Carbenicillin Resistance of *Pseudomonas aeruginosa*

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Four strains of *Pseudomonas aeruginosa* obtained from clinical isolates which are carbenicillin resistant were studied to find the cause(s) of resistance to this β-lactam antibiotic. The electrophoresis patterns of the four strains (PH20610, PH20815, PH4011, and PH4301) were found to be different from those of a wild-type strain, *P. aeruginosa* NCTC 10662, and appeared to lack penicillin-binding protein 2. Affinity of other penicillin-binding proteins from strains PH20610 and PH20815 for carbenicillin seemed to be normal or slightly diminished. Electrophoretic patterns of penicillin-binding proteins from strains PH4011 and PH4301 had more profound differences, since the affinities of their penicillin-binding proteins 1a, 1b, and 4 for carbenicillin were decreased by nearly two orders of magnitude relative to the preparations from the wild-type strain. Kinetic studies on binding of carbenicillin to penicillin-binding proteins both in isolated membrane preparations and in intact cells revealed that carbenicillin penetration into resistant cells was a much slower process than in susceptible cells, suggesting that the outer envelope structures serve as an efficient barrier against carbenicillin entry into our *P. aeruginosa* strains from clinical isolates.

The clinical treatment of *Pseudomonas aeruginosa* infections has become an important priority in hospitals in recent years, because *P. aeruginosa* is highly resistant to many antibiotics that inhibit many other bacteria (1). The importance of certain cell structures that form penetration barriers in *P. aeruginosa* has recently been stressed (15, 16). Furthermore, decreased affinity of β-lactam antibiotics for penicillin-binding proteins (PBPs) and susceptibility to β-lactams of bacterial peptidoglycan biosynthetic reactions have been observed in *P. aeruginosa* isolates shown previously to be particularly resistant to β-lactam antibiotics (3, 4, 8). Hence, we undertook a biochemical study of the PBPs from four clinical isolates of *P. aeruginosa* and a kinetic investigation of carbenicillin penetration into the cells.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The wild-type *P. aeruginosa* NCTC 10662 susceptible to carbenicillin and the clinical isolates of *P. aeruginosa* PH20610, PH20815, PH4011, and PH4301 resistant to the antibiotic were studied. The clinical strains were isolated from patients treated in Clinica Puerta de Hierro, Madrid, Spain. Cells were grown in the medium described by Lennox (7) at 37°C under forced aeration.

Minimal inhibitory concentrations were determined as described by Curtis et al. (3).

**Preparation and isolation of bacterial envelopes.** *P. aeruginosa* strains were grown as described above to a density of approximately 5 × 10⁸ cells per ml. Cells were harvested by centrifugation at 10,000 × g at 0°C for 10 min. They were then washed by centrifugation with 50 mM sodium phosphate buffer (pH 7.0) containing 10 mM MgCl₂ (standard buffer). The procedure for cell disruption and envelope isolation was the same as that described by Zimmermann (16), in which there were five pulses of 1-min ultrasonic treatment, a low-speed centrifugation (10,000 × g for 10 min at 0°C) to remove unbroken cells, and a high-speed centrifugation (100,000 × g for 60 min at 0°C) to sediment cell envelopes. This last centrifugation was repeated two more times after the pellets were suspended in the standard buffer. Membranes were finally suspended in sodium phosphate standard buffer at a final concentration of 10 mg of protein per ml and stored at −70°C.

Chemicals. Carbenicillin, cefoxime, and nitrocefin were provided by Glaxo Laboratories, Middlesex, U.K. Cefaclor was obtained from Eli Lilly & Co., Indianapolis, Ind.; cefotaxime (HR 756) was obtained from Roussel Uclaf, Paris, France; cefoperazone (T 1551) was obtained from Pfizer Inc., New York, and cefonicid was obtained from Smith Kline & French Laboratories, Philadelphia, Pa. Culture media were obtained from Difco Laboratories, Detroit, Mich. benzyl[¹⁴C]penicillin (51 Ci/mol) and Bolton and Hunter reagent [N-succinimidyl-3-(4-hydroxy-5-[¹²⁵I]iodophenyl)propionate; 2,000 Ci/mmol] were obtained from Amersham International Ltd., Amersham, Bucks, England. Chemical reagents used were of analytical grade.

