Pseudomonas aeruginosa biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-translationally by RsmA

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Summary

Extracellular polysaccharides are important components of biofilms. In non-mucoid Pseudomonas aeruginosa strains, the Pel and Psl polysaccharides are major structural components of the biofilm matrix. In this study, we demonstrate that the alternative σ-factor RpoS is a positive transcriptional regulator of psl gene expression. Furthermore, we show that psl mRNA has an extensive 5’ untranslated region, to which the post-transcriptional regulator RsmA binds and represses psl translation. Our observations suggest that upon binding RsmA, the region spanning the ribosome binding site of psl mRNA folds into a secondary stem-loop structure that blocks the Shine–Dalgarno sequence, preventing ribosome access and protein translation. This constitutes a novel mechanism for translational repression by this family of regulators.

Introduction

Pseudomonas aeruginosa is found in a variety of environmental niches and can cause chronic, as well as acute infections in humans. Cystic fibrosis (CF) patients’ airways are chronically infected with P. aeruginosa exhibiting the biofilm mode of growth. Microbial biofilms are surface-associated microorganisms encased within an extracellular matrix. Extracellular polysaccharides are key components of the matrix, critical for building and maintaining biofilm structure (Sutherland, 2001; Branda et al., 2005). P. aeruginosa produces at least three different extracellular polysaccharides that can contribute to the matrix: alginate, Pel and Psl (Ryder et al., 2007). Mucoid variants that overproduce alginate are predominant colony morphotypes usually isolated from older CF patients (Govan and Deretic, 1996). Alginate is an important biofilm matrix component in mucoid strains (Hentzer et al., 2001). In non-mucoid strains, Pel and Psl polysaccharides have been shown to be critical for biofilm formation (Friedman and Kolter, 2004; Jackson et al., 2004; Matsukawa and Greenberg, 2004). Colony morphology variants that overproduce Pel and Psl have also been isolated from CF patients (Drenkard and Ausubel, 2002; Kirisits et al., 2005; Smith et al., 2006). These variants are called rugose small colony variants (RSCVs) and are characterized by a small, wrinkly appearance on solid medium. The prevalence of both mucoid and RSCV phenotypes in the CF environment highlights the importance of alginate, Pel and Psl for chronic infection and further suggests the importance of biofilm growth in this environment.

In contrast to alginate expression, whose regulation has been a focus of research for some time, we know very little regarding the regulation of pel and psl expression. Recent studies have described the transcriptional regulation of pel and psl by a secondary messenger molecule c-di-GMP and a transcription factor FleQ (Hickman and Harwood, 2008). Furthermore, c-di-GMP was recently reported to bind and allosterically regulate the activity of PelD, activating Pel synthesis and secretion (Lee et al., 2007). Finally, quorum sensing has been suggested to...
positively regulate *pel* and *psl* expression (Sakuragi and Kolter, 2007; Gilbert et al., 2009).

There are many examples of transcriptional control of extracellular polysaccharide genes in various species. There is also evidence for control at the level of protein translation. One example is the negative control of poly-β-1,6-N-acetyl-D-glucosamine (PGA) expression by the regulator CsrA in *Escherichia coli* (Wang et al., 2005). PGA is an extracellular polysaccharide important for biofilm formation in *E. coli* (Itoh et al., 2008). The CsrA/RsmA family of RNA binding proteins has been described in a variety of bacterial species to regulate protein translation (Lapouge et al., 2008; Babitzke et al., 2009). All known examples of CsrA/RsmA-mediated translational repression involve the binding of this protein to the mRNA leader and/or proximal coding region, thus blocking the access of ribosomes to the translation initiation region (Baker et al., 2002; Dubey et al., 2003; Wang et al., 2005, 2007; Lapouge, 2008). Previous studies of *rsmA* mutant strains of *P. aeruginosa* revealed possible involvement of RsmA in regulatory pathways important for quorum sensing, virulence, biofilm formation and motility (Pessi et al., 2001; Heurlier et al., 2004; Mulcahy et al., 2006, 2008). Whether these regulatory influences are due to direct regulation by RsmA or pleiotropic effects remains to be demonstrated.

In this study, we demonstrate that *psl* expression is controlled transcriptionally and translationally through two independent mechanisms. First we show that the stationary-phase σ-factor RpoS is a transcription factor that positively regulates *psl* expression. Strains lacking *rpoS* exhibit reduced *psl* transcripts and products. Overexpression of *rpoS* results in elevated *psl* transcription and an RSCV colony morphology on solid medium, which is a phenotype consistent with Psl overproduction. We also demonstrate that the *psl* transcript contains a large 5′ untranslated region (UTR). We then show that the post-transcriptional regulator RsmA directly binds to sequences within the 5′ UTR of the *psl* mRNA, repressing translation of *pslA*. We propose a novel mechanism for RsmA control that involves RsmA stabilization of a stem-loop structure in the mRNA that blocks ribosome access to the *pslA* Shine–Dalgarno (SD) sequence.

