Role of AmpD, OprF and penicillin-binding proteins in β -lactam resistance in clinical isolates of *Pseudomonas aeruginosa*

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In this study, the mechanisms leading to increased chromosomal AmpC β -lactamase expression and the contributory roles of the outer-membrane protein OprF and penicillin-binding proteins were analysed in 33 characterized clinical isolates of *Pseudomonas aeruginosa*. The genes *ampD* and *ampE* were analysed by PCR and DNA sequencing. Expression of the gene *oprF* was assessed using real-time RT-PCR, and penicillin-binding proteins were analysed using a chemiluminescence assay. Several of the isolates with increased *ampC* expression had major deletions affecting *ampD*, although in some isolates the mechanism of increased *ampC* expression could not be ascertained. Occasional isolates had increased expression of both *ampC* and *oprF* but remained susceptible to cephalosporins, suggesting that increased β -lactamase activity could not offset increased outer-membrane permeability. There were no discernible changes in penicillin-binding proteins. Genomic deletions in *ampD* were observed in selected clinical isolates of *P. aeruginosa* with increased expression of the AmpC β -lactamase. For some isolates, cephalosporin resistance was dependent upon the interplay of *ampC* and *oprF* expression.

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen, affecting patients in intensive care areas and those with cystic fibrosis. Both intrinsic and acquired resistance mechanisms contribute to antibiotic resistance, and multidrug resistance is increasingly common. Derepression of the chromosomal AmpC β -lactamase or, less commonly, acquisition of a new β -lactamase, are commonly observed in β -lactam-resistant isolates. AmpR is a positive transcriptional regulator belonging to the LysR family of regulatory proteins (Bagge *et al.*, 2002; Kong *et al.*, 2005). Although laboratory-derived mutants of *P. aeruginosa* involving *ampR* have confirmed the regulatory role of this protein (Kong *et al.*, 2005), mutations involving *ampR* are infrequently seen in clinical isolates overexpressing *ampC* (Campbell *et al.*, 1997; Bagge *et al.*, 2002; Juan *et al.*, 2005; Quale *et al.*, 2006). The gene *ampD* encodes a cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase that represses *ampC* expression; altering mutations in this gene have been noted in only a few clinical isolates with derepressed *ampC* activity (Langaee *et al.*, 2000; Juan *et al.*, 2005). In studies involving the strain PAO1, inactivation of *ampD* resulted in partial derepression of *ampC*, and *ampC* remained inducible with cefoxitin (Juan

et al., 2006). Two homologues of *ampD* have been described recently that are also involved in the regulation of *ampC* expression; loss of all three *ampD*-type genes led to very high-level stably derepressed expression of *ampC* (Juan *et al.*, 2006).

It is also evident that other mechanisms may be involved in β -lactam resistance in isolates of *P. aeruginosa*. The association between reduced production of the porin OprD and carbapenem resistance has been verified. However, the role of OprF in antibiotic resistance remains controversial. OprF is the most common outer-membrane protein in *P. aeruginosa* and is most closely related to OmpA of *Escherichia coli* (Woodruff & Hancock, 1989; Rawling *et al.*, 1998; Brinkman *et al.*, 2000). Although the pore-forming structure of OprF has been questioned (Gotoh *et al.*, 1989; Yoshihara & Nakae, 1989), it appears that the N-terminal half of the molecule can form channels (Nikaido *et al.*, 1991; Bellido *et al.*, 1992; Rawling *et al.*, 1998; Brinkman *et al.*, 2000). OprF-deficient isolates, obtained by either chemical or insertional mutagenesis, demonstrate poor growth in low-osmolarity media and altered morphology (Gotoh *et al.*, 1989; Woodruff & Hancock, 1989; Rawling *et al.*, 1998). In one clinical isolate, loss of OprF following exposure to a fluoroquinolone was associated with a fourfold to tenfold increase in the MICs for ciprofloxacin, carbenicillin and ceftazidime but not imipenem (Piddock *et al.*, 1992). However, diminished

Abbreviations: DFAM, 6-carboxyfluorescein; DTAM, 6-carboxytetramethylrhodamine.

