Hfq-assisted RsmA regulation is central to *Pseudomonas aeruginosa* biofilm and motility

Yasuhiko Irie\textsuperscript{1,2,*}, Jessica E. Geyer\textsuperscript{1} and Victoria Shingler\textsuperscript{2}

\textsuperscript{1} Department of Biology, University of Dayton, Dayton, OH 45469, USA
\textsuperscript{2} Department of Molecular Biology, Umeå University, Umeå, 901 87, Sweden

Short Title: *Pseudomonas aeruginosa* RsmA regulates Vfr
Keywords: *Pseudomonas aeruginosa*, RsmA, Vfr, FleQ, Hfq

*Corresponding author, current mailing address: Department of Biology, University of Dayton, Science Center 234, 300 College Park, Dayton, OH 45469-2320, USA. Tel: +1-937-229-3061. E-mail: yirie1@udayton.edu

ORCID ID for Yasuhiko Irie: 0000-0002-7162-4772
ABSTRACT

Expression of biofilm and motility genes is controlled by multiple regulatory elements, allowing bacteria to appropriately adopt a sessile or motile lifestyle. In Pseudomonas aeruginosa, the post-transcriptional regulator RsmA has been implicated in the control of various biofilm- and motility-associated genes, but much of the evidence for these links is limited to transcriptomic and phenotypic studies. RsmA binds to target mRNAs to modulate translation by affecting ribosomal access and/or mRNA stability. Here we trace a global regulatory role of RsmA to the inhibition of Vfr – a transcription factor required for efficient production of two other transcriptional regulators, namely FleQ and AlgR. FleQ and AlgR, in turn, directly control flagella and pili genes, respectively. FleQ also directly controls biofilm-associated genes that encode the PEL polysaccharide biosynthesis machinery. Furthermore, we show that RsmA cannot bind vfr mRNA alone, but requires the RNA chaperone protein Hfq. This is the first example where a RsmA protein family member is demonstrated to require another protein for RNA binding.

SIGNIFICANCE STATEMENT

Microorganisms are subjected to dynamic changes in their environment and rapidly adapt their gene expressions accordingly. Such environmental cues influence planktonic/biofilm and acute/chronic infection modes of growth. In the opportunistic pathogen Pseudomonas aeruginosa, RsmA facilitates this switch by controlling gene expression on the RNA level. In this study, we present evidence that RsmA lies central to several downstream regulatory cascades that govern biofilm formation, flagellar motility, and Type IV pilus-mediated motility – all previously described as part of the RsmA regulon. Importantly, we show that RsmA-binding to the vfr mRNA requires Hfq. These findings have implications for the global regulatory role of RsmA and necessitates a genetic and biochemical re-evaluation of what is currently thought to be the RsmA regulon.
INTRODUCTION

The opportunistic pathogen *Pseudomonas aeruginosa* can be isolated from a wide range of environmental niches, in large part owing to its versatile metabolic capabilities. It is also proficient in colonizing various eukaryotic organisms and can cause both acute and chronic infections, with the latter often associated with biofilm-like modes of growth at the sites of infection (1). *P. aeruginosa* is also highly competitive against other microbial species, with its adaptability and competitive fitness being aided by the production of various secondary metabolites and virulence factors (2, 3). Many of these cellular processes are inter-regulated by multiple regulatory pathways, presumably to provide appropriate response mechanisms to a wide range of environmental cues. One of the central regulators that determine *P. aeruginosa* behavior is the post-transcription factor RsmA (Regulator of Secondary Metabolite) as evidenced by its null mutant phenotypes linked to motility (4), virulence (5-7), biofilm (5, 8), growth (8), and secreted products (4, 9).

RsmA belongs to the RsmA/CsrA family of dimeric RNA-binding proteins (10-12), homologues of which are found across a wide range of both Gram-negative and Gram-positive bacterial species (13). RsmA/CsrA proteins generally function as translation inhibitors by binding to the ribosome binding sites of target mRNAs, typically overlapping either the Shine-Dalgarno or the start codon (14). However, in a few cases described, they can also serve as positive regulators by altering the secondary structures of bound RNAs (15, 16) or protecting RNAs from degradation (17).

Many RsmA/CsrA-targeted mRNAs are reported to have altered RNA decay rates. Several studies attempted to exploit this property to determine the global *P. aeruginosa* RsmA regulon using microarrays (18-20). However, this approach cannot distinguish directly regulated genes from indirectly regulated gene sets that result from cascade regulation, nor can it identify target RNAs that do not exhibit changes in their stability (21, 22). For example, direct RsmA-mediated translation inhibition has been demonstrated for the *P. aeruginosa* biofilm polysaccharide operon *psl* without any effect on the level of
transcription (8). Hence, the psl operon was not prominently featured in transcriptomic
studies despite a strong biofilm phenotype (18, 19), raising concerns of what is currently
considered to be ‘the RsmA regulon’ of *P. aeruginosa*.

Here, we present evidence that RsmA serves a master regulatory role through several
intermediate transcription factors that are known to control different cellular processes.
Through cascade regulation, originating from a direct effect on vfr mRNA, RsmA indirectly
affects both bacterial motility and biofilm formation. Furthermore, we discovered an
unexpected regulatory interplay between RsmA and the RNA chaperone protein Hfq on the
target mRNA, whereby RsmA could only bind in the presence of Hfq *in vitro*. To our
knowledge, this is the first example where RsmA/CsrA is unable to bind to its target alone.
Because Hfq is known to facilitate RNA degradation (23), these results may provide a
mechanistic insight into RsmA-associated mRNA stability changes.
RESULTS

RsmA is a post-transcriptional regulator of vfr through which fleQ and pel transcripts are indirectly regulated.

P. aeruginosa produces at least three different extracellular biofilm polysaccharides: alginate, PEL, and PSL (24). PEL and PSL are co-regulated by several factors including the secondary messenger molecule c-di-GMP via the c-di-GMP-binding transcriptional regulator FleQ (25) and possibly also quorum sensing (26, 27). Similar to PSL, PEL has long been considered to be regulated by RsmA (28) based on a model originally proposed from a microarray study (29). However, unlike the psl operon transcript, which was genetically and biochemically demonstrated to be directly regulated by RsmA (8), no direct evidence for the pel operon has been presented.

