Roles of β-Lactamases and Porins in Activities of Carbapenems and Cephalosporins against Klebsiella pneumoniae

LUIS MARTÍNEZ-MARTÍNEZ, ALVARO PASCUAL, SANTIAGO HERNÁNDEZ-ALLÉS, DOLORES ALVAREZ-DÍAZ, ANA ISABEL SUÁREZ, JOHN TRAN, VICENTE JAVIER BENEDI, AND GEORGE A. JACOBY

Department of Microbiology, School of Medicine, University of Seville, and Department of Microbiology University Hospital V. Macarena, Seville, and Area of Microbiology, Department of Biology and IMDEEA (CSIC-UIB), University of the Balearic Islands, Palma de Mallorca, Spain, and Veterans Affairs Medical Center, Bedford and Lahey Clinic, Burlington, Massachusetts

Received 9 September 1998/Returned for modification 19 January 1999/Accepted 30 April 1999

Two clinical isolates of extended-spectrum β-lactamase (ESBL)-producing Klebsiella pneumoniae were noted to be less susceptible than expected to imipenem. Both were missing outer membrane proteins that serve as channels for antibiotic entry. The role of β-lactamase in resistance was investigated by eliminating the original ESBL and introducing plasmids encoding various ESBLs and AmpC β-lactamase types, by studying the effect of an increased inoculum, and by evaluating interactions with β-lactamase inhibitors. The contribution of porin deficiency was investigated by restoring a functional ompK36 gene on a plasmid. Plasmids encoding AmpC-type β-lactamasmes provided resistance to imipenem (up to 64 µg/ml) and meropenem (up to 16 µg/ml) in strains deficient in porins. Carbapenem resistance showed little inoculum effect, was not affected by clavulanate but was blocked by BRL 42715, and was diminished if OmpK36 porin was restored. Plasmids encoding TEM- and SHV-type ESBLs conferred resistance to cefepime and cefpirome, as well as to earlier oxyimino-β-lactams. This resistance was magnified with an increased inoculum, was blocked by clavulanate, and was also lowered by OmpK36 porin restoration. In addition, SHV-2 β-lactamase had a small effect on carbapenem resistance (imipenem MIC, 4 µg/ml, increasing to 16 µg/ml with a higher inoculum) when porins were absent. In K. pneumoniae porin loss can thus augment resistance provided either by TEM- or SHV-type ESBLs or by plasmid-mediated AmpC enzymes to include the latest oxyimino-β-lactams and carbapenems.

Carbapenems are usually active against extended-spectrum β-lactamase (ESBL)-producing Klebsiella pneumoniae strains, including porin-deficient ones (13). The MICs of meropenem for clinical isolates of K. pneumoniae strains that produce ESBLs are usually 8 to 16 lower than the MICs of imipenem (4, 27). Carbapenems are also usually active against plasmid-mediated AmpC-type β-lactamase-producing strains (10, 16). Imipenem-resistant K. pneumoniae strains that produce ACT-1 (an AmpC-type β-lactamase) (3) or ESBL SHV-2 (18) and that are deficient in a major outer membrane protein, presumably a porin, have been reported recently.

Plasmid- or chromosome-encoded carbapenemases (17) or the association of reduced outer membrane permeability with increased chromosomal β-lactamase production have been shown to be the major mechanisms of resistance to carbapenems in several species of enterobacteria, including Enterobacter spp. (5, 7, 9, 29), Serratia marcescens (25, 36), Citrobacter freundii (19), Providencia rettgeri (29), and Escherichia coli (6, 28). The association of porin loss and metallo-β-lactamase production in laboratory-constructed strains of E. coli determined resistance to both imipenem and meropenem when a high bacterial inoculum was used (6).

As a part of a study of resistance to expanded-spectrum β-lactams in enterobacteria, two clinical isolates of K. pneumoniae that were resistant to ceftazidime (MICs, >256 µg/ml for both strains) and that presented with reduced sensitivity to imipenem (MICs, 2 µg/ml) have been identified. The objectives of this work were to evaluate the mechanisms for the increased MIC of imipenem for these two clinical isolates and to evaluate the relative roles of porins and β-lactamases (both plasmid-mediated ESBLs and plasmid-mediated AmpC-type enzymes) in the activities of imipenem, meropenem, and cephalosporins against K. pneumoniae.

(This study was presented in part in the 36th and 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, La. [20a], 15 to 18 September 1996 and Toronto, Ontario, Canada, 28 September to 1 October 1997 [21a], respectively.)

