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RESEARCH ARTICLE

The *Pseudomonas aeruginosa* Two-Component Regulator AlgR Directly Activates *rsmA* Expression in a Phosphorylation-Independent Manner

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ABSTRACT *Pseudomonas aeruginosa* is an important pathogen of the immunocompromised, causing both acute and chronic infections. In cystic fibrosis (CF) patients, *P. aeruginosa* causes chronic disease. The impressive sensory network of *P. aeruginosa* allows the bacterium to sense and respond to a variety of stimuli found in diverse environments. Transcriptional regulators, including alternative sigma factors and response regulators, integrate signals changing gene expression, allowing *P. aeruginosa* to cause infection. The two-component transcriptional regulator AlgR is important in *P. aeruginosa* pathogenesis in both acute and chronic infections. In chronic infections, AlgR and the alternative sigma factor AlgU activate the genes responsible for alginate production. Previous work demonstrated that AlgU controls *rsmA* expression. RsmA is a posttranscriptional regulator that is antagonized by two small RNAs, RsmY and RsmZ. In this work, we demonstrate that AlgR directly activates *rsmA* expression from the same promoter as AlgU. In addition, phosphorylation was not necessary for AlgR activation of *rsmA* using *algR* and *algZ* mutant strains. AlgU and AlgR appear to affect the antagonizing small RNAs *rsmY* and *rsmZ* indirectly. RsmA was active in a *mucA22* mutant strain using leader fusions of two RsmA targets, *tssA1* and *hcnA*. AlgU and AlgR were necessary for posttranscriptional regulation of *tssA1* and *hcnA*. Altogether, our work demonstrates that the alginate regulators AlgU and AlgR are important in the control of the RsmA posttranscriptional regulatory system. These findings suggest that RsmA plays an unknown role in mucoid strains due to AlgU and AlgR activities.

IMPORTANCE *P. aeruginosa* infections are difficult to treat and frequently cause significant mortality in CF patients. Understanding the mechanisms of persistence is important. Our work has demonstrated that the alginate regulatory system also significantly impacts the posttranscriptional regulator system RsmA/Y/Z. We demonstrate that AlgR directly activates *rsmA* expression, and this impacts the RsmA regulon. This leads to the possibility that the RsmA/Y/Z system plays a role in helping *P. aeruginosa* persist during chronic infection. In addition, this furthers our understanding of the reach of the alginate regulators AlgU and AlgR.

KEYWORDS *P. aeruginosa*, RsmA, AlgR, mucoid, *Pseudomonas aeruginosa*, cystic fibrosis, *mucA*, two-component regulatory systems

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The opportunistic pathogen *Pseudomonas aeruginosa* possesses multiple virulence factors for causing disease. This allows *P. aeruginosa* to cause both acute and chronic infections. Acute infecting strains are characterized by the presence of type IV pili (T4P), flagella, and a type III secretion system (T3SS) (1–3). In contrast, chronic infecting strains diversify (4, 5) and frequently do not express T3SS, T4P, or flagella (6, 7). Chronic infecting strains often form biofilms composed of exopolysaccharides, such as alginate, and signal a decline in lung function in CF patients (8–11). Alginate

biosynthesis requires activation of the alternative sigma factor AlgU (12, 13) and the two-component transcriptional regulator AlgR (12, 14, 15).

Two-component signal transduction systems are important in regulating the bacterial response to environmental conditions. AlgR controls both acute and chronic virulence genes (16–18). In the case of acute infections, AlgZ phosphorylates AlgR and activates the *fimU* operon encoding minor pilins important in pilus biogenesis (19–21). The production of alginate, indicative of chronic infection, does not require AlgR phosphorylation (22). This fact has been demonstrated by deletion of the histidine sensor kinase gene *algZ* (23) and by the expression of an *algR* mutant allele encoding an asparagine in place of the conserved aspartate (22).

In chronic *P. aeruginosa* infections, the bacteria frequently acquire spontaneous *mucA* mutations that free the alternative sigma factor AlgU (12, 14, 24–26). AlgU and AlgR regulate the alginate biosynthesis genes producing mucoid colonies (12). In addition, AlgR decreases the expression of acute virulence factors, such as the T3SS in chronic infections when *mucA* mutations occur (7, 27). Therefore, AlgR has multiple roles depending on the type of infection and the phosphorylation state of AlgR: phosphorylated in acute infections activating the *fimU* operon and unphosphorylated in chronic infections activating alginate biosynthesis.

Previous work implicated the AlgZ/R system in the control of *rsmA* expression (28), but a mechanism for AlgR activation of *rsmA* was not investigated. RsmA is considered a global regulator that controls the expression of many *P. aeruginosa* genes by binding to mRNAs (29). Two noncoding RNAs, RsmY and RsmZ, counteract RsmA (29, 30). In this study, we sought to determine how AlgR activates *rsmA* and further investigate the role of RsmA in *P. aeruginosa* strains containing an *mucA* mutation. We further examined *rsmY* and *rsmZ* expression in *algR* mutant strains.

We demonstrate that AlgR directly activates *rsmA* expression in a phosphorylation-independent manner and that both AlgR and AlgU are important in activating *rsmA* expression in mucoid strains. The activity of RsmA is not thought to be significant in chronic infecting strains, such as *mucA* mutants. However, we also provide evidence that RsmA is active in mucoid *P. aeruginosa*. Altogether, our work shows that the two-component regulator AlgR can affect gene expression through RsmA, providing another mechanism for how AlgR impacts virulence gene regulation. We postulate that AlgR and RsmA likely function as a rheostat, as opposed to a switch, and suggest that RsmA plays a role in chronic infections.

RESULTS

***mucA* mutant strains require AlgR for increased *rsmA* expression.** Previous studies suggested that AlgU and AlgR control *rsmA* expression (28, 31). However, whether both AlgZ and AlgR were involved in the control of *rsmA* expression was not tested. We constructed and assayed an *rsmA* transcriptional fusion (Fig. 1A) that contains both *rsmA* promoters (rsmATF1-*lacZ*). The rsmATF1-*lacZ* fusion was analyzed in the wild-type strain *P. aeruginosa* PAO1, Δ*algR* and *algZ* mutants, and in the corresponding *mucA22* mutants. The *algZ* mutant has a mutation in the conserved histidine residue, which prevents AlgZ-mediated phosphorylation of AlgR (19, 32) but does not disrupt the internal *algR* promoter (33). As shown in Fig. 1B, there was a slight increase in rsmATF1-*lacZ* activity in a Δ*algR* mutant. When the rsmATF1-*lacZ* fusion was assayed in an *algZ* mutant strain, there was also a slight increase in reporter activity (Fig. 1B). The modest increase in reporter activity suggests that AlgZ and AlgR have a minor role in *rsmA* regulation in strain PAO1.

As previous studies also indicated a role for AlgU in regulating *rsmA* expression, the rsmATF1-*lacZ* transcriptional fusion was assayed in a *mucA22* strain, where AlgU is most active. Reporter activity was increased ~3-fold in a *mucA22* strain, as previously described (31) (Fig. 1B). However, in the *mucA22* Δ*algR* double mutant, there was a drastic decrease (~3-fold) in rsmATF1-*lacZ* activity (Fig. 1B). The *mucA22 algZ* mutant strain had rsmATF1-*lacZ* activity almost identical to that of the *mucA22* mutant strain

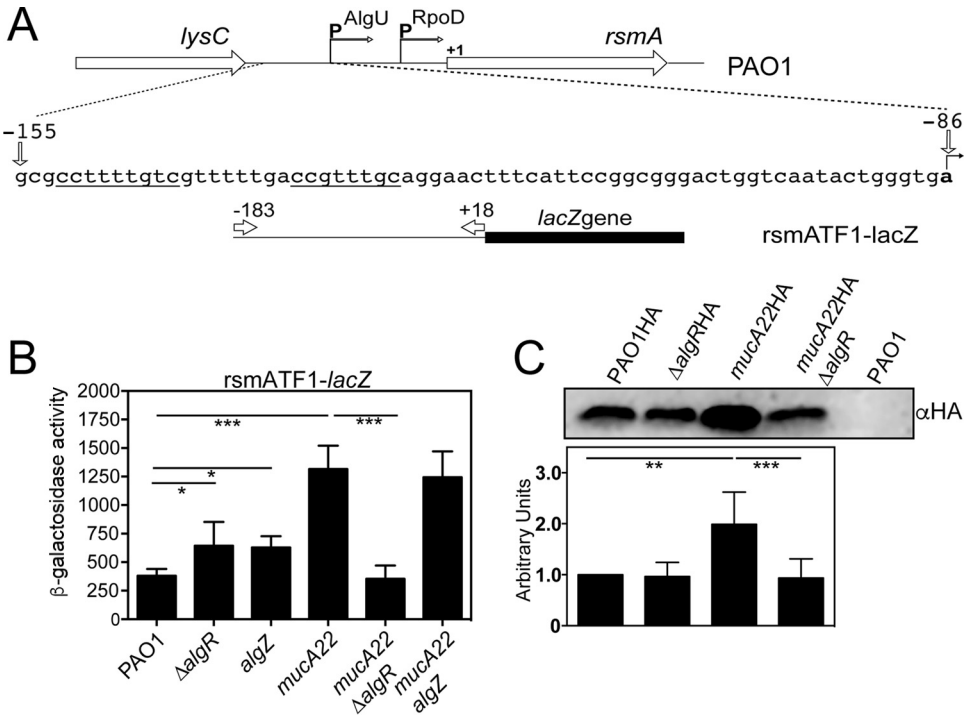

FIG 1 AlgR, but not AlgZ, is necessary for increased *rsmA* expression in mucoid strains. (A) Schematic of *rsmA* genomic region. The sequence below the genomic schematic indicates the AlgU-dependent promoter region. Underlined sequences are potential AlgR-binding sites. The bent arrow and bold nucleotide indicate the transcriptional start sites. The *rsmA* transcriptional fusion rsmATF1-*lacZ* used in this study is listed below. Arrows indicate the primers used and the numbers indicate the primer locations relative to the translational start site. (B) The transcriptional fusion rsmATF1-*lacZ* (Fig. 1A) was assayed in the indicated strains after growth for 8 h in LB broth. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Asterisks indicate *P* values of 0.01 (*) and <0.0001 (***). (C) Western blot analysis of the indicated strains containing an HA-tagged *rsmA* allele. PAO1 without the *rsmAHA* allele was run as a negative control. Densitometry analysis was performed using a duplicate gel and staining for total protein using Coomassie blue, and all strains were normalized to PAO1 containing the *rsmAHA* allele. Western blotting was performed four times, and densitometry analysis is indicated below the Western blot. A representative Western blot is shown above the densitometry. A one-way ANOVA with Tukey's posttest was used to determine statistical significance. **, $P < 0.001$; ***, $P < 0.0001$.