Consistent with a previous microarray study (18), using a chromosomal transcriptional reporter, we found that pel was up-regulated in the ΔrsmA mutant as compared to the wild type (WT) background (Fig. S1A). However, the PSL polysaccharides are also over-expressed in a ΔrsmA strain (8), and as a direct consequence of this over-overexpression, intracellular levels of c-di-GMP were elevated (30). Given that the pel genes are known to be transcriptionally up-regulated by c-di-GMP (25, 31) and ΔrsmA has elevated c-di-GMP, it appeared plausible that the up-regulation of pel could be caused by changes in c-di-GMP levels. To uncouple pel expression from elevated c-di-GMP, we also measured pel transcriptional reporter activities when introduced into a ΔrsmA Δpel Δpsl triple mutant background, which has low c-di-GMP (30). In this strain, pel was still up-regulated (Fig. S1A), indicating that the up-regulation was caused by lack of RsmA rather than changes in intracellular c-di-GMP concentration.

Genes that are directly regulated by RsmA/CsrA often manifest differences that are experimentally observed at the transcriptional reporter level, since bound RNAs frequently have altered half-lives (14). Therefore, we next determined whether the apparent pel
transcriptional changes between the WT and ΔrsmA strains were actually a consequence of post-transcriptional RNA stability changes. However, quantification of pelA transcripts at progressive time points after blocking de novo transcription revealed no differences in stability between the WT and ΔrsmA strains (Fig. S1B). These results suggest that the observed differences in pel expression likely occur at the promoter activity level. Because the RNA-binding protein RsmA is not a transcription factor, we next turned our attention to the possibility of RsmA affecting expression of a known transcriptional regulator of pel, namely FleQ. As a well-characterized transcriptional activator of pel (25, 31), potential RsmA-mediated alterations in expression of FleQ would be anticipated to affect transcription of the pel genes. Using a similar approach as described for pel above, we found that a transcriptional reporter fusion of fleQ gave lower levels in the absence of RsmA (Fig. S2A). This is consistent with previous data showing pel transcript levels are up-regulated in ΔfleQ backgrounds (25).

The data above could lend themselves to the interpretation that RsmA serves as a positive regulator of fleQ. While RsmA is more commonly known to be a translational repressor, this possibility is not without precedence. Examples of RsmA/CsrA proteins stimulating translation of target mRNAs include phz2 and moaA in P. aeruginosa (15, 16) and flhDC in E. coli (17). While P. aeruginosa FleQ and E. coli FlhD4C2 share no sequence or mechanistic similarities, they are functional counterparts, with both being class I master regulators of flagellar biosynthesis in their respective hosts (32). In addition, flagellar motility was previously found to be positively controlled by RsmA in P. aeruginosa (4), while in E. coli, CsrA has been shown to up-regulate flagellar gene expression through protecting flhDC mRNA from degradation (17). It follows that if RsmA regulated fleQ in an analogous manner, fleQ mRNAs would be hyper-stabilized by RsmA and thus, less stable in its absence. However, no evidence of altered mRNA turnover rate was found between WT and ΔrsmA strains (Fig. S2B). Therefore, we reasoned that the direct action of RsmA may lie even further upstream in a regulatory cascade that involved control of fleQ by Vfr.
Vfr acts as a transcriptional repressor of the fleQ promoter (33). Therefore, to be consistent with the above phenotypes, RsmA would have to serve as a repressor of vfr. In line with this idea, comparison of vfr transcriptional reporter activities in WT and ΔrsmA strains revealed up-regulation in the absence of RsmA (Fig. 1A). To ensure that the regulatory cascade functioned as anticipated, we also tested the prediction that over-expression of Vfr should ultimately result in increased activity of the pel transcriptional reporter. As shown in Fig. 1B, this is indeed the case. In contrast to pel (Fig. S1B) and fleQ (Fig. S2B), we found that the transcript stability of vfr was significantly different between WT and ΔrsmA strains, such that vfr mRNAs were more stable in the absence of RsmA (Fig. 1C). These results indicated that RsmA affects the vfr mRNA levels by mediating changes in turnover rates.

**RsmA regulation of Vfr alters swimming and twitching motility.**

*P. aeruginosa* uses at least two major modes of motility: flagellar-driven swimming motility through liquid and surface ‘twitching’ motility using Type IV pilus (34, 35). Bacterial migration is an important aspect during *P. aeruginosa* biofilm development as initial attachment to a surface is thought to require flagella and mature microcolony formation has been attributed to Type IV pili functions (36, 37). Thus, fine-tuned regulatory control of motility and biofilm genes is a necessity for effective *P. aeruginosa* adaptation, particularly when switching between motile and sessile lifestyles.

Because FleQ is a class I master regulator of flagellar genes (32) and RsmA and Vfr lie upstream of FleQ in the regulatory cascade, lack of either protein should predictively impact flagellar motility, but in opposite ways. As would be predicted, ΔrsmA has reduced motility while Δvfr is hyper-motile (Fig. 2A). Mutants in vfr have previously been documented to be defective in Type IV pilus-dependent twitching motility (38, 39) due to Vfr’s positive regulation of the AlgZR two-component system (40, 41) required for
expression of the Type IV pili genes (42, 43). Based on the results that RsmA serves as a repressor of vfr (Fig. 1), we predicted that the over-expression of RsmA and lack of Vfr should have epistatically similar phenotypic effect on twitching motility. As shown in Fig. 2, Δvfr and RsmA over-expressing strains were both defective in twitching motility.

**Hfq is required for RsmA to bind to vfr mRNA.**

Given the evidence that RsmA may post-transcriptionally regulate vfr, we next examined the binding properties of RsmA to vfr mRNA. *P. aeruginosa* RsmA has a binding specificity for the CANGGAYG consensus sequence of its target mRNA (44), which is similar to the *E. coli* CsrA consensus sequence RUACARGGAUGU (45). The transcripional start site for vfr lies 153 bases upstream of the translational start site (46). Inspection of the vfr leader sequence identified one putative RsmA-binding site overlapping the Shine-Dalgarno sequence (Fig. 3A).