OprF had a minimal effect on antibiotic resistance in laboratory-derived strains and in another clinical isolate (Woodruff & Hancock, 1988; Gotoh *et al.*, 1989; Chamberland *et al.*, 1990). Increased permeability due to the disruption of membrane integrity by the loss of OprF may explain the minimal effect that OprF deficiency has on antibiotic susceptibility (Hancock & Woodruff, 1988; Woodruff & Hancock, 1988).

Studies evaluating the role of penicillin-binding protein alterations on β -lactam resistance in *P. aeruginosa* have also been conflicting. The high-molecular-mass penicillin-binding proteins (1a, 1b, 2 and 3) carry essential functions corresponding to those found in *E. coli* (Curtis *et al.*, 1979; Noguchi *et al.*, 1979). The low-molecular-mass penicillin-binding proteins function as DD-carboxypeptidases and are not essential for cell viability (Curtis *et al.*, 1979; Noguchi *et al.*, 1979, 1985). The anti-pseudomonal cephalosporins appear to bind preferentially to PBP3 (Pucci *et al.*, 1991), whilst carbapenems bind to PBP2 (Quinn *et al.*, 1986). Two different serotypes of *P. aeruginosa* isolated from a patient with cystic fibrosis that developed resistance to piperacillin following exposure to that antibiotic had reduced affinity for most penicillin-binding proteins, and one strain appeared to have an altered PBP3 (Godfrey *et al.*, 1981). However, no changes in penicillin-binding proteins were observed in three clinical isolates that developed resistance to imipenem (Quinn *et al.*, 1986).

In this report, we examined the mechanisms contributing to β -lactam resistance in a large number of clinical isolates of *P. aeruginosa*. The roles of AmpD, OprF and penicillin-binding proteins were examined in a large number of previously characterized isolates (Quale *et al.*, 2006).

METHODS

Thirty-three clinical isolates of *P. aeruginosa* with varying degrees of β -lactam susceptibility were selected as described previously (Quale *et al.*, 2006). Susceptibility testing and ribotyping were also performed as described previously (Quale *et al.*, 2006). The isolates have been characterized for expression (measured by real-time RT-PCR) of *ampC* and *oprD*, and have had the regulatory gene *ampR* amplified and sequenced (Quale *et al.*, 2006). For all experiments, *P. aeruginosa* ATCC 27853 was used as the control isolate.

***ampD* and *ampE* studies.** To search for possible mutations affecting important genes involved in *ampC* expression, *ampD* and *ampE* were characterized. Template DNA was obtained using a DNeasy kit (Qiagen). The following primer pairs were used for amplification and sequencing: *ampD* forward, 5'-AAGCACTGCAGC-AATGTCAG-3', and *ampD* reverse, 5'-ATGCCGGGTCTTTCT-TTCT-3'; and *ampE* forward, 5'-GAAATCATGCGGACACGTT-3', and *ampE* reverse, 5'-AGGAAACATGACGTTCTG-3'. The PCR conditions were 94 °C for 15 min, followed by 32 cycles of 94 °C for 60 s, 37 °C for 60 s and 72 °C for 60 s, with a final extension at 72 °C for 10 min.

For isolates in which *ampD* could not be amplified, the following non-overlapping primers were employed: *ampD2* forward, 5'-ACGTATGTTTCCTCCTTGG-3', and *ampD2* reverse, 5'-ATTAG-AGTGGGGGCTCGTTC-3'. To evaluate further the *ampD* operon in

isolates with non-amplifiable *ampD*, primers derived from the adjacent *ampE* gene (*ampE*for 5'-GAGACTGTAGACCACCACC-AGAAGGT-3') and PA4523 (*PA4523rev* 5'-ACAGCCTGTACCAG-AAGATCGAACAG-3') were employed. Because of the possibility of a large insertion sequence (Bagge *et al.*, 2002), PCR was performed using Accutaq DNA polymerase (Sigma).

Amplified products underwent bidirectional sequencing using an automated fluorescent dye-terminator sequencing system (Applied Biosystems). Sequences were compared with those of the PAO1 strain of *P. aeruginosa* using the BLAST program from the National Center for Biotechnology Information.