**Preparation of the ¹²⁵I-ampicillin derivative and protein estimations.** The ¹²⁵I-ampicillin derivative was obtained by reacting radioiodinated Bolton and Hunter reagent with ampicillin, followed by purification of the product on a Sephadex column by the procedure described by Schwarz et al. (13). Protein concentration was determined by using the Folin phenol reagent.

**Binding of β-lactam antibiotics to PBPs by using**
isolated membrane preparations. Competition experiments were carried out between nonradioactive \( \beta \)-lactams and either benzyl\(^{14}\)C]penicillin or the 125I-ampicillin derivative to bind to the PBPs essentially by the procedure of Spratt (14). For this purpose, 150- to 400-\( \mu \)g membrane protein samples were suspended in standard buffer and incubated at 37°C with the required concentration of nonradioactive \( \beta \)-lactam. After 15 min of incubation, saturating concentrations of either benzyl\(^{14}\)C]penicillin (16) or the 125I-ampicillin derivative (14) were added, and incubation at 37°C was continued for 15 min. The binding reaction was terminated by adding a concentrated solution of sodium dodecyl sarcosinate to give 1% (wt/vol) final concentration of the detergent. After 30 min at room temperature the material insoluble to detergent was removed by centrifugation at 30,000 \( \times \) g for 30 min at 30°C. The supernatant fractions were collected and mixed with a 25% volume of a gel sample denaturing buffer, consisting of 100 mM Tris-hydrochloride buffer (pH 7.2), 5% (wt/vol) sodium dodecyl sulfate (SDS), 50% (vol/vol) glycerol, 0.1% (wt/vol) bromophenol blue, and 120 mM \( \beta \)-mercaptoethanol. Samples were immediately heated, held at a temperature of 100°C for 5 min, cooled, and analyzed by SDS-polyacrylamide gel electrophoresis.

Kinetics of carbenicillin binding to PBPs from intact cells of \( P. \) aeruginosa. Cells from strains PH4011 and PH4301 were grown as previously described until the end of the log phase. Cultures were cooled quickly, centrifuged at 10,000 \( \times \) g for 10 min at 0°C, and then resuspended in 5% of the volume of the original culture of the standard buffer supplemented with 150 mM NaCl. Mixtures containing 100 \( \mu \)l of this cell suspension and either 20 or 200 \( \mu \)g of carbenicillin per ml were incubated at 37°C for increasing lengths of time. The 125I-ampicillin derivative was added to the mixture at a final concentration of 8 \( \mu \)M, and incubation was continued for 15 min. Binding was stopped by the addition of a concentrated SDS solution to give a 1% final concentration of SDS (wt/vol). Samples were immediately heated to 100°C, kept at this temperature for 10 min, and then centrifuged at 10,000 \( \times \) g for 45 min at 20°C. Supernatant samples of 50 \( \mu \)l were taken and mixed with 10 \( \mu \)l of gel sample buffer without SDS. Samples were then subjected to SDS-polyacrylamide gel electrophoresis as indicated above, and gels were autoradiographed to detect PBPs.

Carbenicillin binding to \( P. \) aeruginosa PBPs in growing cell cultures. Strains of \( P. \) aeruginosa were grown in 100-ml volumes of L medium (7), supplemented with 0.5% glucose in 500-ml flasks under forced aeration. Three flasks were used for each strain. When the culture reached an absorbancy of approximately 0.25 at 660 nm, a concentrated solution of carbenicillin was added to two of the flasks at a final concentration of 1 and 100 \( \mu \)g/ml, respectively. No carbenicillin was added to the third flask, which was used as a control. The cells were incubated, and no inhibition of growth was observed within the experimental period. Three samples of 20 to 40 \( \mu \)l, depending on the cell density of the culture, were taken at the required times, cooled quickly, and centrifuged at 5,000 \( \times \) g for 5 min at 0°C. The pelleted cells were resuspended in 40 ml of ice-cold standard buffer and centrifuged as described before. The washing procedure was repeated once more, and the final pellet was resuspended in 2 ml of ice-cold standard buffer. The cell suspensions were subjected to ultrasonic treatment in an MSE sonicator at full amplitude with three 1-min cycles alternated with two 1-min cooling intervals. Samples of 70 \( \mu \)l were taken from the sonicate and combined with 20 \( \mu \)l of 125I-ampicillin derivative to obtain a final saturation concentration (8 \( \mu \)M). The mixture was incubated at 37°C for 10 min, and binding was stopped by the addition of 10 \( \mu \)l of 10% (wt/vol) SDS solution. Samples were maintained at 100°C for 10 min and centrifuged at 30,000 \( \times \) g for 45 min at 20°C. Supernatant samples of 50 \( \mu \)l were collected and mixed with 10 \( \mu \)l of gel sample buffer without SDS. Samples were then subjected to SDS-polyacrylamide gel electrophoresis, and gels were autoradiographed to detect PBPs.

