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Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA

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Summary

One of the prokaryotic post-transcriptional regulatory mechanisms involves the CsrA/RsmA family of proteins that act by modulating translation initiation at target mRNAs. In this study, we identified the regulon of RsmA of the *Pseudomonas aeruginosa* PAK strain by using cultures in the stationary phase of growth. The RsmA regulon includes over 500 genes, of which approximately one third were affected by an *rsmA* mutation negatively, while the rest were affected positively. By isolating RsmA/mRNA complexes, analyzing transcriptional and translational fusions, and performing gel-shift analyses, we identified 40 genes in six operons that are regulated by RsmA directly at the level of translation. All of these genes were affected by RsmA negatively and include genes encoding the type VI secretion system HSI-I, which has been implicated in the *P. aeruginosa* chronic infections. On the other hand, we were unable to demonstrate a direct interaction of RsmA with transcripts that are positively affected by this protein, including mRNAs encoding the type III secretion system and the type IV pili genes. Our work supports a model in which RsmA acts as a negative translational regulator, and where its positive effects are achieved indirectly by RsmA-mediated interference with translation of specific regulatory factors.

Introduction

Regulation of gene expression in bacteria operates at several levels, including transcriptional and translational initiation. One type of post-transcriptional control is mediated by the RsmA/CsrA family of proteins (Babitzke and Romeo, 2007; Lapouge *et al.*, 2008; Liu and Romeo, 1997; Romeo, 1998). These small (ca. 7 kD) proteins specifically recognize and bind to a conserved GGA trinucleotide located in the 5' leader sequence of target mRNAs. In addition to the central GGA element, flanking ribonucleotides are also important, presumably because in order to be recognized by RsmA/CsrA the GGA motif must be exposed in the loop of a stem-loop structure (Baker *et al.*, 2007; Dubey *et al.*, 2005; Lapouge *et al.*, 2007; Lapouge *et al.*, 2008; Schubert *et al.*, 2007). However, the relative importance of primary sequence elements and secondary structures in RsmA/CsrA recognition is not completely understood. The GGA motif can be found in several copies per leader sequence of a target mRNA, however, one GGA element always overlaps the ribosome binding site (RBS) sequence (Baker *et al.*, 2002; Baker *et al.*, 2007; Blumer *et al.*, 1999; Dubey *et al.*, 2003; Lapouge *et al.*, 2007). Thus, RsmA/CsrA proteins regulate gene expression by binding

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to target mRNAs and thereby blocking the interaction of the RBS with the 30S ribosomal subunit. This prevents initiation of translation and eventually leads to destabilization and degradation of the transcript.

The activity of RsmA/CsrA proteins is modulated by two apparently redundant small RNAs, called RsmY and RsmZ in *Pseudomonas aeruginosa*, and CsrB and CsrC in *Escherichia coli* (Gottesman, 2005; Heeb *et al.*, 2002; Kay *et al.*, 2006; Liu *et al.*, 1997; Romeo, 1998; Valverde *et al.*, 2003). These regulatory RNAs contain multiple copies of the GGA motif, the majority of which occur in single stranded regions of stem-loops of the predicted secondary structures. Thus, these RNAs inhibit the activity of the translational regulator by competing with target mRNAs for RsmA/CsrA binding.

The members of the RsmA/CsrA family have been identified in many Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, *Pseudomonas fluorescens*, and species of *Salmonella*, *Legionella*, *Proteus*, *Helicobacter*, and *Erwinia*, where they have been implicated in the regulation of phenotypes such as virulence, motility, quorum sensing, and stress response (Altier *et al.*, 2000; Barnard *et al.*, 2004; Fettes *et al.*, 2001; Jonas *et al.*, 2008; Liaw *et al.*, 2003; Romeo *et al.*, 1993; Whitehead *et al.*, 2002). In *E. coli*, CsrA has been shown to directly bind and regulate translation of mRNAs encoding the RNA chaperone Hfq, enzymes involved in carbon starvation and glycogen synthesis, proteins responsible for the production of a biofilm polysaccharide, and most recently, two proteins with GGDEF domains involved in the regulation of motility (Baker *et al.*, 2002; Baker *et al.*, 2007; Dubey *et al.*, 2003; Jonas *et al.*, 2008; Wang *et al.*, 2005). In *P. aeruginosa* and *P. fluorescens*, a direct regulation by RsmA has been demonstrated for a transcript encoding hydrogen cyanide synthesis (*hcn*) (Lapouge *et al.*, 2008; Pessi *et al.*, 2001). In each of these cases, RsmA/CsrA binding results in rapid degradation of the target mRNAs. However, in *E. coli*, CsrA is also involved in the positive regulation of flagellar motility, where binding of CsrA to the 5' region of the *flhDC* mRNA results in stabilization of the transcript (Wei *et al.*, 2001). Other than these few examples, no other direct targets of RsmA/CsrA regulation have been identified. It is therefore still largely unknown how the RsmA/CsrA proteins coordinate the expression of the large number of genes responsible for the diverse phenotypes that are affected by these regulators.

P. aeruginosa is an opportunistic human pathogen that causes infections in individuals with impaired host defense or suffering from cystic fibrosis (CF). In young CF patients, *P. aeruginosa* initially causes what appears to be a mild form of acute infection, which later develops into a chronic respiratory disease. It has been proposed that two different, mutually exclusive sets of virulence factors are associated with the two stages of infection. For example, the type III secretion (T3S) system and the type IV pili genes are associated with the acute disease, while the type VI secretion (T6S) system HSI-I and production of biofilm are important during the chronic infection (Dacheux *et al.*, 2001; Deretic *et al.*, 1995; Luzar and Montie, 1985; Mougous *et al.*, 2006; Parsek and Singh, 2003; Singh *et al.*, 2000; Smith *et al.*, 2006; Yahr and Greenberg, 2004).

Recently, it has been shown that the expression of these virulence determinants is coordinately regulated by a pair of two-component sensor kinases RetS and LadS acting

through the GacS/GacA two component system (Goodman *et al.*, 2004; Ventre *et al.*, 2006; Yahr and Greenberg, 2004). The molecular mechanism of the RetS-mediated regulation has been recently elucidated. RetS is the antagonist of GacS, a transmembrane sensor kinase which activates its cognate response regulator GacA by phosphotransfer. RetS forms a heterodimer with GacS and blocks its autophosphorylation thus preventing GacA phosphorylation (Goodman *et al.*, 2008). Phosphorylated GacA is required for activation of transcription of the regulatory RNAs, RsmY and RsmZ, which in turn antagonize the regulatory activity of RsmA (Heurlier *et al.*, 2004; Kay *et al.*, 2006; Lapouge *et al.*, 2008). Therefore, based on these findings, the coordinate regulation of the *P. aeruginosa* virulence genes is achieved by the regulatory activities of a translational regulator, RsmA. Accordingly, RsmA plays an extremely important regulatory role in the *P. aeruginosa* biology, and particularly in its life style as a human pathogen. These predictions are consistent with the effects that RsmA has been observed to have on the expression of the T3S system and on biofilm development (Mulcahy *et al.*, 2006; Mulcahy *et al.*, 2008; O'Grady *et al.*, 2006).

In this study we have undertaken a global analysis of transcript concentrations of genes that are controlled by RsmA in the *P. aeruginosa* strain PAK. The RsmA regulon that we thus identified consists of over 500 genes and includes most of the known *P. aeruginosa* virulence genes. Transcript levels of approximately one third of these genes were decreased in the *rsmA* mutant, while the concentrations of the rest of the affected transcripts were increased. The simplest model for RsmA regulation involves direct binding of RsmA to target sequences at the site of translational initiation of each of the affected mRNAs. However, given the large number of genes that were affected by an *rsmA* mutation, the known mechanism of RsmA activity, and the rarity of cases where RsmA binding has a positive effect on the bound transcript, it is more likely that the levels of at least some of these transcripts were altered by the action of RsmA on the translation of transcriptional regulators of those genes.

In the second part of this report, we describe a series of experiments that address these questions. First, we attempted to capture and identify *P. aeruginosa* transcripts bound to RsmA. Next, we analyzed the effect of RsmA on expression of several genes selected from the microarray analysis using transcriptional and translational fusions to the β -galactosidase gene. Furthermore, we prepared labeled transcripts and used gel mobility shift assays to verify their interaction with purified RsmA. Overall, we identified six operons that are directly regulated by RsmA. All of these operons are regulated by RsmA negatively and include two that encode the components of the T6S system HSI-I. In contrast, we observed that the T3S genes and the type IV pili genes are affected by RsmA positively and we present evidence that they are regulated by RsmA indirectly. Furthermore, we demonstrate that another group of genes that are affected by RsmA positively, namely the genes involved in iron homeostasis, is also regulated by RsmA indirectly. We show that these mechanisms are likely to involve iron-sensitive regulatory pathways. Finally, we propose a model in which the majority of genes that are affected by RsmA negatively are directly controlled by this protein, while the rest of the genes and the genes that are affected by RsmA positively are regulated indirectly, presumably via direct RsmA regulation of the relevant regulatory factors. A similar model has been proposed previously in the study by Burrowes and

colleagues (Burrowes *et al.*, 2006). The same study also identified the RsmA regulon of the *P. aeruginosa* PAO1 strain which differs significantly from the RsmA regulon of the PAK strain identified here. The differences between the two regulons are analyzed and discussed.

