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Outer Membrane Profiles of Clonally Related *Klebsiella pneumoniae* Isolates from Clinical Samples and Activities of Cephalosporins and Carbapenems

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Fifteen isolates of *Klebsiella pneumoniae* producing extended-spectrum β-lactamases (ESBLs) isolated during a nosocomial outbreak were studied. The strains belonged to the same clonal type, as shown by pulsed-field gel electrophoretic analysis of chromosomal DNA. All the isolates were resistant to extended-spectrum cephalosporins, aztreonam, gentamicin, and fluoroquinolones and were susceptible to carbapenems, tobramycin, netilmicin, and amikacin. None of the isolates expressed the OmpK36 porin. Eight isolates, for which the MICs of cefoxitin were ≥64 μg/ml, showed a diminished level or no expression of a 35-kDa porin. The MICs of meropenem, cefotaxime, and cefpirome were three to eight times higher for porin-deficient isolates than for isolates expressing the 35-kDa porin, but the MICs of imipenem increased two times for porin-deficient isolates compared to those for isolates expressing the porin. This MIC increase reverted to a level similar to that for the parental strain when porin-deficient isolates were transformed with the gene coding for the *K. pneumoniae* porin OmpK36. It is concluded that the high level of resistance to cefoxitin and the increase in the MICs of meropenem, cefotaxime, and cefpirome for the ESBL-producing *K. pneumoniae* isolates studied are associated with porin deficiency.

Klebsiella pneumoniae is an important human pathogen that has been associated in recent decades with nosocomial outbreaks. After the use of extended-spectrum cephalosporins, extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* has become an increasingly serious problem worldwide (3, 11, 12, 25). This class of β-lactamases consists of plasmid-mediated enzymes that are able to hydrolyze expanded-spectrum cephalosporins and monobactams. In *K. pneumoniae* cefoxitin resistance may be due to β-lactamase production (7, 24) or the loss of porins (15, 23, 30).

Porins are outer membrane proteins (OMPs) that allow the nonspecific diffusion of small molecules into the bacterial cell. Most of the studies about OMPs have been carried out with *Escherichia coli*, in which two major porins (OmpC and OmpF) have been characterized. Loss of either of them has been related to antibiotic resistance (21). Decreased permeability can produce significant levels of resistance that may be increased when it is combined with enzymatic inactivation (21). In *K. pneumoniae*, two main porins have been characterized: OmpK35 (the homolog of OmpF) and OmpK36 (the homolog of OmpC) (1, 10). Recently, loss of the OmpK36 porin has been associated with both cefoxitin resistance and increases in cephalosporin and quinolone MICs (15). The association between the loss of porins and increased MICs of carbapenems has recently been described for *K. pneumoniae* producing a plasmid-mediated AmpC-like β-lactamase (2, 16). Expression of OmpK36 and/or inactivation of AmpC abolished carbapenem resistance in this particular type of strain (16).

From May 1993 to June 1995, a nosocomial outbreak due to *K. pneumoniae* producing ESBL involved 150 patients in our hospital (25). During the outbreak, 4% of the ESBL-producing *K. pneumoniae* isolates showed high levels of resistance to cefoxitin (MIC, >64 μg/ml). The aim of this study was to analyze the mechanism of cefoxitin resistance among these strains.

MATERIALS AND METHODS

Bacterial isolates. Fifteen strains of ESBL-producing *K. pneumoniae* isolated from 12 colonized or infected patients during the outbreak period were studied. Eight of them were highly cefoxitin resistant (MICs, ≥64 μg/ml), for four strains cefoxitin MICs were between 16 and 32 μg/ml, and the remaining three strains were cefoxitin susceptible (MICs, between 2 and 4 μg/ml). These last three isolates were recovered together with highly resistant isolates from a pharyngeal swab, catheter, and blood of three patients, respectively.

Susceptibility studies. MICs were determined by the microdilution method (19) and the E-test (AB Biodisk, Solna, Sweden). The following antibiotics were tested: amikacin, amoxicillin, amoxicillin-clavulanic acid (2:1), aztreonam, cefotaxime, cefoxitin, cefoxitin-clavulanic acid (2:1), cefpirome, ceftazidime, ceftazidime-clavulanic acid (2:1), ciprofloxacin, gentamicin, imipenem, meropenem, netilmicin, ofloxacin, piperacillin, piperacillin-tazobactam (2:1), sparfloxacin, and tobramycin.

