Co-regulation of β-lactam resistance, alginate production and quorum sensing in *Pseudomonas aeruginosa*

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Development of β-lactam resistance, production of alginate and modulation of virulence factor expression that alters host immune responses are the hallmarks of chronic *Pseudomonas aeruginosa* infection in cystic fibrosis patients. In this study, we propose that a co-regulatory network exists between these mechanisms. We compared the promoter activities of *ampR*, *algT/U*, *lasR*, *lasI*, *rhlR*, *rhlI* and *lasA* genes, representing the β-lactam antibiotic resistance master regulatory gene, the alginate switch operon, the *las* and *rhl* quorum-sensing (QS) genes, and the LasA staphylolytic protease, respectively. Four isogenic *P. aeruginosa* strains, the prototypic Alg^- PAO1, Alg^- PAO*ampR*, the mucoid Alg^+ PAO*mucA22* (Alg^+ PDO300) and Alg^+ PAO*mucA22ampR* (Alg^+ PDO*ampR*) were used. We found that in the presence of AmpR regulator and β-lactam antibiotic, the extracytoplasmic function sigma factor AlgT/U positively regulated P_{ampR}, whereas AmpR negatively regulated $P_{algT/U}$. On the basis of this finding we suggest the presence of a negative feedback loop to limit *algT/U* expression. In addition, the functional AlgT/U caused a significant decrease in the expression of QS genes, whereas loss of *ampR* only resulted in increased P_{lasI} and P_{lasR} transcription. The upregulation of the *las* QS system is likely to be responsible for the increased *lasA* promoter and the LasA protease activities in Alg^- PAO*ampR* and Alg^+ PDO*ampR*. The enhanced expression of virulence factors in the *ampR* strains correlated with a higher rate of *Caenorhabditis elegans* paralysis. Hence, this study shows that the loss of *ampR* results in increased virulence, and is indicative of the existence of a co-regulatory network between β-lactam resistance, alginate production, QS and virulence factor production, with AmpR playing a central role.

Received 22 April 2010
Accepted 18 October 2010

INTRODUCTION

Pseudomonas aeruginosa, a ubiquitous, versatile saprophytic bacterium, is a major aetiological agent of nosocomial infections and the leading cause of mortality among patients with cystic fibrosis (CF) (Greenberg, 2000; Rahme *et al.*, 1995). This Gram-negative bacillus is equipped with an impressive arsenal of virulence factors to resist host defence mechanisms, counteract antibacterial agents, circumvent nutrient deprivation and withstand harsh environmental changes (Govan & Harris, 1986; Lyczak *et al.*, 2002; Pedersen, 1992). One distinctive feature of *P. aeruginosa* lung isolates of patients with advanced CF is that a higher proportion of them are mucoid as compared to those from other sites of infection (Doggett, 1969; Fick *et al.*, 1992). This mucoid phenotype is the result of an overproduction of the exopolysaccharide alginate (Evans & Linker, 1973). The activation of genes for alginate overproduction occurs primarily through the deregulation of *algT/U* or its product, σ^{22}, a member of the extracytoplasmic function (ECF) sigma factors (DeVries & Ohman, 1994; Hershberger *et al.*, 1995; Martin *et al.*, 1993). Genomic, proteomic and microarray analyses have shown that AlgT/U regulates a diverse group of genes, ranging from extracellular proteases, periplasmic proteins like DsbA and intracellular enzymes (Firoved *et al.*, 2002; Firoved & Deretic, 2003; Malhotra *et al.*, 2000). Mucoid *P. aeruginosa* isolates from CF patients frequently have a defective *mucA* allele, a gene downstream of *algT/U* (Martin *et al.*, 1993). The *mucA* gene product is an anti-sigma factor that negatively regulates the activity of AlgT/U (Hughes & Mathee, 1998).

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Abbreviations: CF, cystic fibrosis; ECF, extracytoplasmic function; qPCR, quantitative real-time PCR; QS, quorum sensing.

P. aeruginosa is intrinsically resistant to most β-lactam antibiotics. One of the factors contributing to the resistance is the existence of enzymes that can deactivate β-lactams, known as β-lactamases (Kong *et al.*, 2010; Rolinson, 1998). Two inducible chromosome-encoded β-lactamases, AmpC and PoxB (Oxa-50), have been identified in *P. aeruginosa* (Girlich *et al.*, 2004; Kong *et al.*, 2005a; Lodge *et al.*, 1990). Expression of the *ampC* and *poxB* genes is tightly controlled by AmpR, a global LysR-like transcriptional regulator (Kong *et al.*, 2005b). In addition, inactivation of *ampR* in the prototypic non-mucoid PAO1 (henceforth referred to as Alg$^-$ PAO1) resulted in high constitutive production of β-lactamases and pyocyanin, increased LasA staphylolytic protease activity and decreased LasB elastase activity (Kong *et al.*, 2005b).

The production of virulence factors in *P. aeruginosa* is under the control of quorum-sensing (QS) systems mediated by diffusible chemical signalling molecules such as acylhomoserine lactones and quinolones. *P. aeruginosa* has three QS systems – *las*, *rhl* and *Pseudomonas* quinolone system that controls many virulence mechanisms (Ng & Bassler, 2009). Transcriptome studies have led to the identification of a large number of virulence factors that are under QS regulation in *P. aeruginosa*, these include proteases and toxins (Hentzer *et al.*, 2003; Schuster & Greenberg, 2006; Wagner *et al.*, 2004).

