ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1997, p. 563–569 Vol. 41, No. 3
0066-4804/97/$04.00+0

Imipenem Resistance in *Klebsiella pneumoniae* Is Associated with the Combination of ACT-1, a Plasmid-Mediated AmpC β-Lactamase, and the Loss of an Outer Membrane Protein

PATRICIA A. BRADFORD,[1]* CARL URBAN,[2,3] NORIEL MARIANO,[2] STEVEN J. PROJAN,[1] JAMES J. RAHAL,[2,4] AND KAREN BUSH[1]†

Wyeth-Ayerst Research, Pearl River, New York[1]; The New York Hospital Medical Center of Queens, Flushing, New York[2]; and Departments of Microbiology[3] and Medicine,[4] Cornell University Medical College, New York, New York

Received 5 August 1996/Returned for modification 26 November 1996/Accepted 23 December 1996

Six *Escherichia coli* and 12 *Klebsiella pneumoniae* isolates from a single hospital expressed a common β-lactamase with a pI of approximately 9.0 and were resistant to cefoxitin and cefotetan (MIC ranges, 64 to >128 and 16 to >128 μg/ml, respectively). Seventeen of the 18 strains produced multiple β-lactamases. Most significantly, three *K. pneumoniae* strains were also resistant to imipenem (MICs, 8 to 32 μg/ml). Spectrophotometric β-lactamase assays with purified enzyme indicated hydrolysis of cephamycins, in addition to cephaloridine and benzylpenicillin. The gene encoding the pI 9.0 β-lactamase (designated ACT-1 for AmpC type) was cloned and sequenced, which revealed an *ampC*-type β-lactamase gene that originated from *Enterobacter cloacae* and that had 86% sequence homology to the P99 β-lactamase and 94% homology to the partial sequence of MIR-1. Southern blotting revealed that the gene encoding ACT-1 was on a large plasmid in some of the *K. pneumoniae* strains as well as on the chromosomes of all of the strains, suggesting that the gene is located on an easily mobilized element. Outer membrane protein profiles of the *K. pneumoniae* strains revealed that the three imipenem-resistant strains were lacking a major outer membrane protein of approximately 42 kDa which was present in the imipenem-susceptible strains. ACT-1 is the first plasmid-mediated AmpC-type β-lactamase derived from *Enterobacter* which has been completely sequenced. This work demonstrates that in addition to resistance to cephamycins, imipenem resistance can occur in *K. pneumoniae* when a high level of the ACT-1 β-lactamase is produced in combination with the loss of a major outer membrane protein.

Functional group 1 β-lactamases are described as cephalosporinases which are not inhibited by clavulanate (6). Originally, these enzymes were naturally occurring, chromosomally encoded AmpC β-lactamases found in genera such as *Enterobacter*, *Citrobacter*, *Serratia*, and *Pseudomonas*. The genes encoding these enzymes were found on the chromosome and are inducible by certain β-lactam antibiotics (36). However, in recent years some of these genes have found their way onto plasmids and are being expressed constitutively at high levels in *Klebsiella pneumoniae* and *Escherichia coli*. Whereas the extended-spectrum β-lactamases in these organisms confer resistance to the expanded-spectrum β-lactam antibiotics such as ceftazidime, cefotaxime, and aztreonam, the plasmid-mediated AmpC-type enzymes also confer resistance to the cephamycins. The first of these to be described was the MIR-1 enzyme which, on the basis of a partial DNA sequence, appears to have originated from the AmpC β-lactamase of *Enterobacter cloacae* (28). Since that time a number of other enzymes such as BIL-1 (11, 29), CMY-1 (2, 4), CMY-2 (3), LAT-1 (40), LAT-2 (13), FOX-1 (14), and MOX-1 (16, 17) have been described. These enzymes are now being found on plasmids with increasing frequency. In a recent survey, Jacoby et al. (18) described transmissible AmpC-type enzymes in isolates from 8 of 20 centers across the United States. Previously, all of the isolates of *K. pneumoniae* and *E. coli* expressing plasmid-mediated AmpC-type β-lactamases have remained susceptible to imipenem.

The New York Hospital Medical Center of Queens (NYH MCQ) in Flushing, N.Y., has had a history of patients' isolates expressing extended-spectrum β-lactamases. In a 19-month period from 1988 to 1990, more than 400 strains of *K. pneumoniae* which expressed the TEM-26 β-lactamase were isolated (26, 41). These strains were resistant to ceftazidime and aztreonam. Subsequently, ceftazidime usage was restricted in this hospital. In 1993 cefotaxime resistance was observed in several strains of *E. coli* which were found to produce the SHV-7 β-lactamase (5). In addition, one strain was also found to express TEM-10, which was the third extended-spectrum β-lactamase to be found in isolates from this hospital (5). In response to the problem of extended-spectrum β-lactamases, the use of cefotetan was continued at NYHMCQ as an alternative to imipenem. However, in this study, we report on an outbreak of *E. coli* and *K. pneumoniae* strains which are resistant to cefoxitin and cefotetan by the acquisition of a novel plasmid-mediated AmpC-type β-lactamase designated ACT-1. In addition, three of the *K. pneumoniae* isolates producing ACT-1 were also resistant to imipenem because of the loss of an outer membrane protein.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* and *K. pneumoniae* strains were cefoxitin- and cefotetan-resistant clinical isolates from NYHMCQ. *E. coli* BAR207 and DH5α were used as recipients in mating and transformation experiments.

Identification and susceptibility tests. Initial identification and susceptibility testing of the clinical isolates were performed by traditional tube biochemical tests and Bauer-Kirby disk diffusion tests in the clinical laboratory. In the reference laboratory identification of the clinical isolates was performed with the API-20E identification system (bioMérieux-Vitek, Hazlewood, Mo.). The iden-

* Corresponding author. Mailing address: Wyeth-Ayerst Research, 401 N. Middletown Rd., Pearl River, NY 10965. Phone: (914) 732-4396. Fax: (914) 732-5671. E-mail: bradfop@war.wyeth.com.

† Present address: Astra Research Center Boston, Cambridge, MA 02139.