RsmA, purified as used here, directly binds psl mRNA (8) in RNA electrophoretic mobility shift assays (EMSA). However, no RsmA-binding was observed in similar assays with vfr RNA spanning the leader sequence (Fig. 3B). This initially perplexing result led us to consider that RsmA may require another factor to bind the vfr RNA. Our attention was drawn to Hfq for three reasons. Firstly, the small RNA RsmY, which binds multiple RsmA proteins at high affinity to sequester and relieve RsmA-bound mRNAs (47), also has the capacity to be bound by Hfq (48), although it was unclear whether co-binding of both proteins occurred. Secondly, Hfq was recently identified to bind to vfr mRNA by global ChIP-Seq analyses (49). Thirdly, *in silico* analysis of the vfr leader sequence revealed two potential Hfq binding sites upstream of the putative RsmA-binding site (Fig. 3A).

Biochemical studies, primarily of *E. coli* Hfq, have shown that this hexameric protein complex has four regions which can all be involved in RNA-binding: the proximal face, the distal face, the rim, and the C-terminal tail (50). *P. aeruginosa* and *P. putida* Hfq have
shortened C-termini compared to *E. coli* Hfq and may lack C-terminal tail-binding altogether (51). The proximal site preferentially binds to U-rich RNA sequence (52), the distal site binds to A-rich (ARN)_n triplet repeats (53), while the rim associates with UA-rich regions (54). The two potential Hfq-binding sites found within the *vfr* leader sequence (Fig. 3A) represent one A-rich ARN repeat region and one U-rich region.

RNA EMSA analyses showed that *P. aeruginosa* Hfq bound *vfr* RNA with high efficiency, resulting in three distinct band shifts (Fig. 3C) indicative of two or more binding sites. Because wild-type *P. putida* Hfq showed identical binding patterns to *P. aeruginosa* Hfq on *vfr* RNA (Fig. S3A), we took advantage of previously characterized derivatives of *P. putida* Hfq, namely, a distal site mutant (Hfq<sub>Y25D</sub>; A-rich ARN-binding deficient) and a proximal site mutant (Hfq<sub>K56A</sub>; U-rich-binding deficient) (55) to further analyze Hfq-binding to the *vfr* RNA. As seen in Fig. S3B, Hfq<sub>Y25D</sub> only recapitulated the second band shift while Hfq<sub>K56A</sub> only recapitulated the first band shift. Notably, the third band shift is absent with both mutant derivatives of Hfq. Based on this set of data, we conclude that the first shift is caused by Hfq-binding to the U-rich region of the *vfr* leader sequence (Hfq-site 2 in Fig. 3A), the second shift is caused when the ARN repeats are bound by Hfq (Hfq-site 1 in Fig. 3A), while the third shift occurs when both sites are occupied simultaneously.

Having ascertained that Hfq does bind *vfr* RNA, we next added RsmA and Hfq simultaneously to the *vfr* RNA. As seen in Fig. 3D, this resulted in super-shifting of all three Hfq band shifts in the presence of RsmA. Because the GG doublet within the consensus sequences for RsmA and CsrA binding are essential for their abilities to bind cognate target RNAs (8, 45), we made a CC substitution of the GG doublet within the predicted RsmA-binding region of *vfr* RNA. No RsmA-mediated super-shifting of Hfq bands shifts was detected with the CC substituted RNA (Fig. 3E).

We infer from the findings above that Hfq-binding is a pre-requisite for RsmA to bind to its target within *vfr* mRNA. Together with the data in preceding sections, these results lead us
to conclude: 1) that the direct binding of RsmA on vfr RNA requires the RNA chaperone protein Hfq and 2) that RsmA is an indirect regulator of biofilm polysaccharide locus pel, flagellar motility and Type IV pili-mediated twitching motility, through its direct action on vfr propagated through a regulatory cascade from Vfr to FleQ and AlgR.
DISCUSSION

In this study, we report that *P. aeruginosa* RsmA requires the RNA chaperone Hfq to assist its binding to *vfr* mRNA to initiate a regulatory cascade that ultimately impacts both motility- and biofilm-associated genes. The requirement for Hfq was unexpected, since all other biochemical analysis of RsmA/CsrA family members document unaided binding to their target RNAs (e.g.: 8, 15-17, 56-59). We identified two independent Hfq-binding sites upstream of the RsmA-binding motif, which overlaps the ribosome-binding site of *vfr* (Fig. 3A). Based on a mFOLD-predicted secondary structure, a large hairpin loop base-pairs the identified U-rich and A-rich Hfq-binding sites, resulting in a dsRNA structure that would predictively block both RsmA and the ribosome from accessing the mRNA (Fig. 4A). As depicted in Fig. 4B, the simplest model that arises from our findings is that Hfq-binding is required to open the stem-loop to expose the RsmA-binding site. Subsequent binding of RsmA would directly block the Shine-Dalgarno sequence, preventing ribosome access and thereby inhibiting translation. We also provide evidence that *vfr* mRNA is hyper-stable in the absence of RsmA (Fig. 1C), indicating that Hfq- and RsmA-bound *vfr* mRNA may undergo more rapid degradation. It was previously suggested that Hfq may be able to directly recruit RNase E for the degradation of bound RNA in *E. coli* (60), and it is possible that a similar mechanism causes *vfr* mRNA instability.

Due to its histidine-rich C-terminus, *E. coli* Hfq is a common contaminant of His-tagged proteins purified by nickel affinity chromatography after over-expression in *E. coli*, and visually undetectable Hfq levels can critically influence *in vitro* analysis of RNA-binding (51, 61). Because the proteins used in this study were purified from either *P. aeruginosa* (RsmA) or a Hfq null strain of *E. coli* (Hfq proteins), our analyses were not complicated by this issue. However, a large majority of analyses performed in past publications use C-terminal His-tagged RsmA/CsrA proteins derived from over-expression in *E. coli*, raising the possibility of *E. coli* Hfq contamination. Given that Hfq binds *vfr* RNA with apparent high affinity (Figs. 3C and S3), it is plausible that Hfq-assisted RsmA-binding may be prevalent. Although such a possibility would not alter major conclusions and remains to be
experimentally verified, it is provocative that comparison of transcriptomic studies done for
RsmA, Hfq, and Vfr regulons in *P. aeruginosa* identify numerous overlaps of genes that
were differentially expressed between WT and the respective null mutants (18, 19, 29, 62,
63).

