An unusual CsrA family member operates in series with RsmA to amplify posttranscriptional responses in *Pseudomonas aeruginosa*

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Members of the CsrA family of prokaryotic mRNA-binding proteins alter the translation and/or stability of transcripts needed for numerous global physiological processes. The previously described CsrA family member in *Pseudomonas aeruginosa* (RsmA) plays a central role in determining infection modality by reciprocally regulating processes associated with acute (type III secretion and motility) and chronic (type VI secretion and biofilm formation) infection. Here we describe a second, structurally distinct RsmA homolog in *P. aeruginosa* (RsmF) that has an overlapping yet unique regulatory role. RsmF deviates from the canonical 5 β-strand and carboxyl-terminal α-helix topology of all other CsrA proteins by having the α-helix internally positioned. Despite striking changes in topology, RsmF adopts a tertiary structure similar to other CsrA family members and binds a subset of RsmA mRNA targets, suggesting that RsmF activity is mediated through a conserved mechanism of RNA recognition. Whereas deletion of *rsmF* alone had little effect on RsmA-regulated processes, strains lacking both *rsmA* and *rsmF* exhibited enhanced RsmA phenotypes for markers of both type III and type VI secretion systems. In addition, simultaneous deletion of *rsmA* and *rsmF* resulted in superior biofilm formation relative to the wild-type or *rsmA* strains. We show that RsmF translation is derepressed in an *rsmA* mutant and demonstrate that RsmA specifically binds to *rsmF* mRNA in vitro, creating a global hierarchical regulatory cascade that operates at the posttranscriptional level.

virulence | signal transduction | RsmY | RsmZ

The CsrA family of RNA-binding proteins is widely dispersed in Gram-negative and Gram-positive bacteria and regulates diverse cellular processes including carbon source utilization, biofilm formation, motility, and virulence (1–3). CsrA proteins mediate both negative and positive posttranscriptional effects and function by altering the rate of translation initiation and/or target mRNA decay (3). The general mechanism of negative regulation occurs through binding of CsrA to the 5′ untranslated leader region (5′ UTR) of target mRNAs and interfering with translation initiation (1). RsmA-binding sites ($^{A}/_{U}$CANGGAN-G$^{U}/_{A}$) usually overlap with or are adjacent to ribosome-binding sites on target mRNAs in which the core GGA motif (underlined) is exposed in the loop portion of a stem-loop structure (4). Direct positive regulation by CsrA is less common but recent studies of *flhDC* and *moaA* expression in *Escherichia coli* offer insight into potential activation mechanisms. Whereas CsrA binding to *flhDC* mRNA stimulates expression by protecting the transcript from RNase E-dependent degradation (5), binding of CsrA to the *moaA* leader region is thought to trigger a conformational change that facilitates ribosome recruitment (6).

The CsrA homolog in *Pseudomonas aeruginosa* (RsmA) plays an important role in the regulation of virulence factors associated with acute and chronic infections (7–9). RsmA positively controls factors associated with acute infections including genes controlled by the cAMP/virulence factor regulator (Vfr) system, a type III secretion system (T3SS), and type IV pili (9). RsmA negatively controls factors associated with chronic colonization including a type VI secretion system (T6SS) and exopolysaccharide production that promotes biofilm formation (9). The phenotypic switch controlled by RsmA is determined by the availability of free RsmA within cells, which is regulated by two small noncoding RNAs (RsmY and RsmZ). RsmY and RsmZ each contain multiple RsmA-binding sites and function by sequestering RsmA from target mRNAs (1). Acute virulence factor expression is favored when RsmY/Z expression is low and free RsmA levels are elevated. Transcription of *rsmY* and *rsmZ* is controlled by a complex regulatory cascade consisting of two hybrid sensor kinases (RetS and LadS) that intersect with the GacS/A two-component regulatory system (10, 11). The RsmA regulatory system is thought to play a key role in the transition from acute to chronic virulence states (12).

In this study, we report the identification of a second CsrA homolog in *P. aeruginosa*, designated RsmF. Whereas the structural organization of RsmF is distinct from RsmA, both evolved a similar tertiary structure. Functionally, RsmA and RsmF have unique but overlapping regulatory roles and both operate in a hierarchical regulatory cascade in which RsmF expression is translationally repressed by RsmA.

Results

Identification of RsmF, a Structurally Distinct Member of the CsrA Family. Although several *Pseudomonas* species possess two CsrA homologs (RsmA and RsmE) (13, 14), only RsmA had been identified in the opportunistic human pathogen *P. aeruginosa* (15). A homology search of the *P. aeruginosa* strain PAO1 genome identified a small ORF located in the intergenic region between genes PA5183 and PA5184 (*SI Appendix*, Fig. S1*A*). The predicted ORF encodes a 71-aa protein bearing 31% identity and 53% similarity to RsmA (Fig. 1*A*). Given the limited homology of the putative gene product with CsrA, RsmA, and RsmE, the gene was designated *rsmF*. All previously characterized CsrA proteins possess a highly conserved secondary structure consisting of 5 β-strands and a carboxyl-terminal (C-terminal) α-helix (4, 13, 16, 17). Analysis of the predicted RsmF sequence revealed a unique insertion between β-strands 2 and 3, and a C-terminal deletion relative to other CsrA family members (Fig. 1*A*).

