ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1982, p. 216–223
0066-4804/82/020216-08$02.00/0

Vol. 21, No. 2

# Mutation of *Pseudomonas aeruginosa* Specifying Reduced Affinity for Penicillin G

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Received 13 August 1981/Accepted 10 November 1981

A mutant of *Pseudomonas aeruginosa* strain PAO503 was isolated after ethane-methane-sulfonate mutagenesis and selection on ticarcillin. The mutant, PCC17, displayed reduced affinity for [$^{14}$C]penicillin G at all of its penicillin-binding proteins as well as a general increase in resistance to all of the β-lactam antibiotics tested. The mutation designated *pbpA* has been mapped by FP-2-mediated conjugation and was located distal to the *proA* locus and 33% linked to it. The two loci were not cotransducible with phage F116L. PCC17 and exconjugants produced from it had similar phenotypes, displayed the reduced affinity for [$^{14}$C]penicillin G, had similar resistance profiles, and had an increased amount of protein corresponding to penicillin-binding protein 6. On back mutation the *pbpA* locus reverted to the PAO503 phenotype.

Penicillins, cephalosporins, and cephamycins collectively described as β-lactam antibiotics inhibit bacteria by interfering with steps in the synthesis of the bacterial peptidoglycan layer (1, 2). This layer comprises the major structural support for the cell, and inhibition of it results in malformation of the cell and eventual lysis (19). The targets through which β-lactams exert their effects have been identified as penicillin-binding proteins (PBPs) responsible for the final stages of peptidoglycan synthesis (2, 11, 12) and affecting cell division, elongation, and shape (19).

PBPs have been observed in all of the genera tested to date (5, 8, 14, 15, 18) and vary in number from 3 to 8 depending upon the particular species. The PBPs of *Pseudomonas aeruginosa* are similar to those found in *Escherichia coli* (5, 13), with the exception that PBP1a and PBP1b of *P. aeruginosa* correspond to PBP1b and PBP1a, respectively, of *E. coli* (13).

Nonenzymatic resistance to β-lactam antibiotics in *P. aeruginosa* has been proposed to be due to differences in the permeability barrier presented by the outer membrane of susceptible and resistant strains (4, 24). Resistance has also been associated with changes in peptidoglycan transpeptidase activity (3) and with changes in the affinity for penicillin G of the PBPs (6). The strains used in the latter study were hospital isolates obtained from sputum samples from a single patient during treatment. Although the serotypes obtained were consistent with a single origin, the strains could not be conclusively shown to be mutants derived from a single line. In an attempt to evaluate the mechanism(s) of nonenzymatic β-lactam resistance, we have isolated a series of nonsibling, β-lactam-resistant mutants in a genetically defined background. The phenotype of one of these is described here.

## MATERIALS AND METHODS

The bacterial strains used and their origins are shown in Table 1. For routine culturing, bacteria were grown in tryptic soy broth (TSB; Difco Laboratories) or, if solid medium was required, in tryptic soy agar (TSA; Difco).

**Mutant isolation.** *P. aeruginosa* PAO503 was treated with ethyl methane sulfonate (EMS; Eastman Kodak Co.). Between 0.03 and 0.05 ml of EMS was added to 1 ml of a washed exponential-phase culture and suspended to approximately $10^9$ cells per ml in fresh prewarmed TSB. The treated culture was incubated at 37°C. Colonies growing on ticarcillin plates (75 μg/ml) were picked and purified by streaking for single colony growth on the selective media. The prospective mutants were characterized for the parental auxotrophic requirement and for the minimal inhibitory concentrations (MICs) of a variety of β-lactam antibiotics.

**β-Lactam resistance: MICs.** The plate-disk method described previously (6) was used for the following β-lactams: carbenicillin (Beecham Laboratories); ticarcillin; piperacillin (Lederle Laboratories); cefsulodin (Ceba-Giegy); cefotaxime (Hoechst-Roussel Pharmaceuticals); and azlocillin (Delbay Research Corp.).

**β-Lactamase assays.** Strains were tested for the presence of β-lactamases by two methods. The phenol red acidimetric method (6) with carbenicillin, ticarcillin, piperacillin, and cefsulodin as substrates and the microiodometric assay (25) with piperacillin and ticarcillin as substrates were used. The presence of the chromosomally specified penicillinase (17) was screened by the phenol red acidimetric assay with penicillin G (Ayerst, Montreal, Canada) as substrate.

