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The AlgZR Two-Component System Recalibrates the RsmAYZ Posttranscriptional Regulatory System To Inhibit Expression of the *Pseudomonas aeruginosa* Type III Secretion System

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Pseudomonas aeruginosa **causes chronic airway infections in cystic fibrosis (CF) patients. A classic feature of CF airway isolates is the mucoid phenotype. Mucoidy arises through mutation of the *mucA* anti-sigma factor and subsequent activation of the AlgU regulon. Inactivation of *mucA* also results in reduced expression of the Vfr transcription factor. Vfr regulates several important virulence factors, including a type III secretion system (T3SS). In the present study, we report that ExsA expression, the master regulator of T3SS gene expression, is further reduced in *mucA* mutants through a Vfr-independent mechanism involving the RsmAYZ regulatory system. RsmA is an RNA binding protein required for T3SS gene expression. Genetic experiments suggest that the AlgZR two-component system, part of the AlgU regulon, inhibits ExsA expression by increasing the expression of RsmY and RsmZ, two small noncoding RNAs that sequester RsmA from target mRNAs. Epistasis analyses revealed that increasing the concentration of free RsmA, through either *rsmYZ* deletion or increased RsmA expression, partially restored T3SS gene expression in the *mucA* mutant. Furthermore, increasing RsmA availability in combination with Vfr complementation fully restored T3SS expression. Recalibration of the RsmAYZ system by AlgZR, however, did not alter the expression of other selected RsmA-dependent targets. We account for this observation by showing that ExsA expression is more sensitive to changes in free RsmA than other members of the RsmA regulon. Together, these data indicate that recalibration of the RsmAYZ system partially accounts for reduced T3SS gene expression in *mucA* mutants.**

Pseudomonas aeruginosa is a Gram-negative bacterium with the ability to cause both acute and chronic infections (1, 2). Immunocompromised individuals often develop acute *P. aeruginosa* infections that can become systemic, with lethal sepsis as a common outcome (3). Isolates from acute *P. aeruginosa* infections are generally characterized by the presence of a functional type III secretion system (T3SS) (4, 5). The T3SS injects multiple effectors directly into the host cell cytoplasm, where they function to deregulate signal transduction mechanisms and promote phagocytic avoidance and host cell killing (6–8). In contrast to acute infections, the airways of most adults with the genetic disorder cystic fibrosis (CF) are chronically colonized with *P. aeruginosa*. CF patients are initially colonized with environmental strains of *P. aeruginosa* that subsequently undergo diversification via mutation and selection by the CF airway environment to generate a population of cells adapted for persistence, host immune defense, antibiotic resistance, and biofilm formation (9–11).

A common phenotype observed in CF isolates is the loss of T3SS gene expression (12, 13). One mechanism that accounts for reduced T3SS expression involves the MucA/AlgU signal transduction system (see Fig. 8 for a model). MucA is a membrane-bound anti-sigma factor that sequesters the alternative sigma factor AlgU to control expression of ~300 genes (14–16). Inactivating mutations in *mucA* are common in CF isolates and result in high-level expression of the AlgU regulon (14, 17, 18). The AlgU regulon includes membrane stress responses and the alginate biosynthetic gene cluster, overproduction of which results in the mucoid phenotype (14, 17, 19). The presence of mucoid *P. aeruginosa* isolates in the CF airways correlates with a decline in lung function, advanced disease, and increased morbidity (20, 21). Multiple regulators, including the AlgZR two-component system, control alginate biosynthesis (22–24). Elevated AlgU activity in the *mucA* mutant results in increased AlgZR activity (22). Previous studies found that *mucA* mutation or overexpression of AlgZR from a plasmid results in a significant decrease in T3SS gene expression (25, 26). One potential explanation for decreased T3SS expression in *mucA* mutants is that the activity of the cyclic AMP (cAMP)-Vfr signaling pathway is also decreased (26). Vfr promotes the expression of multiple acute virulence factors, including the T3SS (27). The mechanism by which Vfr stimulates T3SS gene expression is unknown.

Similar to Vfr, RsmA also has a positive effect on T3SS gene expression (28). RsmA is a member of the CsrA family of RNA binding proteins and functions as a global posttranscriptional regulator of gene expression. The RsmAYZ system inversely controls numerous phenotypes associated with acute and chronic infections (28–30). CsrA/RsmA proteins typically inhibit gene expression by binding to conserved 5′-ANGGAN-3′ motifs presented within stem-loop secondary structures of target mRNAs (31–35). CsrA/RsmA binding sites are often located adjacent to or overlap the Shine-Dalgarno sequences of target genes (31, 35, 36). CsrA/RsmA proteins can also stimulate gene expression by altering

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mRNA stability, mRNA secondary structure, and/or ribosomal recruitment (34, 37). Although RsmA has a positive effect on T3SS gene expression, the RsmA-controlled target has not been identified.

The intracellular concentration of free RsmA dictates the output of the RsmA regulon, including T3SS expression. T3SS expression is reduced when the concentration of free RsmA is low (28, 30). The concentration of free RsmA is regulated by two small noncoding RNAs, RsmY and RsmZ (38, 39). RsmY and RsmZ each possess 4 to 6 binding sites for homodimeric RsmA and act to titrate free RsmA away from mRNA targets (40, 41). Changes in RsmYZ expression, therefore, control the output of the RsmA regulon (39, 42). The GacAS two-component system regulates RsmYZ expression (42). The GacA response regulator directly binds to and activates the P_{rsmY} and P_{rsmZ} promoters when phosphorylated by the GacS sensor kinase (38, 41–44). Two orphan sensor kinases, RetS and LadS, reciprocally control RsmYZ expression through GacAS (45–47). RetS inhibits RsmYZ expression by forming a heterodimer with GacS which prevents phosphorylation of GacA (48). Conversely, LadS activates RsmYZ expression through GacAS, but a mechanism of action has not yet been described (47).

In the present study, we identified a second AlgZR-dependent mechanism that inhibits T3SS gene expression. This mechanism works concurrently with and independently from the documented Vfr defect mediated by AlgZR and inhibits ExsA expression at a posttranscriptional level, reducing the availability of RsmA. In conjunction with restoration of Vfr expression, increased free RsmA allowed for full restoration of T3SS gene expression in the *mucA* mutant. Finally, we propose that ExsA expression responds to smaller alterations in free RsmA than other RsmA-dependent targets.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and sample preparation. The bacterial strains used in this study are listed in Table S1 in the supplemental material. *Escherichia coli* DH5α was used for all cloning and maintained on LB-Miller agar plates with gentamicin (15 μg/ml), tetracycline (15 μg/ml), or ampicillin (100 μg/ml) as necessary. *P. aeruginosa* strains were cultured on Vogel-Bonner minimal medium (49) agar plates and supplemented with gentamicin (100 μg/ml) as required. For transcriptional and translational reporter assays, *P. aeruginosa* strains were cultured in Trypticase soy broth supplemented with 100 mM monosodium glutamate, 1% glycerol, and 1 mM EGTA to an absorbance at 600 nm (A_{600}) of 1.0. In some reporter assays, 0.4% arabinose as added to induce expression of plasmid-borne genes under the transcriptional control of an arabinose-inducible promoter. β-Galactosidase activity was measured using the substrate *ortho*-nitrophenyl-galactopyranoside (ONPG) (Miller units) or chlorophenol red–β-D-galactopyranoside (CPRG) (CPRG units) (50). The reported values for all reporter assays denote the average from at least three independent experiments divided by the activity of a control strain, and error bars represent the standard deviation.

