Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis

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RsmA is a posttranscriptional regulatory protein in *Pseudomonas aeruginosa* that works in tandem with a small non-coding regulatory RNA molecule, RsmB (RsmZ), to regulate the expression of several virulence-related genes, including the *N*-acyl-homoserine lactone synthase genes *lasI* and *rhlI*, and the hydrogen cyanide and rhamnolipid biosynthetic operons. Although these targets of direct RsmA regulation have been identified, the full impact of RsmA on cellular activities is not as yet understood. To address this issue the transcriptome profiles of *P. aeruginosa* PAO1 and an isogenic *rsmA* mutant were compared. Loss of RsmA altered the expression of genes involved in a variety of pathways and systems important for virulence, including iron acquisition, biosynthesis of the *Pseudomonas* quinolone signal (PQS), the formation of multidrug efflux pumps, and motility. Not all of these effects can be explained through the established regulatory roles of RsmA. This study thus provides both a first step towards the identification of further genes under RsmA posttranscriptional control in *P. aeruginosa* and a fuller understanding of the broader impact of RsmA on cellular functions.

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that causes morbidity and mortality in patients with compromised immunity, such as cystic fibrosis sufferers and burns patients (Pruitt *et al.*, 1998; Van Delden & Iglewski, 1998). The ability to sense aspects of the external environment and bring about timed, co-ordinate gene expression ensures that this nosocomial pathogen exploits its ecological adaptability to successfully live in the immunocompromised host. Virulence in *P. aeruginosa* is multifaceted, and involves the production of disease-causing secondary metabolites and virulence factors, nutrient scavenging, motility and biofilm formation. The GacA/RsmA/RsmB (RsmZ) signal transduction system is a conserved pathway involved in a variety of adaptive functions in *P. aeruginosa*. RsmA has numerous

homologues in both Gram-negative and Gram-positive bacteria (Romeo, 1998); posttranscriptional regulatory proteins similar to RsmA have been described in *Pseudomonas fluorescens* CHA0 (RsmA), *Escherichia coli* (CsrA) and *Erwinia carotovora* (RsmA) (Blumer *et al.*, 1999; Cui *et al.*, 1995; Liu & Romeo, 1997). CsrA is a well-documented RNA binding protein of *E. coli* that mediates posttranscriptional control of genes involved in a number of physiological pathways. CsrA represses the genes involved in glycogen biosynthesis in *E. coli* by binding to the 5' end of the *glgCAP* mRNA transcript around the Shine–Dalgarno site; this prevents ribosome binding and translation and leads to rapid degradation of the mRNA (Baker *et al.*, 2002). Positive posttranscriptional regulation has also been described in *E. coli*, in which CsrA binds to a yet-unidentified area in the 5' untranslated leader sequence of the *flhDC* master operon that is involved in flagellum biosynthesis, thus increasing the stability of the mRNA and facilitating its translation (Wei *et al.*, 2001).

In *P. aeruginosa* PAO1, it has been shown that the response regulator GacA has an effect on RsmA levels, as a *gacA* mutant expresses 30 % less RsmA protein than wild-type in late-exponential phase (Pessi *et al.*, 2001). GacA works in tandem with the transmembrane sensor kinase GacS. The GacS/GacA two-component system controls expression of virulence-related genes in response to unidentified external environmental signals. GacA also has a positive posttranscriptional effect on the *hcnABC* gene cluster (Pessi & Haas,

Abbreviations: AHL, *N*-acyl homoserine lactone; C4-HSL, *N*-butanoyl-L-homoserine lactone; 3-oxo-C12-HSL, *N*-(3-oxododecanoyl)-L-homoserine lactone; PQS, *Pseudomonas* quinolone signal; QS, quorum sensing.

Genes with a statistically significant ($P=0.05$), minimum twofold change in expression in the *rsmA* mutant compared to *P. aeruginosa* PAO1 wild-type are shown in Supplementary Table S1, genes commonly altered in expression in the *P. aeruginosa* PAK *vfr* mutant and the *P. aeruginosa* PAO1 *rsmA* mutant are shown in Supplementary Table S2, and genes commonly altered in expression in the *P. aeruginosa* PAK *retS* mutant and the *P. aeruginosa* PAO1 *rsmA* mutant are shown in Supplementary Table S3, available with the online version of this paper.

2001). Recently, both GacA and RsmA have been shown to modulate the levels of RsmB (RsmZ) by regulating its transcription. RsmB is the small, untranslated regulatory RNA molecule that works in tandem with RsmA (Burrowes *et al.*, 2005; Heurlier *et al.*, 2004). RsmB is proposed to bind and sequester RsmA, and hence limit its activity. Therefore, GacA also regulates the activity of the RsmA protein by controlling the levels of its partner molecule, RsmB. The posttranscriptional regulatory protein RsmA has been implicated in secondary metabolite production (Pessi *et al.*, 2001).

Interestingly, in *P. aeruginosa*, RsmA and GacA are also involved in the regulation of quorum sensing (QS) signalling molecule synthesis. The QS system has key roles in activation and co-ordination of virulence gene expression in *P. aeruginosa* PAO1, in response to cell density and environmental signals. QS involves the accumulation of signal molecules, often *N*-acyl homoserine lactones (AHLs), to a threshold level to activate expression of certain genes (Fuqua & Greenberg, 1998). *P. aeruginosa* has two interdependent QS systems, the *las* and *rhl* systems. The LasI AHL synthase produces *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and the RhlI AHL synthase produces *N*-butanoyl-L-homoserine lactone (C4-HSL). These AHLs, in association with their cognate regulators, LasR and RhlR, respectively, control the transcription of a number of virulence-related genes, such as alkaline protease, and the pyocyanin and hydrogen cyanide biosynthetic genes (Gambello *et al.*, 1993; Reimann *et al.*, 1997). RsmA negatively regulates *lasI*, *rhlI* and the hydrogen cyanide biosynthetic genes (Pessi *et al.*, 2001). In contrast, RsmA has a positive posttranscriptional effect on the expression of the genes encoding the secondary metabolites lipase and rhamnolipid (Heurlier *et al.*, 2004). GacA is also a documented hierarchical member of the QS network and has a positive transcriptional effect on the *las* and *rhl* QS systems (Reimann *et al.*, 1997).

P. aeruginosa also produces a third intercellular signal molecule, 3,4-dihydroxy-2-heptylquinoline, known as the *Pseudomonas* quinolone signal (PQS). LasR has been shown to regulate PQS production, and RhlR is required for PQS activity (Pesci *et al.*, 1999). PQS has been shown to induce expression of *lasB*, the gene encoding LasB elastase, and *rhlI* (McKnight *et al.*, 2000; Pesci *et al.*, 1999). PQS has also been implicated in the regulation of the secondary metabolites pyocyanin and hydrogen cyanide (Gallagher *et al.*, 2002). Diggle *et al.* (2003) showed that mutants defective in PQS production or in PQS-mediated response produced substantially lower levels of *rhl*-dependent exoproducts, while C4-HSL levels were unaltered, suggesting that PQS also regulates *rhl*-dependent genes independently of C4-HSL. MvfR (PqsR) is a QS-associated regulator that controls the production of PQS, pyocyanin and elastases, and is required for full *P. aeruginosa* virulence (Deziel *et al.*, 2005; Diggle *et al.*, 2003; Gallagher *et al.*, 2002; Rahme *et al.*, 1997). A recent study has revealed that LasR and RhlR indirectly

regulate PQS production via MvfR, and that MvfR binds directly to the *pqsABCDE* operon, positively regulating its transcription (Wade *et al.*, 2005). So far, a direct impact of RsmA or GacA on PQS synthesis has not been reported.