**Chromosomal mapping.** PCC17 was converted to a conjugal donor by infection with the *Pseudomonas* sex

TABLE 1. Bacterial strains used

| Strain no. | Genetic markers | Origin |
|---|---|---|
| PAO503 | *met-9011* | B. W. Holloway, Monash University, Australia |
| PAO236 Str$^r$ | *his-4 lys-2 met-28 trp-6 pro-82 ilv-226 nalA2* Str$^r$ | EMS mutation to Str$^r$ |
| PAO227 (*met*$^+$) | *ilv-226 his-4 lys-12 trp-6 pro-82* | *met*$^+$ mutant of PAO227 |
| PCC17 | *met-9011 pbpA* | EMS mutation of PAO503 |
| PCC47 | *met*$^-$ *pbpA*$^+$ | EMS mutation of PCC17 |
| PCC1000 | *his-4 lys-12 ilv-226 met-28 trp-6 pbpA* | Exconjugant in the mating PCC17 (FP2) × PAO227 (*met*$^+$) |
| PCC1014 | *pbpA* | PCC17 (FP2) × PAO227 (*met*$^+$) |
| PCC1011 | *his-4 lys-12 met-28 trp-6 pbpA* Str$^r$ | PCC17 (FP2) × PAO236 Str$^r$ |
| PCC1027 | *his-4 lys-12 met-28 trp-6 pbpA* Str$^r$ | PCC17 (FP2) × PAO236 Str$^r$ |
| PCC1028 | *met-28 pbpA* Str$^r$ | PCC17 (FP2) × PAO236 Str$^r$ |

factor, FP2 (9, 10). A fresh overnight culture of PCC17 was mixed 1:1 with an exponential phase culture of PAO8 (FP2). After stationary incubation at 37°C for 30 min, the culture was vigorously mixed. Samples of 0.1 ml were removed to fresh TSB and allowed to express by growth with aeration at 37°C overnight. The overnight cultures were washed in saline (0.89% NaCl in water) and plated onto minimal agar supplemented with 1 mM methionine and 20 μg of mercuric chloride per ml. Mercury-resistant clones were isolated after overnight incubation at 37°C. These clones were screened for their parental methionine requirement and for the MICs of the test β-lactams.

PCC17 (FP2) was used as a donor in conjugal matings using PAO227 or PAO236 Str$^r$ as recipients. Logarithmic-phase cultures of the donor and fresh overnight cultures of the recipient were washed and suspended in saline. When selecting for auxotrophic recombinants, 0.1 ml of each parent was spread onto selective plates and incubated at 37°C for between 30 and 40 h. The selective medium used was minimal agar supplemented with all but one of the auxotrophic requirements of the recipient and with 250 μg of streptomycin (Sigma Chemical Co.) per ml to contraselect the donor. When selecting for transfer of β-lactam resistance, broth matings (21) were used. Exconjugants were selected on TSA plates containing 75 μg of ticarcillin and 250 μg of streptomycin per ml.

Exconjugants were picked from the mating plates and purified by recloning on selective medium similar to that from which they were isolated. After overnight growth at 37°C, the purified clones were imprinted onto sterile velvet pads. These were used as templates to sequentially inoculate media selecting for independence at the other auxotrophic loci and to both TSA and TSA supplemented with ticarcillin (50 μg/ml). In all cases, when screening for coinheritance of unselected markers, TSA was the final plate inoculated for each template.

