AmpC and OprD Are Not Involved in the Mechanism of Imipenem Hypersusceptibility among *Pseudomonas aeruginosa* Isolates Overexpressing the mexCD-oprJ Efflux Pump

Daniel J. Wolter, Nancy D. Hanson, and Philip D. Lister*

Center for Research in Anti-Infectives and Biotechnology, Department of Medical Microbiology and Immunology, Creighton University School of Medicine, 2500 California Plaza, Omaha, Nebraska 68178

Received 20 April 2005/Returned for modification 24 June 2005/Accepted 17 August 2005

*Pseudomonas aeruginosa* strains that overexpress mexCD-oprJ become hypersusceptible to imipenem. Disruption of AmpC induction has been suggested to cause this phenotype. However, data from this study demonstrate that hypersusceptibility to imipenem can develop without changes in ampC expression or AmpC activity. Furthermore, hypersusceptibility is not caused by changes in expression of the outer membrane porin, OprD.

An alternative mechanism may involve increased production of the outer membrane porin, OprD. A relationship between overexpression of the mexEF-oprN efflux system, decreased production of OprD, and imipenem resistance has been established (5, 15). Alternatively, *mexCD-oprJ* hyperexpression may elicit upregulation in oprD expression, thus promoting the entry of more carbapenem molecules and increased susceptibility to imipenem.

To test this hypothesis, *mexCD-oprJ*-overexpressing mutants were selected from four *P. aeruginosa* strains, and the relationship between AmpC, OprD, and imipenem hypersusceptibility was evaluated. An isogenic panel of *P. aeruginosa* isolates exhibiting various phenotypes for AmpC production served as the foundation for evaluating the role of AmpC. The parent, strain 164, was a "wild-type" clinical isolate that does not produce any β-lactamases other than its inherent chromosomal AmpC (17). A partially derepressed mutant, 164M1, and a fully derepressed mutant, 164CD, were previously selected from strain 164 through in vitro exposure to cephalosporins (2). OprD involvement was also investigated with an unrelated clinical isolate, *P. aeruginosa* 244, that was resistant to imipenem through the loss of OprD from its outer membrane.

Fluoroquinolone-resistant mutants were selected from the four *P. aeruginosa* strains using ciprofloxacin as the selecting agent (20), and changes in susceptibility to imipenem were evaluated by agar dilution (14). Two fluoroquinolone-resistant, imipenem-hypersusceptible (≥4-fold decrease in MIC) mutants were selected from each parent strain (Table 1). Overexpression of *mexCD-oprJ* was confirmed by real-time reverse transcription-PCR (RT-PCR) (described below), as a 700- to 1,500-fold increase in *mexC* expression was observed (Table 1).

To investigate the contribution of AmpC to imipenem hypersusceptibility, cell-free lysates were prepared from un-

---

**TABLE 1. Phenotypes, susceptibilities, and gene expression of *P. aeruginosa* parents and mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>MIC (µg/ml)</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CIP</td>
<td>LEV</td>
</tr>
<tr>
<td>164</td>
<td>Wt</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td>164-921C</td>
<td>CDJ</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>164-922C</td>
<td>CDJ</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>164M1-84C</td>
<td>PD</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td>164M1-94C</td>
<td>CDJ</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>164CD-84C</td>
<td>FD</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td>164CD-921C</td>
<td>CDJ</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>244</td>
<td>ΔOprD</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>244-921C</td>
<td>CDJ</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>244-911C</td>
<td>CDJ</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

---

* Corresponding author. Mailing address: Center for Research in Anti-Infectives and Biotechnology, Creighton University School of Medicine, 2500 California Plaza, Omaha, NE 68178. Phone: (402) 280-1224. Fax: (402) 280-1875. E-mail: pdlister@creighton.edu.  
  
**Abbreviations:** CIP, ciprofloxacin; LEV, levofloxacin; IMP, imipenem.  
  
**Transcriptional expression of mexC and oprD as measured by real-time RT-PCR. Values represent the difference (n-fold) in gene expression of the mutants relative to their respective parent strain. ND, not determined.
treated and imipenem-treated (1/4 the MIC) cultures, and AmpC hydrolysis was measured as previously described (16).

