of β-lactamases

β-Lactamase	Relative rate of hydrolysis[a]							
	Penicillin	Ampicillin	Carbenicillin	Cloxacillin	Cephalothin	Cefotaxime	Ceftazidime	Cefoxitin
MIR-1	4	1	<1	1	122	10	3	<1
AmpC[b]	19	2	<1	1	127	<1	3	<1
P99	1	<1	<1	<1	18	<1	<1	<1
TEM-1	126	101	50	2	16	1	2	<1
TEM-6	86	32	40	2	44	4	112	<1

[a] Rate for cephaloridine = 100.
[b] From *E. coli* 87120702.

Double-stranded pMG232 DNA was used for sequencing (6) after purification on a pZ523 column (5 Prime-3 Prime, Inc., West Chester, Penn.). Sequencing was performed by the dideoxy chain termination technique of Sanger et al. (44) by using [α-^{35}S]dATP from Amersham Corp. (Arlington Heights, Ill.), Sequenase from United States Biochemical Corporation (Cleveland, Ohio), and 17-mer M13 sequencing primers from New England BioLabs (10). Sequence comparisons were performed with IBI Pustell sequence analysis programs (IBI, New Haven, Conn.).

Nucleotide sequence accession number. The preliminary sequence obtained for MIR-1 has been submitted to GenBank and has been assigned accession number M37839.

RESULTS

Epidemiological information. The Miriam Hospital is an acute care general hospital of 250 beds. Between September 1988 and June 1989, 11 patients were identified as carrying isolates of *K. pneumoniae* that were resistant to cefoxitin and ceftibuten and resistant or intermediate to cefotaxime and ceftazidime. This pattern of multiple beta-lactam resistance would be typical of an *Enterobacter* sp. or other gram-negative bacillus that can mutate to overproduce its chromosomal β-lactamase (43) but was exceptional for *K. pneumoniae*. All 11 patients appeared to have acquired the isolates in hospital, and 10 of the 11 isolates caused infection. Nine of the patients had one or more surgical procedures. All but one patient had either a prolonged hospitalization or multiple hospital stays within the preceding 6 months. Sources of organisms included four wound, four urine, and three sputum cultures. Nine of the isolates were recovered in mixed cultures with other potential pathogens. *K. pneumoniae* was the sole pathogen in the other two cases. Eight of the 11 patients had received cephalosporins prior to recovery of the resistant organism. Only two of them had received cefoxitin.

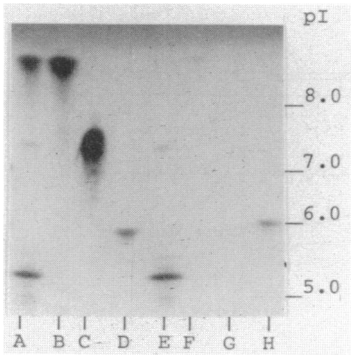

FIG. 3. Isoelectric focusing gel showing the effect of the addition of cefoxitin to the nitrocefin solution used to visualize the β-lactamase bands. Lanes A through D were developed with nitrocefin alone, and lanes E through H were developed with nitrocefin plus cefoxitin. Lanes A and E, *K. pneumoniae* 96D, TEM-1, SHV-1, and MIR-1; lanes B and F, *E. coli* C600(pMG231) MIR-1; lanes C and G, *E. cloacae* P99; lanes D and H, *E. coli* A15R$^+$(HB114-5748) TEM-6.