**Determination of a permeability barrier.** Permeability of the outer membrane for β-lactams was studied by two methods. Penetration of [$^{14}$C]penicillin G to binding sites on the inner membrane and total uptake of [$^{14}$C]penicillin G were assayed by the method of Suginaka et al. (23), with minor modifications. Exponential-phase cultures (30 ml at approximately 1.5 × $10^9$ cells per ml) were washed and resuspended in the same volume of 20% sucrose in Tris buffer (0.05 M, pH 7.8). The culture was then split, and half was distributed into 1-ml samples and incubated with doubling concentrations of [$^{14}$C]penicillin G for 30 min at room temperature. The labeled cells were collected onto 0.22-μm membrane filters (Millipore Corp.), washed with Tris buffer containing 120 mg of penicillin G per ml, and air dried before counting in Omnifluor-toluene (0.4 g/100 ml). The other half of the culture was used to provide spheroplasts. Lysozyme (Sigma) was added to the cell suspension to a final concentration of 50 μg/ml. Incubation at room temperature was carried out for 30 min. Disodium EDTA (Sigma) was added slowly with gentle agitation to a final concentration of 2.5 mM. Spheroplast formation was monitored by phase-contrast microscopy. Greater than 90% spheroplasts were achieved routinely, although 90% spheroplast formation occasionally required slightly higher concentrations of EDTA (up to 3 mM). The spheroplasts were stabilized by the addition of $MgCl_2$ to a final concentration of 0.01 M. The spheroplasts were collected by centrifugation at 2,500 rpm and then suspended to the same concentration in 20% sucrose in Tris buffer (0.05 M, pH 7.8) with 0.01 M $MgCl_2$. Under these conditions the spheroplasts were stable for at least 1 h. Spheroplasts were treated with [$^{14}$C]penicillin G as described above for the whole cells. The second method used was the iodometric β-lactamase technique originated by Zimmerman and Rosselet (25) and discussed previously (6).

**Preparation of cell membranes.** Cell membranes were prepared from fresh overnight cultures as described previously (6, 20).

**Assay of PBPs.** PBPs were assayed as described previously (6), with one modification. After electrophoresis the gels were stained for protein with 0.1% Coomassie blue (Sigma) in 50% methanol–10% acetic acid for 3 h. The gels were destained in several changes of 5% methanol–10% acetic acid. The destained gels were photographed before immersion into 250 ml of En$^3$Hance (New England Nuclear Corp.) for 1 h. The fluor was precipitated by immersing the drained gels in 1 liter of water for exactly 1 h. The gels were dried and fluorographed as described previously (6).

## RESULTS

**Mutant isolation and phenotype.** After EMS mutagenesis, the *P. aeruginosa* PAO503 mutant, PCC17, was isolated on 75 μg of ticarcillin per

TABLE 2. MICs of various β-lactam antibiotics for parental, mutant, recipient, and exconjugant strains of *P. aeruginosa*

| Strain | MIC for β-lactam: | | | | | | |
|---|---|---|---|---|---|---|---|
| | Carbenicillin | Ticarcillin | Piperacillin | Azlocillin | BLP1654 | Cefsulodin | Cefotaxime |
| PAO503 | 31 | 12.5 | 3.125 | 6.25 | 28 | 3.125 | 25 |
| PCC17 | 250 | 250 | 125 | >500 | 2,500 | 25 | >100 |
| PAO236 $Str^r$ | 31 | 12.5 | 1.5 | 6.25 | >28 | 1.5 | 12.5 |
| PAO227 (*met*$^+$) | 31 | 12.5 | 6.25 | 6.25 | >28 | >6.25 | 12.5 |
| PCC47 | 31 | 12.5 | 3.125 | 6.25 | 28 | >3.125 | 12.5 |
| PCC1000 | 250 | 250 | 125 | >500 | >500 | 25 | >50 |
| PCC1014 | 250 | 250 | >50 | >500 | >500 | 12.5 | >50 |
| PCC1011 | 250 | 250 | >50 | >500 | >500 | 12.5 | >50 |
| PCC1027 | 250 | >50 | >50 | >500 | >500 | 25 | ND[a] |
| PCC1028 | 250 | >50 | >50 | >500 | >500 | 25 | ND |

[a] ND, Not determined.

ml. The mutant retained the parental requirement for methionine and displayed an increased resistance to a range of β-lactam antibiotics (Table 2). PCC17 had elevated MICs for all of the β-lactams tested. The resistance level was adjudged low level, since the MICs attained were considerably lower than those attainable by enzymatic inactivation (10). However, the increase in resistance was considerable when compared with that of the parent strain and was presumptively nonenzymatic since no detectable β-lactamase activity was evident on either carbenicillin, ticarcillin, piperacillin, or cefsulodin (data not shown). The chromosomal penicillinase activity (17) was still present as shown by the cleavage of penicillin G by extracts of PCC17.