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Data deposition: The RsmF coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4K59). The RsmF primary sequence has been deposited in the GenBank database [accession no. KF364633 (strain PA103)].

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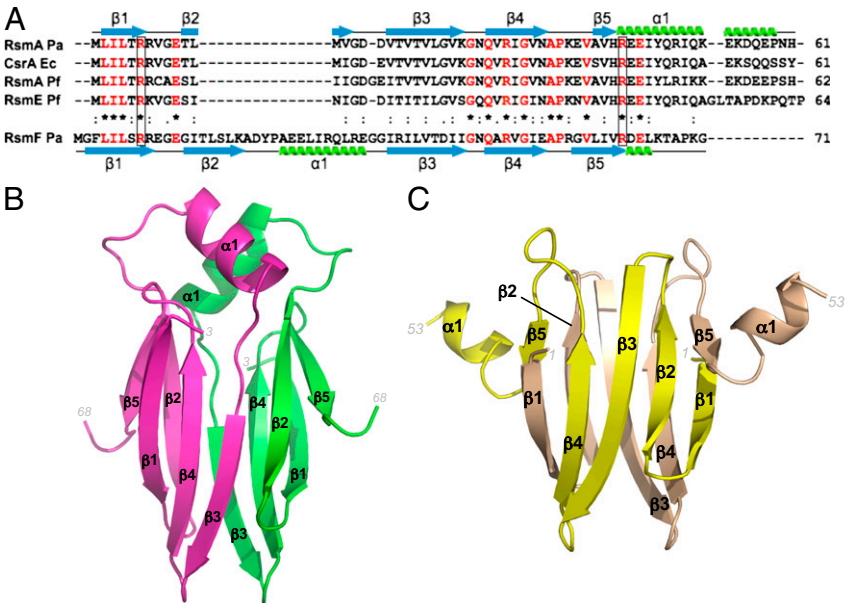

Fig. 1. RsmF structure. (*A*) Primary sequence alignment of *E. coli* (Ec) CsrA, *P. aeruginosa* (Pa) RsmA and RsmF, and *P. fluorescens* (Pf) RsmA and RsmE. All five proteins consist of five β-strands (β1–5) and one primary α-helix (α1), but the organization of those elements is distinct for RsmF. Conserved arginine residues required for maximal CsrA/RsmA RNA-binding activity are boxed. (*B* and *C*) Ribbon diagrams of the RsmF crystal structure as a homodimer (*B*) and the reported solution structure of *P. fluorescens* dimeric RsmE (pdb ID 2JPP), a homolog of *P. aeruginosa* RsmA (*C*).

To determine whether RsmF maintained the overall architecture of other CsrA proteins, we determined the crystal structure at 2.2-Å resolution and refined it to R and R_{free} values of 0.21 and 0.27, respectively (*SI Appendix*, Table S1). RsmF forms a dimer, with residues 1–65 of each monomer ordered in the final structure (Fig. 1*B*). The RsmF dimer is created by two antiparallel β-sheets, each composed of β1, β3, and β4 from one protein monomer, and β2 and β5 from the other (*SI Appendix*, Fig. S2*A*). The α-helices of each RsmF monomer, located between β-strands 2 and 3, interact with each other and are located above the central region of the dimer (Figs. 1*B* and *SI Appendix*, S2*A*). This arrangement differs from CsrA family members of known structure in that the antiparallel β-sheets are composed of β1 and β5 from one monomer and β2, β3, and β4 from the other monomer (Fig. 1*C* and *SI Appendix*, Fig. S2*B*) (4, 13, 16, 17). In addition, the C-terminal α-helices of typical CsrA/RsmA monomers do not interact and are arranged as wings extending from the sides of the dimer (Fig. 1*C*). Despite the topological differences and positioning of the α-helices, the structure of the RsmF β-sandwich is largely similar to other CsrA proteins, suggesting that it may possess an analogous regulatory function (*SI Appendix*, Fig. S2*C*).

Biofilm Formation Is Significantly Elevated in an *rsmAF* Mutant. To determine whether RsmF and RsmA are involved in controlling related virulence-associated functions, and whether RsmF activity is conserved across *P. aeruginosa* lineages, we constructed a set of isogenic *rsmA* and *rsmF* deletion mutants in strains PA103 and PA14, two well-characterized clinical isolates of *P. aeruginosa*. Both PA103 (accession no. KF364633) and PA14 (accession no. NC_008463, PA14_68470) encode proteins that are identical to RsmF in strain PAO1.

Because RsmA inhibits *P. aeruginosa* exopolysaccharide production, *rsmA* mutants typically develop robust biofilms (18). None of the mutations, however, led to an altered biofilm phenotype in strain PA103. This was not unexpected because strain PA103 lacks flagella and has a defect in the Las quorum-sensing system, both of which are required for robust biofilm formation (19–21). In contrast, the PA14 *rsmA* mutant showed a significant increase in biofilm formation (13-fold) compared with wild type (Fig. 2*A*). Although the *rsmF* mutant was indistinguishable from wild type, the PA14 *rsmA, rsmF* (*rsmAF*) double mutant developed a significantly more robust biofilm than either wild type (44-fold increase) or the *rsmA* mutant (3.5-fold increase). The biofilm phenotype was restored to near wild-type levels in the *rsmAF* double mutant when either *rsmA* or *rsmF* were provided *in trans*. Notably, the PA103 and PA14 *rsmA* and *rsmAF* double mutants grew slower than wild type (*SI Appendix*, Fig. S3 *A* and *B*); however, the modest increase in generation times of the PA14 *rsmA* and *rsmAF* mutants (*SI Appendix*, Fig. S3*C*) is unlikely to account for their altered biofilm forming capacity. These