Detection of PBPs by SDS-polyacrylamide gel electrophoresis. We followed essentially the method of Spratt (14), which is a modification of the electrophoresis method described by Laemmli and Favre (6). The composition of the separation gels was 10% (wt/vol) acrylamide and 0.1% (wt/vol) N-methylenebisacrylamide. The best resolution of the PBPs was obtained when the electrophoreses were performed at 50 V for 14 to 15 h.

When the radioactive antibiotic used was benzyl\(^{14}\)C]penicillin, the gels were treated for fluorography by impregnation in 1 M sodium salicylate solution (pH 7.0) for 45 min at room temperature (2). Gels were dried under suction. They were then placed onto a precoated Kodak X-Omat X-ray films and exposed at −70°C for 4 to 6 weeks.

For experiments with the 125I-ampicillin derivative, the gels were dried and exposed to the X-ray films at −20°C for 6 h to 2 weeks. By using different concentrations of the radiiodinated antibiotic, we observed that its saturation concentration was 8 \( \mu \)M under that in our experimental conditions.

Quantification of the amounts of radioactive \( \beta \)-lactam bound to the PBPs was carried out by microdensitometry of the plates, using an Optronic photography P-1700 digital microdensitometer.

\( \beta \)-Lactamase assay. Bacteria were grown as described above, sedimented by centrifugation, and suspended in a small volume of standard buffer. The cell suspension was subjected to ultrasonic treatment as described above, and the cell debris was sedimented by centrifugation. Supernatant fractions were collected and used for the \( \beta \)-lactamase assay. We used nitrocefin, a chromogenic cephalosporin, and measured the appearance of absorbance at 485 nm in a Perkin-Elmer 554 double-beam spectrophotometer at 25°C (10).

To estimate inductible \( \beta \)-lactamase, cells were grown as described above to 0.25 absorbance units, and carbenicillin was added at the required concentrations. Cells were collected before lysis was detected as estimated by absorbancy measurements, centrifuged as described above, and suspended gently in a considerable volume of standard buffer. The washing procedure was repeated twice more, and cells were resuspended in a small amount of standard buffer. The suspension was subjected to sonication and further treated as described. \( \beta \)-Lactamase was measured by the cephalosporin method as described before, and its hydrolyzing capacity was measured by studying the decrease in absorbancy at 249 nm at 25°C. Carbenicillin (100 \( \mu \)M) was used for the assay (9).
RESULTS

Electrophoretic patterns of PBPs from membranes of \textit{P. aeruginosa} strains. Electrophoretic patterns of PBPs from the four clinical isolates differed greatly from the corresponding wild-type strain (\textit{P. aeruginosa} NCTC 10662) patterns. All of the mutants lacked PBPs 2 and 4', although PBPs 1a, 1b, and 4 had the same mobility as their counterparts from the wild-type strain. Even at saturation concentrations of the labeled antibiotics, PBP patterns were dependent on the radioactive \(\beta\)-lactam used to interact with the PBPs, as summarized in Table 1. PBP patterns of strains PH20610, PH20815, PH4011, and NCTC 10662 were similar when benzyl\(^{14}\)Cpenicillin was used, although the amounts of labeling differed among the strains used. When the \(125\)I-ampicillin derivative was used, PBP 5 from PH4011 and PH4301 was scarcely labeled. Labeling was in all cases more pronounced for PBP 1a than for PBP 1b.