(Fig. 1B). These data implicate AlgR but not AlgZ in *rsmA* regulation in a *mucA22* mutant strain and suggest that AlgR phosphorylation is not necessary for *rsmA* activation.

To confirm that AlgR affected *rsmA* expression, an epitope-tagged *rsmA* allele was introduced into the wild-type strain (PAO1), *mucA22* mutant, and respective *algR* mutant strains and analyzed by Western blot analysis. As shown in Fig. 1C, a slight decrease in RsmA levels in the Δ*algR* mutant strain was detected compared to the wild-type strain PAO1. As reported previously (31), a *mucA22* mutant strain had drastically increased RsmA levels (Fig. 1C). The *mucA22* Δ*algR* mutant strain had significantly decreased RsmA compared with the *mucA22* mutant strain (Fig. 1C). The Western blot analysis confirmed the transcriptional fusion analysis and supported a significant role for AlgR activating *rsmA* in the mucoid *mucA22* mutant strain but not in the nonmucoid strain PAO1.

AlgR phosphorylation is not required for activation of *rsmA* expression. To further investigate the role of AlgR phosphorylation in the regulation of *rsmA*, *algR* site-directed mutants were constructed that mimic either the unphosphorylated (D54N) or phosphorylated form of AlgR (D54E). The *algR*D54N and *algR*D54E mutant alleles were also constructed in the *mucA22* mutant background to confirm a phosphorylation-independent mode of AlgR activation of *rsmA*. Both the *mucA22 algR*D54N and the *mucA22 algR*D54E mutant strains were mucoid (data not shown), supporting the notion that phosphorylation is not necessary for alginate production and that the mutant AlgR proteins produced in these strains were still functional. The *mucA22*D54N mutant had

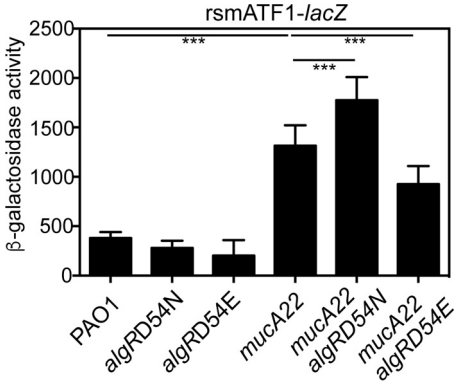

FIG 2 AlgR phosphorylation is not required for *rsmA* activation. Mutants containing either a mutated aspartate to asparagine (D54N) or aspartate to glutamate (D54E) were constructed in both the wild-type background PAO1 and the *mucA22* mutant background. The *rsmA* transcriptional fusion rsmATF1-*lacZ* was introduced into the strains indicated, resulting in single-copy chromosomal transcriptional fusions. The indicated strains were grown for 8 h in LB broth and assayed for β-galactosidase activity. Values indicate the actual β-galactosidase activity minus the vector control (-28 Miller units). Differences from the wild-type strain PAO1 or *mucA22* mutant were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate P values of <0.0001.

increased *rsmA* reporter activity compared to a *mucA22* mutant strain (Fig. 2). The *mucA22 algR*D54E strain had decreased rsmATF1-*lacZ* activity compared to the *mucA22* mutant (Fig. 2). Because the *mucA22 algR*D54N and the *mucA22 algZ* mutant strains had elevated and similar levels, respectively, of rsmATF1-*lacZ* activity (Fig. 2 and 1B, respectively) compared to the *mucA22* mutant strain, this suggests that phosphorylation of AlgR is not required for *rsmA* activation.

AlgR regulates the distal *rsmA* promoter. Previous work determined that *rsmA* has two promoters (31). An RNase protection assay was performed using a probe spanning the upstream region of *rsmA* (Fig. 3A) to determine which *rsmA* message AlgR affected. As we previously reported (31), two *rsmA* messages were seen in both the wild-type strain PAO1 and the *mucA22* mutant strain, with the longer transcript increased in the *mucA22* mutant strain (Fig. 3B). There was little difference in the *rsmA* transcripts between PAO1 and the Δ*algR* mutant (Fig. 3B). In contrast, there was a substantial decrease in the longer *rsmA* transcript in the *mucA22* Δ*algR* mutant strain compared to the *mucA22* parent strain (Fig. 3B). The specificity of the *rsmA* probe was confirmed using the probe incubated with yeast tRNA (+RNase) and using a Δ*rsmA* mutant (Fig. 3B). These results suggest that AlgR is most important for *rsmA* control in the *mucA22* background and that AlgR controls the distal *rsmA* promoter that is also under control by AlgU.

To confirm that AlgR regulates the distal *rsmA* promoter, transcriptional fusions containing individual *rsmA* promoters were used (Fig. 3A; see also Fig. S1A in the supplemental material). The transcriptional fusion rsmATF2-*lacZ* contains only the proximal *rsmA* promoter (Fig. S1A). The transcriptional fusion rsmATF3-*lacZ* (Fig. 3A) contains only the distal promoter that has been shown to be controlled by AlgU (31). The deletion of *algR* had no effect in the rsmATF2-*lacZ* transcriptional fusion in the wild-type or a *mucA22* background (Fig. S1B). The transcriptional fusion rsmATF3-*lacZ* had increased reporter activity in a *mucA22* mutant strain compared to that in PAO1 (Fig. 3C). Compared to PAO1, no change in rsmTF3-*lacZ* activity was seen in a Δ*algR* mutant strain (Fig. 3C). However, *algR* deletion in a *mucA22* mutant strain (*mucA*22 Δ*algR*) resulted in a significant decrease in rsmATF3-*lacZ* activity (Fig. 3C), supporting the RNase protection assay. Taking these results together, we conclude that AlgR activates the distal *rsmA* promoter, and this further suggests that AlgU and AlgR both are required to activate *rsmA* transcription from the distal promoter.

AlgR directly binds the distal *rsmA* promoter. We hypothesized that AlgR directly activated the distal *rsmA* promoter. In support of this idea, two potential AlgR-binding sites are located upstream of *rsmA*. One of the potential AlgR-binding sites is 2

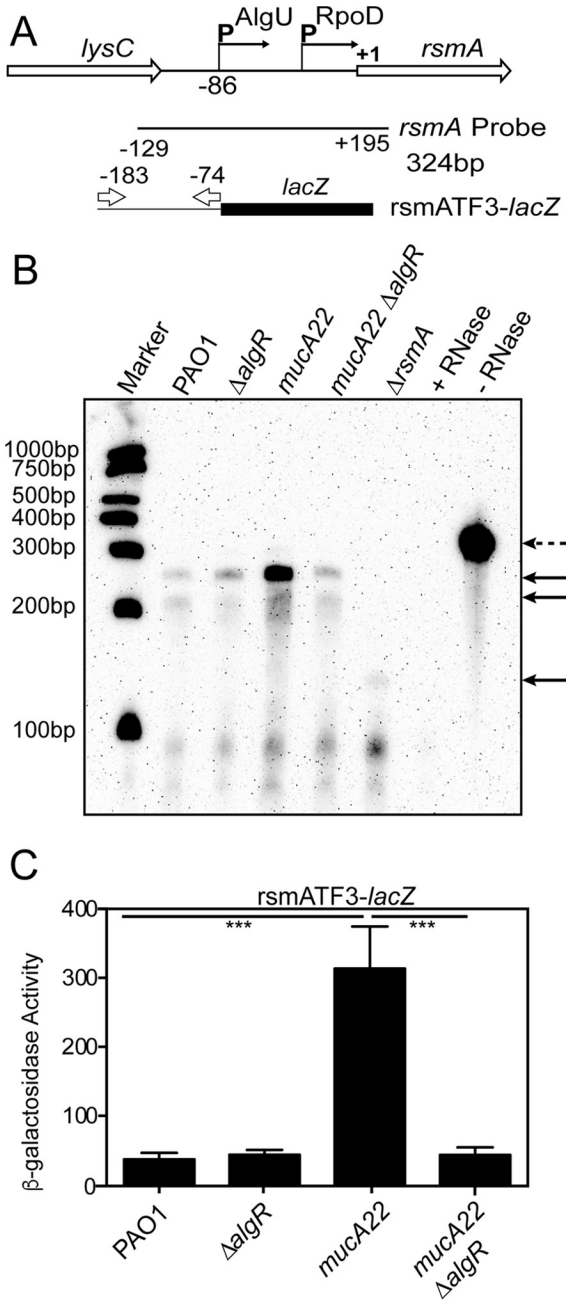

FIG 3 AlgR controls the distal AlgU-dependent *rsmA* promoter. (A) Schematic of the RNase protection assay probe used. Numbers indicate distances from the *rsmA* translational start site. The transcriptional fusion rsmATF3-*lacZ* is indicated at the bottom. Arrows indicate the primers used and their location in reference to the *rsmA* translational start site. (B) A representative RNase protection assay performed on the indicated strains after 8 h of growth in LB broth. Total RNA was isolated from each strain and hybridized to 800 pg of biotinylated *rsmA* probe. After treatment with RNase A/RNase T1, protected probe fragments were detected after electrophoresis through a denaturing polyacrylamide gel and transfer to a nylon membrane. Protected probe fragments are indicated by solid arrows. The broken arrow indicates a full-length probe. Biotinylated molecular size markers are indicated to the left. RNase protection assays were performed three times. (C) The *rsmA* transcriptional fusion rsmATF3-*lacZ* was introduced into the indicated strains to generate strains containing single-copy chromosomal fusions. The strains were grown for 8 h in LB broth and assayed for β-galactosidase activity minus the vector control. Differences from the wild-type strain PAO1 or *mucA22* mutant were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate P values of <0.0001. Fusion analysis was performed in triplicate three times.