It has long been established that the production of proteases is inversely correlated with alginate production (Mathee *et al.*, 1999; Mohr *et al.*, 1990; Ohman & Chakrabarty, 1982). Previous comparison of Alg$^-$ PAO1 and its isogenic *ampR* mutant strain, Alg$^-$ PAO*ampR*, showed differential regulation of virulence factors, including the *las* QS system (Kong *et al.*, 2005b). In the present study, we sought to understand the regulatory network between alginate production, protease activity, β-lactam resistance and QS in *P. aeruginosa*. We hypothesized that AmpR may be differentially regulated in alginate-producing strains with consequent effects on the protease activities. To address this, *ampR* was inactivated in an alginate constitutive producer, Alg$^+$ PDO300, generating an Alg$^+$ PDO*ampR* mutant strain. This mutant produced exceedingly high levels of β-lactamase, extracellular proteases and pyocyanin suggesting that AmpR either directly or indirectly suppresses the expression of many other virulence factors.

METHODS

Bacterial strains, plasmids and media. Table 1 shows the bacterial strains, plasmids and primers used in this study. The bacterial strains of *Escherichia coli* and *P. aeruginosa* were routinely cultured in Luria–Bertani medium. *Pseudomonas* isolation agar (Difco) was used in triparental mating experiments for the selection of *P. aeruginosa*. Antibiotics, when used, were at the following concentrations unless indicated otherwise: ampicillin at 50 μg ml^{-1}, tetracycline at 20 μg ml^{-1}, gentamicin at 30 μg ml^{-1} for *E. coli*; and carbenicillin at 300 μg ml^{-1}, gentamicin at 300 μg ml^{-1}, tetracycline at 60 μg ml^{-1} for *P. aeruginosa*. For induction, 500 μg benzyl-penicillin ml^{-1} was used.

DNA manipulations. All molecular techniques were performed according to standard protocols (Ausubel *et al.*, 1999).

Insertional inactivation of the *ampR* gene. Inactivation of *ampR* in Alg$^+$ PDO300 (PAO*mucA22*) was performed as previously reported using the same constructs (Kong *et al.*, 2005b). The *ampR*::*aacCI* fragment subcloned into pEX100T (Schweizer & Hoang, 1995) was introduced by conjugation into an alginate-overproducing *P. aeruginosa*, Alg$^+$ PDO300 (Mathee *et al.*, 1999), with a helper strain harbouring pRK2013 (Figurski & Helinski, 1979). The merodiploids resulting from homologous recombination were selected with *Pseudomonas* isolation agar containing gentamicin. The colonies were then screened for gentamicin resistance and carbenicillin sensitivity by replica plating. The insertion was confirmed by PCR and restriction analysis of the PCR product. The Alg$^+$ PDO300 isogenic strain with defective *ampR* (PAO*mucA22ampR*) is named Alg$^+$ PDO*ampR* (Table 1). Complementation studies were performed using plasmid pSJ06 that contains a PCR-amplified *ampR* on a low-copy-number, highly stable shuttle vector pME6030 to minimize the effects of gene dosage (Kong *et al.*, 2005b). This plasmid is referred to as pAmpR.

Construction of promoter-*lacZ* fusions. A 330 bp *ampC–ampR* intergenic region with the putative promoters was subcloned into the promoterless *lacZ* in the mini-CTX-*lacZ* reporter plasmid (Becher & Schweizer, 2000), creating pSJ10 (P_{ampC}-*lacZ*) and pSJ11 (P_{ampR}-*lacZ*) (Table 1) (Kong *et al.*, 2005b). The resulting clones were mobilized into Alg$^+$ PDO300 and Alg$^+$ PDO*ampR* (Table 1).

Quantitative real-time PCR (qPCR). RNA extraction was performed with an RNeasy mini kit (Qiagen) following the manufacturer's protocols after treatment of cells with subMIC levels (200 μg ml^{-1}) of penicillin G at OD_{600} 0.6 for 1 h. The samples were stabilized with 5 % phenol/95 % ethanol mixture (pH 4.7) immediately after harvesting and during cell lysis (Brencic *et al.*, 2009). After determining RNA quantity spectrophotometrically (Beckman DU640; Beckman Coulter) and quality by denaturing agarose gel electrophoresis (Northern Max Gly; Ambion), cDNA was synthesized by annealing NS5 random primers to total purified RNA. Subsequent extension was carried out using SuperScript III reverse transcriptase (Invitrogen) (Brencic *et al.*, 2009). The cDNA was quantified and 10 ng cDNA was used per qPCR. We used the ABI 7500 cycler (Applied Biosystems) and Power SYBR Green PCR mastermix with ROX (Applied Biosystems) to test for expression of the *ampR* gene in these strains. The ATP-binding subunit *clpX* (PA1802) of the ATP-dependent protease was used as the internal control. Assays were performed in triplicate. Primer specificity was determined from dissociation profiles using melt curves. The cycling conditions for the qPCR were: 95 °C for 2 min (holding); 40 cycles of 95 °C for 15 s, 60 °C for 1 min (cycling); 95 °C for 15 s, 60 °C for 1 min (melt curve conditions).

Quantification of pyocyanin and LasA protease. Extracellular pyocyanin was quantified as previously described (Kong *et al.*, 2005b). LasA protease activity was measured by determining the ability of *P. aeruginosa* culture supernatants to lyse boiled *Staphylococcus aureus*, as described by Kessler *et al.* (1993).

β-Lactamase assay. The assay of the *P. aeruginosa* chromosomal β-lactamase was performed as previously described using nitrocefin as the colorimetric substrate (Kong *et al.*, 2005b).

β-Galactosidase assay. Assays for β-galactosidase in *P. aeruginosa* were performed as previously described (Mathee *et al.*, 1997) and adapted into a high-throughput 96-well array (Griffith & Wolf, 2002).