Results

Identification of the RsmA regulon of *P. aeruginosa* PAK

In order to assess the full range of *P. aeruginosa* transcripts affected by the translational regulator RsmA, we first measured the kinetics of *rsmA*, *rsmZ* and *rsmY* expression during growth in rich medium. Expression of *rsmY* and *rsmZ* was monitored by measuring β -galactosidase activities of transcriptional *rsmY-lacZ* and *rsmZ-lacZ* fusions in *P. aeruginosa* PAK wild type and *gacA* mutant strains. As expected, transcription of both fusions depended on the presence of the response regulator GacA. In the wild type strain, at all stages of growth, the expression of *rsmY* was higher than that of *rsmZ*, however, the two fusions displayed similar kinetics (Figure 1A and 1B). For both fusions, expression sharply increased in the late exponential phase and peaked in the stationary phase when there was minimal increase in bacterial mass (Figure 1A and 1B). This pattern of expression was similar to that of RsmA whose intracellular concentration also peaked in stationary phase (Figure 1C). Similar observations have been reported previously for the kinetics of *rsmZ*, *rsmY* and *rsmA* expression in the *P. aeruginosa* PAO1 strain (Kay *et al.*, 2006). The fact that all three components of this regulatory system (RsmA, RsmZ and RsmY) follow the same expression pattern suggests that in the absence of environmental signals, the ratio of the amounts of the two small RNAs to the amount of RsmA are constant throughout the course of growth. However, in the stationary phase, the system is expressed at its maximum level. Consequently, we used cultures at 7 hours of growth for RNA isolation and microarray analysis.

Of the 5,678 genes surveyed in the *P. aeruginosa* microarray, 528 showed significantly altered transcript levels in *P. aeruginosa rsmA* as compared to the wild-type strain (Table S1). Many of these genes encode virulence factors. Specifically, the mRNA levels for 22 of the 40 structural and regulatory genes of the T3S system were decreased in the *rsmA* mutant, including the genes encoding the T3S transcriptional regulator ExsA, the components of the secretion machinery and all three secreted effectors (Table 1). A substantial effect (3- to 25-fold) was detected despite the fact that the medium used for culturing bacteria was not calcium-depleted and therefore not optimal for expression of T3S genes (Wolfgang *et al.*, 2003). Furthermore, transcript levels of 8 genes encoding the type IV pili biogenesis apparatus were also decreased in the *rsmA* mutant. On the other hand, the *rsmA* mutation had a positive effect on transcript levels of the *pel* and the *psl* genes encoding exopolysaccharide components of the biofilm matrix and the genes encoding the T6S system HSI-I (Tables 1, S1) (Matsukawa and Greenberg, 2004; Mougous *et al.*, 2006). In total, 187 genes showed a decrease in their transcript levels in the *rsmA* mutant, while 341 genes were affected by the *rsmA* mutation positively (Tables 1, S1).

Identification of mRNAs that co-purify with RsmA

To identify genes in the RsmA regulon that are regulated by RsmA through its direct interaction with the corresponding transcripts, we carried out an RNA-RsmA co-purification experiment. A C-terminally histidine-tagged RsmA was expressed from a plasmid in an *rsmA* deletion mutant (PAK *rsmA* (pMMB67-RsmA-H₆)) and was isolated by nickel affinity chromatography. The bound RNA was released and isolated from the protein by phenol/chloroform extraction followed by ethanol precipitation. A control culture of a *P. aeruginosa* *rsmA* deletion mutant carrying an empty vector (PAK *rsmA* (pMMB67)) was processed in parallel. Although equivalent amounts of total RNA were applied to the nickel affinity columns, the amount of RNA isolated from the control culture was approximately 6% of that which was isolated from the experimental culture of PAK *rsmA* (pMMB67-RsmA-H₆) (3 µg vs 50 µg). This suggested that the majority of RNA that was isolated from the experimental culture was associated with RsmA-H₆. Each RNA sample was analyzed on a denaturing agarose gel (Figure 2). Two bands corresponding to 16S rRNA and 23S rRNA were present in both samples, suggesting that these abundant transcripts were co-purified non-specifically. The sample from the experimental culture contained another very prominent band of approximately 100 nt in length, which was absent in the control sample (Figure 2). The RNAs in this band were identified as RsmY and RsmZ (See below). In addition, a smear between the 16S rRNA band and the 100 nt band was relatively abundant in the experimental sample.

To enrich for RsmA-bound transcripts other than RsmY and RsmZ, or rRNAs, 25 µg of RNA from the experimental sample was separated again on a denaturing polyacrylamide gel and extracted separately from five distinct sections of the gel (Figure 2). Sections corresponding to 16S and 23S rRNA were pooled into sample 1. Sample 2 contained the section between the 16S and 23S rRNA bands, sample 3 the smear between the 16S rRNA and the 100 nt band, and sample 4 the 100 nt band. RNAs isolated from each sample were used separately as templates for cDNA synthesis (Figure 2). The resulting cDNA was converted to double-stranded DNA, which was cloned and sequenced. Initially, we sequenced 12 clones per RNA sample. As expected, sample 1 contained exclusively the 23S rRNA and the 16S rRNA (Figure 2). The three sequences that were identified from sample 2 corresponded to 23S rRNA, while clones obtained from sample 4 corresponded to RsmY (4 clones) and RsmZ (6 clones). Approximately 50% of the clones identified from sample 3 encoded fragments of 23S rRNA, while the rest corresponded to various species of mRNA (Figure 2). Therefore, we sequenced 170 additional clones from sample 3. In these samples, the non-coding RNAs still represented almost half of the identified sequences. Of the 88 sequenced clones that encoded mRNAs (See Supporting Information for description of these sequences), we further considered only those that were identified at least twice. There were 14 such sequences corresponding to a total of 6 genes (Table S2). Only two of these genes, PA0081 and PA4492, were also affected by the *rsmA* mutation in the RsmA transcriptome analysis (Table 1, Table S2). Both of these genes, as well as the genes encoded in their putative resident operons, were affected by the *rsmA* mutation positively (Table 1). Furthermore, the RBSs of both PA0081 and PA4492 genes have features in common with other known CsrA/RsmA targets (see below and Discussion). For these reasons, the PA0081 and PA4492 genes were chosen for further analysis. Other genes that were detected more

than once include PA4611, which was detected four times, and PA2977, PA4489 and PA4744, each of which was detected twice (Table S2). These genes were not identified as members of the *P. aeruginosa* PAK RsmA regulon and their RBSs do not resemble the known RsmA binding sites (See Table S2 and Discussion). Although we do not exclude the possibility that at least some of these genes represent true substrates for RsmA binding (See Discussion), they were not further explored in this study.

Sequences of mRNAs that co-purified with RsmA share features that are common to known RsmA/CsrA targets

Based on gene organization and microarray expression data, both PA0081 and PA4492 are encoded as the first genes in larger operons (Figure 3). The members of the PA0081 operon encode components of the T6S system HSI-I (Mougous *et al.*, 2006), while the PA4492 operon encodes genes with hypothetical functions. PA0082 is the first gene in another putative operon that is adjacent to and divergent from PA0081 (Figure 3A) (Mougous *et al.*, 2006). Like the PA0081 operon, the PA0082 operon encodes components of the T6S system and its transcripts are present in substantially higher amounts in the *rsmA* mutant compared to the wild type (Table 1). For these reasons, in the experiments that follow, PA0082 was investigated in parallel with PA0081 and PA4492.

The likely promoters and transcription start sites of PA4492, PA0081 and PA0082, as well as of all other genes addressed in this study were determined based on previously published data or by using BPROM, a bacterial σ^{70} promoter recognition program. A 5' RACE procedure was carried out to confirm transcription initiation sites and the predicted promoters for PA0081, PA0082, and PA4492 (Figure 3). The procedure confirmed the BPROM-predicted promoters and transcription start sites for all three genes. For PA0081 and PA0082, it identified an additional weak transcription initiation site upstream of the predicted site (Figure 3A), however, in all experiments in this study, the downstream transcription start site was considered as the 5' end.

The sequences corresponding to the PA0081 mRNAs that bound and co-purified with RsmA-H₆ were 67 and 82 nucleotides in length. Both contained parts of the leader sequence and overlapped with the PA0081 RBS (Figure 3A). The two PA4492 mRNA segments that co-purified with RsmA-H₆ were 75 and 45 nucleotides in length. One of them overlapped the RBS of the gene, while the other corresponded to nucleotides 105-149 of the coding sequence (Figure 3B). In the predicted secondary structures of leader sequences of PA0081, PA0082, and PA4492, which were determined by Mfold (Zuker, 2003), a GGA motif overlaps the RBS and resides in the single-stranded region of a stem-loop structure (Figure 4). Similar motifs have previously been identified as recognition elements for RsmA and CsrA. This suggested that PA0081 and PA4492 co-purified with RsmA specifically and that they, as well as the PA0082 gene, represented true targets of direct RsmA regulation (Baker *et al.*, 2007; Dubey *et al.*, 2005; Lapouge *et al.*, 2007; Lapouge *et al.*, 2008; Schubert *et al.*, 2007). Apart from the GGA of the RBS, the leader sequences of PA0081 and PA0082 contain three and two additional GGA motifs, respectively. Based on the predicted secondary structures, some of these additional GGA motifs may also be exposed in loop structures and could serve as substrates for RsmA binding, as has been observed for several

of the known RsmA and CsrA targets (Dubey *et al.*, 2003; Lapouge *et al.*, 2007; Wang *et al.*, 2005).