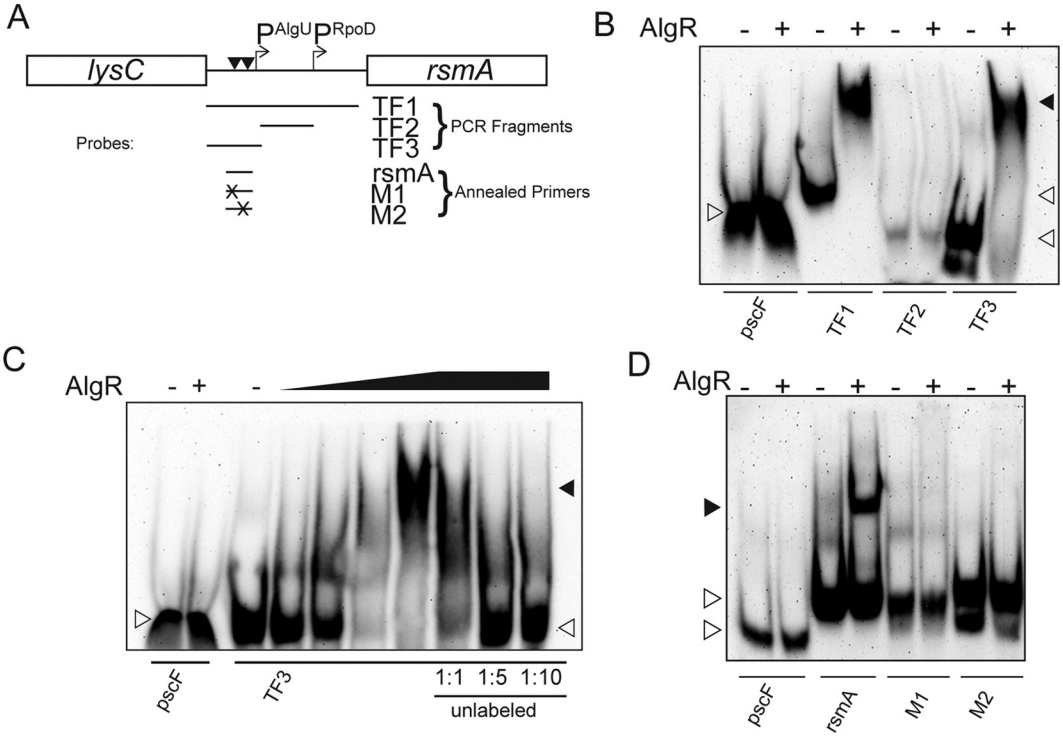

FIG 4 AlgR directly binds the AlgU-dependent *rsmA* promoter and requires both AlgR-binding sites. Purified AlgR was incubated with either biotinylated PCR products or annealed primers. (A). Schematic of the *rsmA* genomic region. Below are fragments or annealed primers and the approximate location upstream of *rsmA*. Promoters are indicated above as bent arrows and are denoted by P^{AlgU} or P^{RpoD}. The arrowheads indicate the approximate location of the putative AlgR-binding sites. (B) Analysis of biotinylated PCR fragments in gel shift studies. The PCR fragment TF1 represents the entire *rsmA* upstream region (see panel A). Fragments TF2 and TF3 correspond to the separated *rsmA* promoters (see Fig. 1). TF2 corresponds to the proximal *rsmA* promoter, and TF3 corresponds to the distal AlgU-dependent promoter. A minus sign indicates probe alone. A plus sign indicates AlgR concentration of 2.5 μM. *pscF* was used as a negative control. Shaded arrowheads indicate shifted fragments. White arrowheads indicate unbound probe. (C) Titration and competition assay using purified AlgR and the TF3 fragment corresponding to the distal *rsmA* promoter. The *pscF* fragment was used as a negative control. TF3 was incubated with different concentrations of AlgR (indicated by the graded triangle; 0.05, 0.25, 1.25, and 2.5 μM). TF3 was also used in a competition assay using unlabeled TF3 as a competitive inhibitor. Ratios below indicate the ratio of labeled to unlabeled probe. Shaded arrowheads indicate shifted fragments. White arrowheads indicate unbound probe. A minus sign indicates probe alone. (D) Two AlgR-binding sites are upstream of the distal *rsmA* promoter. Annealed primers containing either wild-type or two different mutated AlgR-binding sites were tested in gel shift experiments. The *pscF* fragment was used as a negative control. The *rsmA* lanes indicate the probe using annealed primers with both AlgR-binding sites intact. M1 indicates the same as *rsmA*, except that the furthest upstream AlgR-binding site was mutated. M2 indicates the same as *rsmA*, except that the further downstream AlgR-binding site is mutated. Shaded arrowheads indicate shifted fragments. White arrowheads indicate unbound probe. Minus signs indicate probe alone. Plus signs indicate an AlgR concentration of 2.5 μM.

nucleotides away from the AlgU −35 consensus promoter (Fig. 4A). Purified AlgR was tested in gel shift studies with biotinylated *rsmA* promoter fragments (Fig. 4A). A biotinylated *pscF* gene, a component of the T3SS (34, 35), was used as a negative control and did not shift when incubated with purified AlgR (Fig. 4B to D). A PCR fragment representing the entire *rsmA* upstream region containing both promoters was dramatically shifted using purified AlgR (Fig. 4B), suggesting that AlgR directly binds to at least one of the AlgR-binding sites upstream of *rsmA*. To further define the region upstream of *rsmA* that was bound by AlgR, PCR amplicons that corresponded to the transcriptional fusion constructs containing individual *rsmA* promoters (Fig. 4A) were biotinylated and tested via gel shift analysis. A biotinylated probe containing the proximal promoter (TF2) and upstream sequence to the more distal transcriptional start site did not shift when incubated with purified AlgR (Fig. 4B). This result suggested that AlgR does not bind the proximal *rsmA* promoter. As shown in Fig. 4B, AlgR bound the distal promoter sequence fragment, TF3, containing the AlgU-dependent promoter and potential AlgR-binding sites. The TF3 fragment containing the AlgU promoter was tested in a competition assay using increasing concentrations of AlgR and unlabeled

probe. A 1:1 ratio of labeled to unlabeled probe was capable of decreasing the shift of the TF3 probe (Fig. 4C). Competition with the unlabeled specific probe in a 1:5 or 1:10 ratio was able to compete for AlgR binding, as indicated by severe reduction of the probe shift (Fig. 4C). These data suggest that AlgR specifically binds the AlgU-dependent promoter region and strongly suggests that AlgR binds at least one of the AlgR consensus sequences located in this promoter region.

AlgR has previously been shown to bind an 11-bp consensus sequence, with nucleotides 2 to 10 being the most conserved (36). To determine if one, or both, of the putative AlgR-binding sites were required for AlgR binding, we used annealed primers upstream of the distal promoter in gel shift analyses. Approximately 30-bp primers were annealed and biotinylated that contained the predicted AlgR-binding sites. AlgR was able to shift the annealed biotinylated primers containing the two predicted AlgR-binding sites (Fig. 4D). The first putative AlgR-binding site, CCTTTTGTC, was mutated, and this mutation resulted in a loss of AlgR binding (Fig. 4D), suggesting that the first AlgR-binding site is required for AlgR binding to the *rsmA* promoter. The second putative AlgR-binding site, CCGTTTGGC, is located 7 bp downstream and directly adjacent to the AlgU −35 consensus sequence. When the second AlgR-binding site was mutated, AlgR was no longer able to bind to the annealed primers (Fig. 4D). These data suggest that AlgR binds both of the consensus sequences in order to activate *rsmA* expression.

AlgR regulates *rsmY* expression indirectly. A previous study indicated that *rsmY* and *rsmZ* expression was also increased in a *mucA* mutant background and required AlgR (28). The transcriptional start site of *rsmY* was determined in *Pseudomonas fluorescens* using 5′ rapid amplification of cDNA ends (RACE), and this information was used to deduce the transcriptional start site in *P. aeruginosa* (30, 37). To confirm the *P. aeruginosa rsmY* transcriptional start site, we performed primer extension. As indicated in Fig. 5A, a single extension product was obtained after reverse transcription of total RNA that confirmed the *rsmY* transcriptional start site assigned using *P. fluorescens*.

A transcriptional fusion, rsmYTF1-*lacZ* (Fig. 5B), was constructed and assayed in *algR* and *algZ* mutants in both the PAO1 (wild-type) and the *mucA22* background. When a Δ*algR* or *algZ* mutant was tested for *rsmY* reporter activity, there was no decrease compared to PAO1 (Fig. 5C). There was a >3-fold increase in rsmYTF1-*lacZ* activity in a *mucA22* mutant strain compared to PAO1 (Fig. 5C). When tested in a *mucA22* Δ*algR* mutant strain, the increased activity in the *mucA22* mutant strain was reduced to PAO1 levels (Fig. 5C). In the case of the *mucA22 algZ* mutant strain, there was a slight but statistically significant decrease in rsmYTF1-*lacZ* activity (Fig. 5C). Overall, the fusion results suggest that AlgR affects *rsmY* expression, but only in the *mucA22* background.

To confirm that the *rsmY* levels decreased, Northern blotting was performed on the same strains. Two bands hybridized to the *rsmY* probe, as has been seen previously (Fig. 5D) (30, 38). Little difference was seen between a Δ*algR* mutant strain and the wild-type PAO1 (data not shown). However, an increase in both hybridizing RNAs was seen in a *mucA22* mutant strain (Fig. 5D). The *mucA22* Δ*algR* and *mucA22 algZ* mutant strains had decreased hybridizing fragments (Fig. 5D). The loading control used (5S rRNA) was consistently uneven upon repeated attempts, but the Northern blotting does confirm the transcriptional fusion analysis. A second probe using *proC* was also used and gave similar results. A Δ*rsmY* strain had no hybridizing RNA, confirming the specificity of the *rsmY* probe. Overall, these results support AlgR control of *rsmY* expression in the *mucA22* strain.

