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

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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 Δ*rsmA* mutant as compared to the wild type (WT) background (Fig. S1A). However, the PSL polysaccharides are also over-expressed in a Δ*rsmA* strain (8), and as a direct consequence of this over-overexpression, intracellular levels of c-di-GMP are elevated (30). Given that the *pel* genes are known to be transcriptionally up-regulated by c-di-GMP (25, 31) and Δ*rsmA* 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 Δ*rsmA* Δ*pel* Δ*psl* 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 Δ*rsmA* 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 Δ*rsmA* 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 Δ*fleQ* 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* $FlhD_4C_2$ 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 Δ*rsmA* 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 Δ*rsmA* 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 Δ*rsmA* 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, Δ*rsmA* has reduced motility while Δ*vfr* 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, Δ*vfr* 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 transcriptional 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_{Y25D}; A-rich ARN-binding deficient) and a proximal site mutant (Hfq_{K56A}; U-rich-binding deficient) (55) to further analyze Hfq-binding to the *vfr* RNA. As seen in Fig. S3B, Hfq_{Y25D} only recapitulated the second band shift while Hfq_{K56A} 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 37ºC 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 $\mu g \cdot ml^{-1}$ carbenicillin, 10 $\mu g \cdot ml^{-1}$ gentamicin, and 10 $\mu g \cdot ml^{-1}$ tetracycline. For *P. aeruginosa* strains: 300 $\mu g \cdot ml^{-1}$ carbenicillin, 100 $\mu g \cdot ml^{-1}$ gentamicin, and 100 $\mu g \cdot ml^{-1}$ 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 30ºC 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 30ºC. 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 37ºC, 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 37ºC 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 37ºC were treated with 200 $\mu g \cdot ml^{-1}$ 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 1ml 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_2O 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_2O.

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.

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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 Δ*rsmA* 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 Δ*rsmA* 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 (Δ*rsmA*) and enhanced in the absence of Vfr (Δ*vfr*) as compared to the WT strain.

B. Type IV pilus-mediated twitching motility is unaffected when *rsmA* is mutated but is reduced in the Δ*vfr* 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

