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Role of *Pseudomonas aeruginosa* AmpR on β-lactam and non-β-lactam transient cross-resistance upon pre-exposure to subinhibitory concentrations of antibiotics

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Pseudomonas aeruginosa is one of the most dreaded opportunistic pathogens accounting for 10 % of hospital-acquired infections, with a 50 % mortality rate in chronically ill patients. The increased prevalence of drug-resistant isolates is a major cause of concern. Resistance in *P. aeruginosa* is mediated by various mechanisms, some of which are shared among different classes of antibiotics and which raise the possibility of cross-resistance. The goal of this study was to explore the effect of subinhibitory concentrations (SICs) of clinically relevant antibiotics and the role of a global antibiotic resistance and virulence regulator, AmpR, in developing cross-resistance. We investigated the induction of transient cross-resistance in *P. aeruginosa* PAO1 upon exposure to SICs of antibiotics. Pre-exposure to carbapenems, specifically imipenem, even at 3 ng ml^{-1}, adversely affected the efficacy of clinically used penicillins and cephalosporins. The high β-lactam resistance was due to elevated expression of both *ampC* and *ampR*, encoding a chromosomal β-lactamase and its regulator, respectively. Differences in the susceptibility of *ampR* and *ampC* mutants suggested non-AmpC-mediated regulation of β-lactam resistance by AmpR. The increased susceptibility of *P. aeruginosa* in the absence of *ampR* to various antibiotics upon SIC exposure suggests that AmpR plays a major role in the cross-resistance. AmpR was shown previously to be involved in resistance to quinolones by regulating MexEF–OprN efflux pump. The data here further indicate the role of AmpR in cross-resistance between quinolones and aminoglycosides. This was confirmed using quantitative PCR, where expression of the *mexEF* efflux pump was further induced by ciprofloxacin and tobramycin, its substrate and a non-substrate, respectively, in the absence of *ampR*. The data presented here highlight the intricate cross-regulation of antibiotic resistance pathways at SICs of antibiotics and the need for careful assessment of the order of antibiotic regimens as this may have dire consequences. Targeting a global regulator such as AmpR that connects diverse pathways is a feasible therapeutic approach to combat *P. aeruginosa* pathogenesis.

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INTRODUCTION

Treatment of *Pseudomonas aeruginosa* infections poses a clinical challenge due to the extensive spread of multidrug-resistant isolates (Barbier & Wolff, 2010; Curcio, 2013). The alarming development of resistance to almost all clinically relevant antibiotics has led to *P. aeruginosa* being classified as one of the ESKAPE pathogens, which account for 40 % of all nosocomial infections (Rice, 2010; Pendleton *et al.*, 2013). *P. aeruginosa* has adapted to prevail and infect widely, in part due to its high degree of resistance to antibiotics, and

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Abbreviations: AMK, amikacin; AMX, amoxicillin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CLSI, Clinical and Laboratory Standards Institute; DOR, doripenem; ETP, ertapenem; FEP, cefepime; FIC, fractional inhibitory concentration; FICi, FIC index; IPM, imipenem; LVX, levofloxacin; MEM, meropenem; OFX, ofloxacin; PIP, piperacillin; RQ, relative quantity; SAM, ampicillin/sulbactam; SIC, subinhibitory concentration; TIC, ticarcillin; TIM, ticarcillin/clavulanic acid; TOB, tobramycin; TZP, piperacillin/tazobactam.

its expression of a large arsenal of virulence factors posing a serious threat in the clinical setting (Kerr & Snelling, 2009).

The major mechanisms contributing to antibiotic resistance in *P. aeruginosa* include an impermeable outer membrane, expression of efflux pumps, target alteration and production of drug-inactivating enzymes (Livermore, 2002; Zavascki *et al.*, 2010; Alvarez-Ortega *et al.*, 2011). The highly impermeable membrane provides *P. aeruginosa* with natural resistance to many antimicrobials (Angus *et al.*, 1982; Nikaido, 2003). To deal with the antimicrobials that cross the cell-wall barrier, the *P. aeruginosa* PAO1 genome encodes 10 resistance–nodulation–division systems that include MexAB–OprM, MexCD–OprJ, MexEF–OprN and MexXY–OprM contributing to antibiotic resistance (Stover *et al.*, 2000; Lister *et al.*, 2009). Upregulation of the MexAB–OprM efflux pump confers resistance to many different classes of antibiotics including fluoroquinolones, β-lactams, sulfonamides, chloramphenicol and trimethoprim (Li *et al.*, 1995). The MexCD–OprJ pump predominantly expels quinolones, macrolides, tetracycline, chloramphenicol and fourth-generation cephalosporins such as cefepime (FEP) and cefpirome (Masuda *et al.*, 1996; Poole *et al.*, 1996a). The MexEF–OprN pump is involved in efflux of fluoroquinolones, chloramphenicol and trimethoprim (Köhler *et al.*, 1997). The MexXY–OprM pump is involved in the efflux of aminoglycosides, quinolones, tetracyclines and some β-lactams (Morita *et al.*, 2012). Importantly, it is the only pump involved in the efflux of aminoglycosides. It is evident that the efflux pumps share substrates and may involve a coordinated expression to deal with the onslaught of antibiotics.

Another major determining factor contributing to antibiotic resistance in *P. aeruginosa* is the overexpression of antibiotic-hydrolysing enzymes such as β-lactamases that are either chromosomally encoded or acquired (Hennessey, 1967; Lindberg & Normark, 1986; Jacoby, 2009; Castillo-Vera *et al.*, 2012; Yong *et al.*, 2012). The β-lactamases degrade β-lactam antibiotics (Normark *et al.*, 1986; Hanson & Sanders, 1999; Gupta, 2008; Zhao & Hu, 2010; Bonnin *et al.*, 2013). The major chromosomally encoded β-lactamase in *P. aeruginosa* is AmpC, whose expression is positively regulated by a LysR-type transcriptional regulator, AmpR (Lodge *et al.*, 1990, 1993). In addition to AmpC, *P. aeruginosa* expresses a second chromosomal β-lactamase, PoxB (Girlich *et al.*, 2004; Kong *et al.*, 2005a), and can also acquire extended-spectrum metallo-β-lactamases on mobile genetic elements and plasmids (Bradford, 2001; Poirel *et al.*, 2012; Zhu *et al.*, 2013). In recent years, it has become apparent that *P. aeruginosa* co-regulates antibiotic resistance and virulence (Gooderham & Hancock, 2009; Yeung *et al.*, 2011; Balasubramanian *et al.*, 2013b). Our studies show that AmpR plays a major role in this co-regulation (Kong *et al.*, 2005b; Balasubramanian *et al.*, 2011, 2012). In addition to β-lactam resistance, AmpR regulates fluoroquinolone resistance, expression of many different virulence factors, QS-regulated phenotypes and biofilm formation (Balasubramanian *et al.*, 2011, 2012, 2013a).