The failure to demonstrate any additional β-lactamase activity by either phenol red reduction or the iodometric assay to explain the increased resistance of PCC17 prompted investigations into other mechanisms of resistance.

**Permeability barriers to β-lactam antibiotics.** Zimmerman (24) has convincingly demonstrated that the intrinsic relatively high resistance of *P. aeruginosa* to β-lactams is due to a permeability barrier imposed by the outer membrane. To determine whether a change in this mechanism such that higher levels of β-lactams are excluded could explain the resistance in PCC17, the outer membrane permeability of PCC17 was compared with that of its parent, PAO503.

The relative permeabilities to β-lactam antibiotics for R68.45 (a derivative of R68 [10])-infected derivatives of PAO503 and PCC17 are shown in Table 3. The differences reflected in the ratios suggested that PCC17 (R68.45) had a lower permeability barrier than that observed in PAO503 (R.68.45), i.e., the ratio of V intact/V lysed (Table 3) was higher for PCC17 (R68.45) than for PAO503 (R68.45), suggesting either that the intact cells of PCC17 were more permeable to piperacillin or that leakage of β-lactamase from PCC17 (R68.45) had occurred. No inactivation of piperacillin was detected in filtrates of PCC17 (R68.45) whole cells, suggesting that free β-lactamase was not present.

Binding of [$^{14}$C]penicillin G was compared in both whole cells and spheroplasts for both strains. The results (Fig. 1) show that the binding in PCC17 was lower than that observed in PAO503 for both whole cells and spheroplasts. This observation was in keeping with the lower [$^{14}$C]penicillin G binding to membrane preparations of PCC17 (Fig. 2). The binding in both PCC17 and PAO503 spheroplast preparations (Fig. 1) was biphasic (23). The binding found for whole cells was typical of an unsaturated system in both cases, suggesting nonspecific accumulation of label. The data confirmed the presence of a permeability barrier since the binding kinetics were different for whole cells and spheroplasts. The difference, however, was apparent in both PCC17 and PAO503. The increased resistance found for PCC17 could not be explained by a permeability barrier; however, it could have been due to the decreased binding of penicillins to the penicillin targets in the inner membrane.

**Mapping of the resistance mutation.** Although selected after mutagenesis with EMS, PCC17 could be resistant due to multiple mutations. Reversion to the sensitive phenotype was not

TABLE 3. Ratios of the rate of hydrolysis of piperacillin by intact cells and lysed cells of PAO503 (R68.45) and PCC17 (R68.45)[a]

| Strain | V intact/V lysed[b] at the following concn of piperacillin (μg/ml): | | |
|---|---|---|---|
| | 10 | 5 | 2.5 |
| PAO503 (R68.45) | 0.54 | 0.39 | 0.29 |
| PCC17 (R68.45) | 0.65 | 0.49 | 0.41 |

[a] Permeability of piperacillin was assayed by the method of Zimmerman and Rosselet (25).

[b] V intact, Rate of hydrolysis by intact cells; V lysed, rate of hydrolysis by lysed cells.

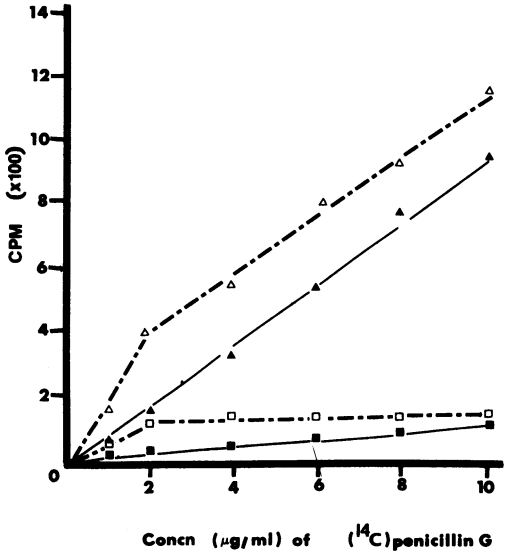


FIG. 1. Binding of [$^{14}$C]penicillin G to whole cells and spheroplasts of PAO503 and PCC17. Whole cells or spheroplasts were incubated with [$^{14}$C]penicillin G for 10 min. Cells were collected onto a membrane filter, washed with a 1,000-fold excess of unlabeled penicillin G, and dried, and residual label was counted. Symbols: △, PAO503 whole cells; ▲, PAO503 spheroplasts; □, PCC17 whole cells; ■, PCC17 spheroplasts.