TABLE 1. Bacterial strains used in study

Strain	Organism	Plasmid	Characteristics	pI(s) of β-lactamases	PFGE type[a]	Reference
MCQ-7	*E. coli*		Clinical isolate, urine	5.4, 5.6, 9.0	1	This study
MCQ-11b	*E. coli*		Clinical isolate, sputum	5.4, 7.6, 9.0	2	This study
MCQ-13	*E. coli*		Clinical isolate, sputum	5.4, 5.6, 9.0	3	This study
MCQ-14	*E. coli*		Clinical isolate, sputum	5.4, 5.6, 9.0	3	This study
MCQ-17	*E. coli*		Clinical isolate, urine	5.4, 5.6, 7.6, 9.0	4	This study
MCQ-21	*E. coli*		Clinical isolate, urine	9.0	5	This study
MCQ-95	*K. pneumoniae*		Clinical isolate, sputum	5.4, 5.6, 7.0, 7.6, 9.0	I	This study
MCQ-102	*K. pneumoniae*		Clinical isolate, blood	5.4, 5.6, 7.6, 9.0	IIa	This study
MCQ-103	*K. pneumoniae*		Clinical isolate, urine	5.4, 5.6, 9.0	IIa	This study
MCQ-110	*K. pneumoniae*		Clinical isolate, blood	7.0, 7.6, 9.0	IIa	This study
MCQ-111	*K. pneumoniae*		Clinical isolate, urine	5.4, 5.6, 7.0, 7.6, 9.0	IIb	This study
MCQ-112	*K. pneumoniae*		Clinical isolate, sputum	5.4, 5.6, 9.0	IIa	This study
MCQ-113	*K. pneumoniae*		Clinical isolate, urine	5.4, 5.6, 9.0	IIb	This study
MCQ-114	*K. pneumoniae*		Clinical isolate, exit site	5.4, 7.0, 7.6, 9.0	III	This study
MCQ-117	*K. pneumoniae*		Clinical isolate, sputum	5.4, 5.6, 7.6, 9.0	IIc	This study
MCQ-120	*K. pneumoniae*		Clinical isolate, fluid	5.4, 5.6, 7.6, 9.0	IId	This study
MCQ-121	*K. pneumoniae*		Clinical isolate, blood	5.4, 7.0, 7.6, 9.0	IV	This study
MCQ-122	*K. pneumoniae*		Clinical isolate, sputum	5.4, 7.0, 7.6, 9.0	IV	This study
PT4875	*K. pneumoniae*		Imipenem- and cephamycin-susceptible clinical isolate, blood	7.6 (SHV-1)		
DH5α	*E. coli*		F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recAI endAI hsdR17* (r_k^-, m_k^+) *supE44 thiI gyrI relAI*	None		15
BAR207	*E. coli*		*galK galE lacZ leu phoR relA strA sucC sucB supD43 supD74 trp, tsx*	None		31a
DH5α	*E. coli*	pCLL2300	Kanamycin-resistant cloning vector	None		33
DH5α	*E. coli*	pCLL3418	Transformant of MCQ-95	5.6		This study
BAR207	*E. coli*	pCLL3419	Transconjugant of MCQ-102	5.4, 9.0 (ACT-1)		This study
BAR207	*E. coli*	pCLL3420	Transconjugant of MCQ-122	5.4, 9.0 (ACT-1)		This study
DH5α	*E. coli*	pCLL3415	15-kb *Eco*RI fragment containing three β-lactamases cloned from MCQ-95 in pCLL2300	5.4, 7.0, 9.0 (ACT-1)		This study
DH5α	*E. coli*	pCLL3414	6-kb *Pst*I fragment subclone from pCLL3415 containing ACT-1	9.0 (ACT-1)		This study

[a] Arabic numerals were used to designate strain types for *E. coli*, and Roman numerals were used to designate strain types for *K. pneumoniae.*

tification of *K. pneumoniae* strains which were resistant to imipenem was confirmed by whole-cell fatty acid analysis with the MIDI system.

The MICs of various β-lactam antibiotics were determined by broth macrodilution tests by standard methods (27). The following antibiotics (obtained from the indicated sources) were used: piperacillin (Lederle Laboratories, Pearl River, N.Y.); tazobactam (Taiho Laboratories, Japan); ticarcillin and potassium clavulanate (Beecham Laboratories, Bristol, Tenn.); sulbactam (Pfizer Inc., New York, N.Y.); ceftazidime (Glaxo Group Research Ltd., Greenford, England); cefotaxime (Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.); imipenem (Merck, Rahway, N.J.); aztreonam, cefepime, and benzylpenicillin (Bristol-Myers Squibb, Princeton, N.J.); cephaloridine (Eli Lilly, Indianapolis, Ind.); cefotetan (Zeneca Pharmaceuticals, Wilmington, Del.); and cefoxitin (Sigma Chemical Company, St. Louis, Mo.). Conditions used for testing the β-lactam–β-lactamase inhibitor combinations were as follows: ampicillin-sulbactam, 2 to 1 ratio of drug to inhibitor; ticarcillin-clavulanate, constant concentration of 2 μg of inhibitor per ml; piperacillin-tazobactam, constant concentration of 4 μg of inhibitor per ml.

Isoelectric focusing and β-lactamase assays. Crude preparations of β-lactamases from clinical isolates were obtained from sonic extracts prepared in 0.05 M phosphate buffer (pH 7.0) (7). Isoelectric focusing (IEF) was performed by the method of Matthew et al. (25) with an LKB Multiphor apparatus with prepared PAGplates, (pH 3.5 to 9.5; Pharmacia LKB, Piscataway, N.J.). The pH gradient of the IEF gel was measured with a surface pH electrode. The isoelectric point of each enzyme was confirmed by activity staining with nitrocefin (Becton Dickinson Microbiology Systems, Cockeysville, Md.) following IEF.

The β-lactamases tested were partially purified by Sephadex G75 chromatography in 0.05 M phosphate buffer (pH 7.0) (37). The ACT-1 β-lactamase was further purified (>95% purity by sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis [PAGE]) by ion-exchange chromatography on QAE Sephadex C-50 (Pharmacia LKB) in 0.05 M phosphate buffer (pH 8.2). Initial hydrolysis rates for each substrate were monitored spectrophotometrically at least twice on separate days as described previously (30), with a coefficient of variation of 15 to 39%. The computer program ENZPACK (Biosoft, Cambridge, England) was used to calculate kinetic parameters by using five methods of calculation. Inhibition studies were as described previously (32).