Whole-cell lysate samples were prepared by pelleting 1.25 ml (A_{600} = 1.0) of cells, suspending the pellet in 0.25 ml SDS-PAGE sample buffer, sonicating for 10 s, and heating for 5 min at 95°C. Samples were analyzed by SDS-PAGE followed by immunoblotting using the indicated antibodies and processed using enhanced chemiluminescent fluorescence detection reagents (Thermo Scientific, Rockford, IL).

Mutant and expression vector construction. The transcriptional and translational reporters, complementation plasmids, and allelic exchange vectors used in this study are listed in Table S2 in the supplemental material. The details of the plasmid construction are provided in Table S3, and the primers are listed in Table S4. All PCR products were amplified from PA103 genomic DNA and verified by DNA sequencing. Allelic exchange vectors were introduced into the desired strains as previously described (51). Transcriptional and translational reporter fusions were introduced into the CTX phage attachment site of the desired strain as previously described (52). To construct p3UY51 (pUY68-$P_{lacUV5\text{-}exsCEBA'\text{-}'lacZ}$), the P_{lacUV5} promoter was generated via PCR using primers 33914045-44895670 and inserted into the HindIII-NcoI sites of pUY68. Subsequent translational fusions (p3UY51-$P_{lacUV5\text{-}exsCEB'\text{-}'lacZ}$, p3UY51-$P_{lacUV5\text{-}exsCE'\text{-}'lacZ}$, p3UY51-$P_{lacUV5\text{-}exsC'\text{-}'lacZ}$, and p3UY51-$P_{lacUV5\text{-}exsD'\text{-}'lacZ}$) were constructed by cloning PCR products into the EcoRI-BamHI sites of p3UY51. The mini-CTX1-$P_{lacUV5\text{-}exsCEBA'\text{-}'lacZ}$ reporter was generated by cloning the $P_{lacUV5\text{-}exsCEBA}$ fragment from p3UY51 into the EcoRI-BamHI sites of mini-CTX1. p3UY71 (pEXG2Δ*vfr*) was constructed by PCR amplifying the *vfr* deletion allele from PA103 *vfr* using primers 7299293-7299296 and cloning into the HindIII-EcoRI sites of pEXG2. The pAlgR and pMucA expression vectors were constructed by cloning the respective genes into the XbaI-SacI or EcoRI-XbaI sites of pJN105, respectively. The p2UY137 (pVfr) expression vector was constructed by cloning *vfr* into the NdeI-SacI sites of P_{35B}. pUY68-$P_{PA4492'\text{-}'lacZ}$ was constructed by cloning the P_{PA4492} promoter region into the EcoRI-BamHI sites of pUY68. pEXG2Δ*gacA* was constructed by PCR amplifying upstream (78241012-78241013) and downstream (78241010-78241011) flanking regions of *gacA* and cloning into the KpnI-XbaI sites of pEXG2. The mini-CTX-$P_{rsmY\text{-}lacZ}$, $P_{rsmZ\text{-}lacZ}$, $P_{gacA\text{-}lacZ}$, $P_{gacS\text{-}lacZ}$, $P_{retS\text{-}lacZ}$, and $P_{ladS\text{-}lacZ}$ reporters were constructed by cloning the desired promoter fragment into the EcoRI-BamHI sites of mini-CTX1. p3UY36 (pEXG2-P_{35B}) was constructed in three steps. First, the *rpoC* transcriptional terminator from pOM90 was amplified using primers 45403337-45420277 and cloned into the SphI restriction site of p2UY36, creating vector p2UY104. Second, the terminator-P_{35B} promoter fragment was excised from p2UY104 and inserted into the HindIII-XhoI sites of pEXG2. Finally, the *exsCEBA* region from p2UY104 was cloned into the SphI-SacI sites of the above-described vector, creating p3UY36.

Statistical analyses. Two-tailed unpaired *t* tests were used for data analyses when comparing two groups. One-way analysis of variance (ANOVA) and Tukey's posttest were used to determine statistical significance when comparing three or more groups. Statistical analyses were performed using Prism 5.0c (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Reduced T3SS gene expression in *mucA* mutants occurs through Vfr-dependent and -independent mechanisms. Previous studies found that Vfr is required for maximal T3SS gene expression (27) and that Vfr expression is reduced in a *mucA* mutant (26). Vfr insufficiency, therefore, is the simplest model to account for reduced T3SS gene expression in a *mucA* mutant and is consistent with the finding that overexpression of Vfr in *trans* restores T3SS gene expression in a *mucA* mutant (26). If reduced T3SS gene expression in the *mucA* mutant were solely attributable to Vfr insufficiency then T3SS gene expression should remain unchanged in a *mucA vfr* double mutant. Using the previously described $P_{exsD\text{-}lacZ}$ transcriptional reporter as a marker for T3SS gene expression (53), however, we found that reporter activity and ExsA expression were significantly reduced in the *mucA vfr* double mutant compared to either of the single mutants alone (Fig. 1A). To further examine the role of Vfr, we exploited the fact that plasmid-expressed AlgR phenocopies a *mucA* mutant with regard to T3SS gene expression (25, 26) (Fig. 1B). We again predicted that if Vfr insufficiency were the sole explanation for decreased T3SS expression, then AlgR expressed in a *vfr* single mutant should result in no further decrease in T3SS expression. Expression of AlgR in the *vfr* mutant, however, resulted in a further decrease