***oprF* expression studies.** Expression of *oprF* was determined using real-time RT-PCR. An overnight culture was diluted 1 : 100 in Mueller-Hinton broth and grown to late exponential phase. RNA was isolated and treated with DNase using an RNeasy kit (Qiagen). A total of 2 μ l RNA adjusted to a concentration of 25 μ g ml⁻¹ was used for all RT-PCR experiments; the housekeeping gene *rpoD* was used as the normalizing gene (Quale *et al.*, 2006). Primers for *oprF* expression were *oprF* forward (5'-CTTCGACAAGTCCAAGGTCA-3') and *oprF* reverse (5'-AAGTGGACGGGTACTGCTTC-3') and the probe for detection was 5'-DFAM-CGCTGACATCAAGAACCTGGCTG-DT-AM-3'. Primer and probe concentrations were adjusted to provide amplification efficiencies of ~90–110% for all experiments. Real-time RT-PCR experiments were performed using the Brilliant QRT-PCR master mix (Stratagene). Samples were run in triplicate; virtually all individual results were within 0.5 C_t (cycle threshold) units of the averaged triplicate value. Controls run without reverse transcriptase confirmed the absence of contaminating DNA in any of the samples. Carboxy-X-rhodamine was included as a reference dye. Normalized expression of *oprF* was calibrated against corresponding mRNA expression by *P. aeruginosa* ATCC 27853; results are given as the relative expression of the mRNA compared with *P. aeruginosa* ATCC 27853.

Penicillin-binding protein analysis. Penicillin-binding proteins were assessed using a chemiluminescence assay, as described previously (Quale *et al.*, 2003). Briefly, isolates were grown to late exponential phase in brain heart infusion broth. Cells were harvested by centrifugation, sonicated and cellular debris was removed by centrifugation. Membranes were collected by ultracentrifugation (100 000 g for 40 min at 4 °C) and resuspended in 10 mM phosphate buffer (pH 7.0). Penicillin-binding proteins were bound with biotinylated ampicillin (200 μ g ml⁻¹) for 10 min, and the reaction stopped by the addition of penicillin (120 mg ml⁻¹) and 20% aqueous Sarkosyl. Following SDS-PAGE on a 12% resolving gel, the proteins were transferred to a nitrocellulose membrane using the Trans-Blot SD transfer cell (Bio-Rad). Following washes in PBS and skimmed milk, the membrane was incubated in a 1 : 5000 dilution of streptavidin-horseradish peroxidase solution. Penicillin-binding proteins were visualized using the Opti-4CN detection kit (Bio-Rad).

RESULTS AND DISCUSSION

A total of 33 clinical isolates was examined; susceptibility and ribotyping data are given in Table 1. Ten isolates were non-susceptible to ceftazidime and had increased expression of *ampC*, defined as a tenfold or greater increase over the control. One other isolate (UL517) was intermediate in its susceptibility to ceftazidime and did not have increased expression of *ampC*. A total of 3 isolates were susceptible to ceftazidime with increased expression of *ampC*, and the remaining 19 isolates were susceptible to ceftazidime without increased *ampC* expression (Table 1).

Table 1. Ribotyping, susceptibility data, AmpD, and AmpE identification, and relative expression of *ampC*, *oprD* and *oprF* in 33 clinical isolates of *P. aeruginosa*