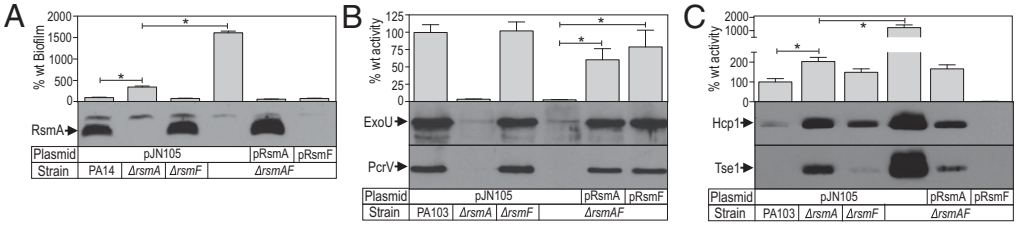

Fig. 2. Contribution of RsmA and RsmF to biofilm formation, T3SS, and T6SS gene expression. (*A*) The indicated PA14 strains were cultured in glass tubes with shaking for 12 h at 37 °C in LB medium. Biofilm formation was measured by crystal violet staining and values are reported normalized to percent wild-type (WT) activity (set at 100%). (*B* and *C*) Wild-type PA103 and the indicated mutants carrying a transcription reporter for (*B*) T3SS gene expression ($P_{exsD\text{-}lacZ}$) or a translational reporter for TssA1 translation ($P_{lacUV5\text{-}tssA'\text{-}'lacZ}$) were transformed with a vector control (pJN105), pRsmA, or pRsmF. Strains were cultured under inducing conditions (low Ca^{2+}) for T3SS gene expression in the presence of 0.4% arabinose to induce *rsmA* or *rsmF* expression from the P_{BAD} promoter and assayed for β-galactosidase activity. Reported values are in Miller units normalized to percent WT activity (set at 100%). Statistical differences were determined using two-tailed unpaired *t* tests. $*P < 0.01$. (*A*–*C*) Whole-cell lysate (*A*) and culture supernatant fluid (*B* and *C*) samples were harvested from an equivalent number of cells and immunoblotted for RsmA (*A*), or secreted proteins of the T3SS (*B;* ExoU and PcrV), or T6SS (*C*; Hcp1 and Tse1).

results show that although both RsmA and RsmF repress biofilm formation, the contribution of RsmF is only revealed in the absence of RsmA.

Expression of Either *rsmA* or *rsmF in Trans* Is Sufficient to Restore T3SS Gene Expression in an *rsmAF* Mutant. Because RsmA is required for maximal T3SS gene expression (7, 9, 22), we hypothesized that RsmF may play a similar role in controlling T3SS gene expression. To test this hypothesis, we introduced a T3SS-dependent reporter gene ($P_{exsD\text{-}lacZ}$) (23) into the ectopic ΦCTX attachment site on the chromosome of wild-type strain PA103 and the *rsmA*, *rsmF*, and *rsmAF* mutants. Under T3SS-inducing conditions (low Ca^{2+}), $P_{exsD\text{-}lacZ}$ reporter activity was significantly reduced in the PA103 *rsmA* mutant, whereas the *rsmF* mutant was indistinguishable from wild type (Fig. 2*B*). Reporter activity was restored in the *rsmAF* mutant when either *rsmA* or *rsmF* were provided *in trans*. Immunoblots of culture supernatant fluid confirmed that secretion of the ExoU effector and PcrV translocator proteins was similar in PA103 wild type and the *rsmF* mutant (Fig. 2*B*). By comparison, ExoU and PcrV secretion was severely reduced in the *rsmA* and *rsmAF* mutants and could be restored to near wild-type levels by providing the *rsmAF* mutant with either plasmid-expressed *rsmA* or *rsmF* (Fig. 2*B*). A similar pattern of PcrV synthesis was detected in the panel of PA14 strains, although complementation with RsmF did not restore PcrV expression (*SI Appendix*, Fig. S4*A*).

T6SS Gene Expression Is Significantly Elevated in an *rsmAF* Double Mutant. Whereas RsmA is required for T3SS gene expression, RsmA inhibits expression of some components of the Hcp secretion island-I-encoded T6SS (H1-T6SS) (7). The *tssA1* operon encodes structural components of the H1-T6SS and is subject to RsmA-mediated regulation at both the transcriptional and posttranscriptional level (7). To compare the effect of RsmA and RsmF on T6SS gene expression, *tssA1* transcriptional ($P_{tssA1\text{-}lacZ}$) and translational ($P_{tssA1'\text{-}'lacZ}$) reporters were integrated into the ΦCTX site. Compared with wild-type PA103, $P_{tssA1\text{-}lacZ}$ transcriptional reporter activity remained unaffected in the *rsmF* mutant, but was slightly derepressed in the *rsmA* mutant and significantly derepressed in an *rsmAF* mutant (13.5-fold) (*SI Appendix*, Fig. S4*B*). Similarly, translational reporter activity was indistinguishable in wild-type PA103 and the *rsmF* mutant, but significantly derepressed in the *rsmA* (7.5-fold) and *rsmAF* double mutant (72-fold) (*SI Appendix*, Fig. S4*C*). Complementation of the *rsmAF* mutant with either plasmid-encoded RsmA or RsmF restored repression of $P_{tssA1\text{-}lacZ}$ and $P_{tssA1'\text{-}'lacZ}$ reporter activities. The same general patterns were seen in strain PA14 (*SI Appendix*, Fig. S4 *D* and *E*). To verify that RsmA and RsmF both regulate TssA1 expression at the posttranscriptional level we constructed a second *tssA1* translational reporter under the transcriptional control of the constitutive P_{lacUV5} promoter ($P_{lacUV5\text{-}tssA1'\text{-}'lacZ}$). Deletion of *rsmA* resulted in modest, but significant translational depression (2.2-fold), whereas deletion of both *rsmA* and *rsmF* (*rsmAF*) had a much greater effect, resulting in 18.3-fold translational derpression of TssA1 (Fig. 2*C*).