Nucleic acid techniques. DNA isolation, restriction enzyme digestions, recombinant DNA manipulations, and transformations of plasmid DNA were performed as described by Sambrook et al. (35). Plasmids conferring cefoxitin resistance were transferred from the clinical isolates to a susceptible *E. coli* host, strain BAR207, by filter mating. Transconjugants were selected on Luria-Bertani agar plates containing 25 μg of cefoxitin per ml and 70 μg of 5-bromo-4 chloro-3-indolyl phosphate (Sigma) per ml. Transconjugants were cefoxitin-resistant blue colonies. Pulsed-field gel electrophoresis (PFGE) was performed as described by Maslow et al. (24) by using a CHEF Mapper apparatus (Bio-Rad Laboratories, Hercules, Calif.). DNA insert blocks were digested overnight with *Xba*I. PFGE strain types were determined by using the criteria of Tenover et al. (38).

To facilitate nucleotide sequencing, a 6-kb *Pst*I fragment encoding the β-lactamase with a pI of 9.0 from strain MCQ-95 was cloned from a chromosomal DNA preparation into pCLL2300, a kanamycin resistance-conferring cloning vector (33), and the resulting plasmid was designated pCLL3414. Sequencing was initiated with an 18-bp degenerate "guessmer" containing dITP at the degenerate bases. The guessmer was derived from a consensus region of AmpC-type proteins. Subsequently, both strands of the entire β-lactamase gene were sequenced with a nested set of custom-synthesized oligonucleotide primers which were specific for the ACT-1 β-lactamase gene. DNA sequencing was performed on double-stranded plasmid DNA by using a Sequenase kit (United States Biochemicals, Cleveland, Ohio) with [^{35}S]dATP label (Amersham, Arlington Heights, Ill.) according to the manufacturer's instructions. Regions of compression were resolved by using C7-deaza deoxynucleoside triphosphates.

Southern blots were performed on plasmid DNA with the Rad-Free kit (Schleicher & Schuell, Keene, N.H.), which uses a psoralin-biotin label to probe nucleic acids, according to the manufacturer's instructions. A 700-bp fragment internal to the coding region for the ACT-1 β-lactamase gene was generated from pCLL3414 (Table 1) by PCR.

TABLE 2. MICs of β-lactam drugs

Strain	MIC (μg/ml)[a]											
	SAM	TIM	PIP	PTZ	ATM	IPM	CTT	FOX	CAZ	CTX	CRO	CPM
MCQ-7	64	512	128	32	16	1	>128	>128	32	64	64	0.5
MCQ-11b	128	128	>512	32	>128	1	128	>128	>128	>64	>64	4
MCQ-13	128	128	128	64	32	≤0.5	>128	>128	128	16	8	4
MCQ-14	64	64	64	32	8	≤0.5	32	>128	32	16	8	2
MCQ-17	128	512	>512	32	>128	1	>128	>128	>128	64	64	8
MCQ-21	64	32	64	8	8	1	64	64	32	16	8	2
MCQ-95	64	>512	512	32	16	2	>128	>128	128	16	8	2
MCQ-102	>256	256	256	64	16	8	>128	>128	64	32	32	4
MCQ-103	>256	>512	>512	128	32	>16	>128	>128	>128	>64	64	16
MCQ-110	256	>512	>512	64	8	2	>128	>128	32	8	4	1
MCQ-111	256	256	512	32	64	2	32	>128	128	8	8	4
MCQ-112	>256	512	>512	32	32	2	>128	>128	>128	8	16	2
MCQ-113	>256	>512	>512	64	>128	2	>128	>128	>128	8	8	2
MCQ-114	128	256	>512	512	>128	≤0.5	16	>128	>128	>64	>64	16
MCQ-117	256	512	>512	64	64	2	32	>128	>128	8	8	4
MCQ-120	256	256	>512	64	32	2	>128	>128	128	8	16	1
MCQ-121	256	>512	>512	128	64	1	>128	>128	64	64	64	2
MCQ-122	>256	>512	>512	256	32	16	>128	>128	32	64	>64	8
DH5α	≤1	4	≤0.5	≤0.5	≤0.13	0.25	0.25	2	0.13	≤1	0.03	≤0.5
BAR207	1	2	≤0.5	≤0.5	0.25	≤0.5	1	4	0.5	≤1	0.13	≤0.5
DH5α(pCLL3418)	2	16	64	≤0.5	4	≤0.5	0.5	4	16	≤1	≤1	≤0.5
BAR207(pCLL3419	32	32	32	2	4	1	16	>256	8	≤2	≤2	≤0.06
BAR207(pCLL3420)	32	32	16	2	4	1	16	>256	4	≤2	≤2	≤0.06
DH5α(pCLL3415)	16	32	16	≤0.5	2	≤0.5	64	128	2	4	4	≤0.5
DH5α(pCLL3414)	16	32	8	2	2	≤0.5	32	>128	4	2	2	≤0.5
PT4875	4	4	16	2	≤0.13	0.25	≤0.25	4	0.5	≤1	0.06	≤0.5

[a] MICs were determined by broth macrodilution tests (27). Abbreviations: SAM, ampicillin-sulbactam; TIM, ticarcillin-clavulanate; PIP, piperacillin; PTZ, piperacillin-tazobactam; ATM, aztreonam; IPM, imipenem; CTT, cefotetan; FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; CPM, cefepime.

Outer membrane protein analysis. Bacterial cell membranes were isolated as described previously (1, 22). The outer membrane proteins were extracted with a 1% solution of *n*-lauryl sarcosine (Sigma) in 0.05 M PO_4 buffer (pH 7.0). Outer membrane samples were separated by SDS-PAGE in a running buffer of 0.025 M Tris, 0.19 M glycine, and 0.1% SDS (pH 8.3) with a 12% acrylamide, 0.12% bisacrylamide gel and were visualized after staining with Coomassie blue.

Nucleotide sequence accession number. The nucleotide sequence data reported here were submitted to the GenBank nucleotide sequence database, and the sequence was assigned the accession number U58495.

RESULTS

Epidemiology. A cefotetan- and cefoxitin-resistant strain of *K. pneumoniae* was first isolated at NYHMCQ in March 1994. Subsequently, additional cefoxitin-resistant *K. pneumoniae* and *E. coli* organisms were isolated from patients' blood, sputum, wounds, urine, and body fluids from August to October 1994. The patients were from different units within the hospital. Cefotetan and imipenem usage had remained constant during 1993 and 1994, and no other cephamycin or carbapenems were used at NYHMCQ. In October 1994 it was noted that three of the cephamycin-resistant *K. pneumoniae* isolates were also resistant to imipenem. Each cefoxitin-resistant isolate except *K. pneumoniae* MCQ-121 and MCQ-122 were isolated from a different patient; strains MCQ-121 and MCQ-122 were obtained from the same patient before and after treatment with imipenem, respectively.