Affinity of carbencillin and other \(\beta\)-lactams for PBPs of \textit{P. aeruginosa} NCTC 10662 and the clinical isolates. Because the \textit{P. aeruginosa} clinical strains were selected for their resistance to carbencillin, experiments were carried out to investigate the affinity of their PBPs for this \(\beta\)-lactam (Table 2). They show that carbencillin affinities for PBPs from the resistant strains PH20610 and PH20815 are very similar to those of the PBPs from the wild-type susceptible strain \textit{P. aeruginosa} NCTC 10662. However, one reason for carbencillin resistance in PH4011 and PH4301 could be the dramatic decrease in the affinity of the antibiotic for PBPs 1a, 1b, 3, and 4. Indeed, the affinities decreased nearly two orders of magnitude in these cases compared with those of the wild-type bacteria. Table 2 also shows that the minimal inhibitory concentrations for carbenicillin were particularly high in PH4011 and PH4301.

We have further studied the affinities of other \(\beta\)-lactams for the PBPs from PH4011, PH4301, PH20610, and PH20815 compared with those from the wild-type bacteria (Table 3). All of the \(\beta\)-lactams that we tested belong to the cephalosporin group and are, therefore, chemically different from carbenicillin. These \(\beta\)-lactams displayed affinities for the PBPs from the clinical resistant strains which were very similar to those from the wild-type susceptible strain. Affinities of those \(\beta\)-lactams for the PBPs of PH20610 and PH20815 were also studied, but no differences were observed from the wild-type susceptible strain (not shown). Therefore, the mutations of PBPs 1a, 1b, 3, and 4 leading to resistance to carbenicillin do not affect either the susceptibility or affinity in the cephalosporins tested. This differential effect might be due either to the penem nucleus, present in carbencillin but not in the other antibiotics tested, or specifically to the structure of carbenicillin, since the mutants studied were responsible for hospital infections in a milieu where carbenicillin was mainly used for treatment against \textit{Pseudomonas} spp. Therefore, an alteration of the PBP patterns can explain the resistance to carbencillin of only PH4011 and PH4301.

Permeability of \textit{P. aeruginosa} strains to \(\beta\)-lactams. To elucidate the importance of this permeability barrier in carbencillin resistance, we have followed a kinetic method to study the permeation of \(\beta\)-lactams through bacterial outer structures (17).

In our method, which is essentially that developed by Zimmermann (17), the transient rates of formation of the \(\beta\)-lactam–PBP intermediate in intact cells compared with those in isolated

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**TABLE 1.** Binding of PBPs from \textit{P. aeruginosa} NCTC 10662 and the clinical isolates PH20610, PH20815, PH4011, and PH4301

<table>
<thead>
<tr>
<th>PBP</th>
<th>NCTC 10662</th>
<th>PH20610</th>
<th>PH20815</th>
<th>PH4011</th>
<th>PH4301</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>1a</td>
<td>9.0</td>
<td>14.1</td>
<td>5.3</td>
<td>20.8</td>
<td>5.4</td>
</tr>
<tr>
<td>1b</td>
<td>9.5</td>
<td>1.2</td>
<td>5.6</td>
<td>1.3</td>
<td>4.9</td>
</tr>
<tr>
<td>2</td>
<td>7.0</td>
<td>3.7</td>
<td>3b</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>4.2</td>
<td>NM</td>
<td>—</td>
<td>NM</td>
</tr>
<tr>
<td>4</td>
<td>15.0</td>
<td>15.8</td>
<td>16.4</td>
<td>10.2</td>
<td>7.6</td>
</tr>
<tr>
<td>4'</td>
<td>15.0</td>
<td>2.3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>37.0</td>
<td>58.7</td>
<td>72.7</td>
<td>67.7</td>
<td>82.1</td>
</tr>
</tbody>
</table>

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\(^a\) Binding was studied by incubating isolated membranes with saturation concentrations of either (A) benzyl\(^{14}\)Cpenicillin (100 \(\mu\)M) or (B) \(125\)I-ampicillin derivative (8 \(\mu\)M). Samples were subjected to electrophoresis, and the gels were either (A) fluorographed or (B) autoradiographed. Values of the intensity of the bands were obtained by densitometry.

\(^b\) —, PBPs were not detectable.