Both *rsmY* and *rsmZ* contain upstream activating sequences (UAS) that GacA controls (39). A potential AlgR-binding site is located 23 nucleotides upstream of the UAS for *rsmY*. An additional AlgR-binding site is present further upstream on the opposite strand (Fig. 5B). Two additional transcriptional fusions, rsmYTF2-*lacZ* and rsmYTF3-*lacZ* (Fig. 5B and S2A), were constructed to determine the sequences required for AlgR control. The rsmYTF2-*lacZ* transcriptional fusion deletes the AlgR-binding sites but retains the UAS sequence (Fig. 5B). When assayed in the *algR* mutant strains in both

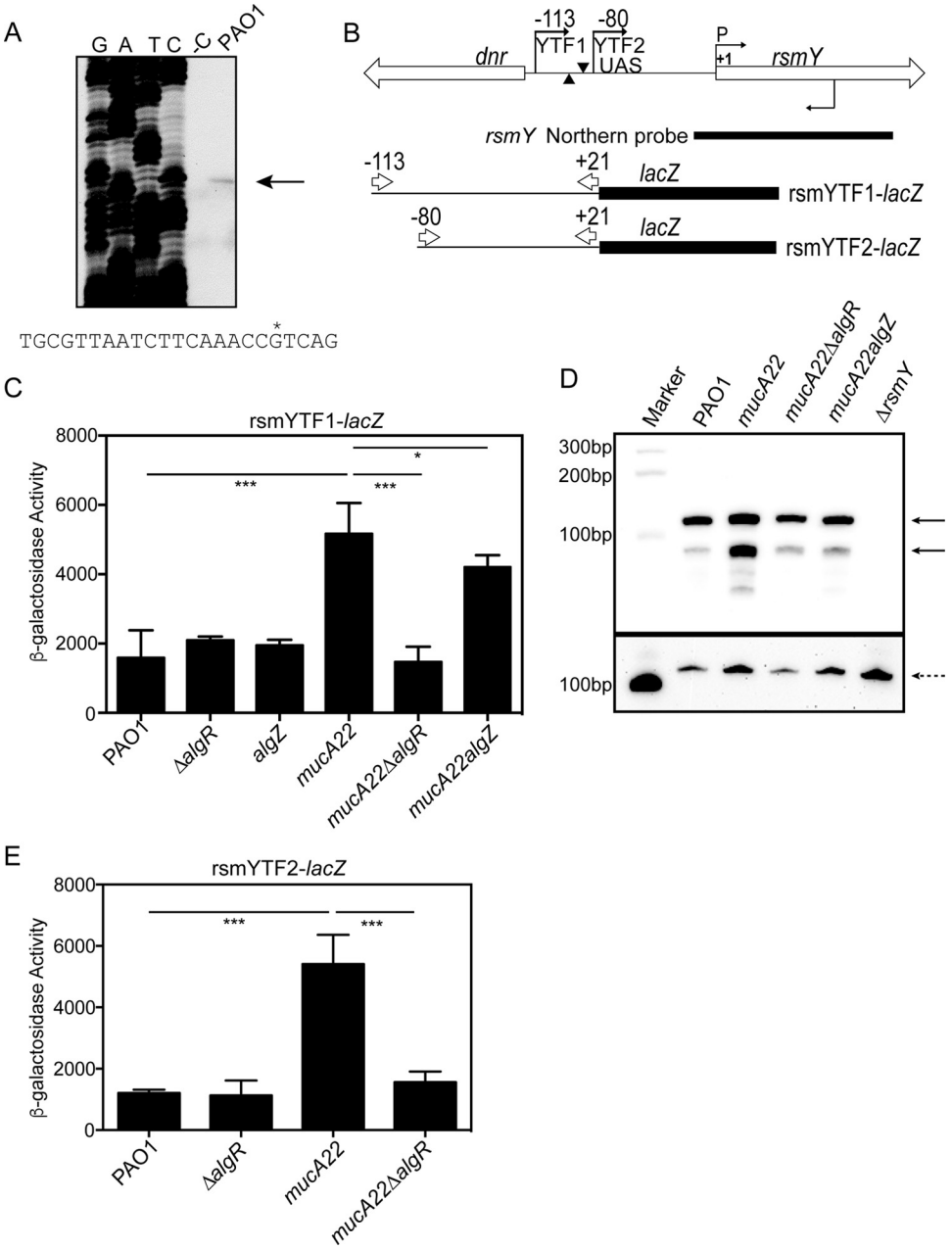

FIG 5 AlgR indirectly increases *rsmY* expression in a *mucA22* mutant strain. (A) Primer extension analysis performed on total RNA from strain PAO1. GATC indicates the sequencing ladder. -C is a control without reverse transcriptase. PAO1 is reverse-transcribed PAO1 mRNA. Below is the sequence of the *rsmY* promoter region. The asterisk denotes the transcriptional start site identified. (B) Schematic of the *rsmY* genomic region. The UAS is indicated between the numbers from the transcriptional start site. The bent arrows above indicate the primers used for the transcriptional fusions, and the numbers correspond to the distance from the transcriptional start site. P and the bent arrow indicate the start site of transcription. The arrowheads represent potential AlgR-binding sites. The bent arrow below indicates the primer used in the primer extension experiment. Indicated below is the probe generated by *in vitro* transcription for Northern analysis. Transcriptional fusions are indicated below. Numbers above the arrows indicate the distance from the transcriptional start site. (C) Transcriptional fusion rsmYTF1-*lacZ* was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for β-galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Asterisks indicate *P* values of 0.001 (**) and <0.0001 (***). (D) Northern blot analysis of RsmY in the indicated strains after growth for 8 h in LB broth. The Δ*rsmY* mutant was used as a control. The top half is a membrane probed with the RsmY probe, and the bottom portion is a second independent blot of the loading control probed with a 5S rRNA probe. Northern blotting was performed at least three times. (E) Transcriptional fusion rsmYTF2-*lacZ* was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for β-galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001. All transcriptional fusion analyses were performed at least three times.

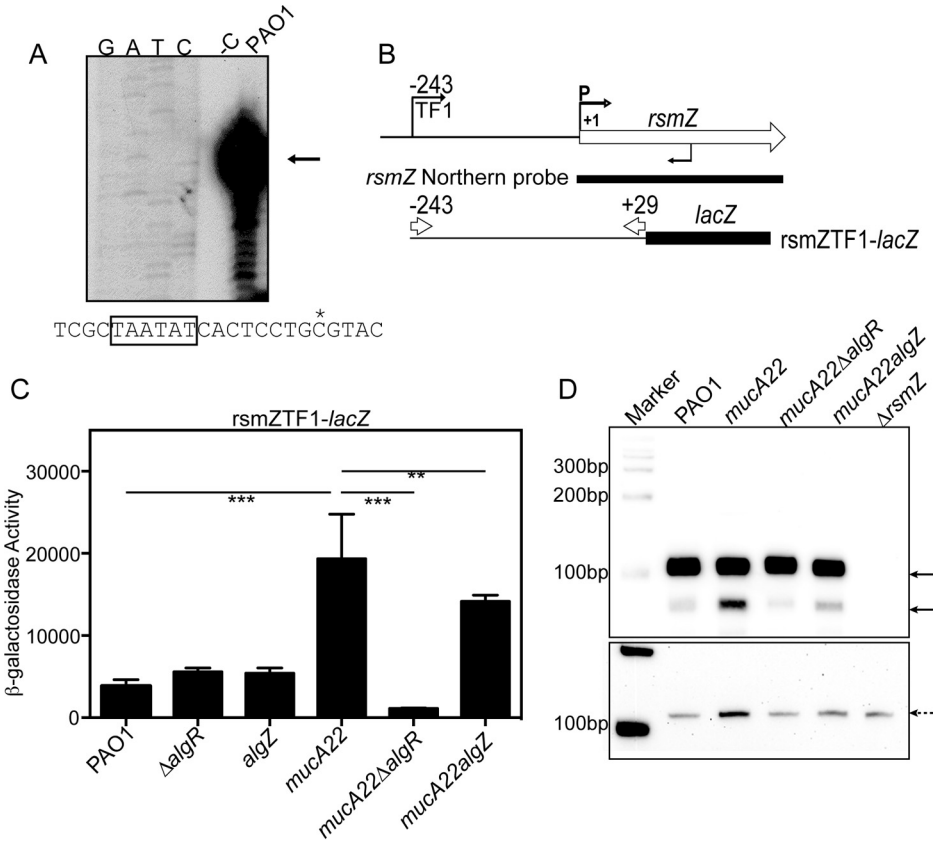

FIG 6 AlgR increases *rsmZ* expression in a *mucA22* mutant strain indirectly. (A) Primer extension analysis using total RNA isolated from PAO1. GATC indicates the sequencing ladder. -C is a control without reverse transcriptase. PAO1 is reverse-transcribed PAO1 mRNA. Below is the sequence of the *rsmZ* promoter region. The asterisk denotes the transcriptional start site identified. (B) Schematic of the *rsmZ* genomic region. The bent arrow on top indicates the primer used for transcriptional fusion construction. The bent arrow indicated by a "P" indicates the transcriptional start site. The bent arrow below indicates the primer used in primer extension. (C) Transcriptional fusion ZTF1-*lacZ* was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for β-galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Asterisks indicate *P* values of 0.001 (**) and <0.0001 (***). Transcriptional fusion analysis was performed in triplicate three times. (D) Northern analysis of the indicated strains grown for 8 h in LB broth and total RNA probed with and *rsmZ* probe. A Δ*rsmZ* mutant strain was used to denote the specificity of the probe. A separate blot of the same strains was probed using a 5S rRNA probe (shown below). Northern blotting was performed three times.

PAO1 and *mucA22* backgrounds, a difference was only seen in the *mucA22* background (Fig. 5E). This result suggests that the potential AlgR-binding sites are not necessary for increased *rsmY* reporter activity.

An additional *rsmY* reporter, rsmYTF3-*lacZ*, was constructed that does not contain the UAS sequence. This fusion had very low activity in both PAO1 and *mucA22* (Fig. S2B). This result suggests that the UAS is required for increased *rsmY* reporter activity in a *mucA22* mutant strain as well as PAO1. Overall, the data suggest that AlgR and AlgU indirectly affect *rsmY* expression.

AlgR regulates *rsmZ* expression indirectly. The regulation of *rsmZ* is more complex than *rsmY* and includes regulators other than GacA (38, 40, 41). Primer extension analysis confirmed the location of the transcriptional start site in *P. aeruginosa* that was predicted from *P. fluorescens* (Fig. 6A) (42, 43). A transcriptional fusion, rsmZTF1-*lacZ*, was assayed in the *algZ* and *algR* mutants (Fig. 6B). There was no difference in the *rsmZ* reporter in the Δ*algR* or *algZ* mutant compared to PAO1 (Fig. 6C). There was a 5-fold increase in rsmZTF1-*lacZ* activity in the *mucA22* mutant strain compared to PAO1 (Fig. 6C). In a *mucA22* Δ*algR* mutant strain, there was a significant decrease in rsmZTF1-*lacZ* activity. In a *mucA22 algZ* mutant strain, there was a significant but slight decrease in

rsmZTF1-*lacZ* activity compared to the *mucA22* mutant (Fig. 6C). In all, these results suggest that AlgR plays a major role and AlgZ plays only a minor role, if any, in terms of *rsmZ* expression in the *mucA* mutant background.

To confirm the transcriptional fusion results, we again wanted to monitor the actual RNA levels. Using Northern blot analysis, a trend was seen similar to that in the transcriptional fusions. As shown in Fig. 6D, there were two bands detected, with the larger fragment being the most intense. Previous studies utilizing Northern blotting suggested that the smaller hybridizing fragment represents the small RNA lacking the stem-loop found at the 3′ end of the small RNA (30). Therefore, the smaller hybridizing bands may represent the small RNAs lacking a transcriptional terminator. A Δ*rsmZ* mutant was used as a negative control. The *mucA22* mutant strain had increased RsmZ compared to PAO1 (Fig. 6D). The *mucA22* Δ*algR* mutant strain had the most drastic decrease in RsmZ levels, and there was a slight decrease in RsmZ levels in the *mucA22 algZ* mutant (Fig. 6D). Altogether, these results suggest that AlgR plays a role in increasing the RsmZ small RNA in the *mucA22* background.