**Results**

**Determining the transcriptional start site of the psl operon**

To initiate our study of the regulation of *psl* expression, we first performed 5′ RACE. The transcriptional start site was found to be 148 bp upstream of the *pslA* open reading frame (ORF) (Fig. 1A). Interestingly, this result was not consistent with a previous study that also employed 5′ RACE (Overhage et al., 2005). Overhage et al. reported the transcriptional start site to be 41 bp upstream of the ATG of *pslA* (Fig. 1A). They predicted the corresponding −10 and −35 promoter regions resembled the consensus sequence for σ^70^L. However, the predicted promoter sequences are positioned with the −10 region centred approximately 30 bp upstream of their transcriptional start site. This was unusual as the typical distance between the

![Fig. 1](image_url). Two possible transcriptional start sites of *psl*.

A. Transcriptional start site (+1 position) was determined by 5′ RACE analysis. Predicted RpoS/σ^70^-like −10 and −35 sites are underlined. The ‘extended −10 region’, a hallmark of σ^70^-dependent promoters, is denoted by positions −13 and −14 as indicated by arrows. A previously reported transcriptional start site is positioned 108 bp downstream of the +1 (Overhage et al., 2005). σ^70^-like −10 and −35 sites predicted by Overhage et al. are also underlined. *pslA* ORF is boxed, and the first nucleotide position of *pslA* is labelled as position +149.

B. Sequence alignment of RpoS-dependent −10 and ‘extended −10’ consensus compiled from previous reports (Schuster et al., 2004; Typas et al., 2007) and the predicted *psl* −10 and ‘extended −10’ sequence (K = T/G). Nucleotides that were 100% conserved in all examined RpoS-dependent promoters by Schuster et al. are labelled with asterisks.

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In P. aeruginosa, RpoS is an alternative σ-factor that regulates expression of a number of genes in the stationary phase (Schuster et al., 2004). Schuster et al. observed that all the genes in the psl operon (pslA–L) were upregulated during stationary-phase growth in wild-type (WT) PAO1, but not in an isogenic ΔrpoS mutant strain. We therefore tested whether RpoS controls psl transcription. As shown by quantitative real-time polymerase chain reaction (PCR) in Fig. 3A, psl transcripts increased about threefold in stationary phase compared with mid-log phase in WT cultures, but ΔrpoS failed to show an increase in psl transcription during stationary phase. To confirm a functional consequence for the RpoS-dependent increase in psl transcription in stationary phase, we analysed relative Psl levels using Psl-specific antisera (Fig. 3D). As predicted from the quantitative real-time PCR data, the amount of Psl produced by WT and ΔrpoS were low during mid-log phase, and only WT increased during stationary phase.

To further investigate the involvement of RpoS in psl expression, we overexpressed RpoS and observed that psl transcription increased significantly (Fig. 3B). Consistent with previous work examining the effects of psl overexpression (Ma et al., 2006), strains overexpressing RpoS exhibited the RSCV morphology (Fig. 3C) and increased Psl production (Fig. 3E). The RSCV phenotype is lost when RpoS is overexpressed in a Δpsl background, verifying that the RSCV phenotype is due to Psl production.

We also observed that our newly predicted promoter sequences of the psl operon strongly resemble that of an RpoS/α5-dependent promoter. The –10 region of psl aligned well with the previously derived consensus sequence (identical to that of E. coli) for the P. aeruginosa RpoS-dependent promoters (Fig. 1B) (Schuster et al., 2004). The ‘extended –10 region’, known as the –13 and –14 positions, is a highly conserved hallmark of α5-dependent promoters in E. coli that distinguishes them from α70 promoters (Typas et al., 2007). The E. coli consensus for the –13 position is a C, and a G/T for the –14 position (Barne et al., 1997). Our predicted psl –10 sequence is a perfect match to the consensus motif (Fig. 1B).

ΔrsmA mutations confer a Psl-dependent RSCV phenotype

Previous work on the RSCV phenotype has linked it to elevated pel and psl expression (Friedman and Kolter, 2004; Hickman et al., 2005; Kirisits et al., 2005; Ueda and Wood, 2009; Malone et al., 2010). In the well-characterized ΔwspF RSCV background, this mutation
results in the hyper-activation of WspR, producing elevated levels of c-di-GMP (Hickman et al., 2005). This results in enhanced transcription of the pel and psl operons (Hickman et al., 2005; Kirisits et al., 2005). Mutations in both pel and psl in a ΔwspF strain convert the RSCV phenotype to a WT smooth colony (Fig. 4A) while no colony morphology changes are observed when pel and psl are mutated in the WT background (Fig. S1).