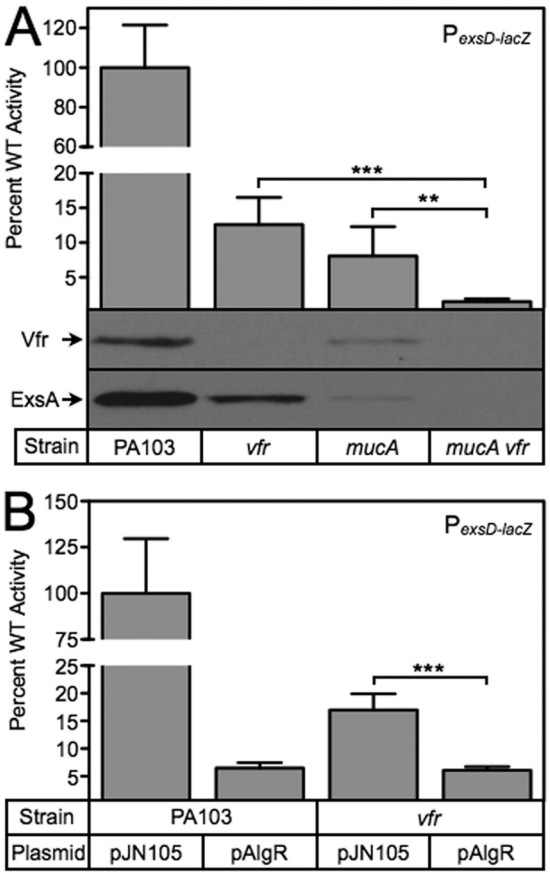

FIG 1 Both Vfr-dependent and -independent mechanisms account for reduced T3SS gene expression in *mucA* mutants. (A) The indicated strains carrying the ExsA-dependent $P_{exsD\text{-}lacZ}$ transcriptional reporter were cultured under low-Ca^{2+} conditions (1 mM EGTA) until the A_{600} reached 1.0. Cells were then harvested, assayed for β-galactosidase activity, and immunoblotted for ExsA and Vfr. The activity of wt PA103 was 2,291 Miller units. All of the values reported in this paper were normalized to those for wt PA103 or wt PA103 carrying the vector control (pJN105) and represent the average from at least three experiments. **, $P < 0.001$; ***, $P < 0.0001$. (B) PA103 and a *vfr* mutant were transformed with a vector control (pJN105) or an arabinose-inducible AlgR expression plasmid (pAlgR). The resulting strains were cultured in the presence of 1 mM EGTA and 0.4% arabinose and assayed for $P_{exsD\text{-}lacZ}$ reporter activity. The activity of wild-type PA103 carrying the vector control was 2,319 Miller units. ***, $P < 0.0001$.

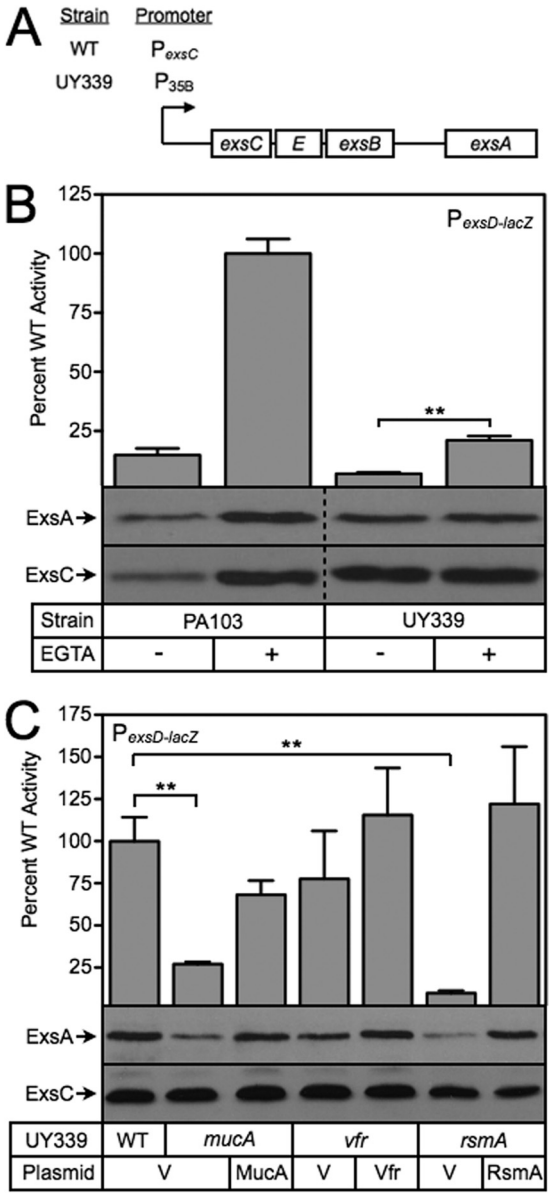

FIG 2 Both *mucA* and *rsmA* mutants have a posttranscriptional defect in T3SS gene expression. (A) Diagram of the *exsCEBA* operon and transcriptional control by the ExsA-dependent P_{exsC} promoter. In strain UY339, P_{exsC} has been replaced with P_{35B}, a constitutive derivative of the *lacUV5* promoter. (B) PA103 and strain UY339 were cultured under noninducing (high Ca^{2+}, without EGTA) or inducing (low Ca^{2+}, with EGTA) conditions for T3SS gene expression and assayed for $P_{exsD\text{-}lacZ}$ reporter activity. The activity of wild-type PA103 cultured under inducing conditions was 2,150 Miller units. For both panels A and B, whole-cell lysates were immunoblotted for ExsC and ExsA. **, $P < 0.001$. (C) The indicated strains carrying either a vector control (pJN105) or MucA, Vfr, or RsmA expression vector (pMucA, pVfr, and pRsmA) were cultured under inducing conditions for T3SS gene expression and assayed for $P_{exsD\text{-}lacZ}$ reporter activity. Values reported in panel C were normalized to those for the wt UY339 strain carrying the vector control (777 Miller units). **, $P < 0.001$.

in $P_{exsD\text{-}lacZ}$ reporter activity relative to that of the vector control (pJN105). These combined data indicate that Vfr insufficiency alone cannot account for reduced T3SS gene expression in the *mucA* mutant and suggest that an additional inhibitory mechanism exists.

***mucA* and *rsmA* mutants have a posttranscriptional defect in ExsA expression.** The ExsECD regulatory cascade serves as the primary means to activate T3SS gene expression in response to calcium limitation or host cell contact and controls ExsA-dependent transcription (54). Transcription of the *exsCEBA* operon is positively autoregulated by ExsA through the P_{exsC} promoter (55). We hypothesized that transcription of the *exsCEBA* operon and/or expression of an *exsCEBA* gene product(s) is disrupted in the *mucA* mutant. To differentiate transcriptional from posttranscriptional effects on expression of the *exsCEBA* operon, we replaced the ExsA-dependent P_{exsC} promoter with a constitutive derivative of the P_{lacUV5} promoter (P_{35B}), resulting in strain UY339 (Fig. 2A). PA103 and UY339 were then cultured under noninducing (high Ca^{2+}, without EGTA) and inducing (low Ca^{2+}, with EGTA) conditions for T3SS gene expression and assayed for $P_{exsD\text{-}lacZ}$ reporter activity and ExsC and ExsA expression. Consis-

tent with a previous report, $P_{exsD\text{-}lacZ}$ reporter activity and ExsC and ExsA expression were induced when the wild-type (wt) strain (PA103) was cultured under low-Ca^{2+} conditions (56) (Fig. 2B). In strain UY339, $P_{exsD\text{-}lacZ}$ reporter activity was only modestly induced in response to low-Ca^{2+} conditions. Importantly, steady-state levels of ExsC and ExsA expression were similar under both high- and low-Ca^{2+} conditions, indicating that *exsCEBA* transcription is constitutive in strain UY339.

Having verified that e*xsCEBA* expression is constitutive in strain UY339, we introduced in-frame *mucA* and *vfr* deletions into the UY339 background and assayed the resulting strains for ExsA-dependent $P_{exsD\text{-}lacZ}$ transcriptional reporter activity. The UY339 *mucA* mutant demonstrated a significant reduction in reporter activity that was partially complemented by MucA expressed from a plasmid (Fig. 2C). Although ExsA expression levels were significantly reduced in the UY339 *mucA* mutant, ExsC expression levels were similar in strain UY339 and the UY339 *mucA* mutant. Since *exsC* and *exsA* are encoded on the same polycistronic mRNA (55) (Fig. 2A), this finding raised the possibility that the *mucA* mutant has a posttranscriptional defect in ExsA expression.