Isolate	Ribotype*	MIC (μg ml ⁻¹)								Amino acid changes in:		Expression rela- tive to <i>P. aerugi- nosa</i> ATCC 27853		
		IPM*	MEM*	ERT*	CAZ*	FEP*	ATM*	CIP	AMK	AmpD	AmpE	<i>ampC</i> *	<i>oprD</i> *	<i>oprF</i>
ATCC 27853		2	0.5	4	1	2	2	1	2	Gln ⁴⁴ →His ⁴⁴ ; Gly ¹⁴⁸ →Ala ¹⁴⁸ ; Asp ¹⁸³ →Tyr ¹⁸³	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹	1	1	1
Isolates non-susceptible to ceftazidime with increased expression of <i>ampC</i>														
BK553	A	32	4	>16	16	>256	2	>32	4	Gly ¹⁴⁸ →Ala ¹⁴⁸ ; Asp ¹⁷³ →His ¹⁷³	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	610	0.10	3.5
HW311	A	4	8	>16	>32	>256	>256	>32	16	None; partial†	Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	970	0.70	1.6
DB322	A	32	16	>16	>32	>256	64	>32	16	Gly ¹⁴⁸ →Ala ¹⁴⁸ ; partial†	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	2400	0.17	1.3
BK545	A	>32	16	>16	>32	32	4	>32	12	Gly ¹⁴⁸ →Ala ¹⁴⁸ ; partial†	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	270	0.13	3.8
BK546	A	>32	16	>16	>32	32	8	>32	16	Gly ¹⁴⁸ →Ala ¹⁴⁸ partial†	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	480	0.04	1.8
DB537	A	>32	16	>16	>32	16	2	>32	8	Gly ¹⁴⁸ →Ala ¹⁴⁸ ; partial†	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	380	0.14	1.6
BK501	A	32	16	>16	>32	64	32	>32	16	Gly ¹⁴⁸ →Ala ¹⁴⁸ ; partial†	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	380	0.14	1.6
CK575	F	32	16	>16	>32	16	4	0.5	32	Gln ⁴⁴ →His ⁴⁴ ; Gly ¹⁴⁸ →Ala ¹⁴⁸ ; Asp ¹⁸³ →Tyr ¹⁸³	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹	170	0.56	1.9
AM602	J	4	8	>16	>32	>256	>256	>32	2	None	Ser ⁶⁹ →Pro ⁶⁹	29	0.71	5.8
AM339	E	32	32	>16	>32	>256	>256	>32	16	Asn ²⁰ →Asp ²⁰	Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	290	0.28	1.1
Isolate non-susceptible to ceftazidime without increased expression of <i>ampC</i>														
UL517	E	>32	32	>16	16	64	16	>32	8	Asn ²⁰ →Asp ²⁰	Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	2.4	0.35	0.76
Isolates susceptible to ceftazidime with increased expression of <i>ampC</i>														
FI506	H	8	2	16	4	1	2	1	4	Gly ¹⁴⁸ →Ala ¹⁴⁸ ; Asp ¹⁸³ →Tyr ¹⁸³	Ser ⁶⁹ →Pro ⁶⁹	66	0.16	8.1
AM609	I	8	0.5	>16	2	16	8	>32	64	Gly ¹⁴⁸ →Ala ¹⁴⁸	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	2646	NA	6.4
DB513	N	2	2	>16	8	64	32	2	16	NA	NA	1500	0.46	1.9
Isolates susceptible to ceftazidime without increased expression of <i>ampC</i>														
DB504	A	2	0.25	2	4	2	2	0.25	8	Ala ¹³⁶ →Val ¹³⁶ ; Gly ¹⁴⁸ →Ala ¹⁴⁸	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹	7.5	0.94	1.1
CK140	B	2	4	>16	8	16	32	1	16	Gly ¹⁴⁸ →Ala ¹⁴⁸ ; Asp ¹⁸³ →Tyr ¹⁸³	Ser ⁶⁹ →Pro ⁶⁹	5.5	11	1.4
CK188	B	2	4	>16	8	16	16	>32	16	Gly ¹⁴⁸ →Ala ¹⁴⁸ ; Asp ¹⁸³ →Tyr ¹⁸³	Ser ⁶⁹ →Pro ⁶⁹	0.31	4.1	2
MD104	B	2	2	>16	8	12	16	>32	16	Ala ¹³⁶ →Val ¹³⁶ ; Gly ¹⁴⁸ →Ala ¹⁴⁸	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹	0.41	10	0.37
MD510	C	2	1	16	1	2	2	>32	8	None	Ser ⁶⁹ →Pro ⁶⁹	2.62	0.81	0.64
UL118	C	8	16	>16	2	8	4	>32	4	None	Ser ⁶⁹ →Pro ⁶⁹	1.3	0.02	1.3

Table 1. cont.