The current treatment regimen for *P. aeruginosa* infections involves fluoroquinolones, aminoglycosides and β-lactams alone or in combination (Cystic Fibrosis Foundation, 2011; Vardakas *et al.*, 2013). The major β-lactams used extensively in clinical settings include carbapenems [imipenem (IPM) or meropenem (MEM)], third-generation cephalosporins [ceftazidime (CAZ)], Gram-negative-specific drugs [monobactam aztreonam (ATM)] and penicillin derivatives [piperacillin (PIP), either alone or in combination with tazobactam (TZP)] (Giamarellou & Kanellakopoulou, 2008; Page & Heim, 2009). The use of combination therapy for treatment of *P. aeruginosa* infections has been controversial. Many studies have dismissed the efficacy and advantages of combination therapy over monotherapy (Boyd & Nailor, 2011; Johnson *et al.*, 2011; Tamma *et al.*, 2012). In fact, adverse effects of combination therapy have been reported frequently (Paul *et al.*, 2004, 2006). In addition, *P. aeruginosa* isolates from patients undergoing combination therapy have a higher rate of resistance, and, with simultaneous exposure, this allows the co-evolution of resistance to multiple classes of antibiotics (McGowan, 2006). However, compelling data on the interplay between antibiotics in the development of resistance is missing. We hypothesize that use of the same mechanisms involving efflux pumps or regulators such as AmpR may contribute to the development of cross-resistance to multiple classes of antibiotics.

Pathogens have adapted to respond to a gradient of antibiotic concentration in the environment as well as within the host during therapy (Baquero & Negri, 1997; Baquero *et al.*, 2008). Whilst much research is focused on studying bacterial resistance to high antibiotic doses, the response to subinhibitory concentrations (SICs) remains largely unexplored. During the last decade, whole-genome studies have enabled identification of the effects of SICs of antibiotics on cellular mechanisms other than their direct targets (Davies *et al.*, 2006). In this study, we explored the role of pre-exposure to SICs of clinically relevant antibiotics on the induction of transient cross-resistance in *P. aeruginosa*. Our studies also provide insights into the role of AmpR in regulating antibiotic cross-resistance in response to SICs of antibiotics.

METHODS

Bacterial strains, growth conditions and antibiotics. The prototypic *P. aeruginosa* strain PAO1 and its isogenic *ampR* deletion mutant, PAOΔ*ampR*, used in this study have been described previously (Stover *et al.*, 2000; Balasubramanian *et al.*, 2012). Luria–Bertani broth (Fisher Scientific) was used for routine cultivation of strains and was supplemented with 1.5% agar when needed. Cation-adjusted Mueller–Hinton (CAMH) broth (Difco) was used for MIC studies and chequerboard assays. All antibiotics were obtained from Sigma-Aldrich. The antibiotics used in the study were: penicillins (amoxicillin, AMX; ampicillin/sulbactam, SAM; PIP; TZP; ticarcillin, TIC; ticarcillin/clavulanic acid, TIM), cephalosporins (CAZ; FEP; cefotaxime, CTX), carbapenems (IPM; MEM; doripenem, DOR; ertapenem, ETP), monobactam (ATM), quinolones (ciprofloxacin, CIP; ofloxacin, OFX;

levofloxacin, LVX) and aminoglycosides (tobramycin, TOB; amikacin, AMK).

Construction of PAOΔ*ampC*. An in-frame deletion of *ampC* was constructed using overlapextension PCR and homologous recombination as described previously (Balasubramanian *et al.*, 2012). Briefly, sequences upstream (855 bp) and downstream (875 bp) of the target deletion were amplified using primer pairs DZampCUF1 (5′-GGAATTCAAGACGATGCTCCGGGTCAGTG-3′) and DZampCUR1 (5′-GATACCAGATTCCCCTGCCTGTCTAGCTAGCTAGAATGCTC-3′), and DZampCDF2 (5′-CTAGCTAGCTAGAATGCTCAAGCG-CGCTCGCGAGGGCGACGGA-3′) and DZampCDR2 (5′-CGGGAT-CCGACCCTGCATACCATCAAGG-3′), respectively. The two amplicons were then ligated through PCR and cloned into the suicide vector pEXG2 (Rietsch *et al.*, 2005). The resultant plasmid was moved into PAO1 for homologous recombination with the genomic DNA. Clones were screened for gentamicin sensitivity (75 μg ml^{-1}) and sucrose resistance (8 % sucrose) corresponding to a double cross-over recombination event and replacement of the target gene with the deletion product. The presence of the deletion in PAOΔ*ampC* (PKM201) was confirmed by PCR amplification and sequencing of the deletion product (data not shown).

