Co-regulation of \(\beta\)-lactam resistance, alginate production and quorum sensing in \textit{Pseudomonas aeruginosa}

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Development of \(\beta\)-lactam resistance, production of alginate and modulation of virulence factor expression that alters host immune responses are the hallmarks of chronic \textit{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 \(\beta\)-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 \textit{P. aeruginosa} strains, the prototypic Alg\(^{-}\)PAO1, Alg\(^{-}\)PAOampR, the mucoid Alg\(^{+}\)PAOmucA22 \((\text{Alg}^{+}\ P\text{DOampR})\) and Alg\(^{+}\)PAOmucA22ampR \((\text{Alg}^{+}\ P\text{DOampR})\) were used. We found that in the presence of AmpR regulator and \(\beta\)-lactam antibiotic, the extracytoplasmic function sigma factor AlgT/U positively regulated \(P_{\text{ampR}}\) whereas AmpR negatively regulated \(P_{\text{algT/U}}\). 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 \(P_{\text{last}}\) and \(P_{\text{lasA}}\) 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\(^{-}\)PAOampR and Alg\(^{+}\)PDAOampR. The enhanced expression of virulence factors in the \(ampR\) strains correlated with a higher rate of \textit{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 \(\beta\)-lactam resistance, alginate production, QS and virulence factor production, with AmpR playing a central role.

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

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  \item Greenberg, 2000; Rahme et al., 1995; Govan & Harris, 1986; Lyczak et al., 2002; Pedersen, 1992.
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\textbf{INTRODUCTION}

\textit{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 \textit{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, \(\sigma^{22}\), 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 (Fioreved et al., 2002; Fioreved & Deretic, 2003; Malhotra et al., 2000). Mucoid \textit{P. aeruginosa} isolates from CF patients frequently have a defective \textit{mucA} allele, a gene downstream of \(algT/U\) (Martin et al., 1993). The \textit{mucA} gene product is an anti-sigma factor that negatively regulates the activity of AlgT/U (Hughes & Mathee, 1998).
**P. aeruginosa** is intrinsically resistant to most \-lactam antibiotics. One of the factors contributing to the resistance is the existence of enzymes that can deactivate \-lactams, known as \-lactamases (Kong et al., 2010; Robinson, 1998). Two inducible chromosome-encoded \-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 \-lactamases and pyocyanin, increased LasA staphyloolig 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). Transcriptional 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- PAOampR, 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, \-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 + PDOampR mutant strain. This mutant produced exceedingly high levels of \-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 benzylpenicillin 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 (PAOmuca22) was performed as previously reported using the same constructs (Kong et al., 2005b). The *ampR::aacCl* fragment subcloned into pEX100T (Schuster & Schweizer, 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* (PAOmuca22ampR) is named Alg + PDOampR (Table 1). Complementation studies were performed using plasmid pS106 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 pS10 (P*ampC-lacZ*) and pS11 (P*ampR-lacZ*) (Table 1) (Kong et al., 2005b). The resulting clones were mobilized into Alg + PDO300 and Alg + PDOampR (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 (Brennic 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 N55 random primers to total purified RNA. Subsequent extension was carried out using SuperScript III reverse transcriptase (Invitrogen) (Brennic 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).

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

**\-Galactosidase assay.** Assays for \-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− φ80lacZΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17(rk−, m−) | New England Biolabs |
| TOP10F’ | F’[lacI, Tn10(TetR)] mcrA (mrr-hsdS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 D(ara-leu)/7697 galU galK rpsL (StrR) endA1 supG | Invitrogen |
| **P. aeruginosa** | | |
| PAO1 | Prototype; Alg− | Holloway & Morgan (1986) |
| PDO300 | PAOmucA22; Alg+ | Mathee et al. (1999) |
| PKM805 | PAOmucA22algT24-1; Alg− | PDO300 derivative; Ramos et al. (2003) |
| PKM300 | PAOampR::aacCl; Alg− | PDOampR; Kong et al. (2005b) |
| PKM307 | PAOmucA22 ampR::aacCl; Alg+ | PDOampR; this study |
| PKM308 | PAOmucA22 attB::PampC-lacZ; TcR, Alg+ | PDO300 derivative; this study |
| PKM309 | PAOmucA22 attB::PampR-lacZ; TcR, Alg+ | PDO300 derivative; this study |
| PKM310 | PAOmucA22 ampR::aacCl attB::PampC-lacZ; GmR, TcR, Alg+ | PDOampR derivative; this study |
| PKM311 | PAOmucA22 ampR::aacCl attB::PampR-lacZ; GmR, TcR, Alg+ | PDOampR derivative; this study |