In contrast to our findings for the UY339 *mucA* mutant, the UY339 *vfr* mutant had $P_{exsD\text{-}lacZ}$ activity similar to that of the parental UY339 strain (Fig. 2C). These data suggest that decreased $P_{exsD\text{-}lacZ}$ activity in the UY339 *mucA* mutant does not result from the loss of Vfr expression and support our hypothesis that AlgZR inhibits T3SS gene expression through a second, Vfr-independent, mechanism.

Previous studies found that RsmA has a positive effect on T3SS gene expression, but the mechanism has not been determined (45). Given the established role of RsmA as a posttranscriptional regulator of gene expression, we hypothesized that the *mucA* defect in ExsA expression might be linked to RsmA function. As an initial test of this hypothesis, we introduced an *rsmA* mutation into strain UY339 and measured $P_{exsD\text{-}lacZ}$ reporter activity and ExsC and ExsA protein levels. Interestingly, the phenotype of UY339 *rsmA* was similar to that of the UY339 *mucA* mutant in that $P_{exsD\text{-}lacZ}$ reporter activity and ExsA expression were reduced while ExsC levels remained unchanged (Fig. 2C). $P_{exsD\text{-}lacZ}$ reporter activity and ExsA expression were fully restored in the *rsmA* mutant by the RsmA complementation plasmid.

To further examine the posttranscriptional defect in ExsA expression and determine whether other components of the ExsCED regulatory cascade are also subject to posttranscriptional control, we integrated $P_{lacUV5\text{-}exsC'\text{-}'lacZ}$, $P_{lacUV5\text{-}exsCE'\text{-}'lacZ}$, $P_{lacUV5\text{-}exsCEB'\text{-}'lacZ}$, $P_{lacUV5\text{-}exsCEBA'\text{-}'lacZ}$, and $P_{lacUV5\text{-}exsD'\text{-}'lacZ}$ translational reporters into the CTX phage attachment sites of the *mucA* and *rsmA* mutants (see Fig. S1 in the supplemental material). Each reporter was transcribed from the constitutive P_{lacUV5} promoter to avoid detection of regulatory events occurring at the transcriptional level. The only reporter in the *exsCEBA* operon to demonstrate a significant change in activity was the $P_{lacUV5\text{-}exsCEBA'\text{-}'lacZ}$ translational fusion, with ~2-fold-decreased activity in both the *mucA* and *rsmA* mutants (Fig. 3A). To verify that the decreased activity of the $P_{lacUV5\text{-}exsCEBA'\text{-}'lacZ}$ reporter was due to a posttranscriptional defect and not reduced activity of a cryptic, unknown promoter within the *exsCEBA* operon, we constructed a transcriptional fusion ($P_{lacUV5\text{-}exsCEBA\text{-}lacZ}$) with the same sequence used to construct the translational fusion. $P_{lacUV5\text{-}exsCEBA\text{-}lacZ}$ transcriptional reporter activity was similar in wt PA103 and the *mucA* and *rsmA* mutants, further confirming

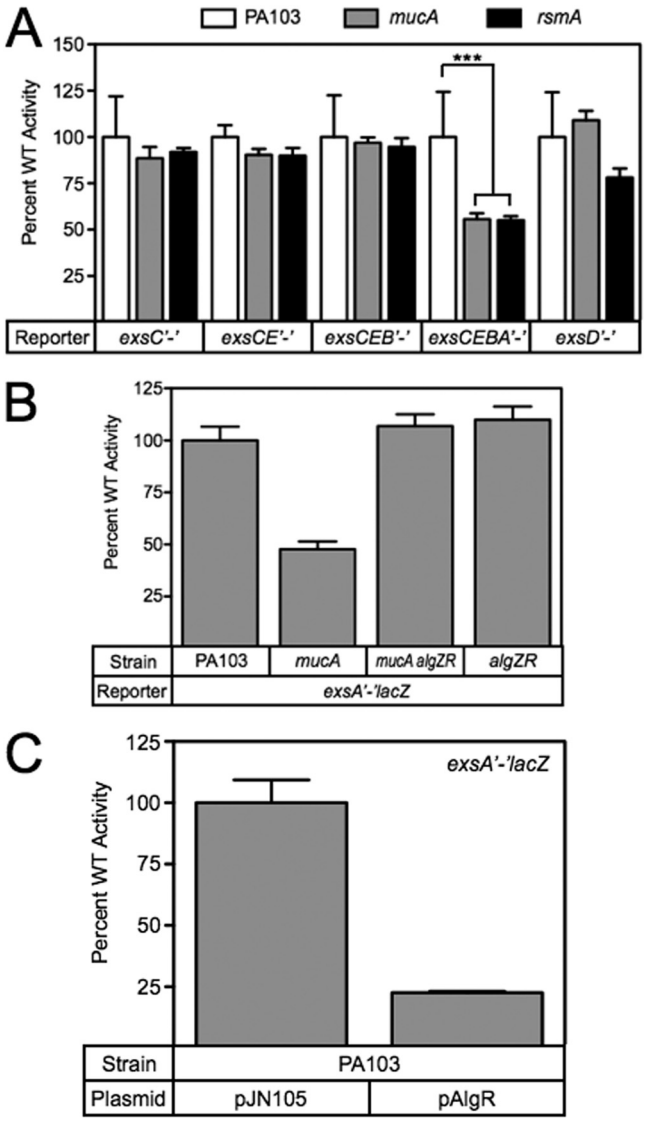

FIG 3 The posttranscriptional defect in *mucA* and *rsmA* mutants is specific to ExsA. (A and B) The indicated strains carrying *exsC'-'lacZ* (371 CPRG units), *exsCE'-'lacZ* (1,379 CPRG units for PA103), *exsCEB'-'lacZ* (292 CPRG units for PA103), *exsCEBA'-'lacZ* (33 CPRG units for PA103), or *exsD'-'lacZ* (256 CPRG units for PA103) translational fusions, driven from the constitutive P_{lacUV5} promoter, were cultured under inducing conditions for T3SS gene expression and assayed for reporter activity. ***, $P < 0.0001$. (C) PA103 carrying the *exsCEBA'-'lacZ* translational reporter was transformed with a vector control (pJN105, 34 CPRG units) or an AlgR expression vector. The resulting strains were cultured under inducing conditions for T3SS in the presence of 0.4% arabinose and assayed for *exsCEBA'-'lacZ* reporter activity.

that the decreased activity of the $P_{lacUV5\text{-}exsCEBA'\text{-}'lacZ}$ translational fusion resulted from a posttranscriptional defect (see Fig. S2 in the supplemental material).