Genetic studies. Three cefoxitin-resistant *K. pneumoniae* isolates were initially tested for ability to transfer cefoxitin resistance to *E. coli* J53-2 by conjugation. No transconjugants were obtained, despite the presence on agarose gel electrophoresis of multiple plasmid bands in the donors, even when matings were performed at 30°C to detect plasmids temperature sensitive for transfer. Accordingly, plasmid DNA from these strains was used to transform *E. coli* C600 to cefoxitin resistance. Transformants were resistant to many beta-lactam antibiotics, to mercuric chloride, and, for one of the three donors (strain 258B), to chloramphenicol. Each contained a plasmid of about 44 kb that could not be transmitted to another *E. coli* strain by conjugation but that could be again transformed into *E. coli* with retention of all resistance markers, whichever marker was used for selection. The cefoxitin and mercuric ion resistance plasmid derived from *K. pneumoniae* 96D was termed pMG230 and was used for further studies.

Cloning the MIR-1 β-lactamase gene. *E. coli* C600 (pMG230) produced two β-lactamases on isoelectric focusing: one with a pI of 5.4 that cofocused with TEM-1 and one with a pI of 8.4. To isolate the gene for the enzyme with a pI of 8.4, the gene was cloned by using the 2.7-kb vector plasmid pMLC28 that determines resistance to chloramphenicol and carries the multiple cloning sites of M13 phage derivatives. pMG230 and pMLC28 were cut with *Kpn*I restriction endonuclease, and the resulting fragments were ligated and transformed into *E. coli* C600 selecting for cefoxitin and chloramphenicol resistance. A 6.8-kb derivative containing a 4.1-kb insert was termed pMG231. On

TABLE 4. I_{50}s of drugs for various β-lactamases

β-Lactamase	I_{50}s (nM)			
	Clavulanate	Tazobactam	Cefoxitin	Cefotetan
MIR-1	210	8.3	6.3	0.6
AmpC	357	19.4	0.2	0.25
P99	79.5	0.26	0.08	0.01
TEM-1	0.24	0.04	350	33
TEM-6	0.01	0.03	3.3	0.2

TABLE 5. Inhibition profiles of MIR-1, AmpC, P99, TEM-1, and TEM-6 β-lactamases[a]

β-Lactamase	pI	Clavulanate		Sulbactam		Tazobactam		Cefoxitin		Cefotetan		Moxalactam		Cefotaxime		Ceftazidime	
		Max	SI	Max	SI	Max	SI	Max	SI	Max	SI	Max	SI	Max	SI	Max	SI
MIR-1 (*E. coli*)	8.4	1		5		3		94	<0.3	98	<0.3	92	<0.3	93	0.7	60	4.9
AmpC (*E. coli*)	8.5	1		1		3		94	<0.3	97	<0.3	96	<0.3	94	<0.3	86	0.2
P99 (*E. cloacae*)	7.66	14		1		15		92	<0.3	100	<0.3	96	<0.3	94	<0.3	90	0.7
TEM-1 (*E. coli*)	5.4	70	0.3	86	0.9	94	<0.3	7		3		3		121	1.0	8	
TEM-6 (*E. coli*)	5.87	80	0.4	91	0.5	98	<0.3	84	<0.3	92	<0.3	78	<0.3	78	1.7	62	2.7

[a] Max, Maximum percent inhibition during first 2 min; SI, slope, i.e., change in percent inhibition over time (in minutes), given when maximum inhibition exceeded 25%.

isoelectric focusing, pMG231 encoded only the β-lactamase with a pI of 8.4 (see below).

Table 2 compares the susceptibility of *E. coli* C600 (pMG231) producing MIR-1 with the same host producing TEM-1 or the extended-spectrum β-lactamase TEM-6 (3). MICs of ampicillin differed little among the strains, but the MIR-1 strain remained conspicuously resistant to ampicillin in combination with any of the β-lactamase inhibitors potassium clavulanate, sulbactam, and tazobactam. Both MIR-1 and TEM-6 conferred resistance to aztreonam, ceftazidime, cefotaxime, and ceftriaxone, but the level of resistance to the last two compounds was higher with MIR-1. MIR-1 provided striking resistance to the α-methoxy beta-lactams cefmetazole, cefotetan, cefoxitin, and moxalactam, unlike TEM-6. Susceptibility to cefepime, cefpirome, and the carbapenems imipenem and meropenem was little affected. For the MIR-1 strain, the MIC of each drug was 1 μg/ml or less but was higher for each beta-lactam than the MIC for plasmid-free C600.