MICs. The MICs of clinically relevant antibiotics were determined for *P. aeruginosa* strains using E-strips following the manufacturer's protocol (bioMérieux). E-test MICs are widely regarded as being reliable and the results are identical to the broth microdilution method (Arendrup *et al.*, 2001; Pankuch *et al.*, 2006; Amsler *et al.*, 2010) recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). To determine whether exposure to SICs of the antibiotics altered the *P. aeruginosa* susceptibility profile, exponential-phase cells (OD_{600} of 0.6) were divided into two pools. One pool was exposed to SICs ($\leqslant$0.25 MIC) of antibiotic (one antibiotic at a time) for 1 h at 37 °C and plated on CAMH plates containing the same antibiotic. The second pool was not pre-exposed to antibiotic and was plated on plain CAMH plates. E-test assays were then performed with a panel of antibiotics to compare unexposed and pre-exposed MICs. The SICs of the various antibiotics used were: 0.1 μg ml^{-1} (IPM, CAZ and TOB), 0.05 μg ml^{-1} (CIP and MEM) and 0.2 μg ml^{-1} (PIP). All the assays were performed at least in duplicate. In general, $\geqslant$2.5-fold difference in the MIC profile was considered significant.

Chequerboard assays. Interactions between antibiotics were determined using chequerboard assays in 96-well plates (Sopirala *et al.*, 2010). Two-dimensional chequerboard assays were performed with twofold serial dilutions of each antibiotic, along the rows (for antibiotic 1) and columns (for antibiotic 2) in a 96-well plate (BD Labware). The bacterial cells were diluted according to CLSI recommendations (CLSI, 2006) and 100 μl dilution (5×10^5 c.f.u. ml^{-1}) was added to each well containing antibiotics, for a total reaction volume of 200 μl per well. Results were observed after 16–18 h static incubation at 37 °C. The first clear well containing both antibiotics was used to calculate the fractional inhibitory concentration (FIC) as follows: FIC of antibiotic A (FICA)=MIC of antibiotic A in combination/MIC of antibiotic A alone; FIC of antibiotic B (FICB)=MIC of antibiotic B in combination/MIC of antibiotic B alone; FIC index (FICi)=FICA+FICB. FICi data were interpreted as follows: <0.5, synergy; >0.5–4.0, indifference; >4.0, antagonism (Odds, 2003). Chequerboard assays were performed with the following antibiotics: IPM (0.003–0.2 μg ml^{-1} and 0.05–3.2 μg ml^{-1}) with CAZ (0.8–51.2 μg ml^{-1}), PIP (12.5–800 μg ml^{-1}), TIC (12.5–800 μg ml^{-1}) and ATM (0.5–32 μg ml^{-1}). The chequerboard assays were performed in triplicate.

RNA isolation, cDNA synthesis and quantitative real-time PCR (qPCR). Total RNA was isolated from PAO1 and PAOΔ*ampR* grown to mid-exponential phase with or without pre-exposure to SICs of antibiotics (as described above). RNA isolation, cDNA synthesis and qPCR assays were performed as described previously (Balasubramanian *et al.*, 2012). The gene-specific primers used for qPCR were: *ampC*: forward, 5′-CGCCGTACAACCGGTGAT-3′; reverse, 5′-CGGCCG-TCCTCTTTCGA-3′; *ampR*: forward, 5′-CATTGGCCTTCATCAC-CGGTTGTA-3′; reverse, 5′-GGTTTCTCATGCAGCCAACGACAA-3′; *poxB*: forward, 5′-AATCGGCCAGGTTGTGGATAA-3′; reverse, 5′-GGAGCAGAAAGCGGGTCTGT-3′; and *mexE*: forward, 5′-AAGTCATCGAACAACCGCTGAACG-3′; reverse, 5′-TTCTTCAC-CAGTGCGCCTTCAT-3′. Ten nanograms of cDNA was used per reaction well in the qPCR assays. As an internal control, the *clpX* gene (*PA1802*) was included to ensure equal amounts of RNA were used in all samples. qPCR assays for each gene were performed at least in biological duplicates, each with technical triplicates. Melting curves were determined to ensure primer specificity. Gene expression values were normalized to the non-antibiotic-treated PAO1 values and are represented as means ± SE.

Statistical analysis. All data were analysed for statistical significance using Student's *t*-test on GraphPad statistical analysis software.

RESULTS AND DISCUSSION

Effect of exposure to SICs of antibiotics on the *P. aeruginosa* PAO1 susceptibility profile

In this study, the effects of pre-exposure to SICs of various clinically relevant antibiotics on the *P. aeruginosa* PAO1 susceptibility profile were determined (Table 1). After pre-exposure, the cells were plated on CAMH plates with and without SICs of antibiotics. The MIC profile was determined by E-test (Table 1). The effect on *P. aeruginosa* PAO1 susceptibility was dependent on the class of antibiotic used for the SIC exposure (Table 1).

Pre-exposure to a SIC of the β-lactam PIP (0.2 μg ml^{-1}) and the aminoglycoside TOB (0.1 μg ml^{-1}) resulted in a significant increase in resistance only to SAM (2.7-fold) and not to the other antibiotics tested (Table 1). In contrast, when PAO1 was pre-exposed to a SIC of the quinolone CIP (0.05 μg ml^{-1}), there was increased susceptibility to almost all the classes of antibiotics tested (SAM, 3-fold; TZP, 2.7-fold; TIM, 3-fold; ETP, 5.3-fold; ATM, 3-fold). SIC exposure to the cephalosporin CAZ (0.1 μg ml^{-1}) marginally enhanced PAO1 susceptibility to all classes of antibiotics except quinolones (Table 1). Although the difference in MICs was only between 1.5- and 2.0-fold, which is typically not considered significant, the trend was consistent over the different classes of antibiotics (Table 1). Such marginal differences are suggestive of creeping baseline MICs, contributing to breakthrough resistance in the clinical setting (Fernández *et al.*, 2011).

Exposure to a SIC of IPM (0.1 μg ml^{-1}) significantly enhanced PAO1 cross-resistance to penicillins (TZP, 32-fold; TIM, 10.7-fold), cephalosporins (CAZ, 5.3-fold; FEP, 3-fold) and monobactams (ATM, 2.7-fold) but had little or no effect on other carbapenems (DOR, MEM and ETP), quinolones and aminoglycosides (Table 1). The gain in resistance to the penicillins was significant enough to cross their MIC breakpoints and classify the strain as clinically

Table 1. Resistance profile of *P. aeruginosa* PAO1 after SIC antibiotic exposure

Results are shown as MIC values (μg ml^{-1}).