***P. aeruginosa–Caenorhabditis elegans* paralysis assays.** The *P. aeruginosa–C. elegans* standard paralysis assay was modified from

Table 1. Bacterial strains, plasmids and primers used in this study

Strain/plasmid	Genotype	Reference
E. coli		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA–argF*) U169 *deoR recA1 endA1 hsdR17*(r_k^-, m_k^+) *phoA supE44* λ- *thi-1 gyrA96 relA1*	New England Biolabs
TOP10F′	F′[*lacI*^{q}, Tn10(Tet^R)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139 D(ara-leu)7697 galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen
P. aeruginosa		
PAO1	Prototype; Alg$^-$	Holloway & Morgan (1986)
PDO300	PAO*mucA22*; Alg$^+$	Mathee *et al.* (1999)
PKM805	PAO*mucA22algT24-1*; Alg$^-$	PDO*algT*; PDO300 derivative; Ramos *et al.* (2003)
PKM300	PAO*ampR*::*aacCI*; Alg$^-$	PAO*ampR*; Kong *et al.* (2005b)
PKM307	PAO*mucA22 ampR*::*aacCI;* Alg$^+$	PDO*ampR*; this study
PKM308	PAO*mucA22 attB*::P$_{ampC}$–*lacZ*; Tc^R, Alg$^+$	PDO300 derivative; this study
PKM309	PAO*mucA22 attB*::P$_{ampR}$–*lacZ*; Tc^R, Alg$^+$	PDO300 derivative; this study
PKM310	PAO*mucA22 ampR*::*aacCI attB*::P$_{ampC}$-*lacZ*; Gm^R, Tc^R, Alg$^+$	PDO*ampR* derivative; this study
PKM311	PAO*mucA22 ampR*::*aacCI attB*::P$_{ampR}$–*lacZ*; Gm^R, Tc^R, Alg$^+$	PDO*ampR* derivative; this study
Plasmids		
Mini-CTX-*lacZ*	Tc^R; integration-proficient vector for single-copy chromosomal *lacZ* fusion	Becher & Schweizer (2000)
pEX100T	Ap^R; *sacB oriT*	Schweizer & Hoang (1995)
pGEMEX-1	Ap^R; ColE1*ori lacZ*α	Promega
pKMG37	Ap^R; pQF50 containing P$_{algT}$–*lacZ* transcriptional fusion	Mathee *et al.* (1997)
pLP170	Ap^R; *lacZ* transcriptional fusion vector that contains an RNase III splice sequence positioned between the MCS and *lacZ*	Preston *et al.* (1997)
pLPLA	Ap^R; pLP170 containing P$_{lasA}$-*lacZ* transcriptional fusion	Preston *et al.* (1997)
pLPLB	Ap^R; pLP170 containing P$_{lasB}$-*lacZ* transcriptional fusion	Preston *et al.* (1997)
pLPR1	Ap^R; pLP170 containing P$_{rhlI}$-*lacZ* transcriptional fusion	Van Delden & Iglewski (1998)
pME6030	Tc^R; *ori*V$_{pVS1}$ *ori*V$_{p15A}$ *oriT*	Heeb *et al.* (2000)
pPCS223	Ap^R; pLP170 containing P$_{lasI}$-*lacZ* transcriptional fusion	Van Delden & Iglewski (1998)
pPCS1001	Ap^R; pLP170 containing P$_{lasR}$-*lacZ* transcriptional fusion	Pesci *et al.* (1997)
pPCS1002	Ap^R; pLP170 containing P$_{rhlR}$-*lacZ* transcriptional fusion	Pesci *et al.* (1997)
pQF50	Ap^R; broad-host-range vector with promoterless *lacZ*	Farinha & Kropinski (1990)
pRK2013	Km^R; ColE1*ori*-Tra (RK2)$^+$	Figurski & Helinski (1979)
pSJ01	Ap^R; pGEMEX-1 with a 1220 bp *Eco*RI–*Bam*HI flanked fragment containing *ampR*	Kong *et al.* (2005b)
pSJ06	Ap^R; pME6030 with a 1220 bp *Eco*RI–*Bam*HI flanked fragment containing *ampR* (referred to as pAmpR)	Kong *et al.* (2005b)
pSJ07	Ap^R; pEX100T derivative with *ampR*::*aacCI*	Kong *et al.* (2005b)
pSJ09	Ap^R, Gm^R; pGEMEX-1 with a 330 bp *Eco*RI–*Bam*HI flanked fragment containing *ampC-ampR* intergenic region	Kong *et al.* (2005b)
pSJ10	Tc^R; CTX-*lacZ* fused with *ampC* promoter, P$_{ampC}$	Kong *et al.* (2005b)
pSJ11	Tc^R; CTX-*lacZ* fused with *ampR* promoter, P$_{ampR}$	Kong *et al.* (2005b)
pUCGm	Ap^R, Gm^R; pUC19 derivative containing gentamicin cassette	Schweizer (1993)
Primers		
SBJ01*ampR*For*	5′-G*GAATTC*TGGCGAACAGCAGTGTGGAAGCGG-3′	
SBJ02*ampR*Rev*	5′-CG*GGATCC*ATTCCAATCACAACCCCAACGCC-3′	
SBJ03*ampCR*For*	5′-G*GAATTC*TGAGGCCGCGCGGCAGACGCTTGAACA-3′	
SBJ04*ampCR*Rev*	5′-CG*GGATCC*CATGAGGATTGGCGTCCTTTG-3′	
DBS_QRTAmpRF	5′-CATTGGCCTTCATCACCGGTTGTA-3′	
DBS_QRTAmpRR	5′-GGTTTCTCATGCAGCCCACGACAA-3′	

*The italicized portion of the sequence indicates a restriction site in a PCR product prepared with the primer.

that of Gallagher & Manoil (2001). Bacterial cultures were grown overnight. A 1 : 1000 dilution was plated onto brain heart infusion agar plates. These plates were incubated for 18–24 h for the formation of bacterial lawns. Meanwhile, a synchronized culture of L4 stage larvae hermaphrodite Bristol N2 *C. elegans* was washed off an *E. coli* OP50-seeded nematode growth medium plate (1.7 % agar, 0.35 % peptone,

0.34 % K_2HPO_3, 0.3 % NaCl, 0.012 % $MgSO_4$, 0.011 % $CaCl_2$, 0.0005 % cholesterol). The worms were centrifuged at 1300 ***g*** for 2 min and washed twice with M9 medium to remove residual *E. coli* bacteria. A total of 30 to 50 worms was then added to the *P. aeruginosa* bacterial lawns. Both live and paralysed worms were scored at 1, 2 and 4 h by microscopic observation. The analysis was performed in triplicate.