We hypothesized that like *rsmY* expression, AlgR indirectly affects *rsmZ* expression. An additional transcriptional fusion, rsmZTF2-*lacZ* (Fig. S3A), was constructed and assayed in the wild-type strain PAO1 and a *mucA22* mutant strain, which lacks the UAS sequence. Both strains had drastically decreased reporter activity (Fig. S3B). Interestingly, the *mucA22* mutant strain had significantly decreased activity compared to PAO1 (Fig. S3B). Further support for an indirect mechanism of AlgR activation was obtained using gel shift analysis of a PCR amplicon of the *rsmZ* upstream region (Fig. S3A). Purified AlgR was not able to shift the *rsmZ* upstream region tested (Fig. S3C), suggesting AlgR does not directly activate *rsmZ* expression. These results suggest that the UAS is required for increased *rsmZ* expression, and we conclude that AlgR affects *rsmZ* expression indirectly.

AlgR is necessary for RsmA activity in a *mucA* mutant background. To ascertain whether AlgR control of *rsmA* was significant in a biological context, the direct RsmA targets *hcnA* and *tssA1* were used to assess RsmA activity (29). A new integrating vector was constructed that contains the *lacUV5* promoter and lacks a ribosome-binding site or a start codon for *lacZ*. When tested in the wild-type strain PAO1, there was no activity of the vector alone.

The *hcnA* gene encodes part of the hydrogen cyanide synthase enzyme that is implicated in virulence (44, 45). RsmA was shown to previously negatively affect a leader fusion using the *tac* promoter and the ribosome-binding site region of *hcnA* (45). An *hcnA* leader fusion was constructed by annealing 33 nucleotides, including one of the predicted RsmA-binding sites, the ribosome-binding site, and the translational start codon of *hcnA*. As shown in Fig. 7A, the *lacUV5 hcnA-lacZ* fusion had activity in strain PAO1. When assayed in a *mucA22* mutant strain, there was an ~2.5-fold decrease in reporter expression (Fig. 7A), consistent with increased RsmA levels in the *mucA22* mutant strain. If *rsmA* expression requires AlgR and AlgU, mutating *algR* and *algU* should result in increased fusion activity in the *mucA22* background due to decreased *rsmA* expression. Both the *mucA22* Δ*algR* and the *mucA22* Δ*algU* mutant strains had at least a 3-fold increase in reporter expression compared to the *mucA22* strain (Fig. 7A). The *mucA22* Δ*rsmA* mutant strain was assayed and had a 3.6-fold increase in reporter activity compared to the *mucA22* mutant (Fig. 7A). Overall, these results are consistent with the conclusion that RsmA is active in the *mucA22* strain and that AlgU and AlgR are important for the decreased posttranscriptional regulation of *hcnA*.

Another RsmA target, *tssA1*, was also analyzed. The *tssA1* gene encodes a portion of the T6SS and is directly regulated by RsmA (29, 46). Previous studies have used a *lacUV5 tssA1* construct containing 227 bp of *tssA1* upstream sequence that had extremely low activity (28, 29, 31). However, this fusion was found to contain additional transcriptional controls that resulted in low activity (data not shown). To more carefully assess RsmA activity using *tssA1*, we constructed a new leader fusion by annealing 31 nucleotides, including the predicted RsmA-binding site, the ribosome-binding site, and the trans-

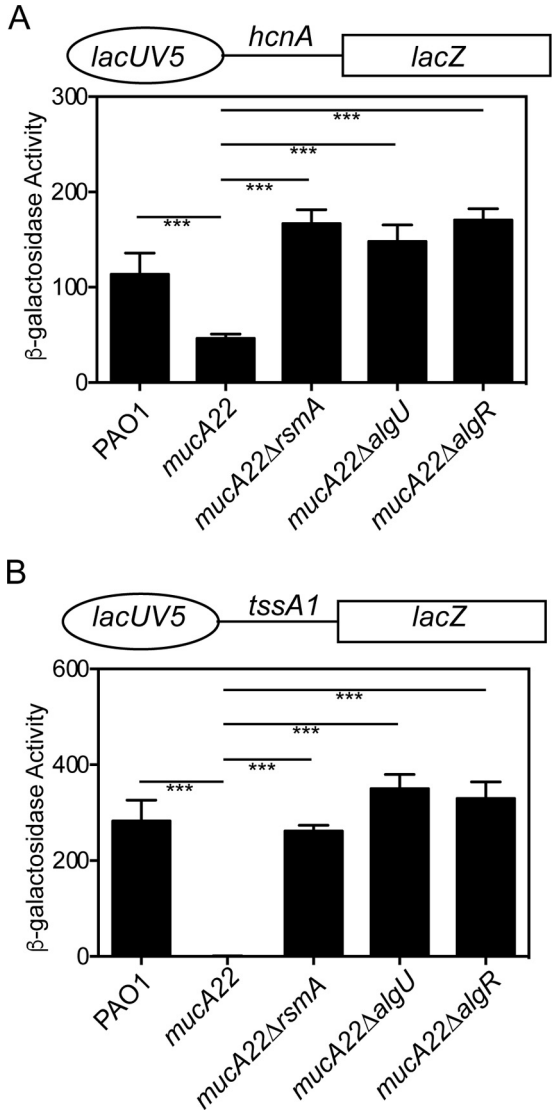

FIG 7 RsmA is active in a *mucA22* mutant strain. (A) Leader/translational fusion *lacUV5 hcnA-lacZ* (above graph) was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for β-galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001. (B) Leader/translational fusion *lacUV5 tssA1-lacZ* (above graph) was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for β-galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001. All leader/translational fusions were assayed in triplicate three times.

lational start codon of *tssA1*. The *lacUV5 tssA1-lacZ* fusion had approximately 200 times the activity as the previously constructed *lacUV5 tssA1-lacZ* fusion containing 227 bp of upstream sequence in the wild-type strain PAO1 (Fig. S4). There was no activity when this fusion was assayed in a *mucA22* mutant strain (Fig. 7B and S4). The *lacUV5 tssA1-lacZ* fusion confirmed our previous result (31), demonstrating increased activity in the *mucA22* Δ*algU* mutant strain, validating the use of this construct. When a *mucA22* Δ*algR* mutant was tested, the fusion was also increased from the *mucA22* mutant strain to levels similar to those of the *mucA22* Δ*algU* mutant strain (Fig. 7B). A *mucA22* Δ*rsmA* mutant strain also had statistically significant activity compared to that of the *mucA22* mutant (Fig. 7B). These results demonstrate that AlgR and AlgU are necessary for posttranscriptional regulation of *tssA1*. From these data, we conclude that AlgU and AlgR are both required for increased *rsmA* expression in the *mucA* mutant background

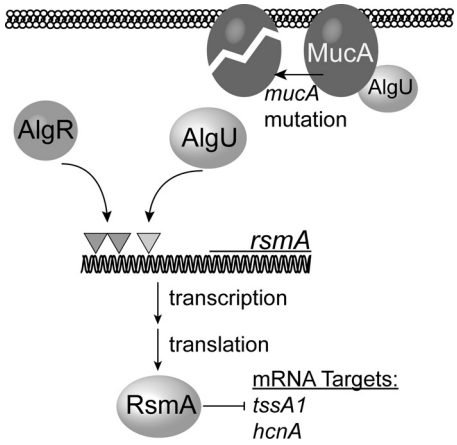

FIG 8 Model for AlgR activation of *rsmA* and RsmA activity in mucoid strains. In chronic infections, *mucA* mutants arise, leading to increased AlgU activity. In the case of *rsmA*, AlgR and AlgU activate the distal *rsmA* promoter. The dark-shaded triangles indicate the AlgR-binding sites located upstream of *rsmA*. The light-shaded triangle indicates the AlgU-dependent promoter. The model predicts that increased expression of *rsmA* leads to increased RsmA activity, as indicated by the negative regulation of the RsmA targets, *hcnA* and *tssA1*.

and that this leads to posttranscriptional regulation on two known RsmA targets, most likely through RsmA.

DISCUSSION

AlgR is an important two-component regulator having roles in both acute and chronic infections. In the case of acute infections, AlgR activates the *fimU* operon, enabling the production of T4P (19, 21, 47). In chronic infections, AlgR, AlgU, and other transcriptional regulators activate the production of alginate (22, 48–50). These and other virulence factors in *P. aeruginosa* are often considered mutually exclusive, depending on whether there is an acute or chronic infection (51–53). This work demonstrates that another effect of a *mucA* mutation is that AlgR activity is directed at increasing the posttranscriptional regulator RsmA. AlgR activation of *rsmA* may help explain how AlgR participates in the mutually exclusive production of some virulence factors depending on acute or chronic infection.

The initial description of AlgR as a regulator of *rsmA* (28) did not evaluate the contribution of AlgR phosphorylation by AlgZ or a mechanism for AlgR activation of *rsmA*. Because AlgR is part of a two-component system that is important for both acute and chronic infections (19, 32), it was necessary to address the mechanism for AlgR activation of *rsmA* in order to create a framework to understand how AlgR regulation of *rsmA* might impact virulence gene expression. AlgR binds a consensus sequence CCGTTCGTC (21, 48, 49), and phosphorylation is thought to enable AlgR to bind potential binding sites that deviate from this consensus, such as the sites found in the *fimU* promoter (32, 47). However, the *rsmA* promoter deviates from the AlgR-binding consensus, and AlgR phosphorylation was not required for *rsmA* expression. This argues against phosphorylation as the sole mechanism for AlgR binding less well-conserved consensus sequences. The studies using the *mucA22* mutant strain, the *algZ* mutant, and the D54N mutant are consistent with the conclusion that AlgR phosphorylation is not required for *rsmA* activation. As AlgR phosphorylation is not required for alginate expression (22), our results are consistent with the conclusion that AlgR activates *rsmA* expression in mucoid strains.

The mechanism for AlgR activation of *rsmA* is by directly binding upstream of the AlgU-dependent promoter, further supporting our model (Fig. 8). Gel shift studies determined that two AlgR-binding sites are required for *in vitro* binding. The location of the AlgR-binding sites, the *in vitro* gel shift analysis, transcriptional fusions, and RNase protection assay all support AlgR binding the distal promoter region of *rsmA* and are in accord with the activation of the AlgU-dependent promoter.

Our data suggest that AlgR plays a more important role in regulating *rsmA* expression in *mucA* mutant strains, such as those found in CF patients. If AlgU and AlgR activate *rsmA* expression, as predicted by our model (Fig. 8), this would explain why AlgR did not appear to play a significant role in regulating *rsmA* in the wild-type PAO1 strain (Fig. 1B and C, 2, and 3). From this work, we conclude that AlgR and AlgU play a greater role in *rsmA* activation in *mucA* mutant strains. However, it is possible that *in vivo* conditions differ and that nonmucoid wild-type strains also have increased regulation of *rsmA* under other conditions.