Antimicrobial susceptibility tests with strains CSUB10R(pSHA19) and CSUB10R(pSHA20) (see below) were performed in Mueller-Hinton broth (Izasa, Barcelona, Spain) supplemented with 50 μg of kanamycin (Sigma, Madrid, Spain) per ml and 25 μg of chloramphenicol (Sigma) per ml.

The presence of broad extended-spectrum β-lactamase production was studied by the double-disk synergy test (14) and by the E-test method (AB Biodisk).

Conjugation experiments. Transfer of resistance to expanded-spectrum cephalosporins and monobactams from *K. pneumoniae* CSUB10S and CSUB10R to *E. coli* J53-2 was carried out by conjugation in broth as described previously (15). Ampicillin and rifampin (100 μg/ml each) were used as selective agents.

Isoelectric focusing. Strains were grown for 4 h in Luria broth. The growing bacteria were pelleted, resuspended in distilled water, and sonicated. Extract purifications were performed by ultracentrifugation (14). Isoelectric focusing of

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TABLE 1. Characteristics of cefoxitin-resistant and -susceptible ESBL-producing *K. pneumoniae* pairs isolated from three patients and the transformed strains of CSUB10R containing plasmids coding for the entire (pSHA19) or truncated (pSHA20) sequence of OmpK36 porin

Antimicrobial agent	MIC (μg/ml)							
	Patient 1		CSUB10R(pSHA19)	CSUB10R(pSHA20)	Patient 2		Patient 3	
	CSUB10R	CSUB10S			CSUB8R	CSUB8S	CSUB9R	CSUB9S
Cefoxitin	128	2	0.6	128	128	4	128	4
Cefotaxime	>256	4	32	>256	>256	64	256	4
Cefpirome	>256	2	8	>256	256	8	128	2
Ceftazidime	>256	>256	>256	>256	>256	>256	>256	>256
Ceftazidime-clavulanic acid	2	0.5	0.5	2	4	1	2	0.5
Aztreonam	>256	>256	>256	>256	>256	>256	>256	>256
Imipenem	0.5	0.12	0.06	0.5	0.5	0.12	0.5	0.12
Meropenem	2	0.06	0.12	2	2	0.03	2	0.03
Ofloxacin	4	2	0.5	4	4	2	8	4
Ciprofloxacin	4	0.5	0.5	4	2	1	2	2
Sparfloxacin	4	1	0.25	4	2	1	2	1
Porin expression	−	+	+	−	−	+	−	+

β-lactamase extracts was done with the PhastSystem apparatus (Pharmacia, Uppsala, Sweden) in polyacrylamide gels with a pH range of 3 to 9 (PhastGel 3-9; Pharmacia). The gels were stained with 500 μg nitrocefin (Oxoid, Hampshire, England) per ml, and pIs were determined by comparison with different β-lactamases with known pIs.

Typing methods. Biotyping was carried out with API 20E galleries (bioMérieux, Balmes les Grottes, France) according to the manufacturer's instructions and with MicroScan NegCombo 6I panels (DADE International, Inc., West Sacramento, Calif.).

Macrorestriction analysis of chromosomal DNA was done by pulsed-field gel electrophoresis (PFGE) by previously described procedures (8). DNA restriction was done with *Xba*I (New England Biolabs, Madrid, Spain) following the manufacturer's recommendations. PFGE was performed in a CHEF-DR III apparatus (Bio-Rad, Hercules, Calif.) for 23 h at 14°C with pulse times ranging from 1 to 30 s at 6 V/cm.

OMP isolation and analysis. For porin isolation, we used a combination of the two methods for the isolation of *E. coli* porins (20, 22). Cell envelopes were treated with trypsin and subjected to differential solubilization as described in detail by Alberti et al. (1). The isolated porins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and were electrophoretically transferred to Immobilon P membranes (Millipore, Barcelona, Spain) by using the buffers and conditions described by Towbin et al. (29). The membranes were stained with Ponceau red, and bands of interest were excised separately, destained, and sequenced in an Applied Biosystems 470 gas-phase sequencer (kindly done by the Servicio de Secuenciación of the Centro de Investigaciones Biológicas del Consejo Superior de Investigaciones Cientificas, Madrid, Spain).

K. pneumoniae strains were grown in Mueller-Hinton broth and sonicated, and cell envelopes were recovered by ultracentrifugation. After treatment with sodium *N*-lauroyl sarcosinate (Sigma, Madrid, Spain), the OMPs were collected by ultracentrifugation (15). Electrophoretic analysis of OMP by SDS-PAGE was performed in 11% acrylamide–0.35% bisacrylamide–0.1% SDS by using Laemmli's buffers. The samples were boiled for 5 min in Laemmli's buffer before electrophoresis. The gels were stained with Coomassie blue.

