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Susceptibility to β-Lactam Antibiotics of *Pseudomonas aeruginosa* Overproducing Penicillin-Binding Protein 3

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By using a broad-host-range vector, pUCP27, the *Pseudomonas aeruginosa* and *Escherichia coli pbpB* genes, which encode penicillin-binding protein 3 (PBP3), were separately overexpressed in a *P. aeruginosa* strain, PAO4089, that is deficient in producing chromosomal β-lactamase. Susceptibility studies indicated that overproduction of the *P. aeruginosa* PBP3 in PAO4089 resulted in twofold-increased resistance to aztreonam, fourfold-increased resistance to cefepime and cefsulodin, and eightfold-increased resistance to ceftazidime, whereas overproduction of the *P. aeruginosa* PBP3 in PAO4089 did not affect susceptibility to PBP1-targeted cephaloridine or PBP2-targeted imipenem. Similar results were obtained with PAO4089 overproducing *E. coli* PBP3, with the exception that there was no influence on the MICs or minimal bactericidal concentrations of cefsulodin and cefepime, which have very low affinities for *E. coli* PBP3. These data are consistent with the conclusion that PBP3 has to achieve a certain level of saturation, with β-lactams targeted to this protein, to result in cell inhibition or death.

Most of the information about how β-lactams kill bacteria has been derived from two types of experiments. The first involves the influence of defined (5, 19) or clinical (4) mutations which lead to the inability of specific penicillin-binding proteins (PBPs) to covalently bind β-lactams on the MICs of a range of β-lactams. The second type of experiment involves an ex vivo determination of the relative affinity of PBPs for specific β-lactams as assessed by the ability of the drugs to block binding of radiolabelled benzylpenicillin. However, despite these data about primary targets, we are still lacking specific information about the relationship between binding of β-lactam to PBP(s) and eventual killing. For example, although there is usually a primary PBP target, must other PBPs also be inhibited partly or totally to permit killing? Also, must the primary PBP target molecules within a cell be totally saturated with β-lactams or partly saturated, or must a given number of molecules of PBP (e.g., those in defined cellular locations) be inhibited to permit β-lactams to have their lethal action? A mathematical relationship (2, 11) between MIC and certain biochemical parameters has been devised in an attempt to characterize the factors involved in β-lactam action on cells. However, this relationship characterized β-lactam interaction with its target by use of a term, S_i, which represents the β-lactam concentration in the periplasm when the external concentration is equal to the MIC. To permit some clarification of the above factors, we decided to test the influence of overexpression of a specific PBP on MIC.

Previous reports had suggested that *Pseudomonas aeruginosa* PBP3 was the primary target for expanded-spectrum and "fourth-generation" cephalosporins (9, 20). To further characterize *P. aeruginosa* PBP3, we previously cloned and sequenced the PBP3-encoding gene, *pbpB*. The *pbpB* gene product was expressed in *Escherichia coli* and characterized by a [^{3}H]penicillin-binding assay (6). It was found that the *pbpB* gene was located upstream of a gene encoding a homolog of the *E. coli* *murE* gene product (6) and that these two genes mapped to the same region as did some other cell division genes, including *ftsA*, *ftsZ*, and *envA* (7), suggesting that *pbpB* might be essential for cell viability. To address the above questions and to examine the hypothesis that *P. aeruginosa* PBP3 plays an important role in susceptibility to β-lactam antibiotics, we have studied here the effects of overproduction of the *P. aeruginosa* and *E. coli pbpB* gene products in *P. aeruginosa* on susceptibility to β-lactam antibiotics.

P. aeruginosa PAO4089, deficient in producing chromosomal β-lactamase (5), was obtained from N. Gotoh and used as the host for gene expression experiments since the wild-type strain of *P. aeruginosa* contains inducible β-lactamase, which had been found to interfere with penicillin-binding assays (12). *E. coli* JM110 was used as the host for DNA cloning experiments. The broad-host-range vector pUCP27 (16) was obtained from H. Schweizer and used for *pbpB* gene expression experiments. Luria-Bertani (Difco, Detroit, Mich.) and Mueller-Hinton (Becton Dickinson, Cockeysville, Md.) media were used for the growth of *E. coli* and *P. aeruginosa*, respectively. Solid media were made by the addition of 2% Bacto-agar (Difco). Antibiotics used in selective media were ampicillin at 75 μg/ml

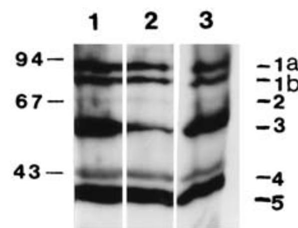

FIG. 1. Autoradiogram of cell membrane proteins after incubation with [^{3}H]penicillin and separation by SDS–8.5% PAGE. Lane 1, PAO4089(pXL506), containing the cloned *P. aeruginosa pbpB* gene; lane 2, PAO4089(pXL546), containing the 300 bp at the 3′ end of the *P. aeruginosa pbpB* gene and used as the control for lanes 1 and 3; lane 3, PAO4089(pXLK20), containing the cloned *E. coli pbpB* gene. Numbers on the left indicate molecular mass in kilodaltons. PBPs are indicated on the right.