A diverse range of phenotypes have been associated with RsmA functions in *P. aeruginosa*
(64), but phenotypic observations do not distinguish direct regulatory activities from
indirect effects through regulatory cascades. Our identification of Hfq and RsmA action on
*vfr* mRNA resulted from an initial aim to determine whether RsmA directly targeted *pel*
RNA, as is the case for *psl* RNA (8). Our back-tracking to trace the ultimate cause of RsmA
effects on *pel* expression revealed a regulatory pathway originating from Vfr to the
transcriptional regulator FleQ and from there to *pel* (Figs. S1, S2, and 1) – a cascade that in
addition to biofilm-associated genes, also impacts bacterial motility (Fig. 2).

As summarized in Fig. 4C, this work places the global post-transcriptional regulators RsmA
and Hfq as a hub at the center of upstream and downstream regulatory pathways and thus
provides a conceptually new framework to evaluate/interpret past (and future) work. The
two component GacS/GacA system lies at the top of the hierarchy that controls RsmA-
mediated regulation. Although the environmental cue(s) for activation is still unknown,
GacS activity is modulated through interactions with RetS and LadS (65-67). GacA, in turn
controls the production of the small RNAs – *rsmY* and *rsmZ* – which possess multiple high
affinity binding sites to titrate RsmA away from target mRNAs (4, 68). In light of a report
indicating that Hfq also binds to *rsmY* (48), it appears plausible that these small RNAs may
also require Hfq to assist their function.

In addition to motility- and biofilm-associated phenotypes, there are numerous other
processes associated with RsmA in *P. aeruginosa*, including acute virulence (5, 69). Many
previously assigned ‘RsmA-regulated factors and phenotypes’ are likely to be indirectly
regulated through Vfr initiated regulatory cascades. Here we present evidence for
swimming motility (Vfr/FleQ/Flagella cascade, Fig. 4C center), twitching motility (Vfr/AlgR/Type IV pili, Fig. 4C right) and PEL (Vfr/FleQ/PEL cascade, Fig. 4C left),

Based on our findings, it will be crucial to determine and distinguish direct versus indirect regulatory routes to gain a greater understanding of the RsmA regulon.
MATERIALS AND METHODS

**Bacterial strains and growth conditions**

Table S1 lists the bacterial strains used in this study. *E. coli* and *P. aeruginosa* strains were grown in lysogeny broth (LB) at 37°C unless specified otherwise. VBMM citrate medium (70) was used for selecting *P. aeruginosa* post-conjugation. For *E. coli*, the following antibiotics concentrations were used: 50 μg·ml⁻¹ carbenicillin, 10 μg·ml⁻¹ gentamicin, and 10 μg·ml⁻¹ tetracycline. For *P. aeruginosa* strains: 300 μg·ml⁻¹ carbenicillin, 100 μg·ml⁻¹ gentamicin, and 100 μg·ml⁻¹ tetracycline were used. Sucrose counter-selection for plasmids carrying the sacB gene used in *P. aeruginosa* strain constructions was performed by streaking colonies on LB agar (no salt) supplemented with 10% w/v sucrose. Plates were incubated at 30°C for 24 hours, after which the counter-selected colonies were confirmed for the loss of antibiotic resistance and mutations confirmed by PCR for double-cross-over genomic mutants.

**Strain constructions**

Transcriptional fusion constructs were generated in single copy on the chromosome of *P. aeruginosa* strains via integration into the chromosomal attB site as previously published (8). In brief, promoter regions were PCR amplified using oligonucleotides in Table S2. PCR products were ligated between the EcoRI and BamHI sites of mini-CTX lacZ (Fig. S4). These plasmids were then introduced into *P. aeruginosa* by conjugation. After double site recombination, plasmid backbones were removed by FLP recombinase, and then strains cured of the pFLP2 plasmid by sucrose counter-selection.

**Motility assays**

Swimming motility assays were performed essentially as previously described (71). LB plates containing 0.3% Bacto agar were inoculated with overnight cultures with a sterile inoculation needle, ensuring the needle tip was inserted approximately halfway into the agar but not to the plastic petri dish bottom, and incubated for 24 hours at 30°C. Swim ring diameters were measured for quantitation. For twitching motility, we followed the protocol
of Haley et al. (72) by using LB plates supplemented with 5 mg/ml porcine gastric mucin (Sigma-Aldrich). A sterile inoculation needle was inserted through the agar until it touched the plastic bottom. Plates were incubated for 48 hours at 37°C, then at room temperature for 48 more hours. Subsequently, the agar was peeled off, and the plates were stained with 1% w/v crystal violet to visualize the twitching zone diameters prior to measurement (73). All motility experiments were performed in biological quadruplicates.

β-galactosidase assays
Quantitative β-galactosidase activities were assayed using Galacto-Light Plus kit (Thermo-Fisher) as previously published (74). All P. aeruginosa cultures were grown in VBMM citrate at 37°C to exponential phase, and lysed using chloroform as previously described (8). β-galactosidase activity units were normalized to total proteins per ml as determined using Bradford assay reagents (Bio-Rad). Assays were performed in biological triplicates.

Quantitative real-time PCR and RNA stability analyses
Real-time PCR was performed as previously described (30), using the oligonucleotides listed in Table S2. For RNA stability experiments, exponential phase P. aeruginosa cultured in VBMM citrate at 37°C were treated with 200 μg·ml⁻¹ rifampicin (75). RNAs were extracted from 1 ml of the cultures at various time points as previously described (8) after the addition of rifampicin. RNA extractions were performed using the RNeasy Mini Kit (Qiagen) after treating the harvested cells with RNAProtect (Qiagen). Genomic DNA was removed using DNase I (Promega) and removal confirmed by PCR using primers designed against the rplU gene (Table S2). SuperScript III First-Strand Synthesis (Invitrogen) was used to synthesize cDNA as per manufacturer’s protocol using random hexamers. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Thermo/Applied Biosystems). The internal control gene used was ampR. All experiments were done in biological quadruplicates.

