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

CARMEN ARDANUY,1* JOSEFINA LINARES,1 MARIA ANGELES DOMINGUEZ,1 SANTIAGO HERNÁNDEZ-ALLES,2 VICENTE J. BENEDI,2 AND LUIS MARTINEZ-MARTINEZ3

Servicio de Microbiología, Hospital de Bellvitge, Universidad de Barcelona, Barcelona,3 Departamento de Biología Ambiental, Universidad de las Islas Baleares and IMEDEA (CSIC-UIB), Palma de Mallorca,2 and Departamento de Microbiología, Facultad de Medicina, Universidad de Sevilla, Seville,3 Spain

Received 19 November 1997/Returned for modification 11 February 1998/Accepted 16 April 1998

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 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 extended-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 cefalosporin 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, cefotaxime-clavulanic acid (2:1), cefpirome, meropenem, imipenem, 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-disc 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 CSUB108 and CSUB10R to E. coli 153-2 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. Extracted purifications were performed by ultracentrifugation (14). Isoelectric focusing of...
β-lactamase extracts was done with the PhastSystem apparatus (Phar- 
macia, Uppsala, Sweden) in polyacrylamide gels with a pH range of 
to 9 (PhastGel 3-9; Pharmacia). The gels were stained with 500 
g nitrocefin (Oxoid, Hampshire, England) per ml, and pIs were 
determined by comparison with different β-lacta-
mases with known pIs.

Typing methods. Biotyping was carried out with API 20E galleries (bio-
Mérieux, Balmes les Grottes, France) according to the manufacturer's in-
structions and with MicroScan NeGtCombo 61 panels (DADE Interna-
tional, Inc., West Sacramento, Calif.).

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

OMIP 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 electro-
phoretically transferred to Immobilon P membranes (Millipore, Bar-
celona, Spain) by using the buffers and conditions described by Towbin et al. (29). The 
membranes were stained with Pronase 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 Científicas, 
Madrid, Spain).

K. pneumoniae strains were grown in Mueller-Hinton broth and sonicated, and 
cell envelopes were recovered by ultrafiltration. After treatment with so-
dium N-lauroyl sarcosinate (Sigma, Madrid, Spain), the OMIs were collected by 
ultracentrifugation (15). Electrophoretic analysis of OMIP by SDS-PAGE was 
performed in 11% acrylamide-0.35% bisacrylamide-0.1% SDS by using Laem-
mli'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 pSU77, 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 
transposon Tn10 derivatives was cloned into the unique XbaI fragment and was cloned into 
the unique XbaI sites of plasmids pSU77 and pFR167. The resulting plasmid 
was transferred to CSUB10R by conjugation of the donor. 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 pro-
cedures (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-resis-
tant 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 pa-

tient and the MICs of these antibiotics for CSUB10R 
(pSHA19) and CSUB10R(pSHA20) containing the entire and 
truncated ompK36 porin genes, respectively. For cefoxitin-resis-
tant isolates, the MICs of cefotaxime (3 to 5 dilution steps), 
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 
cefotaxime resistance and the increased MICs of meropenem, 
ceftazidime, cefpirome, and ceftazidime-clavulanic acid re-
verted 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. pneu-
niae isolates with different degrees of resistance to cefoxitin. 
For isolates for which the cefoxitin MIC was ≥64 
µg/ml, the cefpirome and ceftazidime MICs increased and the mero-
openem MIC was 4 to 32 times higher than those for strains 
for which cefoxitin MICs were <64 µg/ml. The MICs of norfloxa-
in (MIC range, 64 to 128 µg/ml), ofloxacin (MIC range, 4 to 
8 µg/ml), ciprofloxacin (MIC range, 2 to 8 µg/ml), or spar-
floxacin (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-spec-
trum cephalosporins as a result of ESBL production. Cefoxitin 
resistance and increased carbapenem MICs were not trans-
flected to E. coli by conjugation of the donor.

β-Lactamase study. The production of ESBLs was demon-
strated in all the strains by the double-disk synergy test and by 
and 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
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 pIs. Aztreonam MIC range, 0.5 to 32 μg/ml)

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

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSUB3</td>
<td>CSUB7</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>16</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.25</td>
</tr>
<tr>
<td>Cepiprole</td>
<td>4</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>16</td>
</tr>
<tr>
<td>Ceftazidime-clavulanic acid</td>
<td>1</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>1</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.12</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.03</td>
</tr>
<tr>
<td>Oloxacain</td>
<td>8</td>
</tr>
<tr>
<td>Ciprofloxacain</td>
<td>4</td>
</tr>
<tr>
<td>Sparfloxacain</td>
<td>4</td>
</tr>
</tbody>
</table>

Porin expression + + + + + + + + +

* +, reduced level of porin expression.

**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 carbapenam resistant as a result of porin deficiency and plasmid-mediated AmpC-like β-lactamases (2, 16).
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.

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 rettgeri, 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).

ACKNOWLEDGMENTS

This work was supported in part by the grant 95/1234 from the Fondo de Investigación Sanitaria (FIS) of the National Health Institute of Spain, by grant P96-0197 from Comisión Interministerial de Ciencia y Tecnología (CICYT), and by a grant from Merck Sharp & Dohme de España S.A. C.A. and M.A.D. were supported by predoctoral fellowship from CICYT (fellowship FP94-419723).

REFERENCES