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TABLE 1. MICs of β-lactam antibiotics for PAO4089 expressing the *P. aeruginosa* and *E. coli pbpB* gene products

Strain	PBP gene expressed	MIC (MBC), μg/ml[a]					
		Ceftazidime	Cefsulodin	Cefepime	Aztreonam	Imipenem	Cephaloridine
PAO4089	wt[b]	0.63 (1.25)	0.8 (1.6)	0.5 (1)	2 (4)	0.25 (0.5)	8 (16)
PAO4089(pXL546)	wt	0.63 (1.25)	0.8 (1.6)	0.5 (1)	2 (4)	0.25 (0.5)	8 (16)
PAO4089(pXL506)	*pbpB*[c]	5.0 (10)	3.2 (6.4)	1.0 (4)	4 (8)	0.25 (0.5)	8 (16)
PAO4089(pXLK20)	*pbpB*[d]	2.5 (5)	0.8 (1.6)	0.5 (1)	4 (8)	0.25 (0.5)	8 (16)

[a] Results and trends observed were reproducible and are a compilation of three to six measurements. Sources for β-lactam antibiotics used in this study are as follows: cefepime, Bristol-Myers-Squibb Inc., Wallingford, Conn.; cefsulodin, Ciba Geigy, Basel, Switzerland; ceftazidime, Glaxo Canada Inc.; cephaloridine, Sigma; and imipenem, Merck, Rahway, N.J.
[b] wt, wild type.
[c] *P. aeruginosa* gene.
[d] *E. coli* gene.

and tetracycline at 12 μg/ml for *E. coli* growth and tetracycline at 100 μg/ml for *P. aeruginosa* growth.

All DNA cloning techniques were performed as described previously (6). To construct the plasmid for the expression of the *P. aeruginosa pbpB* gene in PAO4089, a 1.7-kb *Nde*I-*Bam*HI DNA fragment which contained the T7 ribosome-binding site and the *P. aeruginosa pbpB* gene was isolated from pXL706 (6) and cloned into pUCP27 behind the *lac* promoter to generate pXL506. A stable negative-control plasmid, pXL546, was obtained by cloning a 300-bp fragment (which contained only the portion of the *pbpB* gene corresponding to the C terminus, without a translational start site) into pUCP27, since in our hands the vector plasmid alone was not stable in *P. aeruginosa*. To determine whether *E. coli* PBP3 functioned in *P. aeruginosa*, an attempt was made to express the *E. coli pbpB* gene product in PAO4089. The expression plasmid pXLK20 was constructed by cloning a 2.6-kb *Bam*HI-*Eco*RI fragment, which contained the *E. coli pbpB* gene from pPH125 (18) (obtained from B. Spratt), into pUCP27 behind the *lac* promoter. Plasmids pXL506, pXL546, and pXLK20 were transformed into PAO4089 by the $CaCl_2$ procedure (10) as modified by Schweizer (15).

PAO4089 cells were grown in Mueller-Hinton broth and harvested in the mid-log phase of growth, followed by washing in 10 mM Tris-HCl buffer, pH 8.0. All clones grew at wild-type rates and did not form filaments under these conditions. The washed cells were lysed in a French press, and the membrane proteins were prepared by the procedure described previously (6). Membrane proteins of PAO4089(pXL506) and PAO4089 (pXLK20) were examined for the expression of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, no novel proteins were visualized after Coomassie blue staining (data not shown). Subsequently, samples of membrane proteins from PAO4089(pXL506) and PAO4089 (pXLK20) were used in [^{3}H]penicillin-binding protein assays, which were carried out essentially by the method of Spratt (17) and described previously (6). The expressed products of the *P. aeruginosa* and *E. coli pbpB* genes were detected with [^{3}H]penicillin (Fig. 1). By using a scanning densitometer, the amounts of *P. aeruginosa* and *E. coli* PBP3 produced from the recombinant clones were estimated to be sevenfold higher than the amount of native PBP3 present in PAO4089(pXL546). The negative-control plasmid, pXL546, failed to direct the expression of any novel product.

