Co-regulation of $\beta$-lactam resistance, alginate production and quorum sensing in 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 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 P. aeruginosa strains, the prototypic Alg− PAO1, Alg− PAOampR, the mucoid Alg+ PAOmucA22 (Alg+ PDO300) and Alg+ PAOmucA22ampR (Alg+ PDOampR) 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_{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− PAOampR and Alg+ PDOampR. 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 $\beta$-lactam resistance, alginate production, QS and virulence factor production, with AmpR playing a central role.

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, $\sigma^{22}$, 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 (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).

†These authors contributed equally to this work.

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 staphylocltytic 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− 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, 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+ PDOampR 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 (PAOmuacA22) 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 carbencilin 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 (PAOmuacA2ampR) is named Alg+ PDOampR (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 (PampC::lacZ) and pSJ11 (PampR::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 OD600 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 (Brenic 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) (Brenic 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 (PAI802) of the ATP-dependent protease was used as the internal control. Assays were performed in triplicate. Primers 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

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F⁻ 808lacZAM15 Δ(lacZYA-argF) U169 deor recA1 endA1 hsdR17(rK−, mK+)</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td></td>
<td>phoA supE44 λ- thi-1 gyrA96 relA</td>
<td></td>
</tr>
<tr>
<td>TOP10F'</td>
<td>F'[lacI, Tn10[TeF']3] mcrA Δ(ntrC-hsdS-MCS-argRBC) 808lacZAM15 ΔlacX74 deor</td>
<td>Invitrogen</td>
</tr>
<tr>
<td></td>
<td>recA1 araD139 D(ara-leu)7697 galU galK rpsL (StrR) endA1 supG</td>
<td></td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Prototype; Alg−</td>
<td>Holloway &amp; Morgan (1986)</td>
</tr>
<tr>
<td>PDO300</td>
<td>PAO mucA22; Alg+</td>
<td>Mathee et al. (1999)</td>
</tr>
<tr>
<td>PKM805</td>
<td>PAO mucA22algT24-1; Alg−</td>
<td>PDOAlgT; PDO300 derivative; Ramos et al. (2003)</td>
</tr>
<tr>
<td>PKM300</td>
<td>PAO2ampR::aacCl; Alg−</td>
<td>PDO2ampR; Kong et al. (2005b)</td>
</tr>
<tr>
<td>PKM307</td>
<td>PAO2ampR::aacCl; Alg+</td>
<td>PDO2ampR; this study</td>
</tr>
<tr>
<td>PKM308</td>
<td>PAO2ampR::aacCl; Alg+</td>
<td>PDO2ampR; this study</td>
</tr>
<tr>
<td>PKM309</td>
<td>PAO2ampR::aacCl; Alg+</td>
<td>PDO2ampR; this study</td>
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<td>PDO2ampR; this study</td>
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<tr>
<td>PKM311</td>
<td>PAO2ampR::aacCl; Alg+</td>
<td>PDO2ampR; this study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEX100T</td>
<td>ApR; sacB oriT</td>
<td>Schweizer &amp; Hoang (1995)</td>
</tr>
<tr>
<td>pGEMEX-1</td>
<td>ApR; ColElori lacZx</td>
<td>Promega</td>
</tr>
<tr>
<td>pKMG37</td>
<td>ApR; pQS50 containing Palg-lacZ transcriptional fusion</td>
<td>Mathee et al. (1997)</td>
</tr>
<tr>
<td>pLP170</td>
<td>ApR; lacZ transcriptional fusion vector that contains an RNase III splice sequence positioned between the MCS and lacZ</td>
<td>Preston et al. (1997)</td>
</tr>
<tr>
<td>pLPLA</td>
<td>ApR; pLP170 containing Pala-lacZ transcriptional fusion</td>
<td>Preston et al. (1997)</td>
</tr>
<tr>
<td>pLPLB</td>
<td>ApR; pLP170 containing Pala-lacZ transcriptional fusion</td>
<td>Preston et al. (1997)</td>
</tr>
<tr>
<td>pLPR1</td>
<td>ApR; pLP170 containing Pala-lacZ transcriptional fusion</td>
<td>Van Delden &amp; Iglewski (1998)</td>
</tr>
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<td>pME6300</td>
<td>TcR; oriV&lt;sub&gt;Fvs1&lt;/sub&gt;, oriV&lt;sub&gt;P13A&lt;/sub&gt;, oriT</td>
<td>Heeb et al. (2000)</td>
</tr>
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<td>pPCS223</td>
<td>ApR; pLP170 containing Pala-lacZ transcriptional fusion</td>
<td>Van Delden &amp; Iglewski (1998)</td>
</tr>
<tr>
<td>pPCS1001</td>
<td>ApR; pLP170 containing Pala-lacZ transcriptional fusion</td>
<td>Pesci et al. (1997)</td>
</tr>
<tr>
<td>pPCS1002</td>
<td>ApR; pLP170 containing Pala-lacZ transcriptional fusion</td>
<td>Pesci et al. (1997)</td>
</tr>
<tr>
<td>pQ50</td>
<td>ApR; broad-host-range vector with promoterless lacZ</td>
<td>Farinha &amp; Kropinski (1990)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>KmR; ColElori-Tra (RK2)</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pS101</td>
<td>ApR; pGEMEX-1 with a 1220 bp EcoRI-BamHI flanked fragment containing ampR</td>
<td>Kong et al. (2005b)</td>
</tr>
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<td>pS106</td>
<td>ApR; pME6300 with a 1220 bp EcoRI-BamHI flanked fragment containing ampR (referred to as pAMPPr)</td>
<td>Kong et al. (2005b)</td>
</tr>
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<td>pS107</td>
<td>ApR; pEX100T derivative with ampR::aacCl</td>
<td>Kong et al. (2005b)</td>
</tr>
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<td>pS109</td>
<td>ApR; GmR; pGEMEX-1 with a 330 bp EcoRI-BamHI flanked fragment containing ampC-ampR intergenic region</td>
<td>Kong et al. (2005b)</td>
</tr>
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<td>pS110</td>
<td>ApR; CTX-lacZ fused with ampC promoter, PampC</td>
<td>Kong et al. (2005b)</td>
</tr>
<tr>
<td>pU6Gm</td>
<td>ApR; GmR; pUC19 derivative containing gentamicin cassette</td>
<td>Schweizer (1993)</td>
</tr>
</tbody>
</table>