RsmA binds leader sequences of PA4492 and the T6S genes *in vitro*

To confirm the specificity of RsmA binding to mRNAs encoding PA0081 and PA4492, and to test RsmA binding to PA0082, we carried out gel mobility shift assays. C' terminally histidine-tagged RsmA of *P. aeruginosa* was purified from *E. coli* and tested for binding to *in vitro* synthesized and ³²P-end-labelled fragments of RNA encoding leader sequences of PA0081, PA0082, and PA4492. Full length RsmY and RsmZ were also synthesized and ³²P-end-labelled, and served as positive controls, while the leader mRNA of the *lolB* gene, which was unaffected by the *rsmA* deletion in our transcriptome analysis, served as a negative control. The *in vitro* synthesized RNAs were homogenous in size and migrated in a single band when subjected to electrophoresis under denaturing conditions (data not shown). However, following native gel electrophoresis each RNA species migrated in multiple bands in the presence and absence of RsmA (Figure 5). We conclude that the multiple bands were likely due to formation of secondary structures in the RNA that were preserved under native conditions that were used in the experiment.

The purified RsmA-H₆ was able to retard the PA0081, PA0082, and PA4492 mRNA fragments (Figure 5A). The protein also shifted both RsmY and RsmZ, but not the negative control *lolB*. Furthermore, RsmY and RsmZ could efficiently compete with both PA0081 and PA0082 for RsmA binding (Figure 5B and data not shown), while the negative control, *lolB*, could not. These results confirm that RsmA is able to specifically bind to the leader RNA of PA0081, PA0082, and PA4492, and in combination with the microarray results suggest that expression of these genes is regulated by RsmA directly via RsmA-mRNA binding.

Expression of PA4492 and the T6S genes is regulated by RsmA at a post-transcriptional level

If RsmA regulates the PA0081, PA0082, and PA4492 genes by binding to mRNA, the effect of RsmA on expression of these genes is expected to be manifested at a post-transcriptional level. To compare the effect of RsmA on transcription and translation of PA0081, PA0082, and PA4492, we constructed transcriptional and translational chromosomal *lacZ* fusions to these genes, and measured their expression in the wild type and the *rsmA* mutant strain. The sequences encoding the promoter, the 5' leader sequence, and several nucleotides of the 5' coding sequence of each gene were cloned into vector mini-CTX-*lacZ* (Becher and Schweizer, 2000), and into vector pUC18-mini-Tn7-*lacZ*20-Gm (Choi and Schweizer, 2006), to construct transcriptional and translational fusions, respectively (Figure 6D). Vector mini-CTX-*lacZ* carries an RNaseIII processing site that is located between the multiple cloning site and the RBS of the *lacZ* reporter gene (Figure 6D). This ensures construction of transcriptional fusions that are translated independently of the cloned sequence (Choi and Schweizer, 2006). In the pUC18-mini-Tn7-*lacZ*20-Gm vector, the multiple cloning site is positioned directly upstream of the eighth codon of the *lacZ* gene (Figure 6D).

For all three genes, PA0081, PA0082, and PA4492, the *rsmA* mutation had only a marginal effect on the expression of the transcriptional *lacZ* fusions, while it strongly affected expression of the translational fusions (Figure 6A and 6B). Furthermore, translational fusions remained under RsmA control when the promoter of each gene was replaced by the constitutively expressed *PlacUV5* promoter (Figure 6C and 6D). Taken together, these results demonstrate that RsmA regulates expression of these genes at a post-transcriptional level, and are consistent with RsmA regulation of these genes via direct RsmA-mRNA binding.

Since the amounts of transcript of all three operons, PA0081, PA0082 and PA4492, were elevated in the *rsmA* mutant, we next determined whether or not any other genes that were similarly affected by the *rsmA* mutation were also regulated by RsmA directly. In the microarray analysis, transcripts encoding genes in the PA0277, PA2541 and PA3732 operons were among those that showed the highest increase in concentration in the *rsmA* mutant relative to the wild type (Table 1 and S1). Furthermore, Mfold analysis of leader sequences of these operons predicts that for all three genes the GGA motif that overlaps the RBS is located in the single-stranded region of a stem-loop structure (Figure S1). According to the *Pseudomonas* genome database, PA0277 encodes a hypothetical Zn-dependent protease and is likely transcribed as a single gene. PA2541 and PA3732 are the first genes in two unrelated putative six-gene operons of unknown functions. We constructed transcriptional and *PlacUV5*-driven translational *lacZ* fusions to PA0277, PA2541 and PA3732 genes and tested their expression in the wild type and *rsmA* backgrounds. While the *rsmA* mutation had a significant positive effect on the expression of the *PlacUV5*-driven translational fusions to all three of these genes, its effect on the transcriptional fusions was negligible (Table 2). Based on these results, we conclude that the PA0277, PA2541, and PA3732 are directly controlled by RsmA at a post-transcriptional level and represent additional new targets of direct RsmA regulation.

The effect of RsmA on the expression of the T3S and the type IV pili genes is indirect

All of the genes that we have identified thus far as direct targets of RsmA regulation were affected by the *rsmA* mutation positively. To test whether or not any of the genes that were affected by the *rsmA* mutation negatively were also regulated by RsmA directly, we constructed transcriptional fusions to *exsC*, *exsD*, *exoS*, and *pilM*, and *PlacUV5*-driven translational *lacZ* fusions to *exsD*, *exoS*, and *pilM*. *exsC* is the first gene in an operon encoding T3S genes with regulatory functions. *ExsD* is a negative regulator of the T3S genes and is encoded by the first gene in an operon that includes a number of T3S structural genes. *exoS* is a cistron and encodes a T3S effector, while *pilM* is the first gene in an operon encoding type IV pili biogenesis genes. Expression of all four of these genes was negatively affected by the *rsmA* mutation in the microarray analysis (Table 1). The transcriptional and translational *lacZ* fusions that we constructed were introduced into the wild type and *rsmA* mutant strains and tested for expression. The *rsmA* mutation had a strong negative effect on expression of all four transcriptional fusions, but did not significantly affect the expression of the *PlacUV5*-driven translational fusions (Table 2). These results show that RsmA affects these genes at the level of transcription rather than translation, suggesting that the RsmA effect on expression of these genes is indirect.

We also carried out gel mobility shift assays to test the binding of purified RsmA-H₆ to leader sequences of *exsD*, *exoS*, *pilM*, and *exsC* (Figure 5A). Although we observed heterogeneity in the mobility of the various RNA species under native conditions, none of the bands altered their mobility following incubation and electrophoresis with RsmA-H₆ (Figure 5A). The failure of RsmA to bind these mRNAs *in vitro* further confirms that RsmA regulates *exsD*, *exoS*, *pilM* and *exsC* indirectly, possibly by controlling translation of mRNAs encoding transcriptional regulators of these genes.

RsmA and RetS are parts of the same global regulatory pathway

Comparison of the microarray profile of the *P. aeruginosa* PAK *rsmA* mutant to the previously published microarray profile of a *P. aeruginosa* PAK *retS* mutant (Goodman *et al.*, 2004), showed that 35 percent (139 genes) of all genes that were altered in the *retS* mutant were also altered in the same direction in the *rsmA* mutant (Table S3). This number is relatively high, taking into account that the two analyses were performed using different media and cultures in different phases of growth. The genes that were common to both transcriptomes include PA0277, the genes encoding the T6S system HSI-I, and the genes encoded in the PA2541, PA3732, and PA4492 operons. Transcript levels of all of these genes were increased in both mutants. Furthermore, the common genes include the T3S and the type IV pili, which were downregulated in both mutants (Table S3). These results are consistent with the model in which RsmA and RetS are parts of the same global regulatory pathway in which RetS positively affects RsmA activity by negatively regulating the ability of the GacS/A two component system to activate transcription of the small RNAs RsmY and RsmZ (Goodman *et al.*, 2004; Burrowes *et al.*, 2006). Also consistent with this model is the effect that the *retS* mutation had on the expression of *rsmY* and *rsmZ* (Figure S2). Throughout the growth phase, the expression of both *rsmY* and *rsmZ* was strongly elevated in the *retS* mutant, while the two small RNAs were not expressed in strains where either *gacA* or *gacS* were mutated.

To test this model further and to verify the post-translational mechanism of regulation supported by the experiments above, we examined the dependence of expression of two RsmA-regulated genes on the entire RetS/GacS/A/RsmY/Z/RsmA signal transduction pathway. From the genes whose mRNA concentrations increased or decreased in the absence of RsmA, we selected the T6S gene PA0081 and the T3S gene *exsD* to represent each class. Transcriptional and translational PA0081-*lacZ* and *exsD-lacZ* fusions were tested in the *retS*, *gacS*, *gacA*, *rsmZ*, *rsmY*, and *rsmZY* backgrounds, as well as in the control wild type and *rsmA* strains. The transcriptional PA0081-*lacZ* fusion was expressed at similar levels in the wild type strain and all mutant backgrounds (Figure 7A). In contrast, the expression of the translational PA0081-*lacZ* fusion was strongly affected in *P. aeruginosa retS* and *rsmA* strains, where it was expressed at elevated levels (Figure 7B). These results confirm the model in which PA0081, and possibly other genes that are negatively regulated by RsmA, are reciprocally controlled by the RetS/GacS/A regulatory system through regulation of transcription of the small RNAs RsmZ and RsmY, which in turn antagonize the RsmA's activity as a direct translational repressor of PA0081 (Figure 11). In contrast, the *exsD-lacZ* expression was affected at both transcriptional and translational levels (Figures 7C and 7D); the expression was moderately elevated in the *gacS*, *gacA* and *rsmYZ* mutant backgrounds,

and strongly repressed in the *retS* and *rsmA* mutants. These results show that *exsD* and possibly other genes that are positively affected by RsmA are also controlled by the RetS/GacS/A/RsmY/Z/RsmA pathway, however, they are regulated primarily at the transcriptional level and thus their dependence on RsmA is indirect.