To investigate the effect of overproduction of *P. aeruginosa* and *E. coli* PBP3 on antibiotic susceptibility, the MICs of several β-lactam antibiotics (including the PBP3-targeted compounds cefsulodin [PBP1 targeted in *E. coli*], ceftazidime, cefepime, and aztreonam; PBP2-targeted imipenem; and PBP1-targeted cephaloridine) were determined by standard broth microdilution assay (1) and their minimal bactericidal concentrations (MBCs) were determined by plating on growth media the contents of wells containing concentrations of antibiotic above the MIC. Table 1 summarizes the MICs of these antibiotics for *P. aeruginosa* PAO4089 overproducing *P. aeruginosa* or *E. coli* PBP3. Overproduction of PBP3 from the cloned *P. aeruginosa pbpB* gene led to two- to eightfold increases in the MICs and MBCs of PBP3-targeted antibiotics. The MICs and MBCs of the PBP2-targeted and PBP1-targeted antibiotics were not influenced by the presence of the extra copies of this PBP3 protein. In contrast, cells containing the negative-control plasmid, pXL546, had unaltered susceptibility to all the β-lactams tested. The increased MICs for the strains that overproduced PBP3 indicated that the recombinant PBP3 was biologically functional. Similar results were observed for PAO4089, which overproduced the *E. coli pbpB* gene product, except that cefsulodin and cefepime susceptibility were not affected. This result indicated that *E. coli* PBP3 retained its biological function in *P. aeruginosa.*

Competition binding experiments have previously been per-

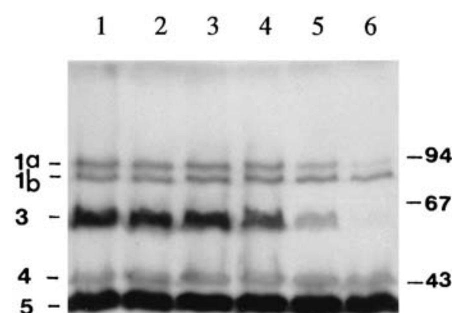

FIG. 2. Autoradiogram of cell membrane proteins illustrating competition of ceftazidime with [^{3}H]penicillin for the PBPs of PAO4089 expressing the *P. aeruginosa pbpB* gene product. The antibiotic concentrations increase from 0.0125 to 3.2 μg/ml (fourfold increase per lane) from lanes 2 to 6, and lane 1 is the control (no ceftazidime). Numbers on the right indicate molecular mass in kilodaltons. PBPs are indicated on the left. Membrane proteins (100 μg) were incubated with ceftazidime or, for control experiments, with distilled water at 25°C for 10 min. Subsequently, 4 μCi [^{3}H]penicillin G (20 μCi/μmol; Amersham Life Sciences, Oakville, Ontario, Canada) was added to the solution. After incubation at 25°C for 10 min, the reaction was stopped by the addition of an excess (1,000-fold) of nonradioactive penicillin G. The proteins were resolved by SDS–7.5% PAGE. The resultant gel was treated with 1 M sodium salicylate, dried, and exposed to X-ray film (X-Omat K XK-1; Kodak) for 3 days at −70°C. The intensities of the bands on the fluorograms were quantitated with a scanning densitometer (Studio Scan II; Agfa) in combination with a Macintosh computer and the public domain NIH Image program. The software was used to integrate scan peaks for quantitation of the binding of PBP to [^{3}H]penicillin. Binding inhibition was evaluated as the concentration of the antibiotic inhibiting binding by 50% relative to the level of binding in the absence of competing antibiotic.

TABLE 2. I_{50}s of β-lactam antibiotics for PBPs from PAO4089 expressing the *P. aeruginosa* and *E. coli pbpB* gene products

Strain[a]	PBP	I_{50} (μg/ml)[b] of:				
		Ceftazidime	Cefsulodin	Cefepime	Aztreonam	Cephaloridine
PAO4089(pXL506)	1A	0.8 (0.8)[c]	>9.6 (19)	0.14 (0.03)	>1.25	0.5 (0.8)
	1B	>3.2 (6.0)	1.6 (2)	0.4 (0.75)	>1.25	0.2 (0.4)
	3[d]	0.2 (0.1)	0.6 (0.3)	0.01 (0.003)	0.05 (0.05)	2.5 (0.9)
	4	>3.2 (6.4)	>9.6 (39)	>0.06 (0.04)	>1.25	0.05 (0.04)
	5/6	>3.2 (50)	>9.6 (>250)	>0.06	>1.25	>5.0 (>10)
PAO4089(pXLK20)	3[e]	0.3 (0.06)	>25 (>250)	0.15 (0.03)	0.25 (0.01)	8.0 (8)