We observed that ΔrsmA mutant strains produced a RSCV colony morphology similar to that of ΔwspF (Fig. 4A). We therefore hypothesized that both Pel and Psl contribute to the RSCV phenotype in the ΔrsmA strain. However, unlike ΔwspF, a ΔrsmA mutant strain only required a psl mutation to convert the RSCV to a smooth phenotype (Fig. 4A). This observation suggested that only Psl contributes to the RSCV phenotype of a ΔrsmA strain and that Psl expression is elevated in this background. We subsequently confirmed elevated Psl levels in a ΔrsmA strain by performing α-Psl immunoblots (Fig. 4B).

Differences in the Pel and Psl dependence of the RSCV phenotypes in ΔrsmA and ΔwspF strains suggested that the RSCV formation followed separate molecular mechanisms. RsmA overexpression in a ΔwspF background and WspF overexpression in a ΔrsmA background failed to reverse the RSCV phenotypes to WT colony morphologies (Fig. 5), further supporting that Wsp-dependent and RsmA-dependent RSCV pathways are epistatically independent of each other.

psl translation, not transcription, is elevated in a ΔrsmA mutant strain

As Psl expression was elevated in a ΔrsmA strain, we hypothesized that RsmA was acting as a translational repressor of psl expression. In order to test this, we
generated pslA chromosomal transcriptional and translational lacZ fusion reporter strains. We compared expression of psl transcription and translation in stationary-phase cultures of a WT and ΔrsmA.

Translational fusion data showed that psl translation was threefold to fourfold higher in ΔrsmA than WT (Figs 6B and S2B), despite psl transcriptional activity being similar (Figs 6A and S2A). Elevated psl translational activity in ΔrsmA could be complemented by overexpressing RsmA (Fig. 6C). RsmA overexpression further reduced the activity below the WT level, providing additional evidence that RsmA acts as a repressor of psl.

Previous reports have concluded that RsmA expression is elevated in stationary phase (Pessi et al., 2001; Brencic and Lory, 2009). This is somewhat paradoxical, as RpoS activates psl transcription maximally at stationary phase, while RsmA represses psl translation. Therefore we sought to discern whether RsmA levels are influenced by RpoS. Western blot analyses revealed no substantial changes of RsmA expression levels in ΔrpoS compared with WT (Figs 7 and S3). Furthermore, the levels of RsmA protein did not vary significantly over the course of growth of either strains, inconsistent with the aforementioned previous studies. We conclude that RpoS does not regulate RsmA, and that psl transcriptional and translational regulation by RpoS and RsmA respectively, represent two distinct modes of control.

Characterization of RsmA binding site to the 5′ UTR of psl mRNA

P. aeruginosa RsmA and E. coli CsrA share 85.2% sequence identity, and heterologous expression of P. aeruginosa RsmA has previously been shown to complement an E. colicsrA mutant (Pessi et al., 2001). Thus, it appears likely that RsmA binding sites in P. aeruginosa mRNAs should resemble the E. coli CsrA consensus sequence generated by SELEX and other studies (Baker et al., 2002; Dubey et al., 2003, 2005; Gutiérrez et al., 2005; Wang et al., 2005). We identified only one probable RsmA binding site within the 5′ UTR of the psl transcript, spanning the region 24–37 bases upstream of the start codon (Fig. 8) and 12–25 bases upstream of the predicted SD sequence.
To determine whether RsmA binds to the predicted binding site, we purified RsmA with a His₆ C-terminal tag (RsmA–His₆; Fig. S4) to perform RNA gel mobility shift assays. RsmA–His₆ was demonstrated before the assay to be functional in vivo, as it could successfully complement ∆rsmA (Fig. S5). As seen in Fig. 9A, C and E, RsmA–His₆ demonstrated specific, high affinity binding to the psl mRNA. Interestingly, two distinct complexes were formed. The slower moving complex appeared at RsmA concentrations higher than 160 nM (Fig. 9A). This raises the possibility that there is a second binding site on the psl mRNA, at which RsmA could bind at a lower affinity.