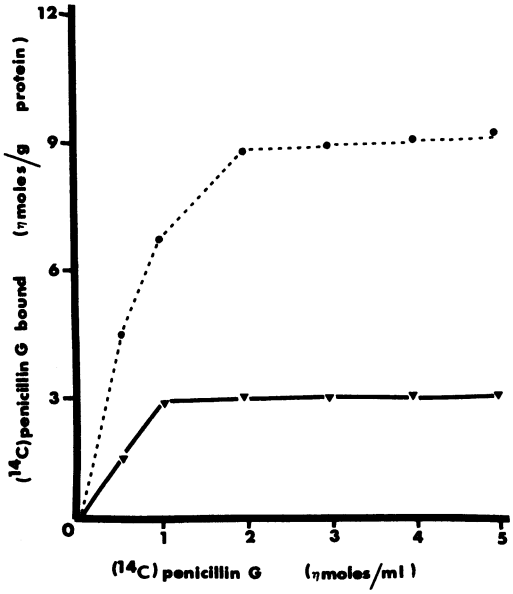


FIG. 2. Binding of [$^{14}$C]penicillin G to isolated inner membrane of PAO503 and PCC17. Inner membrane prepared from logarithmic-phase cultures of PAO503 (●) or PCC17 (▼) was treated with various concentrations of [$^{14}$C]penicillin G, washed, and counted. Data are expressed as moles of bound radioactivity per gram of membrane protein.

observed spontaneously (12,200 clones screened), but did occur at approximately $10^{-4}$ to $10^{-5}$ per clone screened upon back mutation with EMS. The original mutation was probably in a single locus, designated *pbpA*, rather than a deletion or a multiple mutation.

In an attempt to define genetically this mutation, PCC17 was infected with the *Pseudomonas* sex factor, FP2, and used as a donor in matings with a streptomycin-resistant derivative of a multiply auxotrophic recipient, PAO236. The exconjugants were selected for independence at each of the recipient auxotrophic loci and screened for coinheritance of the other markers and for ticarcillin resistance (75 μg/ml). The results (Table 4) show that the ticarcillin resistance (Tic$^r$) was 33% coinheritable with the *pro-82* allele at 40 min on the *P. aeruginosa* chromosome map (17). Tic$^r$ in exconjugants, which were resistant, was associated, with one exception, with the *pro-82* marker. The observation that *pro*$^+$ *trp*$^+$ recombinants were not always Tic$^r$ suggested that the resistance locus was distal to the *pro-82* locus. The proposed gene order, then, was *trp-6 pro-82 pbpA*. An order supported for a further mating selecting for Tic$^r$ and scored for independence at the *pro-82*, *trp-6*, and *met-28* loci which gave 34% coinheritance of *pro*$^+$, 16% coinheritance of *trp*$^+$, and 1% coinheritance of *met*$^+$.

Selected exconjugants (for genotypes, see Table 1) were screened for their resistance profile on the test β-lactams. Their profiles (Table 2) were similar to that observed for PCC17.

**PBPs.** The preceding data showed that the increased β-lactam resistance of PCC17 was not associated with either enzymatic inactivation or exclusion of the antibiotic by the outer membrane. Another possibility to explain this resistance was a change in the target proteins in the inner membrane (6). Inner membrane preparations from PCC17, PAO503, PAO236 Str$^r$, and the exconjugants PCC1000, PCC1014, PCC1027, and PCC1028 were prepared and adjusted to a standard protein concentration of 8 mg/ml, and samples were fractioned by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Fluorographs of the [$^{14}$C]penicillin G labeled proteins fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis are shown in Fig. 3 and 4. The patterns obtained for PCC17 (Fig. 3, wells 8 and 9) and for exconjugants selected for Tic$^r$, PCC1000 (Fig. 3, wells 1 and 2), and PCC1014 (Fig. 3, wells 5 and 6) were all similar in that patterns, although faint, were observed at [$^{14}$C]penicillin G concentrations of 195 μg/ml, but were not observed at concentrations of 39 μg/ml (Fig. 3, wells 2, 6, and 9). The parent of PCC17 (PAO503) and the conjugal recipient PAO227 are shown in Fig. 3, wells 3, 4