Class	Antibiotic	None*	SIC antibiotic exposure				
			IPM (0.1 μg ml^{-1})	CAZ (0.1 μg ml^{-1})	PIP (0.2 μg ml^{-1})	CIP (0.05 μg ml^{-1})	TOB (0.1 μg ml^{-1})
Penicillin	AMX	>256	>256	>256	>256	>256	>256
	SAM	96	96	64	256	32	256
	TZP	4	128	3/2	4	1.5	4
	TIM	24	256	16	24	8	16
Cephalosporin	CAZ	1.5	8	1	2	0.75	1
	FEP	2.0	6	1.5	2	1	3
Carbapenem	IPM	1.5	3	1.5	1.5	0.75	1.5
	DOR	0.38	0.5	0.19	0.25	0.19	0.5
	MEM	0.38	0.25	0.25	0.5	0.75	0.5
	ETP	>32	ND	ND	>32	6	>32
Monobactam	ATM	3	6/8	1.5	3	1	3
Quinolone	OFX	1	1.5	1	1.5	0.5	1.5
	CIP	0.25	0.25	0.25	0.25	0.125	0.38
	LVX	0.5	0.5	0.5	0.5	0.25	0.75
Aminoglycoside	AMK	4	6	4	4	4	4
	TOB	1	0.75	0.75	0.75	0.75	0.75

ND, Not determined.
*MIC of PAO1 with no antibiotic exposure.

resistant, in accordance with CLSI standards (TZP and TIM: ⩾128 μg ml^{-1}; CLSI, 2006). This is in agreement with previous findings where exposure to a SIC of IPM led to clinical resistance in *P. aeruginosa* (Livermore, 1987). To determine whether the trend was true of other carbapenems, PAO1 was exposed to a SIC of MEM (0.05 μg ml^{-1}) before determining the MIC (Table 2). Compared with unexposed PAO1, SIC MEM-exposed cells displayed a marginal increase in resistance towards TZM (3-fold) and CAZ (2-fold; Table 2) but showed no effect on the rest of the antibiotics (data not shown). Thus, it seems that, although carbapenems in general have the potential to induce resistance to other antibiotics, the extent varies and IPM is a much more effective inducer of transient cross-resistance compared to MEM (Table 2).

The susceptibility profile of the pre-exposed and unexposed cells was similar on plates containing no antibiotic (data not shown). Also, the difference seen in the profile of the pre-exposed cells on plates contacting antibiotics (Table 1) was lost when subcultured on plates without any antibiotics (data not shown). These results showed that the changes conferred are transient and that the altered susceptibility is observed only in the presence of SICs of antibiotics. This implies the existence of a dynamic interplay between different classes of antibiotics at SICs in *P. aeruginosa*. The findings also necessitate a careful assessment of both combinatorial therapy and the antibiotic treatment history of a patient, because trailing concentrations of one antibiotic such as IPM can provide resistance to other subsequently used antibiotics. The role of carbapenems in the transient cross-resistance was explored further.

A concentration as low as 3 ng IPM ml^{-1} can induce β-lactam cross-resistance

The data from cells exposed to a SIC of IPM suggested a widespread antagonistic effect with other antibiotics (Table 1). In order to determine the extent of combinatorial cross-resistance, chequerboard assays were performed for IPM (0.05–3.2 μg ml^{-1}) with PIP, TIC, CAZ and ATM as described in Methods. The FIC for each antibiotic and the FICi for each combination were calculated (Fig. 1). Any two drugs were considered synergistic, indifferent (or non-synergistic) or antagonistic if the FICi scores were <0.5, >0.5 but <4.0 or>4.0, respectively (Odds, 2003).

The FICi for the IPM/PIP combination was 8.06, suggesting that the antibiotic pair was antagonistic (Fig. 1). When IPM was combined with TIC, CAZ or ATM, the FICi was 2.06 in each case, suggesting indifference. However, a closer assessment of the FIC for individual antibiotics in the combinations tested revealed that the FICi was heavily skewed in one direction. For example, the FIC of PIP in the presence of IPM was 8, whereas when reversed, the score was 0.06, suggesting an antagonistic and synergistic effect, respectively (Fig. 1). Similarly, the FIC of TIC, CAZ and ATM with IPM (FIC=2.0) suggested a non-synergistic interaction, whereas the FIC

Table 2. MICs of penicillins and cephalosporins in response to SIC carbapenem exposure

Results are shown as MIC values (μg ml^{-1}).

Class	Antibiotic	None*	SIC antibiotic exposure	
			IPM (0.1 μg ml^{-1})†	MEM (0.05 μg ml^{-1})
Penicillin	TZP	4	128	12
	TIM	24	256	32
Cephalosporin	CAZ	1.5	8	3
	FEP	2	6	2

*MIC of PAO1 with no antibiotic exposure.
†IPM-exposed PAO1 MIC values are from Table 1 for comparison.

of IPM with these three antibiotics was 0.06, indicating synergy (Fig. 1). Thus, the chequerboard data suggested that IPM became more potent at killing cells at lower concentrations in the presence of other antibiotics like PIP, TIC, CAZ and ATM. More importantly, the other antibiotics became less effective (high MIC) in the presence of low doses of IPM (Fig. 1). These results highlight the significance of the order of antibiotic treatment. A better treatment output can be expected if PIP, CAZ or TIC therapy is followed by IPM, whereas the reverse order could lead to high resistance to PIP, CAZ or TIC at diminishing IPM concentrations.