Transfer and expression of *ompK36* gene. Plasmid pSUV7, containing the gene coding for the OmpK36 porin, and plasmid pFR167, containing a truncated *ompK36* gene, have been described previously (15). These plasmids include a kanamycin resistance cassette to allow their selection in the multidrug-resistant background of strain CSUB10R. Briefly, the kanamycin resistance cassette of plasmid pCSI2 (6) was obtained as an *Xba*I-*Xba*I fragment and was cloned into the unique *Xba*I sites of plasmids pSUV7 and pFR167. The result was plasmid pSHA19, which contains the *ompK36* gene, and plasmid pSHA20, which contains a truncated *ompK36* gene. The modified plasmids were introduced by electroporation into strain CSUB10R, and the transformed CSUB10R strains carrying the cloned porin genes were selected as kanamycin-resistant strains. DNA isolation, enzyme restrictions, and ligation were performed by standard procedures (28).

RESULTS

Susceptibility testing. All the strains tested were resistant to amoxicillin (MIC, >256 μg/ml) and piperacillin (MICs, 128 to >256 μg/ml). The amoxicillin-clavulanic acid MICs ranged from 4 to 16 μg/ml, whereas the piperacillin-tazobactam MIC range was from 2 to >256 μg/ml. For cefoxitin-resistant strains, the addition of clavulanic acid did not result in a reversion to cefoxitin susceptibility. Amikacin (MIC, 2 μg/ml), tobramycin (MIC range, 0.5 to 1 μg/ml), and netilmicin (MIC range, 0.5 to 2 μg/ml) were active against all *K. pneumoniae* isolates. All the isolates were resistant to gentamicin (MIC range, 8 to 16 μg/ml).

Table 1 presents the MICs of 11 antibiotics for cefoxitin-susceptible and -resistant isolates cultured from the same patient and the MICs of these antibiotics for CSUB10R (pSHA19) and CSUB10R(pSHA20) containing the entire and truncated *ompK36* porin genes, respectively. For cefoxitin-resistant isolates, the MICs of cefotaxime (3 to 5 dilution steps), cefpirome (5 to 7 dilution steps), meropenem (5 to 6 dilution steps), imipenem (2 dilution steps), and ceftazidime-clavulanic acid (2 dilution steps) were higher than those for the cefoxitin-susceptible strains. The quinolone MICs for cefoxitin-resistant strains were always from 1 to 3 dilution steps higher than those for cefoxitin-susceptible strains. For strain CSUB10R, the cefoxitin resistance and the increased MICs of meropenem, cefotaxime, cefpirome, and ceftazidime-clavulanic acid reverted to MICs which were similar to those for strain CSUB10S after cloning of the *ompK36* gene into the strain [strain CSUB10R(pSHA19)].

Table 2 presents the MICs of 11 antibiotics for *K. pneumoniae* isolates with different degrees of resistance to cefoxitin. For isolates for which the cefoxitin MIC was ≥64 μg/ml, the cefpirome and cefotaxime MICs increased and the meropenem MIC was 4 to 32 times higher than those for strains for which cefoxitin MICs were <64 μg/ml. The MICs of norfloxacin (MIC range, 64 to 128 μg/ml), ofloxacin (MIC range, 4 to 8 μg/ml), ciprofloxacin (MIC range, 2 to 8 μg/ml), or sparfloxacin (MIC range, 1 to 4 μg/ml) cannot be related to the degree of cefoxitin resistance.

E. coli transconjugants, with *K. pneumoniae* CSUB10R or CSUB10S used as donors, were resistant to expanded-spectrum cephalosporins as a result of ESBL production. Cefoxitin resistance and increased carbapenem MICs were not transferred to *E. coli* by conjugation of the donor.