TABLE 4. Coinheritance of resistance to ticarcillin with selected markers after mixed culture of *P. aeruginosa* strains PAO236 Str$^r$ × PCC17 (FP2)[a]

| Selection | % Coinheritance of: | | | | | | | Transmission frequency[b] |
|---|---|---|---|---|---|---|---|---|
| | *ilv* | *his* | *lys* | *met* | *trp* | *pro* | Tic$^r$ | |
| *ilv* | 100 | 12 | 12 | 4 | 1 | 2 | 1[c] | $5.08 \times 10^{-5}$ |
| *his* | 53 | 100 | 50 | 4 | 6 | 7 | 8[c] | $1.42 \times 10^{-5}$ |
| *lys* | 35 | 75 | 100 | 12 | 2 | 0 | 0 | $3.33 \times 10^{-6}$ |
| *met* | 90 | 38 | 66 | 100 | 3 | 2 | 2[c] | $3.50 \times 10^{-7}$ |
| *trp* | 75 | 65 | 52 | 33 | 100 | 38 | 21[c] | $2.75 \times 10^{-7}$ |
| *pro* | 90 | 55 | 48 | 9 | 39 | 100 | 33[d] | $2.83 \times 10^{-7}$ |

[a] Tic$^r$ was unselected. Exconjugants selected for independence at one of the auxotrophic markers were then screened for coinheritance of other markers and for Tic$^r$.
[b] Recombinant frequency per donor cell.
[c] All except one were *pro*$^+$.
[d] Eight were *trp*$^-$; all others were *trp*$^+$.

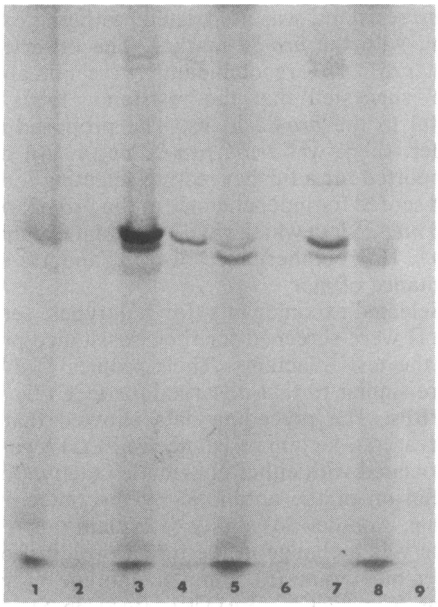


FIG. 3. PBPs from inner membrane preparations of PCC17, PAO227, and exconjugants. Fluorograph of the PBPs of *P. aeruginosa* PCC17 and derivatives. Partially purified inner membrane preparations (8 mg of protein per ml) were treated with either 39 or 195 μg of [$^{14}$C]penicillin G per ml for 10 min at 26°C. [$^{14}$C]penicillin G binding was stopped by the addition of a 1,000-fold excess of unlabeled penicillin G and 50 μl of Sarkosyl NL-97 per ml. The inner membrane protein was loaded onto vertical 11% sodium dodecyl sulfate-polyacrylamide gels and run at 20 mA for 6 to 7 h. Gels were fixed in methanol-acetic acid-water (50:10:40, vol/vol/vol) and treated with a fluor (En$^3$Hance) for 1 h before drying. Dried gels were used to expose prefogged X-ray film (Kodak, X-Omat) at −70°C for 32 days. Membranes treated with [$^{14}$C]penicillin G at 39 μg/ml: PCC1000 (2); PAO227 (4); PCC1014 (6); PAO503 (7); PCC17 (9). Membranes treated with [$^{14}$C]penicillin G at 195 μg/ml: PCC1000 (1); PAO227 (3); PCC104 (5); PCC17 (8).

and 7, for comparison. PAO503 (Fig. 3, well 7) when incubated with 39 μg of [$^{14}$C]penicillin G per ml gave a pattern consistent with that observed for *P. aeruginosa* strain 18$^s$ (4). *P. aeruginosa* PAO227 (Fig. 3, wells 3 and 4) had a PBP pattern similar to that observed in PAO503. In this case the pattern obtained with 39 μg of [$^{14}$C]penicillin G per ml (Fig. 3, well 4) has been