In the Masuda bioassay, the MIR-1, AmpC, and P99 β-lactamases inactivated both cefoxitin and cefotaxime, whereas TEM-6 inactivated only cefotaxime (Fig. 1). TEM-1 did not inactivate either of the compounds. Aztreonam was inactivated by TEM-6 but not by MIR-1, AmpC, or P99.

Analytical isoelectric focusing. On isoelectric focusing *K. pneumoniae* 96D produced predominant β-lactamase bands with pIs of 5.4, 7.6, and 8.4 (Fig. 2, lane C). The band with a pI of 5.4 cofocused with TEM-1 (Fig. 2, lane A), while that with a pI of 7.6 resembled SHV-1 or SHV-2 (Fig. 2, lane B). *E. coli* C600(pMG230) (Fig. 2, lane D) made the bands with pIs of 5.4 and 8.4, while C600(pMG231) (Fig. 2, lane E) had only the β-lactamase with a pI of 8.4, MIR-1, which focused at a pI distinct from chromosomal *E. coli* AmpC (Fig. 2, lane F) or *E. cloacae* P99 (Fig. 2, lane G) enzymes. The other *K. pneumoniae* isolates had the same isoelectric focusing pattern as strain 96D.

When the nitrocefin overlay by which the gel was developed contained cefoxitin, the bands of MIR-1, AmpC, and P99 β-lactamases were visualized weakly or not at all (Fig. 3). TEM-1 and SHV-1 β-lactamases were unaffected by the presence of cefoxitin, thus illustrating their low affinity for this agent as inhibitor.

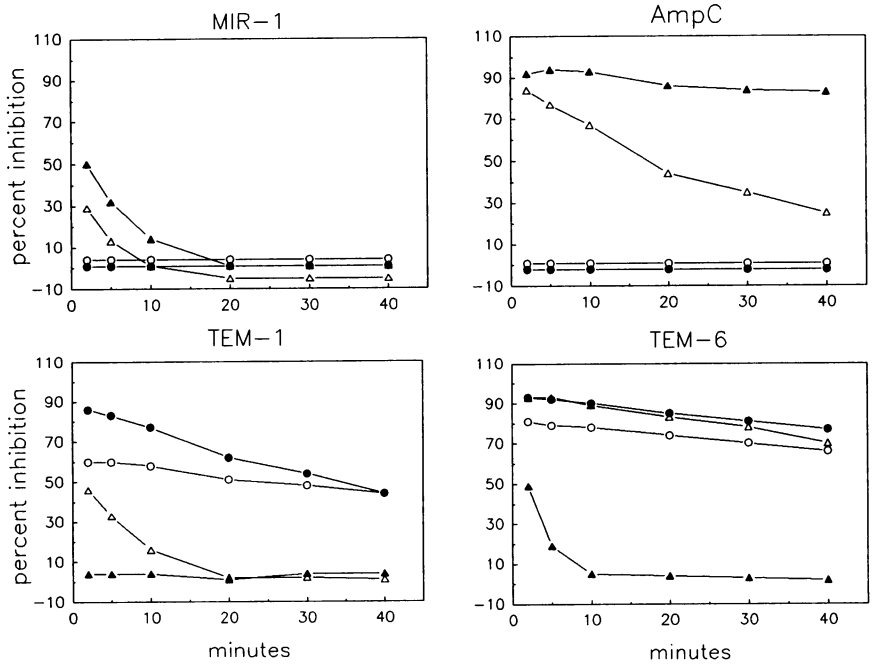

FIG. 4. Inhibition profiles of MIR-1, AmpC, TEM-1, and TEM-6 β-lactamase. Symbols: ○, clavulanate; ●, sulbactam; △, cefoperazone; ▲, ceftazidime.

