RsmV, a Small Noncoding Regulatory RNA in *Pseudomonas aeruginosa* That Sequesters RsmA and RsmF from Target mRNAs

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ABSTRACT The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* has distinct genetic programs that favor either acute or chronic virulence gene expression. Acute virulence is associated with twitching and swimming motility, expression of a type III secretion system (T3SS), and the absence of alginate, Psl, or Pel polysaccharide production. Traits associated with chronic infection include growth as a biofilm, reduced motility, and expression of a type VI secretion system (T6SS). The Rsm posttranscriptional regulatory system plays important roles in the inverse control of phenotypes associated with acute and chronic virulence. RsmA and RsmF are RNA-binding proteins that interact with target mRNAs to control gene expression at the posttranscriptional level. Previous work found that RsmA activity is controlled by at least three small, noncoding regulatory RNAs (RsmW, RsmY, and RsmZ). In this study, we took an *in silico* approach to identify additional small RNAs (sRNAs) that might function in the sequestration of RsmA and/or RsmF (RsmA/RsmF) and identified RsmV, a 192-nucleotide (nt) transcript with four predicted RsmA/RsmF consensus binding sites. RsmV is capable of sequestering RsmA and RsmF *in vivo* to activate translation of *tssA1*, a component of the T6SS, and to inhibit T3SS gene expression. Each of the predicted RsmA/RsmF consensus binding sites contributes to RsmV activity. Electrophoretic mobility shifts assays show that RsmF binds RsmV with >10-fold higher affinity than RsmY and RsmZ. Gene expression studies revealed that the temporal expression pattern of RsmV differs from those of RsmW, RsmY, and RsmZ. These findings suggest that each sRNA may play a distinct role in controlling RsmA and RsmF activity.

IMPORTANCE The members of the CsrA/RsmA family of RNA-binding proteins play important roles in posttranscriptional control of gene expression. The activity of CsrA/RsmA proteins is controlled by small noncoding RNAs that function as decoys to sequester CsrA/RsmA from target mRNAs. *Pseudomonas aeruginosa* has two CsrA family proteins (RsmA and RsmF) and at least four sequestering sRNAs (RsmV [identified in this study], RsmW, RsmY, and RsmZ) that control RsmA/RsmF activity. RsmY and RsmZ are the primary sRNAs that sequester RsmA/RsmF, and RsmV and RsmW appear to play smaller roles. Differences in the temporal and absolute expression levels of the sRNAs and in their binding affinities for RsmA/RsmF may provide a mechanism of fine-tuning the output of the Rsm system in response to environmental cues.

KEYWORDS *Pseudomonas aeruginosa*, RsmA, RsmF, RsmV, RsmW, RsmY, RsmZ, type III secretion, type VI secretion

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Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that can cause acute infections in the immunocompromised and chronic infections in individuals with cystic fibrosis (CF) (1, 2). Acute *P. aeruginosa* maladies include skin and soft tissue infections, ventilator-associated pneumonia (VAP), and urinary tract infections. *P. aeruginosa* bacteria isolated from acute infections are typically motile, nonmucoid, and toxigenic. Acute infections by multidrug-resistant *P. aeruginosa* are difficult to resolve and can progress to sepsis resulting in a high rate of morbidity and mortality (3). Chronic *P. aeruginosa* infections are most common in CF patients and result from a variety of mutations in the CF transmembrane conductance regulator (CFTR) ion channel. The clearance defect in CF results from dehydrated and thickened mucus and from physiochemical changes in the airway surface fluid (4). The persistence of *P. aeruginosa* in the CF airways is associated with adaptive changes, including loss of motility, growth as a biofilm, mucoidy, and loss of some acute virulence functions (5, 6). The coordinate transition from an acute to a chronic infection phenotype is regulated by a variety of global regulatory networks, including the Rsm system (7).

The Rsm system controls ~10% of the *P. aeruginosa* genome, including type III secretion (T3S) and type VI secretion (T6S), exopolysaccharides important for biofilm formation, and motility (8, 9). The Rsm system includes two small RNA-binding proteins (RsmA and RsmF/RsmN [RsmA and RsmF]) and at least three small noncoding RNAs (RsmW, RsmY, and RsmZ) that function by sequestering RsmA and RsmF from mRNA targets. RsmA and RsmF are part of the CsrA family and regulate gene expression at the posttranscriptional level. RsmA and RsmF are 31% identical at the amino acid level, and both rely on a conserved arginine residue for RNA-binding activity (10, 11). RsmA and RsmF directly interact with mRNA targets to positively or negatively alter translation efficiency and/or mRNA stability (8, 12, 13). The RsmA and RsmF binding sites on target mRNAs commonly overlap the ribosome binding site and consist of a conserved 5′-CANGGAYG sequence motif (where N is any nucleotide, the underlined GGA is 100% conserved, and Y is either a cytosine or uracil) that presents the GGA sequence in the loop portion of a stem-loop structure (10, 14–16). While RsmA is able to bind mRNA targets with a single CANGGAYG sequence (14), RsmF differs in that high-affinity binding is observed with mRNA targets possessing at least two CANGGAYG consensus binding sites (14). Although RsmA and RsmF have some targets in common, the full extent of the regulons is unknown (10, 11). The RsmF regulon, however, was recently determined using a pulldown method to identify 503 target RNAs (17).

The RNA-binding activity of RsmA is controlled by the small noncoding RNAs RsmW, RsmY, and RsmZ (10, 18). RsmW, RsmY, and RsmZ each have multiple CANGGAYG binding sites that allow sequestration of RsmA from target mRNAs (19). It is unclear whether RsmW, RsmY, and RsmZ are the only small RNAs (sRNAs) that function in the sequestration of RsmA or whether RsmW, RsmY, or RsmZ are the primary sRNAs that function in sequestration of RsmF. The affinity of RsmF for RsmY and RsmZ is 10-fold lower than that of RsmA. In this study, we sought to identify additional sRNAs that regulate RsmA and RsmF activity and identified RsmV, a 192-nucleotide (nt) transcript that has four CANGGAYG sequences presented in stem-loop structures. We demonstrate that RsmV is able to sequester RsmA and RsmF *in vivo*, that full RsmV activity is dependent upon each of the four CANGGAYG sequences, and that RsmV demonstrates a temporal expression pattern that is distinct from those seen with RsmW, RsmY, and RsmZ. We propose a model wherein each sRNA plays differential and distinct roles in control of the Rsm system.