Recent studies have shed more light on the regulation of *rsmA*. Deziel *et al.* (2005) reported that in *P. aeruginosa* PA14, *rsmA* is posttranscriptionally regulated by MvfR. Another regulatory factor known to influence the levels of free, active RsmA is the recently identified hybrid sensor kinase/response regulator RetS (RtsM) (Goodman *et al.*, 2004; Laskowski *et al.*, 2004). Transcriptome profiling of the *P. aeruginosa* PAK *retS* mutant revealed a decrease in the expression of genes involved in type III secretion and virulence and an increase in the biosynthetic genes (the *psl* and *pel* operons) involved in the formation of the mannose- and glucose-rich exopolysaccharide component of biofilms (Goodman *et al.*, 2004). Interestingly, these phenotypes were suppressed by transposon insertions in *gacS*, *gacA* and *rsmB*. The authors propose that, under conditions of acute infection, environmental signals favour activation of RetS, resulting in repression of the GacS/GacA/RsmB pathway. A mutation in *retS* results in de-repression of the levels of GacS, GacA and RsmB, and hence a decrease in the level of active RsmA. A *retS* mutation is, therefore, thought to cause alterations in gene expression via decreased levels of free, active RsmA (Goodman *et al.*, 2004).

Although RsmA may be an element in signal transduction pathways with key roles in virulence, the full regulatory impact of RsmA on cellular activities in *P. aeruginosa* PAO1 is not understood. Here we have used transcriptome profiling to examine the global effects of an *rsmA* mutation on gene expression in *P. aeruginosa* PAO1. Thus, we have identified a set of genes whose expression is most probably indirectly affected by the absence of RsmA. Validation of the transcriptome data using *lacZ* transcriptional fusions confirmed that, amongst other targets, RsmA is involved in the modulation of *pvdS*, *vfr* and *pilM* transcription. Analysis of these data identified a number of candidate genes, including some encoding transcriptional regulators, that may be novel targets for direct RsmA action.

METHODS

Bacterial strains, plasmids and culture conditions. The *P. aeruginosa* strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1 strains and *E. coli* strains were routinely cultured at 37 °C in Luria-Bertani (LB) broth or on LB plates. Antibiotics were used at the following concentrations: for *P. aeruginosa*, 70 µg tetracycline ml⁻¹, 500 µg chloramphenicol ml⁻¹, 200 µg carbenicillin ml⁻¹; for *E. coli*, 25 µg tetracycline ml⁻¹, 30 µg chloramphenicol ml⁻¹.

DNA manipulations. Restriction-enzyme digests, ligations, transformations and agarose gel electrophoresis were performed as previously described (Sambrook *et al.*, 1989). Small-scale plasmid DNA isolation was performed using the Qiagen Plasmid Mini kit according to the manufacturers' specifications. Restriction fragments were purified from agarose gels using the Qiagen Gel Extraction kit. PCR

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

The sequences for primers pvdSFZ, pvdSRZ, pilMFZ, pilMRZ, vfrFZ and vfrRZ include the recognition sequences (*italics*) for the restriction enzymes *Xba*I and *Kpn*I. Tc^R, tetracycline resistant.

Strain, plasmid or primer	Description or sequence (5'→3')	Reference or source
Strains		
<i>P. aeruginosa</i>		
PAO1	Wild-type	Holloway collection
PAZH13	<i>rsmA</i> deletion mutant of PAO1	Pessi <i>et al.</i> (2001)
MP551	<i>pqsR</i> ::TnIS <i>phoA</i> /hah-Tc; derived from PAO1	Gallagher <i>et al.</i> (2002)
PAO1 <i>phnA</i>	<i>phnA</i> ::Tn5, Tc ^R	Jacobs <i>et al.</i> (2003)
Plasmids		
pMP220	Transcriptional reporter, Tc ^R	Spaink <i>et al.</i> (1987)
pCU705	<i>rsmA</i> in pBBR1MCS	Burrowes <i>et al.</i> (2005)
pLP0996	<i>pqsA-lacZ</i> transcriptional fusion in pLP170	McGrath <i>et al.</i> (2004)
pCUB24	<i>pilM-lacZ</i> transcriptional fusion in pMP220	This study
pCUB25	<i>pvdS-lacZ</i> transcriptional fusion in pMP220	This study
pCUB26	<i>vfr-lacZ</i> transcriptional fusion in pMP220	This study
pRTL1-1	<i>rpoS-lacZ</i> translational fusion in pMP77	Bertani <i>et al.</i> (2003)
Primers		
proCF	CAGGCCGGGCAGTTGCTGTC	Savli <i>et al.</i> (2003)
proCR	GGTCAGGCGCGAGGCTGTCT	Savli <i>et al.</i> (2003)
pilUF	TGCGCCTGATGGTGGAAAAG	This study
pilUR	TTCCGGGAGCTTCAGCTCTT	This study
pilMF	GCTGGTTTTCCAGTTCATCCT	This study
pilMR	GACATCAGCTCGACCTCGG	This study
oprNF	GTTCAGTCTGCTGGCGCTC	This study
oprNR	TTGCTGGCCCTTGCCGATG	This study
pvdSF	CCGTACGATCCTGGTGAAGA	This study
pvdSR	GATCACCACGTTCAAGCCTT	This study
gloA2F	TGCGAATCCTGCACAGCATG	This study
gloA2R	ATCACGCTGCGTCCGTGCT	This study
pilMFZ	CCCGGGGTACCCGACCAATTCCTATTAGCG	This study
pilMRZ	CCCGGGTCTAGAGAGTTAAGCGAATAGGACAGG	This study
pvdSFZ	CCCGGGGTACCGGAACGATAACAACGGCAAA	This study
pvdSRZ	CCCGGGTCTAGATGGAAATCACCTTGCTGCGG	This study
vfrFZ	CCCGGGGTACCCGGGATCACAGTCCTGATAG	This study
vfrRZ	CCCGGGTCTAGACATGCCCCGAGTCCCGAAAAGA	This study

products were purified using the High Pure PCR Product Purification kit (Boehringer). Plasmids were introduced into *Pseudomonas* strains via electroporation.