TABLE 5—*Continued*

Aztreonam		Ceftriaxone		Ceftibuten		Cefoperazone		Cefpirome		Cefsulodin		Cefuroxime		Imipenem		Cloxacillin	
Max	SI	Max	SI	Max	SI	Max	SI	Max	SI	Max	SI	Max	SI	Max	SI	Max	SI
96	<0.3	97	<0.3	86	0.8	63	2.9	52	0.9	7		89	0.5	98	<0.3	98	<0.3
95	<0.3	95	<0.3	90	<0.3	79	1.4	73	1.2	23	2.0	94	<0.3	99	<0.3	98	<0.3
94	<0.3	100	<0.3	87	<0.3	90	1.3	70	0.5	37	3.3	94	<0.3	99	<0.3	98	<0.3
1		3		19		46	3.0	4		1		18		92	0.7	84	1.2
53	2.0	72	1.4	28	2.0	94	0.5	44	3.5	15		16		92	<0.3	96	<0.3

Characterization of MIR-1 β-lactamase. Table 3 shows the substrate profiles of MIR-1, chromosomal β-lactamases AmpC and P99, and plasmid-mediated β-lactamases TEM-1 and TEM-6. MIR-1 readily hydrolyzed cephaloridine and cephalothin. In contrast, penicillins were poor substrates. Cefotaxime and ceftazidime were hydrolyzed much less than cephalothin. AmpC and P99 β-lactamases also hydrolyzed cephaloridine more rapidly than penicillins. However, AmpC hydrolyzed cephalothin at a rate similar to cephaloridine and cleaved penicillin G more readily than P99 β-lactamase did.

TEM-1 primarily hydrolyzed penicillin G, ampicillin, and carbenicillin. Rapid hydrolysis of ceftazidime distinguished TEM-6 from the other β-lactamases tested. MIR-1 and AmpC were the only β-lactamases that hydrolyzed cephalothin more rapidly than cephaloridine. None of the β-lactamases tested were shown to hydrolyze cefoxitin to a significant degree by this technique.

The concentrations of potassium clavulanate, tazobactam, cefoxitin, and cefotetan that inhibited 50% of the activity of these enzymes are shown in Table 4. MIR-1 and the chromosomal β-lactamases AmpC and P99 required higher amounts of clavulanate and tazobactam for inhibition than TEM-1 or TEM-6 did. In contrast, the amount of cefoxitin or cefotetan required for 50% inhibition of MIR-1 was closer to the value for TEM-6 than for the chromosomal enzymes. The P99 enzyme was the most susceptible to inhibition by cefoxitin or cefotetan, while TEM-1 and TEM-6 had the lowest I_{50}s for clavulanate and tazobactam.

The β-lactamase activity of *K. pneumoniae* 96D or *E. coli* C600(pMG231) did not increase after growth in broth containing cefoxitin or imipenem, in contrast to *E. cloacae* P99,

```
                      10         20         30         40         50         60
MIR-1        GGATAGCTTT TATTCGCCAG CATCACAATG CCGAGCTGCT TTTCAGGAAT AAATGCCACG
                      |          |          |          |          |          |
ENTPAMPC     <GGATAGCTTg TATTCGCgAG CATCACAATa CCGAtCTGCT TTTCAGGAAT AAAgGCCACG
                      |          |          |          |          |          |
ECOAMPCFR    <GGATAGtTTT TgTTtGCCAG CATCACgATa CCcAGCTctT TTTCtGGAAT AAAcGCgACa


                      70         80         90        100        110        120
MIR-1        TAGCTGCCAA ACCCGCCCGT CGAGCCTGTT TTATGGACCC AGGAGGCCTT GACCGGCGGC
                      |          |          |          |          |          |
ENTPAMPC     <TAGCTGCCAA ACCCGCCaGT aGAGCCcGTT TTATGGACCC AGGAcGCtTT GACCGGgGGa
                      |          |          |          |          |          |
ECOAMPCFR    <TAGCTaCCAA AtCCGCCgGT CGccCCTGTT TTATGtACCC AtGAtGCgcg tACtGcaGGa


                     130        140        150
MIR-1        GCGGGTGGAT TCACTTCTGC CACGGGCAAT
                      |          |          |
ENTPAMPC     <GCcGGTGGAT TCACTTCTGC CACGGGCAAc
                      |          |          |
ECOAMPCFR    <GttGGgGGcg TaAtcgCTtt tACGGGgcgT
```