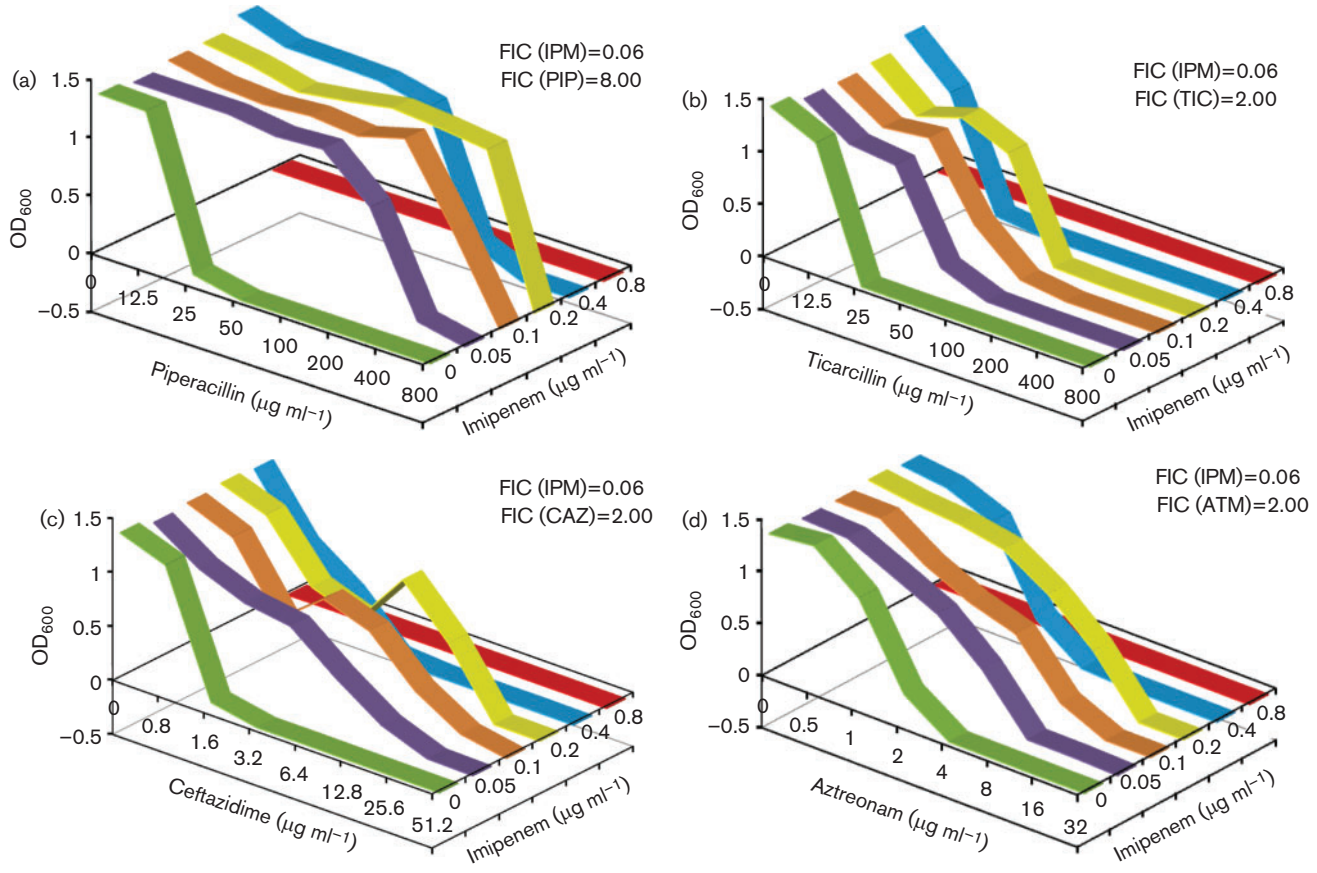

Fig. 1. IPM chequerboard assays. The effects of combinatorial cross-resistance of IPM with PIP (a), TIC (b), CAZ (c) and ATM (d) on *P. aeruginosa* PAO1 was determined. The FIC and FICi values were calculated as described in Methods. The antibiotics were used at the following concentrations: IPM, 0.05–3.2 μg ml^{-1}; PIP, 12.5–800 μg ml^{-1}: TIC, 12.5–800 μg ml^{-1}; CAZ, 0.8–51.2 μg ml^{-1}; ATM, 0.5–32 μg ml^{-1}. All assays were performed in triplicate.

The susceptibility to various antibiotics in the presence of a SIC of IPM was concentration dependent (Fig. 1), the effect being more pronounced at 0.1 μg ml^{-1} and starting to drop at 0.4 μg ml^{-1} as it approached the MIC for IPM. To further explore the lowest concentration limit, another chequerboard assay was carried out with 0.003–0.2 μg IPM ml^{-1}, whilst the other β-lactams were kept at the same concentrations as before. The results clearly demonstrated that, even at concentrations as low as 3–6 ng ml^{-1}, IPM was able to alter the resistance to PIP, CAZ, TIC and ATM (Fig. 2).

IPM is often used to treat multidrug-resistant *P. aeruginosa* infections (Page & Heim, 2009). SICs of IPM are known to confer clinical resistance (Livermore, 1987). However, this is the first study to reveal that a concentration as low as 3 ng IPM ml^{-1} can enhance resistance to clinically used penicillins and cephalosporins (Table 1, Fig. 2). The biphasic response that shows low-dose stimulation and high-dose inhibition is termed hormesis (Stebbing, 1982; Davies *et al.*, 2006). An earlier study also linked the use of IPM with the risk of emergence of antibiotic resistance (Carmeli *et al.*, 1999). A SIC of IPM has also been shown to induce genes coding for alginate synthesis and biofilm formation, hallmarks of chronic infection (Bagge *et al.*, 2004). Together, these findings indicate that the use of IPM as an anti-pseudomonal drug comes with a high cost in terms of increased resistance to other clinically used antibiotics, and favours chronic infection at its lingering low concentrations.

Induction of *ampC* and *ampR* expression by SICs of clinically used antibiotics

In *P. aeruginosa*, AmpC β-lactamase is the primary mediator of β-lactam resistance, whose expression is modulated by the AmpR in response to the inducers (Lodge *et al.*, 1990, 1993). In order to determine the most proficient inducer of *ampC*, mRNA levels were quantified using qPCR after exposure of PAO1 to SICs of various antibiotics (Fig. 3). As expected, the most potent inducer of *ampC* expression was IPM [relative quantity (RQ): 221 ± 16, $P=0.003$], corroborating previous findings (Livermore & Yang, 1987) and further supporting the enhanced resistance seen in the MIC and chequerboard assays (Table 1, Fig. 1). A similar induction of *ampC* expression in response to a SIC of IPM was also seen in *P. aeruginosa* biofilm (Bagge *et al.*, 2004). The qPCR assays also identified MEM as another moderately strong inducer of *ampC* (RQ: 58 ± 3, $P=0.001$). However, the poor induction of *ampC* with SICs of other β-lactams (CAZ and PIP) was similar to the non-β-lactams (CIP and TOB; Fig. 3).