The *mucA* mutation leads to AlgU and AlgR activation of *rsmA*, suggesting that RsmA may regulate specific targets in *mucA* mutant strains. RsmA is a posttranscriptional regulator that binds mRNAs, in many cases, at or near the ribosome-binding site of targets (54). It is likely that RsmA has unknown targets in a *mucA* mutant background due to transcriptional differences between nonmucoid and mucoid strains (55–59). Therefore, RsmA will bind only to targets that are present at a given time, supporting RsmA working as a rheostat. An alternative explanation is that our conditions of growth in the laboratory environment do not represent the conditions in the CF lung. While this is quite obvious, it is known that the mutation in *mucA* leads to alginate production *in vivo* (11, 60, 61). In addition, nonmucoid *P. aeruginosa* also has active AlgU and AlgR during infection because of alginate secretion by nonmucoid *P. aeruginosa* from CF patients (62, 63). Therefore, it is highly probable that AlgU and AlgR activity *in vivo* would increase RsmA levels during infection. What role RsmA plays due to AlgU and AlgR regulation *in vivo* is not known.

The significance of AlgU and AlgR control of *rsmA* is demonstrated by the analyses of RsmA targets. The posttranscriptional activity of the RsmA targets, *tssA1* and *hcnA*, was greater in the *mucA22* background than in the wild-type PAO1 strain (Fig. 7). We also observed a lack of posttranscriptional activity on RsmA targets when *algU* or *algR* was inactivated in a *mucA22* mutant strain (Fig. 7), due to the decreased expression of *rsmA*. Therefore, we conclude that RsmA is active in an *mucA22* mutant strain and that RsmA activity requires AlgU and AlgR to increase *rsmA* gene expression in this background. The correlation between increased AlgR activity and increased RsmA activity in *mucA* mutants also supports these systems acting as a rheostat to fine-tune gene expression, as opposed to an on/off switch. When AlgU and AlgR increase RsmA levels, the indirect effects of these two regulators may be due to RsmA. Further work is necessary to pursue this exciting discovery that could help explain the exclusive expression of particular virulence genes in a given background.

The role of AlgU and AlgR on the RsmA-antagonizing small RNAs *rsmY* and *rsmZ* is likely indirect. While our Northern blot data were not strong, they did support our transcriptional fusion analysis. Our transcriptional fusions mirrored what was seen in a previous study (28), supporting the idea of AlgR indirectly controlling *rsmY* and *rsmZ* expression. We hypothesize that AlgU and AlgR activities may coincide with factors that lead to increased GacA phosphorylation, resulting in the increased expression of *rsmY* and *rsmZ*. Further work is necessary to establish the mechanism for increased expression of the small RNAs in a *mucA* background.

Another question raised by our study is how RsmA remains active when the antagonizing small RNAs are increased. One possibility is that RsmA preferentially binds its targets in the *mucA* mutant strains better than the antagonizing small RNAs. This might also result from other regulators, either protein or other small RNAs that are currently unknown and affect the ability of RsmA to interact with the antagonizing small RNAs RsmY and RsmZ.

Altogether, our work demonstrates that AlgR is required for increased *rsmA* expression in *mucA* mutant strains. Phosphorylation of AlgR was not required for *rsmA* activation. The increased RsmA levels in a *mucA22* mutant strain result in increased RsmA activity, even though the antagonizing small RNAs are also increased. What roles RsmA plays and how RsmA functions in chronic infecting strains are not known. To better understand the role of RsmA, we are currently investigating new possible RsmA targets in AlgU-active strains. A further understanding of the RsmA regulon in *mucA*

mutants may provide additional insight into how *P. aeruginosa* becomes such a successful CF pathogen and has implications for the important role of RsmA in all types of *P. aeruginosa* infections.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains used in this study are presented in Table 1. *Escherichia coli* strains were maintained on LB (Difco) plates or broth without or with antibiotics as appropriate. *Pseudomonas aeruginosa* strains were grown on *Pseudomonas* isolation agar (PIA), LB, or Vogel-Bonner minimal medium (64) and supplemented with the appropriate concentration of antibiotics. For *E. coli*, antibiotics were used at the following concentrations when appropriate: 10 μg/ml tetracycline, 15 μg/ml gentamicin, 100 μg/ml ampicillin, and 35 μg/ml kanamycin. For *Pseudomonas* strains, antibiotics were used at the following concentrations: 150 μg/ml gentamicin, 50 μg/ml tetracycline, and 300 μg/ml carbenicillin. For allelic exchange, sucrose was supplemented at 10% in YT (1% tryptone and 0.5% yeast extract) medium.

Mutant construction. All PCR products were amplified from *P. aeruginosa* PAO1, unless otherwise noted, using Q5 polymerase (New England BioLabs). Crossover PCR (SOE'ing) (65) was used to construct deletion mutations and to clone into the suicide vector pEX18Tc or pEX18Gm (66). All cloned constructs were confirmed via sequencing. *P. aeruginosa* strains were conjugated with *E. coli* as a donor strain and the pRK2013-containing helper strain (67). Conjugations were performed overnight on LB plates at 30°C, and conjugations were plated for single-crossover mutants on the appropriate selective media. Merodiploids were grown without selection and then screened for sucrose sensitivity on YT–10% sucrose plates. Mutations were confirmed using PCR with primers containing the suffix intF and intR shown in Table 1 and sequencing of the resulting PCR fragment. Hemagglutinin (HA) tagging of proteins was accomplished using primers containing the HA tag at the 3′ end of the gene and introduced as described above using the suicide vector pEX18Gm.

***algR* mutant construction.** The wild-type *algR* genomic region was amplified using Q5 (NEB) and primers algRXbaIR and algRHindIIIF and cloned into pEX18Tc. Site-directed mutagenesis was performed using the algRD54XbaIF/algRD54NR primers for the D54N allele or the algRD54EF/algRD54ER primer pair for the D54E mutation. The *algZ* mutant was constructed using the algZHSDMF/algZHSDMR primers. The primers were phosphorylated and used in site-directed mutagenesis, in accordance with the manufacturer's instructions, using Q5 (NEB). Constructs were analyzed by restriction enzyme analysis and sequencing. Mutant strains were constructed using homologous recombination, as described above, and were checked using PCR and the algRintF/algRintR primer pair and digestion with the appropriate restriction enzyme. Additional PCR amplicons were sequenced to confirm the mutation in each strain using the same primers. Further confirmation of mutants was done using phenotypic assays.

Transcriptional and translational leader fusion analysis. Upstream DNA fragments containing promoter regions were generated by using primers listed in Table 1 in conjunction with Q5 polymerase (New England BioLabs). PAO1 genomic DNA was used as the template. PCR products were cloned into pMiniT (NEB) and then subcloned into miniCTX*lacZ* using the restriction enzymes HindIII and BamHI, HindIII and EcoRI, or KpnI and BamHI (NEB). The *rsmA* transcriptional fusions have been previously described (31). To construct *rsmY* and *rsmZ* transcriptional fusions, the rsmYTFFEcoRI/rsmYTFR and rsmZTFF/rsmZTFR primer pairs, respectively, were used. PCR products were purified, cut with restriction enzymes, and inserted into the EcoRI and BamHI sites of miniCTX*lacZ* using T4 DNA ligase (NEB). The leader fusion vector was constructed by annealing the lacUV5NotI/lacUV5BamHI primer pair together and cloning into the NotI/BamHI site of CTXCP (31). Translational/leader fusions were constructed using the tssA1annealF/R and hcnAannealF/R primer pairs (Table 1) and were cloned into the ScaI/BamHI site of the leader fusion vector. Fusion constructs were confirmed by sequencing and conjugated into *P. aeruginosa* strains by triparental conjugation. Strains were selected for tetracycline resistance and then conjugated with pFLP2 to remove vector sequences (66). Strains were selected for carbenicillin resistance, grown overnight without selection, and plated on YT medium with 10% sucrose to select for the loss of pFLP2. Individual colonies were patch-plated onto VBMM CB300 and PIA to ensure the loss of pFLP2. To confirm the presence of the fusion constructs, PCR was performed using the forward primer used to construct the fusion and the reverse primer lacZRforTF (Table 1). β-Galactosidase activity was determined by incubating cell extracts with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml), as described by Miller (68). A strain carrying the empty vector miniCTX*lacZ* was also conjugated into PAO1 and assayed, and this background (28 Miller units) was subtracted from all transcriptional fusions. The translational/leader fusion backbone CTXCPlacUV5 had no background activity. All mucoid strains were confirmed mucoid at the end of each experiment by plating on PIA plates to ensure all colonies were mucoid. Three biological replicates were reproduced for all assays.

AlgR purification. The *algR* gene was PCR amplified using Q5 (NEB) and PAO1 chromosomal DNA using oligonucleotides algRSal1F and algRNot1R (Table 1). A 754-bp SalI/NotI fragment was cloned into pGEX-4T-3 (Novagen) to be expressed as a glutathione *S*-transferase–AlgR (GST-AlgR) fusion protein. The resulting plasmid (pGEX-4TAlgR) was transformed into *E. coli* BL21(DE3) (NEB) cells and incubated overnight. The colonies from this transformation were collected, inoculated into LB supplemented with 100 μg/ml ampicillin, and grown to an optical density at 600 nm (OD_{600}) of 0.6; 0.2 mM isopropyl-β-D-galactopyranoside (IPTG) was added to induce AlgR expression for 4 h at 15°C. The cells were collected by centrifugation (6,740 $\times$ *g*, 15 min), washed once in 20 ml of phosphate-buffered saline (PBS), and resuspended in 1 ml of PBS containing protease inhibitors (Thermo Fisher). AlgR was purified from this supernatant using the GST spin purification kit (Thermo Fisher). After binding of the fusion protein and