The GGA triplet that is centrally located in the consensus sequence is essential for high affinity CsrA binding (Dubey et al., 2005). We therefore constructed an altered psl mRNA where GG was replaced with CC (Fig. 10A). The GG → CC substitution abolished the fast moving RsmA–RNA complex (Fig. 9B, D and F), indicating that the predicted binding site was indeed a direct target of RsmA. The GG → CC mutation did not eliminate the secondary shift complex seen at 320 nM RsmA, suggesting that the putative second binding site is still functional in the absence of the high affinity binding site. Replacing the WT sequence with the GG → CC mutation in the translational and transcriptional reporter strains resulted in a loss of RsmA control of translation (Fig. 10C).

Model for RsmA repression of psl translation

The CsrA/RsmA family typically represses gene expression by binding to the mRNA leader and directly blocking access of the ribosome to the ribosome binding site (RBS) (Lapouge et al., 2008). In addition, CsrA/RsmA-bound RNAs assume stem-loop secondary structures (Schubert et al., 2007). The high affinity RsmA binding site of psl, however, does not appear to overlap with the RBS, as is usually the case. We noted that there was an alternative potential translational start site GTG, located 21 bases upstream of the PAO1 genome annotated translational start site ATG, which would add 7 amino acids to the N-terminus of PslA without frameshifting the ORF (Fig. S6A). Furthermore, placing GTG as the translational start site would position the SD sequence overlapping with the experimentally determined RsmA binding site, similar to other known systems. In order to determine the true translational start site of psl, we constructed fusion strains that were fused to lacZ immediately upstream of the AUG codon. The construct without AUG showed equal
transcriptional activity (Fig. S6B) as the transcriptional fusion constructs contain a synthetic translational start site, but the translational fusion activity was abolished (Fig. S6C). These results demonstrate that the psl AUG codon is an indispensable element in translation, and suggest that the translational start site of pslA is the AUG codon, and not the GUG codon.

We noticed that there was a perfect inverted complement region to the SD sequence (termed ‘anti-SD sequence’ here) between 10 and 15 bases upstream of the RsmA binding site. Furthermore, prediction of the secondary structure of the pslA leader by mFOLD software (Zuker, 1989) revealed a large stem-loop structure in which the anti-SD sequence and the SD sequence are base-paired at the base of the stem (Fig. 11A). We hypothesized that RsmA binding to the target site on the psl mRNA stabilizes the large stem-loop structure, which sterically hinders the ribosome access to the SD sequence by base-pairing with the anti-SD sequence (Fig. 11B). We therefore attempted to destabilize this structure by mutating the anti-SD sequence UGCUCU into GACGUC. The mutation was predicted to prevent base-pairing and free the SD sequence to allow ribosome access, resulting in derepression of psl in the presence of RsmA (Fig. 11B). The anti-SD mutation completely alleviated RsmA repression of psl in the WT background, elevating psl translational activity to an identical level as that of ΔrsmA (Fig. 11D). Furthermore, psl translational activity of the anti-SD mutant was indistinguishable between WT and ΔrsmA, suggesting that RsmA repression of psl translation acts through the anti-SD sequence. This model of translational repression is, to our knowledge, novel for CsrA/RsmA regulatory mechanisms.

Discussion

Extracellular polysaccharides comprise major components of the biofilm matrix in many bacterial species (Sutherland, 2001; Branda et al., 2005). In P. aeruginosa PAO1, Psl is an important component of the matrix. Without the capacity to produce Psl, PAO1 exhibits a profound attachment and biofilm development defect (Ma...
et al., 2006). Psl appears to be indispensable for biofilm formation, and understanding its regulation informs us about the environmental signals influencing biofilm formation. In this paper, we present evidence that Psl regulation is complex. We show that the psl operon is transcriptionally regulated by stationary-phase σ-factor RpoS and translationally by the post-transcriptional regulator RsmA. Furthermore, we demonstrate that these mechanisms of control are mediated independently of one another.

RpoS/σ^S regulation has previously been linked to biofilm formation in P. aeruginosa and other bacterial species (Adams and McLean, 1999; Xu et al., 2001; Corona-Izquierdo and Membrillo-Hernández, 2002). The prevalence of a slow-growing subpopulation within biofilm communities has been demonstrated numerous times for P. aeruginosa (Walters et al., 2003). An earlier study demonstrated that Psl was important not only for cell–surface attachment and cell–cell adhesion, but had to be actively produced in order to maintain biofilm structure (Ma et al., 2006). This might suggest that stationary-phase populations within the biofilm are important for producing Psl. This is supported by Overhage et al., who demonstrated that biofilm cells are transcriptionally expressing the psl operon (Overhage et al., 2005). Another key point is that transition to the stationary phase of growth by liquid

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**Fig. 9.** RsmA protein binds to the 5′ UTR of psl mRNA.