Immunoblots of culture supernatant fluid confirmed that secretion of the T6SS effector proteins Hcp1 and Tse1 was similar in PA103 wild type and the *rsmF* mutant (Fig. 2*C*). By comparison, Hcp1 and Tse1 expression was severely derepressed in *rsmA* and *rsmAF* mutants, with substantially more accumulation of these proteins in the *rsmAF* mutant. Repression of Hcp1 and Tse1 production could be restored in the *rsmAF* mutant by providing either *rsmA* or *rsmF in trans*. In contrast to strain PA103, Hcp1 and Tse1 expression were only detected in the PA14 *rsmAF* mutant (*SI Appendix*, Fig. S4*A*). Taken together, these results demonstrate that deletion of both *rsmA* and *rsmF* significantly enhances phenotypes exhibited by the *rsmA* mutant alone.

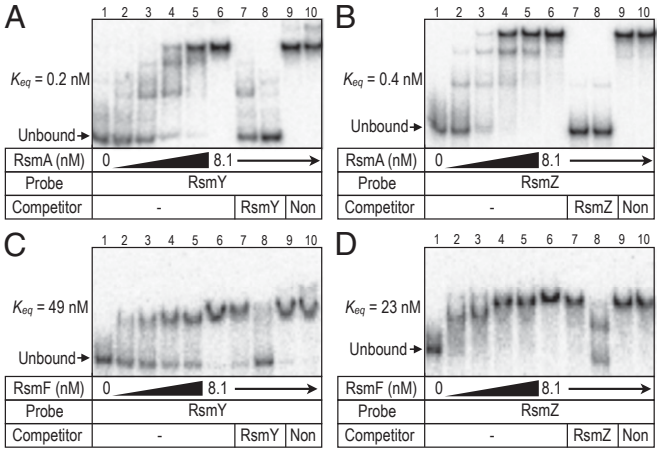

Fig. 3. Role of RsmY/Z in controlling RsmF activity. (*A–D*) Binding of $RsmA_{His}$ (*A* and *B*) and $RsmF_{His}$ (*C* and *D*) to the small noncoding RNAs RsmY (*A and C*) and RsmZ (*C and D*). Radiolabeled RNA (100 pmols) was incubated with $RsmA_{His}$ (0, 0.1, 0.3, 0.9, 2.7, and 8.1 nM) or $RsmF_{His}$ (0, 20, 40, 60, 80, and 100 nM) for 30 min at 37 °C and analyzed by native gel electrophoresis and phosphorimaging. Competition experiments were performed by including a 100- (lanes 7 and 9) or 1,000-fold (lanes 8 and 10) molar excess of unlabeled RsmY, RsmZ, or a nonspecific competitor RNA (Non) in the binding reaction as indicated. The positions of the unbound probes are marked with arrows.

RsmF Binds the Small Regulatory RNAs RsmY and RsmZ with Reduced Affinity and Stoichiometry Compared with RsmA. RsmA activity is controlled by two small regulatory RNAs (RsmY and RsmZ), which antagonize RsmA activity through direct binding. To determine whether RsmF is also regulated by RsmY/Z, C-terminal hexahistidine–tagged versions of RsmA and RsmF ($RsmA_{His}$ and $RsmF_{His}$) were individually expressed in *E. coli* and purified to homogeneity (*SI Appendix*, Fig. S5). RNA probes, corresponding to the full-length RsmY/Z transcripts were synthesized in vitro, radiolabeled, and incubated with purified $RsmA_{His}$ or $RsmF_{His}$ before electrophoresis on nondenaturing polyacrylamide gels (Fig. 3 *A–D*). Similar to previous reports (7, 24), RsmA formed high-affinity complexes with both RsmY/Z (Fig. 3 *A* and *B*). The apparent equilibrium constant (K_{eq}) for RsmA binding to RsmY and RsmZ was 0.2 nM and 0.4 nM, respectively. Compared with RsmA, the apparent K_{eq} for RsmF binding to RsmY and RsmZ was significantly reduced at 49 nM (245-fold lower) and 23 nM (58-fold lower), respectively (Fig. 3 *C* and *D*). Interestingly, the RsmA– and RsmF–RNA complexes exhibited different migration patterns. Previous reports found that RsmY and RsmZ can each sequester two to six copies of homodimeric RsmA (1, 24, 25). Consistent with those studies, RsmA binding to either RsmY or RsmZ exhibited a laddering pattern with at least three distinct shift products (Fig. 3 *A* and *B*). In contrast, the RsmF EMSAs showed one distinct shift product for both RsmY and RsmZ (Fig. 3 *C* and *D*), indicative of a single binding event. Competition experiments, performed to assess the specificity of RsmA and RsmF for RsmY/Z binding, indicated that unlabeled RsmY or RsmZ were efficient competitors for complex formation, whereas a nonspecific probe (Non) was unable to competitively inhibit binding (Fig. 3 *A–D*). These data demonstrate that RsmF binds RsmY/Z with high specificity but with reduced affinity and at a lower stoichiometric ratio than RsmA.