RESULTS

Identification of RsmV as a sequestering RNA for RsmA and RsmF. We took an *in silico* approach to identify candidate sRNAs that might control RsmA activity and/or RsmF activity *in vivo*. A prior SELEX (systematic evolution of ligands) study concluded that optimal RNA-binding activity by RsmF requires RNA targets with at least two GGA sequences presented in the loop portion of stem-loop structures. Transcriptome sequencing (RNA-seq) studies have identified ~500 potential sRNAs

in *P. aeruginosa* (20, 21). The secondary structure of each sRNA was predicted using mFold and then examined for the presence of ≥2 GGA sequences presented in stem-loop structures. One sRNA candidate had six GGA sequences (Fig. 1A). Each GGA sequence demonstrated a ≥60% match to the full RsmA/RsmF consensus binding site (CAnGGAyG) (Fig. 1B). Four of the GGA sequences (designated sites 2, 3, 5, and 6) are predicted by mFold to be presented in stem-loop structures (Fig. 1A). The gene encoding the sRNA is located in the intergenic region between *mucE* and *apqZ* and has been designated *rsmV* (Fig. 1C). A search of the Pseudomonas Genome Database indicated that the *rsmV* sequence is highly conserved in >100 sequenced *P. aeruginosa* genomes and is absent from the genomes of other pseudomonads.

The RNA-seq study that identified *rsmV* concluded that the RNA is 192 nucleotides (nt) long (21). To verify the *rsmV* transcription start site, cDNA was generated using a primer within the gene (Fig. 1D). The cDNA was then used in a PCR with primers positioned just upstream of and at the predicted start site. Whereas the primer positioned at the start site generated the expected product, the primer located just upstream did not generate a product. This finding is consistent with the *rsmV* transcription start site identified in the Wurtzel RNA-seq study (21). There is no identifiable transcriptional terminator downstream of *rsmV*. To verify the 3′ boundary, cDNA was generated from total cellular RNA with primers positioned at the predicted 3′ end of *rsmV* and at several downstream positions as shown in Fig. 1C. The resulting cDNAs were then used as templates in PCRs with primers (Fprimer and Rprimer) positioned within the gene (Fig. 1C). The cDNA primer positioned within the *aqpZ* coding region (primer 4) did not yield a product. The cDNA primers positioned upstream of *aqpZ* (primers 1, 2, and 3) all yielded products, and the strongest product was observed with cDNA generated at the predicted 3′ terminus of *rsmV* using primer 2 (Fig. 1E). The weaker PCR products produced from cDNA generated with primers 2 and 3 may represent transcriptional readthrough.

RsmV interacts with and controls RsmA and RsmF activity. The presence of four predicted GGA sequences in stem-loop structures is consistent with RsmV serving as a sequestering sRNA for RsmA and/or RsmF. To test this prediction, we measured binding using electrophoretic mobility shift assays (EMSAs). Full-length RsmV was synthesized *in vitro*, radiolabeled at the 5′ end, and incubated with purified $RsmA_{His}$ or $RsmF_{His}$ prior to electrophoresis on nondenaturing gels. $RsmA_{His}$ formed high-affinity binding products with RsmV (equilibrium constant [K_{eq}] = 14 nM), and two distinct binding complexes were evident (Fig. 2A). Those products could reflect binding of multiple $RsmA_{His}$ dimers or differential interactions with multiple sites on the RsmV probe. RsmF also bound the RsmV probe with high affinity (K_{eq} = 2 nM), but only a single binding complex was detected (Fig. 2A).

With evidence indicating that both RsmA and RsmF interact with RsmV, we next examined whether RsmV can sequester RsmA/RsmF *in vivo*. RsmA and RsmF have inverse effects on expression of the type VI secretion system (T6SS) and the type III secretion system (T3SS) (10). We used the previously described $P_{lac\text{-}tssA1'\text{-}'lacZ}$ translational reporter as a surrogate for regulatory control of the T6SS (10) and the $P_{exsD\text{-}lacZ}$ transcriptional reporter as a marker for the T3SS (22). RsmA/RsmF directly bind the *tssA1* leader region to inhibit translation (10, 14) and positively regulate T3SS gene expression through a mechanism that remains to be defined (10). In a mutant lacking *rsmV*, *rsmY*, and *rsmZ*, RsmA/RsmF availability is high, resulting in repression of $P_{lac\text{-}tssA1'\text{-}'lacZ}$ reporter activity and high levels of $P_{exsD\text{-}lacZ}$ reporter activity (Fig. 2B and C). The presence of plasmid-expressed RsmV resulted in significant activation of $P_{lac\text{-}tssA1'\text{-}'lacZ}$ reporter activity and inhibition of the $P_{exsD\text{-}lacZ}$ reporter. Both of these findings are consistent with RsmV serving a role in RsmA/RsmF sequestration. Compared to the previously identified sequestering RNAs RsmW, RsmY, and RsmZ (10, 23), RsmV demonstrated activity comparable to RsmY in experiments using the $P_{lac\text{-}tssA1'\text{-}'lacZ}$ reporter