Statistical analysis. All data were analysed with one-way ANOVA using the statistical software package SPSS (SPSS).

RESULTS

P_{ampC} expression in *ampR* mutants

We have previously reported that in the non-mucoid strain, AmpR positively regulates *ampC* expression but negatively controls the expression of *poxB* (Kong *et al.*, 2005a, b). To test whether such opposing controls remain true in the Alg^+ background, strains were constructed with a single copy of the *ampC* promoter fused to a promoterless reporter gene, *lacZ* (P_{ampC}-*lacZ*). This was integrated into the Alg^+ PDO300 and the Alg^+ PDO*ampR* chromosomes via *attB-attP* site-specific recombination, thus allowing mimicking of the chromosomal regulation. In the absence of inducer, the P_{ampC}-*lacZ* activity remained at a basal level in Alg^+ PDO300 and Alg^+ PDO*ampR* strains (Table 2). A significant ninefold induction of the *ampC* promoter was observed in Alg^+ PDO300 upon challenge with β-lactams (Table 2). However, the inducibility of the P_{ampC} was lost in Alg^+ PDO*ampR*.

Based on the above analysis, we expected to observe a loss of β-lactamase activity concomitant with the loss of *ampR*. However, the Alg^+ PDO*ampR* expressed a statistically significant sixfold higher β-lactamase compared to the parent Alg^+ PDO300 in the absence of antibiotics (Fig. 1). No further induction was demonstrated in the presence of the inducer. This phenotype varied from the parental strain Alg^+ PDO300, which showed only a threefold inducible phenotype (Fig. 1). The inducible phenotype was restored in Alg^+ PDO*ampR* mutant by complementation with pAmpR. The high β-lactamase activity in an *ampR* mutant has been shown previously to be due to the uninhibited expression of an oxacillinase *poxB* gene, rather than the elevated expression of *ampC* gene (Kong *et al.*, 2005b).

ampR transcription in alginate-overproducing strains

The LysR family of transcriptional regulators is known to repress their own transcription as in the case of *Citrobacter freundii* AmpR (Lindquist *et al.*, 1989). However, we have previously reported that *P. aeruginosa* AmpR does not autoregulate in the prototypic strain Alg^- PAO1 (Kong *et al.*, 2005b). To determine if there is a change in the AmpR autoregulation in Alg^+ strains, a single-copy fusion of P_{ampR}-*lacZ* was introduced at the *attP* site in Alg^+ PDO300 and Alg^+ PDO*ampR*. In the absence of inducers, the *ampR* transcription remained at low levels in both strains (Table 2). In the presence of inducers, a significant increase in P_{ampR} expression was seen in Alg^+ PDO300 (Table 2). Comparing the genotypes of the isogenic Alg^- PAO1 and Alg^+ PDO300 strains, this significant increase was likely due to the uninhibited activity of the ECF sigma factor AlgT/U in the latter. This suggests that AlgT/U activates the *ampR* promoter in the presence of inducers. Due to loss of *ampR*, no significant induction of P_{ampR} was seen in Alg^+ PDO*ampR*. In order to test AlgT/U regulation of *ampR*, mRNA levels of *ampR* were determined by qPCR with the Alg^- PAO1, Alg^+ PDO300 and Alg^- PDO*algT* strains. The Alg^+ PDO300 strain showed an increase in the *ampR* mRNA levels (relative quantity of 2.1 ± 0.2 compared to 1.0 ± 0 in Alg^- PAO1) indicating positive regulation of *ampR* by AlgT/U. Mutation in *algT/U* in Alg^- PDO*algT* led to a decrease in this expression (relative quantity 1.4 ± 0.1 compared to 2.1 ± 0.2 in Alg^+ PDO300) supporting our hypothesis of positive regulation of *ampR* by AlgT/U and concurs with the transcriptional fusion assays. These results suggest that both AlgT/U and AmpR

Table 2. β-Galactosidase activities of *ampC* and *ampR* promoters

Strain	P_{ampC}-*lacZ* (Miller units)		*P* value*	P_{ampR}-*lacZ* (Miller units)		*P* value*
	Non-induced	Induced		Non-induced	Induced	
Alg^- PAO1†	124.1 ± 11.6	1644.2 ± 33.7	<0.05	77.1 ± 8.7	123 ± 1.2	NS
Alg^- PAO*ampR*†	113.2 ± 7.5	122.3 ± 7.4	NS	96.3 ± 15.2	106.0 ± 16.0	NS
P value‡	NS	<0.05		NS	NS	
Alg^+ PDO300	104.2 ± 4.5	957.5 ± 161.4	<0.05	79.5 ± 26.3	332.8 ± 14.3	<0.05
Alg^+ PDO*ampR*	142.4 ± 4.9	143.8 ± 6.9	NS	155.8 ± 0.8	153.0 ± 2.1	NS
P value§	NS	<0.05		NS	<0.05	

NS, Not significant (*P* values >0.05).

*ANOVA compares the activity values between the presence (+) and absence (−) of inducers.