\(^c\) NM, PBPs were detectable, but so faint that they were not measurable.
expected that PH20815, with P. aeruginosa NCTC 10662, PH20610, and the higher, interacts with the different PBPs of carbenicillin, preparations. Indeed, benzyl[4Clpenicillin have PH4301, quite high concentrations of those membranes from TABLE 2.

Table 2. Carbenicillin concentrations required for 50% reduction in binding of the 125I-ampicillin derivative to PBPs from P. aeruginosa strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbenicillin concn (μg/ml) for 50% reduction in binding of 125I-ampicillin derivative to PBP:</th>
<th>Carbenicillin MICb (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1a</td>
<td>1b</td>
</tr>
<tr>
<td>NCTC 10662</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>PH20610</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>PH20815</td>
<td>0.2</td>
<td>0.7</td>
</tr>
<tr>
<td>PH4011</td>
<td>6.0</td>
<td>10.0</td>
</tr>
<tr>
<td>PH4301</td>
<td>15.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

* Values were obtained by calculating the decrease in densitometry of the peaks corresponding to each PBP.

b MIC, Minimal inhibitory concentration of carbenicillin were determined in a liquid medium (3).

PBPs were not detectable.

membrane preparations were measured, since we expected that there would be no delay in the interaction of β-lactams with PBPs in the latter preparations. Indeed, we have observed that carbenicillin, at concentrations of 1 μg/ml or higher, interacts with the different PBPs of P. aeruginosa NCTC 10662, PH20610, and PH20815, with a half-time lower than 3 min, this being the shortest incubation time studied. In those membranes from P. aeruginosa PH4011 and PH4301, quite high concentrations of carbenicillin have to be used (20 and 200 μg/ml) to saturate most of the PBPs. We have indicated above that the affinity of carbenicillin for PBPs 1a, 1b, and 4 from these strains was greatly diminished. By using these concentrations, half-times of the formation of carbenicillin-PBP intermediates were also lower than 3 min in membrane preparations from P. aeruginosa PH4011 and PH4301. Therefore, it was concluded that the formation of the carbenicillin-PBP complexes is a very rapid process indeed.

Carbenicillin binding to PBPs in intact cells was studied by using bacterial cultures growing in liquid media. Time course experiments were carried out on carbenicillin interaction to PBPs in these cultures under conditions in which cell lysis was not detected. Therefore, the antibiotic certainly had to pass through outer cell structures to reach its PBP targets. Time course-dependent saturation of PBPs by carbenicillin has been seen to be clearly much slower in P. aeruginosa PH20610 and PH20815 than in P. aeruginosa NCTC 10662 (Fig. 1). Similar experiments were also performed with P. aeruginosa PH4011 and PH4301 by using 100 μg of carbenicillin per ml (not shown), but binding of the antibiotics with the PBPs was not detected under these conditions. Therefore, in further experiments, we used washed concentrated bacterial suspensions of P. aeruginosa PH4011 and PH4301. Incubation was carried out at different times in the presence of quite high concentrations of carbenicillin. The results obtained clearly showed that the antibiotic was capable of binding PBP 4 at high concentrations in these strains compared with the results obtained with P. aeruginosa NCTC 10662 (not shown). Moreover, no accurate data could be obtained on the interaction of carbenicillin with other PBPs of P.

Table 3. Concentrations of β-lactams required for 50% reduction in binding of benzyl[14C]penicillin to the PBPs of P. aeruginosa strains

<table>
<thead>
<tr>
<th>β-Lactam antibiotic</th>
<th>β-Lactam concn (μg/ml) for 50% reduction in binding of benzyl[14C]penicillin to PBP:</th>
<th>MICb (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCTC 10662 PH4011</td>
<td>NCTC 10662 PH4011</td>
</tr>
<tr>
<td>Cefonicid</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Cefachlor</td>
<td>200.0</td>
<td>200.0</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>1.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Values were obtained by calculating the decrease in densitometry of the peaks corresponding to each PBP.

b MICs were determined in a liquid medium.
Aeruginosa PH4011 and PH4301, since its affinity and permeability appear to be very low indeed (not shown).