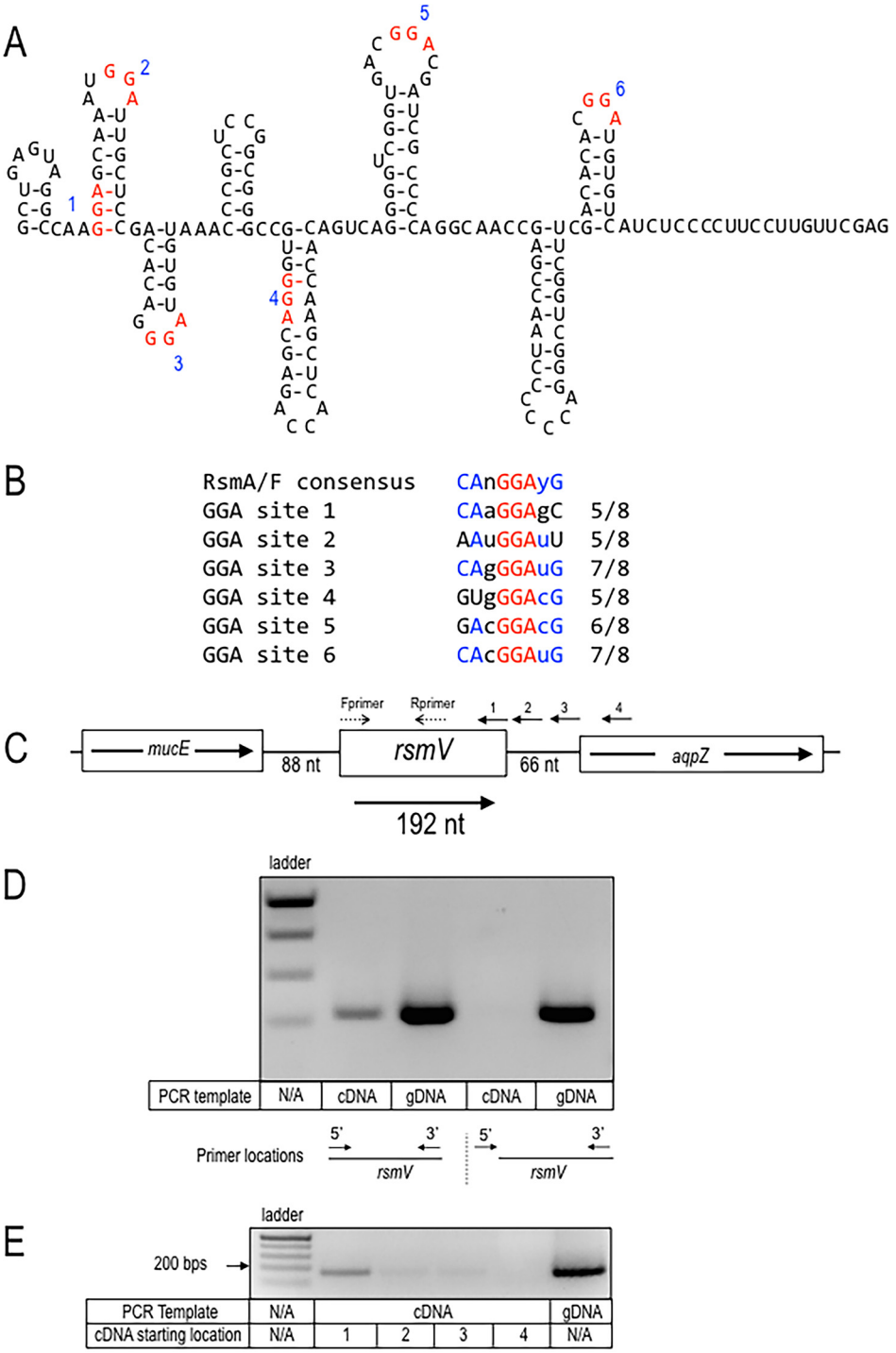

FIG 1 Predicted structure of RsmV and genomic context. (A) Predicted mFold structure of RsmV. The *P. aeruginosa* RsmV secondary structure was determined by mFold modeling. Each of the six GGA sequences is highlighted in red and numbered by order of appearance from the 5′ end of the sequence. (B) Alignment of each GGA site to the full RsmA/RsmF consensus binding site. The GGA sites are 100% conserved (red), and other conserved portions of the consensus sequence are highlighted in blue. (C) The genome context of *rsmV*, located between *mucE* and *aqpZ*. The positions of the primers used to generate cDNA for the experiment represented in Fig. 2B are labeled 1 to 4. The primers used to generate PCR products in Fig. 2B are labeled Fprimer and Rprimer. (D and E) Verification of the *rsmV* 5′ and 3′ boundaries. (D) RNA purified from wt cells was used to generate cDNA using the indicated 3′ primer. The cDNA was then used in PCRs with the same 3′ primer and 5′ primers positioned just upstream of or at the predicted start of *rsmV* transcription. Genomic DNA (gDNA) served as a positive control. N/A, not applicable. (E) Verification of the *rsmV* termination site. cDNA was generated using primers 1 to 4 as shown in panel C. The cDNA was then used in PCRs with the primer sets indicated in panel C. Genomic DNA (gDNA) served as a positive control.

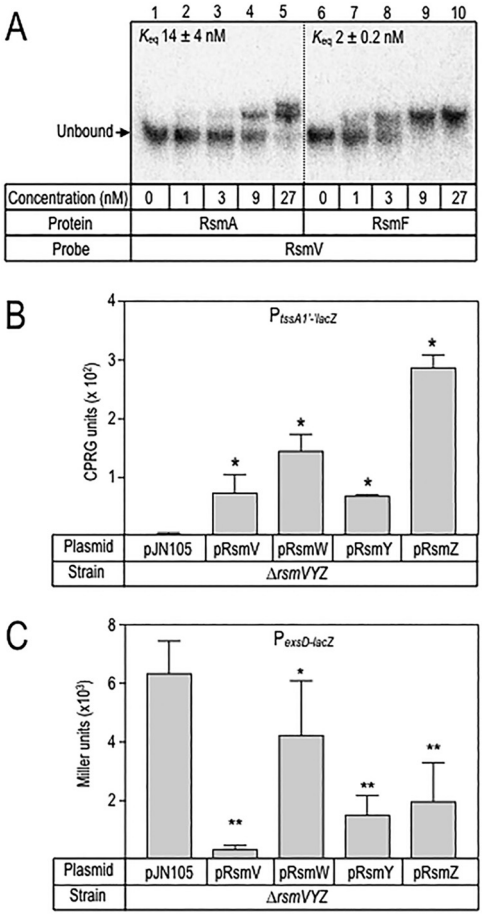

FIG 2 RsmV binding and regulatory activity. (A) RsmV was radiolabeled and used in electrophoretic mobility shift assays with purified RsmA (lanes 2 to 5) and RsmF (lanes 7 to 10) at the indicated concentrations. The position of the unbound RsmV probe is indicated. (B and C) Effect of RsmV on $P_{lac\text{-}tssA1'\text{-}'lacZ}$ ($P_{tssA1'\text{-}'lacZ}$) translational reporter (B) and $P_{exsD\text{-}lacZ}$ transcriptional reporter (C) activities. Strains consisting of a Δ*rsmVYZ* mutant transformed with either a vector control (pJN105) or the indicated sRNA expression plasmids were cultured in the presence of 0.4% arabinose to induce expression of the respective RNAs and were assayed for β-galactosidase activity. The reported values represent the averages of results from at least three experiments, with the standard error indicated. *, *P* value of <0.05; **, *P* value of 0.01 (relative to the vector control data).