Microarray sample preparation and analysis. Duplicate cultures of *P. aeruginosa* PAO1 wild-type and the *rsmA* mutant were grown in LB broth to an OD₆₀₀ of 0.8, and 4 ml of each culture was treated with RNeasy protect bacterial reagent (Qiagen), according to the manufacturers' instructions. Cells were harvested by centrifugation and stored at -80 °C. Total RNA was extracted using the RNeasy Midi RNA Isolation kit (Qiagen) according to the manufacturers' instructions. The RNA was treated with RQ1 RNase-free DNase (Promega) for 1 h at 37 °C and purified using the Clean-Up protocol of the RNeasy Midi RNA Isolation kit according to the manufacturers' instructions. The RNA was precipitated with ethanol and the RNA pellet was resuspended in sterile distilled H₂O to a final volume of 20 µl; the integrity of the RNA was checked on an agarose gel and quantified using a spectrophotometer. cDNA was synthesized according to the Affymetrix Expression Analysis Protocol

guide; spike RNA transcripts from *Bacillus subtilis* genes were included in the reaction. The cDNA was treated with 2 µg DNase-free RNase (Roche) in a final volume of 100 µl for 1 h at 37 °C, purified using the QIAquick PCR Purification kit (Qiagen) and eluted in 45 µl sterile distilled H₂O. The cDNA was fragmented using RQ1 RNase-free DNase for 1 h at 37 °C, and biotin-labelled using the BioArray Terminal Labelling kit with biotin-ddUTP (Enzo Life Sciences). The cDNA was snap-frozen on dry ice and analysed at the MRC Geneservice (Cambridge, England) on Affymetrix GeneChip *P. aeruginosa* Genome arrays. A Student's *t* test was carried out on the data generated from the microarray analysis using GeneDirector software (BioDiscovery) to give a list of genes with altered expression greater than twofold and a *P* value ≤ 0.05.

Semi-quantitative RT-PCR analysis. To confirm the microarray data, semi-quantitative RT-PCR analysis was carried out. RNA was isolated from cultures of PAO1 wild-type and the *rsmA* mutant grown to an OD₆₀₀ of 0.8 in LB broth, and converted to cDNA using the procedure already described. The primers used to amplify

the genes *gloA2*, *pilM*, *pvdS*, *oprN*, *pilU* and *proC* are listed in Table 1. PCRs were carried out using PCR Master Mix (Promega) and 50 ng cDNA template for 20–25 cycles. The constitutively expressed control gene *proC* (Savli *et al.*, 2003) was used as the internal control to verify the absence of significant variation in cDNA levels in all samples.

Construction and analysis of transcriptional fusions. The promoters and start sites of *pvdS*, *pilM* and *vfr* were amplified from *P. aeruginosa* PAO1 DNA using primers listed in Table 1. The primers (*pvdSFZ*, *pvdSRZ*, *pilMFZ*, *pilMRZ*, *vfrFZ*, *vfrRZ*) include the recognition sequences (Table 1, italics) for the restriction enzymes *Xba*I and *Kpn*I. The resultant PCR products were digested using *Xba*I or *Kpn*I, PCR-purified, and cloned into the reporter fusion pMP220 (Spaink *et al.*, 1987), upstream of the promoterless *lacZ* gene. β -Galactosidase activity was measured by standard methods (Sambrook *et al.*, 1989). Due to the clumping phenotype of the *rsmA* mutant, total protein was extracted from 2 ml of cells at each time-point analysed, and this number was substituted for OD₆₀₀ to calculate β -galactosidase activity.

Western blot analysis. Total protein was extracted from strains of *P. aeruginosa* PAO1, grown in LB broth to an OD₆₀₀ of 0.8 and quantified using the Bio-Rad Protein Assay Dye Reagent. Proteins and All Blue Precision Plus Protein standard (Bio-Rad) were electrophoresed on 5% polyacrylamide SDS stacking gels and 15% polyacrylamide SDS resolving gels at 130 V using the Mini-PROTEAN II cell (Bio-Rad). Proteins were either stained with Coomassie brilliant blue (BDH Chemicals) or transferred to a nitrocellulose membrane using a Mini Trans-Blot cell (Bio-Rad). The membrane was blocked with 5% non-fat dried milk, 0.1% (v/v) Tween 20 in Tris-buffered saline (TBS) for 1 h, washed three times in TBS/Tween and incubated with anti-PvdS monoclonal antibodies (received from Professor M. Vasil, University of Colorado) for 2 h at room temperature. The membrane was washed three times in TBS/Tween and incubated with anti-mouse secondary antibodies (Dakocytomation) for 1 h. Detection was performed using an ECL+ kit (Amersham Pharmacia Biotech) and chemiluminescent film (Kodak).

Pyocyanin quantification. Production of the phenazine pyocyanin by *P. aeruginosa* PAO1 wild-type and the *rsmA* mutant was measured as described elsewhere (Essar *et al.*, 1990).

Motility assay. Swimming was analysed on tryptone swim plates (1%, w/v, tryptone, 0.5%, w/v, NaCl, 0.3%, w/v, agar), which were inoculated using a sterile toothpick with bacteria from colonies grown overnight on LB agar at 37 °C. The plates were incubated at 37 °C, and motility was assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation.

Adhesion assay. Adhesion on polyvinyl chloride (PVC) was tested using non-coated 24-well tissue-culture flat-well plates (Sarstedt). LB (1 ml) was added to each well and inoculated to a starting OD₆₀₀ of 0.05. The plates were incubated at 37 °C without agitation for 12 h. The wells were rinsed with water and the bacterial film was stained with 1% crystal violet. Six replicates were performed per sample. The wells were then washed with 1 ml 95% ethanol and the OD₅₇₀ of a 1:100 dilution in sterile distilled H₂O was obtained.

PQS extraction and analysis. Cultures of *P. aeruginosa* PAO1 wild-type, PAZH13 (*rsmA* mutant), PAO1 pCU705 (*rsmA* overexpressing strain), the *P. aeruginosa* *pqsR* mutant (Gallagher *et al.*, 2002) and the *P. aeruginosa* *phnA* mutant (Jacobs *et al.*, 2003) were grown to early stationary phase in LB broth. Culture (3 ml) was extracted with 3 ml acidified ethyl acetate, the solvent removed by rotary evaporation and the extract resuspended in 1:1 acidified ethyl acetate/acetonitrile. Silica gel 60 F₂₅₄ aluminium TLC plates (Merck) were activated by soaking for 15 min in 5% KH₂PO₄,

wrapping in tin foil and heating to 100 °C for 1 h. Samples, PQS standard (Diggle *et al.*, 2003) and anthranilate standard (Sigma) were separated on the TLC plates using 95:5 dichloromethane/methanol in a solvent chamber. PQS appeared as a blue/purple spot under UV light.

RESULTS

Microarray analysis of *P. aeruginosa* PAO1 wild-type and the *rsmA* mutant

It has been shown previously that RsmA levels peak during late-exponential phase, which is the time-point at which RsmA maximally affects target gene expression (Pessi *et al.*, 2001). Therefore, in order to study the impact of the loss of *rsmA* on gene expression, duplicate transcriptome analyses were carried out on *P. aeruginosa* PAO1 wild-type and PAZH13, the *rsmA* mutant, grown to late-exponential phase in LB broth at 37 °C. Under these conditions, the growth rate and final OD₆₀₀ of PAO1 wild-type and the *rsmA* mutant were identical (data not shown). The transcriptome analysis was carried out using four Affymetrix GeneChip *P. aeruginosa* Genome arrays (see Methods). A Student's *t* test was carried out on the comparative data generated from the microarrays using GeneDirector software (BioDiscovery) to give a list of genes with a statistically significant ($P \leq 0.05$), minimum twofold, change in expression (Table 2 and Supplementary Table S1).