Comparison of the RsmA transcriptomes of the *P. aeruginosa* strains PAK and PAO1 and regulation of iron homeostasis genes

In the previously published study, Burrowes and colleagues described the RsmA-dependent transcriptome of the *P. aeruginosa* strain PAO1 (Burrowes *et al.*, 2006). The transcriptome consists of 506 genes, of which only a small number (67 or ~13%) are also found in the RsmA transcriptome of the *P. aeruginosa* PAK strain that was identified in this study (Table S4). Moreover, of the 67 genes that are common to both transcriptomes, only 36 were affected by the *rsmA* mutation in the same direction. However, despite the modest overlap between the two regulons, many of the groups of genes that were among the most strongly affected in the *rsmA* mutant of the *P. aeruginosa* PAK strain are also represented in the RsmA regulon of the PAO1 strain, although in smaller numbers. For example, in this study we detected an *rsmA*-dependent decrease in transcript levels of 22 T3S genes (Table 1), while only five T3S genes were affected in a similar way in the previous study (Burrowes *et al.*, 2006). Similarly, in the case of the type IV pili genes, transcript levels of the *pilM* operon were decreased in both studies, while downregulation of the genes in the *pilV* operon was only detected in the current study. Similar observations can also be made for all of those operons that we identified in this study as direct targets of RsmA (Table 1, Table S4, Burrowes *et al.*, 2006), with one exception. Many of the T6S genes that are encoded in the putative PA0081 and PA0082 operons were significantly upregulated in the *rsmA* mutant of the PAK strain (Table 1), while none of these genes were identified as members of the PAO1 RsmA regulon (Burrowes *et al.*, 2006).

The fundamental difference between the two microarray experiments is the growth stage at which the cultures were analyzed, notwithstanding the different genomic backgrounds (PAO1 vs PAK) which also may have contributed to the discrepancies between the two identified regulons. The cultures that were used for RNA isolation and analysis of the PAO1 transcriptome were in the exponential phase of growth (OD=0.8), while in this study we used cultures in the stationary phase (OD=6.0). To test whether or not the RsmA regulation of the T6S genes is growth phase dependant, we measured the effect of the *rsmA* mutation on the expression of the PA00082-*lacZ* translational fusion at different stages of growth (Figure S3). As a control, the PA4492 gene, which is the first gene of an operon whose members were affected by the *rsmA* mutation in both studies, was also included in the assay. The *rsmA* mutation positively affected the expression of both genes at all stages of growth, demonstrating that the RsmA regulation of the T6S genes is not dependent on growth phase (Figure S3). These results are further substantiated by the fact that the T6S genes encoded in the PA0081 and PA0082 operons were among the most strongly affected genes in the microarray analysis of the *P. aeruginosa* PAK RetS regulon, which was conducted with cultures in the exponential phase of growth (Goodman *et al.*, 2004). It is unclear why these genes were not detected in the transcriptome analysis of the *P. aeruginosa* PAO1 *rsmA* mutant (Burrowes *et al.*, 2006). It is unlikely that in the PAO1 background the T6S genes are

not affected by the regulatory activity of RsmA, especially in light of the fact that a *retS* deletion in this genomic background resulted in an increased expression of the T6S system (Mougous *et al.*, 2006).

A large group of genes that was affected by the *rsmA* mutation in both PAK and PAO1 parental backgrounds encodes proteins that are involved in iron homeostasis. However, while in this study the expression of these genes was strongly reduced in the *rsmA* mutant, in the previously published study that used the PAO1 strain, the expression of these same genes was increased in the mutant (Table 3). To explore this discrepancy, we constructed transcriptional and translational *lacZ* fusions to two randomly-selected iron homeostasis genes and tested the effect of the *rsmA* deletion on their expression. PA1300 is a sigma factor of the ECF subfamily that is predicted to have a role in the regulation of iron transport (Potvin *et al.*, 2006). PA4228 is the first gene in an operon encoding biosynthesis of the siderophore pyochelin (Serino *et al.*, 1997). Interestingly, the *rsmA* mutation had a positive effect on the expression of these two genes (Figure 8A). This is in agreement with the results of the PAO1 *rsmA* transcriptome analysis (Table 3). However, the expression of genes involved in iron homeostasis, including PA1300 and PA4228, is affected by the concentration of iron in the culture medium (Ochner *et al.*, 2002). When we tested the expression of PA1300 and PA4228 in the presence of the iron chelator 2,2'-dipyridyl, the *rsmA* mutation had a negative effect on the expression of both genes (Figure 8A). This result was in agreement with the results of our microarray analysis (Table 3). Addition of the chelator had no effect on RsmA-dependent expression of *lacZ* translational fusions to the control genes PA0081 and *exsD* (Figure 8A). Under both conditions, both of these genes were affected by the *rsmA* mutation in the same direction; PA0081 positively, and *exsD* negatively. Based on these results we conclude that the effect of RsmA on the expression of genes involved in iron homeostasis is dependent on the concentration of iron in growing cultures; RsmA has a negative effect when iron is abundant and positive effect when the concentration of iron is reduced. Both this (PAK parental background) and the previous (PAO1 parental background) study used poorly-defined complex media (LB) for culturing the bacteria and no particular steps were taken in either study to control the levels of iron in the media. Furthermore, more iron was likely consumed from the media of the cultures in the stationary phase (this study) compared to the cultures in the exponential phase of growth (previous study). Therefore, it is likely that the amount of iron available to the bacteria in the two experiments differed sufficiently to influence the outcome of the result. The fact that transcriptional and translational fusions to the PA1300 and PA4228 genes were affected by the *rsmA* mutation similarly, suggests that the effect of RsmA on expression of these genes is indirect (Figure 8A and 8B). It is possible that these genes are regulated by RsmA via a direct effect of RsmA on translation of a component in an iron-responsive regulatory pathway that controls transcription of these genes. Clearly more work needs to be carried out to unravel the involvement of RsmA in the complex, highly regulated expression of iron homeostasis genes in *P. aeruginosa*.

In addition to iron availability, expression of at least some of the genes involved in iron homeostasis is regulated by quorum sensing (Bollinger *et al.*, 2001; Hassett *et al.*, 1999; Oglesby *et al.*, 2008). Since the two microarray analyses used cultures at different cell densities, quorum sensing could have contributed to some of the differences in the effects

that RsmA had on the expression of iron homeostasis genes. However, in this study, this possibility was not investigated. We did, however, test the effects of the mutations in two quorum sensing regulators LasR and RhlR on the expression of *rsmY* and *rsmZ*. Neither *lasR* nor *rhlR* mutation had any effect on the expression of transcriptional *rsmY-lacZ* and *rsmZ-lacZ* fusions (Figure S2), suggesting that the LasR- or RhlR-dependent quorum sensing systems do not effect amounts of RsmY and RsmZ and hence do not affect the activity of RsmA.

Effect of RsmA on levels of selected *P. aeruginosa* proteins

To compare the wild type and *rsmA* transcriptomes with actual amounts of proteins produced by *P. aeruginosa* PAK, we carried out Western immunoblot analysis to determine the amounts of selected proteins in cells and extracellular fractions of the *P. aeruginosa* PAK wild type and *rsmA* mutant cultures (Figure 9A). The results show that the influence of the *rsmA* mutation was variable, ranging from modest effects on the relative amounts of PilA and PilQ, to very strong effects on the amounts of the Type VI and Type III proteins Hcp1 and ExoS/ExoT, respectively. The differences in the degree of influence could be explained by the fact that the levels of each of these proteins may be influenced by multiple factors, some of which are controlled by RsmA. Thus, the total effect of this regulator may be determined by its ability to control expression of several factors in a regulatory pathway, including those involved in the regulation of gene expression and/or protein targeting. However, its effect on extracellular proteins appears to be particularly pronounced.

We also tested the effect of RsmA on the production of exotoxin A, whose expression is dependent on an alternative sigma factor PvdS and is induced under iron-limiting conditions (Lamont *et al.*, 2002; Ochsner *et al.*, 1995; Ochsner *et al.*, 1996). In the *rsmA* transcriptome analysis, amounts of the *pvdS* transcript were 32-fold lower in the *rsmA* mutant compared to the wild type (Table 3). Accordingly, our Western blot analysis showed that when cells were grown in the presence of the iron chelator 2,2'-dipyridyl, exotoxin A accumulated in the supernatant of the wild type strain while it could not be detected in the supernatant of the *rsmA* mutant (Figure 9B). In the secreted fraction of wild type cells, in addition to the predicted full-length protein (Mr of 68 Kd), two additional immunoreactive bands were seen. These very likely represent fragments of exotoxin A generated by the action of *P. aeruginosa* elastase (Sanai *et al.*, 1980). We also tested the levels of exotoxin A in the cell-associated fractions of cultures containing 2,2'-dipyridyl, and in cells and supernatants of cells grown in iron replete medium. In line with previous reports, we could not detect any exotoxin A in those samples (data not shown). Overall, this result further substantiates the model proposed above in which the effect of RsmA on iron regulated genes is dependent on the concentration of iron in the growing cultures.

Discussion

The members of the RsmA family of regulators are found in numerous Gram negative bacteria where they control diverse phenotypes ranging from metabolism and stress response to virulence and quorum sensing (Altier *et al.*, 2000; Barnard *et al.*, 2004; Fettes *et al.*, 2001; Liaw *et al.*, 2003; Romeo *et al.*, 1993; Whitehead *et al.*, 2002). However, only very few

direct targets of this family of proteins have been identified. Thus, it is still largely unknown how these RNA-binding translational regulators control these various phenotypes and what fraction of these phenotypes are affected by RsmA indirectly via RsmA influence on various regulatory systems.