Gel mobility shift analyses of RsmA–pslA (A) and RsmA–pslA GG→CC (B) interactions in the absence of RNA competitor. 5′-end labelled WT pslA or pslA GG→CC transcripts (50 pM) were incubated with RsmA at the indicated concentrations. The positions of free (f) and bound (b) RNA are shown. WT pslA RNA displays two shifts, while the mutated pslA GG→CC only has one apparent shift. The K_d values of RsmA binding to the WT pslA RNA (C) and to the pslA GG→CC RNA (D) were calculated using non-linear least-square analysis of data from three independent gel mobility shift assays. Competition reactions using specific (pslA or pslA GG→CC) or non-specific (phoB from E. coli) unlabelled RNA competitors (E and F). The concentrations of RsmA and competitor RNA are shown at the bottom of the corresponding lanes.
cultures of *P. aeruginosa* may promote biofilm production through Psl expression. This indirectly suggests that a response to cessation of growth may be to coordinate initiation of biofilm formation, perhaps as a protection mechanism for starved cells. Intriguingly, a recent report placed a quorum sensing regulator LasR binding site upstream of the −35 region of psl (Gilbert *et al.*, 2009). More detailed studies will need to be conducted in order to investigate whether LasR-mediated regulation involves RpoS.

We also provide evidence that *psl* is regulated post-transcriptionally by the RNA binding protein RsmA. This family of proteins has been shown to repress translation of target mRNAs by competing with the ribosome for binding to the mRNA leader (Babitzke *et al.*, 2009). This often, though not invariably, results in decreased stability and steady state levels of target mRNAs. An exception to this trend is the *E. coli* hfq gene, which is translationally repressed without concomitant effects on its stability (Baker *et al.*, 2007). We observed that RsmA post-transcriptionally represses *psl* without affecting transcription. We also noted that minor *psl* transcriptional changes (approximately twofold) due to a mutation in *rsmA* were documented in one recent microarray study (Brencic and Lory, 2009), but not in another (Burrowes *et al.*, 2006). It is unclear why these inconsistencies exist.

In *E. coli* and many other species, small regulatory RNAs (sRNAs) CsrB and CsrC bind multiple copies of CsrA, and thereby sequester and antagonize its activity (Liu *et al.*, 1997; Weilbacher *et al.*, 2003). These non-coding RNAs require a two-component signal transduction system, BarA–UvrY, for transcription (Suzuki *et al.*, 2002; Weilbacher *et al.*, 2003), and are targeted for degradation by RNase E via CsrD (Suzuki *et al.*, 2006). The BarA sensor kinase responds to metabolic by-products of glycolysis, including formate and acetate (Chavez *et al.*, 2007).

**Fig. 10.** GG→CC mutation within the RsmA binding site abolishes RsmA-mediated regulation of *pslA* translation. A. Translational fusion constructs of full length and GG→CC constructs. The GG nucleotides in the RsmA binding site (boxed) important for RsmA binding were mutated to CC. B. Transcriptional and C. translational lacZ fusion constructs assayed for β-galactosidase activities show deregulation of *pslA* translation in the Δ*rsmA* compared with WT when GG pair was mutated into CC.
CsrA, in turn, activates glycolysis and downregulates gluconeogenesis and glycogen biosynthesis (Sabnis et al., 1995). Thus, CsrA both governs and responds to the metabolic status of the cell. The complex genetic feedback circuitry of this system permits CsrA to regulate expression of the csrB, csrC and csrD genes (Suzuki et al., 2006; Romeo and Babitzke, 2010). Elements of this circuitry are present and appear to function similarly in Pseudomonas species: a homologous two-component system GacS–GacA transcribes the sRNAs that antagonize RsmA (Valverde et al., 2004; Humair et al., 2010). Nevertheless, it does not appear that Pseudomonas species encode a conserved homologue for CsrD, and it is premature to conclude that the overall strategy for circuitry design is conserved in these species.