(Fig. 2B) and had strong inhibitory activity with respect to the $P_{exsD\text{-}lacZ}$ reporter (Fig. 2C).

To determine whether RsmV preferentially sequesters either RsmA or RsmF, the $P_{lac\text{-}tssA1'\text{-}'lacZ}$ translational reporter was introduced into Δ*rsmAVYZ* and Δ*rsmFVYZ* mutant backgrounds. RsmA is more active than RsmF, resulting in stronger repression of $P_{lac\text{-}tssA1'\text{-}'lacZ}$ reporter activity in the Δ*rsmFVYZ* mutant than in the Δ*rsmAVYZ* background for strains carrying the vector control (pJN105) (Fig. 3A versus B). In the Δ*rsmFVYZ* mutant, where repression of $P_{lac\text{-}tssA1'\text{-}'lacZ}$ activity is attributable to RsmA, RsmV demonstrated relatively weak suppressive activity compared to RsmW, RsmY, and RsmZ (Fig. 3A). A similar picture emerged in experiments using the Δ*rsmAVYZ* background to examine RsmF sequestration in that RsmV demonstrated the weakest suppressive activity (Fig. 3B). Thus, RsmV is capable of sequestering both RsmA and RsmF but appears to lack a strong preference for one versus the other under the conditions tested.

Contribution of GGA sites 2, 3, 5, and 6 to RsmV activity. The RsmV primary sequence contains six GGA sequences, four of which (GGA2, GGA3, GGA5, and GGA6) may be presented in the loop portions of stem-loop structures (Fig. 1A). To determine which GGA sites are important for RsmV regulatory activity, each of the GGA sequences in stem-loop structures was changed to CCU. mFold predictions of the single GGA

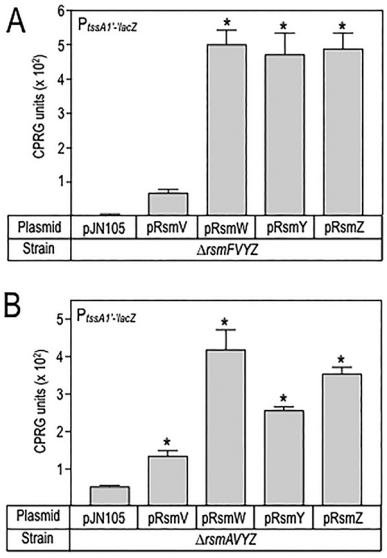

FIG 3 Sequestration of RsmA or RsmF by the RsmV, RsmW, RsmY, and RsmZ regulatory RNAs. Either Δ*rsmAVYZ* (A) or Δ*rsmFVYZ* (B) quadruple mutants carrying the $P_{lac\text{-}tssA1'\text{-}'lacZ}$ ($P_{tssA1'\text{-}'lacZ}$) translational reporter were transformed with either a vector control (pJN105) or RsmV, RsmW, RsmY, and RsmZ expression plasmids. The resulting strains were cultured in the presence of 0.4% arabinose to induce expression of the respective RNAs and assayed for β-galactosidase activity. Reported values represent averages of results from at least three experiments, with the standard error indicated. *, *P* value of <0.05 relative to the vector control.

mutants indicated that each is expected to adopt the same secondary structure shown in Fig. 1A for wild-type (wt) RsmV. The activity of each mutant RNA was tested using the $P_{lac\text{-}tssA1'\text{-}'lacZ}$ translational and $P_{exsD\text{-}lacZ}$ transcriptional reporters. The GGA3 and GGA6 mutant RNAs demonstrated a significant loss of regulatory activity for the $P_{lac\text{-}tssA1'\text{-}'lacZ}$ translational reporter compared to wt RsmV (Fig. 4A). In contrast, each of the GGA sites was required for full regulatory control of the $P_{exsD\text{-}lacZ}$ transcriptional reporter (Fig. 4B). Differences in the activities of the mutant RNAs were not attributable to altered RNA stability as determined by quantitative reverse transcription-PCR (qRT-PCR) (Fig. 4C). The most likely explanation for the differential requirement for the GGA2 and GGA5 sites is that the $P_{exsD\text{-}lacZ}$ reporter is more sensitive to changes in RsmA availability than the $P_{lac\text{-}tssA1'\text{-}'lacZ}$ reporter.

The simplest interpretation of the reporter findings is that the mutant RNAs with altered activity have a reduced capacity to sequester RsmA/RsmF. To test this prediction, binding assays were performed with radiolabeled RNA probes. RsmA bound each of the single GGA substitution mutants with affinities similar to or greater than those seen with wt RsmV (Fig. 4D and Table 1) (see also Fig. S1 in the supplemental material). We consistently observed two distinct products upon RsmA binding to the wt, GGA2, and GGA5 probes. Only a single product was observed for the GGA3 and GGA6 probes. This is noteworthy, as the mutant GGA3 and GGA6 RNAs also demonstrated a defect in activation of the $P_{lac\text{-}tssA1'\text{-}'lacZ}$ translational reporter (Fig. 4A). RsmF also bound each of the mutant probes with high affinity, with the exception of GGA6, where binding affinity was significantly reduced (Fig. 4E and Table 1; see also Fig. S1). Given that RsmA and RsmF are homodimers with two RNA binding sites (one from each monomer) and that each mutant RNA still has three potential GGA interaction sites, high-affinity binding to the mutant probes was not unexpected. We thus generated a probe bearing CCU substitutions at all four sites (Quad) and found that RsmA (K_{eq}, >27 nM) and RsmF (K_{eq}, >243 nM) were unable to bind (Fig. 4D and E and Table 1; see also Fig. S1) or exert regulatory control over the $P_{lac\text{-}tssA1'\text{-}'lacZ}$ translational and $P_{exsD\text{-}lacZ}$ transcriptional reporters. We conclude that the primary sites for RsmA/RsmF binding are GGA2, GGA3, GGA5, and GGA6.