To confirm and validate the microarray data, semi-quantitative RT-PCR was carried out on five genes (*pilM*, *pilU*, *oprN*, *pvdS* and *gloA2*). These genes were chosen for RT-PCR analysis because their fold change in expression in the *rsmA* mutant compared to PAO1 wild-type varied across a relatively broad range (+1173 for *gloA2* expression and –3 for *pilM* expression) and they were physiologically relevant candidates for further analysis. The constitutively expressed gene *proC* (Savli *et al.*, 2003), which encodes a pyrroline-5-carboxylate reductase, and whose expression was not altered in the *rsmA* mutant, was used as an internal control in these reactions. The RT-PCR analyses correlated with the microarray alterations in gene expression: *pilM* and *pilU* showed reduced expression, and *gloA2*, *pvdS* and *oprN* showed increased expression in the *rsmA* mutant compared to wild-type (Fig. 1).

Of the 5570 genes present on the array, 506 genes (9%) showed significantly altered expression in the *rsmA* mutant compared to PAO1. Most altered genes ($n = 365$ or 6.5%) were decreased in expression in the *rsmA* mutant compared to PAO1. All genes of known function, with altered expression in the *rsmA* mutant compared to PAO1, are listed in Table 2. A large number of altered genes ($n = 222$ or 44%) were categorized as Class 4 or of unknown function according to the *Pseudomonas* website classification system (<http://www.pseudomonas.com>; Supplementary Table S1). Where possible, genes of altered expression were classified into functional groups (Table 2). The expression of RNA complementary to 29 intergenic regions was also significantly

Table 2. Genes of altered expression in *P. aeruginosa* *rsmA* compared to *P. aeruginosa* PAO1 wild-type, arranged into functional groups

Positive values represent genes increased in expression in the *rsmA* mutant compared to PAO1; negative values represent genes decreased in expression in the *rsmA* mutant compared to PAO1. PA numbers and annotations are from the *Pseudomonas* website (<http://www.pseudomonas.com>).

PA no. and gene name	Function	Fold change
Genes involved in motility		
PA0173	Putative response regulator for chemotaxis	−10
PA0396 <i>pilU</i>	Twitching motility protein	−2
PA0994 <i>cupC3</i>	Usher protein	−3
PA1092 <i>fliC</i>	Flagellin type B	−2
PA1251	Probable chemotactic transducer	−8
PA2129 <i>cupA2</i>	Chaperone protein	−9
PA4307 <i>pctC</i>	Chemotactic transducer	−3
PA4310 <i>pctB</i>	Chemotactic transducer	−3
PA4527 <i>pilC</i>	Still frameshift type 4 fimbrial biogenesis protein	−2
PA4953 <i>motB</i>	Chemotaxis protein	−2
PA4954 <i>motA</i>	Chemotaxis protein	−2
PA5041 <i>pilP</i>	Type 4 fimbrial biogenesis protein	−2
PA5042 <i>pilO</i>	Type 4 fimbrial biogenesis protein	−2
PA5043 <i>pilN</i>	Type 4 fimbrial biogenesis protein	−2
PA5044 <i>pilM</i>	Type 4 fimbrial biogenesis protein	−3
Iron-regulated genes		
PA0672 <i>hemO/pigA</i>	Haem oxygenase	−4
PA0673 <i>pigB</i>	Hypothetical protein	−39
PA2386 <i>pvdA</i>	L-Ornithine N5 oxygenase	+25
PA2394 <i>pvdN</i>	Pyoverdine biosynthesis	+4
PA2396 <i>pvdF</i>	Pyoverdine synthetase	+3
PA2397 <i>pvdE</i>	Pyoverdine synthetase	+2
PA2398 <i>fpvA</i>	Ferric-pyoverdine receptor	+17
PA2413 <i>pvdH</i>	L-2,4-Diaminobutyrate : 2-ketoglutarate 4-aminotransferase	+14
PA2424 <i>pvdL</i>	Pyoverdine biosynthesis	+2
PA2425 <i>pvdG</i>	Pyoverdine biosynthesis	+4
PA2426 <i>pvdS</i>	Iron-regulated sigma factor	+11
PA3531 <i>bfrB</i>	Bacterioferritin B	−3
PA4158 <i>fepC</i>	Ferric-enterobactin transport protein	+5
PA4221 <i>fptA</i>	Fe(III)-pyochelin outer-membrane receptor	+7
PA4226 <i>pchE</i>	Dihydroaeruginic acid synthetase	+12
PA4228 <i>pchD</i>	Pyochelin biosynthesis	+20
PA4229 <i>pchC</i>	Pyochelin biosynthesis	+8
PA4230 <i>pchB</i>	Salicylate biosynthesis protein	+9
PA4231 <i>pchA</i>	Salicylate biosynthesis isochorismate synthase	+29
PA4468 <i>sodM</i>	Superoxide dismutase	+10
PA4470 <i>fumC</i>	Fumarate hydratase	+2
Genes encoding the MexEF-OprN pump		
PA2493 <i>mexE</i>	RND multidrug efflux membrane fusion protein precursor	+224
PA2494 <i>mexF</i>	RND multidrug efflux transporter	+73
PA2495 <i>oprN</i>	Multidrug efflux outer-membrane protein precursor	+4
Ungrouped genes of known function		
PA0105 <i>coxB</i>	Cytochrome <i>c</i> oxidase, subunit II	−4
PA0108 <i>coIII</i>	Cytochrome <i>c</i> oxidase, subunit III	−4
PA0129 <i>gabP</i>	γ-Aminobutyrate permease	−3
PA0210 <i>mdcC</i>	Malonate decarboxylase δ subunit	−3
PA0212 <i>mdcE</i>	Malonate decarboxylase γ subunit	−6
PA0229 <i>pcaT</i>	Dicarboxylic acid transporter	−2

Table 2. cont.