PFGE showed that there were five unique strain types among the 6 clinical isolates of *E. coli* and four unique strain types among the 12 isolates of *K. pneumoniae* (Table 1). For the isolates of *K. pneumoniae*, PFGE strain type II was common to eight strains and consisted of four different subtypes. Sequential patient isolates MCQ-121 and MCQ-122 had identical PFGE strain types, indicating that the strains were identical except for imipenem resistance in MCQ-122.

Susceptibility. The MICs of selected β-lactam antibiotics for the clinical isolates, transconjugants, transformants, and clones are given in Table 2. When using the criteria of the National Committee for Clinical Laboratory Standards for intermediate and resistance as conferring clinical resistance, the clinical isolates of *E. coli* and *K. pneumoniae* were resistant to the penicillins, the β-lactam–β-lactamase inhibitor combinations, aztreonam, cefoxitin, cefotetan, and all cephalosporins except cefepime. In addition, *K. pneumoniae* MCQ-102, MCQ-103, and MCQ-122 were also resistant to imipenem. Also, several of the clinical isolates of *K. pneumoniae* were resistant to all commercially available non-β-lactam drugs except polymyxin (data not shown).

The pI 9.0 β-lactamase plasmid in transconjugant strains BAR207(pCLL3419) and BAR207(pCLL3420) conferred resistance to ampicillin-sulbactam, cefoxitin, and cefotetan. The transconjugants remained susceptible to piperacillin-tazobactam, aztreonam, ceftazidime, and cefepime. Strain DH5α(pCLL3414) containing the cloned pI 9.0 β-lactamase showed a similar pattern of susceptibility, which showed that this β-lactamase provided selective resistance to penicillins and cephamycins.

β-Lactamase characterization. The pIs of the β-lactamases present in crude extracts obtained from the clinical isolates are shown in Table 1. All of the clinical isolates expressed a β-lactamase with a pI of approximately 9.0. In addition, most strains produced multiple enzymes, with as many as five different enzymes in two strains (strains MCQ-95 and MCQ-111). The enzymes produced included a β-lactamase with a pI of 5.4, which correlated with the TEM-1 β-lactamase; a β-lactamase with a pI of 5.6, which was consistent with a TEM-10 or TEM-26 enzyme; a β-lactamase with a pI of 7.0; and a β-lactamase with a pI of 7.6, which corresponded to an SHV-type

TABLE 3. Substrate and inhibition profiles of purified ACT-1 β-lactamase

Substrate	Relative V_{max}	K_m (μM)	Relative V_{max}/K_m	IC_{50} (nM)[a]
Cephaloridine	100	380	100	ND[b]
Benzylpenicillin	7.1	10	240	ND
Cefoxitin	0.11	3.7	9.9	ND
Cefotetan	0.014	2.5	2.1	ND
Imipenem	0.006	7.5	0.26	ND
Cefepime	0.059	1,040	0.02	ND
Cefotaxime	0.006	7.0	0.29	ND
Aztreonam	<0.1	NC[c]	NC	ND
Clavulanate	ND	ND	ND	52,000
Sulbactam	ND	ND	ND	2,600
Tazobactam	ND	ND	ND	1,300

[a] IC_{50}, 50% inhibitory concentration.
[b] ND, not determined.
[c] NC, not calculated (rates were too slow to obtain reliable values).

β-lactamase. The presence of the pI 9.0 β-lactamase in the transconjugants correlated with resistance to cefoxitin.

Following SDS-PAGE, the homogeneous pI 9.0 enzyme had an apparent molecular mass of 43 kDa. On the basis of the V_{max} values (Table 3), this enzyme hydrolyzed cephaloridine 14 times faster than benzylpenicillin. Cefoxitin was hydrolyzed 8-fold faster than cefotetan and 18-fold faster than imipenem. This β-lactamase had lower K_m values for cefoxitin, cefotetan, and imipenem than for cephaloridine or benzylpenicillin. Furthermore, the affinity of the pI 9.0 β-lactamase for cefepime was quite low (K_m = 1,040 μM) and resulted in the lowest hydrolytic efficiency for the substrates with measurable rates of hydrolysis. The inhibition profile showed that the pI 9.0 β-lactamase was not inhibited well by any of the β-lactamase inhibitors tested. Therefore, the pI 9.0 β-lactamase is a cephalosporinase which is not inhibited by clavulanic acid and therefore belongs in functional group 1 (6).

Nucleotide sequencing. In an initial attempt to clone the pI 9.0 β-lactamase, a 15-kb *Eco*RI fragment from a preparation of chromosomal DNA was cloned into pCLL2300 and was designated pCLL3415. IEF revealed that this clone produced three β-lactamases with pIs of 5.4, 7.0, and 9.0 (Table 1). Subsequently, a subclone was made by using *Pst*I. A strain with the resulting plasmid, pCLL3414, containing a 6-kb insert expressed only the pI 9.0 β-lactamase. This clone was then used for nucleotide sequencing.

The nucleotide sequence for the cloned pI 9.0 β-lactamase from *K. pneumoniae* MCQ-95 in *E. coli* DH5α(pCLL3414) (Fig. 1) revealed an open reading frame 1,143 bp in length. The derived amino acid sequence of the β-lactamase (Fig. 2) shows the presence of a novel AmpC-type β-lactamase 381 amino acids in length that was named ACT-1, for AmpC-type β-lactamase. The bla_{ACT-1} β-lactamase gene is 94% homologous to the 150 bp of sequence available for the MIR-1 β-lactamase gene (28). It also shares 86, 73, and 71% identities with the *ampC* β-lactamase genes from *E. cloacae* P99 (12), *Citrobacter freundii* OS60 (21), and *E. coli* K-12 (19), respectively. There was similar homology on the amino acid level. Thus, it appears that ACT-1 originated from an *E. cloacae* AmpC β-lactamase.