RsmA repression of psl mRNA is novel, in that the target binding site of RsmA does not appear to overlap the RBS. *E. coli* CsrA binds to six sites on the pga mRNA, two of which overlap the SD sequence and start codon, effectively blocking ribosome access (Wang et al., 2005). The high affinity RsmA binding site of psl, however, is located 12 bases upstream of the SD sequence. High affinity binding sites for CsrA are found in the single-stranded loop of a stem-loop structure (Dubey et al., 2005), and a recent structural study of RsmA homologue RsmE in complex with RNA revealed that protein binding induces the formation of a secondary stem-loop structure in the RNA (Schubert et al., 2007). mFold software analysis of the 5′ UTR region of psl predicted a large, imperfect stem-loop structure in which the RsmA binding site is located within the loop region, consistent with the binding properties of this protein. Furthermore, the predicted structure contains a double-stranded RNA base-pairing event between the SD sequence and an anti-SD sequence, which form the base of the stem (Fig. 11A). Disruption of the anti-SD–SD pairing led to an increase of psl translational activity, which was no longer responsive to RsmA (Fig. 11D). These findings suggest that the
RsmA-mediated repression of psl translation involves the anti-SD sequence. One possible mechanism that can be envisioned is that RsmA binding helps to stabilize base-pairing between the SD and the anti-SD in the imperfect stem-loop. PA0081, PA0082 and PA4492 have recently been identified as direct targets of RsmA (Brencic and Lory, 2009). Unlike pslA, RsmA binding sites appear to overlap with either the start codon, putative SD sequence, or both, consistent with previous models in the literature for other described RsmA/CsrA targets. Without specific experimentation, we do not know the exact lengths of their 5’ UTRs and therefore cannot accurately rule out the possibility of similar anti-SD sequences, although there are no obvious anti-SD sequences upstream of the SD of these genes. It will be interesting to pursue whether anti-SD sequences are more common in RsmA/CsrA regulated genes in P. aeruginosa and other organisms.

Gel shift assays revealed that the presence of a possible second RsmA binding site within the leader transcript of psl (Fig. 9). Other than the primary binding site determined in this study (Figs 9 and 10), another GGA triplet is located 70 bases upstream of the RsmA binding site. While the flanking region does not share any homology with the SELEX-derived E. coli consensus sequence, we cannot rule out potential weak binding by RsmA to this region. In support of this possibility, it was recently demonstrated that dual-site binding of CsrA to target RNAs strengthens regulation. Molecular bridging of the CsrA homodimer occurs when a high affinity binding site is positioned adjacent to a lower affinity binding site on the mRNA (Mercante et al., 2009). Future experiments will explore the nature of this second binding site.

The ability to enter or leave the biofilm lifestyle would be vital to P. aeruginosa’s adaptations to new or changing environments. One way to regulate this lifestyle change would be through controlling the production of extracellular matrix components such as Psl. Our study indicates that there are at least two levels of Psl regulation. Clearly, transcriptional regulation by RpoS ties Psl expression to the growth phase of the cell. This suggests that cessation of active growth would promote biofilm formation. What is not so clear are the signals governing translation control of Psl by RsmA. As RsmA levels were not observed to differ dramatically over the growth curve, translational control of Psl expression may be subject to environmental signals controlling RsmY and RsmZ sRNA expression. Additionally, perhaps an increase in stationary-phase levels of psl mRNA exceeds the binding capacity of RsmA leading to a relief in translational repression. Unfortunately, the environmental signals controlling the Gac–RsmA system in P. aeruginosa are yet to be identified. Nevertheless, a more thorough understanding of how bacteria regulate biofilm matrix production may be the key for developing strategies targeting biofilms in industry and medicine.

**Experimental procedures**

**Bacterial strains and growth conditions**

The bacterial strains used in this study are listed in Table S1. E. coli and P. aeruginosa strains are propagated in LB medium at 37°C unless otherwise specified. Selective medium for P. aeruginosa used throughout this study is VBMM with citrate as the carbon source (Hoang and Schweizer, 1997). Concentrations of antibiotics used for E. coli were: 100 μg ml⁻¹ ampicillin or 50 μg ml⁻¹ carbenicillin, 10 μg ml⁻¹ gentamicin and 10 μg ml⁻¹ tetracycline. For P. aeruginosa: 300 μg ml⁻¹ carbenicillin, 100 μg ml⁻¹ gentamicin and 100 μg ml⁻¹ tetracycline were used. Sucrose counter-selection for P. aeruginosa containing sacB gene was performed by streaking a colony on LB (no salt) + 10% sucrose at 30°C for 24 h.

**Strain constructions**

**ΔrsmA construction.** Primers rsmA-1 and rsmA-2 amplify a 400 bp fragment, which was cloned into pEX18 Tc using an EcoRI site on the primer, and a BamHI in the genomic DNA sequence. Primers rsmA-3 and rsmA-4 amplify a 200 bp sequence, which was cloned into the vector via BamHI and XbaI. The Gm/GFP cassette from pPS858 was inserted into the BamHI site. Upon allelic replacement, the resistance cassette was subsequently excised with FLP recombinase via introduction of pFLP2 plasmid (Hoang et al., 1998). The resulting mutation deletes the first 70 bp of rsmA, and 55 bp of the upstream region.

**Δpel and Δpsl constructions.** Construction of Δpel and Δpsl strains were performed as previously described (Kirisits et al., 2005; Starkey et al., 2009).