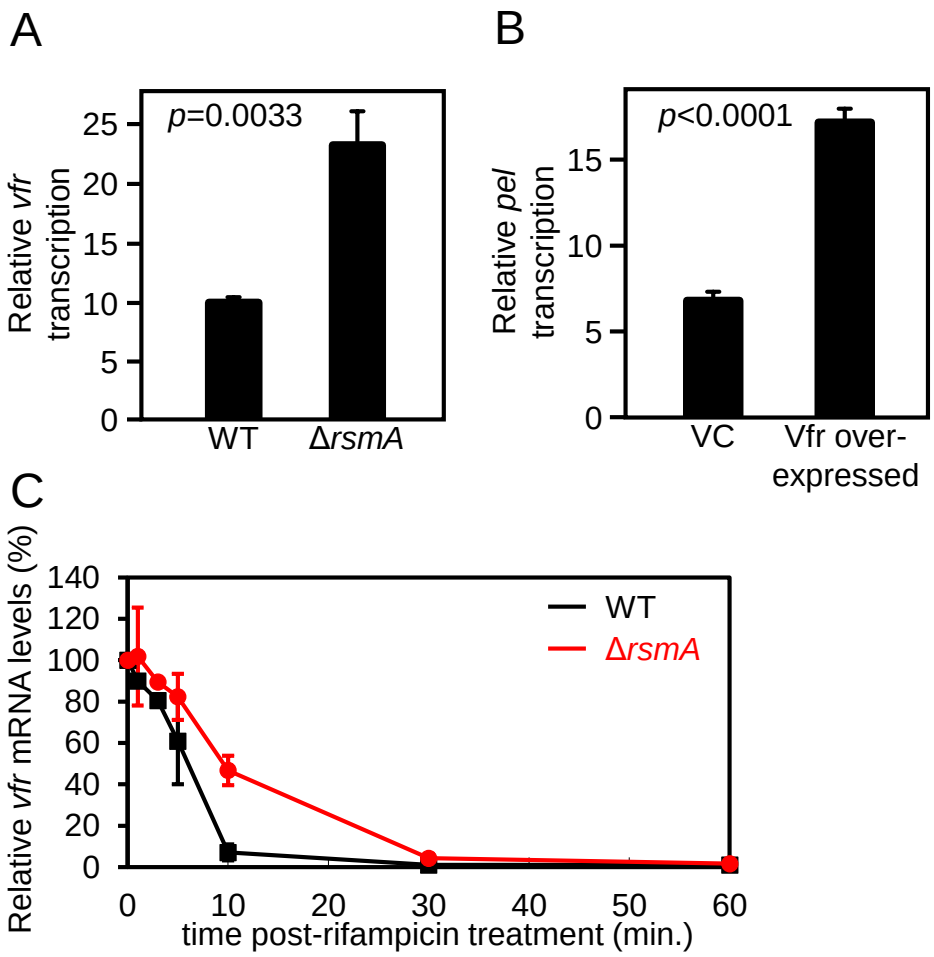

A
p=0.0033
Relative vfr transcription
25
20
15
10
5
0
WT
ΔrsmA
B
p<0.0001
Relative pel transcription
15
10
5
0
VC
Vfr over-expressed
C
Relative vfr mRNA levels (%)
140
120
100
80
60
40
20
0
WT
ΔrsmA
0
10
20
30
40
50
60
time post-rifampicin treatment (min.)

Fig. 2

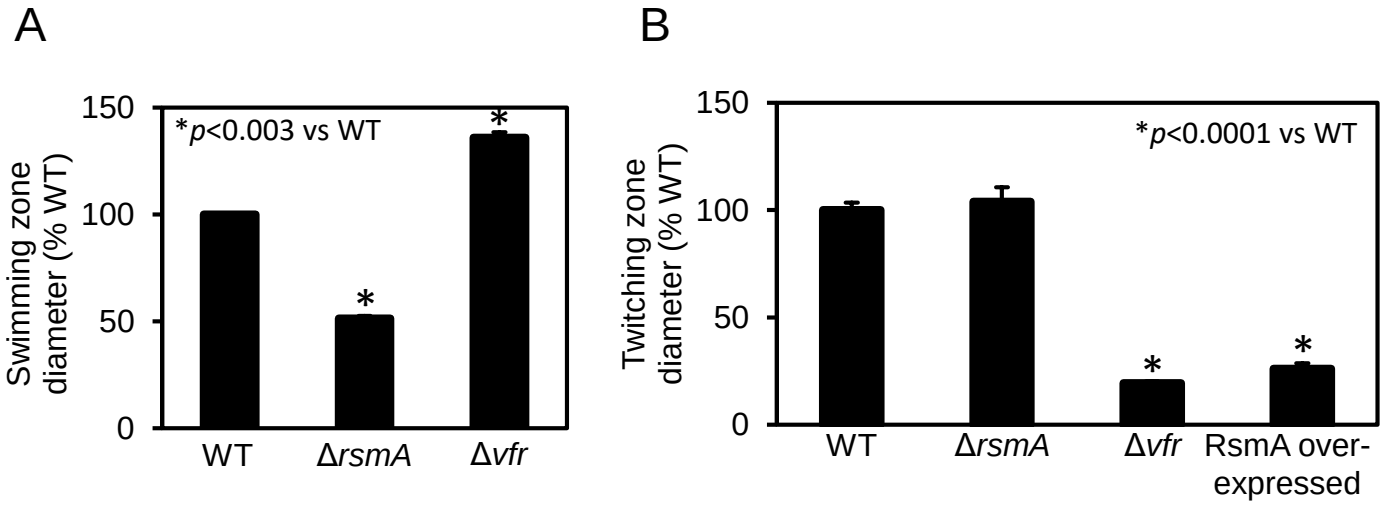

A
150
100
50
0
Swimming zone diameter (% WT)
*p<0.003 vs WT
*
*
WT
ΔrsmA
Δvfr
B
150
100
50
0
Twitching zone diameter (% WT)
*p<0.0001 vs WT
*
*
WT
ΔrsmA
Δvfr
RsmA over-expressed