***bla*$_{ACT-1}$ gene location.** To determine if the gene encoding the ACT-1 β-lactamase was located on a plasmid, Southern blot analysis was performed with DNA obtained from plasmid and also from chromosomal preparations. The ACT-1 probe hybridized to the chromosomal preparations of all of the *E. coli* and *K. pneumoniae* clinical isolates (data not shown) but not to control strains PT4875 and DH5α. In addition, the probe hybridized to a large (>50 kb) plasmid in *K. pneumoniae* strains of PFGE strain types II and IV. There was no hybridization to plasmids in any of the *E. coli* strains or in *K. pneumoniae* strain type I or III.

```
CCCTTGAACT GCTATTACGG AAGATAAATG ATGATGACTA AATCCCTTTG   50
TTGTGCCCTG CTGCTCAGCA CCTCCTGCTC GGTATTGGCT ACCCCGATGT  100
CAGAAAAACA GCTGGCTGAG GTGGTGGAAC GGACCGTTAC GCCGCTGATG  150
AAAGCGCAGG CCATTCCGGG TATGGCGGTG GCGGTGATTT ATGAGGGTCA  200
GCCGCACTAC TTCACCTTCG GTAAAGCCGA TGTTGCGGCG AACAAACCTG  250
TCACTCCACA AACCTTGTTC GAACTGGGTT CTATAAGTAA AACCTTCACC  300
GGCGTACTCG GTGGCGATGC CATTGCTCGC GGTGAAATAT CGCTGGGCGA  350
TCCGGTGACA AAATACTGGC CTGAGCTGAC AGGCAAGCAG TGGCAGGGGA  400
TCCGCATGCT GGATCTGGCA ACCTATACCG CAGGAGGTTT GCCGTTACAG  450
GTACCGGATG AGGTCAAGGA TAACGCCTCT CTGTTGCGCT TTTATCAAAA  500
CTGGCAGCCG CAGTGGAAGC CGGGCACCAC GCGTCTTTAC GCCAATGCCA  550
GCATCGGTCT TTTTGGCGCG CTGGCGGTCA AACCTTCCGG CATGAGCTAT  600
GAGCAGGCCA TGACTACAAG GGTCTTTAAG CCGCTCAAGC TGGACCATAC  650
GTGGATTAAC GTTCCGAAAG CGGAAGAGGC GCATTACGCC TGGGGATACC  700
GCGACGGTAA AGCAGTACAC GTTTCGCCAG GAATGCTGGA CGCTGAAGCC  750
TATGGCGTAA AAACCAACGT GCAGGATATG GCAAGCTGGG TGATGGTCAA  800
CATGAAGCCG GACTCCCTTC AGGATAATTC ACTCAGGAAA GGCCTTACCC  850
TGGCGCAGTC TCGCTACTGG CGCGTGGGGG CCATGTATCA GGGGTTAGGC  900
TGGGAAATGC TTAACTGGCC GGTCGATGCC AAAACCGTGG TTGAAGGTAG  950
CGACAATAAG CTTGCACTGG CACCGCTGCC TGCGAGAGAA GTGAATCCAC 1000
CAGCGCCCCC GGTCAACGCA TCCTGGGTCC ATAAAACAGG CTCTACCGGC 1050
GGGTTTGGCA GCTACGTGGC ATTTATTCCT GAAAAGCAGC TCGGTATTGT 1100
GTGCTGGCAA ATAAAAGCTA TCCGAACCCG GCACGCGTTG AGGCGGCATA 1150
CCGTATTTTG AGCGCGCTGT AGTAAACATT GCCGGGTGGC GCTAACGCTT 1200
```

FIG. 1. Nucleotide sequence of bla_{ACT-1} β-lactamase gene. Underlined nucleotides indicate start and stop codons.

In a further attempt to demonstrate the presence of the bla_{ACT-1} gene on a plasmid, filter mating was performed with two strains of *K. pneumoniae* from each of the four PFGE strain types. Transconjugants could be selected from strain types II [designated BAR207(pCLL3419)] and IV [designated BAR207(pCLL3420)], which produced a pI 5.4 β-lactamase plus ACT-1 (Table 1). No transconjugants were obtained from the *E. coli* strains or *K. pneumoniae* strain types I and III. Plasmid DNAs from the *E. coli* strains and *K. pneumoniae* strain types I and III were transformed into a susceptible *E. coli* host, and transformants [represented by DH5α(pCLL3418)] selected with ampicillin expressed only the pI 5.6 β-lactamase. These studies indicated that ACT-1 is plasmid mediated in some, but not all, of the *K. pneumoniae* strains.