Isolate	Ribotype*	MIC ($\mu\text{g ml}^{-1}$)								Amino acid changes in:		Expression relative to <i>P. aeruginosa</i> ATCC 27853		
		IPM*	MEM*	ERT*	CAZ*	FEP*	ATM*	CIP	AMK	AmpD	AmpE	ampC*	oprD*	oprF
CK505	C	2	0.5	8	4	2	4	0.25	4	None	Ser ⁶⁹ →Pro ⁶⁹	0.70	0.19	0.53
CK558	D	16	2	>16	4	16	4	8	16	None	Ser ⁶⁹ →Pro ⁶⁹ ; Leu ²¹⁵ →Phe ²¹⁵	2.0	0.38	0.55
CK550	D	8	2	>16	4	16	4	4	16	None	Ser ⁶⁹ →Pro ⁶⁹ ; Leu ²¹⁵ →Phe ²¹⁵	6.2	0.27	4.0
DB311	D	2	2	>16	2	4	4	0.5	4	None	Ser ⁶⁹ →Pro ⁶⁹ ; Leu ²¹⁵ →Phe ²¹⁵	1.4	1.8	3.2
BK555	G	32	4	>16	2	8	4	>32	8	Gly ¹⁴⁸ →Ala ¹⁴⁸	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	2.4	NA	2.1
AM128	K	2	4	16	2	4	8	0.25	8	Gln ⁴⁴ →His ⁴⁴ ; Gly ¹⁴⁸ →Ala ¹⁴⁸	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Leu ²¹⁵ →Phe ²¹⁵	3.6	NA	1.4
UL140	L	2	4	>16	4	16	8	>32	8	None	Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	5.1	2.4	0.89
MD119	M	2	4	>16	4	8	8	0.25	4	None	None	2.7	1.2	0.23
MD519	O	2	≤0.12	1	2	8	2	0.5	16	Gly ¹⁴⁸ →Ala ¹⁴⁸	Ser ⁶⁹ →Pro ⁶⁹	2.1	NA	1.1
MD504	P	2	0.25	2	2	2	4	0.25	8	Gly ¹⁴⁸ →Ala ¹⁴⁸	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹	1.8	NA	0.89
MD521	Q	2	0.25	2	2	2	4	0.25	8	Gly ¹⁴⁸ →Ala ¹⁴⁸	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹ ; Leu ¹⁹⁰ →Phe ¹⁹⁰	3.8	NA	2.2
CK516	R	2	≤0.12	2	2	1	2	0.12	4	Gly ¹⁴⁸ →Ala ¹⁴⁸	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹ ; Glu ¹²⁹ →Asp ¹²⁹ ; Leu ¹⁹⁰ →Phe ¹⁹⁰	3.5	NA	1.4
CK503	S	1	≤0.12	2	2	2	4	0.25	4	Ala ¹³⁶ →Val ¹³⁶ ; Gly ¹⁴⁸ →Ala ¹⁴⁸	Ser ³³ →Gly ³³ ; Ser ⁶⁹ →Pro ⁶⁹	4.0	1.0	0.19

AMK, Amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; ERT, ertapenem; FEP, cefepime; IPM, imipenem; MEM, meropenem; NA, not amplifiable.

*Data from Quale *et al.* (2006).

†Only the terminal 240 nt of the gene could be amplified.

ampD and ampE studies

Most of the 33 clinical isolates had one or two amino acid substitutions involving *ampD* and *ampE*; these did not seem to affect expression of *ampC*, as they were found in isolates with and without increased expression of *ampC* and were found in the control strain. Of the ten ceftazidime-resistant isolates with increased *ampC* expression, six (all from the same ribotype) had a deletion affecting the initial segment of *ampD* (Table 1). Attempts at amplification of the *ampD* operon using primers derived from the adjacent genes were also unsuccessful, suggesting genetic rearrangement involving this region. It is noteworthy

that these six isolates did not have an inducible AmpC, consistent with constitutively derepressed mutants (Quale *et al.*, 2006). A seventh isolate (BK553) in this group possessed a unique mutation causing an Asp¹⁷³→His¹⁷³ change in AmpD; the significance of this mutation is unknown. One unique isolate (DB513) that was susceptible to ceftazidime, despite markedly increased expression of *ampC*, had completely non-amplifiable *ampD* and *ampE* genes. Overall, of the 13 isolates with increased expression of *ampC*, nearly half did not have changes in AmpD to explain the increased β -lactamase activity, results similar to those in another report involving clinical isolates (Juan *et al.*, 2005).