FIG. 5. Comparison of MIR-1 with *ampC* genes from *E. cloacae* (ENTPAMPC) and *E. coli* (ECOAMPCFR). Mismatches are shown in lowercase letters.

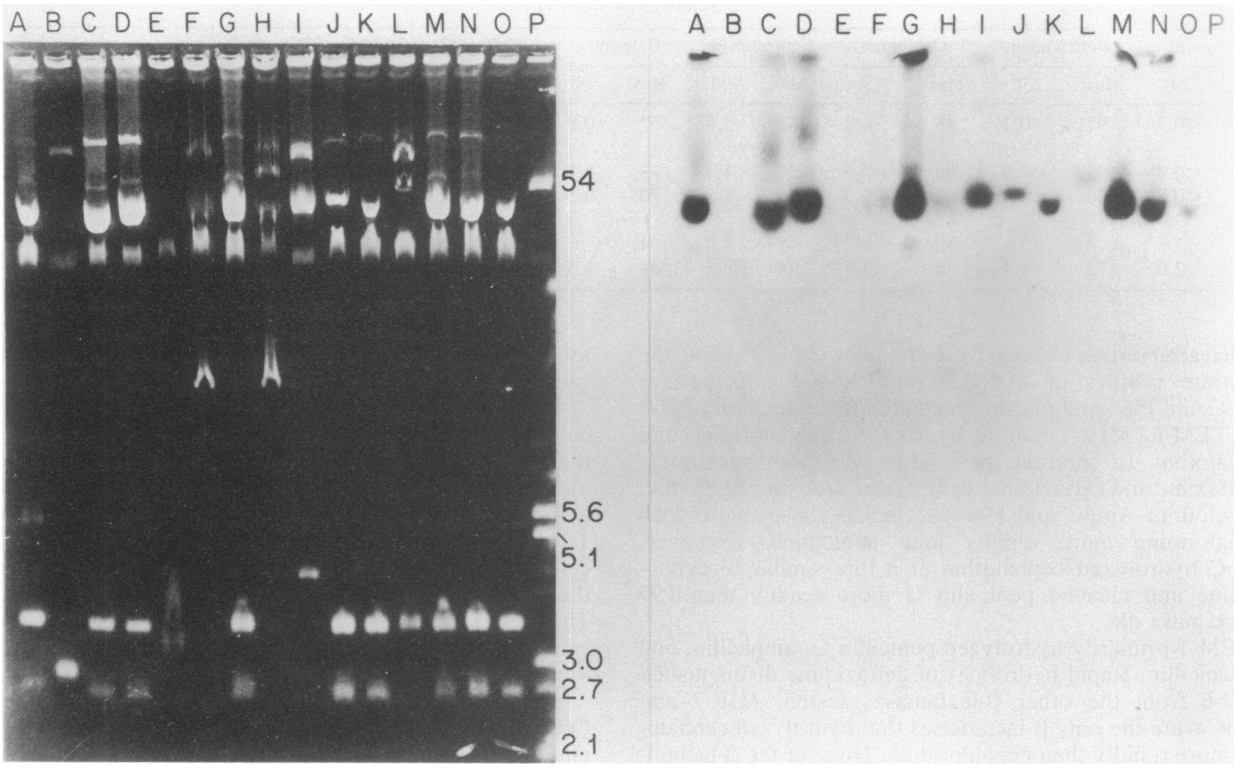

FIG. 6. Agarose gel electrophoresis of plasmid DNA from *K. pneumoniae* isolates (left panel) and Southern blot of the same gel probed with the MIR-1-encoding 1.4-kb *Acc*I-*Pst*I insert in pMG232 (right panel). Lanes A, strain 258B; lanes B, strain 758B; lanes C, strain 96D; lanes D, strain 928B; lanes E, strain 265D; lanes F, strain 636C; lanes G, strain 834C; lanes H, strain 280B; lanes I, strain 1293D; lanes J, strain 244C; lanes K, strain 45C; lanes L, strain 844C; lanes M, strain 1162D; lanes N, strain 1429D; lanes O, strain 561E; lanes P, plasmid size standards in *E. coli* V517 (27) with molecular masses (in kilodaltons) as shown. Strains 758B and 265D were cefoxitin resistant but cefotaxime, ceftazidime, and ceftibuten susceptible. Strains 258B, 265D, and 1429D were repeat isolates from the same patient, and strains 928B and 1162D were repeat isolates from another patient.

suggesting that regulatory genes either were not associated with the MIR-1 structural gene or were not functional.

Inhibition profile. Table 5 shows the inhibition profiles of MIR-1, the chromosomal enzymes AmpC and P99, and the plasmid-mediated β-lactamases TEM-1 and TEM-6. The inhibition profile of MIR-1 was very similar to the profiles of β-lactamases AmpC and P99. All were readily inhibited by most cephalosporins, and all three were little affected by clavulanate, sulbactam, or tazobactam, in contrast to the plasmid-mediated enzymes. MIR-1 was, however, more like TEM-1 than *E. coli* AmpC in response to cefoperazone and more like TEM-6 in response to ceftazidime (Fig. 4).