*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 hermaphroditic Bristol N2 C. elegans was washed off an E. coli OP50-seeded nematode growth medium plate (1.7% agar, 0.35% peptone,
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

PampC 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
ture in the Alg+ background, strains were constructed with
a single copy of the ampC promoter fused to a promoterless
reporter gene, lacZ (PampC-lacZ). This was integrated into the Alg+
PDAO300 and the Alg+ PDOampR
chromosomes via attB-attP site-specific recombination,
thus allowing mimicking of the chromosomal regulation.
In the absence of inducer, the PampC-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 PampC 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
pAMP. 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 PampR-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 PampR 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 PampR
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

<table>
<thead>
<tr>
<th>Strain</th>
<th>PampC-lacZ (Miller units)</th>
<th>P value*</th>
<th>PampR-lacZ (Miller units)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-induced</td>
<td>Induced</td>
<td></td>
<td>Non-induced</td>
</tr>
<tr>
<td>Alg− PAO1</td>
<td>124.1 ± 11.6</td>
<td>1644.2 ± 33.7</td>
<td>&lt;0.05</td>
<td>77.1 ± 8.7</td>
</tr>
<tr>
<td>Alg+ PAOampR†</td>
<td>113.2 ± 7.5</td>
<td>122.3 ± 7.4</td>
<td>NS</td>
<td>96.3 ± 15.2</td>
</tr>
<tr>
<td>P value‡</td>
<td>NS</td>
<td>&lt;0.05</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Alg− PDO300</td>
<td>104.2 ± 4.5</td>
<td>957.5 ± 161.4</td>
<td>&lt;0.05</td>
<td>79.5 ± 26.3</td>
</tr>
<tr>
<td>Alg+ PDOampR</td>
<td>142.4 ± 4.9</td>
<td>143.8 ± 6.9</td>
<td>NS</td>
<td>155.8 ± 0.8</td>
</tr>
<tr>
<td>P value§</td>
<td>NS</td>
<td>&lt;0.05</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

*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 Alg⁺ 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.
To address this, all the four QS promoter fusions, P_D and respective parental strains (Table 3). These results suggest in line with previous observations (Kong et al., 1999), we postulated that the slight increase of las expression in mucoid Alg 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_las-lacZ, P_rhl-lacZ, P_las-lacZ and P_rhl-lacZ. The promoterless lacZ vector has a low basal level of activity of <20 Miller units.](image)

**QS gene expression in mucoid ampR mutants**

In line with previous observations (Kong et al., 2005b), we postulated that the slight increase of las expression in Alg PDO AmpR could be due to upregulation of the las system. To address this, all the four QS promoter fusions, P_las-lacZ, P_las-lacZ, P_rhl-lacZ and P_rhl-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_las expression in Alg PDO300 and Alg PDO AmpR (Fig. 3). However, the P_las 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_rhl and P_rhl expression. Thus, AmpR negatively regulates lasl 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 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

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**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).

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**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 PAO\(ampR\) at 2 h, and PDO300 and PDO\(ampR\) at 4 h).
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 polymerases, 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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