Reduced T3SS gene expression in *mucA* mutants is mediated through the AlgZR two-component regulatory system (25, 26). To determine whether this was also true for ExsA expression, the $P_{lacUV5\text{-}exsCEBA'\text{-}'lacZ}$ translational reporter was introduced into the *mucA algZR* mutant. As predicted, deletion of *algZR* in the *mucA* mutant background restored $P_{lacUV5\text{-}exsCEBA'\text{-}'lacZ}$ activity to wt levels (Fig. 3B). We also found that expression of AlgR in the wt

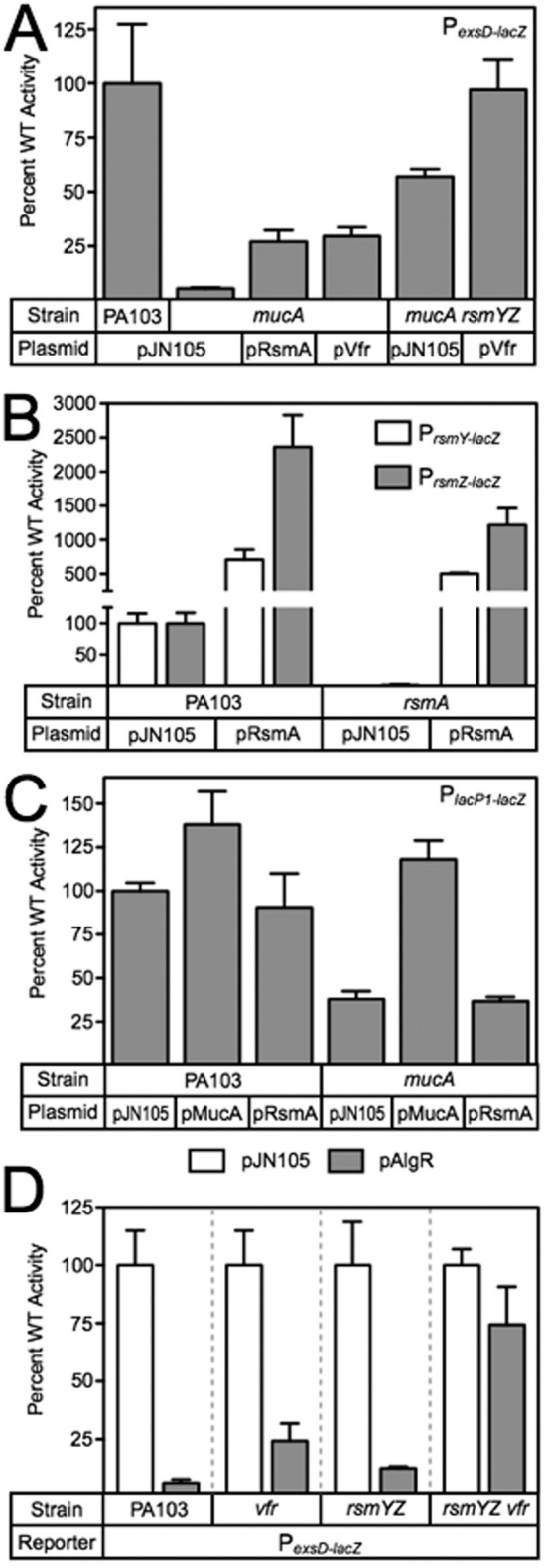

FIG 4 Combined restoration of RsmA and Vfr activities restores T3SS gene expression in the *mucA* mutant. (A) The indicated strains transformed with either a vector control (pJN105) or Vfr and RsmA expression vectors were cultured under T3SS-inducing conditions and assayed for $P_{exsD-lacZ}$ reporter activity. The activity of PA103 carrying the vector control was 1,548 Miller units. (B) PA103 and an *rsmA* mutant carrying the $P_{rsmY-lacZ}$ or $P_{rsmZ-lacZ}$ transcriptional reporter were transformed with a vector control (pJN105) or an RsmA expression vector. The resulting strains were cultured under T3SS-inducing conditions until the A_{600} reached 1.0 and were assayed for reporter activity. The activities of the $P_{rsmY-lacZ}$ and $P_{rsmZ-lacZ}$ reporters in PA103 carrying the vector control were 3,031 and 491 Miller units, respectively. (C) The indicated strains transformed with either a vector control (pJN105) or MucA and RsmA expression vectors (pMucA or pRsmA) were cultured under T3SS-inducing conditions and assayed for expression of the Vfr-dependent $P_{lacP1-lacZ}$ reporter. The activity of PA103 carrying the vector control was 792 Miller units. (D) The indicated strains were transformed with either a vector control (pJN105) or an AlgR expression vector and were assayed for $P_{exsD-lacZ}$ reporter activity under T3SS-inducing conditions and in the presence of 0.4% arabinose. The activities of PA103 and the *vfr*, *rsmYZ*, and *rsmYZ vfr* mutants carrying the vector control were 1,875, 266, 6,652, and 1,356 Miller units, respectively.

background reduced $P_{lacUV5-exsCEBA'-'lacZ}$ reporter activity ~4-fold compared to that of a vector control strain (Fig. 3C). Together, these data indicate that *rsmA* and *mucA* mutants have a similar posttranscriptional defect in ExsA expression and that the defect in the *mucA* mutant is AlgZR dependent.

Increasing the level of free RsmA partially restores T3SS gene expression in a *mucA* mutant. The finding that *mucA* and *rsmA* mutants both possess a posttranscriptional defect specific to ExsA suggested that the phenotypes are linked to one another. We hypothesized that the concentration of free RsmA is reduced in the *mucA* mutant. To test this idea, we introduced an arabinose-inducible RsmA expression vector (pRsmA) into the *mucA* mutant and found that $P_{exsD-lacZ}$ reporter activity increased 3- to 4-fold (Fig. 4A). Compared to the case in the parental strain carrying the vector control, however, plasmid-expressed RsmA was unable to fully restore T3SS gene expression in the *mucA* mutant.

A previous study reported that expression of the RsmY and RsmZ regulatory RNAs is significantly reduced in an *rsmA* mutant (Fig. 4B) (57). We observed the same using $P_{rsmY-lacZ}$ and $P_{rsmZ-lacZ}$ transcriptional reporters and also saw that plasmid-expressed RsmA results in a significant increase in $P_{rsmY-lacZ}$ and $P_{rsmZ-lacZ}$ reporter activities (Fig. 4B) relative to that of the vector control. Based on those findings, we hypothesized that plasmid-expressed RsmA results in only a modest increase in the level of free RsmA, owing to negative feedback by increased expression of the RsmA-sequestering RsmY and RsmZ RNAs. Such an outcome could account for the limited restoration of $P_{exsD-lacZ}$ reporter activity by pRsmA in the *mucA* mutant (Fig. 4A). To test this possibility, we constructed a *mucA rsmYZ* triple mutant and found that $P_{exsD-lacZ}$ reporter activity was increased 2-fold relative to that in the *mucA* mutant (Fig. 4A). These combined data indicate that increased RsmA activity, whether it be through increased RsmA expression or elimination of the RsmA-antagonizing RsmYZ small RNAs (sRNAs), partially restores T3SS expression in the *mucA* mutant.

