

# Roles of Porin and $\beta$ -Lactamase in $\beta$ -Lactam Resistance of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa demonstrates high intrinsic resistance to most  $\beta$ -lactam antibiotics. Two factors that are interrelated appear to be important in this intrinsic resistance: an inducible, chromosomally encoded type Id  $\beta$ -lactamase and low outer-membrane permeability.  $\beta$ -Lactamase-noninducible mutants are supersusceptible to many  $\beta$ -lactam agents, whereas constitutively derepressed mutants are considerably more resistant even to so-called  $\beta$ -lactamase-stable  $\beta$ -lactams. For the latter mutants, by analysis of kinetics, it can be demonstrated that synergy between slow permeation across the outer membrane and slow hydrolysis of the  $\beta$ -lactamase-stable  $\beta$ -lactam can explain resistance. Wild-type *P. aeruginosa* allows outer membrane permeation of  $\beta$ -lactam agents at rates 1%-8% of those measured for *Escherichia coli*. The majority of trans-outer-membrane channels formed by *P. aeruginosa* porin protein F are too small to allow passage of  $\beta$ -lactam antibiotics. Nevertheless, this porin is apparently a conduit for  $\beta$ -lactam agents. This low outermembrane permeability to certain  $\beta$ -lactam agents. This low outermembrane permeability acting in synergy with  $\beta$ -lactamase is probably responsible for intrinsic  $\beta$ -lactam resistance in *P. aeruginosa*.

## Pseúdomonas aeruginosa Chromosomal β-Lactamase: Inducibility and Role in Resistance

All wild-type Pseudomonas aeruginosa strains studied contain an inducible type Id chromosomal βlactamase [1]. This enzyme is induced in the presence of  $\beta$ -lactam agents [2]. Some  $\beta$ -lactam agents are more potent in their ability to induce  $\beta$ -lactamase than others [3]. This ability is not related to the susceptibility of the inducing  $\beta$ -lactam to hydrolysis by the  $\beta$ -lactamase (a point that is illustrated by imipenem) [3], and there is no obvious relation between the target of individual  $\beta$ -lactam antibiotics and their ability to induce β-lactamase. In contrast, an interrelation between outer-membrane permeability and *P. aeruginosa* chromosomal β-lactamase inducibility has been suggested [2, 4]. There have been some reports [5] that non- $\beta$ -lactam compounds can induce  $\beta$ -lactamase, but we have been unable to confirm these results using P. aeruginosa PAO1 (R. E. W. H., unpublished results); thus we attribute previous observations to specific strain-related effects.

Some of the strongest support for the role of the

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Please address requests for reprints to Dr. R. E. W. Hancock, Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5. type Id  $\beta$ -lactamase in intrinsic  $\beta$ -lactam antibiotic resistance of *P. aeruginosa* comes from measurements of MICs for mutants with altered  $\beta$ -lactamase inducibility. Mutants that are noninducible for chromosomal  $\beta$ -lactamase are supersusceptible to  $\beta$ lactam antibiotics [6–8] (table 1). Conversely, constitutively derepressed mutants are considerably more resistant to most  $\beta$ -lactam agents than are their inducible parent strains [3, 8] (table 1). Thus, resistance to  $\beta$ -lactam antibiotics is strongly influenced by the inducibility and the level of *P. aeruginosa*  $\beta$ -lactamase. These data argue powerfully for a role of  $\beta$ -lactamase in the intrinsic resistance of *P. aeruginosa* to almost all  $\beta$ -lactams.