Outer membrane protein analysis. Because the substrate profile of ACT-1 indicated that hydrolysis of imipenem by the β-lactamase alone should not confer resistance to *K. pneumoniae*, outer membrane protein profiles were analyzed. This was done because the combination of an AmpC β-lactamase plus the loss of an outer membrane protein has been shown to confer imipenem resistance in *Enterobacter* (9, 10, 20, 31, 39). As shown in Fig. 3, the susceptible strains of *K. pneumoniae* expressed two outer membrane proteins, one major band of approximately 42 kDa and a minor band of 40 kDa. *K. pneumoniae* MCQ-102, MCQ-103, and MCQ-122 were missing the major 42-kDa protein band. The loss of this outer membrane

```
ACT-1:       MMMTKSLCCALLLSTSCSVLATPMSEKQLAEVVERTVTPLMKAQA 45
AmpC Ent cl: -..RK.......GI...A....V.........AN.I.......S
AmpC Cit fr: -..K..I......TA.F.TF.AAKT.Q.I.DI.N..I....QE..
AmpC E.coli: -.FK.T..-...ITA...TF.A.---Q.INDI.H..I...IEQ.K

ACT-1:       IPGMAVAVIYEGQPHYFTFGKADVAANKPVTPQTLFELGSISKTF 90
AmpC Ent cl: ..........Q.K...Y......I.....................
AmpC Cit fr: .......I....K.Y...W....I...H...Q........V....
AmpC E.coli: ..........Q.K.Y...W.Y..I.KKQ...Q........V....

ACT-1:       TGVLGGDAIARGEISLGDPVTKYWPELTGKQWQGIRMLDLATYTA 135
AmpC Ent cl: ................D.A..R...Q...................
AmpC Cit fr: N......R......K.S..............R..SL.H......
AmpC E.coli: ..............K.S..T........A...N..TL.H......

ACT-1:       GGLPLQVPDEVKDNASLLRFYQNWQPQWKPGTTRLYANASIGLFG 180
AmpC Ent cl: ..............................................
AmpC Cit fr: ......I.GD.T.K.E............T..AK.....S......
AmpC E.coli: ............SSSD..........A.A...Q.....S......

ACT-1:       ALAVKPSGMSYEQAMTTRVFKPLKLDHTWINVPKAEEAHYAWGYR 225
AmpC Ent cl: .........P.........L..........................
AmpC Cit fr: .....S......E......LQ....A.......QS.QKN.....L
AmpC E.coli: ........L.F....Q....Q....N.......P...KN......

ACT-1:       DGKAVHVSPGMLDAEAYGVKTNVQDMASWVMVNMKPDSLQDNSLR 270
AmpC Ent cl: .....R........Q............N...A..A.ENVA.A..K
AmpC Cit fr: .G.P......Q.........SS.I...R..QA..DASHV.EKT.Q
AmpC E.coli: E.........A.........STIE...R..QS.L..LDINEKT.Q

ACT-1:       KGLTLAQSRYWRVGAMYQGLGWEMLNWPVDAKTVVEGSDNKVALA 315
AmpC Ent cl: Q.IA........I.S..............E.N.............
AmpC Cit fr: Q.IE........I.D.............LK.DSIIN...S.....
AmpC E.coli: Q.IQ.......QT.D..........D...NPDSIIN.....I...

ACT-1:       PLPAREVNPPAPPVNASWVHKTGSTGGFGSYVAFIPEKQLGIVML 360
MIR-1:       *..VA.........K..............................
AmpC Ent cl: ...VA.........K.......................I.....
AmpC Cit fr: A...V.......A.K...................V...N......
AmpC E.coli: AR.VKAIT..T.A.R........A..............E......

ACT-1:       ANKSYPNPARVEAAYRILSAL-                        381
MIR-1:       ......***************
AmpC Ent cl: ..T............H..E..Q
AmpC Cit fr: ..K...........W...EK.Q
AmpC E.coli: ...N.......D..WQ..N..Q
```

FIG. 2. Derived amino acid sequence of the ACT-1 β-lactamase compared to those of selected other group 1 enzymes. Alignment of the deduced amino acid sequence of the ACT-1 β-lactamase with the AmpC β-lactamases of *E. cloacae* (AmpC Ent cl) (12), *C. freundii* (AmpC Cit fr) (21), and *E. coli* (AmpC E. coli) (19) and the MIR-1 β-lactamase (28). Dots indicate identical amino acids at that residue, hyphens indicate no amino acid residue present at that position, whereas asterisks indicate no amino acid sequence data available for those residues.

protein band correlated with imipenem resistance in these strains (Table 2).

DISCUSSION

Several plasmid-mediated AmpC β-lactamases have been described previously; however, only the MIR-1 (28) and the ACT-1 enzymes appear to have originated from the AmpC enzyme of *E. cloacae*. The BIL-1 (11, 29), CMY-2 (3), LAT-1 (40), and LAT-2 (13) β-lactamases were derived from *C. freundii*, whereas CMY-1 (4), FOX-1 (14), and MOX-1 (16, 17) originated from the *Pseudomonas aeruginosa* AmpC β-lactamase. The increasing frequency of occurrence of clinical isolates expressing a plasmid-mediated group 1 β-lactamase (18) is troublesome because the ACT-1 enzyme found in our *K. pneumoniae* and *E. coli* isolates conferred resistance to the cephamycins. This was in addition to resistance to the expanded-spectrum β-lactam drugs provided by the presumed extended-spectrum TEM and SHV β-lactamases also found in these strains, resulting in resistance to all cephalosporins except cefepime.

The fact that the ACT-1 β-lactamase gene is found on the chromosomes of some strains but on a plasmid in other strains suggests that the bla_{ACT-1} gene is located on a transposon which is easily mobilized from plasmid to chromosome and vice versa. In addition, the ACT-1 β-lactamase was initially cloned on a 15-kb piece of DNA which also contained genes

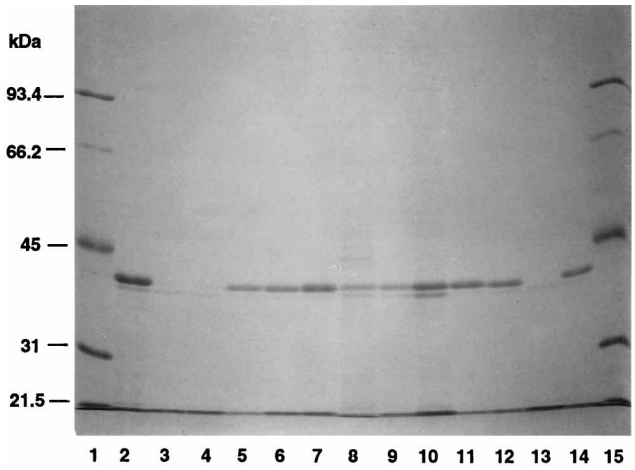

FIG. 3. Outer membrane protein profile of *K. pneumoniae* clinical isolates expressing ACT-1. Outer membrane proteins were run by SDS-PAGE in 12% acrylamide. Lane 1, Bio-Rad low-molecular-weight standard; lane 2, MCQ-95; lane 3, MCQ-102 (imipenem resistant); lane 4, MCQ-103 (imipenem resistant); lane 5, MCQ-110; lane 6, MCQ-111; lane 7, MCQ-112; lane 8, MCQ-113; lane 9, MCQ-114; lane 10, MCQ-117; lane 11, MCQ-120; lane 12, MCQ-121; lane 13, MCQ-122 (imipenem resistant); lane 14, PT 4875 (imipenem-susceptible control strain); lane 15, molecular size standard.

for two other β-lactamases having pIs of 5.4 and 7.0. It is possible that the three β-lactamase genes are all contained within a single transposable element.

In this study the loss of a single major outer membrane protein in clinical isolates of *K. pneumoniae* correlated with resistance to imipenem when it occurred in a strain expressing the ACT-1 β-lactamase. The PFGE experiments demonstrated that strains MCQ-121 and MCQ-122, which were obtained from a single patient before and after imipenem therapy, respectively, were indistinguishable. Therefore, imipenem resistance in strain MCQ-122 is most likely due to the outer membrane protein alteration, which is the only observed difference between the two strains. This result is consistent with previous reports in which the imipenem-resistant strains *Enterobacter aerogenes*, *E. cloacae*, and *Providencia rettgeri* constitutively produced high levels of the AmpC β-lactamase and lacked a major outer membrane protein (9, 10, 20, 31, 39).