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant properties[a]	Reference or source
E. coli strains		
NEB5α	*fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44* ϕ*80*Δ(*lacZ*)M15 *gyrA96*	New England BioLabs
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r	72
pRK2013	Helper strain	67
Plasmids		
pEX18Tc	Allelic exchange vector	66
pEX18Gm	Allelic exchange vector	66
Δ*rsmA* pEX18Tc	Allelic exchange for *rsmA* nonpolar deletion	31
Δ*algR* pEX18Tc	Allelic exchange for *algR* nonpolar deletion	47
algZHSDM/pEX18Gm	Allelic exchange for making *algZ* mutant	This study
*rsmA*HA/pEX18Gm	Allelic exchange for *rsmA* HA allele	31
pGEX4T-3	GST fusion vector	GE Healthcare
pGEX4T-3 *algR*	AlgR purification	This study
miniCTX*lacZ*	Transcriptional fusion vector	73
rsmATF1-*lacZ*	Transcriptional fusion	31
rsmATF3-*lacZ*	Transcriptional fusion	31
rsmATF2-*lacZ*	Transcriptional fusion	31
rsmYTF1-*lacZ*	Transcriptional fusion	This study
rsmYTF2-*lacZ*	Transcriptional fusion	This study
rsmYTF3-*lacZ*	Transcriptional fusion	This study
rsmZTF1-*lacZ*	Transcriptional fusion	This study
rsmZTF2-*lacZ*	Transcriptional fusion	This study
*lacUV5*CTXCP	Leader fusion vector	This study
lacUV5 tssA-lacZ	*tssA1* leader fusion	This study
lacUV5 hcnA-lacZ	*hcnA* leader fusion	This study
P. aeruginosa strains		
PAO1	Wild type	74
Δ*algR* mutant	*algR* mutant	75
D54N mutant	*algR* with asparagine instead of aspartate at residue 54	This study
D54E mutant	*algR* with glutamate instead of aspartate at residue 54	This study
algZ mutant	*algZ* mutant	This study
mucA22 mutant	*mucA22* mutant	76
mucA22 Δ*algR* mutant	*mucA22* Δ*algR* mutant	This study
*mucA22*D54N mutant	*mucA22* mutant strain with *algR* with asparagine instead of aspartate at residue 54	This study
*mucA22*D54E mutant	*mucA22* mutant strain with *algR* with glutamate instead of aspartate at residue 54	This study
mucA22 algZ mutant	*mucA22* mutant strain, *algZ* mutation	This study
Δ*rsmA* mutant	*rsmA* nonpolar deletion	31
mucA22 Δ*rsmA* mutant	*rsmA* nonpolar deletion in *mucA22* strain	31
Δ*rsmY* mutant	*rsmY* nonpolar deletion mutant	This study
Δ*rsmZ* mutant	*rsmZ* nonpolar deletion mutant	This study
PAO1 rsmATF1-l*acZ*	*rsmA* transcriptional fusion strain	31
Δ*algR* TF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
D54N rsmATF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
D54E rsmATF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
*mucA22*D54N rsmATF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
*mucA22*D54E rsmATF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
mucA22 algZ mutant	*rsmA* transcriptional fusion strain	This study
mucA22 TF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	31
mucA22 Δ*algR* rsmATF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
PAO1 rsmATF3-*lacZ*	*rsmA* transcriptional fusion strain	31
Δ*algR* rsmATF3-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
mucA22 TF3-*lacZ* mutant	*rsmA* transcriptional fusion strain	31
mucA22 Δ*algR* rsmATF3-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
PAO1 rsmATF2-*lacZ*	*rsmA* transcriptional fusion strain	31
Δ*algR* rsmATF2-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
mucA22 rsmATF2-*lacZ* mutant	*rsmA* transcriptional fusion strain	31
mucA22 Δ*algR* rsmATF2-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
PAO1 rsmYTF1-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
Δ*algR* rsmYTF1-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
algZ rsmYTF1-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
mucA22 rsmYTF1-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study

(Continued on next page)

TABLE 1 (Continued)

Strain or plasmid	Genotype or relevant properties[a]	Reference or source
mucA22 Δ*algR* rsmYTF1-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
mucA22 algZ mutant	*rsmY* transcriptional fusion strain	This study
PAO1 rsmYTF2-*lacZ*	*rsmY* transcriptional fusion strain	This study
Δ*algR* rsmYTF2-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
mucA22 rsmYTF2-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
mucA22 Δ*algR rsm*YTF2-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
PAO1 rsmYTF3-*lacZ*	*rsmY* transcriptional fusion strain	This study
mucA22 rsmYTF3-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
PAO1 rsmZTF1-*lacZ*	*rsmZ* transcriptional fusion strain	This study
Δ*algR* rsmZTF1-*lacZ* mutant	*rsmZ* transcriptional fusion strain	This study
algZ rsmZTF1-*lacZ* mutant	*rsmZ* transcriptional fusion strain	This study
mucA22 mutant	*rsmZ* transcriptional fusion strain	This study
mucA22 Δ*algR* rsmZTF1-*lacZ* mutant	*rsmZ* transcriptional fusion strain	This study
mucA22 algZ rsmZTF1-*lacZ* mutant	*rsmZ* transcriptional fusion strain	This study
PAO1 rsmZTF2-*lacZ*	*rsmZ* transcriptional fusion strain	This study
mucA22 rsmZTF2-*lacZ* mutant	*rsmZ* transcriptional fusion strain	This study
PAO1HA	PAO1 with epitope-tagged RsmA	31
Δ*algR*HA mutant	Δ*algR* mutant with epitope-tagged RsmA	This study
*mucA22*HA mutant	*mucA22* mutant with epitope-tagged RsmA	31
mucA22 Δ*algR*HA mutant	*mucA22* Δ*algR* mutant with epitope-tagged RsmA	This study
PAO1 *lacUV5 hcnA-lacZ* mutant	PAO1 with *hcnA* leader fusion	This study
mucA22 lacUV5 hcnA-lacZ	*mucA22* mutant with *hcnA* leader fusion	This study
mucA22 Δ*rsmA lacUV5 hcnA-lacZ* mutant	*mucA22* Δ*rsmA* mutant with *hcnA* leader fusion	This study
mucA22 Δ*algU lacUV5 hcnA-lacZ* mutant	*mucA22* Δ*algU* mutant with *hcnA* leader fusion	This study
mucA22 Δ*algR lacUV5 hcnA-lacZ* mutant	*mucA22* Δ*algR* mutant with *hcnA* leader fusion	This study
PAO1 *lacUV5 tssA1-lacZ*	PAO1 with *tssA1* leader fusion	This study
mucA22 lacUV5 tssA1-lacZ mutant	*mucA22* mutant with *tssA1* leader fusion	This study
mucA22 Δ*rsmA lacUV5 tssA1-lacZ* mutant	*mucA22* Δ*rsmA* mutant with *tssA1* leader fusion	This study
mucA22 Δ*algU lacUV5 tssA1-lacZ* mutant	*mucA22* Δ*algU* mutant with *tssA1* leader fusion	This study
mucA22 Δ*algR lacUV5 hcnA-lacZ* mutant	*mucA22* Δ*algR* mutant with *tssA1* leader fusion	This study

[a]Km^r, kanamycin resistance.

washing the column, AlgR was cleaved away from GST with 10 U of thrombin/column overnight at 22°C. Purified protein was dialyzed using a Slide-A-Lyzer (Thermo Fisher) and storage buffer (20% glycerol, 20 mM Tris [pH 7.5], 5 mM $MgCl_2$, and 1 mM dithiothreitol [DTT]) overnight at 22°C. The identity of the purified protein was determined by mass spectrometry. The purity of AlgR was visually determined in a Coomassie-stained 7.5% electrophoresis gel (SDS-PAGE).

EMSAs. Purified AlgR was used in electrophoretic mobility shift assays (EMSAs) using either PCR amplicons or annealed primers using the LightShift kit, in accordance with the manufacturer's instructions (Thermo Fisher Scientific). For *rsmA*, the rsmAPE3F/rsmAPE3R, rsmAPE1F/rsmAEcoRIR, or rsmAPE3F/rsmAEcoRIR primer pair was used to produce PCR amplicons using *Taq* polymerase (NEB). Amplicons were gel extracted and biotinylated using the 3′ biotinylation kit (Thermo Fisher Scientific). Annealed primers were heated to 95°C for 5 min and cooled to room temperature. The annealed primers were biotinylated and incubated with purified AlgR. Purified AlgR was incubated at increasing concentrations to determine the suitable concentrations to be used. The nonspecific competitor poly(dI-dC) at 25 ng/μl was used for all gel shift reactions. DNA and protein were electrophoresed through 5% or 10% native polyacrylamide gels, transferred to a nylon membrane using a semidry apparatus (Hoefer), UV cross-linked, developed using the chemiluminescence detection kit (Thermo Fisher Scientific), and visualized using a charge-coupled-device (CCD) camera (ProteinSimple). The gel shift assays were repeated at least two times.

Western blot analysis. *P. aeruginosa* strains were grown in LB at 37°C for 8 h. The bacteria were collected by centrifugation, resuspended in sterile PBS, and lysed by sonication using a Branson sonifier. Total protein concentrations were determined by the Bradford protein assay (Bio-Rad, Carlsbad, CA). Cell extracts (5 to 10 μg) were separated by 15% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked and probed using a 1:2,500 dilution of anti-HA monoclonal antibody in blocking buffer (Thermo Fisher, Pittsburgh, PA), followed by a 1:30,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (69). Densitometry of Coomassie-stained SDS-PAGE gels was used to standardize the Western blots using total protein analysis, as described previously (70, 71). Detection was performed using the ECL Plus kit (Thermo Fisher) and chemiluminescence detection (ProteinSimple, Santa Clara, CA). The blots and Coomassie-stained SDS-PAGE were analyzed using ImageJ analysis. All Western blotting was repeated at least four times.