# Importance of the Porin Pathway in $\beta$ -Lactam Susceptibility and Intrinsic Resistance

A role for the outer membrane of *P. aeruginosa* in  $\beta$ -lactam resistance has been suggested on the basis of the function of the outer membrane as a permeability barrier. *P. aeruginosa* demonstrates moderate to high intrinsic resistance to almost all known hydrophilic antibiotics [10]. Even these newly developed  $\beta$ -lactam compounds, which demonstrate good activity against *P. aeruginosa*, have substantially higher MICs for this species than for most other gram-negative organisms (in the absence of constitutive  $\beta$ -lactamases) [10]. This observation has led



Mutant	Mutant-to-parent ratio of β-lactamase levels*	Mutant-to-parent MIC ratio for indicated β-lactam					
		Freely β-lactamase-hydrolyzable			β-Lactamase-stable		
		PenG	Amp	Clor	Carb	Czid	Ctax
Noninducible							
2126-8	0.05	0.07	0.12		1.2		
5	0.01	0.03	0.08		1.2		
1F03080L	0.01	0.01	0.01	0.01	0.25		
Tid53L	0.01	0.12	0.01	0.03	1.0		
185A1-	0.01	0.12	0.5	0.03	1.0	0.5	0.5
50SAI-	0.01	0.01	0.01	0.01	1.0	0.5	0.5
Derepressed							
18sH	913					16	>4
50DR	1,433					16	256
PA-48	296					>128	
CD64		• • •		• • •	1	>32	>4

**Table 1.**  $\beta$ -Lactamase levels and MICs for noninducible and constitutively derepressed  $\beta$ -lactamase mutants of *Pseudomonas aeruginosa* compared with those for parent strains.

NOTE. Abbreviations: PenG = penicillin G; Amp = ampicillin; Clor = cephaloridine; Carb = carbenicillin; Czid = ceftazi-dime; Ctax = ceftazime. Ellipses indicate measurement not done.

\* Relative levels of  $\beta$ -lactamase are compared after induction for noninducible mutants and before induction for derepressed mutants. All data in this table come from [3, 6–10].

researchers to propose that the outer membrane constitutes a barrier to the diffusion of hydrophilic antibiotics [11, 12].

Direct measurements of bacterial outer-membrane permeability revealed that rates of penetration into P. aeruginosa were only 1%-8% of those into Escherichia coli [4, 13, 14]. Purification of a major outer-membrane protein F and its reconstitution into lipid bilayer membranes showed that this protein could form hydrophilic channels of two types [15]. The greater percentage (>99%) of channels were small and assumed to be impermeable to  $\beta$ -lactam antibiotics [15]. Large channels, observed at a frequency of <1%, were considered to be responsible for the measurable uptake of  $\beta$ -lactam antibiotics by P. aeruginosa [15-17]. The existence of large channels is consistent with the known ability of P. aeruginosa to grow on pentamethionine as the sole methionine source [18] (cf. E. coli [19]) and the observation that the  $\beta$ -lactams that tend to be highly effective against P. aeruginosa often have high molecular weights [10] - e.g., piperacillin (mol wt, 644) cefsulodin (544), cefpiramide (600), cefpirome (511), cefpimizole (688), and ceftazidime (545).

Heterogeneity in channel formation is an intrinsic property of protein F, since protein F purified from an *E. coli* strain containing the cloned protein F gene demonstrates channel-size heterogeneity similar to that of native protein F purified from P. aeruginosa [15]. We have proposed a model involving two different arrangements of the four protein F cysteines in cystine disulfides to account for these observations [20]. Since both types of channels were formed by protein F purified from an E. coli porin-deficient strain containing the cloned protein F gene [15], we believe that the small proportion of large channels did not likely result from contamination of protein F by another P. aeruginosa porin. In addition, aggregation would not seem to account for these data because we saw distinct Poisson distributions of channel conductances for both the small and large channels (rather than a series of channel conductances with magnitudes twofold, threefold, fourfold, and so on greater than that of the small channel).

Further evidence for the role of porins in  $\beta$ -lactam permeability came from the analysis of protein F-deficient mutants. A protein F-deficient mutant of *P. aeruginosa* demonstrated a six- to eightfold reduction in the rate of diffusion of the chromogenic  $\beta$ -lactam nitrocefin across the outer membrane [13] (table 2). Presumably, the residual diffusion of nitrocefin in the protein F-deficient mutant was due either to an alternative, less efficient porin with reduced ability to mediate nitrocefin diffusion across the outer membrane or to an alternative, nonporin pathway.