MATERIALS AND METHODS

Bacterial strains. K. pneumoniae 143098-3 was cultured from sputum at the Massachusetts General Hospital, Boston, in December 1993. It was resistant to ceftazidime, cefotaxime, aztreonam, cefotetan, cefoxitin, chloramphenicol, cephalothin, gentamicin, kanamycin, sulfonamide, tetracycline, tobramycin, and trimethoprim and had a 16-mm zone diameter around a 10-µg imipenem disk. On isoelectric focusing (22) K. pneumoniae 143098-3 had β-lactamase bands at pIs 5.4, 7.6, and 8.2, consistent with TEM-1 and SHV-1, and SHV-5 respectively, and could transfer a plasmid (designated pMG257) encoding the pl 5.4 and 8.2 β-lactamases to E. coli J53 Rif r Lahey Clinic, Burlington, Massachusetts, by selection with ceftazidime. Strain C1 was obtained from strain 143098-3 after overnight growth at 43°C by replica plating for a ceftazidime susceptible derivative. It was also ceftazidime and aztreonam sensitive but retained the parent’s resistance to the other drugs except that the imipenem disk diameter was 24 mm. On isoelectric focusing, β-lactamase bands at pl 5.4 and 7.6 were retained, but the band at pl 8.2 was no longer present, suggesting that the putative SHV-5 enzyme was no longer expressed. Attempts to cure the entire resistance phenotype with acridine orange, ethidium bromide, novobiocin, or sodium dodecyl sulfate (SDS) were unsuccessful.

K. pneumoniae NEDH1 is a urine isolate obtained at the New England Deaconess Hospital, Boston, Mass., in August 1994. The isolate was resistant to ceftazidime, cefotaxime, aztreonam, chloramphenicol, kanamycin, streptomycin, sulfonamide, tobramycin, and trimethoprim and could transfer a plasmid (named pMG258) encoding resistance to these drugs on mating to E. coli. On isoelectric focusing, β-lactamase bands at pl 5.4 and 7.6 consistent with TEM-1 and...
zymes were introduced by conjugation, as described previously (14), into C1 and cated resistance to tellurite and which is equivalent to pSHA2 but which has a trun-
cassette (for selection purposes) (20). Plasmid pSHA4 (which also codes for
encodes the OmpK36 porin and which contains a potassium tellurite resistance
ase-producing strains was tested by transformation with plasmid pSHA2, which
P. aeruginosa chromosomal enzyme of
(30
produced
growth in ethidium bromide and screening for a ceftazidime-susceptible deriva-
tives. The combinations of cefoxitin, imipenem, and meropenem with the serine-β-lactam-
ase inhibitor BRL 42715 (SmithKline Beecham) were also tested against strains
that express AmpC-type enzymes. BRL 42715 is able to inhibit not only class A
β-lactamases but also class C enzymes, and for this reason it was expected that
it would inhibit the plasmid-mediated AmpC-type enzymes evaluated in the
present study. MICs were determined in cation-adjusted Mueller-Hinton broth,
according to the guidelines of the National Committee for Clinical Laboratory
Standards (23). The activities of cefepime, cefpirome, imipenem, and meropenem were compared at inocula of 10^6 and 10^7 CFU/ml. The E test was also
used for some assays. Media were supplemented with potassium tellurite (30 µg/ml) when strains carrying pSHA plasmids were studied in order to avoid
plasmid loss.

**Isoelectric focusing of β-lactamases.** Cells were grown to the logarithmic
phase and were disrupted by sonication. After removal of nonbroken cells and
debris, the supernatant was used to determine pIs by isoelectric focusing as
described previously (20). Plasmid pSHA4 (also which codes for
resistance to tellurite and which is equivalent to pSHA2 but which has a trun-
cated ompK36 gene) was used as a control. Transformants were obtained on
Mueller-Hinton agar containing ceftazidime (20 µg/ml) and potassium tellurite
(30 µg/ml).

SHV-2, respectively, were present. C2 was derived from strain NEDH-1 by
growth in ethidium bromide and screening for a ceftazidime-resistant deriva-
tive. It lost the resistance of the parent to all drugs listed above and no longer
produced β-lactamase, suggesting that pMG258 had been completely eliminated.

Plasmids coding for different TEM- or SHV-type ESBLs or AmpC-type en-
zeymes were introduced by conjugation, as described previously (14), into C1 and C2 (Table 1).