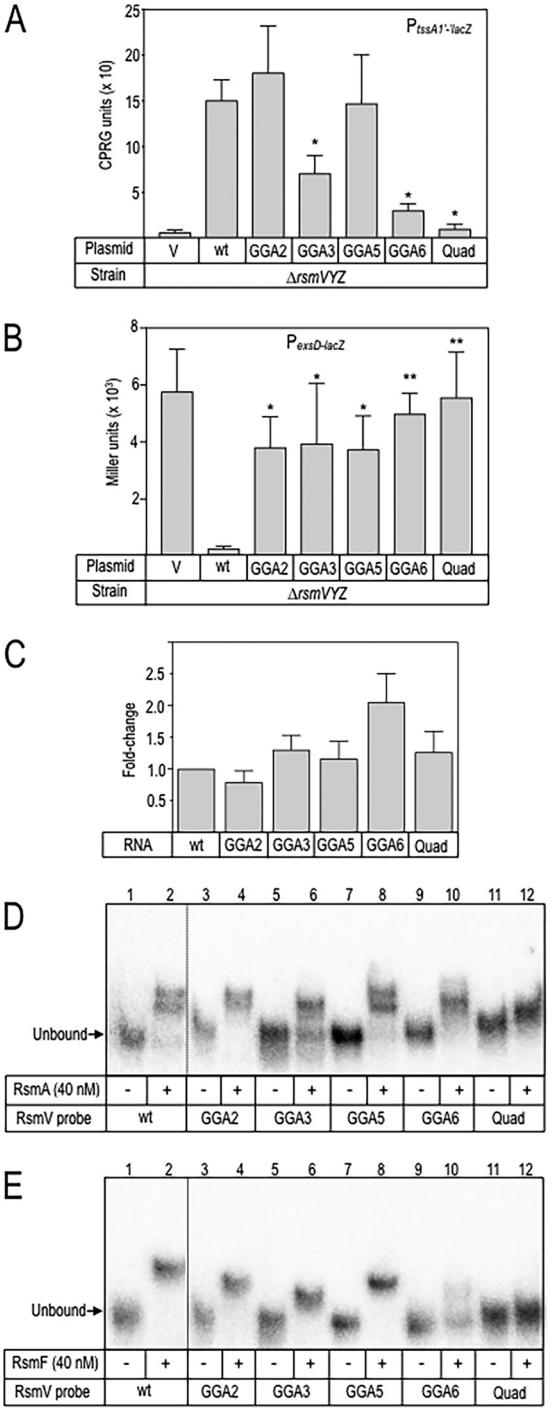

FIG 4 Functional analyses of the RsmV mutants. (A and B) The PA103 Δ*rsmVYZ* mutant carrying the $P_{lac\text{-}tssA1'\text{-}'lacZ}$ ($P_{tssA1'\text{-}'lacZ}$) translational reporter (A) or $P_{exsD\text{-}lacZ}$ transcriptional reporter (B) was transformed with either a vector control (V) (pJN105) or the indicated RsmV expression plasmids. The resulting strains were cultured in the presence of 0.4% arabinose to induce expression of the respective RNAs and assayed for β-galactosidase activity. Reported values represent averages of results from at least three experiments, with the standard error indicated. *, *P* value of <0.05 relative to cells expressing wt RsmV. (C) RNA levels of native RsmV and the indicated GGA mutants were determined by qRT-PCR. Each RNA sample was normalized to *rimM*. The reported values represent averages of results from three experiments. (D and E) EMSAs with wt RsmV and the indicated mutant radiolabeled probes. A 40 nM concentration of RsmA (D) or RsmF (E) was incubated with the indicated probes, and the reaction mixture was subjected to nondenaturing gel electrophoresis and phosphorimaging. The positions of the unbound probes are indicated.

TABLE 1 RsmA and RsmF affinities for wt and mutant RsmV

RNA	Affinity (nM)	
	RsmA	RsmF
wt RsmV	14 ± 4[a]	2 ± 0.2
GGA2	3 ± 3	1 ± 0.6
GGA3	1 ± 0.4	5 ± 3
GGA5	3 ± 3	8 ± 13
GGA6	9 ± 11	>243
Quad	>27	>243

[a]The indicated value represents the apparent equilibrium binding constant.

Role of RsmV *in vivo*. Data presented thus far have relied upon plasmid-expressed RsmV, the expression of which may result in RNA levels that exceed the native level expressed by cells under physiologically relevant conditions. To address the effect of RsmV expressed at native levels on the output of the Rsm system, we generated an in-frame *rsmV* deletion mutant (Δ*rsmV*) and measured $P_{exsD\text{-}lacZ}$ reporter activity. Compared to wt cells, the Δ*rsmV* mutant cells demonstrated a modest but significant increase in reporter activity (Fig. 5A). This increase in reporter activity is consistent with reduced sequestration of RsmA and RsmF, both of which have a positive effect on T3SS gene expression. By comparison, $P_{exsD\text{-}lacZ}$ reporter activity was also elevated in an Δ*rsmYZ* double mutant.

A second approach to test the relevance of RsmV *in vivo* involved precipitation experiments with histidine-tagged RsmA or RsmF. A Δ*rsmAF* double mutant transformed with either $RsmA_{His}$ or $RsmF_{His}$ expression plasmids was cultured to mid-log phase and then rapidly subjected to precipitation with Ni^{2+}-agarose beads and isolation of bound RNA. The presence of specific RNAs was detected from the entire pool of bound RNAs by qRT-PCR. The positive controls were the known RsmA/RsmF targets RsmY and RsmZ and the *tssA1* leader region (10). Negative controls included (i) two mRNAs (*lolB* and *rnpB*) that are not known targets of RsmA or RsmF and (ii) Ni^{2+}-agarose beads alone. Whereas no enrichment of the *lolB* or *rnpB* mRNAs was detected,

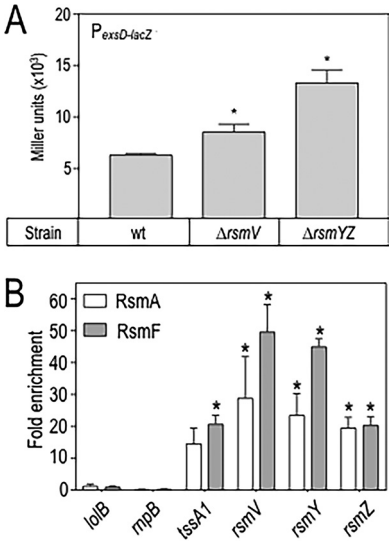

FIG 5 *In vivo* activity of RsmV. (A) Strain PA103 (wt) and the Δ*rsmV* and Δ*rsmYZ* mutants carrying the $P_{exsD\text{-}lacZ}$ transcriptional reporter were cultured under inducing conditions for T3SS gene expression and assayed for β-galactosidase activity. *, *P* value of <0.05 relative to wt. (B) A Δ*rsmAF* mutant transformed with either a vector control or $pRsmA_{His}$ or $pRsmF_{His}$ expression vectors was cultured and subjected to rapid purification of $pRsmA_{His}$ or $pRsmF_{His}$ and bound RNAs. Select RNAs (as indicated) were quantified from the purified RNA pool by qRT-PCR and are reported as fold change relative to the vector control. The coding sequences for *lolB* and *rnpB* were included as negative controls, and *tssA1* served as a positive control. Reported values represent averages of results from at least three replicates, with the standard error reported. *, *P* value of < 0.05 (compared to expression of the wild-type vector).