Increased β-lactam resistance due to outer membrane protein mutations in *K. pneumoniae* have been reported previously (8, 23, 34, 42, 43). Cefamandole and cefoxitin MICs were increased for strains expressing a broad-spectrum β-lactamase and lacking a 40-kDa outer membrane protein (42). Chen and Livermore (8) used cefoxitin to select mutants of *K. pneumoniae* expressing SHV-1 which had lost a 40-kDa doublet of outer membrane proteins (8). The loss of a 41-kDa outer membrane protein resulted in resistance to cefoperazone-sulbactam in strains expressing TEM-2, as reported by Rice et al. (34). In studies by Martínez-Martínez et al. (23) and Vatopoulos et al. (43), SHV-5-producing strains of *K. pneumoniae* with altered 35- to 40-kDa outer membrane proteins had increased resistance to cefotaxime and cefoxitin. However, in each of those studies the MICs of imipenem remained low. Therefore, it appears that the type of β-lactamase present in the parent strain dictates the spectrum of resistance which will occur in the outer membrane protein-deficient strain. In the imipenem-resistant strains in this study, the ACT-1 β-lactamase was not sufficient to confer resistance, as demonstrated by susceptibility tests with transconjugant strains and clones. However, when combined with the presumed decreased permeability provided

by the outer membrane protein mutation, the enzyme can sufficiently protect the cell from the killing action of imipenem. This outer membrane protein may have different rates of uptake of the various β-lactam antibiotics and may even be a preferential porin for carbapenems.

Imipenem resistance in *K. pneumoniae* is a particularly disturbing development in the world of antibiotic resistance. For many strains expressing multiple AmpC types and extended-spectrum β-lactamases, imipenem was the only therapeutic option for serious infections. Now *Klebsiella* has been added to the growing list of pathogens which are susceptible to a very limited number of antibiotic therapies.

ACKNOWLEDGMENTS

We thank B. A. Rasumussen for providing *E. coli* BAR207 and cloning vector pCLL2300, E. Calcagni for assistance with the PFGE experiments, and V. Bernan for help with the MIDI identification.

C. Urban, N. Mariano, and J. Rahal were supported by the BMA Medical Foundation, Inc.

REFERENCES

1. **Ames, G. F.** 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. **249:**634–644.
2. **Bauernfeind, A., Y. Chong, and S. Schweighart.** 1989. Extended broad spectrum β-lactamase in *Klebsiella pneumoniae* including resistance to cephamycins. Infection **17:**316–321.
3. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, and H. Giamarellou.** 1996. Characterization of the plasmidic β-lactamase CMY-2 which is responsible for cephamycin resistance. Antimicrob. Agents Chemother. **40:**221–224.
4. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, R. Wilhelm, and Y. Chong.** 1996. Comparative characterization of the cephamycinase bla_{CMY-1} gene and its relationship with other β-lactamase genes. Antimicrob. Agents Chemother. **40:**1926–1930.
5. **Bradford, P. A., C. Urban, A. Jaiswal, N. Mariano, B. A. Rasmussen, S. J. Projan, J. J. Rahal, and K. Bush.** 1995. SHV-7, a novel cefotaxime-hydrolyzing β-lactamase, identified in *Escherichia coli* isolates from hospitalized nursing home patients. Antimicrob. Agents Chemother. **39:**899–905.
6. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
7. **Bush, K., and S. B. Singer.** 1989. Effective cooling allows sonication to be used for liberation of β-lactamases from gram-negative bacteria. J. Antimicrob. Chemother. **24:**82–84.
8. **Chen, H. Y., and D. M. Livermore.** 1993. Activity of cefepime and other β-lactam antibiotics against permeability mutants of *Escherichia coli* and *Klebsiella pneumoniae*. J. Antimicrob. Chemother. **32**(Suppl. B)**:**63–74.
9. **Chow, J. W., and D. M. Shlaes.** 1991. Imipenem resistance associated with the loss of a 40 kDa outer membrane protein in *Enterobacter aerogenes*. J. Antimicrob. Chemother. **28:**499–504.
10. **Ehrhardt, A. F., C. C. Sanders, K. S. Thomson, C. Watanakunakorn, and I. Trujillano-Martin.** 1993. Emergence of resistance to imipenem in *Enterobacter* masquerading as *Klebsiella pneumoniae* during therapy with imipenem/cilastatin. Clin. Infect. Dis. **17:**120–122.
11. **Fosberry, A. P., D. J. Payne, E. J. Lawlaor, and J. E. Hodgson.** 1994. Cloning and sequence analysis of bla_{BIL-1}, a plasmid-mediated class C β-lactamase gene in *Escherichia coli*. Antimicrob. Agents Chemother. **38:**1182–1185.
12. **Galleni, M., F. Lindberg, S. Normark, S. Cole, N. Honore, B. Joris, and J.-M. Frere.** 1988. Sequence and comparative analysis of three *Enterobacter cloacae ampC* β-lactamase genes and their products. Biochem. J. **250:**753–760.
13. **Gazouli, M., L. S. Tzouvelekis, E. Prinarakis, V. Miriagou, and E. Tzelepi.** 1996. Transferable cefoxitin resistance in enterobacteria from Greek hospitals and characterization of a plasmid-mediated group 1 β-lactamase (LAT-2). Antimicrob. Agents Chemother. **40:**1736–1740.
14. **Gonzalez-Leiza, M., J. C. Perez-Diaz, J. Ayala, J. M. Casellas, J. Martinez-Beltran, K. Bush, and F. Baquero.** 1994. Gene sequence and biochemical characterization of FOX-1 from *Klebsiella pneumoniae*, a new AmpC-type plasmid-mediated β-lactamase with two molecular variants. Antimicrob. Agents Chemother. **38:**2150–2157.
15. **Hanahan, D.** 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. **166:**557–580.
16. **Horii, T., Y. Arakawa, M. Ohta, S. Ichiyama, R. Wacharotayankun, and N. Kata.** 1993. Plasmid-mediated AmpC-type β-lactamase isolated from *Klebsiella pneumoniae* confers resistance to broad-spectrum β-lactams, including moxalactam. Antimicrob. Agents Chemother. **37:**984–990.
17. **Horii, T., Y. Arakawa, M. Ohta, T. Sugiyama, R. Wacharotayankun, H. Ito, and N. Kato.** 1994. Characterization of a plasmid-borne and constitutively expressed bla_{MOX-1} gene encoding AmpC-type β-lactamase. Gene **139:**93–98.
18. **Jacoby, G. A., P. Han, M. Alvarez, and F. Tenover.** 1995. Survey of extended-spectrum β-lactamase production in US clinical isolates, abstr. C40, p. 46. *In* Program and abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
19. **Jaurin, B., and T. Grundström.** 1981. *ampC* cephalosporinase of *Escherichia coli* K-12 has a different evolutionary origin from that of β-lactamases of the penicillinase type. Proc. Natl. Acad. Sci. USA **78:**4897–4901.
20. **Lee, E. H., M. H. Nicolas, M. D. Kitzis, G. Pialoux, E. Collatz, and L. Gutmann.** 1991. Association of two resistance mechanisms in a high-level imipenem-resistant clinical isolate of *Enterobacter cloacae*. Antimicrob. Agents Chemother. **35:**1093–1098.
21. **Lindberg, F., and S. Normark.** 1986. Sequence of the *Citrobacter freundii* OS60 chromosomal *amp C* β-lactamase gene. Eur. J. Biochem. **156:**441–445.
22. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. V. Alphen.** 1975. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli*. FEBS Lett. **58:**254–258.
23. **Martínez-Martínez, L., S. Hernández-Allés, S. Albertí, J. Tomás, V. Benedi, and G. Jacoby.** 1996. In vivo selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded-spectrum cephalosporins. Antimicrob. Agents Chemother. **40:**342–348.
24. **Maslow, J. N., A. M. Slutsky, and R. D. Arbeit.** 1993. Application of pulsed-field gel electrophoresis to molecular epidemiology, p. 563–572. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, D.C.
25. **Matthew, M. A., A. M. Harris, M. J. Marshall, and G. W. Ross.** 1975. The use of isoelectric focusing for detection and identification of beta-lactamases. J. Gen. Microbiol. **88:**169–178.
26. **Meyer, K. S., C. Urban, J. A. Eagan, B. J. Berger, and J. J. Rahal.** 1993. Nosocomial outbreak of *Klebsiella* infection resistant to late generation cephalosporins. Ann. Intern. Med. **119:**353–358.
27. **National Committee for Clinical Laboratory Standards.** 1996. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
28. **Papanicolaou, G., A. A. Medeiros, and G. A. Jacoby.** 1990. Novel plasmid-mediated β-lactamase (MIR-1) conferring resistance to oxyimino- and α-methoxy β-lactams in clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **34:**2200–2209.
29. **Payne, D. J., N. Woodford, and S. G. B. Amyes.** 1992. Characterization of the plasmid mediated β-lactamase BIL-1. J. Antimicrob. Chemother. **30:**119–127.
30. **Quinn, J. P., D. Miyashiro, D. Sahm, R. Flamm, and K. Bush.** 1989. Novel plasmid-mediated β-lactamase (TEM-10) conferring selective resistance to ceftazidime and aztreonam in clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **33:**1451–1456.
31. **Raimondi, A., A. Traverso, and H. Nikaido.** 1991. Imipenem- and meropenem-resistant mutants of *Enterobacter cloacae* and *Proteus rettgeri* lack porins. Antimicrob. Agents Chemother. **35:**1174–1180.