†These data are presented in a previous paper (Kong *et al.*, 2005b); they are included here for comparison.

‡ANOVA compares the activity values between the Alg^- PAO1 and the mutant Alg^- PAO*ampR*.

§ANOVA compares the activity values between the Alg^+ PDO300 and the mutant Alg^+ PDO*ampR*.

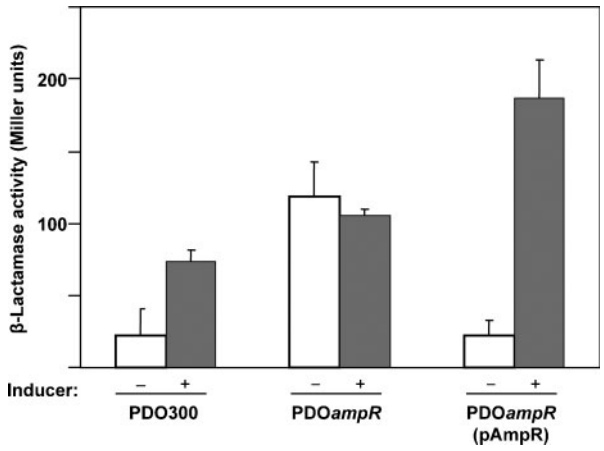

Fig. 1. β-Lactamase expression in the Alg$^+$ PDO*ampR* mutant. Assays were performed using the parent strain Alg$^+$ PDO300, the mutant Alg$^+$ PDO*ampR* and Alg$^+$ PDO*ampR* (pAmpR) in the absence (–) and presence (+) of an inducer. The plasmid pAmpR carries the wild-type *ampR* gene on a broad-host-range low-copy-number plasmid pME6030 (Heeb *et al.*, 2000). Fresh cultures of OD_{600} 0.6–0.8 were induced with 100 μg benzylpenicillin ml^{-1} for 3 h before harvesting. Assays were performed on sonicated lysate using nitrocefin as a chromogenic substrate. Assays were performed in triplicate. One Miller unit of β-lactamase is defined as 1 nmol nitrocefin hydrolysed min^{-1} (μg protein)$^{-1}$.

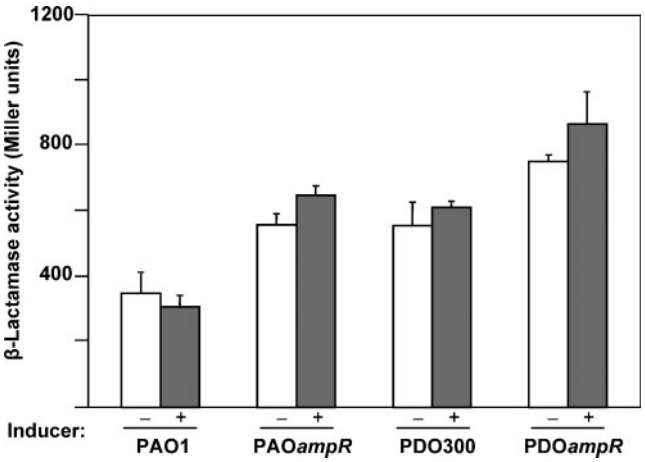

Fig. 2. The effects of the *ampR* mutation on *algT/U* transcription. The promoter fusion $P_{algT/U}$-*lacZ* was introduced into Alg$^-$ PAO1, Alg$^-$ PAO*ampR*, Alg$^+$ PDO300 and Alg$^+$ PDO*ampR*. Induction was carried out using 500 μg benzylpenicillin ml^{-1} and the β-galactosidase activity was determined in Miller units after 30 min incubation. The basal level of expression was detected in the promoterless *lacZ* vector, pLP170.

are required for the induction of the *ampR* promoter in the presence of inducers.

ampR mutation affects *algT/U* transcription

The loss of inducibility of *ampR* transcription in the Alg$^+$ PDO*ampR* background provided us with the first clue of the existence of a co-regulatory network involving β-lactam resistance and alginate production. To determine if this relationship is bidirectional, a $P_{algT/U}$-*lacZ* fusion construct was introduced into Alg$^-$ PAO1, Alg$^+$ PDO300 and the corresponding *ampR* mutant strains. As expected, the expression of *algT/U* promoter is constitutive in Alg$^-$ PAO1 and increased in Alg$^+$ PDO300 (Fig. 2). Insertional inactivation of *ampR* in Alg$^-$ PAO1 and Alg$^+$ PDO300 resulted in an approximately twofold increase in $P_{algT/U}$ activity in the absence of inducer. The effect of *ampR* mutation in Alg$^-$ PAO*ampR* is the same as the known AlgT/U repressor *mucA* mutation (Alg$^+$ PDO300) with respect to $P_{algT/U}$ expression, indicating negative regulation of $P_{algT/U}$ by AmpR. The AmpR-regulation of *algT/U* promoter in these strains was not significantly affected by β-lactam antibiotic. The consistent increase in $P_{algT/U}$ in the absence of *ampR* suggests that AmpR is a negative modulator of the ECF sigma factor, AlgT/U.

AlgT/U-dependent regulation of pyocyanin

Our quantitative analysis showed that the Alg$^+$ PDO300 produced threefold less pyocyanin than Alg$^-$ PAO1 in the absence of β-lactam antibiotics (Table 3). This finding confirms that the AlgT/U sigma factor suppresses the production of pyocyanin. The presence of inducer resulted in an increase in pyocyanin production, albeit at low levels in Alg$^+$ PDO300. However, the Alg$^+$ PDO*ampR* mutant produced a significantly high basal level of pyocyanin, which was inducible in the presence of β-lactam antibiotics (Table 3). Expressing *ampR in trans* in Alg$^+$ PDO*ampR* on a low-copy-number plasmid restored the phenotype to the parental strain, Alg$^+$ PDO300 (data not shown). On the basis of this data we further argue that AmpR acts as a negative regulator of pyocyanin production.