Restoration of Vfr expression in conjunction with increased free RsmA restores T3SS expression in a *mucA* mutant. The observations that Vfr expression is reduced in a *mucA* mutant (26) and that increased free RsmA partially restores T3SS gene expression in a *mucA* mutant (Fig. 4A) prompted us to test whether plasmid-expressed RsmA would also restore Vfr expression in the *mucA* background. To examine this possibility, we used the previously described Vfr-dependent $P_{lacP1-lacZ}$ transcriptional reporter integrated at the chromosomal CTX phage attachment site (52). Whereas pMucA provided in *trans* increased $P_{lacP1-lacZ}$ reporter activity to wt levels in the *mucA* mutant, pRsmA had no

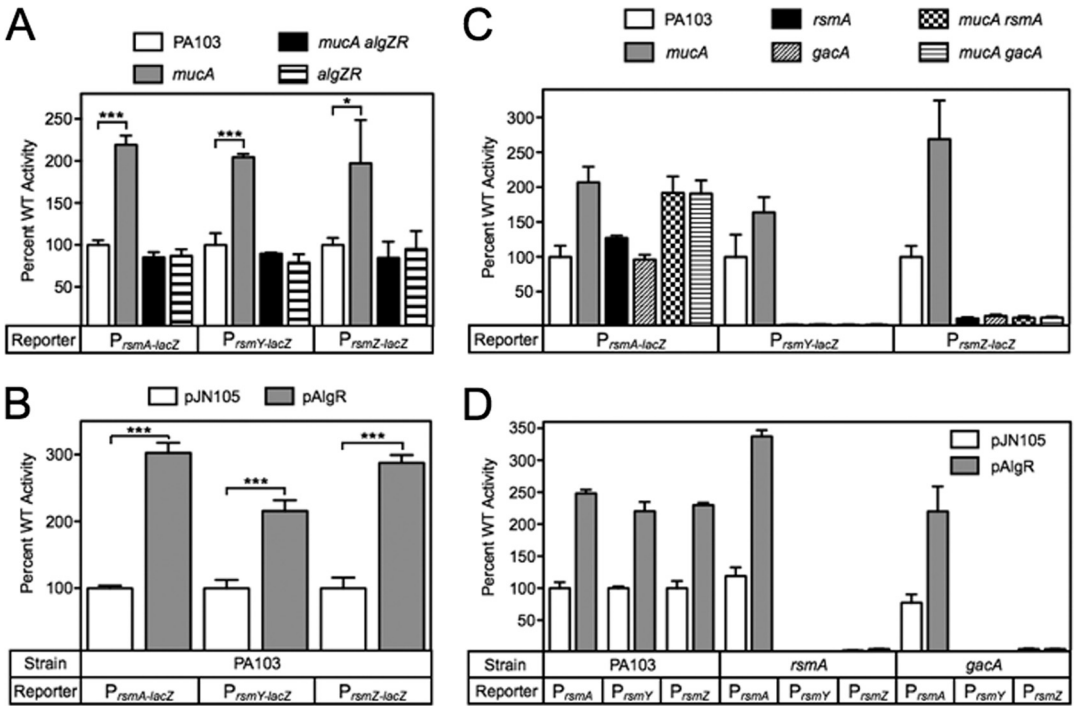

FIG 5 $P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, and $P_{rsmZ\text{-}lacZ}$ transcriptional reporter activities are elevated in the *mucA* mutant. (A to C) The indicated strains carrying either a $P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, or $P_{rsmZ\text{-}lacZ}$ transcriptional reporter were cultured under inducing conditions for T3SS gene expression until the A_{600} reached 1.0 and were assayed for reporter activity. (A) The activities of the $P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, and $P_{rsmZ\text{-}lacZ}$ reporters in PA103 were 45, 828, and 104 Miller units, respectively. (B) The activities of the $P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, and $P_{rsmZ\text{-}lacZ}$ reporters in PA103 carrying the vector control were 50, 3,370, and 464 Miller units, respectively. (C) The activities of the $P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, and $P_{rsmZ\text{-}lacZ}$ reporters in PA103 were 48, 670, and 151 Miller units, respectively. (D) The indicated strains carrying either a vector control (pJN105) or an AlgR expression vector were assayed for $P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, or $P_{rsmZ\text{-}lacZ}$ transcriptional reporter activities under T3SS-inducing conditions and in the presence of 0.4% arabinose. The activities of the $P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, and $P_{rsmZ\text{-}lacZ}$ reporters in PA103 carrying the vector control were 53, 3,277, and 602 Miller units, respectively. *, $P < 0.05$; ***, $P < 0.0001$.

effect on reporter activity (Fig. 4C). The latter finding suggests that the defects in ExsA and Vfr expression observed in the *mucA* mutant occur through distinct mechanisms and that Vfr expression is reduced in the *mucA* mutant independently of RsmA.

Our data to this point suggested that the availabilities of Vfr and free RsmA are both limiting in the *mucA* mutant and that plasmid-expressed RsmA is unable to correct the Vfr expression defect (Fig. 4C). We reasoned, therefore, that simultaneous restoration of both Vfr and RsmA activity in the *mucA* mutant would restore T3SS gene expression to wt levels. Increasing Vfr expression in the *mucA* mutant or elevating the level of free RsmA (through *rsmYZ* deletion) stimulated $P_{exsD\text{-}lacZ}$ reporter activity 5-fold or 10-fold, respectively, relative to the vector controls (Fig. 4A). Combining both activities by expressing Vfr in the *mucA rsmYZ* mutant restored $P_{exsD\text{-}lacZ}$ reporter activity to wt levels (Fig. 4A). We conclude, therefore, that the loss of T3SS gene expression in the *mucA* mutant results from the combined loss of Vfr and RsmA activity.

Based upon the above findings, our working model is that AlgR reduces both Vfr expression and free RsmA levels to inhibit T3SS expression in a *mucA* mutant. One prediction of that model is that plasmid-expressed AlgR should no longer inhibit T3SS expression in an *rsmYZ vfr* triple mutant. Relative to the vector control, the pAlgR expression plasmid resulted in strong inhibition of $P_{exsD\text{-}lacZ}$ reporter activity in the *vfr* and *rsmYZ* mutants (Fig. 4D). In contrast, inhibition by pAlgR was significantly attenuated in the *rsmYZ vfr* triple mutant (Fig. 4D).

Expression of RsmA, RsmY, and RsmZ is increased in a *mucA* mutant. The reduction in the concentration of free RsmA in the *mucA* mutant could result from decreased RsmA expression and/or increased RsmYZ expression. To examine this, transcriptional reporters derived from the *rsmA*, *rsmY*, and *rsmZ* promoter regions ($P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, and $P_{rsmZ\text{-}lacZ}$) were introduced into wt PA103 and the *mucA*, *algZR*, and *mucA algZR* deletion strains. To our surprise, the activity of all three reporters was elevated ~2-fold in the *mucA* mutant compared to wt PA103 (Fig. 5A). The 2-fold increase in $P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, and $P_{rsmZ\text{-}lacZ}$ reporter activities was suppressed in the *mucA algZR* mutant, and this correlates with the finding that reduced T3SS gene expression in the *mucA* mutant also requires AlgR. This conclusion is further supported by the finding that expression of AlgR in wt PA103 increased the activity of the $P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, and $P_{rsmZ\text{-}lacZ}$ reporters 2- to 3-fold relative to that of the vector control (Fig. 5B). These data indicate that transcription of *rsmA*, *rsmY*, and *rsmZ* is increased in response to elevated AlgR activity.