β-Lactamase study. The production of ESBLs was demonstrated in all the strains by the double-disk synergy test and by at least a threefold reduction in the ceftazidime MIC when clavulanic acid was added. Isoelectric focusing of β-lactamase extracts showed that all but one of the isolates produced a

TABLE 2. Characteristics of nine *K. pneumoniae* strains with different degrees of resistance to cefoxitin isolated from nine patients

Antimicrobial agent	MIC (μg/ml)								
	CSUB3	CSUB7	CSUB1	CSUB6	CSUB4	CSUB5	CSUB2	CSUB11	CSUB12
Cefoxitin	16	16	32	32	64	64	128	128	128
Cefotaxime	0.25	4	2	2	128	64	4	>256	32
Cefpirome	4	2	1	2	64	32	16	>256	32
Ceftazidime	16	64	4	128	>256	64	8	>256	>256
Ceftazidime-clavulanic acid	1	1	0.5	2	2	1	0.5	4	2
Aztreonam	1	128	1	128	32	16	2	>256	>256
Imipenem	0.12	0.12	0.06	0.12	0.25	0.12	0.12	0.5	0.5
Meropenem	0.03	0.03	0.03	0.03	0.12	0.12	0.5	2	1
Ofloxacin	8	8	8	8	8	8	8	4	4
Ciprofloxacin	4	4	4	8	4	4	8	2	2
Sparfloxacin	4	2	4	4	4	2	4	1	1
Porin expression	+	+	+	+	±[a]	±	−	−	−

[a] ±, reduced level of porin expression.

single enzyme: nine produced an enzyme with a pI of 8.2, and five produced an enzyme with a pI of 7.6. The remaining isolate produced two β-lactamases with pIs of 7.6 and 8.2. No relationship between pI and the degree of cefoxitin resistance was found. The cefoxitin-resistant and -susceptible strains isolated from the same patients had β-lactamases with identical pIs. Aztreonam MICs showed variations according to the β-lactamase pI. For *K. pneumoniae* isolates producing a β-lactamase with a pI of 8.2 the aztreonam MIC (MIC range, 128 to >256 μg/ml) was higher than that for those producing a β-lactamase with a pI of 7.6 (MIC range, 0.5 to 32 μg/ml).

Typing methods. All 15 isolates exhibited the same biotype by testing both with the API 20E and the MicroScan systems. A major PFGE pattern after chromosomal DNA restriction with *Xba*I was found (Fig. 1). This pattern showed only five minor variations (subtypes A1 to A5), and overall, the isolates were considered to be clonally related to the dominant strain found during the outbreak. There was no relationship between the PFGE subtypes of the strains and porin expression. Pairs of cefoxitin-resistant and cefoxitin-susceptible isolates from three patients also had identical PFGE patterns.

OMP analysis. SDS-PAGE analysis of the OMPs showed that all the clinical isolates expressed two OMPs of about 32 and 45 kDa and that isolates for which cefoxitin MICs were <128 μg/ml also expressed an additional OMP of about 35 kDa (Fig. 2 and 3). The 32-kDa protein is probably the *K. pneumoniae* homolog of *E. coli* OmpA because of its increased mobility (molecular mass, about 22 kDa) in samples solubilized at 37°C (data not shown). The 45-kDa band was isolated from strain CSUB10R (Fig. 4) by a porin isolation method, and N-terminal analysis of its first 12 amino acids showed that it had complete identity with *K. pneumoniae* LamB. The porin isolation method, when applied to strain CSUB10S, produced the 35- and 45-kDa proteins (Fig. 4). After SDS-PAGE separation, they were transferred to a polyvinylidene difluoride membrane, and their N termini were sequenced. Sequence analysis confirmed that 45-kDa protein corresponds to LamB, while the 35-kDa sequence demonstrated that it is a nonspecific pore protein (porin). The complete identity of the first amino acids of the OmpK36 and OmpK35 porins from *K. pneumoniae* and other enterobacterial porins (13) prevented assignment of the 35-kDa porin to either one of the two porins of the species. This 35-kDa porin was absent from isolates for which the cefoxitin MIC was 128 μg/ml (Fig. 2 and 3). Expression of this porin, as judged by SDS-PAGE, was reduced in isolates for which cefoxitin MICs were 64 μg/ml, while sufficient expression of this porin was found in isolates for which cefoxitin MICs were 32 μg/ml (Fig. 2 and 3).

DISCUSSION

Nosocomial outbreaks due to ESBL-producing enterobacteria have become a serious problem worldwide (12, 17, 18). Treatment of infections due to these microorganisms is a difficult task because β-lactamase production inactivates most of the β-lactam antibiotics, and these microorganisms are usually resistant to other antibiotic groups such as aminoglycosides and quinolones. Cephamycins such as cefoxitin are active in vitro against these strains, but this agent can select porin-deficient mutants with increased levels of resistance to cefoxitin and other cephalosporins (15, 23, 30). Combinations of a β-lactam and a β-lactamase inhibitor are not always active against these microorganisms (27). Carbapenems also remain a good option, but the emergence of imipenem-resistant strains of *Pseudomonas aeruginosa* and other gram-negative bacilli could occur when imipenem is widely used (17). In addition, it has recently been described that *K. pneumoniae* becomes carbapenem resistant as a result of porin deficiency and plasmid-mediated AmpC-like β-lactamases (2, 16).