Primer extension. Total RNA (15 μg) from *P. aeruginosa* was isolated using the PureLink RNA minikit (Life Technologies, Grand Island, NY). Radiolabeled primers rsmYPE1 and rsmZPE1 (see Table 1) were used in a reverse transcription reaction using ThermoScript (Life Technologies). A temperature of 42°C was used for the extension. The same primer used for primer extension was also used in a sequencing reaction with the *rsmY* or *rsmZ* upstream sequence in pGEM-T (Promega, Madison, WI) using the

TABLE 2 Primers used in this study

Primer name	Sequence	Use[a]
algRintF	GCAACTGGACTGGCAGGTGC	Mutant
algRintR	CGCGACTGGTCATCGGCAG	Mutant
algRBamHIR	GCGCGGATCCGTCAGAGCTGATGCATCAGACG	Mutant
algZHindIIIF	GCGCAAGCTTCTCTCGCTGCAACAAGAAACGG	Mutant
algZSDMcheckF	CAGCTGGGCGGAGAACTGAC	Mutant
algRD54XbaIF	AACATCCGCATGCCCGGTCTAGACGGC	Mutant
algRD54NR	CAGCAGGACGATATCGGGCTTGA	Mutant
algRD54EF	GTCCTTCTAGAGATCCGCATGC	Mutant
algRD54ER	GATATCGGGCTTGAGGCTGTCG	Mutant
algZHSDMF	GAATTCCTGTTCAACAGCCTGAACAG	Mutant
algZHSDMR	CGGGCGAATCCGCGCCTGCA	Mutant
algRSalIF	CCTGTCGACATGAATGTCCTGATTGTCGATGAC	AlgR purification
algRNotIR	AGCCGCGGCCGCTCAGAGCTGATGCATCAGACG	AlgR purification
rsmARXbaI	GCGCTCTAGAGCACGGTGATCCTGCAGACC	Mutant
rsmAFHindIII	GCGCAAGCTTCGGCAACATCACCACGCTGGG	Mutant
rsmASDMcheckF	GCCAAGGTTTCCATCGTCGG	PCR check
rsmYRPAF	CTTGGACGTCAGGACATTGCGCAGGAAG	Northern blot
rsmYT7RPAR	TAATACGACTCACTATAGGGAGACAATAAAAAACCCCGCCTTTTGGG	Northern blot
rsmZRPAF	GGGCCCCACTCCTGCGTACAGGGAACAC	Northern blot
rsmZRPART7	TAATACGACTCACTATAGGGAGACAAAAAAAAGGGGCGGGGTATTAC	Northern blot
rsmYTFFEcoRI	GCGCGAATTCGTGTTGCCGTCGGTCCGGC	TF
rsmYTFRBamHI	GCGCGGATCCTTCCTGCGCAATGTCCTGACG	TF
rsmYTFFUAS	GCGCGCGGCCGCCAGCGTGTAAGCCAAGGCTTAC	TF
rsmYshortEcoRIF	GCGCGAATTCAAACGGCGGTGGTTTTGGCTG	TF
dnrTFRBamHI	GCGCGGATCCCCATGCTGGGAAGGCTCGC	TF
rsmZKpnIF	GCGCGGTACCCGTCTGGCGCAGAAGGGCG	Mutant
rsmZXbaIR	GCGCTCTAGAGAGCGAAACCGCCAACATCC	Mutant
rsmZSOEF	GTTTTCTGGCAGGTTGCGGGATTTGCCTGCCGTTTTACTCGTC	Mutant
rsmZSOER	GACGAGTAAAACGGCAGGCAAATCCCGCAACCTGCCAGAAAAC	Mutant
rsmZintF	GCGCGCGGCCGCACCGATCCGTGCGAGCTG	Mutant
rsmZintR	CATGCAGGAATTCATCGAGCTG	Mutant
rsmYKOF	CGCGGAGCTCCTGTTCACTCGAAGCACTCCAG	Mutant
rsmYKOR	CGCGTCTAGATTCGCCAACTCCGCTATTTCG	Mutant
rsmYSOEF	GCGTAATCTTCAAACCGTCAGGATCCTGCGGCCCGAGGAAAACCGCGTCG	Mutant
rsmYSOER	CGACGCGGTTTTCCTCGGGCCGCAGGATCCTGACGGTTTGAAGATTACGC	Mutant
rsmYintF	CCTGGAGCTGGACGGGAG	Mutant
rsmYintR	GGAATTCAGGAAGGTGTCCC	Mutant
lacUV5NotI	GGCCGCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGGAG	Leader fusion vector
lacUV5BamHI	GATCCTCCACACATTATACGAGCCGGAAGCATAAACTGTAAAGC	Leader fusion vector
tssA1annealF	GATCCTTCGATGATAGGGAGATCGTCACCGTGCTG	Leader fusion
tssA1annealR	CAGCACGGTGACGATCTCCCTATCATCGAAG	Leader fusion
hcnAannealF	GATCCACTCTCTCTCACGGATGAAAGGGCAATGCAC	Leader fusion
hcnAannealR	GTGCATTGCCCTTTCATCCGTGAGAGAGAGTG	Leader fusion
5SrRNAI	TTGGACAGGATGGGGTTGGA	Northern blot
5SrRNA2T7	TAATACGACTCACTATAGGGA GACGATTGTGTGTTGTAAGG	Northern blot
lacZScaIF	GCTATGACCATGATTAGTACTGATTCACTGGCCGTC	TLF vector
lacZRXhoI	CCCCTCGAGCAGACATGGCCTGCCCGGTTATTA	TLF vector
lacZRforTF	GATGTGCTGCAAGGCGATTAAG	SEQ
rsmASDMAlgRF	GAATTCGGCAGGAACTTTCATTCCGGC	RPA
rsmARPAR	TAATACGACTCACTATAGGGAGAGATAAAAATTAATGGTTTGGCTCTTG	RPA
rsmARSDMF	CCTTTTGTCGTTTTTGAGAATTCGGCAGGAACTTTCATTC	Gel shift
rsmARSDMR	GAATGAAAGTTCCTGCCGAATTCTCAAAAACGACAAAAGG	Gel shift
algDBS1F	CGCCTCCTGGCGCTACCGTTCGTCCCTCCGACACCCCTGCTGCGTCGC	Gel shift
algDBS1R	GCGACGCAGCAGGGGTGTCGGAGGGACGAACGGTAGCGCCAGGAGGCG	Gel shift
rsmARBS1F	GCGCCTTTTGTCGTTTTTGACCGTTTGGCAGGAACTTTCATTCCG	Gel shift
rsmARBS1R	CGGAATGAAAGTTCCTGCCAAACGGTCAAAAACGACAAAAGGCGC	Gel shift
pscFRBSF	GCTGGCGGAGTGTCGCCGGGAACTGGCCAGAGG	Gel shift
pscFRBSR	CCTCTGGCCAGTTCCCGGCGACACTCCGCCAGC	Gel shift
rsmAPE1F	GCGCGGTACCAAGGATCGCGCTCTTGATTTCTGCGGATCCGCCGCCATTTCTT	Gel shift
rsmAPE3F	GCGCGGTACCGCTGACAGGCGAAAGGCG	Gel shift
rsmAPE3R	GCGCGGATCCTCACCCAGTATTGACCAGTCC	Gel shift
rsmAEcoRIR	GCGCGAATTCACGAGTCAGAATCAGCATTCCTTTC	Gel shift
rsmAR1SDMannealF	CCGCGGAATTCGTCGTTTTTGACCGTTTGGCAGGAACTTTC	Gel shift
rsmAR1SDMannealR	GAAAGTTCCTGCCAAACGGTCAAAAACGACGAATTCCGCGG	Gel shift
rsmAR2SDMannealF	CCGCGGAATTCGTCGTTTTTGAGAATTCGGCAGGAACTTTC	Gel shift
rsmAR2SDMannealR	GAAAGTTCCTGCCGAATTCTCAAAAACGACGAATTCCGCGG	Gel shift
rsmAGSWTannealF	CCGCGCCTTTTGTCGTTTTTGACCGTTTGGCAGGAACTTTC	Gel shift
rsmAGSWTannealR	GAAAGTTCCTGCCAAACGGTCAAAAACGACAAAAGGCGCGG	Gel shift

[a]TF, transcriptional fusion; TLF, translational fusion; SEQ, sequencing primer; RPA, RNase protection assay.

Sequenase 7-deaza-deoxy-GTP (7-deaza-dGTP) kit (Affymetrix, Cleveland, OH). Both primer extensions and sequencing reactions were run on a 6% acrylamide-8 M urea gel. The gel was dried and extension products detected using autoradiography.

***In vitro* transcription and RPA.** The *rsmA*, *rsmY*, and *rsmZ* probes for RNase protection assay (RPA) were synthesized using a PCR product generated with primers that incorporated a T7 promoter sequence at the 5′ end and using the MAXIscript T7 kit (Life Technologies) (Table 1). The reverse primer containing the T7 promoter sequence contained a nonhomologous sequence to discriminate between the full-length probe and protected fragments. The primers rsmASDMAlgRF and rsmARPAR were used to produce the PCR product for *rsmA*. The rsmYRPAT&R/rsmYRPAF primer pair for *rsmY* or rsmZRPAT7RPAF/rsmZRPAF primer pair for *rsmZ* was used to produce probes for the appropriate target. Probes were labeled by biotin-11-UTP (Life Technologies) at a ratio of 4:6 with UTP and were gel purified after *in vitro* transcription. Five micrograms of total RNA from each *P. aeruginosa* strain was precipitated with 800 pg of labeled probe and resuspended in hybridization buffer [80% formamide, 400 mM NaCl, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 1 mM EDTA], heated 95°C for 5 min, and incubated overnight at 42°C. Negative controls using the probe incubated with 10 μg of yeast RNA were also included. Digestion of unhybridized RNA was performed using RNase A at 25 μg/ml and RNase T1 at 10 U/ml. A biotinylated RNA ladder (Kerafast) was also used to determine the size of protected probe fragments. The reactions were run on a 5% acrylamide-8 M urea gel for *rsmA* and a 10% acrylamide-8 M urea gel for *rsmY* and *rsmZ*. RNA was transferred to a positively charged membrane (Hybond N+; Amersham Biosciences) using a semidry blotting system and 0.5× Tris-borate-EDTA (TBE; Hoefer, Holliston, MA). Nucleic acid was UV cross-linked and probes detected using the chemiluminescent nucleic acid detection module (Thermo Scientific). After washing, the blot was incubated for 5 min and developed using a FluorChem M (ProteinSimple). RNase protection assays were performed at least three times.

Northern blot analysis. Total RNA was extracted from strains using the PureLink RNA minikit (Thermo Fisher). RNA was quantitated using a NanoDrop (Thermo Fisher). Two to 5 μg of total RNA was electrophoresed through a 10% acrylamide-8 M urea gel using formaldehyde loading buffer. After electrophoresis, RNA was transferred using a semidry blotter (Hoefer, Holliston, MA) at 300 mAmp for 45 min and the membranes cross-linked using UV light (Bio-Rad). Probes produced by *in vitro* transcription were biotin labeled and hybridized to the membrane using ULTRAhyb buffer (Thermo Fisher) at 65°C overnight. The primers used to make probes for *rsmY* and *rsmZ* are listed in Table 2. For *rsmY*, the rsmYRPAF/rsmYT7RPAR primer pair was used. For *rsmZ*, the rsmZRPAF/rsmZRPART7 primer pair was used. Membranes were washed with two 5-min low-stringency washes at room temperature and 2 high-stringency washes for 15 min at 65°C. Blots were developed using the chemiluminescence detection kit (Thermo Fisher), in accordance with the manufacturer's instructions. For normalization, 5S RNA probes (Table 2) made by *in vitro* transcription were used using primers 5SrRNAI and 5SrRNA2T7. Northern blotting was repeated more than three times.

Statistical analyses. Statistical analyses were performed using Prism 6.0 (GraphPad software, La Jolla, CA). Transcriptional and translational fusions and densitometry analysis were compared using a one-way analysis of variance (ANOVA) with Tukey's posttest.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00048-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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