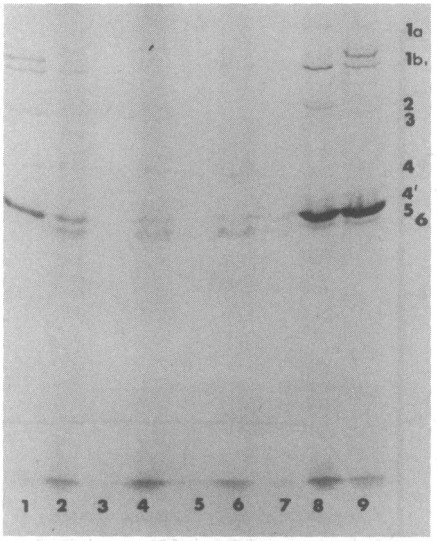


FIG. 4. PBPs from the parents and exconjugants in the cross PCC17 (FP2) × PAO236 Str$^r$. Inner membranes treated with [$^{14}$C]penicillin G at 39 μg/ml: PAO236 Str$^r$ (1); PCC1027 (3); PCC1028 (5); PCC17 (7); PCC47 (8). The same membrane preparations treated with [$^{14}$C]penicillin G at 195 μg/ml: PCC1027 (2); PCC1028 (4); PCC17 (6); PAO236 Str$^r$ (9). X-ray film was exposed for 39 days at −70°C. Other electrophoretic conditions were as stated previously. PBP nomenclature is as proposed by Curtis et al. (3).

enhanced by reaction with 195 μg of [$^{14}$C]penicillin G per ml (Fig. 3, well 3).

Similar observations were made when sample exconjugants selected for independence at an auxotrophic locus concomitantly ticarcillin resistant were screened for their PBP pattern (Fig. 4).

The exconjugants PCC1027 (Fig. 4, wells 2 and 3) and PCC1028 (Fig. 4, wells 4 and 5) show the differential response to [$^{14}$C]penicillin G already reported for PCC17 and exconjugants selected for by their resistance to ticarcillin. A repeat of the PBP pattern for PCC17 is shown in Fig. 4, wells 6 and 7. PBP patterns for the recipient in this set of experiments, PAO236 Str$^r$, are shown with [$^{14}$C]penicillin G at 39 μg/ml (Fig. 4, well 1) and 195 μg/ml (Fig. 4, well 9). The response to 39 μg of [$^{14}$C]penicillin G per ml for a back mutant (revertant?) of PCC17 (Fig. 4, well 8) showed a pattern similar to that observed for the original parent strain, PAO503 (Fig. 3).

Before fluorography, the gel shown in Fig. 4 was stained for protein. The results (Fig. 5) revealed similar protein patterns for PCC17 and the exconjugants PCC1027 and PCC1028 (Fig. 5, wells 2 to 7). The recipient strain PAO236 Str$^r$ and the back mutant PCC47 (Fig. 5, wells 1, 8, and 9) had profiles similar to each other, but different from that observed for the β-lactam-resistant strains. The major difference noted between the two resistant classes was the enhancement of the protein band corresponding to PBP6 in the resistant strain profiles.

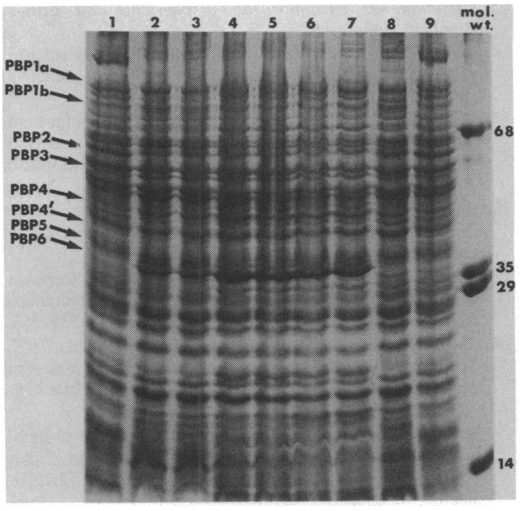


FIG. 5. Inner membrane preparations of PCC17 (FP2), PAO236 Str$^r$, PCC47, and exconjugants stained for protein. The gel shown is the same gel used to provide the fluorograph shown in Fig. 4. Before incorporation of the fluor, the gel was stained with 0.1% Coomassie blue, destained in 5% methanol and 10% acetic acid, and photographed. The protein bands shown correspond to the strains shown in the legend to Fig. 4. Molecular weight standards were bovine serum albumin (68,000), lactate dehydrogenase (35,000), carbonic anhydrase (29,000), and lysozyme (egg white, 14,300).