oprF expression studies

Most isolates had expression of *oprF* that approximated that of the control strain, *P. aeruginosa* ATCC 27853. The single isolate (UL517) that was non-susceptible to ceftazidime but without increased *ampC* expression had the lowest expression of *oprF* among the ceftazidime-resistant isolates (although it was comparable to many of the isolates in the ceftazidime-susceptible group). This isolate was intermediately susceptible to ceftazidime and aztreonam and resistant to cefepime, and did not over-express efflux systems (Quale *et al.*, 2006). Of the three isolates susceptible to ceftazidime with overexpression of *ampC*, two (AM609 and FI506) had markedly increased expression of *oprF* (6.4 and 8.1 times the control, respectively), suggesting that even increased β -lactamase activity could not overcome the increased permeability. The third isolate (DB513) had a markedly increased *ampC* expression but only marginally increased *oprF* expression; this isolate was resistant to cefepime and aztreonam, and the MIC for ceftazidime was at the breakpoint for susceptibility. It appears that, for selected isolates, the balance between *ampC* and *oprF* expression can affect the susceptibility to cephalosporins, although this interplay does not fully explain all of the susceptibility results we obtained.

For the 26 isolates in which there was both *oprD* and *oprF* expression data, there was a marked decrease in *oprD* in the isolates non-susceptible to imipenem, as expected (0.21 ± 0.15 versus 2.7 ± 3.6 , $P=0.03$). These isolates also tended to have greater expression of *oprF*, although this did not reach statistical significance (2.6 ± 2.1 versus 1.5 ± 1.5 , $P=0.15$). There was no relationship with *oprF* expression and meropenem or ertapenem susceptibility.

Penicillin-binding protein analysis

The penicillin-binding proteins of 17 clinical isolates and *P. aeruginosa* 27853 were characterized (Fig. 1). All of the isolates appeared to have similar patterns regarding the

essential high-molecular-mass proteins. Due to the close molecular masses of PBP4 and PBP5, there was poor resolution of these two non-essential proteins. Three isolates (including the control strain) had evidence of PBP6; the presence or absence of this protein did not appear to correlate with β -lactam susceptibility.

Resistance to cephalosporins in isolates of *P. aeruginosa* revolves around β -lactamase expression. Our findings suggest that expression of *ampC* generally correlates with cephalosporin resistance, although exceptions did occur. In some isolates, generally belonging to the same ribotype, major changes in *ampD* were found that could explain the increased expression of *ampC*, although none had the previously reported insertion sequence or point mutation (Bagge *et al.*, 2002). Many of our isolates did not have a readily identifiable reason for increased *ampC* expression, which has been noted in other clinical isolates (Juan *et al.*, 2005).

Our findings also suggested that, for some isolates, there is a relationship between *ampC* and *oprF* expression and cephalosporin susceptibility. Whilst most of our isolates had *oprF* expression comparable to the control strain, occasional isolates had markedly increased expression of *oprF*. For these isolates, increased expression of *ampC* did not result in cephalosporin resistance, suggesting that increased membrane permeability might offset the increase in β -lactamase activity. A modest inverse relationship between *oprF* and *oprD* was also noted in our clinical isolates. In one report, disruption of the regulatory gene *sigX* resulted in diminished *oprF* expression and increased production of a 47 kDa outer-membrane protein (Brinkman *et al.*, 1999); this may explain the slight decrease in the MIC of imipenem seen in one laboratory-derived *OprF*-deficient isolate (Woodruff & Hancock, 1988).

In conclusion, our results suggest the following: (i) rearrangements in *ampD* are observed in some isolates overexpressing *ampC*, although these isolates were generally clonally related, and for several unrelated isolates

Fig. 1. Penicillin-binding protein patterns in 17 clinical isolates and in *P. aeruginosa* ATCC 27853 (lane 10).

there was no apparent explanation for increased *ampC* expression; (ii) overexpression of *oprF* may offset increased *ampC* expression and isolates may retain susceptibility to cephalosporins; and (iii) penicillin-binding protein alterations did not appear to contribute to cephalosporin or carbapenem resistance.

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