The role of porin OmpK36 expression in the resistance of β-lactamase-
producing strains was tested by transformation with plasmid pSHA2, which
encodes the OmpK36 porin and which contains a potassium tellurite resistance
cassette (for selection purposes) (20). Plasmid pSHA4 (also which codes for
resistance to tellurite and which is equivalent to pSHA2 but which has a trun-
cated ompK36 gene) was used as a control. Transformants were obtained on
Mueller-Hinton agar containing ceftazidime (20 µg/ml) and potassium tellurite
(30 µg/ml).

**RESULTS**

*K. pneumoniae* C1 and C2 were deficient in both OmpK35 and OmpK36 porins, as determined by SDS-PAGE (data not shown). Tables 2 and 3 show that introduction of TEM- or
SHV-type ESBLs into these strains increased the MICs of

### TABLE 1. Plasmids used in the study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Enzyme</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMG226</td>
<td>TEM-6</td>
<td>Munich, Germany</td>
<td>2, 11</td>
</tr>
<tr>
<td>pMG223</td>
<td>TEM-10</td>
<td>Boston, Mass.</td>
<td>11, 31</td>
</tr>
<tr>
<td>pMG224</td>
<td>TEM-12</td>
<td>Cambridge, Mass.</td>
<td>30, 31</td>
</tr>
<tr>
<td>pMG225</td>
<td>TEM-26</td>
<td>Cambridge, Mass.</td>
<td>30, 31</td>
</tr>
<tr>
<td>pMG229</td>
<td>SHV-2</td>
<td>Paris, France</td>
<td>11, 13</td>
</tr>
<tr>
<td>pMG258</td>
<td>SHV-2*</td>
<td>Boston, Mass.</td>
<td>This paper</td>
</tr>
<tr>
<td>pDU18</td>
<td>SHV-3</td>
<td>Paris, France</td>
<td>24</td>
</tr>
<tr>
<td>pMG257</td>
<td>SHV-4</td>
<td>Boston, Mass.</td>
<td>This paper</td>
</tr>
<tr>
<td>pMG233</td>
<td>AmpC</td>
<td>Providence, R.I.</td>
<td>26</td>
</tr>
<tr>
<td>pMG245</td>
<td>AmpC</td>
<td>Boston, Mass.</td>
<td>12</td>
</tr>
<tr>
<td>pMG248</td>
<td>AmpC</td>
<td>Durham, N.C.</td>
<td>12</td>
</tr>
<tr>
<td>pMG250</td>
<td>AmpC</td>
<td>Ann Arbor, Mich.</td>
<td>12</td>
</tr>
<tr>
<td>pMG252</td>
<td>AmpC</td>
<td>Birmingham, Ala.</td>
<td>21</td>
</tr>
</tbody>
</table>

*Presumptive identification based on pl determination.

**Abbreviations:** FOX, cefoxitin; IPM, imipenem; MEM, meropenem; FEP, cefepime; PIR, cefpirome; CIP, ciprofloxacin.

### TABLE 2. MICs of antimicrobial agents determined by microdilution for ESBL- or AmpC-producing strains of *K. pneumoniae* at an inoculum of 10^6 or 10^7 CFU/ml

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Enzyme</th>
<th>FOX, 10^6</th>
<th>FEP, 10^6</th>
<th>PIR, 10^7</th>
<th>IPM, 10^7</th>
<th>MEM, 10^7</th>
<th>CIP, 10^5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>No ESBL</td>
<td></td>
<td>128</td>
<td>128</td>
<td>32</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>C1-T6</td>
<td>pMG226</td>
<td>TEM-6</td>
<td>128</td>
<td>128</td>
<td>32</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>C1-T10</td>
<td>pMG223</td>
<td>TEM-10</td>
<td>128</td>
<td>128</td>
<td>32</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>C1-T12</td>
<td>pMG224</td>
<td>TEM-12</td>
<td>128</td>
<td>128</td>
<td>32</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>C1-S2</td>
<td>pMG229</td>
<td>SHV-2</td>
<td>128</td>
<td>128</td>
<td>32</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>C1-33</td>
<td>pUD18</td>
<td>SHV-3</td>
<td>128</td>
<td>128</td>
<td>32</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>C1-S5</td>
<td>pMG257</td>
<td>SHV-5</td>
<td>128</td>
<td>128</td>
<td>32</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>C1-AC233</td>
<td>pMG233</td>
<td>AmpC</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>C1-AC245</td>
<td>pMG245</td>
<td>AmpC</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>C1-AC248</td>
<td>pMG248</td>
<td>AmpC</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>C1-AC252</td>
<td>pMG252</td>
<td>AmpC</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>NEDH-1</td>
<td>pMG258</td>
<td>SHV-2</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>C2</td>
<td>No ESBL</td>
<td></td>
<td>64</td>
<td>64</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>C2-S2</td>
<td>pMG258</td>
<td>SHV-2</td>
<td>64</td>
<td>64</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>C2-AC248</td>
<td>pMG252</td>
<td>AmpC</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

Abbreviations: FOX, cefoxitin; IPM, imipenem; MEM, meropenem; FEP, cefepime; PIR, cefpirome; CIP, ciprofloxacin.