## DISCUSSION

Penicillin and other β-lactam resistances have traditionally been associated with enzymatic inactivation of the β-lactam by enzymes carried by resistant bacteria (10, 22). In the past decade several authors have shown that resistance can arise by other mechanisms. Changes in the permeability barrier posed by the outer membrane in gram-negative bacteria can either increase or decrease the level of resistance (24). Similarly, changes in the β-lactam targets have been shown to provide resistance to some of the newer β-lactams (4, 15). In a previous paper (6), we demonstrated that β-lactam resistance can be associated with changes in the affinity for penicillin at a number of target proteins. The strains used in this case were isolated from a patient during treatment. In this paper we have described a laboratory-induced mutant of *P. aeruginosa*, whose phenotype is similar to that described previously.

The mutation in PCC17 has been shown to be revertable, strongly suggesting a point mutation. Mapping the mutation by FP2-mediated conjugation placed the lesion at around 45 min on the PAO chromosomal map (16). This region of the chromosome is sparsely marked and attempts to more precisely localize the mutation by transduction have shown no linkage to the *proA* allele. Similar mating experiments utilizing different conjugative plasmids (16) may more exactly define this lesion.

The observation that PCC17 still retains the methionine requirement found in its parent, PAO503, leaves little doubt of its lineage. The decreased affinity of several PBPs was associated with an increase in the amount of a small-molecular-weight PBP. Available evidence is in favor of a single mutation causing these changes.

There are several possible models to explain the mutation in PCC17. One model is that PBP6 acts as a precursor of the other PBPs. This view would suggest a common PBP containing the penicillin-binding site undergoing at least one modification to give rise to the other PBPs. Nguyen-Disteche et al. (11, 12) suggested a model in which a limited number of enzyme proteins catalyzed several distinct cell wall cross-linking activities by the same enzyme protein, the functioning of which was moderated by its microenvironment in the cytoplasmic membrane. In the model suggested above, the mutation in PCC17 could affect the post-translational

modification of the PBPs forming one or both of the enzyme groups proposed (12).

Alternatively, PBP6 could be a degradation product of other PBPs reducing their number through post-translational degradation. Gutmann et al. (7) described the accumulation of low-molecular-weight protein bands binding [$^3$H]penicillin in *Streptococcus pyogenes* pretreated with the aminoglycoside gentamicin. The low-molecular-weight protein accumulation was interpreted as rapid PBP degradation caused by the protein synthesis inhibitor. The mutation in PCC17 may affect the normal turnover rate of PBP1 through 5, resulting in the reduced binding at these proteins. This model would suggest a decreased amount of protein corresponding to the PBPs (other than PBP6) in polyacrylamide gels stained for protein. As seen in Figure 5, the protein bands corresponding to PBP1 through 4 are very minor constituents in the protein pattern obtained. Only PBP5 in sensitive cells and PBP5 and 6 in the resistant cells are readily recognized. There was no apparent decrease in the amount of the protein corresponding to PBP5 in mutant strains. However, reduction in the number of other PBPs could contribute to the protein banding at PBP6. This model still suggests a common subunit, at least in terms of molecular weight.

Yet another alternative is that *pbpA* is a control mutation reducing the activity of several PBPs and concomitantly increasing translation of a single protein, PBP6. The observation that PCC17 grows normally (i.e., is motile with a rod shape) and has a generation time similar to the parent, PAO503, suggests that the enzymes necessary for peptidoglycan synthesis are present in sufficient quantity to effect normal cell wall synthesis.

At the moment these alternative models have not been differentiated. Several experimental approaches are being tried in attempts to differentiate among them.

## ACKNOWLEDGMENTS

This research was supported by a grant from the Medical Research Council of Canada.

We express our appreciation to Cecilia Po for technical assistance and to Shirley Eikerman for typing the manuscript.

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