LasA protease activity and *lasA* promoter expression in Alg$^+$ PDO*ampR*

The inverse relationship seen between alginate production and proteases is presumed to be AlgT/U-dependent (Mathee *et al.*, 1999; Mohr *et al.*, 1990; Ohman & Chakrabarty, 1982). Thus, a significant increase in *algT/U* expression in Alg$^+$ PDO*ampR* (Fig. 2) should result in downregulation of LasA protease expression. As expected, in comparison to the wild-type Alg$^-$ PAO1, Alg$^+$ PDO300 produced 2.3-fold less LasA protease. However, loss of *ampR* resulted in a marginal increase in the LasA protease activity (Table 3) in an inducer-independent manner. To further confirm the above hypothesis, a P_{lasA}-*lacZ* transcriptional fusion plasmid was introduced into Alg$^-$ PAO1, Alg$^+$ PDO300 and the *ampR* mutant strains (Table 3). In concordance to the LasA activity analysis, the P_{lasA} levels were low in all mucoid strains, suggesting that the transcription of these promoters was suppressed (Table 3). Furthermore, the P_{lasA}-*lacZ* fusion expression was increased twofold to threefold in Alg$^-$

Table 3. Pyocyanin, LasA and P_{lasA}-*lacZ* activities

Inducer*	Pyocyanin [μg (μg total protein)$^{-1}$]†		LasA [ΔOD_{600} h^{-1} (μg protein)$^{-1}$]‡		P_{lasA}-*lacZ* (Miller units)§
	−	+	−	+	−
Alg$^-$ PAO1‖	0.285 ± 0.219	2.293 ± 0.216¶	0.310 ± 0.065	0.317 ± 0.059	790.0 ± 128.9
Alg$^-$ PAO*ampR*‖	2.934 ± 0.761#	3.317 ± 0.638	1.109 ± 0.099#	0.951 ± 0.045#	1970.5 ± 312.6#
Alg$^+$ PDO300	0.102 ± 0.017**	0.324 ± 0.051	0.135 ± 0.011	0.147 ± 0.024	246.5 ± 26.5
Alg$^+$ PDO*ampR*	0.538 ± 0.026††	2.518 ± 0.640††	0.268 ± 0.017	0.143 ± 0.025	536.0 ± 19.0

*Induction was carried out using 500 μg benzylpenicillin ml^{-1} for *P. aeruginosa*.
†Pyocyanin concentrations were expressed as μg pyocyanin produced (μg total protein)$^{-1}$.
‡LasA activities were determined as the reduction of OD_{600} over a period of 1 h (μg total protein)$^{-1}$.
§β-Galactosidase assays were performed in a high-throughput 96-well array and the results expressed in Miller units.
‖These data are presented in a previous paper (Kong *et al.*, 2005b); they are included here for comparison.
¶$P<0.05$ between non-induced and induced in the same strain.
#$P<0.05$ between Alg$^-$ PAO1 and Alg$^-$ PAO*ampR* under the same conditions.
**$P<0.05$ between Alg$^-$ PAO1 and Alg$^+$ PDO300 under the same conditions.
††$P<0.05$ between Alg$^+$ PDO300 and Alg$^+$ PDO*ampR* under the same conditions.

PAO*ampR* and Alg$^+$ PDO*ampR*, as compared to their respective parental strains (Table 3). These results suggest that AmpR is a negative regulator of *lasA* expression.

QS gene expression in mucoid *ampR* mutants

In line with previous observations (Kong *et al.*, 2005b), we postulated that the slight increase of *lasA* expression in Alg$^+$ PDO*ampR* could be due to upregulation of the *las* system. To address this, all the four QS promoter fusions, P_{lasI}-*lacZ*, P_{lasR}-*lacZ*, P_{rhlI}-*lacZ* and P_{rhlR}-*lacZ* were introduced into Alg$^-$ PAO1, Alg$^-$ PAO*ampR*, Alg$^+$ PDO300 and Alg$^+$ PDO*ampR*. As we postulated, the Alg$^+$ PDO300 exhibited significantly lower QS gene expression as compared to Alg$^-$ PAO1. There was no difference in the P_{lasR} expression in Alg$^+$ PDO300 and Alg$^+$ PDO*ampR* (Fig. 3). However, the P_{lasI} activity was significantly increased in Alg$^+$ PDO*ampR*. Similar to the Alg$^-$ PAO*ampR*, the loss of *ampR* in Alg$^+$ PDO300 resulted in minimal alteration of P_{rhlI} and P_{rhlR} expression. Thus, AmpR negatively regulates *lasI* expression in alginate-overproducing strains.

Role of AmpR in virulence

The nematode *C. elegans* has been used as a bacterial pathogenesis model for the determination of virulence in *P. aeruginosa* (Gallagher & Manoil, 2001; Sifri *et al.*, 2005; Tan *et al.*, 1999). This simple host–pathogen interaction model was used to ascertain the virulence of Alg$^-$ PAO1, Alg$^-$ PAO*ampR*, Alg$^+$ PDO300 and Alg$^+$ PDO*ampR*. As expected, there was no observable paralysis in the negative control (*E. coli* OP50) plates (Fig. 4) and during the first hour of incubation with all the four isogenic *P. aeruginosa* strains. Consistent with the molecular and biochemical data, Alg$^-$ PAO*ampR* paralysed *C. elegans* at a significantly ($P<0.05$) faster rate than the wild-type Alg$^-$ PAO1 (Fig. 4). The lowest survival was seen at the second hour, 19 % with Alg$^-$ PAO*ampR*, as compared to 34 % with Alg$^-$ PAO1 (Fig. 4). In addition, Alg$^+$ PDO*ampR* also showed a higher virulence than Alg$^+$ PDO300 with 85 and 98 % survival at 4 h post-incubation, respectively (Fig. 4). The increase in virulence in Alg$^-$ PAO*ampR* and Alg$^+$ PDO*ampR* could be restored using pAmpR (Fig. 4), suggesting that AmpR acts as a negative regulator of *P. aeruginosa* virulence.