Table 2. (	Duter-membrane permeability of <i>Pseudomonas</i>
aeruginosa	PAO1 strain H103 compared with that of its
chemically	induced protein F-deficient mutant, H283,
and with th	at of an Escherichia coli K12 strain.

Strain	Relative outer-membrane permeability*	Molecules of major porin/cell <sup>†</sup>
E. coli K12	1.00	100.000
P. aeruginosa PAO1 P. aeruginosa protein	0.08	200,000
F-deficient mutant	0.01	No protein F

\* Outer-membrane permeability, assessed as described in [13], is expressed as the nitrocefin diffusion rate relative to that for  $E. \ coli$ , which is assigned a value of 1.00.

<sup>†</sup> Approximate number of copies per cell of the outer membrane protein F and C porins for *E. coli* and the protein F porin for *P. aeruginosa*.

Unfortunately, this chemically mutagenized, protein F-deficient mutant was quite unstable and reverted during MIC experiments. Therefore, we set about isolating insertion mutants carrying a transposon in the protein F gene. We mutagenized the cloned protein F gene in E. coli with the transposon Tn1, transferred the mutated plasmid into P. aeruginosa using a helper plasmid, and forced the transposon-mutagenized gene to recombine with and replace the chromosomal gene using the phage F116L packaging method of Ohman et al. [21, 22]. The resulting protein F-deficient Tnl mutant was analyzed by MIC assays. We observed increased resistance to ceftazidime and aztreonam using the protein F-deficient Tn1 mutant (table 3). In contrast, we observed no increase in resistance to tobramycin-a result consistent with our demonstration that aminoglycosides utilize an alternative, nonporin pathway for uptake across the outer membrane [20].

The presence of Tn1 (TEM-2)  $\beta$ -lactamase in the previously mentioned protein F-deficient mutant of *P. aeruginosa* limited the range of  $\beta$ -lactam antibiotics that could be utilized in these experiments; therefore, we set out to make protein F-deficient mutants by an alternative technique. The commercially available (Amersham; Oakville, Ontario) DNA fragment  $\Omega$  (encoding streptomycin resistance and containing multiple transcriptional stop signals) was engineered into a unique *SmaI* site in the protein F gene in vitro [22]. This  $\Omega$  fragment-mutated protein F gene was then inserted into plasmid pRZ102, a plasmid that utilizes the instability of Tn5 in *P. aerugi* 

nosa to drive recombination with the chromosome [23]. The resulting *P. aeruginosa* protein F-deficient  $\Omega$  mutant was examined for resistance to many of the known  $\beta$ -lactam antibiotics that have reasonable effectiveness against *P. aeruginosa* (table 4). MIC changes due to the protein F mutation were small, as assessed on the basis of five separate trials. Nevertheless, the degree of change in MIC was similar to that observed when *E. coli* porin-deficient mutants were compared with wild-type *E. coli* (table 4). Given the proposed role of porins in  $\beta$ -lactam resistance, the observation that the difference in MIC between porin-sufficient strains and porin-deficient mutants is small is an issue that must be addressed.

We think that there are at least two possible explanations for these data. First, it is possible that other porins can be utilized in  $\beta$ -lactam uptake. In *P. aeruginosa* the candidates include protein D1, a glucose-inducible porin [24], and protein D2, which has been implicated in imipenem uptake [25, 26]. Second, protein F-deficient mutants have lost a major outer-membrane and cellular structural component. Consequently, they seem to have acquired an additional nonporin pathway, as judged by their increased permeability to a hydrophobic probe, NPN [22]. It is possible that this nonporin pathway can be utilized for uptake of  $\beta$ -lactam antibiotics in the protein F-deficient mutant but not in the protein F-sufficient wild-type strain. The existence of such

**Table 3.** Comparison of mean MICs for protein F-sufficient (H309) and protein F-deficient (H608/F<sup>-</sup>) Tn1-containing strains of *Pseudomonas aeruginosa* PAO1.