To determine whether the induction of *ampC* is a function of increased expression of *ampR*, its transcript levels were also quantified. Pre-exposure to SICs of both IPM (RQ: 77 ± 2, $P<0.0001$) and MEM (RQ: 49 ± 1, $P<0.0001$) were found to induce the expression of *ampR*, although expression was lower compared with *ampC* expression (Fig. 3). Induction of *ampR* expression in *P. aeruginosa* PAO1 at SIC exposure to antibiotics has not been shown before. Although *ampR* expression is induced by a β-lactam in the mucoid *P. aeruginosa* variant PDO300, this is due to

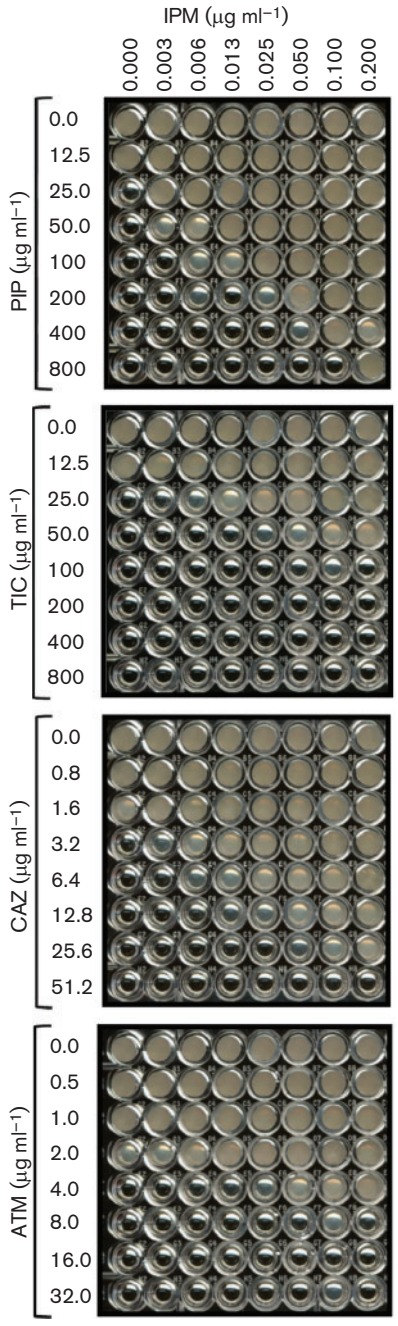

Fig. 2. Low-range IPM chequerboard assays. The effects of co-exposure of very low concentrations of IPM with PIP (a), TIC (b), CAZ (c) and ATM (d) on *P. aeruginosa* PAO1 was determined. The antibiotics were used at the following concentrations: IPM, 0.003–0.2 μg ml^{-1}; PIP, 12.5–800 μg ml^{-1}; TIC, 12.5–800 μg ml^{-1}; CAZ, 0.8–51.2 μg ml^{-1}; ATM, 0.5–32 μg ml^{-1}. All assays were performed at least in triplicate.

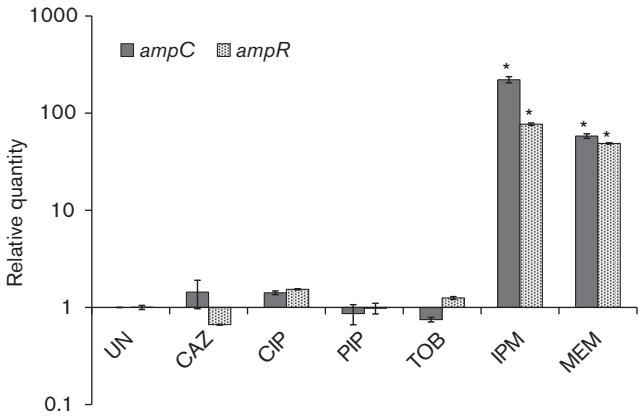

Fig. 3. Induction of *ampR* and *ampC* expression. qPCR was used to quantify the relative quantities of *ampR* and *ampC* mRNA after *P. aeruginosa* PAO1 cells were treated with SICs of various antibiotics. Data were normalized to expression in the untreated cells. The *clpX* gene (*PA1802*) was used as the housekeeping control. *$P \leqslant 0.003$ compared with expression in the uninduced (UN) condition.

its regulation by the alginate master regulator AlgT/U (Balasubramanian *et al.*, 2011). A previous transcriptional fusion study from our group showed that *ampR* expression in *P. aeruginosa* PAO1 is not significantly affected in the presence of inducer (Kong *et al.*, 2005b). Even *Citrobacter freundii ampR* is known to be expressed constitutively in a heterologous host, irrespective of the inducer (Lindquist *et al.*, 1989). However, the above studies used benzylpenicillin and 6-aminopenicillanic acid as inducer, respectively. We showed here that IPM was far more effective than the other β-lactams at inducing *ampC* and *ampR* expression (Fig. 3). What makes carbapenems and specifically IPM a strong inducer, even at very low concentrations, is still not known and needs to be explored further.

Absence of AmpR leads to enhanced susceptibility following SIC antibiotic exposure

The role of AmpR in antibiotic resistance is well established (Balasubramanian *et al.*, 2011, 2012; Cabot *et al.*, 2012). To determine the role of AmpR in transient cross-resistance, specifically in the presence of IPM, an E-test was performed on PAOΔ*ampR* with and without exposure to SICs of antibiotics (Table 3).

In the absence of SIC exposure, the loss of *ampR* resulted in increased sensitivity to most of the β-lactams that were tested such as penicillins (AMX and SAM), carbapenems (IPM and ETP) and monobactam (ATM) but not the cephalosporins (CAZ and FEP; Table 3). The increased sensitivity could be due to loss of *ampC* expression in PAOΔ*ampR*.