Genetic analysis of the MIR-1 β-lactamase gene. To localize the MIR-1 β-lactamase gene further, it was subcloned into plasmid pBGS131 as a *Kpn*I-*Eco*RI fragment and recloned into pMLC28 on a 2.5-kb *Pst*I fragment. This derivative was again subcloned into pMLC28 with *Acc*I and *Pst*I to yield pMG232 carrying a 1.4-kb insert encoding cefoxitin resistance.

DNA probes were prepared for SHV-1 and TEM-1 genes and the *E. coli ampC* genes and labeled with digoxigenin. Under stringent conditions none of the probes hybridized with plasmids containing cloned fragments encoding MIR-1 β-lactamase (data not shown).

Accordingly, nucleotide sequencing of the insert in pMG232 was initiated. By using double-stranded plasmid DNA as template and a sequencing primer that initiated from the *Acc*I end, a provisional sequence of 150 bp was obtained and tested for homology to known β-lactamase sequences. The MIR-1 gene fragment proved to be 90% identical to a sequence near the C terminus of the *ampC* gene from *E. cloacae* P99 (8) but only 71% identical to the corresponding region in *ampC* of *E. coli* (15) (Fig. 5).

Molecular epidemiology of MIR-1 production. Figure 6 shows the plasmid content of the 11 MIR-1-producing *K. pneumoniae* isolates and of two *K. pneumoniae* strains isolated during the outbreak which were resistant to cefoxitin but susceptible to cefotaxime. At least five plasmid profiles were evident among the MIR-1-producing strains. When the 1.4-kb MIR-1 gene-containing insert in pMG232 was used as a probe, all except the cefotaxime-susceptible control strains in lanes B and E demonstrated a hybridizing plasmid band in the range of 40 to 60 kb (Fig. 6).