Presumptive identification based on pl determination.
ceftazidime, ceftazidime, and ceftazidime but not that of cefoxitin. The MICs of imipenem and meropenem were not increased except for a fourfold increase with the SHV-2 β-lactamase. Introduction of plasmids encoding AmpC-type β-lactamases also increased the MICs of cefepime and cefpirome, but to a lesser extent, especially for cefepime. Cefoxitin MICs were higher by acquisition of AmpC-type enzymes, and all except the enzyme determined by pMG252 increased imipenem MICs as much as 64-fold and meropenem MICs up to 16-fold. Table 2 shows that increasing the test inoculum from 10⁵ to 10⁷ CFU/ml resulted in higher MICs of cefepime and ceftazidime for both ESBL- and AmpC-producing strains. The higher inoculum did not enhance the carbapenem resistance of the ESBL-producing strains except for those with SHV-2, for which the imipenem MIC reached 16 µg/ml. With AmpC strains carbapenem resistance showed a two- to fourfold inoculum effect, with MICs of 64 µg/ml for imipenem and 16 µg/ml for meropenem.

Clavulanic acid blocked resistance to ceftazidime, cefepime, and cefpirome produced by TEM- and SHV-type ESBLs in strains C1 and C2 (Table 3). The resistance to cefoxitin, imipenem, and meropenem produced by AmpC-type β-lactamases was reduced by BRL 42715 but not by clavulanic acid (Table 3).

SDS-PAGE analysis documented that the missing OmpK36 porin was restored by transformation with plasmid pSHA2. Control strains transformed with plasmid pSHA4 (which encodes a truncated ompK36 gene) remained OmpK36 deficient (data not shown). Table 4 shows that restoration of OmpK36 lowered the MICs of cefoxitin, cefepime, ceftazidime, and cefpirome and, for AmpC-producing strains, the MICs of imipenem and meropenem. The MICs of imipenem (32 to 64 µg/ml) and meropenem (16 µg/ml) were reduced to 0.5 to 2 and 0.25 µg/ml, respectively, by a functional OmpK36 porin. The MICs of cefoxitin, imipenem, and meropenem in the presence of BRL 42715 for strain C1-AC248 that expressed or that did not express porin OmpK36 were 4 and 128 µg/ml, respectively, for cefoxitin, 1 and 1 µg/ml, respectively, for imipenem,
and 0.25 and 4 μg/ml, respectively, for meropenem. The corresponding values for strain C2-AC248 that expressed or that did not express OmpK36 were 4 and 64 μg/ml, respectively, for cefoxitin, 0.125 and 0.25 μg/ml, respectively, for imipenem, and 0.03 and 0.25 μg/ml, respectively, for meropenem.

**DISCUSSION**

Results from this study confirm previous observations (20, 35) indicating that resistance to expanded-spectrum cephalosporins increases in *K. pneumoniae* strains that produce TEM- or SHV-type ESBLs that lack the two major porins of the species (OmpK35 and OmpK36). The level of resistance further increased when a high inoculum (107 CFU/ml) was used, and this may be clinically relevant in situations in which the number of bacteria at the site of infection is particularly high.

Restoration of OmpK36 function in porin-deficient strains producing ESBLs caused significant decreases in the MICs of ceftazidime, cefepime, and cephirome. In *E. coli* we have observed that the expression of OmpF in strains that are deficient in both OmpF and OmpC porins and that hyperproduce chromosomal β-lactamase is more efficient than expression of OmpC in reverting the resistant phenotype (28). It has been previously shown that the OmpK36 porin of *K. pneumoniae* is homologous to OmpC of *E. coli* (1). Cloning of the ompK35 gene of *K. pneumoniae* is in progress and will allow evaluation of the role of this other porin, in comparison with that of OmpK36, in the resistance of *K. pneumoniae* to antimicrobial agents.

ESBL production did not affect the activities of carbapenems except when imipenem was tested against strain C1-S2 (which produces SHV-2), for which MICs of 4 μg/ml (107 cfu/ml) and 16 μg/ml (107 cfu/ml) were obtained. It has previously been reported that a clinical isolate of *K. pneumoniae* that produces SHV-2 was resistant to imipenem (MIC, 8 μg/ml) and meropenem (MIC, 16 μg/ml) (18).