Pre-exposure to SICs of antibiotics made PAOΔ*ampR* more susceptible to penicillins, cephalosporins and monobactam (Table 3). Specifically, exposure to a SIC of IPM increased the susceptibility of PAOΔ*ampR* to many penicillins (SAM, fourfold; TZP, threefold; TIM, threefold), cephalosporins (CAZ, fourfold; FEP, threefold) and monobactam (ATM, threefold). The data indicated that AmpR plays a key role in subinhibitory IPM-mediated β-lactam resistance in *P. aeruginosa* (Table 3).

Apart from β-lactams, loss of *ampR* also resulted in increased susceptibility to the aminoglycosides AMK and TOB (Table 3). The sensitivity to aminoglycosides was enhanced upon pre-exposure to various β-lactams and non-β-lactams. The change in TOB susceptibility of PAOΔ*ampR* upon SIC antibiotic exposure was also confirmed by the broth dilution method (data not shown). Regulation of aminoglycoside resistance by AmpR is not AmpC-mediated because the resistance profile of PAOΔ*ampC* remained unaltered (Table 3). The data clearly suggest that AmpR plays a critical role in regulating antibiotic cross-resistance in *P. aeruginosa*.

Loss of *ampR*, in addition to rendering *P. aeruginosa* sensitive to many β-lactam antibiotics, also leads to reduced production of acute virulence factors (Balasubramanian *et al.*, 2012, 2013a). This finding further augments the suggestion that AmpR is a viable drug candidate as it would make *P. aeruginosa* less virulent, and sensitive to β-lactams and aminoglycosides, without confounding their efficacy at SIC pre-exposure to antibiotics.

AmpR-mediated cross-resistance to β-lactams is partially contributed by AmpC

Largely, AmpR regulates β-lactam resistance through AmpC as both PAOΔ*ampR* and PAOΔ*ampC* became sensitive to β-lactams compared with PAO1 (Table 3). To investigate whether AmpR could regulate β-lactam resistance an in AmpC-independent manner, the MIC profile was compared between PAOΔ*ampR* and PAOΔ*ampC* with and without exposure to SICs of antibiotics. Compared with PAOΔ*ampC*, pre-exposure to various β-lactams and TOB made PAOΔ*ampR* more susceptible to many penicillins (SAM, TZP and TIM), cephalosporins (CAZ and FEP) and monobactam (ATM). The difference in susceptibility showed that AmpR also regulates β-lactam resistance by AmpC-independent mechanisms. One such mechanism could involve MexR, the regulator of the MexAB–OprM pump involved in the efflux of β-lactam antibiotics (Poole *et al.*, 1996b). This is further supported by previous qPCR studies that show positive regulation of *mexR* expression by AmpR (Balasubramanian *et al.*, 2012). This would argue that, in the absence of *ampR*, the expression of the MexAB–OprM pump should confer increased resistance. However, the data from our laboratory show that deleting *ampC* in PAO1 (Table 3) or in PAOΔ*ampR* (D. Zincke, unpublished data) diminishes β-lactam resistance, in spite of having a functional MexAB system. This observation suggests that the MexAB pump by itself is not enough to confer β-lactam resistance. Although induction of *ampC* by AmpR seems to

Table 3. Susceptibility profile of *P. aeruginosa* PAOΔ*ampR* and PAOΔ*ampC* after SIC antibiotic exposure

Results are shown as MIC values (μg ml^{-1}).

Class	Antibiotic	None*	None†		SIC antibiotic exposure											
					IPM (0.1 μg ml^{-1})		MEM (0.05 μg ml^{-1})		CAZ (0.1 μg ml^{-1})		PIP (0.2 μg ml^{-1})		CIP (0.05 μg ml^{-1})		TOB (0.1 μg ml^{-1})	
			ampR	*ampC*	*ampR*	*ampC*	*ampR*	*ampC*	*ampR*	*ampC*	*ampR*	*ampC*	*ampR*	*ampC*	*ampR*	*ampC*
Penicillin	AMX	>256	12	12	12	12	12	12	12	12	8	24	8	6	12	16
	SAM	96	12	16	3	12	8	8	6	16	8	32	6	6	4	24
	TZP	(4)	3	4	1	1.5	2	3	1.5	3	3/2	4	2	1.5	2	3
	TIM	24	24	48	8	16	6	12	4	16	8	24	4	6	6	12
Cephalosporin	CAZ	1.5	1.5	2	0.38	1	1	1	1	1.5	1.5	2	1	0.75	1	1.5
	FEP	2	1.5	2	0.5	1.5	1	0.75	0.75	1.5	1.5	3	1	1	2	3
Carbapenem	IPM	1.5	0.38	0.5	0.38	0.38	0.38	0.25	0.38	0.25	0.5	0.5	0.5	0.5	0.5	0.5
	DOR	0.38	0.25	0.125	0.25	0.125	0.19	0.064	0.25	0.094	0.25	0.19	0.38	0.064	0.5	0.125
	MEM	0.38	0.5	0.3	0.25	0.25	0.5	0.19	0.25	0.125	0.5	0.25	0.38	1	0.5	1.5
	ETP	>32	6	8	ND	ND	ND	ND	ND	ND	8	8	6	ND	8	ND
Monobactam	ATM	3	1.5	3	0.5	1.5	1.5	3	0.75	1.5	1.5	3	1	1	1	3
Quinolone	CIP	0.25	1.5	0.25	1.5	0.25	1.5	0.25	1.5	0.25	1.5	0.38	1.5	0.125	1.5	0.38
	LVX	0.5	6	0.5	6	0.5	3	0.5	4	0.5	4	0.5	4	0.25	4	0.75
Aminoglycoside	AMK	4	3	4	3	4	3	3	2	4	3	4	3	6	3	4
	TOB	1	0.5	1	0.38	0.75	0.38	0.5	0.38	0.75	0.38	0.75	0.25	0.75	0.38	0.75

ND, Not determined.