Purified proteins
Proteins were purified by previously established protocols. Over-expressed RsmA-His$_{6}$ was purified from _P. aeruginosa_ bearing pUCP18::rsmA-His$_{6}$ (8), while all Hfq-His$_{6}$ proteins were purified from a Hfq null derivative of _E. coli_ BL21(DE3) (55) carrying P$_{T7}$ expression plasmids (pVI2344-2346 or pVI2357). All proteins were purified through Ni-NTA affinity with HisTrap HP 1 ml and further by ion exchange (HiTrap SP HP 5 ml for RsmA and ResourceQ 1 ml for Hfq) by ÄKTA avant FPLC (GE Healthcare). Final protein concentrations were determined using the BCA Protein Assay Kit (Pierce/Fisher).

**RNA synthesis**

Wild-type and mutant _vfr_ mRNAs were generated using Ambion’s MEGAscript kit as recommended for _in vitro_ transcription from the P$_{T7}$ promoter of linearized plasmids. Reactions (total 40 µl) containing 1 µg of EcoRV-linearized pVI2358 or pVI2359, were incubated for 6 hours prior to 15 minutes DNase I treatment. Reactions were terminated by adding 230 µl RNase-free H$_{2}$O and 30 µl stop solution (5 M ammonium acetate; 100 mM EDTA). The resulting 233 nt RNAs (encompassing co-ordinates -106 to +116 relative to the A of the initiation codon of _vfr_) were extracted twice with phenol:chloroform:IAA (25:24:1) and once with chloroform prior to precipitation and resuspension in 50 µl RNase-free H$_{2}$O.

**RNA electrophoretic mobility shift assays (EMSAs)**

Reactions (total 10 µl) contained 32 nM _in vitro_ transcribed RNA with the indicated concentrations of RsmA and/or Hfq. RsmA and Hfq molarities given here are for the dimeric and hexameric complexes respectively. RNA was first heated at 80°C for 5 min and then immediately chilled in an ice-water bath. Additional final reaction mixture components were as follows: 10 mM HEPES pH 7.9, 2 mM MgCl$_{2}$, 90 ng yeast total RNA (Fisher), 4U RNasin, and 35 mM KCl. Binding reactions were performed at 20°C for 40 min prior to adding 2.5 µl loading buffer (40% sucrose). Reactions were placed on ice and analyzed on 5% TBE Criterion pre-cast gels (Bio-Rad) with electrophoresis at 100V for ~130 min at 4°C. Gels were stained at room temperature with 1:10,000 TBE-diluted SYBR
Gold (Fisher) in a light-protected container with agitation. Images were captured using Typhoon FLA 9500 (GE Healthcare).

**Statistics**

Presented data are the mean with standard deviations of the data collected. Student’s $t$-test using the GraphPad Prism software was used to calculate $p$-values.
ACKNOWLEDGEMENTS

The authors would like to thank Victoria Murina for her assistance with the FPLC. Plasmid pMMBGW:RBS-vfr was a kind gift from Joe J. Harrison. YI would like to thank Matt Parsek for his more than a decade of scientific and moral support that led to the culmination of this project. The authors declare no conflict of interests.

FUNDING INFORMATION

This work was supported by the J C Kempe & S M Kempe Foundation (JCK-1523 to VS).
REFERENCES

1. Gellatly SL & Hancock RE (2013) *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog. Dis. 67(3):159-173.

2. Khare A & Tavazoie S (2015) Multifactorial Competition and Resistance in a Two-Species Bacterial System. PLoS Genet. 11(12):e1005715.

3. Tashiro Y, Yawata Y, Toyofuku M, Uchiyama H, & Nomura N (2013) Interspecies interaction between *Pseudomonas aeruginosa* and other microorganisms. Microbes Environ. 28(1):13-24.

4. Heurlier K, et al. (2004) Positive control of swarming, rhamnolipid synthesis, andlipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. 186(10):2936-2945.

5. Mulcahy H, O'Callaghan J, O'Grady EP, Adams C, & O'Gara F (2006) The posttranscriptional regulator RsmA plays a role in the interaction between *Pseudomonas aeruginosa* and human airway epithelial cells by positively regulating the type III secretion system. Infect. Immun. 74(5):3012-3015.

6. Mulcahy H, et al. (2008) *Pseudomonas aeruginosa* RsmA plays an important role during murine infection by influencing colonization, virulence, persistence, and pulmonary inflammation. Infect. Immun. 76(2):632-638.

7. O'Grady EP, Mulcahy H, O'Callaghan J, Adams C, & O'Gara F (2006) *Pseudomonas aeruginosa* infection of airway epithelial cells modulates expression of Kruppel-like factors 2 and 6 via RsmA-mediated regulation of type III exoenzymes S and Y. Infect. Immun. 74(10):5893-5902.

8. Irie Y, et al. (2010) *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. Mol. Microbiol. 78(1):158-172.

9. Pessi G, et al. (2001) The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*. J. Bacteriol. 183(22):6676-6683.

10. Gutiérrez P, et al. (2005) Solution structure of the carbon storage regulator protein CsrA from *Escherichia coli*. J. Bacteriol. 187(10):3496-3501.

11. Rife C, et al. (2005) Crystal structure of the global regulatory protein CsrA from *Pseudomonas putida* at 2.05 A resolution reveals a new fold. Proteins 61(2):449-453.

12. Schubert M, et al. (2007) Molecular basis of messenger RNA recognition by the specific bacterial repressing clamp RsmA/CsrA. Nat. Struct. Mol. Biol. 14(9):807-813.

13. White D, Hart ME, & Romeo T (1996) Phylogenetic distribution of the global regulatory gene *csrA* among eubacteria. Gene 182(1-2):221-223.

14. Romeo T, Vakulskas CA, & Babitzke P (2013) Post-transcriptional regulation on a global scale: form and function of Csr/Rsm systems. Environ. Microbiol. 15(2):313-324.
15. Patterson-Fortin LM, Vakulskas CA, Yakhnin H, Babitzke P, & Romeo T (2013) Dual posttranscriptional regulation via a cofactor-responsive mRNA leader. J. Mol. Biol. 425(19):3662-3677.