In this study, we identified the RsmA regulon of *P. aeruginosa* PAK. The regulon consists of over 500 genes, of which approximately one third were affected by the *rsmA* mutation negatively, while the rest were affected positively. Until now, the *hcn* operon encoding components of hydrogen cyanide biosynthesis was the only known direct target of the *P. aeruginosa* RsmA (Pessi *et al.*, 2001). Therefore, it was completely unknown, how this protein achieves the coordinate expression of such a large number of genes, many of which are virulence factors.

To address these questions, we conducted a series of experiments that lead to the identification of six genes whose expression is directly regulated by RsmA. These include PA0081, PA0082, PA0277, PA2541, PA3732 and PA4492. Based on gene organization, microarray expression data, and predicted gene functions, some of these genes are encoded in larger operons, bringing the number of the newly identified, directly regulated genes up to 40. According to the *Pseudomonas* genome database, the genes encoded in the PA2541 operon are predicted to be involved in fatty acid and phospholipid metabolism. One of these genes, PA2539, is predicted to encode a tyrosine phosphatase with regulatory functions. Of the genes in the PA3732 operon, PA3728 is predicted to be involved in cell division and chromosome partitioning. PA0277 is a cistron encoding a hypothetical Zn-dependent protease. The genes encoded in the PA0081 and PA0082 operons have been characterized recently and encode the components of a novel bacterial secretion system, the T6S system (Mougous *et al.*, 2006; Shrivastava and Mande, 2008). This system is used by *P. aeruginosa* for secretion of effectors that apparently play a role during chronic infection (Lehoux *et al.*, 2002; Potvin *et al.*, 2003; Mougous *et al.*, 2006). Since in the *rsmA* mutant the expression of the PA0081 and PA0082 operons is coordinately activated with the PA0277, PA2541, PA3732 and PA4492 operons, it is possible that these operons encode novel virulence factors that are, like the T6S genes, also important during chronic infection. Indeed, genes encoded in the putative PA4492 operon have been shown to be required for *P. aeruginosa* virulence in the rat chronic lung infection model (Lehoux *et al.*, 2002; Potvin *et al.*, 2003).

Expression of all of the operons that are directly regulated by RsmA was strongly upregulated in the *rsmA* mutant. This is consistent with the model in which RsmA acts as a negative regulator by binding to an mRNA target sequence near the RBS and preventing translational initiation. In order to characterize the putative RNA binding site of *P. aeruginosa* RsmA, we analyzed the sequences around the RBS sites of PA0081, PA0082, PA4492, PA0277, PA2541, PA3732 and *hcnA*. As shown in Figure 10, all 7 sequences contain a motif ANGGA, which is identical to the binding motif of CsrA in *E. coli* and RsmA in *P. fluorescens* (Dubey *et al.*, 2005; Lapouge *et al.*, 2007; Schubert *et al.*, 2007). Five sequences also contain a C on the 5' end of this motif, which also is typical for the *E. coli* and the *P. fluorescens* recognition sequences (Dubey *et al.*, 2005; Lapouge *et al.*, 2007; Schubert *et al.*, 2007). In addition, six sequences contain a purine immediately upstream of GGA. On the 3' side of GGA, six of the seven sequences contain a G or a T at positions 1

and 2, followed by a purine at position 3 (Figure 10). Based on these alignments, we used the sequence CARGGAKKR (R = A or G, K = G or T) to search the intergenic regions of the *P. aeruginosa* PAO1 genome for additional candidate targets of RsmA. The search resulted in the identification of 87 such sequences. In 47 of these, the putative consensus RsmA binding sequence was located in the appropriate orientation and within 150 nucleotides upstream of the start codon of a gene, and could therefore represent a potential RsmA binding site. Among these 47 sequences, six corresponded to the genes that were also identified as members of the RsmA regulon in the PAK *rsmA* transcriptome analysis. Transcript levels of all six of these genes, PA0041, PA2568, PA2581, PA3484, PA3536, and PA3850, were increased in the *rsmA* mutant (Table S1). For PA0041, PA2581, PA3484, and PA3850, the putative RsmA binding sequence overlaps with the RBSs, while in PA2581 and PA3536, the sequence is located in the leader sequence 50 and 126 bases upstream of the RBS, respectively. It is plausible that PA0041, PA2568, PA2581, PA3484, PA3536, and PA3850 are also regulated by RsmA directly.

In addition to the PA0081 and PA4492 genes, four other genes (PA4611, PA2977, PA4498, and PA4744) were detected in the RNA-RsmA co-purification experiment more than once (Table S2). The RBS sequences of these genes do not contain the minimal ANGGA motif, which is conserved not only in the RBSs of all known targets of RsmA, but also in the majority of the CsrA targets in *E. coli* (Table S2, Figure 10). However, based on the hybridization intensity values, these genes were not expressed at very high levels (data not shown). It is therefore unlikely that they co-purified with RsmA nonspecifically because of their high relative abundance, such as is the case with the ribosomal RNAs. And although expression of these four genes was not affected by the *rsmA* mutation in the microarray analysis of the PAK strain, the levels of the PA4498 transcript were significantly elevated in the *rsmA* mutant in the analysis of the PAO1 strain (Table S2) (Burrowes *et al.*, 2006). Further experiments will be required to determine whether or not RsmA binds mRNAs of any of these genes specifically and to determine the consequence of any such interaction.

Among the genes that we identified as members of the RsmA regulon, 187 are regulated by RsmA positively. Although we do not exclude a possibility of RsmA having a positive effect on translation, we did not identify any such examples in our study. Specifically, none of the genes that were positively affected by RsmA were identified in the RNA-RsmA co-purification experiment or in the search using the consensus RsmA binding motif. Furthermore, we directly examined the role of RsmA in expression of several of these genes, including those encoding the T3S system and those required for the assembly of the type IV pili. Based on the results we have concluded that these genes are not controlled by RsmA directly. Moreover, we have provided evidence suggesting that another large group of approximately 40 genes that encode proteins involved in iron homeostasis and whose transcript levels were strongly reduced in the *rsmA* mutant, is also regulated by RsmA indirectly. Therefore, these results support the hypothesis that positive regulation by RsmA is indirect and not the result of binding of this regulatory protein to a specific sequence at the 5' region of the mRNAs.

In conclusion, RsmA appears to achieve its global control by integrating its regulatory activity at various levels into the *P. aeruginosa* global regulatory network (Figure 11). In

some cases, such as in the case of the T6S genes, RsmA directly regulates expression of multiple operons that collectively encode a specific phenotype. In other instances, RsmA integrates its regulatory effect into a regulatory cascade, by directly controlling the expression of a regulatory factor. We propose that this pattern is exemplified by the RsmA regulation of the T3S and the type IV pili genes (Figure 11). Furthermore, we propose that the mechanisms of indirect regulation are specific to individual groups of genes and may involve overlapping regulatory circuits. For example, we propose that the genes involved in iron homeostasis are regulated by RsmA indirectly via a direct effect of RsmA on translation of a component in an iron-responsive regulatory pathway that controls transcription of these genes. Among the genes in the RsmA regulon whose transcript concentrations were increased in the *rsmA* mutant, there are 2 two-component sensor kinases, 14 transcriptional regulators, 4 DNA-binding proteins with regulatory functions and a synthase of the secondary messenger c-di-GMP (Table S5). Any of these genes is a candidate for direct regulation by RsmA and could play a role in the indirect effects that RsmA has on the expression of genes that are affected by RsmA positively. Recently, a CsrA-dependent regulatory cascade involving *Yersinia pseudotuberculosis* transcriptional regulators of virulence was reported (Heroven *et al.*, 2008). The expression of virulence genes in this organism is regulated by the transcription factor RovA, which in turn is regulated by a LysR type regulator RovM. CsrA controls the levels of RovM by a mechanism that does not involve translational initiation and it is likely affecting the expression of one or several upstream regulators of *rovM*.

The next challenge in understanding RsmA global regulation will be to identify all of the direct targets of RsmA, particularly those that constitute pathways connecting RsmA with genes that are indirectly regulated by this protein. A sensible approach to address this question involves identification of the RNA species that associate specifically with RsmA. We used this approach by performing the RsmA-RNA co-purification experiment. However, although it allowed us to successfully identify two direct targets of RsmA, we found that due to a high non-specific background the efficiency of this method was very low.

Another possibility is to use consensus binding sequences and computational approaches to search bacterial genomes for additional RsmA targets. However, in a similar approach described above, we identified numerous genes in which the RsmA consensus sequence overlaps the RBS, but which were not affected by the *rsmA* mutation in the microarray analysis. Therefore, the presence of the putative RsmA consensus sequence in the region around RBS is not sufficient for RsmA binding and/or regulation. A more extensive analysis of the target sequences, including the dynamics of secondary structure formation, will be required to establish the full range of factors that determine the RsmA binding site. More refined computational tools could then be developed and used for the identification of additional RsmA targets, which could then be validated by direct analyses of RsmA-RNA interactions.

Finally, the biological significance of the RsmA regulation of a subset of genes at the post-transcriptional level needs to be addressed. It is interesting that a substantial fraction of genes controlled by RsmA encode proteins that function on the surface or the exterior of the cell. Therefore, RsmA, via its regulatory RNAs RsmZ and RsmY, may be responding to

external signals and coordinating synthesis of extracellular proteins that may be important to survival in a particular environment. This environment may include an infected host, accounting for a disproportionately large number of virulence factors that are regulated by this protein.