*PAO1 MIC values are from Table 1 for comparison.

†PAOΔ*ampR* and PAOΔ*ampC* with no antibiotic exposure.

be the primary mechanism that leads to enhanced resistance, we are far from understanding the role of various converging pathways in the regulation of antibiotic resistance.

AmpR is involved in cross-resistance to quinolones

The MIC data comparison showed that the increase in resistance to quinolones (CIP and LVX) in PAOΔ*ampR* was AmpC-independent because PAOΔ*ampC* had no effect on resistance to quinolones (Table 3). This finding is in agreement with a previously shown role of AmpR in quinolone resistance through regulation of *mexEF–oprN* encoding the efflux pump and its regulator *mexT*, in the absence of antibiotic induction (Köhler *et al.*, 1999; Balasubramanian *et al.*, 2012). To investigate the role of AmpR in the expression of *mexEF–oprN* efflux pump and cross-induction by clinically used antibiotics, we checked the expression of *mexE*, the first gene of the *mexEF–oprN* operon, in PAO1 and PAOΔ*ampR* after exposure to SICs of antibiotics (Fig. 4).

In the absence of any pre-exposure to antibiotics, PAOΔ*ampR* had a high *mexE* expression compared with PAO1 (RQ in PAOΔ*ampR*: 2253 ± 251, $P=0.0001$; Fig. 4), which is in agreement with previous findings (Balasubramanian *et al.*, 2012). However, *mexE* expression could not be induced in PAO1 with any of the antibiotics including the MexEF–OprN substrate CIP (Fig. 4). The absence of induction was not surprising, as PAO1 has a functional AmpR that represses *mexE* expression (Balasubramanian *et al.*, 2012). In addition, PAO1 harbours an 8 bp insertion in *mexT* encoding the positive regulator of *mexEF–oprN* transcription, rendering it non-inducible (Maseda *et al.*, 2000).

In the presence of a SIC of CIP, a MexEF–OprN substrate, expression of *mexE* in PAOΔ*ampR* was further induced twofold (RQ: untreated 2253 ± 251, CIP treated 5241 ± 246, $P=0.0001$; Fig. 4). This suggested that there is an additional level of transcriptional control in the presence of CIP, which, unlike AmpR, positively regulates *mexE* transcription. In addition, there was a twofold induction of *mexE* expression upon treatment with a SIC of the aminoglycoside TOB, a non-MexEF substrate (RQ in PAOΔ*ampR*: untreated 2253 ± 251, TOB treated 5207 ± 192, $P=0.0008$; Fig. 4). The mechanism resulting in the induction of a quinolone-specific efflux pump due to exposure to a SIC of an aminoglycoside is not clear.

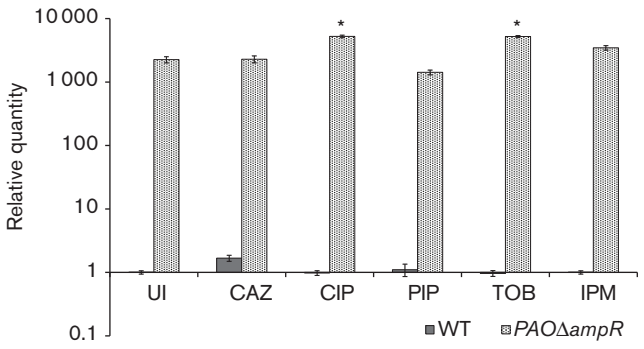

Fig. 4. *mexE* expression in response to SICs. Expression of *mexE* was determined in PAO1 (WT) and PAOΔ*ampR* cells, with and without SIC treatment with various antibiotics. Data were normalized to expression in the PAO1 untreated cells. The *clpX* gene (*PA1802*) was used as the housekeeping control. UI, uninduced. *$P \leqslant 0.0008$ compared with the WT untreated (UI) strain.

The efflux pumps MexAB, MexEF and MexXY are predominantly involved in efflux of β-lactams, fluoroquinolones and aminoglycosides, respectively. However, cross-induction of these by different pump substrates is not uncommon. Expression of *mexXY* is induced by the quinolone OFX in the absence of *mexAB* (Masuda *et al.*, 2000). Our study showed that expression of *mexE* is induced by an aminoglycoside in the absence of *ampR* (Fig. 4), where *mexAB* expression increases significantly (Balasubramanian *et al.*, 2012). Together, these findings suggest that all three efflux pumps share regulation, and AmpR seems to play an important role in this process.

CONCLUSIONS

The development and persistence of antibiotic resistance is a complicated and multifactorial phenomenon necessitating dedicated research to understand and control this problem. Both high and low concentrations of antibiotics have been identified to result in the development of resistance (reviewed by Andersson & Hughes, 2011). Our data showed that exposure to SICs of antibiotics has a widespread role in inducing transient cross-resistance and expression of antibiotic resistance genes. Residual amounts of IPM could be enough to provide resistance to subsequent β-lactam treatment. Whilst the increase in resistance upon SIC exposure to carbapenems is evident and expected, the increase in susceptibility with CIP was unexpected. The unexpected SIC sensitization to CIP warrants further investigation.

We also showed that AmpR has a wider role in regulating antibiotic resistance than previously thought. The data confirmed that AmpR plays a major role in the development of transient cross-resistance upon SIC induction. Additionally, AmpR was shown to regulate β-lactam resistance in an AmpC-dependent and -independent manner as well as non-β-lactam resistance such as that of quinolones and aminoglycosides. Together with our transcriptomic studies that established AmpR as a major regulator of virulence (Balasubramanian *et al.*, 2012, 2013a), AmpR may be a suitable drug target for combating *P. aeruginosa* infections.

In conclusion, a strict and guided use of antibiotics and monitoring patient treatment history along with a careful assessment of the usefulness of therapeutic approaches is needed. With fewer new antibiotics being discovered, the focus should also be on developing new therapeutic

strategies involving important players of resistance and virulence, such as AmpR. Although PAOΔ*ampR* displays fluoroquinolone resistance, targeting AmpR is still a good proposition because strains will become susceptible to β-lactams and have reduced virulence factor production.

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