[a] pXL506, *P. aeruginosa pbpB* gene cloned in the vector pUCP27; pXLK20, *E. coli pbpB* gene cloned in the vector pUCP27.
[b] Results marked ">" are the highest concentrations used in the competition assay. PBP2 was not observed in these experiments, since under the conditions utilized here PBP2 was observed only if the autoradiogram was overexposed, making the other data more difficult to assess, or if cells were grown to stationary phase.
[c] Numbers in parentheses are data from the literature (3, 13, 14). In those studies, the I_{50} for PBP2 was >250 μg/ml.
[d] *P. aeruginosa* PBP3.
[e] *E. coli* PBP3. Since only the cloned PBP was unique (Fig. 1), only these data are presented. However, I_{50} data for other PBPs were obtained and were essentially the same as those observed in strain PAO4089(pXL506).

formed for most of the antibiotics for which data are presented in Table 1 (3, 13, 14). To confirm the preferences of these antibiotics for binding to certain PBPs, we performed [^{3}H] penicillin competition binding assays. The parameter for evaluating the affinity of a selected β-lactam antibiotic for a PBP was I_{50}, which was defined as the concentration of the β-lactam antibiotic that reduced [^{3}H]penicillin binding by 50%. Therefore, the lower the I_{50}, the higher the affinity. The results of competition binding assays indicated that the PBP3-targeted β-lactams ceftazidime (Fig. 2) and aztreonam reacted preferentially with the *P. aeruginosa* and *E. coli* PBP3s (Table 2). In contrast, cefsulodin bound preferentially to *P. aeruginosa* PBP3 but did not influence binding of penicillin to *E. coli* PBP3 (Table 2), a result consistent with its known binding properties, whereas cefepime bound equally to *E. coli* PBP3 and *P. aeruginosa* PBP1a. The I_{50}s for cloned *P. aeruginosa* PBP3 mirrored the results achieved for PBP3 in wild-type cells. The PBP-binding patterns observed were thus consistent with the conclusion that increases in the MICs of the PBP3-targeted antibiotics were due to the overproduction of the *P. aeruginosa* or *E. coli pbpB* gene product. Taken together with data for mutants showing that the loss of binding of a particular β-lactam to PBP3 leads to resistance to that β-lactam (4, 5, 17), this seems to indicate that inhibition of a single PBP target may be sufficient to result in cell killing.

The efficacy of β-lactam antibiotics against gram-negative bacteria, as measured by their MICs, depends on their rate of penetration across the outer membrane, their degree of resistance to inactivation by β-lactamase, and their ability to inhibit their target proteins, the PBPs. Alteration of either of the first two parameters can result in resistance to β-lactam antibiotics (8, 19), which is experimentally detected by an increase in the MIC of the tested antibiotic for the relevant bacterial strain. However, interaction with target PBPs has been expressed as S_i (the periplasmic concentration of β-lactam antibiotics at the MIC) (2, 11). The relationship between this factor and the kinetic constants of β-lactam interaction with PBPs, and the influence of these constants on the MIC, is unknown.

The reaction of a β-lactam antibiotic with a PBP involves the formation of a reversibly bound enzyme-inhibitor complex followed by covalent modification (acylation) and hence irreversible inhibition. The reaction scheme is analogous to that of the Michaelis-Menten mechanism, $E + I = E \cdot I \rightarrow E\text{-}I^*$, and should yield saturation kinetics with increasing inhibitor concentrations. If the level of a critical PBP were to increase, this could influence the MIC and MBC in one of three ways. If the level of peptidoglycan synthesis had to be reduced below a certain basal level to effect cell inhibition and killing, then we would anticipate that increased PBP levels would require increased periplasmic antibiotic concentrations to inhibit peptidoglycan synthesis to that level compared to the concentrations needed in the wild-type strain, i.e., we would expect to observe resistance. Alternatively, if inhibition of even a small number of target PBP molecules was sufficient for cell inhibition and death, one might see an increase in susceptibility to antibiotic accompanying an increase in PBP copy number (reflecting an increase in PBP availability). If, however, a set fraction of PBP3 (e.g., 90%) needed to be inhibited for cell death to occur, or if inhibition of another PBP was rate limiting, we would anticipate no change in MIC or MBC. The first scenario leading to resistance appears to best fit our data. In this scenario the overexpression of PBP3 should result in an increased S_i of PBP3-targeted antibiotic, which consequently would require an increased concentration of the antibiotic in the medium to inhibit cell growth. This corresponded to the observed increase in MIC (Table 1). It should be noted, however, that the MIC is not a simple function of the periplasmic β-lactam concentration (2, 11). The differential increases in MIC among the four different PBP3-targeted antibiotics may have been due in part to this, and also due to their different binding affinities for PBP3, as indicated by their different I_{50}s (Table 2). For example, ceftazidime and cefsulodin had lower affinities (i.e., higher I_{50}s) for PBP3 than cefepime and aztreonam, and higher concentrations of these two antibiotics were thus required to inhibit the overproduced PBP3 (i.e., higher MICs).

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