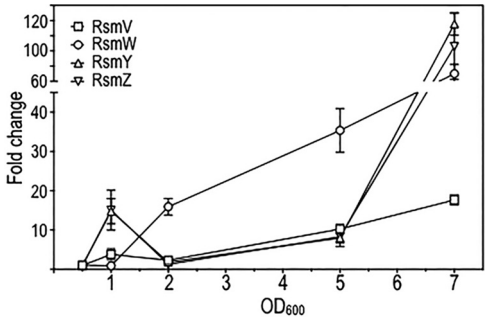

FIG 6 Expression profiles of RsmV, RsmW, RsmY, and RsmZ shown by a growth curve analysis. RNA was isolated from wt cells harvested at the indicated A_{600} readings and used as the template in RT-qPCR experiments with primers specific to *rsmV*, *rsmW*, *rsmY*, and *rsmZ*. Each RNA sample was normalized to *rimM*. The values for each RNA are reported relative to the measurement of the sample collected at an A_{600} of 0.5. The data represent averages of results from at least three replicates.

there was significant enrichment of the *tssA1* mRNA and of the RsmV, RsmY, and RsmZ sRNAs by both RsmA and RsmF (Fig. 5B).

Differential expression of RsmV, RsmW, RsmY, and RsmZ. The *in vivo* data demonstrate that RsmV, RsmW, RsmY, and RsmZ are each capable of sequestering RsmA and RsmF. We hypothesized that differential expression of the RNAs might allow cells to fine-tune the output of the Rsm system. To test for differential expression, RNA samples were collected from cells cultured at optical densities at 600 nm (OD_{600}) of 0.5 (mid-log phase), 1.0 (late log phase), and 2.0, 5.0, and 7.0 (late stationary phase) and readings were performed. The amount of each RNA detected by qRT-PCR at each growth phase was normalized to cells harvested at an OD_{600} of 0.5, and the values for each subsequent time point are reported relative to those values (Fig. 6). Both RsmY and RsmZ showed a transient increase in expression at late-log phase, followed by a decrease at OD_{600} of 2.0 and 5.0, and then a significant increase in late stationary phase (OD_{600} of 7.0). Expression for RsmW was delayed until the OD_{600} reached 2.0, but the highest fold changes in expression were demonstrated at OD_{600} values of 2.0 and 5.0 and then approached the fold changes observed for RsmY and RsmZ at an OD_{600} of 7.0. In contrast, RsmV demonstrated a slow but steady increase in expression throughout the growth curve but was the least dynamic of the four RNAs. The observed differences in expression patterns are consistent with the hypothesis that the sRNAs may serve distinct roles in RsmA/RsmF sequestration on the basis of their timing of expression.

One mechanism to account for the differential expression of RsmV, RsmW, RsmY, and RsmZ is transcriptional control by distinct transcription factors. Transcription of *rsmY* and *rsmZ* is controlled by the GacAS two-component system (24). A previous study found that GacAS does not control *rsmW* transcription (23). To determine whether *rsmV* transcription is regulated by GacA, a $P_{rsmV\text{-}lacZ}$ transcriptional reporter was integrated at the CTX phage attachment site of wt cells and a Δ*gacA* mutant. Whereas $P_{rsmY\text{-}lacZ}$ and $P_{rsmZ\text{-}lacZ}$ reporter activity demonstrated strong *gacA* dependence, $P_{rsmV\text{-}lacZ}$ activity showed no difference between the wild-type strain and the *gacA* mutant (Fig. S2).

DISCUSSION

The primary RsmA/RsmF-sequestering RNAs in *Pseudomonas aeruginosa* are RsmY and RsmZ. In addition to RsmY/RsmZ, RsmW plays a smaller role in the sequestration of RsmA (23) and can also sequester RsmF (Fig. 3). RsmV represents a fourth RsmA/RsmF-sequestering RNA in *P. aeruginosa*. RsmV shares sequence and structural characteristics with RsmY and RsmZ, including multiple GGA motifs (6), four of which are likely presented in stem-loop structures. RsmY and RsmZ are also the primary sequestering RNAs in *P. fluorescens* (now *P. protegens*) (18). At least one additional sRNA, RsmX, also contributes to Rsm control in *P. protegens* (25). The involvement of multiple seques-

tering sRNAs in the control of CsrA/RsmA activity is common. CsrB is the primary CsrA-sequestering RNA in *Escherichia coli* and contains 18 GGA motifs (26). Other *E. coli* sRNAs can also sequester CsrA, including CsrC and McaS (27, 28). CsrC has a structure similar to that of CsrB but with fewer GGA motifs (27). McaS is an sRNA that shows base pairing with some mRNAs involved in curli and flagellum synthesis and can also sequester CsrA via two GGA motifs (28). In addition to sRNAs, the 5′ untranslated region of mRNAs can also function in the sequestration of CsrA (29).