	MIC (µg/mL) for indicated strain				
Antibiotic	H309/ RP1 (Tn <i>1</i> )	H608∕ F⁻::Tn <i>l</i>	Ratio (F⁻/F⁺)		
Ceftazidime	1.0	3.4*	3.4		
Aztreonam	1.7	4.0*	2.3		
Tobramycin	2.0	0.7	0.4		

NOTE. Strain H309 contains Tn1 by virtue of the presence of a plasmid, RP1. Strain H608, which was constructed by recombination of the transposon-mutated protein F gene from plasmid pWW13::Tn1 into the chromosome of *P. aeruginosa* PAO1, contains one copy of Tn1 that has insertionally inactivated the protein F gene; thus, this strain is protein F-deficient. The levels of Tn1  $\beta$ -lactamase expressed were as follows: H309/RP1, 2.2 nmol of nitrocefin hydrolyzed/(min·mg [cell dry weight]); H608/F<sup>-</sup>::Tn1, 1.5 nmol of nitrocefin hydrolyzed/ (min·mg). Five independent trials yielded the above MICs.

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\* Significantly different from H309/RP1 (P < .05, Fisher's exact test).

	Ratio of MICs of porin-deficient mutant to porin-sufficient parent*				
	P. aeruginosa	E. coli K12			
	PAO1	OmpF⁻,			
Antibiotic	F-::Ω	OmpC <sup>-</sup>	OmpF⁻	OmpC <sup>-</sup>	
Cefpirome	3.0†	ND	ND	ND	
Cefotaxime	3.2	2	1-4	1-2	
Cefopeme	2.5	ND	ND	ND	
Aztreonam	1.7	4	4	1	
Carbenicillin	1.8†	8	8-16	1-2	
Ceftazidime	1.6‡	2	4	<1	
Piperacillin	1.4	ND	2	ND	
Imipenem	0.8	1	1	1	
Cefoxitin	ND§	16-32	2-8	1	
Cefazolin	ND§	8-16	1-4	1	
Ampicillin	ND§	4-16	2-4	1	

**Table 4.** Influence of porin deficiencies on  $\beta$ -lactam resistance in *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* K12.

NOTE. Omp = outer-membrane protein; ND = not determined.

\* Data are from [22, 36-39].

<sup>†</sup> Significantly different (P < .05, Fisher's exact test).

<sup>‡</sup> P < .1; all other results, P > .2 unless otherwise indicated. <sup>§</sup> P. *aeruginosa* wild-type strains are resistant to >128 µg of these antibiotics/mL; thus, resistance of the protein F-deficient mutants was not tested.

nonporin pathways has been strongly implicated in studies of other *P. aeruginosa* mutants [27, 28]; such pathways would tend to result in a preferential decrease in antibiotic MICs for the protein F-deficient mutant, thus partially or fully reversing the effect of protein F deficiency. In support of this argument, Godfrey and Bryan [29] isolated a putative protein F-altered strain that retained normal levels of protein F and had much higher increases in antibiotic resistance than our protein F-deficient mutants.

## Synergy Between Low Outer-Membrane Permeability and $\beta$ -Lactamases as a Defense Against $\beta$ -Lactam Antibiotics

Despite the relatively low outer-membrane permeability of *P. aeruginosa* to  $\beta$ -lactams (table 2), an antibiotic like nitrocefin, at an external concentration of 50 µg/mL, will equilibrate to the same periplasmic concentration within 21 seconds in wild-type *P. aeruginosa* [30]. This time is far shorter than the generation time of *P. aeruginosa* in vitro or in vivo [31]; thus, low outer-membrane permeability is insufficient by itself to account for the high intrinsic resistance of *P. aeruginosa* to antibiotics. An additional resistance mechanism is required to take advantage of the slower influx of  $\beta$ -lactam into the periplasm; we believe that this mechanism involves the inducible  $\beta$ -lactamase activity present in the periplasm of wildtype cells [4]. Provided that the rate of influx of  $\beta$ lactam agent is not too high (resulting in enzyme saturation) and that the rate of hydrolysis by periplasmic  $\beta$ -lactamase of the  $\beta$ -lactam is sufficient to inactivate incoming  $\beta$ -lactam molecules, synergy between low outer-membrane permeability and  $\beta$ -lactamase hydrolysis should result in resistance.