Despite the reduced affinity of RsmF for RsmY/Z in vitro, we hypothesized that these sRNAs may play a regulatory role in controlling RsmF activity in vivo. To test this hypothesis, we measured the activity of the $P_{exsD\text{-}lacZ}$ transcriptional and $P_{tssA1'\text{-}'lacZ}$ translational reporters in a triple mutant lacking *rsmA*, *rsmY*, and *rsmZ* (*rsmAYZ*). If free RsmY/Z were capable of inhibiting RsmF activity through titration, we predicted that *rsmYZ* deletion would result in increased free RsmF and a corresponding increase in $P_{exsD\text{-}lacZ}$ reporter activity and reduction in $P_{tssA1'\text{-}'lacZ}$ reporter activity relative to an *rsmA* mutant. There was, however, no significant change in $P_{exsD\text{-}lacZ}$ or $P_{tssA1'\text{-}'lacZ}$ reporter activities between

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the *rsmA* and the *rsmAYZ* mutants, suggesting that RsmY/Z play no major role in controlling RsmF activity in vivo (*SI Appendix*, Fig. S6 *A* and *B*).

RsmA Directly Binds the *rsmF* Transcript and Represses RsmF Translation. Given that RsmF phenotypes were only apparent in strains lacking *rsmA*, we hypothesized that *rsmF* transcription and/or translation is directly or indirectly controlled by RsmA. A transcriptional start site (TSS) was identified 155 nucleotides upstream of the *rsmF* translational start codon using 5′ RACE (*SI Appendix*, Fig. S1*B*). Examination of the 5′ UTR of *rsmF* revealed a putative RsmA-binding site (GCAAGGACGC) that closely matches the consensus ($^{A}/_{U}$CANGGANG$^{U}/_{A}$), including the core GGA motif (underlined) and overlaps the putative Shine–Dalgarno sequence (*SI Appendix*, Fig. S1*B*). The *rsmA* TSS was previously identified by mRNA-seq (26), which we confirmed by 5′ RACE. The 5′ UTR of *rsmA* also contains a putative RsmA-binding site, although it is a weaker match to the consensus (*SI Appendix*, Fig. S1*C*). Transcriptional and translational *lacZ* fusions for both *rsmA* and *rsmF* were integrated into the ΦCTX site. In general, deletion of *rsmA*, *rsmF*, or both genes had little impact on $P_{rsmA\text{-}lacZ}$ or $P_{rsmF\text{-}lacZ}$ transcriptional reporter activities in strains PA103 and PA14 (*SI Appendix*, Fig. S7 *A–D*). In contrast, the $P_{rsmA'\text{-}'lacZ}$ and $P_{rsmF'\text{-}'lacZ}$ translational reporters were both significantly repressed by RsmA (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S7 *E* and *F*). Deletion of *rsmF* alone or in combination with *rsmA* did not result in further derepression compared with either wild type or the *rsmA* mutants, respectively. To corroborate the above findings we also examined the effect of RsmZ overexpression on the $P_{rsmA'\text{-}'lacZ}$ and $P_{rsmF'\text{-}'lacZ}$ reporter activity. As expected, depletion of RsmA through RsmZ expression resulted in significant derepression of $P_{rsmA'\text{-}'lacZ}$ and $P_{rsmF'\text{-}'lacZ}$ reporter activity (Fig. 4*C*).

To determine whether RsmA directly binds *rsmA* and *rsmF* to affect translation, we conducted RNA EMSA experiments. $RsmA_{His}$ bound both the *rsmA* and *rsmF* probes with a K_{eq} of 68 nM and 55 nM, respectively (Fig. 4 *D* and *E*). Binding was specific, as it could not be competitively inhibited by the addition of excess nonspecific RNA. In contrast, $RsmF_{His}$ did not shift either the *rsmA* or *rsmF* probes (*SI Appendix*, Fig. S7 *G* and *H*). These results demonstrate that RsmA can directly repress its own translation as well as *rsmF* translation. The latter finding suggests that *rsmF* translation may be limited to conditions where RsmA activity is inhibited, thus providing a possible mechanistic explanation for why *rsmF* mutants have a limited phenotype in the presence of RsmA.