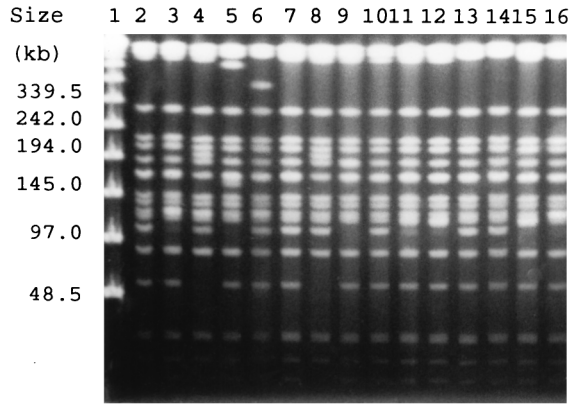

FIG. 1. PFGE of total DNA from *K. pneumoniae* cut with *Xba*I. Lane 1, PFGE Molecular Weight Marker (New England Biolabs); Lanes 2 and 3 and lanes 6 and 7, porin-sufficient isolates; lanes 4 and 5, strains with diminished expression of porin; lanes 8 to 10, porin-deficient isolates; lanes 11 and 12, lanes 13 and 14, and lanes 15 and 16, pairs of porin-deficient and porin-sufficient isolates (in the respective pairs of lanes) from three patients.

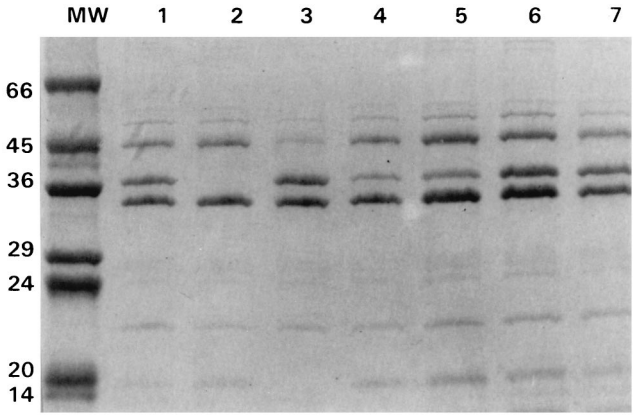

FIG. 2. SDS-PAGE analysis of outer membrane proteins of *K. pneumoniae* isolates. Lane MW, molecular weight standard (in kilodaltons); lanes 1, 3, 6, and 7, porin-expressing isolates; lanes 4 and 5, isolates with diminished levels of porin expression; lane 2, porin-deficient isolate.

The loss of porins OmpC and OmpF as a cause of antibiotic resistance has been noted in several reports, especially for *E. coli* and *Salmonella typhimurium* (21). In *K. pneumoniae*, loss of both the OmpK35 and the OmpK36 porins has been shown to cause increased levels of resistance to cefoxitin and extended-spectrum cephalosporins and probably contributes to ciprofloxacin resistance (4, 15, 30). This resistance phenotype reverted when the strain expressed OmpK36 porin in its outer membrane after cloning of the *ompK36* gene (15).

During an outbreak caused by *K. pneumoniae* producing ESBLs in our hospital (25), 4% of the isolates were highly resistant to cefoxitin (MICs, ≥128 μg/ml). Porin deficiency was associated with this resistance phenotype, and the diminished level of expression of this protein was related to a cefoxitin MIC of 64 μg/ml. In addition, porin deficiency was associated with increased MICs of cefotaxime and cefpirome, probably because in porin-deficient mutants the uptake of extended-spectrum cephalosporins is less effective than that in porin-sufficient strains (23). Thus, the combination of the decreased outer membrane permeation and the hydrolytic effect of ESBLs increased the MICs of expanded-spectrum cephalosporins for the resistant strains studied.

The six porin-deficient strains showed 8- to 32-fold decreased susceptibilities to meropenem but only 2-fold decreased susceptibilities to imipenem. The MICs of meropenem were four times higher than those of imipenem. When isolate CSUB10R was transformed with a gene coding for the OmpK36 *K. pneumoniae* porin, this resistance phenotype reverted and the MICs of carbapenems for this strain were similar to those for the CSUB10S strain that expresses the OmpK36 porin and that was isolated from the same clinical sample as strain CSUB10R. These findings suggest a main role of this porin in the decreased susceptibility of *K. pneumoniae* to meropenem.