PA no. and gene name	Function	Fold change
PA0235 <i>pcaK</i>	4-Hydroxybenzoate transporter	-3
PA0652 <i>vfr</i>	Transcriptional regulator	-2
PA0706 <i>cat</i>	Chloramphenicol acetyltransferase	-23
PA0710 <i>gloA2</i>	Lactoylglutathione lyase	+1173
PA0723 <i>coaB</i>	Coat protein B of bacteriophage Pf1	+5
PA0843 <i>plcR</i>	Phospholipase accessory protein precursor	-2
PA0844 <i>plcH</i>	Haemolytic phospholipase C precursor	+3
PA0905 <i>rsmA</i>	Regulator of secondary metabolites	-192
PA0958 <i>oprD</i>	Basic amino acid, basic peptide and imipenem outer-membrane porin OprD precursor	-4
PA1001 <i>phnA</i>	Anthranilate synthase component I	-3
PA1148 <i>toxA</i>	Exotoxin A precursor	-3
PA1151 <i>imm2</i>	Pyocin S2 immunity protein	-2
PA1173 <i>napB</i>	Cytochrome <i>c</i> -type protein NapB precursor	-2
PA1247 <i>aprE</i>	Alkaline protease secretion protein	-3
PA1338 <i>ggt</i>	γ -Glutamyltranspeptidase precursor	-2
PA1393 <i>cysC</i>	Adenosine 5'-phosphosulfate (APS) kinase	-11
PA1409 <i>aphA</i>	Acetylpolyamine aminohydrolase	-6
PA1635 <i>kdpC</i>	Potassium-transporting ATPase, C chain	-5
PA1691 <i>pscT</i>	Translocation protein in type III secretion	-2
PA1695 <i>pscP</i>	Translocation protein in type III secretion	-11
PA1698 <i>popN</i>	Type III secretion outer-membrane protein PopN precursor	-4
PA1758 <i>pabB</i>	<i>para</i> -Aminobenzoate synthase component I	+4
PA1770 <i>ppsA</i>	Phosphoenolpyruvate synthase	+2
PA1781 <i>nirB</i>	Assimilatory nitrite reductase large subunit	-3
PA1947 <i>rbsA</i>	Ribose transport protein	-3
PA1949 <i>rbsR</i>	Ribose operon repressor	-2
PA1983 <i>exaB</i>	Cytochrome <i>c</i> ₅₅₀	-3
PA2007 <i>maiA</i>	Maleylacetoacetate isomerase	-4
PA2009 <i>hmgA</i>	Homogentisate 1,2-dioxygenase	-4
PA2064 <i>pcoB</i>	Copper resistance protein B precursor	-3
PA2247 <i>bkdA1</i>	2-Oxoisovalerate dehydrogenase (α subunit)	-2
PA2248 <i>bkdA2</i>	2-Oxoisovalerate dehydrogenase (β subunit)	-3
PA2337 <i>mtlR</i>	Transcriptional regulator	-2
PA2509 <i>catB</i>	Muconate cycloisomerase I	-3
PA2926 <i>hisP</i>	Histidine transport protein	-5
PA3540 <i>algD</i>	GDP-mannose 6-dehydrogenase	-3
PA3543 <i>algK</i>	Alginate biosynthetic protein	-3
PA3544 <i>algE</i>	Alginate production outer-membrane protein	-5
PA3549 <i>algJ</i>	Alginate <i>o</i> -acetyltransferase	-3
PA3603 <i>dgkA</i>	Diacylglycerol kinase	+3
PA4110 <i>ampC</i>	β -Lactamase precursor	-2
PA4127 <i>hpcG</i>	2-oxo-hept-3-ene-1,7-dioate hydratase	-4
PA4138 <i>tyrS</i>	Tyrosyl-tRNA synthetase	-2
PA4205 <i>mexG</i>	Hypothetical protein	-3
PA4210 <i>phzA1</i>	Probable phenazine biosynthetic protein	-3
PA4356 <i>xenB</i>	Xenobiotic reductase	+8
PA4522 <i>ampD</i>	β -Lactamase expression regulator	-2
PA4855 <i>purD</i>	Phosphoribosylamine-glycine ligase	+2
PA5243 <i>hemB</i>	δ -Aminolevulinic acid dehydratase	-2
PA5355 <i>glcD</i>	Glycolate oxidase subunit	-4
PA5361 <i>phoR</i>	Two-component sensor	-5
PA5501 <i>znuB</i>	Permease of ABC zinc transporter	-2

Fig. 1. Semi-quantitative RT-PCR analysis of *gloA2*, *pilM*, *pvdS*, *oprN*, *pilU* and *proC* in *P. aeruginosa* PAO1 wild-type (lanes 1, 3, 5, 7, 9 and 11) and the *rsmA* mutant (lanes 2, 4, 6, 8, 10 and 12).

altered by inactivation of *rsmA*. This may indicate yet-unidentified ORFs or the presence of untranslated regulatory RNA molecules. Indeed, RsmA has been shown previously to positively regulate the transcription of the gene encoding its cognate regulatory untranslated RNA, RsmB (Burrowes *et al.*, 2005; Heurlier *et al.*, 2004). However, the *rpoS/fdxA* intergenic region, in which *rsmB* is located, is not represented on the Affymetrix *P. aeruginosa* PAO1 GeneChip.

Functional categories of genes with increased expression in the *rsmA* mutant compared to PAO1 wild-type

Iron acquisition. The expression of genes involved in iron acquisition was increased in the *rsmA* mutant compared to PAO1 wild-type. The expression of a number of *pvd* and *pch* genes, which direct the biosynthesis of the siderophores pyoverdine and pyochelin, respectively, was increased in the *rsmA* mutant compared to wild-type. In addition, expression of *fpvA*, the ferric-pyoverdine receptor, and *fptA*, the ferric-pyochelin receptor, was increased in the *rsmA* mutant compared to PAO1 wild-type. Interestingly, the microarray analysis revealed that expression of the gene encoding the iron-regulated sigma factor PvdS was increased 11-fold in the *rsmA* mutant compared to wild-type. The fact that PvdS levels were increased in the *rsmA* mutant may explain the increase in expression of the *pvd* genes (Beare *et al.*, 2003; Visca *et al.*, 2002) but not the *pch* operon. Transcription of *pchABCDE* and *fptA* is activated by the AraC-like regulator PchR in the presence of pyochelin and repressed in the absence of pyochelin (Heinrichs & Poole, 1996). Although expression of the *pch* genes was increased in the *rsmA* mutant, the levels of *pchR* transcript were not altered. Therefore, the increase in *pch* transcript levels may reflect a change in mRNA stability rather than in transcription rate, or the implementation of an alternate regulator. To confirm that the observed increase in *pvdS* levels in the *rsmA* mutant compared to PAO1 wild-type was due to a change in transcription rate, a *pvdS-lacZ* transcriptional fusion (pCUB25) was constructed in the fusion vector pMP220. Transcription of *pvdS* was increased in the *rsmA* mutant compared to PAO1 wild-type at all time-points analysed (Fig. 2a). To ascertain if this increase in transcription corresponded to an increase in PvdS protein levels, Western blot analysis was carried out on total protein isolated from PAO1 wild-type and from the *rsmA* mutant grown to an OD₆₀₀ of 0.8 in LB broth. The 21.2 kDa PvdS protein was detected with an anti-PvdS antibody via chemiluminescence, and PvdS levels were found to be higher in the *rsmA* mutant compared to PAO1 wild-type, albeit by a

moderate increase (Fig. 3). Band intensities were quantified using GeneTools software (version 3.05.03, Syngene). The mean band intensity in the *rsmA* mutant sample, as a percentage of mean band intensity of the wild-type sample, was $155 \pm 20\%$ ($n=3$).

Both PvdS and Vfr have been shown previously to positively regulate *toxA* expression (Lamont *et al.*, 2002; West *et al.*, 1994). Although PvdS levels were increased in the *rsmA* mutant, *toxA* showed a 2.8-fold decrease in expression in the *rsmA* mutant. This may be due to the drop in *vfr* levels in the *rsmA* mutant (see below), suggesting that under the conditions of this experiment, the loss of the positive influence of Vfr on *toxA* transcription is more potent than the increase in PvdS levels on *toxA* transcription.

The expression of genes involved in resistance to oxidative stress, namely *sodM* and *fumC* (Hassett *et al.*, 1997) was also increased. This is consistent with an increase in iron uptake, which generates reactive hydroxyl radicals via the Fenton reaction.