**pRsmA ox construction.** A 281 bp fragment of rsmA gene and 66 bp upstream of the start codon generated by PCR using primers rsmA EcoRI and rsmA XbaI was cloned into pUCP18. Resulting pRsmA ox plasmid and its vector control pUCP18 were electroporated into P. aeruginosa as described previously (Choi et al., 2006).

**lacZ translation fusion plasmid mini-CTX lacZ EB construction.** mini-CTX lacZ EB was constructed by ligating Smal/SacI digested mini-CTX lacZ backbone with Smal/SacI digested pMC1403 fragment.

**Transcriptional and translational fusion construction.** Upstream promoter region of pslA was amplified by PCR using oligonucleotides listed in Table S2. PCR products, mini-CTX lacZ (for transcriptional fusion) and mini-CTX lacZ EB (for translational fusion) were digested with EcoRI/BamHI, and ligated. The resulting plasmids were conjugated into P. aeruginosa strains and the constructs integrated into the attB site as described previously (Hoang et al., 2000). mini-CTX plasmid backbone was removed upon expressing FLP recombinase via pFLP2, as β-galactosidase was not efficiently expressed when the backbone DNA was present for unknown reasons (data not shown).

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RsmA–His6 overexpression vector construction. rsmA–His6 was amplified by PCR using oligonucleotides rsmA–His6 for and rsmA–His6 rev. EcoRI/XbaI digests of rsmA–His6 and pUCP18 were ligated and introduced into P. aeruginosa by electroporation (Choi et al., 2006).

5′ RACE
The Invitrogen 5′ RACE System was utilized for cDNA synthesis and PCR as per manufacturer’s instructions for the high GC content genome using primers pslA–GSP1 and pslA–GSP2 (Table S2). RNA was isolated from PAO1 cultures grown in LB to OD600 ≈ 1.0.

β-Galactosidase assays
β-Galactosidase activity was quantitatively assayed using a Galacto-Light Plus kit as described elsewhere (Whiteley et al., 1999; Lequette et al., 2006). P. aeruginosa strains were grown in 15 ml of VBMM at 37°C with 250 r.p.m. shaking. One millilitre culture aliquots were extracted, and 200 μl chloroform was added immediately and vortexed for approximately 10 s. Cell lysates were assayed for both β-galactosidase activities, as well as for protein content by Bradford assay (Bio-Rad). All β-galactosidase activity units are normalized by total protein per ml aliquots. All assays were done in triplicates.

Quantitative real-time PCR
Pseudomonas aeruginosa strains were grown in VBMM at 37°C with aeration and monitored for OD600 during growth. A 0.5 ml culture was harvested at mid-log phase (OD600 = 0.3) and at stationary phase (OD600 = 0.9) and treated with RNAsafe reagent (Qiagen) before storing at −80°C. RNA was extracted using RNaseasy Mini Kit (Qiagen) according to the manufacturer’s protocol (enzymatic lysis and mechanical disruption protocol) with on-column DNase digestion option. RNA concentration and purity were determined by measuring OD260, OD280 and OD230. One microgram RNA was further subjected to DNase I treatment (Promega) at 37°C for 2 h. Complete digestion of genomic DNA was confirmed by PCR using rplU forward and rplU reverse primers as described previously (Schuster et al., 2004). cDNA synthesis was performed using SuperScript III First-Strand Synthesis (Invitrogen) according to the manufacturer’s protocol using random hexamers. Eighty-four microlitres of H2O was added to the 10 min at 4°C. Resulting pellet was resuspended in 6 M urea, boiled for 60 min and centrifuged. The supernatant fraction was subjected to the crude polysaccharide extraction as previously described, while the pellet was resuspended in 1 ml of 6 M urea, boiled for 60 min and assayed for protein concentration by Bradford assay (Bio-Rad). Polysaccharide preparations were diluted and normalized with 0.5 M EDTA to equal protein contents as assayed by Bradford. Five microlitres of polysaccharide preparations were spotted onto a nitrocellulose membrane. Blocking step was performed using 5% milk solution in TBST for 1 h at room temperature, and then probed using α-Psl antibodies in TBST + 1% milk for 1 h at room temperature. Secondary antibodies used in this study were horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG used at 1:10 000 in TBST (Pierce) for 1 h at room temperature. Densitometry analysis was done using ImageQuant software (Molecular Dynamics).

Colonies morphology photographs
For determination of colony morphologies, strains were streaked on VBMM 1% Noble agar plates supplemented with 40 μg ml−1 Congo Red and 15 μg ml−1 Coomassie Brilliant Blue R at 37°C for 2 days. Colonies were photographed using a digital camera mounted on a dissection microscope (Olympus SZX-ILLK100).