Fig. 3

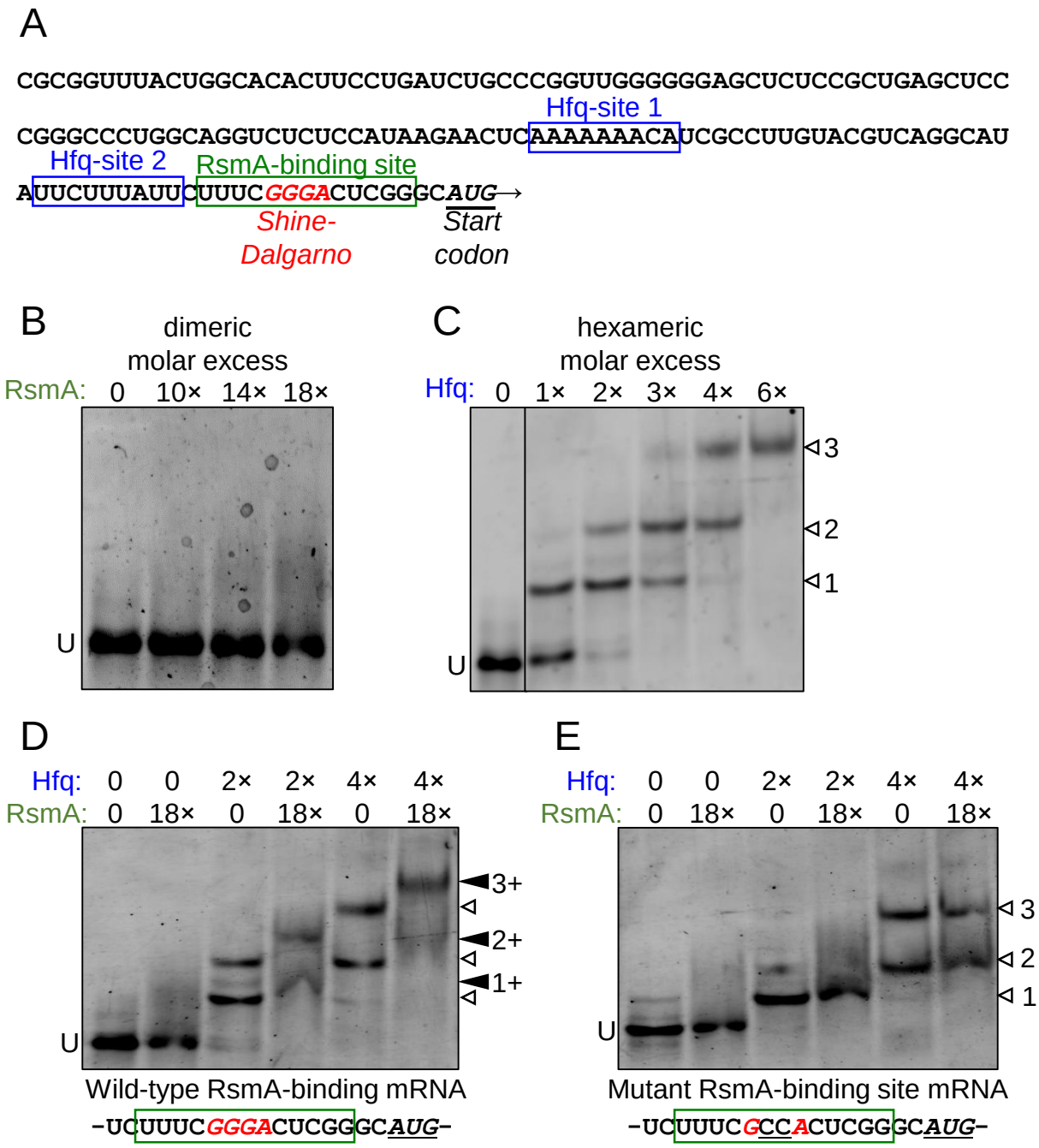
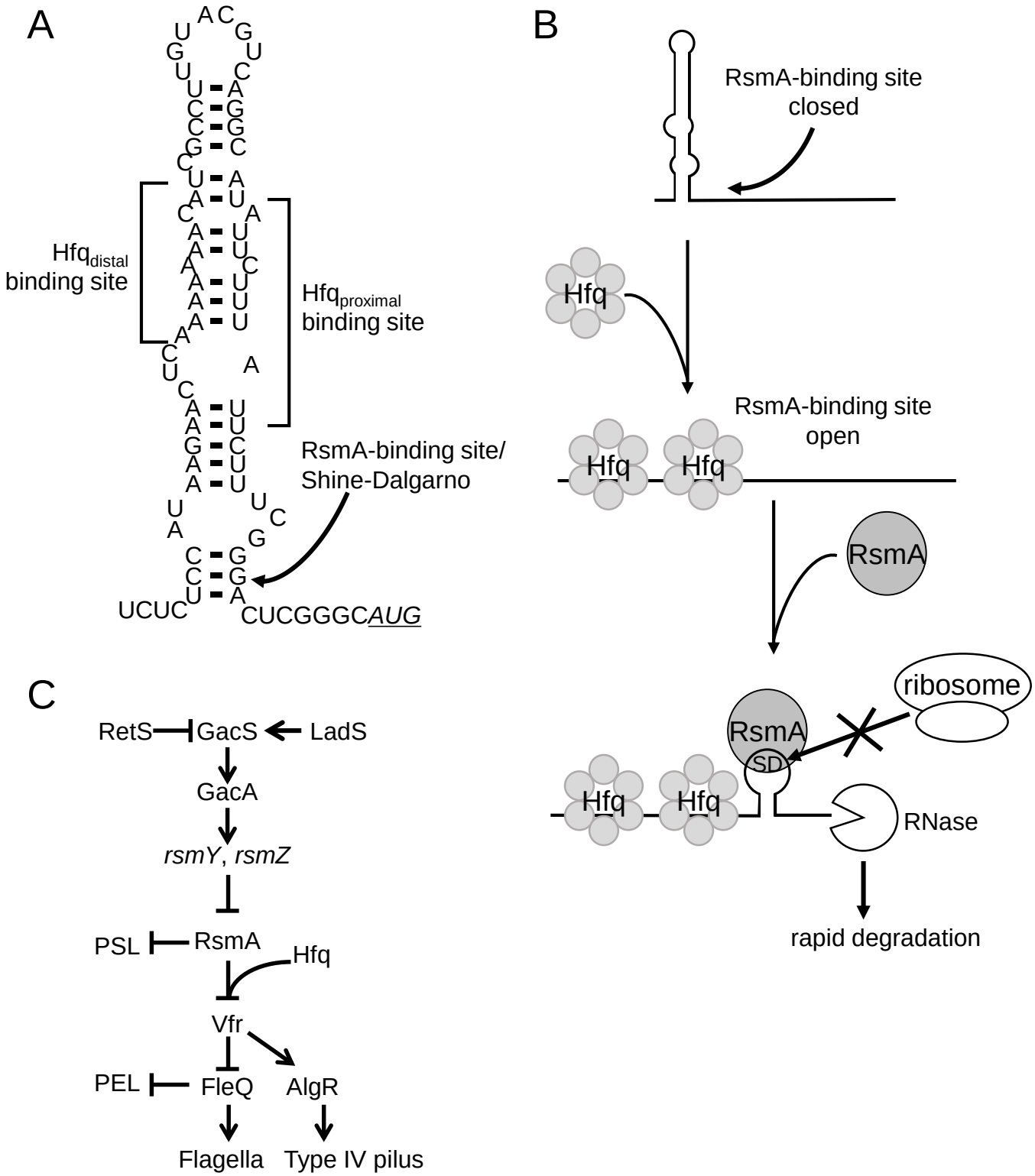