The expression of AmpC-type enzymes affected the activities of cefoxitin (which is in contrast to the effects of ESBLs) and ceftazidime, as has been shown for other plasmid-mediated AmpC-type β-lactamas (10, 16). On the other hand, only moderate increases were observed when cefpirome or cephirome were tested, with cephirome MICs being two to eight times lower than those of cephirome. The relative better activities of both cephirome and cephirome in comparison with those of older cephalosporin derivatives relates to their increased stability to chromosomal AmpC enzymes (from which plasmid-mediated AmpC-type enzymes have been shown to be derived) and to their relatively higher level of permeation through the outer membranes of gram-negative bacteria (33). These data suggest that cephirome and cephirome could represent therapeutic alternatives against AmpC-type producing organisms resistant to multiple antimicrobial agents, as has already been shown for infections caused by cephirome-resistant *Enterobacter* (34). Caution is suggested, however, from the significant increases in the MICs of both cephirome and cephirome when a high inoculum was used in the susceptibility assay.

The production of AmpC-type enzymes significantly increased the MICs of carbapenems, in agreement with the report by Bradford et al. (3) with the ACT-1 β-lactamase. BRL 42715 was able to revert the resistance conferred by AmpC-type enzymes, as expected for class C-related β-lactamas (16). Although BRL 42715 is not available for clinical use, it would be interesting to develop compounds with activity similar to that of BRL 42715 to assess their therapeutic potential. Resistance to carbapenems also depends on the absence of porins, because restoration of OmpK36 significantly reduced the level of resistance. The results obtained after simultaneous inhibition of the AmpC enzyme and expression of OmpK36 in strains C1-AC248 and C2-AC248 suggest that β-lactamase production is the most important factor for resistance to imipenem, while loss of porins seems more important or as important for resistance to meropenem.

The apparent existence of different mechanisms of resistance to imipenem and meropenem in *K. pneumoniae* is also supported by the observation that inhibition of the AmpC enzymes of strains C1-AC248 and C2-AC248 with the same amount of BRL 42715 resulted in a larger decrease in the MICs of imipenem for C2-AC248 than for C1-AC248. Similarly, although both strains present similar levels of resistance to meropenem, the decrease in the MIC of this agent in the presence of BRL 42715 was also higher for strain C2-AC248. It is unlikely that this was caused by a poorer inhibition of the enzyme in strain C1-AC248, because an increase in the concentration of BRL 42715 to 16 μg/ml did not result in an additional decrease in the MIC of either imipenem or meropenem (data not shown).

Although the main objective of this study was to evaluate the mechanisms that lead to resistance to carbapenems and cephalosporins in *K. pneumoniae*, ciprofloxacin was also included as a control for experiments on OmpK36 expression, because its activity is slightly increased when this porin is produced (20) and possible interactions between β-lactamase expression and fluoroquinolone activity were of interest. Surprisingly, one of the plasmids coding for an AmpC-type enzyme (pMG252) was able to increase the resistance to ciprofloxacin in the two *K. pneumoniae* strains evaluated. Details about this finding have been published elsewhere (21). It is noteworthy that pMG252 was the only plasmid of those evaluated in the present study that codes for an enzyme that determined resistance to cefoxitin but not to carbapenems. The AmpC-type enzyme encoded by this plasmid belongs to the group of plasmid-mediated AmpC-type β-lactamases (CMY-1, FOX-1, MOX-1, and others) distantly related to the chromosomal enzyme of *P. aerugi- nosa* (unpublished data). More enzymes of this type need to be tested to determine if this is a general and potentially distinguishing property of this class of enzymes.

Plasmid-mediated AmpC-type enzymes have increasingly been recognized in recent years. Data from previous reports and from this work suggest that these bacterial enzymes may represent a new threat against the more recently introduced antimicrobial agents. The spread of strains that lack porins and that express these new plasmid-mediated enzymes may create serious therapeutic problems in the future.

**ACKNOWLEDGMENTS**

This study was supported by grants from Consolidation of Research Groups, Consejería de Educación, Junta de Andalucía (to L.M.-M. and A.P.), and from the Comisión Interministerial de Ciencia y Tecnología, Ministerio de Educación (grant PB96-0197) (to V.J.B.). G.A.J. was supported by a VA/DoD Mechanisms of Emerging Pathogens award.

**REFERENCES**