Experimental Procedures

Plasmids, Strains and Culture Conditions

All strains used in this study were derived from *P. aeruginosa* strain PAK (D. Bradley). The *P. aeruginosa* PAK *rsmA* strain has been previously described (Ventire *et al.*, 2006). Primer sequences and a detailed description of cloning procedures used for construction of transcriptional and translational fusions are provided in Supporting Information. *P. aeruginosa* strains were maintained in Luria-Bertani (LB) broth with antibiotics as required (150 µg/ml Carbenicillin, 75 µg/ml Gentamicin, 25 µg/ml Irgasan, 50 µg/ml Tetracycline). Where appropriate, the iron chelator 2,2'-dipyridyl (Sigma) was used to reduce levels of iron in culture media (Hassett *et al.*, 1996). Growth incubations were performed at 37°C at 300 rpm shaking in baffled flasks for the transcriptional profiling and western blotting experiments, and in culture tubes for β-galactosidase assays.

Western Blot Analysis

Detection of RsmA. *P. aeruginosa* PAK was grown in LB at 37°C at 300 rpm shaking. Bacterial cells were sampled at 1 hour intervals and lysed by sonication. After standardizing for total protein content, SDS loading buffer was added to each sample. Samples were heated at 95°C for 5 minutes and subjected to electrophoresis on a 15% SDS-polyacrylamide gel. Gel was electroblotted onto a PVD membrane (Biorad) and RsmA was detected using rabbit anti-RsmA polyclonal antibodies and horseradish peroxidase-conjugated secondary antibodies (Biorad).

Detection of PilA, PilQ, ExoS, ExoT, and exotoxin A. *P. aeruginosa* PAK wild type and *rsmA* mutant strains were grown in LB at 37°C at 300 rpm shaking. For the detection of exotoxin A, a parallel culture of *P. aeruginosa* PAK wild type was also grown in the presence of 600 µM iron chelator 2,2'-dipyridyl (Sigma). After 6.5 hours of growth, cells were harvested and pelleted at 9000 × g for 3 min. The supernatant was removed and centrifuged again for 5 min at 10,000 × g; proteins were precipitated from the resulting supernatant by the addition of ice-cold trichloroacetic acid (TCA). TCA precipitation was allowed to proceed for 10 min on ice before a 15 min centrifugation at 16,000 × g. The supernatant was removed, and precipitated proteins were washed once with acetone and then resuspended in final sample buffer. Cell fractions were prepared by removing the remaining culture supernatants and resuspending cell pellets in final sample buffer. Supernatant and cell pellet samples were boiled for 5 min, centrifuged for 2 min at 16000 × g, and loaded onto 12% polyacrylamide gels. Gels were electroblotted onto PVD membranes (Biorad) and detected using rabbit polyclonal anti-PilQ, anti-PilA, anti-ExoS, anti-ExoT antibodies or polyclonal mouse anti-exotoxin A antibodies in combination with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000, Biorad).

Transcriptional Profiling

Duplicate cultures were grown overnight in LB. Next morning, the cultures were inoculated at an OD₆₀₀ of 0.01 into LB and grown for 7 hours. OD₆₀₀ of the cultures at that time point was approximately 6.0. RNA was extracted, reverse-transcribed, fragmented, and labeled as described (Wolfgang et al., 2003), with one exception; at the time of harvesting, stop solution (95% ethanol, 5% phenol, pH 4.7) was added to the cultures in 20% of culture volume. Cultures were kept on ice for 20 minutes before they were centrifuged. The processed samples were hybridized to Affymetrix GeneChip *P. aeruginosa* Microarrays (Affymetrix), and the chips were washed and scanned according to the provided protocol. The 5,678 probe sets specific to strain PAK were filtered for statistically significant differences (Student's t test p value < 0.05), signal above noise level (Present call in at least one sample), and a minimum 2-fold change using commercially available software (Genespring).

RNA-RsmA-H₆ co-purification

A C-terminally histidine-tagged RsmA (RsmA-H₆) was cloned downstream of the inducible *Plac* promoter of a low copy vector pMMB67HE. The construct and the empty vector were introduced into *P. aeruginosa* PAK *rsmA* mutant. The histidine-tagged protein was tested for complementation of two phenotypes associated with *rsmA*, namely, biofilm formation and expression of one of the T3S genes, *exoS*. In both cases, RsmA-H₆ complemented the *rsmA* deletion, confirming that the tagged protein is functional. Cultures of *P. aeruginosa* PAK *rsmA* (pMMB67-RsmA-H₆), and of the negative control *P. aeruginosa* PAK *rsmAYZ* (pMMB67), were grown in the presence of 1 mM IPTG for 6 hours. Cultures were pelleted at 4°C and resuspended in 50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 10 mM imidazole, 150 U of RNaseIN, and 1 mg/ml lysozyme. Cells were chilled on ice for 30 minutes before they were ruptured by sonication. Cell free extracts were prepared by centrifugation at 15 000 *g* at 4°C for 45 min. RsmA-H₆ was purified by Ni-NTA affinity chromatography (Qiagen) according to the manufacturer's recommendations. The protein-containing fractions and equivalent fractions from the control procedure using the control strain were pooled and extracted twice by acidic phenol:chloroform (pH=4.5) (Ambion). RNA was precipitated with 100% ethanol and washed three times with 70% ethanol. RNA was resuspended in distilled water (dH₂O) and passed through a NucAway column (Ambion) to remove any remaining salts. 5% of each, the experimental and the control sample, was analyzed on a denaturing agarose gel and the quantity of the purified RNA was measured by a spectrophotometer (260 nm/280 nm). Finally, 25 ug (50%) of RNA from the experimental sample was separated on a native 5% polyacrylamide gel containing 8M urea and using Tris/Borate/EDTA (TBE) as running buffer. RNA was excised from the gel in five separate sections as described in the results section. Each section was immersed in 200 µl of dH₂O and RNA was eluted from the gel slices by overnight incubation at room temperature. The resulting RNA preparations were used in reverse transcription reactions using random hexamer primers and Superscript II Reverse Transcriptase (Invitrogen) following the protocol provided by the manufacturer. Due to the use of random primers, the resulting cDNA fragments were shorter than the template RNA fragments and did not necessarily represent the exact location of RsmA binding. Second strand synthesis was carried out using Dna Pol I and RnaseH (both Invitrogen) following the protocol provided by the

manufacturer. Blunt ends were created using T4 DNA polymerase (NEB) following the protocol provided by the manufacturer. The resulting DNA was precipitated with 100% ethanol following a phenol-chloroform extraction. Pellets were washed with 70% ethanol and resuspended in dH₂O. EcoRI adaptors (Promega) were ligated to the ends of DNA fragments using T4 DNA ligase (NEB). Finally, DNA was phosphorylated using polynucleotide kinase (PNK) (NEB) following the protocol provided by the manufacturer. The phosphorylated DNA was purified using Qiagen PCR purification kit and cloned into a dephosphorylated (Antarctic phosphatase, NEB), EcoRI-digested cloning vector pUC19 (NEB). Ligation reactions were transformed into *E. coli* DH5 α and dilutions were plated on LB plates containing Ampicillin (100 μ g ml⁻¹) and X-gal. Plasmids were isolated from white colonies and sequenced using M13 primers.

Purification of histidine-tagged RsmA (RsmA-H₆) from *E. coli*

Histidine-tagged RsmA (RsmA-H₆) was overproduced in *E. coli* BL21 lysS-(Novagen) by IPTG induction; 1 mM IPTG was added to cells grown to mid-exponential phase and cells were harvested 4 hours later. Cell pellets were resuspended in 50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 1 mg/ml lysozyme. Cells were ruptured by sonication. Cell-free extract was prepared by centrifugation at 15,000 $\times g$ at 4°C for 45 min. RsmA-H₆ was purified by Ni-NTA affinity chromatography (Qiagen) according to the manufacturer's recommendations. The protein-containing fractions were concentrated and the buffer was exchanged with 10 mM Tris-HCl (pH 7.4), 33% glycerol. The protein concentration was estimated using a Bradford assay (BioRad) using bovine serum albumin as the standard. RsmA-H₆ was estimated to be ~95% pure based on analysis of the preparation with polyacrylamide gel electrophoresis. Final protein preparations were stored at -20°C.

RNA gel mobility shift assay

The PA0081, PA0082, PA4492, *exsD*, *exsC*, *exoS*, *pilM*, *rsmZ*, *rsmY* and *lolB* transcripts, were synthesized from PCR products using T7 RNA polymerase. The primers used for the synthesis of these PCR products are shown in Supporting Information. The sequence of the T7 promoter was engineered into each of the 5' primers. The PCR products were gel purified and used as templates for *in vitro* transcription using MEGAscript kit (Ambion). Transcripts were ³²P-labeled with PNK (NEB) according to instructions provided by the manufacturer. The RNA binding reaction mixtures (10 μ l) included RsmA-H₆ at various concentrations, labeled transcript (10 nM), 10 mM Tris-acetate (pH 7.5), 10 mM MgCl₂, 50 mM NaCl, 50 mM KCl, 10 mM DTT and 5% glycerol. The reactions were incubated at room temperature for 30 min, mixed with 1 μ l of loading dye (97% glycerol, 0.01% bromophenol blue, 0.01% xylene cyanol) and immediately loaded and separated on 8% native polyacrylamide gel using TBE as running buffer. The resulting gels were scanned using Typhoon PhosphorImager. The same protocol was followed in the competition assays.