RsmA and RsmF Have Overlapping yet Distinct Regulons. The reduced affinity of RsmF for RsmY/Z suggested that RsmA and RsmF may have different target specificity. To test this idea, we compared $RsmA_{His}$ and $RsmF_{His}$ binding to additional RsmA targets. In particular, our phenotypic studies suggested that both RsmA and RsmF regulate targets associated with the T6SS and biofilm formation. Previous studies found that RsmA binds to the *tssA1* transcript encoding a H1-T6SS component (7) and to *pslA*, a gene involved in biofilm formation (18). $RsmA_{His}$ and $RsmF_{His}$ both bound the *tssA1* probe with high affinity and specificity, with apparent K_{eq} values of 0.6 nM and 4.0 nM, respectively (Fig. 5 *A* and *B*), indicating that purified $RsmF_{His}$ is functional and highly active. Direct binding of $RsmF_{His}$ to the *tssA1* probe is consistent with its role in regulating *tssA1* translation in vivo (Fig. 2*C*). In contrast to our findings with *tssA1*, only $RsmA_{His}$ bound the *pslA* probe with high affinity (K_{eq} of 2.7 nM) and high specificity, whereas RsmF did not bind the *pslA* probe at the highest concentrations tested (200 nM) (Fig. 5 *C* and *D* and *SI Appendix*, Fig. S8).

To determine whether RsmA and RsmF recognized the same binding site in the *tssA1* transcript, we conducted EMSA experiments using rabiolabeled RNA hairpins encompassing the previously identified *tssA1* RsmA-binding site (AUAGGGAGAT) (*SI Appendix*, Fig. S9*A*) (7). Both RsmA and RsmF were capable of shifting the probe (*SI Appendix*, Fig. S9 *B* and *C*) and RsmA showed a 5- to 10-fold greater affinity for the probe than RsmF, although the actual K_{eq} of the binding reactions could not be determined. Changing the central GGA trinucleotide to CCU in the loop region of the hairpin completely abrogated binding by both RsmA and RsmF, indicating that binding was sequence specific.

Key RNA-Interacting Residues of RsmA/CsrA Are Conserved in RsmF and Necessary for RsmF Activity in Vivo. The RNA-binding data and in vivo phenotypes suggest that RsmA and RsmF have similar yet distinct target specificities. Despite extensive rearrangement in the primary amino acid sequence, the RsmF homodimer has a fold similar to other CsrA/RsmA family members of known structure, suggesting a conserved mechanism for RNA recognition (*SI Appendix*, Fig. S10 *A* and *D*). Electrostatic potential mapping indicates that the $\beta1_a$ to $\beta5_a$ interface in RsmF is similar to the $\beta1_a$ to $\beta5_b$ interface in typical CsrA/RsmA family members, which serves as a positively charged RNA–protein interaction site (*SI Appendix*, Fig. S10 *B* and *E*) (4). Residue R44 of RsmA and other CsrA family members plays a key role in coordinating RNA binding (4, 13, 27, 28) and corresponds to RsmF R62,

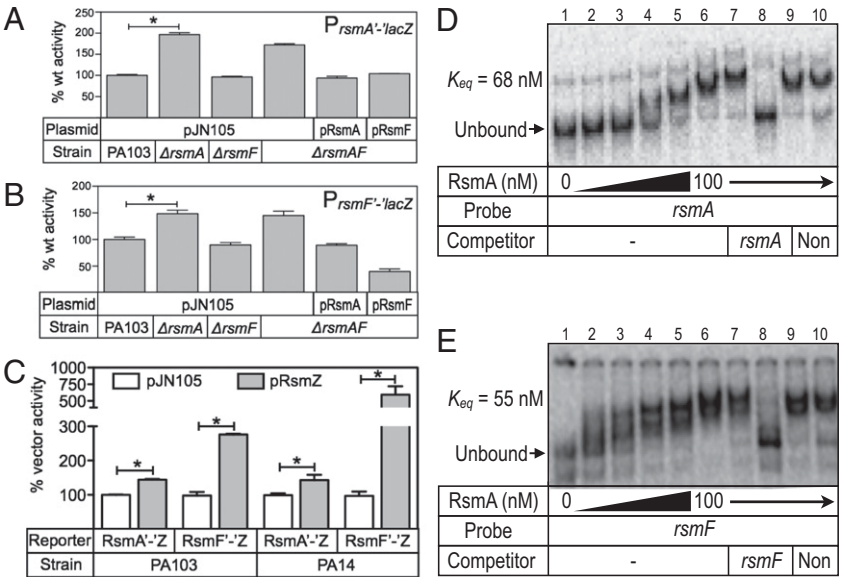

Fig. 4. RsmA inhibits in vivo translation of *rsmA* and *rsmF*. (*A* and *B*) The indicated PA103 strains carrying (*A*) $P_{rsmA'\text{-}'lacZ}$ or (*B*) $P_{rsmF'\text{-}'lacZ}$ translational reporters were cultured in the presence of 0.4% arabinose to induce RsmA or RsmF expression. Reported values are normalized to percent WT activity (set at 100%). *$P <$ 0.001. (*C*) Overexpression of RsmZ (pRsmZ) results in significant derepression of $P_{rsmA'\text{-}'lacZ}$ and $P_{rsmF'\text{-}'lacZ}$ translational reporters in both strains PA103 and PA14. (*D* and *E*) RsmA binding to the (*D*) *rsmA* and (*E*) *rsmF* RNA probes was examined as described in Fig. 3, using 0, 10, 20, 40, 60, and 100 nM $RsmA_{His}$. The competition reactions contained 100- (lanes 7 and 9) or 1,000-fold (lanes 8 and 10) molar excess of unlabeled *rsmA* or *rsmF* RNA or a nonspecific competitor RNA (Non). The position of the unbound probes is indicated with an arrow.