Antibiotic resistance. Loss of *rsmA* resulted in increased expression of the genes encoding the MexEF-OprN pump. In *P. aeruginosa*, the MexEF-OprN pump removes numerous antibiotics and PQS from the cell (Poole, 2001). Several proteins have been implicated in the positive regulation of expression of this Mex system, including MexT, an activator of MexEF-OprN (Maseda *et al.*, 2000). In addition, spontaneous *nfxC* mutants show an increase in expression of the MexEF-OprN pump (Kohler *et al.*, 2001). Examination of MexT protein levels in the *rsmA* mutant showed that they were not altered (data not shown). However, levels of *mexS* mRNA, which encodes a transcriptional repressor of MexEF-OprN, (Sobel *et al.*, 2005) were increased fourfold in the *rsmA* mutant compared to PAO1. These results reflect the complexity of Mex pump regulation.

Functional categories of genes with decreased expression in the *rsmA* mutant compared to PAO1 wild-type

PQS biosynthesis. PQS is a third signal molecule in addition to C4-HSL and 3-oxo-C12-HSL produced by *P. aeruginosa*, and PQS production occurs at the onset of stationary phase (Diggle *et al.*, 2003; Pesci *et al.*, 1999). Microarray analysis revealed that *phnA* expression was decreased by 2.6-fold in the *rsmA* mutant compared to PAO1 wild-type. The *phnAB* operon encodes an anthranilate synthase, which converts chorismate to anthranilate, a precursor of PQS and pyocyanin. PhnA is essential for

Fig. 2. Kinetics of expression of (a) *pvdS* (pCUB25), (b) *pqsA* (pLP0996), (c) *pilM* (pCUB24) and (d) *vfr* (pCUB26) transcriptional reporter *lacZ* fusions in *P. aeruginosa* PAO1 (black bars) and in the *rsmA* mutant (grey bars). Values are the mean and standard deviation of triplicate measurements.

PQS production in *P. aeruginosa*, as a *phnA* mutant does not make PQS (Gallagher *et al.*, 2002). However, levels of the *pqsABCDE* transcripts, which encode the PQS biosynthetic enzymes, did not appear to be altered in the comparative microarray analyses. To validate a transcript recorded as 'no change' on the microarray, confirmatory analysis was performed with a *pqsA-lacZ* transcriptional fusion (pLP0996) (Fig. 2b). As expected, there was no significant difference in the transcription of the *pqsABCDE* operon in PAO1 wild-type and the *rsmA* mutant. To examine the impact of the decrease in expression of *phnA* on PQS levels in the *rsmA* mutant, PQS extractions were carried out on supernatants of PAO1, the *rsmA* mutant, PAO1 pCU705 (*rsmA*⁺⁺), MP551 (PAO1 *pqsR*) and PAO1 *phnA* grown to stationary phase (OD₆₀₀ of 3.0) in LB broth. Fig. 4 shows that the *rsmA* mutant produced less PQS than PAO1 wild-type in stationary phase and that PAO1 overexpressing *rsmA* produced more PQS than PAO1 wild-type. This was accompanied by a parallel reduction in the intensity of the upper band, namely compound B; this compound has not yet been identified, but

its levels have been found to parallel PQS levels in certain *P. aeruginosa* strains, including PAO1 (Collier *et al.*, 2002). The large spot present in the TLC analysis of the *rsmA*, *pqsR* and *phnA* mutants is a precursor of PQS, namely anthranilate (Baysse *et al.*, 2005). As expected, neither PQS nor compound B was present in the *pqsR* mutant, as this gene product is essential for their synthesis. In addition, it was interesting to note that the PQS profile of the *rsmA* mutant was similar to that of the *phnA* mutant, supporting the transcriptome finding that expression of *phnA* was reduced in the *rsmA* mutant.

The PhnAB system also provides the anthranilate required for the biosynthesis of the phenazine pyocyanin (Gallagher *et al.*, 2002); levels of pyocyanin in stationary-phase cultures of PAO1 and the *rsmA* mutant were examined. This revealed that loss of RsmA substantially reduced the production of pyocyanin when the bacteria were cultured in LB; PAO1 wild-type produced 4.2 $\mu\text{g ml}^{-1}$ (± 0.1 , $n=3$) of pyocyanin, while the *rsmA* mutant only produced 0.3 $\mu\text{g ml}^{-1}$ (± 0.05 , $n=3$) of pyocyanin in stationary phase (OD₆₀₀ of

Fig. 3. Western blot analysis of PvdS levels in total protein extracted from *P. aeruginosa* PAO1 and the *rsmA* mutant grown to an OD₆₀₀ of 0.8 in LB broth.

4). However, under the same experimental conditions in glycerol/alanine medium, which was used as a control, the *rsmA* mutant showed an increase in production of pyocyanin compared to wild-type: PAO1 wild-type produced $0.23 \mu\text{g ml}^{-1}$ (± 0.03 , $n=3$) of pyocyanin, while the *rsmA* mutant produced $3.27 \mu\text{g ml}^{-1}$ (± 0.01 , $n=3$) of pyocyanin, which is in accordance with the previous observation by Pessi *et al.* (2001). The result obtained in LB is in agreement with the observed accumulation of the metabolic precursor anthranilate and the drop in levels of PQS in the *rsmA* mutant. Indeed, PQS has been shown to be required for phenazine gene expression in PAO1 (Diggle *et al.*, 2003). Interestingly, expression of *phzA1*, the first gene in one of the pyocyanin biosynthetic operons (Mavrodi *et al.*, 2001), was decreased by approximately threefold in the *rsmA* mutant compared to PAO1 wild-type. The other genes in the operon were recorded as 'absent' for the *rsmA* mutant, which is most likely due to mRNA or cDNA degradation during sample preparation for microarray analysis.

Motility. RsmA positively regulates fimbrial biosynthetic genes in *P. aeruginosa* PAO1. Transcriptome analysis revealed that expression of genes involved in the formation of type IV pili, fimbriae and chemotaxis was decreased in the *rsmA* mutant compared to PAO1 wild-type (Table 2). Expression of the *pilMNOPQ* operon and *pilU*, which

encode proteins of unknown function that are thought to be involved in the biosynthesis of pili (Mattick *et al.*, 1996), was decreased in the *rsmA* mutant compared to wild-type. Expression of a third *pil* gene, *pilC*, was also decreased in the *rsmA* mutant compared to PAO1. To examine the altered transcription of the *pilMNOPQ* operon, a *pilM-lacZ* transcriptional fusion was constructed in pMP220 (pCUB24) and analysed over time in PAO1 and the *rsmA* mutant (Fig. 2c). This confirmed that transcription of the *pilMNOPQ* operon was decreased in the mutant throughout all phases of growth. Depending on the carbon source used, type IV pili are not always necessary for the initial attachment of *P. aeruginosa* PAO1, but they are always necessary for normal biofilm development, irrespective of growth medium (Klausen *et al.*, 2003). Under the conditions used for this transcriptome study (i.e. LB broth), the *P. aeruginosa rsmA* mutant (OD₅₇₀ 0.20 ± 0.06 , $n=5$) was significantly impaired for biofilm formation on PVC compared to wild-type (OD₅₇₀ 0.34 ± 0.05 , $n=5$) (Fig. 5a).