α-RsmA Western blots
α-RsmA Western blots were performed using antibodies raised against E. coli CsrA (Gudapaty et al., 2001). Briefly, P. aeruginosa strains were grown in LB shaking cultures at 37°C to their respective growth phases and lysed by addition of perchloric acid to 0.6 M final concentration. Lysed cells were stored on ice for 30 min and centrifuged at 15 000 g for 10 min at 4°C. Resulting pellet was resuspended in 6 M urea, boiled and assayed for protein concentration by Bradford assay (Bio-Rad) for normalization before being loaded onto an 18% Tris-HCl polyacrylamide gel. Following electrophoresis onto a PVDF membrane, RsmA proteins were detected using α-CsrA antibodies at 1:5000 dilution and 1:20 000 diluted goat α-rabbit HRP-conjugated secondary antibodies (Pierce).

RsmA overexpression and purification
As BL21(DE3) cells expressed RsmA–His6 at a poor level, PAO1 expressing RsmA–His6 was grown to stationary phase in 1 litre 2× YT (16 g tryptone, 10 g yeast extract, 5 g NaCl per litre) supplemented with 300 μg ml−1 carbenicillin at 37°C with shaking in a baffled 2 l flask. Cells were pelleted at 4°C and stored at −20°C. Cell pellets were resuspended in 10 ml of buffer (50 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol, pH 7.5) and treated with 1 mg ml−1 lysozyme for 30 min on ice. Cells were not
treated with DNase I or RNase A as the addition of these enzymes severely reduced the yield of RsmA–His6. Further lysis was performed using microtip sonicator, and cell lysates were centrifuged at 13 000 g at 4°C for 1 h. The soluble fractions were loaded onto a HisTrap HP 1 ml column (GE Healthcare) and eluted using the Äkta FPLC system (GE Healthcare). RsmA–His6 typically eluted in two fractions: between 160–240 and 300–500 mM imidazole. The elution fractions from higher imidazole concentration was found to be pure RsmA–His6 and confirmed by Western blotting to the fractions from higher imidazole concentration. The elution was performed using microtip sonicator, and cell lysates were centrifuged at 13 000 g at 4°C for 1 h. The soluble fractions were loaded onto a HisTrap HP 1 ml column (GE Healthcare) and eluted using the Äkta FPLC system (GE Healthcare). RsmA–His6 typically eluted in two fractions: between 160–240 and 300–500 mM imidazole. The elution fractions from higher imidazole concentration was found to be pure RsmA–His6 and confirmed by Western blotting to the His6 tag using HisProbe kit (Pierce) and CsrA antisera (Liu et al., 1997) (Fig. S4). The elution fractions were pooled and dialysed against 1 litre 10 mM MgCl₂, 10 mM Tris-HCl, 100 mM KCl, 25% glycerol, pH 8.0 for 2 h, 3 h and overnight at 4°C. A large portion of RsmA–His6 was found to be precipitated post dialysis. RsmA–His6 was quantitated by Bradford assay (Bio-Rad) before being aliquoted to 20 µl volumes and stored at −80°C.

**RNA gel mobility shift assay**

Quantitative gel mobility shift assays followed a previously published procedure (Yakhnin et al., 2007). DNA templates for generating *psl* and *psl* GG→CC RNA transcripts were PCR amplified using primers *pslA*-T7-F and *pslA*-T7-R resulting in a 171 bp product. RNA was synthesized in vitro using the MEGAscript kit (Ambion) using the PCR products for *psl* and *psl* GG→CC. *phoB* RNA was synthesized as previously described (Jonas et al., 2009). After gel purification, transcripts were 5’-end labelled using T4 polynucleotide kinase and [γ-32P]-ATP. Radiolabelled RNA was gel purified and resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), heated to 85°C and chilled on ice. Increasing concentrations of purified RsmA–His6 recombinant protein were combined with 80 pM radiolabelled RNA in 10 µl binding reactions [10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 100 mM KCl, 3.25 ng total yeast RNA, 20 mM DTT, 7.5% glycerol, 4 U SUPERasasin (Ambion)] for 30 min at 37°C to allow for RsmA–RNA complex formation. Competition assays were performed in the absence or presence of unlabelled RNA specific and non-specific competitor. Binding reactions were separated using 10% native polyacrylamide gels, and radioactive bands were visualized with a phosphorimager (Molecular Dynamics). Free and bound RNA species were quantified with ImageQuant Software (Molecular Dynamics), and an apparent equilibrium binding constant (K₀) was calculated for RsmA–RNA complex formation according to a previously described cooperative binding equation (Mercante et al., 2006).

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