For gene expression studies, total RNA was prepared using the TRIzolMax method (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed using 250 ng of DNase-treated RNA, the QIAGEN QuantiTect SYBR green RT-PCR kit (QIAGEN Inc., Valencia, CA) and specific internal ampC primer pairs (0.5 μM in 50-μl final volume) (Table 2). The removal of contaminating DNA was verified by PCR in the absence of reverse transcriptase. Expression of the endogenous control gene, rpsL, was used to normalize data. Real-time RT-PCRs were carried out using an ABI Prism 7000 sequence detection system, and results were analyzed with the ABI Prism 7000 sequence detection system software. Relative quantification was determined by the $2^{-ΔΔCT}$ or delta-delta cycle threshold ($C_T$) method (9).

FIG. 1. Transcriptional expression of ampC and AmpC β-lactamase activity in untreated and imipenem-treated cultures of parent strains 164 (A and B), 164M1 (C and D), and 164CD (E and F) and their respective imipenem-hypersusceptible mutants. Results from untreated cultures are displayed as lightly dotted bars, and results from imipenem-treated cultures are represented as solid gray bars. For ampC transcriptional expression studies (A, C, and E), values represent the amount of change in expression relative to untreated cultures of the parent strains (set at a value of 1.0). AmpC hydrolysis data (B, D, and F) reflect the actual nanomoles of cephalothin hydrolyzed per minute per mg of protein for cell extracts from each strain. The numbers above the bars represent the average values for two independent experiments, and error bars represent the standard deviations.
no differences were observed in penem-hypersusceptible mutants (164–921C and 164–922C),
MexCF1 GCTGTTCCAGATCGATCCG 160 U57969
VOL. 49, 2005 NOTES 4765
OprDRTF CTACGCAATCACCGATAACC 189 Z14065
PAERUGF TTACTACAAGGTCGGCGACATGACC 267 X54719
MexCRTR GGTATCGAAGTCCTGCTGG
onstrated the continued absence of OprD among imipenem-
dodecyl sulfate-polyacrylamide gel electrophoresis (22) dem-
outer membrane. Outer membrane protein analysis by sodium
would have to be restored to produce a functional porin in the

These data support the earlier findings of Masuda et al. (13),
Mstraints with fully derepressed strain 164CD and its imipenem-
tications of OprD and efflux systems. Antimicrob. Agents Che-
MexAB-OprM. Another potential mechanism includes
changes in the overall composition of the outer membrane. Studies
have shown that modifications in lipopolysaccharide can affect outer membrane integrity, dramatically decreasing resistance to toxic agents (1, 7). It is possible that the compo-
sion or architecture of the outer membrane of mexCD-oprJ-
overexpressing mutants may be altered, rendering it more permeable to imipenem. A final alternative mechanism involves changes in the penicillin-binding protein (PBP) targets. Studies with both P. aeruginosa and Staphylococcus aureus have dem-
strated that the overproduction of PBP3 and PBP4, respec-
tively, can significantly decrease susceptibility to certain β-lac-
tams (4, 8). In contrast, a decrease in the production of an imipenem-targeted PBP may lead to hypersusceptibility.

In summary, these data confirm that the mechanism of imi-
penem hypersusceptibility exhibited by mexCD-oprJ-overexpressing P. aeruginosa does not involve either AmpC or OprD. Understanding the mechanism of imipenem hypersusceptibility offers intriguing possibilities for discovering new drug tar-
gets capable of restoring susceptibility to carbapenems, even in strains lacking a functional OprD porin for their penetration.