16. Ren B, Shen H, Lu ZJ, Liu H, & Xu Y (2014) The phzA2-G2 transcript exhibits direct RsmA-mediated activation in Pseudomonas aeruginosa M18. PloS one 9(2):e89653.

17. Yakhnin AV, et al. (2013) CsrA activates flhDC expression by protecting flhDC mRNA from RNase E-mediated cleavage. Mol. Microbiol. 87(4):851-866.

18. Brecic A & Lory S (2009) Determination of the regulon and identification of novel mRNA targets of Pseudomonas aeruginosa RsmA. Mol. Microbiol. 72(3):612-632.

19. Burrowes E, Baysse C, Adams C, & O’Gara F (2006) Influence of the regulatory protein RsmA on cellular functions in Pseudomonas aeruginosa PAO1, as revealed by transcriptome analysis. Microbiology 152(Pt 2):405-418.

20. Lawhon SD, et al. (2003) Global regulation by CsrA in Salmonella typhimurium. Mol. Microbiol. 48(6):1633-1645.

21. Baker CS, et al. (2007) CsrA inhibits translation initiation of Escherichia coli hfq by binding to a single site overlapping the Shine-Dalgarno sequence. J. Bacteriol. 189(15):5472-5481.

22. Pannuri A, et al. (2012) Translational repression of NhaR, a novel pathway for multi- tier regulation of biofilm circuitry by CsrA. J. Bacteriol. 194(1):79-89.

23. Bandyra KJ & Luisi BF (2013) Licensing and due process in the turnover of bacterial RNA. RNA Biol. 10(4):627-635.

24. Ryder C, Byrd M, & Wozniak DJ (2007) Role of polysaccharides in Pseudomonas aeruginosa biofilm development. Curr. Opin. Microbiol. 10(6):644-648.

25. Hickman JW & Harwood CS (2008) Identification of FleQ from Pseudomonas aeruginosa as a c-di-GMP-responsive transcription factor. Mol. Microbiol. 69(2):376-389.

26. Gilbert KB, Kim TH, Gupta R, Greenberg EP, & Schuster M (2009) Global position analysis of the Pseudomonas aeruginosa quorum-sensing transcription factor LasR. Mol. Microbiol. 73(6):1072-1085.

27. Sakuragi Y & Kolter R (2007) Quorum-sensing regulation of the biofilm matrix genes (pel) of Pseudomonas aeruginosa. J. Bacteriol. 189(14):5383-5386.

28. Goodeham WJ & Hancock RE (2009) Regulation of virulence and antibiotic resistance by two-component regulatory systems in Pseudomonas aeruginosa. FEMS Microbiol. Rev. 33(2):279-294.

29. Goodman AL, et al. (2004) A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in Pseudomonas aeruginosa. Dev. Cell 7(5):745-754.

30. Irie Y, et al. (2012) Self-produced exopolysaccharide is a signal that stimulates biofilm formation in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U. S. A. 109(50):20632-20636.

31. Baraquet C, Murakami K, Parsek MR, & Harwood CS (2012) The FleQ protein from Pseudomonas aeruginosa functions as both a repressor and an activator to
control gene expression from the pel operon promoter in response to c-di-GMP.

Nucleic Acids Res. 40(15):7207-7218.

32. Dasgupta N, et al. (2003) A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in Pseudomonas aeruginosa. Mol. Microbiol. 50(3):809-824.

33. Dasgupta N, Ferrell EP, Kanack KJ, West SE, & Ramphal R (2002) fleQ, the gene encoding the major flagellar regulator of Pseudomonas aeruginosa, is σ70 dependent and is downregulated by Vfr, a homolog of Escherichia coli cyclic AMP receptor protein. J. Bacteriol. 184(19):5240-5250.

34. Burrows LL (2012) Pseudomonas aeruginosa twitching motility: type IV pili in action. Annu. Rev. Microbiol. 66:493-520.

35. Kazmierczak BI, Schniederberend M, & Jain R (2015) Cross-regulation of Pseudomonas motility systems: the intimate relationship between flagella, pili and virulence. Curr. Opin. Microbiol. 28:78-82.

36. Klausen M, et al. (2003) Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Mol. Microbiol. 48(6):1511-1524.

37. O'Toole GA & Kolter R (1998) Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol. Microbiol. 30(2):295-304.

38. Beatson SA, Whitchurch CB, Sargent JL, Levesque RC, & Mattick JS (2002) Differential regulation of twitching motility and elastase production by Vfr in Pseudomonas aeruginosa. J. Bacteriol. 184(13):3605-3613.

39. Whitchurch CB, Alm RA, & Mattick JS (1996) The alginate regulator AlgR and an associated sensor FimS are required for twitching motility in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U. S. A. 93(18):9839-9843.

40. Luo Y, et al. (2015) A hierarchical cascade of second messengers regulates Pseudomonas aeruginosa surface behaviors. mBio 6(1).

41. Pritchett CL, et al. (2015) Expression analysis of the Pseudomonas aeruginosa AlgZr two-component regulatory system. J. Bacteriol. 197(4):736-748.

42. Belete B, Lu H, & Wozniak DJ (2008) Pseudomonas aeruginosa AlgR regulates type IV pilus biosynthesis by activating transcription of the fimU-pilVWXUY2E operon. J. Bacteriol. 190(6):2023-2030.

43. Lizewski SE, et al. (2004) Identification of AlgR-regulated genes in Pseudomonas aeruginosa by use of microarray analysis. J. Bacteriol. 186(17):5672-5684.

44. Schulmeyer KH, et al. (2016) Primary and Secondary Sequence Structure Requirements for Recognition and Discrimination of Target RNAs by Pseudomonas aeruginosa RsmA and RsmF. J. Bacteriol. 198(18):2458-2469.

45. Dubey AK, Baker CS, Romeo T, & Babitzke P (2005) RNA sequence and secondary structure participate in high-affinity CsrA-RNA interaction. Rna 11(10):1579-1587.

46. Fuchs EL, et al. (2010) The Pseudomonas aeruginosa Vfr regulator controls global virulence factor expression through cyclic AMP-dependent and -independent mechanisms. J. Bacteriol. 192(14):3553-3564.