β -galactosidase Assays

All β -galactosidase experiments were carried out in duplicates starting with overnight cultures. Each experiment was performed at least twice. Overnight cultures were grown in LB with antibiotics as appropriate. In the morning, cultures were diluted 1:100 into LB, and grown for 4-6 hrs. At the time of assay, cultures had similar optical density values (Standard

relative deviation $\pm 25\%$). Although the absolute units for a particular fusion varied from assay to assay (for example, compare Miller units for the PA0081 translational fusion in Figures 6 and 7), the overall trends (units detected in the mutant relative to units detected in the wild type) were observed consistently. Translational fusions (using vector pUC18-miniTn7-*lacZ*10-Gm) were consistently expressed at levels that were lower than those detected with transcriptional fusions (using mini-CTX-*lacZ* vector). Invariably, however, the translational fusions exhibited a very low background activity which permitted us to measure the low levels of β -galactosidase activity accurately. It is unclear why the absolute units measured for the *P**lacUV5*-driven PA4492 and PA0082 translational fusions were lower than those measured in strains where these same fusions were under the control of their native promoter. However, the overall trends (units detected in the mutant relative to units detected in the wild type) were observed consistently. For the time course of *rsmY* and *rsmZ* expression, cultures were sampled every hour, and assayed immediately. The amounts of β -galactosidase activity were quantified as described (Sambrook *et al.*, 1989). In experiments involving the T3S genes, assay cultures were grown under calcium-depleted conditions which were achieved by addition to the LB medium of nitrilotriacetic acid and CaCl_2 , each to a final concentration of 10 mM. In experiments involving iron homeostasis gene expression, iron levels were reduced in the medium by the addition of 2,2'-dipyridyl to a final concentration of 300 μM .

Mapping of the 5' ends of PA0081, PA0082, and PA4492 transcripts

To establish the 5' ends of PA0081, PA0082, and PA4492 transcripts, a 5' RACE procedure was carried out following a published protocol (Scotto-Lavino *et al.*, 2006), with a few exceptions: Superscript III reverse transcriptase (Invitrogen) and Pfu TURBO polymerase (Stratagene) were used instead of the recommended enzymes. DMSO (5% final concentration) was added to all PCR reactions. The PAK *rsmA* total RNA sample that was used for the transcriptome analysis was used as a template for reverse transcription reactions. For each gene, a total of three gene-specific 3' primers and three universal 5' primers were used in the procedure. Sequences of these primers are provided in Supporting Information. The final PCR products were gel purified using Qiagen PCR clean-up kit and sequenced directly using the 3' primer that was used in the final PCR amplification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Growth phase dependent expression of RsmZ, RsmY and RsmA. **A.** Growth curves of the various *P. aeruginosa* strains; **B.** Time course of *rsmY* and *rsmZ* expression as determined by using transcriptional *lacZ* fusions and measuring levels of β -galactosidase activity; **C.** Time course of RsmA accumulation as determined by a Western immunoblot analysis using anti-RsmA antibodies.

Figure 2.

Isolation and identification of transcripts bound to RsmA-H₆. RsmA-H₆ was purified from a culture of *P. aeruginosa* PAK *rsmA* (pMMB67-RsmA-H₆) using nickel-affinity chromatography. 5% of total RNA that was isolated from RsmA-H₆-containing fractions was visualized on a denaturing agarose gel (Upper panel, lane A). 5% of total RNA that was isolated from equivalent fractions obtained using the control strain *P. aeruginosa* PAK *rsmA* (pMMB67) was loaded in lane B. Lane M – Molecular size marker. Sections 1-4 of lane A of the denaturing agarose gel indicate equivalent sections of a denaturing polyacrylamide gel (not shown) that was used to separate the rest of the total RNA isolated from the RsmA-H₆-containing fractions. RNA was extracted separately from each section of the polyacrylamide gel, converted to cDNA, followed by second strand synthesis and cloning. Shown are the identities of the isolated RNAs as determined by sequencing of 12 clones per gel section (Lower panel).

Figure 3.

Organization of operons and features of leader sequences of the PA0081 and PA0082 operons (A); and the PA4492 operon (B). Asterisks represent the transcription initiation sites (+1 sites) identified by a bacterial sigma70 promoter recognition program BPROM, and confirmed by the 5' RACE procedure. -10 and -35 represent the putative promoters corresponding to the indicated +1 sites. Open stars represent additional transcription initiation sites identified in the 5' RACE procedure. RBS: ribosome binding site. Dashed arrows: Sequences identified in RNA-RsmA-H₆ co-purification assay. GGA motifs are

encircled, start codons are underlined. In A., the putative promoter and RBS are shown on the top strand for PA0081 and on the bottom strand for PA0082.

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Figure 4. Secondary structures of leader sequences of genes identified as direct targets of RsmA regulation as predicted by M-fold (Zuker, 2003). Highlighted are the locations of the GGA sequences (thick lines) and of the ribosome binding sites (RBS).

Figure 5.

RNA mobility shift assays with purified RsmA-H₆. **A.** Binding of RsmA-H₆ to RsmY, RsmZ, and the leader sequences of transcripts encoding PA0081, PA0082, PA4492, *exsD*, *exsC*, *exoS*, *pilM*, and the negative control *lolB*. Transcripts were radiolabeled and incubated in the absence or presence of various concentrations of RsmA-H₆ for 30 minutes at room temperature, before they were analyzed on an 8% native polyacrylamide gel. For each panel, reactions in lanes 1-3 contained 10 nM of radiolabeled RNA and 0, 1, and 3 μ M RsmA-H₆ monomer, respectively. **B.** Competition experiments were conducted using 10 nM labeled PA0081 transcript and 0 (lane 1) or 2.5 μ M (lanes 2-8) RsmA-H₆ monomer. The reaction shown in lane 2 was incubated in the absence of any competitor RNA. Reactions shown in lanes 3-8 were incubated in the presence of the unlabeled transcript of RsmY (lanes 3, 4),

RsmZ (lanes 5, 6) and *loliB* (lanes 7, 8) at 2-fold (lanes 3, 5, 7) and 200-fold (lanes 4, 6, 8) excess relative to the labeled transcript.

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Figure 6.

Effect of RsmA on expression of PA0081, PA0082, and PA4492. Levels of β -galactosidase activity were determined in the *P. aeruginosa* PAK wild type and *rsmA* mutant strains carrying chromosomal *lacZ* fusions to PA0081, PA0082, and PA4492. **A.** Transcriptional fusions; **B.** Translational fusions; and **C.** Translational fusions driven by the constitutively active *PlacUV5* promoter. **D.** Schematic representation of the transcriptional (i), translational (ii) and *PlacUV5*-driven translational (iii) *lacZ* fusions used in the study. See text for details.

Figure 7.

Effects of various mutations in the RetS/GacSA/RsmYZ/RsmA regulatory pathway on the expression of PA0081 and *exsD*. Levels of β-galactosidase activity were determined in the *P. aeruginosa* PAK wild type and mutant strains carrying transcriptional (A and C) or translational (B and D) chromosomal *lacZ* fusions to PA0081 (A and B) or *exsD* (C and D).

Figure 8.

Effect of RsmA on the expression of genes involved in iron homeostasis. Levels of β -galactosidase were measured in *P. aeruginosa* PAK wild type (white bars) and *P. aeruginosa* PAK *rsmA* mutant (black bars) carrying translational *lacZ* fusions to PA4228, PA1300, PA0081, and *exsD* (A), and transcriptional *lacZ* fusions to PA4228 and PA1300 (B). All assays were carried out after 4 hours of growth in LB with or without 300 μ M iron chelator 2,2'-dipyridyl as indicated in the figure.

Figure 9.

Immunoblot analysis of RsmA-regulated proteins. **A.** Cultures of *P. aeruginosa* PAK wild type and *rsmA* mutant were incubated for 7 hrs in LB, at 37°C and at 300 rpm shaking. Aliquots of cell and secreted fractions were analyzed by Western immunoblotting using antibodies against Hcp1, ExoS, ExoT, PilA and PilQ. **B.** Immunoblot analysis of secreted exotoxin A (ToxA). *P. aeruginosa* PAK wild type and *rsmA* mutant were incubated for 7 hrs in LB with 600 μ M 2,2'-dipyridyl, at 37°C and at 300 rpm shaking. Secreted fractions were analyzed by Western immunoblotting using antibodies against ToxA.

Gene	Number of GGA motifs in leader sequence	Sequence adjacent to RBS
PA0081	4	ATGCAAGGAGTAGCA
PA0277	3	TCGCAAGGAGCACAC
PA2541	1	GCACAAGGACTGGCT
<i>hcnA</i>	5	TTTCACGGATGAACC
PA3732	9	GGAGAAGGAGGAAGA
PA4492	1	GACCAGGGATTGATC
PA0082	3	TGATAGGGAGATCGT
		C A R G G A K K R

Figure 10.

Alignment of sequences around the ribosome binding site (RBS) that represent the putative RsmA binding site in the leader sequences of the seven known direct targets of the *P. aeruginosa* RsmA. The sequence that was used to screen the intergenic sequences of the *P. aeruginosa* PAO1 genome to identify additional candidate targets of direct RsmA regulation is shown below the alignments. The putative RBS sequences are boxed.

Figure 11.