The GacAS two-component system (42) and RsmA (Fig. 4B) are both required for RsmYZ expression. To determine whether the AlgR-dependent increase in RsmAYZ expression requires GacA and RsmA, we measured $P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, and $P_{rsmZ\text{-}lacZ}$ reporter activities in *gacA*, *mucA gacA*, *rsmA*, and *mucA rsmA* mutants. $P_{rsmY\text{-}lacZ}$ and $P_{rsmZ\text{-}lacZ}$ reporter activities were significantly decreased in the *gacA* and *rsmA* mutants and in the *mucA gacA* and *mucA rsmA* mutants (Fig. 5C). Additionally, expression of pAlgR in the *gacA* and *rsmA* mutants did not result in increased $P_{rsmY\text{-}lacZ}$

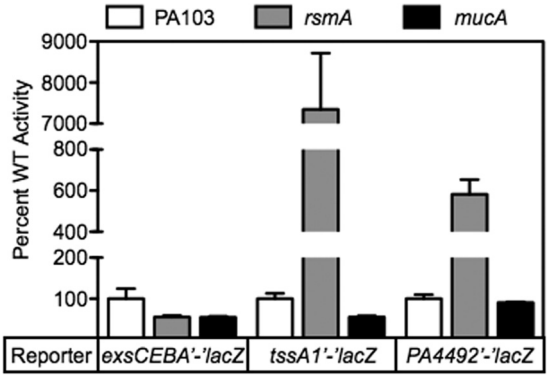

FIG 6 The RsmA regulon is not globally disrupted in the *mucA* mutant. The indicated strains carrying the $P_{lacUV5\text{-}exsCEBA'\text{-}'lacZ}$ (PA103 = 33 CPRG units), $P_{tssA1'\text{-}'lacZ}$ (PA103 = 3 CPRG units), or $P_{PA4492'\text{-}'lacZ}$ (PA103 = 19 CPRG units) translational reporter were cultured under T3SS-inducing conditions (with EGTA) until the A_{600} reached 1.0 and were assayed for reporter activity.

and $P_{rsmZ\text{-}lacZ}$ reporter activity (Fig. 5D), indicating that *rsmA* and *gacA* are epistatic to the *mucA* effect on $P_{rsmY\text{-}lacZ}$ and $P_{rsmZ\text{-}lacZ}$ expression. $P_{rsmA\text{-}lacZ}$ activity, however, showed a much different pattern and was unaffected by deletion of either *rsmA* or *gacA* in the context of a *mucA* mutant (Fig. 5C). In addition, the pAlgR-dependent increase in $P_{rsmA\text{-}lacZ}$ activity occurred independently of *rsmA* and *gacA* (Fig. 5D). Together, these data demonstrate that GacA and RsmA are required for increased RsmYZ expression in the *mucA* mutant but not for increased RsmA expression.

We hypothesized that increased RsmYZ expression in the *mucA* mutant may result from altered *gacA*, *gacS*, *retS*, and/or *ladS* expression. RetS and LadS are hybrid sensor kinase/response regulators that inhibit and promote RsmYZ expression, respectively, through GacAS (45–47). To test this idea, we constructed *gacA*, *gacS*, *retS*, and *ladS* transcriptional reporters ($P_{gacA\text{-}lacZ}$, $P_{gacS\text{-}lacZ}$, $P_{retS\text{-}lacZ}$, and $P_{ladS\text{-}lacZ}$) and measured activity in wt PA103 and *mucA* strains. Compared to in wt PA103, the $P_{gacA\text{-}lacZ}$, $P_{gacS\text{-}lacZ}$, $P_{retS\text{-}lacZ}$, and $P_{ladS\text{-}lacZ}$ reporters were unchanged in the *mucA* mutant (see Fig. S3A in the supplemental material). Additionally, expression of pAlgR in wt PA103 did not alter reporter activity compared to that in PA103 carrying a vector control, with the exception of the $P_{gacS\text{-}lacZ}$ reporter, which increased ~50% (see Fig. S3B in the supplemental material). Together, these data suggest that while RsmYZ expression is increased in the *mucA* mutant, expression of the GacAS/LadS/RetS regulatory components is unaffected.

RsmA targets display differential sensitivity to the level of free RsmA. To determine whether other RsmA-dependent phenotypes are altered in the *mucA* mutant, we constructed translational fusions with genes *tssA1* and PA4492. Both genes were placed under the control of their native promoters. A previous study found that RsmA inhibits expression of both genes at the posttranscriptional level through a direct interaction with their respective mRNAs (28). Consistent with the previous report, the $P_{tssA1'\text{-}'lacZ}$ and $P_{PA4492'\text{-}'lacZ}$ reporter activities were significantly elevated in the *rsmA* mutant relative to in wt PA103 (Fig. 6). In the *mucA* mutant, however, both reporter activities were similar to that in wt PA103. These data suggest that *mucA* mutation does not result in a global shift in expression of the RsmA regulon.

One model to account for our data is that the level of free RsmA

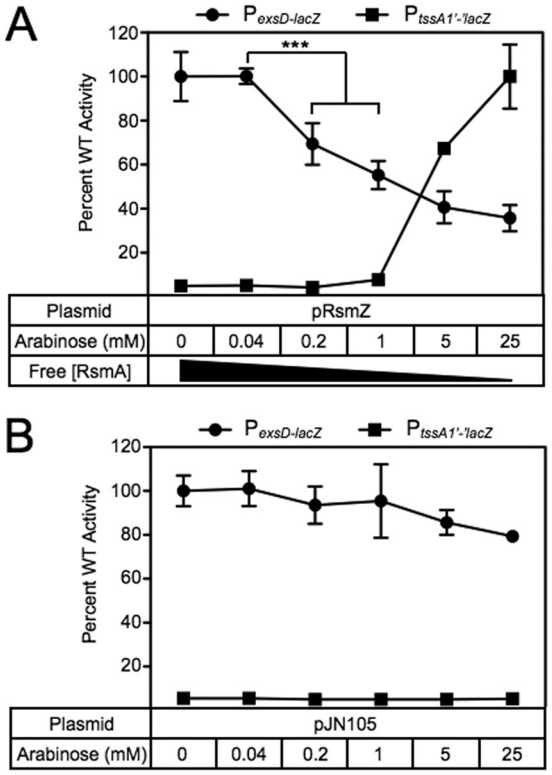

FIG 7 T3SS gene expression is sensitive to smaller changes in RsmA availability compared to TssA1. (A) PA103 transformed with an RsmZ expression vector (pRsmZ) was cultured under T3SS-inducing conditions (with EGTA) in the absence or presence of increasing concentrations of arabinose as indicated and was assayed for reporter activity. The activity of the $P_{exsD\text{-}lacZ}$ reporter was 2,374 Miller units in the absence of arabinose, and the activity of the $P_{tssA1'\text{-}'lacZ}$ reporter was 961 CPRG units in the presence of 25 mM arabinose. ***, $P < 0.0001$. (B) PA103 transformed with a vector control (pJN105) was cultured as described above and assayed for $P_{exsD\text{-}lacZ}$ and $P_{tssA1'\text{-}'lacZ}$ reporter activity.

is only modestly reduced in the *mucA* mutant and may be within a range that alters ExsA expression while leaving other RsmA targets (e.g., *tssA1*) unaffected. To test this model, we introduced an arabinose-inducible RsmZ expression vector into wt PA103 with the expectation that an increase in RsmZ would reduce the availability of free RsmA. The resulting strains were cultured under low-Ca^{2+} conditions over a range of arabinose concentrations and assayed for $P_{exsD\text{-}lacZ}$ and $P_{tssA1'\text{-}'lacZ}$ reporter activities. As expected, $P_{exsD\text{-}lacZ}$ reporter activity decreased when cells were cultured with increasing concentrations of arabinose (Fig. 7A). Conversely, the activity of the RsmA-repressible $P_{tssA1'\text{-}'lacZ}$ reporter increased in response to elevated arabinose. Importantly, pRsmZ expression with several arabinose concentrations (Fig. 7A, 0.2 mM and 1 mM arabinose) resulted in a significant decrease in $P_{exsD\text{-}lacZ}$ activity without significantly altering $P_{tssA1'\text{-}'lacZ}$ reporter activity. Neither $P_{exsD\text{-}lacZ}$ nor $P_{tssA1'\text{-}'lacZ}$ expression was altered significantly by the vector control (Fig. 7B). Together, these data suggest that ExsA expression is sensitive to smaller alterations in free RsmA levels compared to TssA1.