A
CGCGGUUUACUGGCACACUUCCUGAUCUGCCCGGUUGGGGGGAGCUCUCCGCUGAGCUCC
Hfq-site 1
CGGGCCCUGGCAGGUCUCUCCAUAAGAACUCAAAAAAACAUCGCCUUGUACGUCAGGCAU
Hfq-site 2
RsmA-binding site
AUUCUUUAUUCUUUCGGGACUCGGGCAUG→
Shine-Dalgarno
Start codon
B
dimeric molar excess
RsmA: 0 10× 14× 18×
U
C
hexameric molar excess
Hfq: 0 1× 2× 3× 4× 6×
3
2
1
U
D
Hfq: 0 0 2× 2× 4× 4×
RsmA: 0 18× 0 18× 0 18×
3+
2+
1+
U
Wild-type RsmA-binding mRNA
–UCUUUCGGGACUCGGGCAUG–
E
Hfq: 0 0 2× 2× 4× 4×
RsmA: 0 18× 0 18× 0 18×
3
2
1
U
Mutant RsmA-binding site mRNA
–UCUUUCGCCACUCGGGCAUG–

Fig. 4


A
Hfq distal binding site
Hfq proximal binding site
RsmA-binding site/ Shine-Dalgarno
UCUC
CUCGGGCAUG
B
RsmA-binding site closed
Hfq
RsmA-binding site open
Hfq
Hfq
RsmA
ribosome
RsmA
SD
Hfq
Hfq
RNase
rapid degradation
C
RetS
GacS
LadS
GacA
rsmY, rsmZ
PSL
RsmA
Hfq
Vfr
PEL
FleQ
AlgR
Flagella
Type IV pilus