When the remaining nine *K. pneumoniae* isolates were tested for ability to transfer resistance to *E. coli* J53-2, strains 244C and 844C were discovered to contain conjugative cefoxitin resistance plasmids. These plasmids, termed respectively pMG233 and pMG234, were 58 kb long and carried resistance to chloramphenicol, mercuric chloride, and streptomycin in addition to cefoxitin. On testing with donor-specific bacteriophage, J53-2(pMG233) and J53-2 (pMG234) were found susceptible to phage IKe, specific for plasmids of IncN (18). In confirmation of this assignment, when IncN plasmid R269N-1 was introduced into J53-2 (pMG233) or J53-2(pMG234), cefoxitin resistance was lost. J53-2 isolates carrying plasmids from strain R96D(pMG230) or 258B were insusceptible to IKe (because these transfer-deficient plasmids failed to make a pilus required for

IKe absorption), but they were eliminated by introduction of R269N-1, indicating that they also belonged to the IncN group.

DISCUSSION

Since the introduction of broad-spectrum cephalosporins, resistance to these antibiotics that is due to production of plasmid-mediated extended-spectrum β-lactamases has appeared in *K. pneumoniae* (41). These enzymes, however, do not confer resistance to cephamycins and are inhibited by clavulanate or sulbactam. Over the past few years, an additional selective pressure has been exerted on the bacterial population as clavulanate and sulbactam have been increasingly employed. One counterresponse would be for bacteria to produce class I β-lactamases, since these enzymes confer resistance both to cephamycins and to broad-spectrum cephalosporins and are poorly inhibited by clavulanate or sulbactam. However, the chromosomal location of the genes determining these enzymes has limited their mobility.

We describe the discovery in *K. pneumoniae* of MIR-1, a novel plasmid-mediated β-lactamase that has many of the properties of a class I enzyme. MIR-1 conferred resistance to such penicillins as ampicillin, carbenicillin, and ticarcillin and to such cephalosporins as cephalothin, cefazolin, cefamandole, and cefuroxime. In addition, it provided resistance to such broad-spectrum drugs as aztreonam, cefotaxime, ceftazidime, ceftizoxime, and ceftriaxone and especially to such α-methoxy beta-lactams as cefmetazole, cefotetan, cefoxitin, and moxalactam. Furthermore, resistance was virtually unaffected by β-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam. In vitro, the substrate profile of MIR-1 was typical for a cephalosporinase, as was its response to various inhibitors. In particular, the affinity of MIR-1 for cephamycins was reflected in low I_{50}s of cefoxitin and cefotetan, and its resistance to β-lactamase inhibitors was mirrored by high I_{50}s of clavulanate and tazobactam.

Inactivation of cefoxitin by the MIR-1, AmpC, and P99 β-lactamases was demonstrated only by the microbiological assay. Conceivably, this inactivation could be due to binding of cefoxitin at the farthest reaches of the zone of inhibition. However, inactivation was not seen with aztreonam, which binds very well to most cephalosporinases and is hydrolyzed only minimally (5), suggesting that the inactivation of cefoxitin by MIR-1 was due to cefoxitin destruction.

β-Lactamases can provide resistance to broad-spectrum beta-lactams by mutations in either structural or regulatory genes (43, 46). Mutations in genes for TEM-1, TEM-2, and SHV-1 have resulted in enhanced hydrolytic activity against broad-spectrum cephalosporins but not against cefoxitin (41). On the other hand, hyperproduction of the common plasmid-mediated β-lactamases has little effect on resistance to broad-spectrum beta-lactams or cephamycins (14). Resistance to these agents does appear, however, if the *E. coli* AmpC enzyme is overproduced. Hyperproduction can be the result of mutations or insertions in the *ampC* promoter (16, 17), but in clinical isolates it more commonly involves replacement of the native promoter region with one from *Shigella* spp. that allows a higher level of gene expression (38).