47. Kay E, et al. (2006) Two GacA-dependent small RNAs modulate the quorum-sensing response in Pseudomonas aeruginosa. J. Bacteriol. 188(16):6026-6033.
531 48. Sorger-Domenigg T, Sonnleitner E, Kaberdin VR, & Bläsi U (2007) Distinct and
532 overlapping binding sites of *Pseudomonas aeruginosa* Hfq and RsmA proteins on
533 the non-coding RNA RsmY. *Biochem. Biophys. Res. Commun.* 352(3):769-773.
534 49. Kambara TK, Ramsey KM, & Dove SL (2018) Pervasive Targeting of Nascent
535 Transcripts by Hfq. *Cell Rep.* 23(5):1543-1552.
536 50. Updegrove TB, Zhang A, & Storz G (2016) Hfq: the flexible RNA matchmaker.
537 *Curr. Opin. Microbiol.* 30:133-138.
538 51. Milojевич T, Grishkovskaya I, Sonnleitner E, Djinovic-Carugo K, & Bläsi U (2013)
539 The *Pseudomonas aeruginosa* catabolite repression control protein Crc is devoid of
540 RNA binding activity. *PloS one* 8(5):e64609.
541 52. Møller T, et al. (2002) Hfq; a bacterial Sm-like protein that mediates RNA-RNA
542 interaction. *Mol. Cell.* 9(1):23-30.
543 53. Link TM, Valentin-Hansen P, & Brennan RG (2009) Structure of *Escherichia coli*
544 Hfq bound to polyriboadenylate RNA. *Proc. Natl. Acad. Sci. U. S. A.*
545 106(46):19292-19297.
546 54. Schu DJ, Zhang A, Gottesman S, & Storz G (2015) Alternative Hfq-sRNA
547 interaction modes dictate alternative mRNA recognition. *EMBO J.* 34(20):2557-
548 2573.
549 55. Madhushani A, Del Peso-Santos T, Moreno R, Rojo F, & Shingler V (2015)
550 Transcriptional and translational control through the 5'-leader region of the *dmpR*
551 master regulatory gene of phenol metabolism. *Environ. Microbiol.* 17(1):119-133.
552 56. Baker CS, Morozov I, Suzuki K, Romeo T, & Babitzke P (2002) CsrA regulates
553 glycogen biosynthesis by preventing translation of *glgC* in *Escherichia coli*. *Mol.
554 Microbiol.* 44(6):1599-1610.
555 57. Dubey AK, et al. (2003) CsrA regulates translation of the *Escherichia coli* carbon
556 starvation gene, *cstA*, by blocking ribosome access to the *cstA* transcript. *J.
557 Bacteriol.* 185(15):4450-4460.
558 58. Lapouge K, et al. (2007) Mechanism of *hcnA* mRNA recognition in the Gac/Rsm
559 signal transduction pathway of *Pseudomonas fluorescens*. *Mol. Microbiol.*
560 66(2):341-356.
561 59. Wang X, et al. (2005) CsrA post-transcriptionally represses *pgaABCD*, responsible
562 for synthesis of a biofilm polysaccharide adhesin of *Escherichia coli*. *Mol.
563 Microbiol.* 56(6):1648-1663.
564 60. Morita T, Maki K, & Aiba H (2005) RNase E-based ribonucleoprotein complexes:
565 mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs.
566 *Genes Dev.* 19(18):2176-2186.
567 61. Moreno R, et al. (2015) The Crc and Hfq proteins of *Pseudomonas putida* cooperate
568 in catabolite repression and formation of ribonucleic acid complexes with specific
569 target motifs. *Environ. Microbiol.* 17(1):105-118.
570 62. Sonnleitner E, et al. (2018) Interplay between the catabolite repression control
571 protein Crc, Hfq and RNA in Hfq-dependent translational regulation in
572 *Pseudomonas aeruginosa*. *Nucleic Acids Res.* 46(3):1470-1485.
63. Wolfgang MC, Lee VT, Gilmore ME, & Lory S (2003) Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. *Dev. Cell* 4(2):253-263.

64. Vakulskas CA, Potts AH, Babitzke P, Ahmer BM, & Romeo T (2015) Regulation of bacterial virulence by Csr (Rsm) systems. *Microbiol. Mol. Biol. Rev.* 79(2):193-224.

65. Chambonnier G, et al. (2016) The Hybrid Histidine Kinase LsdS Forms a Multicomponent Signal Transduction System with the GacS/GacA Two-Component System in *Pseudomonas aeruginosa*. *PLoS Genet.* 12(5):e1006032.

66. Goodman AL, et al. (2009) Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. *Genes Dev.* 23(2):249-259.

67. Kong W, et al. (2013) Hybrid sensor kinase PA1611 in *Pseudomonas aeruginosa* regulates transitions between acute and chronic infection through direct interaction with RetS. *Mol. Microbiol.* 88(4):784-797.

68. Sonneleitner E, Schuster M, Sorger-Domenigg T, Greenberg EP, & Bläsi U (2006) Hfq-dependent alterations of the transcriptome profile and effects on quorum sensing in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 59(5):1542-1558.

69. Intile PJ, Diaz MR, Urbanowski ML, Wolfgang MC, & Yahr TL (2014) The AlgZR two-component system recalibrates the RsmAYZ posttranscriptional regulatory system to inhibit expression of the *Pseudomonas aeruginosa* type III secretion system. *J. Bacteriol.* 196(2):357-366.

70. Hoang TT & Schweizer HP (1997) Fatty acid biosynthesis in *Pseudomonas aeruginosa*: cloning and characterization of the fabAB operon encoding β-hydroxyacyl-acyl carrier protein dehydratase (FabA) and β-ketoacyl-acyl carrier protein synthase I (FabB). *J. Bacteriol.* 179(17):5326-5332.

71. Shrout JD, et al. (2006) The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. *Mol. Microbiol.* 62(5):1264-1277.

72. Haley CL, Kruczek C, Qaisar U, Colmer-Hamood JA, & Hamood AN (2014) Mucin inhibits *Pseudomonas aeruginosa* biofilm formation by significantly enhancing twitching motility. *Can. J. Microbiol.* 60(3):155-166.