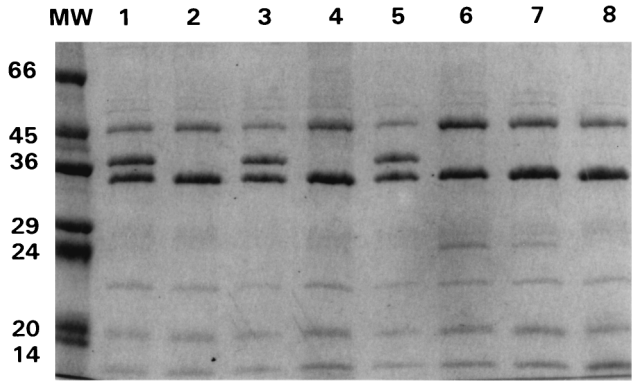

FIG. 3. SDS-PAGE analysis of outer membrane proteins of *K. pneumoniae* isolates. Lane MW, molecular mass standard (in kilodaltons); lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6, porin-sufficient and porin-deficient isolates (in the respective pairs of lanes) from the same patients; lanes 7 and 8, porin-deficient isolates.

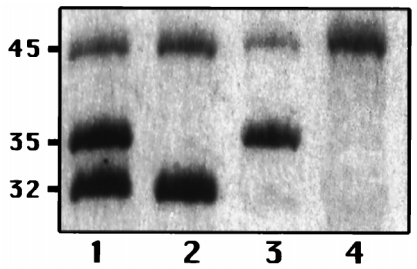

FIG. 4. SDS-PAGE analysis of OMPs and porins from strains CSUB10S (lanes 1 and 3) and CSUB10R (lanes 2 and 4). Lanes 1 and 2, OMP; lanes 3 and 4, porins. Numbers on the left side correspond to the approximate molecular masses of the proteins (in kilodaltons).

The association between the loss of porins and imipenem resistance has recently been described in *K. pneumoniae* producing plasmid-mediated AmpC-like β-lactamase (2, 16). In other members of the family *Enterobacteriaceae* such as *Enterobacter cloacae* and *Proteus rettgerii*, resistance to carbapenems has been related to diminished outer membrane permeability and hydrolysis by the overproduced chromosomal β-lactamase (5, 26). It seems that, like in *E. cloacae*, the level of meropenem susceptibility in *K. pneumoniae* is more dependent on porin expression, whereas imipenem susceptibility is less affected by this resistance mechanism and is more dependent on the production of secondary β-lactamases of the AmpC type (2, 5, 16, 26). It is difficult to determine the exact mechanism by which the loss of porins results in decreased meropenem susceptibility. In independent studies, we have shown that strain CSUB10R produces an active efflux mechanism causing decreased levels of accumulation of fluoroquinolones in the cell (unpublished observation). A similar mechanism has been observed in *P. aeruginosa* with reduced susceptibility to meropenem when the efflux system MexAB-OprM is expressed. Experiments are in progress to determine a possible link between the efflux of meropenem and decreased susceptibility to this carbapenem.

In a comparison of the three pairs of cefoxitin-susceptible and -resistant strains isolated from the same patient, from two- to eightfold increases in the quinolone MICs were found. In addition, for strain CSUB10R from patient 1, a two- to fourfold decrease in the quinolone MICs was observed after the OmpK36 porin was introduced into this strain. This relationship has been reported previously (9, 15, 30), suggesting that loss of porin can contribute to quinolone resistance. However, this cross-resistance did not correlate with the degree of cefoxitin resistance in the other *K. pneumoniae* isolates studied. The relative importance of the possible mechanisms involved in quinolone resistance are under investigation; however, mutations have been detected in the quinolone resistance-determining region of *gyrA* but not that of *parC* in both CSUB10R and CSUB10S strains (unpublished observations); in addition, an active efflux mechanism causing a decreased level of accumulation of fluoroquinolones was detected in strain CSUB10R.

By PFGE there was a clonal relationship among the highly cefoxitin-resistant *K. pneumoniae* isolates; moreover, these isolates belonged to the same clone as the epidemic strain (which was cefoxitin susceptible) responsible for the outbreak. This suggests the in vivo selection of porin-deficient mutants from a common ancestor, i.e., the epidemic strain, as reported previously (15, 23).

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