Cefoxitin resistance due to *E. coli* AmpC β-lactamase hyperproduction has been reported (28, 48). However, among the different types of species-specific *E. coli* chromosomal β-lactamases, certain types seem to be overproduced

TABLE 6. Comparison between chromosomal and plasmid-mediated cephalosporinases

Cephalosporinase	Host	pI	Clavulanate inhibition	% Inhibition by:								Reference
				Cephaloridine	Cephalothin	Penicillin G	Ampicillin	Carbenicillin	Cloxacillin	Cefotaxime	Ceftazidime	
Chromosomal												
P99	*E. cloacae*	7.66	−	100	18	1.5	0.02	0.01	0.01	<0.01	<0.01	8
AmpC	*E. coli*	8.5	−	100	127	19	2	<1	<1	<1	<1	15
Plasmid mediated												
CEP-1	*Proteus mirabilis*	7.5	−	100	ND	31	1.5	ND	<0.01	ND	ND	4
CEP-2	*Achromobacter* sp.	8.1	+	100	106	92.6	<1	44	<1	ND	ND	24
FEC-1	*E. coli*	8.2	+	100	200	ND	17	ND	ND	23	0.13	30
CMY-1	*K. pneumoniae*	8.0	−	ND	ND	ND	ND	ND	ND	ND	ND	2
MIR-1	*K. pneumoniae*	8.4	−	100	122	4	1	<1	1	10	3	This work

more frequently (28). Additionally, the prevalence of *E. coli* strains hyperproducing AmpC seems highly variable in different geographical regions (33).

It has been previously suggested that plasmid-mediated β-lactamases evolved from chromosomal genes (1, 21). For example, plasmid-mediated SHV-1 shares extensive nucleotide similarity with LEN-1 β-lactamase found encoded on the chromosome in *K. pneumoniae* (1). However, the origins of the plasmid-mediated cephalosporinases described to date have been little studied.

Cephalosporinases previously reported to be plasmid encoded make up a diverse group in terms of biochemical properties (Table 6). The first plasmid-determined cephalosporinase (CEP-1) was reported before the introduction of newer cephalosporins (4). CEP-2, another plasmid-mediated enzyme, has only a slight preference for cephalosporins, hydrolyzes carbenicillin efficiently, and is inhibited by clavulanate (24). Two other enzymes, FEC-1 (30) and CMY-1 (2), have pI values similar to that of MIR-1, but FEC-1 was readily inhibited by clavulanate and sulbactam and did not confer resistance to cefoxitin. Like the MIR-1 enzyme, CMY-1 β-lactamase provided resistance to cefoxitin and was little affected in activity by clavulanate or sulbactam. Biochemical characterization of CMY-1 is not yet available, but on the basis of susceptibility data, CMY-1 and MIR-1 appear to differ. CMY-1, unlike MIR-1, was at least partially inhibited by sulbactam and provided the same level of resistance to cefotaxime but less resistance to aztreonam, carumonam, or ceftazidime than did MIR-1.

Genetic studies confirmed that MIR-1 was derived from an *ampC* gene. Although hybridization with an *ampC* gene probe from *E. coli* was negative, preliminary sequence data indicated 90% similarity with the *ampC* gene from *E. cloacae* but only 71% identity with the corresponding *ampC* region from *E. coli*, a degree of similarity insufficient to allow hybridization under the stringent conditions employed.

The variety of plasmid profiles found in the cefoxitin-resistant *K. pneumoniae* isolates indicates that spread of a single strain was not responsible for the outbreak. Plasmids varying in size, transfer proficiency, and associated resistance markers were involved, but those tested all belonged to the IncN group. Probably a conjugative IncN plasmid carrying the *ampC* gene spread to different *K. pneumoniae* strains and then evolved further by deletion or other events to become Tra^-. Where and how the *ampC* gene was acquired by the plasmid and whether the gene underwent additional mutations to facilitate expression of cephamycin resistance will be interesting topics for further studies. Since the selective pressure imposed by use of cephamycins and β-lactamase inhibitors will favor strains making AmpC-type β-lactamases, now that the genes for such enzymes have been demonstrated on plasmids, the occurrence of plasmid-mediated cephalosporinase production is likely to increase.

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