General model for the RsmA-mediated global regulation in *P. aeruginosa*. *P. aeruginosa* RsmA is a part of the LadS/RetS/GacS/A/RsmY/Z/RsmA regulatory pathway in which LadS, RetS, and GacS/A modulate RsmA activity by regulating transcription of two small regulatory RNAs, RsmY and RsmZ. RsmY and RsmZ antagonize RsmA activity by competing with target mRNAs for RsmA binding. RsmA controls expression of more than 500 genes. Transcript levels of about two thirds these genes increase in the *rsmA* mutant or when RsmA is antagonized by RsmY and RsmZ. We propose that many of these genes are regulated by RsmA directly. On the other hand, we propose that the genes that are downregulated in the *rsmA* mutant or in the presence of high levels of RsmY and RsmZ are controlled by RsmA via indirect mechanisms. We propose that these mechanisms are specific to individual groups of genes and involve regulatory factors that are regulated by RsmA directly. Dashed lines – indirect regulation; Solid lines – direct regulation; white background – directly regulated genes; grey background – indirectly regulated genes.

Table 1

Genes of altered expression in *P. aeruginosa* PAK *rsmA* compared to *P. aeruginosa* PAK wild type, as determined by microarray analysis. Positive values represent genes whose mRNA levels increased in the *rsmA* mutant relative to wild type; negative values represent genes with decreased mRNA levels in the *rsmA* mutant relative to wild type. Only the genes addressed in this study are shown. The complete data set is provided in Table S1.

Gene number_name	Fold Change (<i>rsmA</i> /wt)	Gene Function
Type VI Secretion genes		
PA0074_ppkA	2.2	serine/threonine protein kinase PpkA
PA0075_ppA	2.8	phosphoprotein phosphatase
PA0076	3.1	hypothetical protein
PA0077	2.2	hypothetical protein
PA0079	2.1	hypothetical protein
PA0081_fhA1	1.9 *	T6S scaffolding protein
PA0082	5.6	hypothetical protein
PA0083	7.4	conserved hypothetical protein
PA0084	4.8	conserved hypothetical protein
PA0085_hcp1	8.5	secreted protein Hcp
PA0089	3.9	hypothetical protein
PA0090_clpVI	10.7	ClpA/B-type chaperone
PA0277 cistron		
PA0277	121.3	predicted Zn-dependent protease
PA3732 operon **		
PA3728	8.5	hypothetical protein
PA3729	16.9	conserved hypothetical protein
PA3730	14.4	hypothetical protein
PA3731	4.9	conserved hypothetical protein
PA3732	6.7	conserved hypothetical protein
PA2541 operon **		
PA2536	3.4	probable phosphatidate cytidyltransferase
PA2537	8.8	probable acyltransferase
PA2539	31.0	conserved hypothetical protein
PA2540	28.6	conserved hypothetical protein
PA2541	11.9	probable CDP-alcohol phosphatidyltransferase
PA4492 operon **		
PA4487	3.2	conserved hypothetical protein
PA4489	4.4	conserved hypothetical protein

Gene number_name	Fold Change (<i>rsmA</i> /wt)	Gene Function
Type VI Secretion genes		
PA4491	4.9	conserved hypothetical protein
PA4492	5.8	conserved hypothetical protein
Type III Secretion Genes		
PA0044_exoT	-3.3	exoenzyme T
PA1689	-3.2	conserved hypothetical protein
PA1694_pscQ	-24.6	translocation protein in T3S
PA1699	-7.1	conserved hypothetical protein in T3S
PA1701	-11.6	conserved hypothetical protein in T3S
PA1706_pcrV	-10.6	T3S protein PcrV
PA1707_pcrH	-13.4	regulatory protein PcrH
PA1708_popB	-10.8	translocator protein PopB
PA1709_popD	-9.8	translocator outer membrane protein PopD precursor
PA1710_exsC	-11.8	regulatory protein ExsC
PA1711_exsE	-5.9	regulatory protein ExsE
PA1712_exsB	-4.3	hypothetical protein
PA1713_exsA	-2.8	transcriptional regulator ExsA
PA1714_exsD	-19.1	anti-activator of T3S
PA1718_pscE	-10.2	T3S export protein PscE
PA1719_pscF	-6.5	T3S export protein PscF
PA1720_pscG	-3.7	T3S export protein PscG
PA1721_pscH	-5.8	T3S export protein PscH
PA1722_pscI	-3.2	T3S export protein PscI
PA1723_pscJ	-2.7	T3S export protein PscJ
PA2191_exoY	-6.3	adenylate cyclase ExoY
PA3841_exoS	-6.3	exoenzyme S
Type IV pili biogenesis genes		
PA0410_pilI	-3.1	twitching motility protein PilI
PA4528_pilD	-2.7	type 4 prepilin peptidase PilD
PA4551_pilV	-3.4	type 4 fimbrial biogenesis protein PilV
PA4553_pilX	-3.5	type 4 fimbrial biogenesis protein PilX
PA4554_pilYI	-4.3	type 4 fimbrial biogenesis protein PilYI
PA5040_pilQ	-2.7	type 4 fimbrial biogenesis outer membrane protein PilQ
PA5042_pilO	-4.3	type 4 fimbrial biogenesis protein PilO
PA5043_pilN	-3.0	type 4 fimbrial biogenesis protein PilN
PA5044_pilM	-1.7 *	type 4 fimbrial biogenesis protein PilM

* Although the gene did not pass the 2-fold cut-off it was included in the table because expression of the rest of the members of its resident operon was significantly altered.

** These are putative operons that have not been experimentally verified.

Table 2

Expression of selected *P. aeruginosa* PAK genes in wild type and *rsmA* backgrounds as measured by the use of transcriptional and *PlacUV*-driven translational *lacZ* fusions. The numbers are based on duplicate experiments. Positive values represent genes whose expression increased in the *rsmA* mutant compared to wild type; negative values represent genes whose expression decreased in the *rsmA* mutant compared to wild type. n/d–not determined.

<i>rsmA</i> /wt ratio		
Gene	Transcriptional fusions	<i>PlacUV5</i> -driven translational fusions
PA0277	1.8	4.6
PA2541	-1.7	3.8
PA3732	-1.3	4.9
<i>exoS</i>	-11.4	1.3
<i>exsD</i>	-20.9	1.6
<i>exsC</i>	-15	n/d
<i>pilM</i>	-3.1	-1.3

Table 3

Effect of *rsmA* deletion on the expression of genes involved in iron homeostasis. Shown is the comparison of the effects of the *rsmA* deletion on the mRNA levels in strain PAO1 during exponential growth (Burrowes *et al.*, 2006) and in strain PAK in stationary phase of growth (this study). Positive values represent genes with increased transcript levels in the *rsmA* mutant compared to wild type; negative values represent genes with decreased transcript levels of expression in the *rsmA* mutant compared to wild type. n/a – data not available.

<i>rsmA</i> /wt			
Gene Number	PAO1	PAK	Gene function
PA0472	n/a	-3	probable sigma-70 factor, ECF subfamily
PA0672	-4	-13	heme oxygenase
PA0673	-39	-3	hypothetical protein
PA1300	n/a	-13	probable sigma-70 factor, ECF subfamily
PA1912	n/a	-9	probable sigma-70 factor, ECF subfamily
PA2426_ <i>pvdS</i>	11	-32	sigma factor PvdS
PA2466	-3	-4	probable TonB-dependent receptor
PA2467	3	-3	probable transmembrane sensor
PA2468	n/a	-6	probable sigma-70 factor, ECF subfamily
PA3407_ <i>hasAp</i>	n/a	-38	heme acquisition protein HasAp
PA3408_ <i>hasR</i>	n/a	-5	haem uptake outer membrane receptor precursor
PA3899	n/a	-7	probable sigma-70 factor, ECF subfamily
PA3901_ <i>fecA</i>	n/a	-6	Fe(III) dicitrate transport protein FecA
PA4155	n/a	-5	hypothetical protein
PA4156	n/a	-9	probable TonB-dependent receptor
PA4158_ <i>fepC</i>	5	-18	ferric enterobactin transport protein FepC
PA4161_ <i>fepG</i>	n/a	-8	ferric enterobactin transport protein FepG
PA4168	n/a	-25	probable TonB-dependent receptor
PA4220	n/a	-7	hypothetical protein
PA4221_ <i>fptA</i>	7	-5	Fe(III)-pyochelin outer membrane receptor precursor
PA4222	n/a	-4	probable ATP-binding component of ABC transporter
PA4223	n/a	-6	probable ATP-binding component of ABC transporter
PA4224	n/a	-4	pyochelin biosynthetic protein PchG
PA4226_ <i>pchE</i>	12	-5	dihydroaeruginosic acid synthetase
PA4228_ <i>pchD</i>	20	-3	pyochelin biosynthesis protein PchD
PA4229_ <i>pchC</i>	8	-3	pyochelin biosynthetic protein PchC
PA4230_ <i>pchB</i>	9	-3	salicylate biosynthesis protein PchB
PA4231_ <i>pchA</i>	29	-7	salicylate biosynthesis isochorismate synthase
PA4358	n/a	-4	probable ferrous iron transport protein
PA4467	n/a	-27	hypothetical protein
PA4468_ <i>sodM</i>	10	-39	superoxide dismutase
PA4469	n/a	-25	hypothetical protein
PA4470_ <i>fumC1</i>	2	-30	fumarate hydratase
PA4471	n/a	-24	hypothetical protein

rsmA/wt

Gene Number	PAO1	PAK	Gene function
PA4514	n/a	-7	probable outer membrane receptor for iron transport
PA4706	n/a	-4	probable ATP-binding component of ABC transporter
PA4707	n/a	-4	probable permease of ABC transporter
PA4708	n/a	-8	hypothetical protein
PA4709	n/a	-6	probable heme degrading factor
PA4710	4	-15	heme/hemoglobin uptake outer membrane receptor precursor
PA4880	n/a	3	probable bacterioferritin