| Plasmids | | |
| --- | --- | --- |
| Mini-CTX-lacZ | TcR; integration-proficient vector for single-copy chromosomal lacZ fusion | Becher & Schweizer (2000) |
| pEX100T | ApR; sacB oriT | Schweizer & Hoang (1995) |
| pGEMEX-1 | ApR; ColEl ori lacZx | Promega |
| pKMG37 | ApR; pQF50 containing Palg−-lacZ transcriptional fusion | Mathee et al. (1997) |
| pLP170 | ApR; lacZ transcriptional fusion vector that contains an RNase III splice sequence positioned between the MCS and lacZ | Preston et al. (1997) |
| pLPLA | ApR; pLP170 containing Palg−-lacZ transcriptional fusion | Preston et al. (1997) |
| pLPLB | ApR; pLP170 containing Palg−-lacZ transcriptional fusion | Preston et al. (1997) |
| pLPRI | ApR; pLP170 containing Palg−-lacZ transcriptional fusion | Van Delden & Iglewski (1998) |
| pME6030 | TcR; oriV<sub>PVs</sub>, oriV<sub>pl5α</sub> oriT | Heeb et al. (2000) |
| pPC5223 | ApR; pLP170 containing Palg−-lacZ transcriptional fusion | Van Delden & Iglewski (1998) |
| pPC1001 | ApR; pLP170 containing Palg−-lacZ transcriptional fusion | Pesci et al. (1997) |
| pPC1002 | ApR; pLP170 containing Palg−-lacZ transcriptional fusion | Pesci et al. (1997) |
| pQF50 | ApR; broad-host-range vector with promoterless lacZ | Farinha & Kropinski (1990) |
| pRK2013 | KmR; ColEl-ori-Tra (RK2)+ | Figurski & Helinski (1979) |
| pSV1 | ApR; pGEMEX-1 with a 1220 bp EcoRI–BamHI flanked fragment containing ampR | Kong et al. (2005b) |
| pSV6 | ApR; pME6030 with a 1220 bp EcoRI–BamHI flanked fragment containing ampR (referred to as pAmpR) | Kong et al. (2005b) |
| pSV7 | ApR; pEX100T derivative with ampR::aacCl | Kong et al. (2005b) |
| pSV9 | ApR; GmR; pGEMEX-1 with a 330 bp EcoRI–BamHI flanked fragment containing ampC-ampR intergenic region | Kong et al. (2005b) |
| pSV10 | TcR; CTX-lacZ fused with ampC promoter, P<sub>ampC</sub> | Kong et al. (2005b) |
| pSV11 | TcR; CTX-lacZ fused with ampR promoter, P<sub>ampR</sub> | Kong et al. (2005b) |
| pUCGm | ApR; GmR; pUC19 derivative containing gentamicin cassette | Schweizer (1993) |

*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 % K₂HPO₄, 0.3 % NaCl, 0.012 % MgSO₄, 0.011 % CaCl₂, 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⁻ PDOampR 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⁺ PDOampR 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⁺ PDOampR.

Based on the above analysis, we expected to observe a loss of β-lactamase activity concomitant with the loss of ampR. However, the Alg⁺ PDOampR 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⁺ PDOampR 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⁺ PDOampR. 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⁻ PDO300 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⁺ PDOampR. 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⁻ PDOalgT strains. The Alg⁺ PDO300 strain showed an increase in the ampR mRNA levels (relative quantity of 2.1 ± 0.2 compared to 1.0 ± 0.0 in Alg⁻ PAO1) indicating positive regulation of ampR by AlgT/U. Mutation in algT/U in Alg⁻ PDOalgT 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⁺ PAOampR†| 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⁺ PDOampR | 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⁺ PAOampR.
§ANOVA compares the activity values between the Alg⁺ PDO300 and the mutant Alg⁺ PDOampR.
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 Alg² PDOampR 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⁻ PAOampR 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⁺ PDOampR 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⁺ PDOampR 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⁺ PDOampR**

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⁺ PDOampR (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⁻ PDOampR.
Induction was carried out using 500 μg benzylpenicillin ml⁻¹ for P. aeruginosa.

Inducer* Pyocyanin [μg (μg total protein)^−1]| LasA [ΔOD₆₀₀ h⁻¹ (μg protein)^−1]| P₉₅₅- 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^- PAOampR || 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^+ PDOampR || 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 OD₆₀₀ over a period of 1 h (μg total protein)^−1.
§LasA activities were determined as the reduction of OD₆₀₀ over a period of 1 h (μg total protein)^−1.
†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^- PAOampR 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^+ PDOampR under the same conditions.

PAOampR and Alg^+ PDOampR, 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^+ PDOampR could be due to upregulation of the las system. To address this, all the four QS promoter fusions, P₉₅₅- lacZ, P₆₀₀- lacZ, P₆₀₀- lacZ and P₆₀₀- lacZ were introduced into Alg^- PAO1, Alg^- PAOampR, Alg^+ PDO300 and Alg^+ PDOampR. As we postulated, the Alg^+ PDO300 exhibited significantly lower QS gene expression as compared to Alg^- PAO1. There was no difference in the P₆₀₀ expression in Alg^- PDO300 and Alg^- PDOampR (Fig. 3). However, the P₆₀₀ activity was significantly increased in Alg^+ PDOampR. Similar to the Alg^- PAOampR, the loss of ampR in Alg^- PDO300 resulted in minimal alteration of P₆₀₀ and P₆₀₀ expression. Thus, AmpR negatively regulates lasA 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^- PAOampR, Alg^+ PDO300 and Alg^+ PDOampR. 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^- PAOampR 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^- PAOampR, as compared to 34 % with Alg^- PAO1 (Fig. 4). In addition, Alg^+ PDOampR 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^- PAOampR and Alg^+ PDOampR 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
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previously that in addition to regulating AmpC and PoxB \(\beta\)-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 \(\beta\)-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 \(\beta\)-lactamases upon \(\beta\)-lactam chemotherapy. However, this non-mucoid strain is unable to autoregulate *ampR*, indicating that the production of \(\beta\)-lactamases is induced only upon contact with \(\beta\)-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 \(\beta\)-lactam resistance by the increased production of AmpR, and hence \(\beta\)-lactamases, upon contact with \(\beta\)-lactam antibiotics. This observation should be further verified using clinical strains with commonly used \(\beta\)-lactams.

**AmpR is a negative regulator of algT/U**

The simultaneous presence of \(\beta\)-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 \(\beta\)-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 \(\beta\)-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⁻ PAOampR and Alg⁺ PDOampR 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*.

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