The transcriptome analysis also showed that components of the chaperone/usher (*cup*) pathways in *P. aeruginosa* were down-regulated in the *rsmA* mutant compared to PAO1 (Table 2). The *cup* genes are thought to be involved in the assembly of novel *P. aeruginosa* fimbrial structures that are different from type IV pili; the *cupA* gene cluster is required for cell adhesion and initiation of biofilm formation (Vallet *et al.*, 2001). In the *rsmA* mutant, expression of *cupA2*, which encodes a fimbrial chaperone protein, was down ninefold and expression of *cupC3*, which encodes an usher protein, was down approximately threefold compared to PAO1. Interestingly, mutations in *cupA2* have been shown to cause cell clumping in *P. aeruginosa* PAK (Vallet *et al.*, 2001), so it is possible that the clumping phenotype observed for the *rsmA* mutant (Heurlier *et al.*, 2004) is caused by the drop in *cupA2* levels. The expression of a number of genes involved in chemotaxis was also decreased in the *rsmA* mutant compared to PAO1. PA0173, which encodes a putative response regulator for chemotaxis, was decreased tenfold, and expression of PA1251, which encodes a probable chemotaxis

Fig. 4. PQS extracts from *P. aeruginosa* PAO1 wild-type (lane 1), the *rsmA* mutant (lane 2), PAO1 pCU705 (*rsmA*⁺⁺) (lane 3), *P. aeruginosa phnA* (lane 4) and *P. aeruginosa pqsR* (lane 5) grown to an OD₆₀₀ of 0.8 in LB broth.

Fig. 5. (a) Biofilm formation on PVC by *P. aeruginosa* PAO1 wild-type and the *rsmA* mutant grown in LB, as revealed by crystal violet staining; (b) Swimming motility of *P. aeruginosa* PAO1 wild-type and the *P. aeruginosa rsmA* mutant.

transducer, was down eightfold in the *rsmA* mutant compared to wild-type. The decreased expression of genes involved in chemotaxis may explain the drop in swimming motility in the *rsmA* mutant compared to PAO1 (Fig. 5b). The *motA* and *motB* genes encode proteins that make up the stator or stationary component of the flagellar motor (Toutain *et al.*, 2005). Expression of *motA* and *motB* was down twofold in the *rsmA* mutant compared to PAO1 (Table 2). From these data, it is evident that RsmA is involved in a complex regulatory cascade that controls the expression of genes involved in motility in *P. aeruginosa*.

Vfr transcription. Microarray analysis revealed that expression of *vfr* was decreased twofold in the *rsmA* mutant compared to PAO1 wild-type. Vfr is a homologue of the *E. coli* cAMP receptor protein Crp. Crp is activated by binding cAMP and then binds to a specific sequence in the promoter of Crp-controlled genes. In *P. aeruginosa*, Vfr has been implicated in the regulation of twitching motility, QS, pyocyanin production, repression of flagellar biosynthesis and the expression of numerous proteins secreted by the type II pathway, including exotoxin A (Albus *et al.*, 1997; Beatson *et al.*, 2002; Dasgupta *et al.*, 2002; West *et al.*, 1994). As mentioned previously, *toxA* expression was decreased approximately 2.8-fold in the *rsmA* mutant compared to PAO1. The expression of *regA*, *lasR* and *fleQ*, other known Vfr targets, was not altered in the *rsmA* mutant. However, the transcription of *regA*, *lasR* and *fleQ* is also controlled by numerous other regulators (Dasgupta & Ramphal, 2001; Dasgupta *et al.*, 2003; Hunt *et al.*, 2002; Juhas *et al.*, 2005), which could compensate

for the drop in *vfr* levels. To confirm the novel impact of RsmA on Vfr levels, a *vfr-lacZ* transcriptional fusion (pCUB26) was constructed and analysed in PAO1 and the *rsmA* mutant (Fig. 2d). This analysis showed that *vfr* expression peaked after 10 h of growth, corresponding to late-exponential/early stationary phase, in both the wild-type and the *rsmA* mutant. Expression of *vfr* was decreased in the *rsmA* mutant compared to PAO1 throughout the growth cycle until late stationary phase, in which *vfr* transcription was the same in both backgrounds.

Iron storage. Finally, the expression of *bfrB*, the gene encoding the bacterioferritin iron-storage protein, was down-regulated in the *rsmA* mutant compared to PAO1, which is in agreement with the increase in expression of genes involved in siderophore-mediated iron uptake (Table 2). Therefore, although grown in iron-rich LB medium, the *rsmA* mutant behaved partly as though it was cultured in iron-deplete conditions.

DISCUSSION

RsmA exerts its effect at the posttranscriptional level. Commonplace methods for identifying targets of posttranscriptional regulators include gene-by-gene transcriptional and translational fusion analyses, RNA binding studies, and proteomics. However, to provide a comprehensive list of genes whose expression is influenced by RsmA, we chose the somewhat unconventional approach of transcriptome profiling to examine the global effects of an *rsmA* mutation on gene expression and to identify novel functions influenced by RsmA in *P. aeruginosa* PAO1. As regulatory networks in *P. aeruginosa* have been intensively documented, changes in gene expression enable identification of known transcriptional regulators as potential direct targets for RsmA.

A notable feature of this transcriptome analysis was the far-reaching consequences of an *rsmA* mutation, emphasizing the role of RsmA as a global regulator. RsmA is known to indirectly control the transcription of numerous genes through regulation of the QS network (Pessi *et al.*, 2001). Hence, some of the observed alterations in the transcriptome profile of the *rsmA* mutant may be due to the direct effect of the *rsmA* mutation on the QS network, since RsmA negatively regulates *lasI* and *rhII* (Pessi *et al.*, 2001). For example, the increase in 3-oxo-C12-HSL in the *rsmA* mutant (Pessi *et al.*, 2001) may contribute to the increase in expression of the pyoverdine biosynthetic genes (Stintzi *et al.*, 1998; Whiteley *et al.*, 1999) and *sodM* (Hassett *et al.*, 1999), both regulated by the QS system. However, although inactivation of *rsmA* affects AHL synthesis, this does not appear to have a dramatic effect on expression of all QS-regulated genes. Moreover, alteration in AHL levels in the *rsmA* mutant cannot explain all of the changes in gene expression brought about by the loss of RsmA, so further explanations must be considered. An important feature of this study, which cannot be overlooked, is that microarray

experiments measure mRNA steady-state levels, which are influenced by transcription rate and mRNA stability and decay. The only known method of RsmA-mediated regulation of gene expression occurs through alteration of mRNA stability and subsequent translation. However, the known influence of RsmA on the stability of certain mRNAs may not be sufficient to explain the extent of changes observed in the comparative transcriptome profile. Therefore, given that several phenotypes were greatly affected by inactivation of RsmA, we believe that RsmA posttranscriptionally regulates one or more transcriptional regulators, which in turn affect the transcription of a number of genes.