DISCUSSION

AmpR is the master transcriptional regulator involved in β-lactam antibiotic resistance. We have demonstrated

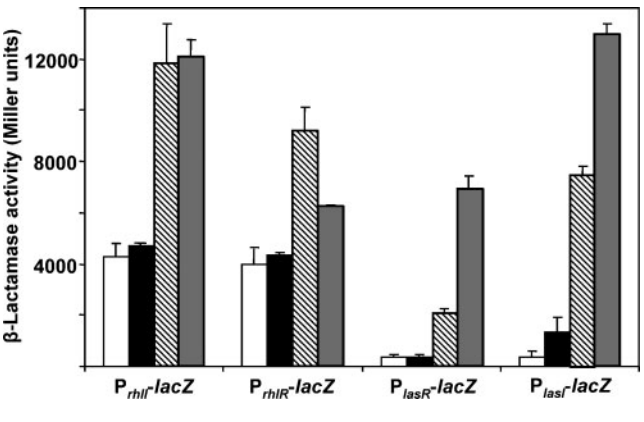

Fig. 3. The effects of the *ampR* mutation on QS *las* and *rhl* gene transcription. The alteration in the transcription of the QS systems in Alg$^-$ PAO1 (hatched bars), Alg$^-$ PAO*ampR* (grey bars), Alg$^+$ PDO300 (white bars) and Alg$^+$ PDO*ampR* (black bars) was monitored using four transcriptional fusions, P_{lasI}-*lacZ*, P_{lasR}-*lacZ*, P_{rhlI}-*lacZ* and P_{rhlR}-*lacZ*. The promoterless *lacZ* vector has a low basal level of activity of <20 Miller units.

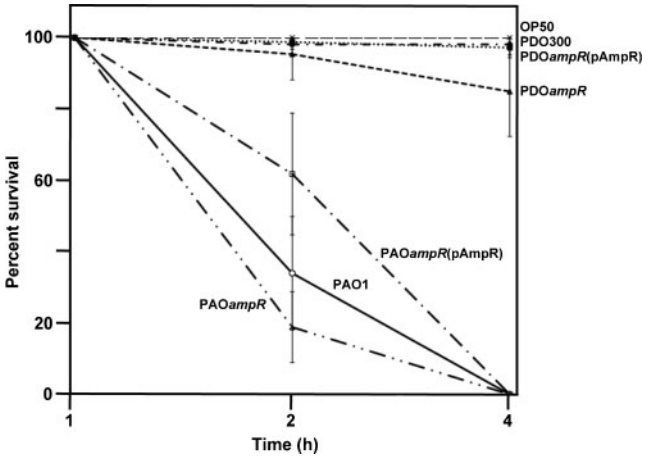

Fig. 4. Kinetics of the paralysis of *C. elegans* by *P. aeruginosa* Alg$^-$ PAO1 wild-type strain (—), Alg$^-$ PAO*ampR* (– · · –), complemented Alg$^-$ PAO*ampR*(pAmpR) (– · –), Alg$^+$ PDO300 (-··-), Alg$^+$ PDO*ampR* (--) and complemented Alg$^+$ PDO*ampR*(pAmpR) (··). L4 stage larval hermaphrodite Bristol N2 *C. elegans* were placed on each brain heart infusion agar plate containing a bacterial lawn and scored for dead worms by microscopic examination. *E. coli* OP50 (— —) was used as a negative control. Values are the mean ± SD of triplicate analyses. Results were statistically significant ($P<0.05$ for PAO1 vs PAOΔ*ampR* at 2 h, and PDO300 and PDO*ampR* at 4 h).

previously that in addition to regulating AmpC and PoxB β-lactamases, *P. aeruginosa* AmpR plays a role in controlling the expression of some virulence factors (Kong *et al.*, 2005b). In this study, we have shown that there is a complex regulatory network between β-lactam resistance, alginate production, and QS and virulence gene expression, factors determining the establishment of both acute and chronic *P. aeruginosa* infection.

ampR autoregulation requires AlgT/U

Previous studies in *Enterobacteriaceae* spp. have demonstrated that the transcription of *ampR* is autoregulatory (Lindquist *et al.*, 1989); but, we reported otherwise for the non-mucoid strain of *P. aeruginosa* (Kong *et al.*, 2005b). Data presented here show that the autoregulatory mechanism of *ampR* could be seen in the presence of inducers in an alginate overproducing strain, Alg$^+$ PDO300, but was lost in the absence of *ampR* (Table 2). This suggests that the regulation of *ampR* transcription requires AlgT/U, and is AmpR-dependent in Alg$^+$ PDO300. The requirement of these factors for autoregulation explains the inconsistency seen with the *Enterobacteriaceae* models: in an *in vivo* system using the heterologous host *E. coli*, the P_{ampR}-*lacZ* activity was repressed threefold in the presence of *Citrobacter freundii* AmpR (Lindquist *et al.*, 1989). However, this mode of regulation was lost in a minicell system with *Enterobacter cloacae* AmpR (Lindberg & Normark, 1987).