It follows from the above arguments that four strategies can be utilized to overcome intrinsic resistance. First, raising the concentrations of the  $\beta$ lactam antibiotic added to cells will result in increased permeation, according to Fick's first law of diffusion [32], and thus will increase periplasmic concentrations. This approach is not always possible in patients. Second, we can attempt to increase permeation across the outer membrane by synthesizing antibiotics with properties that will aid passage through porin protein F-namely, small size and strongly hydrophilic as well as zwitterionic or cationic character (since the protein F channel is waterfilled and cation-selective [16]). A third strategy is to increase antibiotic stability to  $\beta$ -lactamases, and fourth is the development of antibiotics (like aminoglycosides [30] and imipenem [26]) that use alternative uptake pathways.

The best substrates for *P. aeruginosa* type Id  $\beta$ lactamase are hydrolyzed rapidly with a large measured turnover number (molecules hydrolyzed per molecule of  $\beta$ -lactamase per second, e.g., 3,000 for cephaloridine) [33]. Many of the more recent antipseudomonal  $\beta$ -lactam agents are hydrolyzed at low or undetectable rates and consequently have low MICs against most P. aeruginosa strains. Nevertheless, P. aeruginosa mutants derepressed for β-lactamase have been isolated in the clinical setting [26] and during  $\beta$ -lactam treatment of model infections [8] as well as in the laboratory [3]. Such strains demonstrate resistance even to  $\beta$ -lactamase-stable  $\beta$ lactams, e.g., ceftazidime (table 1). Vu and Nikaido [34] demonstrated for analogous strains of Enterobacter cloacae that the rate of diffusion of  $\beta$ lactams like ceftazidime and the rate of β-lactamase synthesis were inconsistent with the concept that a nonhydrolytic barrier (also called enzyme trapping) was responsible for resistance in such mutants. We utilized similar measurements to arrive at the same conclusion for *P. aeruginosa*  $\beta$ -lactamase-derepressed mutants [9]. A combination of a low outer-membrane permeation rate and slow hydrolysis by periplasmic  $\beta$ -lactamase was considered the most likely explanation for resistance to  $\beta$ -lactamasestable  $\beta$ -lactams [9, 34]. Despite the extremely low rates of hydrolysis of ceftazidime by *P. aeruginosa* type Id  $\beta$ -lactamase, we calculated that a turnover number of 0.01 (i.e.,  $10^{-5}$  times that for cephaloridine) would be sufficient to account for resistance to ceftazidime in *P. aeruginosa* mutants with derepressed  $\beta$ -lactamase [9].

#### Conclusions

The studies described indicate that outer-membrane permeability constitutes a resistance mechanism only in cooperation with at least one other factor. In the case of  $\beta$ -lactam antibiotics, the other factor that operates in synergy with low outer-membrane permeability appears to be  $\beta$ -lactamase. However, at least one class of  $\beta$ -lactam compounds is apparently capable of overcoming both of these cooperating defense mechanisms. Imipenem, a carbapenem β-lactam agent, is apparently highly resistant to hydrolysis by type Id  $\beta$ -lactamase [3]; this conclusion is based on the observation that P. aeruginosa strains derepressed for this  $\beta$ -lactamase are unaffected in their resistance to imipenem, despite demonstrated enhanced resistance to all other classes of  $\beta$ -lactam antibiotics [3]. Furthermore, it has been suggested that imipenem is taken up by a pathway that does not involve protein F [25, 26], and this proposal is consistent with our data (table 4) showing that a protein F-deficient  $\Omega$  insertion mutant does not exhibit increased resistance to imipenem. Unfortunately, since the development of imipenem-resistant mutants of P. aeruginosa during therapy has proven a clinical problem [35], this  $\beta$ -lactam does not provide a complete answer to the problems of antipseudomonal therapy. Nevertheless, the existence of such a  $\beta$ -lactam antibiotic offers hope that the twin problems of low outer-membrane permeability and inducible  $\beta$ -lactamase can be overcome.

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