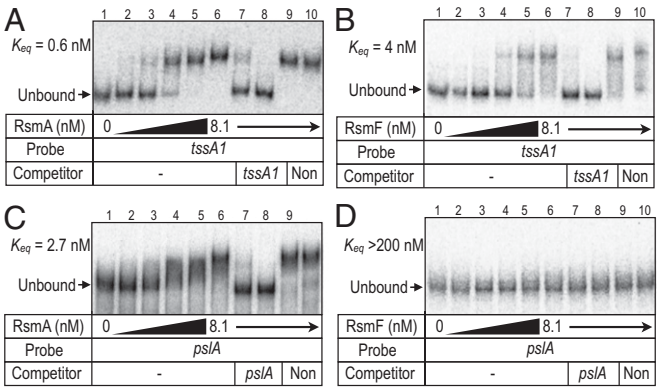

Fig. 5. Binding to the *tssA1* (*A* and *B*) and *pslA* (*C* and *D*) probes was examined as described in Fig. 3, using 0, 0.1, 0.3, 0.9, 2.7, and 8.1 nM $RsmA_{His}$ (*A* and *C*) or $RsmF_{His}$ (*B* and *D*) (lanes 1–6). The competition reactions contained 100- (lanes 7 and 9) or 1,000-fold (lanes 8 and 10) molar excess of unlabeled *tssA1* (*A* and *B*), or *pslA* (*C* and *D*) RNA, or a nonspecific competitor RNA (Non). The position of the unbound probes is indicated with an arrow.

located at the C-terminal end of β5 (Fig. 1*A*). The R44 side chain in RsmE (a representative CsrA/RsmA protein) from *Pseudomonas fluorescens* contacts the conserved GGA sequence and coordinates RNA–protein interaction (4). Modeling of the tertiary structure suggested that the R62 side chain in RsmF is positioned similarly to R44 in RsmA (*SI Appendix*, Fig. S10 *C* and *F*). To test the role of R44 in *P. aeruginosa* RsmA, and the equivalent residue in RsmF (R62), both were changed to alanine and the mutant proteins were assayed for their ability to repress $P_{tssA1'\text{-}'lacZ}$ reporter activity. When expressed from a plasmid in the PA103 *rsmAF* mutant, wild-type $RsmA_{His}$ and $RsmF_{His}$ reduced *tssA1* translational reporter activity 680- and 1,020-fold, respectively, compared with the vector control strain (Fig. 6). The R44A and R62A mutants, however, were unable to repress *tssA1* reporter activity. Immunoblots of whole cell extracts indicated that neither substitution affects protein stability (Fig. 6). The loss of function phenotype for RsmA–R44A is consistent with prior studies of RsmA, CsrA, and RsmE (4, 13, 27, 28). The fact that alteration of the equivalent residue in RsmF resulted in a similar loss of activity suggests that the RNA-binding region of RsmA and RsmF are conserved.

Discussion

CsrA/RsmA regulators integrate disparate signals into global responses and are common in pathogens requiring timely expression of virulence factors (2). In *P. aeruginosa*, RsmA assimilates sensory information and functions as a rheostat that permits a continuum of phenotypic responses (7, 8). In the current study, we describe RsmF as a structurally distinct RsmA homolog whose discovery adds another level of complexity to posttranscriptional regulation in *P. aeruginosa*. Although other *Pseudomonads* have two CsrA homologs, they function in a largely redundant manner. In *P. fluorescens* deletion of either *rsmA* or *rsmE* results in similar levels of derepression for regulatory targets, whereas deletion of both regulators has a synergistic effect (14). Our analyses of RsmA/F regulation, however, found that deletion of *rsmF* alone had little effect on T3SS and T6SS gene expression, or biofilm formation. A synergistic effect was observed in the *rsmAF* double mutant relative to the *rsmA* mutant. We attribute this to RsmA-mediated repression of *rsmF* translation, consistent with our findings that *rsmF* translation is derepressed in an *rsmA* strain, and that $RsmA_{His}$ binds to *rsmF* mRNA in vitro. RsmF translation, therefore, is indirectly influenced by the GacS/A signaling pathway, which controls RsmA activity through the RsmY/Z regulatory RNAs. This model predicts that RsmF is not a primary regulatory target of RsmY/Z, because RsmY/Z levels would be elevated under conditions in which RsmA is sequestered and RsmF is expressed. This hypothesis is supported by observations that $P_{exsD\text{-}lacZ}$ and $P_{tssA1'\text{-}'lacZ}$ reporter activities were unaltered between the *rsmA* and *rsmAYZ* mutants, and that RsmF-binding affinity to RsmY/Z was greatly reduced relative to RsmA. Whether RsmF is sequestered by an alternative regulatory RNA remains to be determined. The hierarchical organization of RsmA and RsmF is reminiscent of other cascades, such as the *P. aeruginosa* Las and Rhl quorum-sensing systems, which also serve to amplify and fine tune global gene expression patterns (29).