The activities of RsmV, RsmW, RsmY, and RsmZ were compared by expressing each sRNA from an arabinose-inducible expression vector. Plasmid-expressed RsmV activated $P_{lac\text{-}tssA1'\text{-}'lacZ}$ reporter activity and inhibited $P_{exsD\text{-}lacZ}$ reporter activity (Fig. 3B and C). RsmV had activity comparable to that shown by RsmY for activation of the $P_{lac\text{-}tssA1'\text{-}'lacZ}$ reporter activity and a strong effect on activation of $P_{exsD\text{-}lacZ}$ reporter activity. RsmA and RsmF both bind to RsmV with high affinity *in vitro* (Fig. 2A), and the affinity of RsmF for RsmV is at least 10-fold higher than for RsmY and RsmZ (10, 14). Although the affinity of RsmF for RsmV is higher *in vitro*, RsmV does not seem to show preferential activity toward RsmF over RsmA *in vivo* (Fig. 3). The reason for this is unclear but may reflect differences between the *in vitro* and *in vivo* binding conditions. A difference between the results seen under *in vitro* and *in vivo* conditions was also evident in examination of the RsmV GGA mutants. Whereas each of single GGA substitution mutants demonstrated altered regulatory control of $P_{exsD\text{-}lacZ}$ and/or $P_{lac\text{-}tssA1'\text{-}'lacZ}$ reporter activity (Fig. 4A and B), the binding affinity of RsmA and RsmF was relatively unaffected by the single GGA substitutions (Table 1). A similar trend was observed in a previous mutagenesis study of RsmY and RsmZ wherein the *in vivo* activity did not strictly correlate with *in vitro* binding (30). It was speculated that other RNA binding proteins, such as Hfq, may prevent binding to suboptimal sites *in vivo*.

RsmV activity was clearly evident when it was expressed from a plasmid (Fig. 2). A role for RsmV when expressed at native levels from the chromosome was also detected. Deletion of *rsmV* resulted in a modest but significant increase in T3SS reporter activity (Fig. 5A), and copurification experiments found that RsmV interacts with RsmA and RsmF (Fig. 5B). Both of these findings suggest that RsmV can compete with RsmY and RsmZ for RsmA/RsmF binding in wt cells (Fig. 5B). Whether conditions exist where *rsmV* transcription is elevated and might result in more-pronounced phenotypes is unclear. Transcription of *rsmY* and *rsmZ* is directly controlled by the GacA/S two-component system, a highly conserved system in *Gammaproteobacteria* (24, 31, 32). GacS is a sensor kinase whose activity is controlled by two orphan kinases, RetS and LadS (33–36). Additional regulators interact with and alter the effect of RetS on GacS (37, 38). SuhB regulates *rsmY* and *rsmZ* transcription indirectly by altering *gacA* levels (39). The HptB phosphotransfer protein regulates *rsmY* and *rsmZ* transcription when *P. aeruginosa* is grown on a surface (40, 41). Other regulators contribute to *rsmY* and *rsmZ* transcription through mechanisms that do not alter GacS/GacA activity. MvaT, a H-NS like protein, binds A+T-rich regions of DNA and silences *rsmZ* transcription, while BswR, a transcriptional regulator, counteracts negative regulation of *rsmZ* by MvaT (24, 42). Recently, MgtE, a magnesium transporter, was shown to alter *rsmY* and *rsmZ* transcription, though a mechanism of action is yet to be defined (43).

Neither *rsmV* nor *rsmW* is under positive transcriptional control of the GacAS system (23) (see Fig. S2 in the supplemental material). GacA may repress *rsmW* transcription through an indirect mechanism (30). RsmW expression appears to be highest during the stationary phase in minimal media, which may be more biologically representative of a biofilm (23). RsmW is encoded directly downstream of PA4570, a protein of unknown function. RsmW and PA4570 are likely cotranscribed and separated by an RNase cleavage event. Determining the transcriptional regulation of PA4570 may provide insight into *rsmW* transcriptional control. A search for potential promoters upstream of *rsmV* predicted binding sites for the transcriptional activators RhlR, AlgU, and FleQ. mRNA levels for *rsmV*, however, were unaffected in PA14 transposon mutants within each of those genes relative to the wild-type results as measured by qRT-PCR

(data not shown). Additional studies are required to determine how *rsmV* and *rsmW* transcription is controlled and if RsmV plays a larger role in regulating RsmA and/or RsmF activity under a different set of growth conditions.

RsmX, RsmY, and RsmZ in *P. protogens* are differentially expressed, thus contributing to a mechanism of fine-tuning RsmA and RsmE activity (25). Expression of *P. protogens* RsmX and expression of RsmY occur in parallel during exponential growth, while RsmZ expression is delayed (25). This may allow cells to fine-tune expression of these sRNAs based on the environmental conditions. We propose a similar scenario for expression of RsmV, RsmW, RsmY, and RsmZ in *P. aeruginosa*. The differences in binding affinities for RsmA/RsmF, timing of gene expression, and expression levels of the sRNAs may provide a mechanism for fine-tuning the expression of genes under the control of the Rsm system.

MATERIALS AND METHODS

Strain and plasmid construction. The strains and plasmids used in the study are listed in Table S1 in the supplemental material. Routine cloning was performed with *E. coli* DH5α cultured in LB-Lennox medium with gentamicin (15 μg/ml) as required. *P. aeruginosa* strain PA103 and the Δ*gacA* and Δ*rsmYZ* mutants were reported previously (Table 1) (10, 14, 44, 45). The in-frame Δ*rsmV* deletion mutant was constructed by allelic exchange. The upstream and downstream flanking regions (~800 bp) of *rsmV* were generated by PCR using primer pair 118845409 and 118845410 and primer pair 118845411 and 118845412. The PCR products were cloned into pEXG2 (46), and the resulting construct was mobilized into wild-type PA103 and the Δ*rsmYZ*, Δ*rsmAYZ*, and Δ*rsmFYZ* mutants by conjugation. Merodiploids were resolved by sucrose counterselection as previously described (47). The RsmV expression plasmid was constructed by positioning the *rsmV* transcription start site immediately downstream of the P_{BAD} promoter start site using the Gibson assembly method (New England Biolabs). Briefly, the P_{BAD} promoter regions from pJN105 (primer pair 117830775 and 117830776) and *rsmV* (primer pair 118845423 and 118845424) were amplified by PCR and then assembled into the MluI- and SacI-digested pJN105 plasmid (48). pRsmV vectors bearing single GGA-to-CCT substitutions, or various combinations thereof, were assembled using the Gibson method from gene blocks listed in Table S2 and cloned into the NruI and PvuI sites of pJN105 as outlined in Table S3. The *rsmV* transcriptional reporter (primer pair 150592489 and 150592490) includes 500 nucleotides upstream of the *rsmV* transcription start site. The *rsmV* reporter was integrated into the CTX phage attachment site in wt and *gacA* strains.