β-Lactamases in P. aeruginosa. P. aeruginosa NCTC 10662 and the four clinical isolates did not appear to have constitutive β-lactamases, since we were not able to detect them (not shown) by using the very sensitive nitrocefin method described by O'Callaghan et al. (10).

Attempts were made to induce β-lactamases in P. aeruginosa NCTC 10662, PH4301, and PH20815 by using carbenicillin. However, moderate levels of β-lactamase-hydrolyzing activity of nitrocefin (74 μmol/liter per min per mg of protein in crude extract) and carbenicillin (390 μmol/liter) were detected only in P. aeruginosa PH4011. In NCTC 10662, the activity was 11.0 μmol/liter for nitrocefin and was not determined.
for carbenicillin. In PH20815, the activity was 11.2 μmol/liter for nitrocefin and was not determined for carbenicillin.

**DISCUSSION**

In this work, we tried to discover the mechanism underlying the resistance of four clinical strains of *P. aeruginosa* to carbenicillin. There are already several reports concerning biochemical studies on *P. aeruginosa* PBPs and the basis for β-lactam resistance or hypersusceptibility in mutants or clinical isolates (3, 4, 8, 16). Alterations of transeptidases (5, 8), PBPs (4, 8), and the permeability barrier (5, 8, 16) have been observed and associated with changes in carbenicillin susceptibility of the bacteria. The results presented above show that in *P. aeruginosa* PH4011 and PH4301, there was an alteration of PBP patterns and a drastic decrease in the affinity of the PBPs for carbenicillin. However, their affinity for several of the cephalosporins tested was not affected. In addition, it appears that carbenicillin entry was impaired in these two strains, which are resistant to carbenicillin. Clinical isolates *P. aeruginosa* PH20610 and PH20815 also showed an alteration in the permeability barrier for carbenicillin, in accordance with the lower susceptibility of these strains to the antibiotics. It should be pointed out that our *P. aeruginosa* wild-type and clinically resistant strains are not isogenic. Therefore, it is theoretically possible that their differences in PBPs and permeability to carbenicillin are because of factors not associated with susceptibility or resistance to the antibiotics, although all of the evidence is against this. In fact, PBP patterns and affinities of the PBPs from *P. aeruginosa* PH20610 and PH20815 carbenicillin-resistant strains are very similar to each other and, at the same time, to the wild-type susceptible strain NCTC 10662, although the two former strains are not isogenic with respect to the latter.

Several methods for studying permeation of β-lactams have been described based on the estimation of β-lactam degradation by β-lactamases located in the periplasmic bacterial space (11, 12, 17). However, these methods are not applicable to our studies, since some of our strains have very low levels of β-lactamases, and manipulation to increase these levels was not desirable. Therefore, we followed a kinetic method to measure carbenicillin binding to PBPs in intact bacteria and isolated membrane preparations. We think this is a useful method to measure β-lactam permeation, since interaction of a β-lactam with the PBPs is the very final stage in its process of permeation. Furthermore, it is the first reaction leading finally to induction of bacterial lysis. By this method, we have shown that there is a certain impairment in the penetration of carbenicillin in *P. aeruginosa* PH20610, PH20815, PH4011, and PH4301 compared with the wild-type susceptible strain *P. aeruginosa* NCTC 10662.

The very low levels of inducible β-lactamase of *P. aeruginosa* PH20815 as assayed with nitrocefin have led us to discard this activity as an important cause for resistance to carbenicillin. However, higher levels of nitrocefin hydrolysis by *P. aeruginosa* PH4301 were observed. These high levels of carbenicillin-induced β-lactamase were confirmed when carbenicillin was used as a substrate, suggesting that this enzyme activity might be relevant in the very high resistance of PH4301 to carbenicillin.

In summary, the results presented here on our *P. aeruginosa* carbenicillin-resistant clinical strains show that a permeability barrier is the cause for resistance in some cases. On the other hand, in other *P. aeruginosa* strains, several pleiotropic effects (a decrease in the affinity of the PBPs for the antibiotic, a permeability barrier, and an inducible β-lactamase) working in combination can be responsible for their resistance to carbenicillin.

**LITERATURE CITED**

for measuring the outer membrane-permeability of beta-lactam antibiotics in gram-negative bacteria. J. Antbiot. 30:1134-1136.