The profound derepression of *tssA1* translation observed in the *rsmAF* mutant relative to either single mutant results from loss of direct regulation by both RsmA and RsmF. Despite substantial differences in secondary structure, both proteins bound the *tssA1* RNA probe containing the predicted RsmA-binding motif, which was abrogated by mutation of the core GGA trinucleotide. Recognition of the consensus GGA is determined by hydrogen bonding of the main chain of residues in the loop between β4 and β5 as well as in β5 (4). This region is highly conserved across all known CsrA/RsmA family homologs, although the size of the loop in RsmF is two residues shorter (Fig. 1*A*). Thus, these regions of RsmF are likely involved in specific recognition of the consensus GGA as in typical RsmA/CsrA family members. Whereas RsmA bound both *tssA1* and *pslA* probes (containing predicted RsmA-bound hexaloops AGGGAG (*tssA1*) and AUGGAC (*pslA*), RsmF did not bind the *pslA* probe. Recent studies of RsmE binding to pentaloops demonstrated a G/A requirement at the position preceding the GGA core trinucleotide for strong binding (30). Interestingly the authors speculated that this preference might also relate to hexaloops, noting that the SELEX-derived CsrA consensus sequence indicated a G/A preference at this position for hexaloop configurations (31). Further studies of RsmF target preferences may reveal this to be a shared feature among RsmF targets.

The decreased binding affinity of RsmF to a subset of RsmA targets may result from variation among equivalent residues that coordinate RNA binding via side-chain interactions. Furthermore, because the α-helix "wings" of RsmA contribute to the formation of a positively charged RNA-binding pocket, the loss of these helices in RsmF likely contributes to the decreased affinity seen for the RsmA-binding targets tested in this work. Differential binding affinity and target specificity of RsmA and RsmF likely provide a mechanism for diversification of RsmA and RsmF responses.

Our results indicate that RsmF recognizes only a subset of RsmA-binding sites in vivo and in vitro (*tssA1* versus *rsmA/B* and *pslA*), suggesting that RsmA and RsmF may have overlapping and independent regulons. A perplexing outcome of our studies is the apparent discrepancy between the dramatic increase in biofilm formation observed in the *rsmAF* mutant, relative to the wild-type and *rsmA* strains, and the lack of in vitro binding of RsmF to the *pslA* transcript. We envision a few scenarios that could explain this inconsistency. RsmF binding in vivo may require

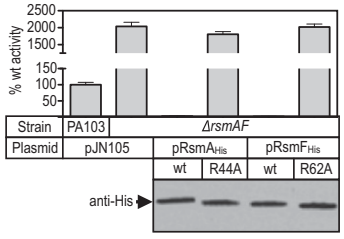

Fig. 6. The conserved arginine residue R62, located in the RNA-binding pocket of RsmF, is required for activity. Wild-type PA103 and the indicated mutants carrying the $P_{tssA'\text{-}'lacZ}$ translational reporter were transformed with either a vector control (pJN105) or the indicated $RsmA_{His}$ and $RsmF_{His}$ expression plasmids and assayed for β-galactosidase activity. The reported values are in Miller units normalized to percent WT activity (set at 100%). Whole-cell extracts were blotted for $RsmA_{His}$ and $RsmF_{His}$ expression using anti-hexahistidine antibody.

additional factors such as a regulatory RNA or accessory binding proteins such as Hfq (24). Alternatively, the effect on biofilm formation may occur indirectly through RsmF regulation of an intermediate factor or through the control of other determinants of biofilm formation, such as flagella.

The benefit of having dual posttranscriptional regulators is not entirely clear. Although we demonstrate that RsmA has both distinct and overlapping targets with RsmF, it remains to be determined whether RsmF recognizes targets distinct from the RsmA regulon. An interesting possibility is that each regulon is partitioned to create target subsets that are: (*i*) positively or negatively regulated by RsmA or RsmF or (*ii*) convergently or divergently positively or negatively regulated by RsmA and RsmF. Such a multitiered system would be an advantageous means of generating complex posttranscriptional response programs for a multitude of circumstances.

Materials and Methods

Bacterial Strains and Growth Conditions. Strains used in this study are listed in *SI Appendix*, Table S2. *E. coli* strains DH5α or GS162 were used for routine cloning, SM10 for conjugation, and Tuner (DE3) for protein expression. *E. coli* was grown on LB-Lennox (LB) agar or broth with antibiotics as required. *P. aeruginosa* strains PA103 and PA14 were maintained on Vogel–Bonner minimal (VBM) medium (32) or LB-agar plates with antibiotics as required.

Genetic Methods and Rapid Amplification of cDNA Ends. Plasmids, primers, and construction details are provided in *SI Appendix*, Tables S2–S4. Transcriptional start site mapping was performed with the primers listed in *SI Appendix*, Table S4 by 5′ RACE as previously described (33). cDNA was poly-T tailed and used as the template in a PCR with a poly-A primer and a nested gene-specific primer. PCR products were sequenced to identify start sites.

β-Galactosidase Assays. PA103 strains grown overnight at 37 °C on VBM agar plates were used to make cell suspensions. PA14 strains were grown overnight at 37 °C in LB. Cells were diluted at an absorbance (A_{600}) of 0.1 in trypticase soy broth supplemented with 100 mM monosodium glutamate, 1% glycerol, and 1 mM ethylene glycol tetraacetic acid (EGTA) as indicated. For PA103 complementation experiments, 0.4% arabinose was added to induce RsmA or RsmF expression. Strains were grown at 30 °C to an A_{600} of 1.0 and β-galactosidase activity was determined as previously described (23). β-Galactosidase activities reported in this study are averages of three or more independent experiments and error bars correspond to SEM. Student two-tailed unpaired *t* tests were performed using Prism 5 GraphPad.

Note. During manuscript preparation, a thesis describing identification of RsmF (designated RsmN) was published online (34).

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