31a. **Rasmussen, B. A.** Personal communication.

32. **Rasmussen, B. A., P. A. Bradford, J. P. Quinn, J. Wiener, R. A. Weinstein, and K. Bush.** 1993. Genetically diverse ceftazidime resistant isolates from a single center: biochemical and genetic characterization of TEM-10 β-lactamases encoded by different nucleotide sequences. Antimicrob. Agents Chemother. **37:**1989–1992.
33. **Rasmussen, B. A., Y. Gluzman, and F. P. Tally.** 1990. Cloning and sequencing of the class B β-lactamase gene (*ccrA*) from *Bacteroides fragilis* TAL3636. Antimicrob. Agents Chemother. **34:**1590–1592.
34. **Rice, L. B., L. L. Carias, L. Etter, and D. M. Shlaes.** 1993. Resistance to cefoperazone-sulbactam in *Klebsiella pneumoniae*: evidence for enhanced resistance resulting from the coexistence of two different resistance mechanisms. Antimicrob. Agents Chemother. **37:**1061–1064.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
36. **Sanders, W. E., Jr., and C. C. Sanders.** 1988. Inducible β-lactamases: clinical and epidemiologic implications for use of newer cephalosporins. Rev. Infect. Dis. **10:**830–838.
37. **Sykes, R. B., D. P. Bonner, K. Bush, and N. H. Georgopapadakou.** 1982. Azthreonam (SQ 26,776), a synthetic monobactam specifically active against aerobic gram-negative bacteria. Antimicrob. Agents Chemother. **21:**85–92.
38. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
39. **Thomson, K. S., C. C. Sanders, and H. Chmel.** 1993. Imipenem resistance in *Enterobacter*. Eur. J. Clin. Microbiol. Infect. Dis. **12:**610–613.

40. **Tzouvelekis, L. S., E. Tzelepi, and A. F. Mentis.** 1994. Nucleotide sequence of a plasmid-mediated cephalosporinase gene (bla_{LAT-1}) found in *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **38:**2207–2209.
41. **Urban, C., K. S. Meyer, N. Mariano, J. J. Rahal, R. Flamm, B. A. Rasmussen, and K. Bush.** 1994. Identification of TEM-26 β-lactamase responsible for a major outbreak of ceftazidime-resistant *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **38:**392–395.
42. **van de Klundert, J. A. M., M. H. van Gestel, G. Meerdink, and S. de Marie.** 1988. Emergence of bacterial resistance to cefamandole in vivo due to outer membrane protein deficiency. Eur. J. Clin. Microbiol. Infect. Dis. **7:**776–778.
43. **Vatopoulos, A. C., A. Philippon, L. S. Tzouvelekis, Z. Komninou, and N. J. Legakis.** 1990. Prevalence of a transferable SHV-5 type β-lactamase in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Greece. J. Antimicrob. Chemother. **26:**635–648.