REFERENCES
1. Angus, B. L., A. M. Carey, D. A. Caron, A. M. Kropinski, and R. E. Hancock. 1982. Outer membrane permeability in Pseudomonas aeruginosa: compar-
production increases beta-lactam resistance in Staphylococcus aureus. Anti-
7. Kropinski, A. M., J. Kurzio, B. L. Augus, and R. E. Hancock. 1982. Chemical and chromatographic analysis of lipopolysaccharide from an antibiotic-sup-

With untreated cultures of wild-type strain 164 and its imi-
penem-hypersusceptible mutants (164–921C and 164–922C),
no differences were observed in ampC expression or AmpC
hydrolysis (Fig. 1A and B). However, after imipenem treat-
ment, ampC expression and hydrolysis activity were 2.4- to
3.4-fold lower for the imipenem-hypersusceptible mutants.
These data support the earlier findings of Masuda et al. (13),
but these modest decreases in ampC induction fail to explain
hypersusceptibility, since far greater increases in AmpC activity
do not significantly decrease imipenem susceptibility.

In contrast to P. aeruginosa 164 and its mutants, ampC ex-
pression and AmpC hydrolysis were significantly higher in un-
treated cultures of the imipenem-hypersusceptible mutants
164M1–94C and 164M1–84C from strain 164M1 than for imi-
penem-treated cultures (Fig. 1C and D). Following treatment
with imipenem, these differences were no longer observed. In
studies with fully derepressed strain 164CD and its imipenem-
hypersusceptible mutants (164CD-921C and 164CD-822C), no
differences were observed in ampC expression or AmpC hy-
drolysis activity, regardless of imipenem treatment (Fig. 1E
and F). These data demonstrate that imipenem hypersus-
cptibility can develop without decreases in ampC expression or
AmpC hydrolysis activity and suggest that the observations
reported by Masuda et al. (13) relate only to “wild-type” P.
aeruginosa. More importantly, the mechanism of imipenem
hypersusceptibility does not appear to involve the AmpC
cephalosporinase, as previously believed.

To evaluate the potential role of OprD, oprD transcription was
examined by real-time RT-PCR (primers shown in Table
2). Steady-state levels of oprD expression were similar among
all strains evaluated (Table 1), suggesting OprD is not involved
in imipenem hypersusceptibility. These data do not rule out
potential changes in posttranscriptional events. Therefore, imi-
penem-hypersusceptible mutants were selected from an OprD-
deficient clinical isolate, P. aeruginosa 244. Sequence analysis
(21) of oprD revealed a base transition from C→T at nucleo-
tide 1438 (GenBank accession number Z14065), creating a
premature translational stop codon (Gln235→stop). If OprD
participated in hypersusceptibility to imipenem, this mutation
would have to be restored to produce a functional porin in the
outer membrane. Outer membrane protein analysis by sodium
dodecyl sulfate-polyacrylamide gel electrophoresis (22) dem-
monstrated the continued absence of OprD among imipenem-

TABLE 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Product size</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MexCF1</td>
<td>GCTGTTCCAGATCGATCCG</td>
<td>160</td>
<td>U57969</td>
</tr>
<tr>
<td>MexCRTR</td>
<td>GGTATCGAATCACCGATAACC</td>
<td>267</td>
<td>X54719</td>
</tr>
<tr>
<td>PAERUGF</td>
<td>TTACTACAAGGTCGGCGACATGACC</td>
<td>189</td>
<td>Z14065</td>
</tr>
<tr>
<td>PAERURG</td>
<td>GGCTATGGGATAGTGGCGGTTG</td>
<td>267</td>
<td>X54719</td>
</tr>
<tr>
<td>OpsDRTF</td>
<td>GACGCTAATCACCGATAACC</td>
<td>189</td>
<td>Z14065</td>
</tr>
<tr>
<td>PAOOpDR2</td>
<td>GTGGTGGTTGCTGATGTCGC</td>
<td>230</td>
<td>AE004842</td>
</tr>
</tbody>
</table>

a Size of PCR products (bp).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Product size</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RpsLF1</td>
<td>GCACTACATCACCGATAGGTG</td>
<td>230</td>
<td>AE004842</td>
</tr>
<tr>
<td>RpsLR1</td>
<td>GCTGTTCCAGATCGATCCG</td>
<td>160</td>
<td>U57969</td>
</tr>
</tbody>
</table>

b Accession numbers for GenBank.