β-Galactosidase assays. PA103 strains were grown overnight at 37°C in LB containing 80 μg/ml gentamicin as required. The next day, strains were diluted to an absorbance (A_{600}) of 0.1 in tryptic soy broth (TSB) for measurement of *tssA1′-′lacZ* reporter activity or TSB supplemented with 100 mM monosodium glutamate and with 1% glycerol for measurement of $P_{exsD\text{-}lacZ}$ reporter activity. Arabinose (0.4%) was also added to induce *rsmV* expression from the P_{BAD} promoter. The cultures were incubated at 37°C and harvested when the A_{600} reached 1.0. β-Galactosidase activity was assayed with *ortho*-nitrophenyl-galactopyranoside (ONPG) substrate as previously described (49) or with chlorophenol red-β-D-galactopyranoside (CPRG) substrate. CPRG activity was determined by measuring product formation at 578 nM and using an adaptation of the Miller equation as follows: CPRG units = (A_{578}/culture A_{600}/time/culture volume [in milliliters]) ± 1,000. CPRG and Miller unit values are reported as averages of results from at least three independent experiments, with error bars representing standard deviations (SD).

Electrophoretic mobility shift assays. DNA templates harboring wild-type *rsmV* or *rsmV* bearing point mutations within the GGA sequences were PCR amplified and used as templates for *in vitro* generation of RNA probes. RNA probes were end labeled with [γ-^{32}P]ATP as previously described (10). Histidine-tagged RsmA and RsmF were purified by Ni^{2+} affinity chromatography as described previously (10). RsmA or RsmF was incubated with the RNA probes at the indicated concentrations in 1± binding buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 100 mM KCl), 3.25 ng/μl total yeast tRNA (Life Technologies), 10 mM dithiothreitol (DTT), 5% (vol/vol) glycerol, and 0.1 unit RNase Out (Life Technologies). Reaction mixtures were incubated at 37°C for 30 min and then mixed with 2 μl of gel loading buffer II (Life Technologies) and immediately subjected to electrophoresis on 7.5% (wt/vol) native polyacrylamide glycine gels (10 mM Tris-HCl [pH 7.5], 380 mM glycine, 1 mM EDTA) at 4°C. Imaging was performed using an FLA-7000 phosphorimager (Fujifilm) and analyzed using MultiGuage v3.0 software. Binding properties were determined with Prism 6.0e using the binding saturation equation for specific binding. The apparent K_{eq} represents the molar concentration of RsmA/RsmF required to shift 50% of the RNA probe. The apparent K_{eq} values reported in the text are averages of results from at least three independent experiments.

qRT-PCR. Steady-state levels of native RsmV and the GGA mutants were determined by quantitative reverse transcription-PCR (qRT-PCR). Using SYBR green as previously described (37). cDNA was generated with an *rsmV*-specific primer (138770592) (Table S2) in reaction mixtures containing 100 ng of RNA, 9 μl RNase-free water, 1 μl oligonucleotide mix (2 pmol/μl of specific primer), and 1 μl deoxynucleoside triphosphate (dNTP) (10 mM) mix. Reaction mixtures were heated at 65°C for 5 min and then placed on ice for 1 min. A 4-μl volume of 5± first-strand buffer, 1 μl of 0.1 m DTT, 1 U of RNase Out, and 1 μl of Superscript III reverse transcriptase (Life Technologies, Grand Island, NY) were added to each reaction mixture, and the mixture was incubated at 50°C for 1 h. Each reaction mixture was heat inactivated at 70°C for 15 min. cDNA (2 ng) and 1.8 μl of forward and reverse primers (primers 138770593 and 138770594, respectively) (Table S2) were then added to Power SYBR green PCR master mix (Life

Technologies, Grand Island, NY). qPCRs were conducted by the Iowa Institute of Human Genetics (IIHG) core of the University of Iowa.

Time course experiments measuring RsmV, RsmW, RsmY, and RsmZ levels were performed by qRT-PCR using TaqMan as previously described (50). cDNA (2 μl) prepared as described above was added to reaction mixtures containing 10 μl 2 $\pm$ TaqMan universal PCR master mix (Life Technologies, Grand Island, NY), 2 μl of a gene-specific probe set (*rimM*, 126317493; *rsmW*, 149204991; *rsmX*, 149204995; *rsmY*, 122503154, 122503155, and 122503156; and *rsmZ*, 122501195, 122501196, and 122503154 (Table S2), and 6 μl H_2O. PCRs were performed by the Iowa Institute of Human Genetics.

RNA enrichment experiments. Strain PA14 Δ*rsmAF* carrying an empty vector control, $pRsmA_{His6}$, or $pRsmF_{His6}$ was grown in TSB supplemented with 20 mM $MgCl_2$, 5 mM EGTA, 15 μg/ml gentamicin, and 0.1% arabinose to mid-log phase, chilled, and pelleted for immediate lysis. Cells were lysed under native conditions to retain protein structure (Qiagen QIAexpressionist manual native purification buffer recipe) using supplementation with 2.5 mM vanadyl ribonucleoside complex (NEB) (to inhibit RNase activity), 1 mg/ml lysozyme, and 0.1% Triton X-100. Lysis was completed by freeze-thaw cycles. Lysates were treated with 10 μl RQ-1 RNase-free DNase and cleared by centrifugation. An aliquot was removed from the cleared lysate for total RNA isolation and preserved in TRIzol, and the remaining lysate was incubated with nickel-nitrilotriacetic acid (Ni-NTA)–agarose at 4°C for 1 h under nondenaturing binding conditions. Ni-NTA–agarose was then loaded into a column and washed 3 times with nondenaturing binding buffer containing 10 mM imidazole. Protein and associated RNAs were eluted in 4 fractions with 250 mM imidazole and 4 fractions with 500 mM imidazole. Protein-containing fractions from the $RsmA_{His6}$- or $RsmF_{His6}$-expressing strains and an equivalent volume from the vector control strain were treated with TRIzol (Thermo Fisher), and RNA was extracted according to the manufacturer's protocol. RNA was treated with RQ-1 RNase-free DNase and concentrated using a RNA Clean and Concentrator kit (Zymo). First-strand cDNA was synthesized using Superscript II (Thermo Fisher) according to the manufacturer's protocol with Random Primer 9 (NEB). The copy number of the indicated genes was determined by qPCR using SYBR green master mix (Bio-Rad).

Statistical analyses. One-way analysis of variance (ANOVA) was performed using Prism 6.0 (GraphPad Software, Inc., La Jolla, CA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00277-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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