RsmA is involved in the expression of *pvdS* and siderophore biosynthetic genes. A recently identified link between the QS network and iron acquisition in *P. aeruginosa* is VqsR (Juhas *et al.*, 2004). VqsR is a regulator of the QS network in *P. aeruginosa* TB, and has been shown to regulate genes involved in iron acquisition. *vqsR* mutants have reduced C4-HSL and 3-oxo-C12-HSL production, reduced expression of both the *phnAB* and *pqsABCDE* genes, and reduced expression of *pvdS*, along with reduced transcription of other genes in the pyoverdine and pyochelin biosynthetic loci (Juhas *et al.*, 2004). As both RsmA and VqsR influence AHL production, and the expression of *phnA* and the genes involved in iron acquisition, it is possible to consider a potential link between these two global regulators, which has not been previously recognized. Clearly more work needs to be carried out to unravel the involvement of RsmA in the complex, highly regulated iron acquisition and storage system in *P. aeruginosa*.

During late-exponential and stationary phase, cultures of the *rsmA* mutant produced less PQS than PAO1 wild-type. The QS network has been implicated in the regulation of PQS production: the *las* system positively controls the transcription of *pqsA*, while the *rhl* system negatively controls the transcription of *pqsA* (McGrath *et al.*, 2004), via the impact of both systems on *pqsR* expression (Wade *et al.*, 2005). However, as neither *pqsR* nor *pqsA* transcription was altered in the *rsmA* mutant, it is unlikely that the increased AHL levels (Pessi *et al.*, 2001) influence the decrease in PQS production in the *rsmA* mutant. Therefore, another explanation for this drop in PQS levels was sought. In *P. aeruginosa*, there are two anthranilate synthase systems: PhnA and PhnB (anthranilate synthase II) and TrpE and TrpG (anthranilate synthetase I) (Essar *et al.*, 1990). Both systems have different functions, in that anthranilate synthetase I converts chorismate to the anthranilate used in tryptophan biosynthesis, while anthranilate synthase II converts chorismate to the anthranilate used in phenazine biosynthesis (Essar *et al.*, 1990). It has been suggested recently that anthranilate produced by anthranilate synthetase I (the Trp pathway) is not available for PQS biosynthesis (Aendekerk *et al.*, 2005). As *trpG* and *trpE* expression was not altered in the *rsmA* mutant compared to PAO1, the anthranilate present in the *rsmA* mutant extract is likely to be an accumulation of anthranilate formed via the anthranilate synthetase I system. As *phnA*

transcription was decreased by approximately threefold in the *rsmA* mutant compared to PAO1 wild-type, while *pqsABCDE* levels were not altered, it is possible that the anthranilate required for PQS formation is not available in the *rsmA* mutant, hence the reduction in PQS levels. Interestingly, loss of RsmA brought about a dramatic drop in levels of the phenazine pyocyanin. This finding is consistent with the decrease in *phnA* expression in the *rsmA* mutant compared to PAO1 wild-type, since a *phnA* mutant has been shown elsewhere to have reduced pyocyanin levels (Essar *et al.*, 1990). In addition, the decrease in expression of *phzA1* in the *rsmA* mutant may contribute to the decrease in pyocyanin production. Other studies have shown that loss of RsmA results in an increase in pyocyanin production compared to *P. aeruginosa* PAO1 wild-type, when the bacteria are cultured in glycerol/alanine broth (Pessi *et al.*, 2001). However, our data show that this is not the case when the bacteria are cultured in LB broth. It is possible that the different carbon sources used in the two experiments alter pyocyanin production, via the activation of the PhnAB versus the TrpEG pathway, which would explain the discrepancy in the results.

We have shown that RsmA affects expression of *vfr*, encoding a homologue of the *E. coli* cAMP receptor protein. Comparison of the transcriptome profiles of the *P. aeruginosa* PAK *vfr* mutant (Wolfgang *et al.*, 2003) and the *P. aeruginosa* PAO1 *rsmA* mutant showed that 19 genes and one intergenic region were commonly altered in expression (Supplementary Table S2). The influence of RsmA on the expression of this subset of genes may therefore be through regulation of *vfr*, particularly with respect to the *pil* genes involved in the biosynthesis of pili. Vfr-null mutants have been shown to exhibit severely reduced twitching motility with barely detectable levels of type IV pili, as the mutant is defective in type IV pilus assembly (Beatson *et al.*, 2002). Beatson *et al.* (2002) propose that it is most likely that Vfr acts as a transcriptional regulator of a gene(s) encoding functional elements of type IV pili or signal transduction pathways that control twitching motility. If this is indeed the case, the drop in *vfr* levels in the *rsmA* mutant could explain the reduced expression of genes involved in pilus biosynthesis. Another candidate target of direct RsmA control is the regulatory protein MvaT. Recently, MvaT has been shown to be a negative regulator of the transcription of *cupA* genes and, to a lesser extent, of *cupB* and *cupC* expression (Vallet *et al.*, 2004). Expression of *cupA2* and *cupC3* was decreased in the *rsmA* mutant compared to PAO1 wild-type. As a regulator of the *cup* genes, it is therefore possible that RsmA has posttranscriptional control over *mvaT*. Since *mvaT* transcript levels were not altered in the *rsmA* mutant, as revealed by the transcriptome assay, it is likely that RsmA affects the translation of *mvaT* rather than the stability of its mRNA.

Since the *retS* mutant is thought to alter gene expression in part by controlling the level of free, active RsmA (Goodman *et al.*, 2004), we assessed the similarities between the

transcriptome profiles of the *P. aeruginosa* PAK *retS* mutant and the *P. aeruginosa* PAO1 *rsmA* mutant, and genes of altered expression in both backgrounds were compared. This revealed that 53 altered genes were common to both mutants (Supplementary Table S3), hence strengthening the proposal put forward by Goodman *et al.* (2004) that RsmA is part of the RetS regulon.

Recent studies conducted by Heeb *et al.* (2005) have shown that in *P. fluorescens* CHA0, RsmA posttranscriptionally regulates *rpoS*. The stationary-phase sigma factor RpoS has been implicated in the regulation of numerous functions in *P. aeruginosa*, including QS-regulated processes (Schuster *et al.*, 2004). To assess the posttranscriptional impact of the loss of RsmA on *rpoS*, an *rpoS*–*lacZ* translational fusion was analysed in *P. aeruginosa* PAO1 wild-type and the *rsmA* mutant over time. However, there was no difference in the translation of *rpoS* between the two strains (data not shown). Therefore, in contrast to *P. fluorescens* CHA0, *P. aeruginosa* PAO1 RsmA does not posttranscriptionally regulate *rpoS*, under the experimental conditions outlined.

Interestingly, the gene with the highest fold increase in expression (1173-fold) (Table 2) in the *rsmA* mutant compared to PAO1 wild-type was *gloA2*, which encodes an enzyme involved in the breakdown of the toxic electrophile methylglyoxal, a by-product of glycolysis. This suggests that, like CsrA in *E. coli* (Romeo, 1998), RsmA posttranscriptionally regulates genes involved in carbon metabolism in *P. aeruginosa*.

In conclusion, this study has revealed the impact of RsmA on gene expression and the regulation of key cellular processes in *P. aeruginosa*. In addition, evidence is provided suggesting that RsmA may function as a downstream effector of hierarchical regulatory proteins, such as RetS and MvfR.

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