73. Déziel E, Comeau Y, & Villemur R (2001) Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *J. Bacteriol.* 183(4):1195-1204.

74. Lequetter Y, Lee J-H, Ledgham F, Lazdunski A, & Greenberg EP (2006) A distinct QscR regulon in the *Pseudomonas aeruginosa* quorum-sensing circuit. *J. Bacteriol.* 188(9):3365-3370.

75. Lory S (1986) Effect of iron on accumulation of exotoxin A-specific mRNA in *Pseudomonas aeruginosa*. *J. Bacteriol.* 168(3):1451-1456.
FIGURE LEGENDS

Fig. 1  RsmA is a repressor of vfr.
A. Single-copy transcriptional lacZ fusion constructs reveal up-regulation of vfr transcripts in the ΔrsmA background as compared to WT.
B. The single-copy P_{pelA}::lacZ transcriptional fusion has a higher activity in a Vfr over-expressing strain compared to the same strain harboring the vector control (VC).
C. Real-time quantitative PCR at different time points after rifampicin treatment show that vfr mRNA is less stable in WT as compared to the ΔrsmA strain.

Fig. 2  RsmA regulation of Vfr indirectly regulates flagellar and Type IV pilus-mediated motilities.
A. Flagellar-mediated swimming motility is decreased in the absence of RsmA (ΔrsmA) and enhanced in the absence of Vfr (Δvfr) as compared to the WT strain.
B. Type IV pilus-mediated twitching motility is unaffected when rsmA is mutated but is reduced in the Δvfr and RsmA over-expression strains.

Fig. 3  Direct targeting of vfr mRNA by RsmA requires Hfq.
A. The leader sequence of the vfr transcript (46) possesses two putative Hfq-binding sites (blue boxes) and a RsmA-binding site (green box) overlapping the predicted Shine-Dalgarno sequence (red italics) upstream of the translational start site (underlined italics).
B. RNA Electrophoretic mobility shift assay (EMSA) shows that RsmA does not bind to the vfr RNA when RsmA is present alone. Dimeric molar excess relative to RNA; U = unbound RNA.
C. The vfr RNA produces three distinct band shifts (open arrow heads 1, 2, and 3) with increasing concentrations of Hfq. Hexameric molar excess relative to RNA.
D. The presence of RsmA causes all Hfq-shifted bands to super-shift (filled arrow heads 1+, 2+, and 3+), indicating that RsmA binds to Hfq-bound RNA. Note that the RsmA
concentration used (36× molar excess of RNA) is incapable of producing a shift when present alone (panel B).

E. A GG→CC substitution within the RsmA-binding site abolishes RsmA super-shifts but does not alter Hfq-binding.

**Fig. 4** Model for Hfq-assisted RsmA regulation of vfr and its consequences

A. RNA secondary structure of the leader sequence region of vfr as predicted by mFOLD. The locations of in silico-detected Hfq- and RsmA-binding sites are indicated. Note that the RsmA-binding site located at the base of the stem loop structure overlaps with the Shine-Dalgarno sequence of the ribosome binding site. The AUG start codon is shown underlined in italics.

B. Schematic illustration of a model for Hfq-assisted RsmA regulation of vfr. Upon Hfq-binding of vfr RNA, the stem loop structure shown in panel A is disrupted, exposing the RsmA-binding site. RsmA-binding then directly blocks the Shine Dalgarno (SD) sequence. RsmA-binding is depicted as producing a smaller stem-loop structure (10-12), preventing ribosome access and thereby inhibiting translation. *In vivo*, the Hfq- and RsmA-bound mRNAs are presumed to be subsequently processed for degradation.

C. Simplified overview of the Gac/Rsm pathway that controls RsmA levels and pertinent parts of its downstream regulon.
Fig. 1

(A) Relative vfr transcription

(B) Relative pel transcription

(C) Relative vfr mRNA levels (%) over time post-rifampicin treatment (min.)
**Fig. 2**

**A**

Swimming zone diameter (% WT)

|          | WT | ΔrsmA | Δvfr |
|----------|----|-------|------|
| WT       | 100| 50    | 150  |
| ΔrsmA    |    |       | *    |
| Δvfr     |    |       | *    |

*<p<0.003 vs WT

**B**

Twitching zone diameter (% WT)

|          | WT | ΔrsmA | Δvfr | RsmA over-expressed |
|----------|----|-------|------|---------------------|
| WT       | 100|       |      |                     |
| ΔrsmA    |    |       | *    |                     |
| Δvfr     |    |       |      | *                   |
| RsmA over-expressed | | | | *               |

*p<0.0001 vs WT*
Fig. 3

A

CGCGGUUUACUGGCACACUUCCUGAUCUGCCCGUUGGGGGGAGCUCUCCCGCUGAGCUCC
CGGGCCCGGCGGAGUCUCUCAUAGAAGACUCAAAAAAACAUCGCCUUGUACGUCAGGCAU
RsmA-binding site
Hfq-site 1
Hfq-site 2

AAUUCUUUAUUUCUGGACUCGGCGGCAUG

Shine-
Dalgarno

Start
codon

B

molar excess
RsmA: 0 10x 14x 18x
dimeric

C

molar excess
Hfq: 0 1x 2x 3x 4x 6x
hexameric

D

RsmA: 0 18x 0 18x 0 18x
Hfq: 0 0 2x 2x 4x 4x

E

RsmA: 0 18x 0 18x 0 18x
Hfq: 0 0 2x 2x 4x 4x

Wild-type RsmA-binding mRNA
-UCUUUCGGAUCGGCGGAUG-

Mutant RsmA-binding site mRNA
-UCUUUCGCCACUCCGGCGGAUG-
Fig. 4

A

Hfq_distal binding site

Hfq_proximal binding site

RsmA-binding site/Shine-Dalgaro

UCUC CUCGGGC AUG

B

RsmA-binding site closed

Hfq

RsmA

ribosome

rapid degradation

C

RetS GacS LadS GacA

rsmY, rsmZ

PSL RsmA Hfq Vfr

FleQ AlgR

Flagella Type IV pilus

Hfq

PSL

Flagella Type IV pilus

Hfq

PSL

Flagella Type IV pilus