The intriguing autoregulatory mechanism seen in the alginate-overproducing PAO1 derivative may have important clinical implications: the data with Alg$^-$ PAO1 suggest that this early colonizer is able to induce the production of β-lactamases upon β-lactam chemotherapy. However, this non-mucoid strain is unable to autoregulate *ampR*, indicating that the production of β-lactamases is induced only upon contact with β-lactam antibiotics. This phenomenon may be disadvantageous during antibiotic selections. Persistence of the organism in the lungs of patients with CF will ultimately result in the selection of mucoid strains that hyperproduce alginate (Høiby, 1975). This phenotypic alteration is accompanied by resistance to antibiotics and immune clearance (Giwercman *et al.*, 1991). Data from Alg$^+$ PDO300 suggest that the selected mucoid *P. aeruginosa* strains are primed to β-lactam resistance by the increased production of AmpR, and hence β-lactamases, upon contact with β-lactam antibiotics. This observation should be further verified using clinical strains with commonly used β-lactams.

AmpR is a negative regulator of *algT/U*

The simultaneous presence of β-lactam resistance and alginate-overproduction suggests a possible co-regulation of these phenomena. We have shown here that autoregulation of *ampR* is AlgT/U-dependent. Loss of the *ampR* gene in Alg$^-$ PAO1 resulted in a significant increase in the promoter activity of *algT/U* operon (Fig. 2). However, this did not phenotypically alter Alg$^-$ PAO1 to an Alg$^+$ phenotype due to the post-transcriptional control of AlgT/U by the anti-sigma factor, MucA, expressed downstream of *algT/U* (Hughes & Mathee, 1998). In Alg$^+$ PDO300, like in Alg$^-$ PAO1, there is an increase of *algT/U* expression upon loss of *ampR*. Data from these two strain backgrounds suggest that AmpR suppresses the expression of *algT/U*.

The possible mechanistic interaction between the *alg* and *amp* regulons has been reported in *Azotobacter vinelandii*, where a mutation in *ampDE*, encoding negative regulators of β-lactamases, resulted in elevated expression of alginate biosynthetic genes (Núñez *et al.*, 2000). In addition, microarray data also have demonstrated that alginate production is induced upon antibiotic challenge (Bagge *et al.*, 2004), and a later study identified AlgT, AlgW and Prc proteases as being involved in this process (Wood *et al.*, 2006). Our results are further supportive of their findings in which β-lactam resistance and alginate production of *P. aeruginosa* are co-regulated. This co-regulation is likely mediated by AmpR-AlgT/U interaction. Future studies will address this potential interaction.

AlgT/U and AmpR are regulators of virulence factors

Multiple QS-dependent phenotypes, including LasA and pyocyanin production, are differentially regulated in an *ampR* mutant, and are probably an indirect effect of AmpR on the QS system. We have previously shown that deletion

of *ampR* gene increased the production of LasA protease in an Alg^- strain, suggesting that *lasA* expression is suppressed by AmpR (Kong *et al.*, 2005b). We postulated that a similar observation should be obtained in an Alg^+ strain. As expected, the absence of *ampR* in the presence of functional AlgT/U elevated the promoter expression of *lasA* and the production of LasA protease (Table 3). This alteration in LasA synthesis suggests that both AlgT/U and AmpR negatively impact transcription of the *lasA* gene. Although the inverse correlation between alginate and protease production has been repeatedly reported, our results establish that this correlation is mediated through the downregulation of QS in Alg^+ strains. Comparing two isogenic strains, Alg^- PAO1 and Alg^+ PDO300, the see-saw effect is brought upon by the ECF sigma factor, AlgT/U. Since sigma factor, an essential component of RNA polymerase, is unlikely to be involved in the repression of gene expression, AlgT/U-mediated downregulation of QS genes is probably indirect.

To determine whether the *in vitro* alterations in virulence factor expression could be translated into significant *in vivo* killing, the *C. elegans–P. aeruginosa* interaction model was employed. As predicted, loss of *ampR* strongly correlated with an increase in virulence with both Alg^- PAO*ampR* and Alg^+ PDO*ampR* showing higher rates of *C. elegans* paralysis as compared to their parent strains (Alg^- PAO1 and Alg^+ PDO300, respectively). The significantly higher amounts of pigmentation produced by *ampR* mutants compared to the isogenic wild-type strain explains the higher killing rate, which is in agreement with other studies (Tan *et al.*, 1999).

Concluding remarks

The data presented here reveal a complex co-regulatory network between β-lactam resistance, alginate production, QS and virulence gene expression. We have previously shown that AmpR regulates AmpC and PoxB β-lactamases and QS-dependent proteases (Kong *et al.*, 2005a, b). In this paper, that observation is further extended to include the alginate master regulator, AlgT/U. Importantly, we show that the positive autoregulation of *ampR* requires AlgT/U, whereas AmpR negatively regulates *algT/U* expression (Fig. 2) serving as a negative feedback loop to limit the AlgT/U expression. We propose that this intimate crosstalk between these two global regulators provides a potential molecular framework for the simultaneous occurrence of β-lactam resistance and alginate-overproducing strains in chronic CF lung infections. Further studies on clinical isolates are warranted to understand the complex regulatory network linking all these critical factors in establishing infections. Delineating the interplaying factors and regulatory network is of fundamental significance to understanding the pathogenesis of *P. aeruginosa*.

ACKNOWLEDGEMENTS

This work was partially supported by the NIH (MBRS SCORE grant S06 GM08205 to K. M.), NIGMS (RISE grant R25 GM61347 to R. T. S.), Florida International University teaching assistantships to D. B., K.-F. K., S. R. J. and R. T. S.), Biomedical Research Initiative student research award (R25 GM61347 to R. T. S) and Cystic Fibrosis Foundation student traineeship (KONG05HO to K.-F. K.; LEAL05HO to S. M. L.). We thank Barbara Iglewski for generously sharing all the promoter-fusion plasmids.

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