DISCUSSION

Clinical isolates from acute and chronic *P. aeruginosa* infections can be differentiated from one another by their patterns of viru-

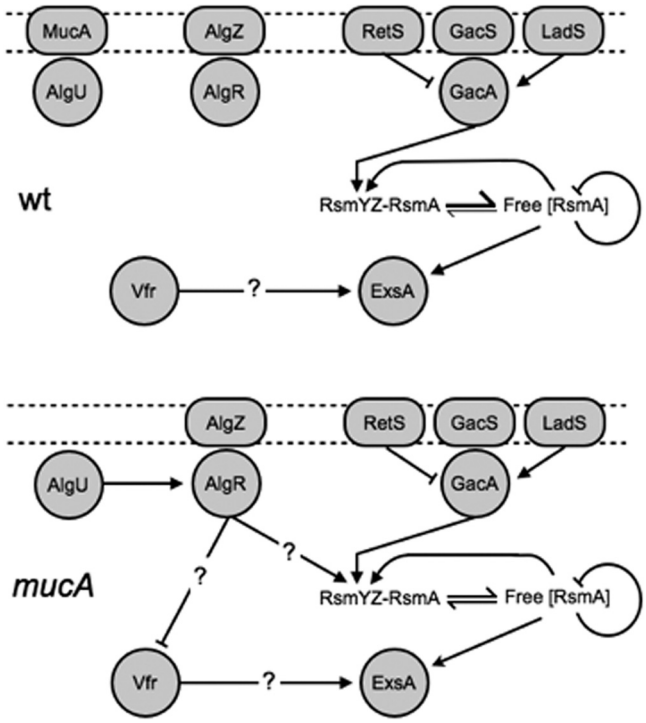

FIG 8 Model for AlgR-dependent inhibition of T3SS gene expression in the *mucA* mutant (see Discussion for details). Dashed lines depict the inner membrane. Question marks indicate regulatory mechanisms that have been observed but remain undefined by mechanism.

lence gene expression. Those patterns are controlled, in part, through global regulatory systems, including the AlgU-MucA alternative sigma factor, cAMP-Vfr signaling, and RsmAYZ systems. Previous studies established that *mucA*, *vfr*, and *rsmA* mutants each have a significant reduction in T3SS gene expression (25–28). In this study, we demonstrate that cross talk between these pathways coordinates T3SS gene expression. A key mediator of this cross talk is the AlgZR two-component system. Our data indicate that AlgR inhibits T3SS gene expression through two distinct mechanisms (Fig. 8). First, as reported previously, AlgR inhibits Vfr expression (26). Interestingly, a strain engineered to constitutively express the *exsCEBA* operon (UY339) was no longer dependent upon Vfr for maximal T3SS gene expression (Fig. 1A versus 2C). This finding suggests that Vfr may regulate transcription of the *exsCEBA* operon through the P_{exsC} promoter. Regulation by Vfr is likely indirect, however, as we have been unable to demonstrate direct binding of Vfr to the P_{exsC} promoter region (data not shown). The second AlgR-dependent inhibitory mechanism involves recalibration of the RsmAYZ regulatory system to inhibit ExsA expression at a posttranscriptional level. Although previous studies reported that T3SS expression is RsmA dependent, our finding that RsmA positively regulates ExsA expression at the posttranscriptional level is novel. Whether the effect of RsmA on *exsA* expression is direct or indirect and working at the level of translation, the level of mRNA stability, or in some other way will be the subject of future studies.

An alternative interpretation of our findings that we considered relates to the negative-feedback feature of the ExsCEDA regulatory cascade. Under noninducing conditions (high Ca^{2+}), the ExsD antiactivator forms a complex with ExsA to inhibit T3SS gene expression. We have proposed that modest changes in ExsA expression (perhaps as small as 2-fold) are sufficient to overcome ExsD-mediated inhibition (58). Increasing Vfr and RsmA activity in the *mucA* mutant, both of which have positive effects on ExsA expression, could nonspecifically suppress the true nature of the *mucA* defect. Nevertheless, we do not believe this is occurring, as neither Vfr nor RsmA alone (i.e., deletion of *rsmYZ*) is sufficient to restore T3SS gene expression in the *mucA* mutant. We propose, therefore, that it is the combined loss of Vfr and RsmA activity in the *mucA* mutant that accounts for reduced *exsA* expression and the corresponding lack of T3SS gene expression (Fig. 8). In support of that model, simultaneous restoration of both RsmA and Vfr activity in the *mucA* mutant fully restored T3SS activity to wt levels.

RsmAYZ expression is increased in *mucA* mutants through an AlgR-dependent mechanism (Fig. 5A and B). We used epistasis analyses to investigate the relationships between AlgR, the GacAS/LadS/RetS regulatory system, and RsmA itself. We found that previously known regulatory networks (GacAS and RsmA) were necessary for RsmYZ expression in the *mucA* mutant (Fig. 5C and D). These data suggest that AlgR works together with known regulatory systems to modify RsmYZ expression levels but does control them independently. For this reason, we do not believe that AlgR directly interacts with the *rsmY* and *rsmZ* promoter regions to control expression. AlgR also does not seem with work through regulation of GacAS/LadS/RetS expression (see Fig. S3A and B in the supplemental material). These data suggest that AlgR is a novel regulator of RsmYZ expression, but the mechanism of action remains undefined. Future studies will focus on whether AlgR directly activates P_{rsmA} transcription or whether AlgR acts to increase RsmA expression indirectly.

Although our data demonstrate that expression of the $P_{rsmA\text{-}lacZ}$, $P_{rsmY\text{-}lacZ}$, and $P_{rsmZ\text{-}lacZ}$ transcriptional reporters increased 2- to 3-fold in the *mucA* mutant (Fig. 5A), it is unclear how this could result in a net reduction in the level of free RsmA. Several lines of evidence indicate that RsmA homeostasis is tightly controlled. Data from this study and others have shown that *rsmYZ* transcription requires RsmA (Fig. 4B) (57). We also found that plasmid-expressed RsmA results in a significant increase in expression of the $P_{rsmY\text{-}lacZ}$ and $P_{rsmZ\text{-}lacZ}$ reporters. When RsmA expression is induced, therefore, there is a compensatory increase in RsmYZ that governs the level of free RsmA. In addition, a recent study found that RsmA autoregulates its own translation through a direct interaction with the *rsmA* mRNA (59). Increased RsmA expression in the *mucA* mutant, therefore, could result in a net decrease in free RsmA through inhibition of its own translation and through elevated production of RsmYZ, both of which are able to sequester 4 to 6 RsmA dimers (40, 41). Such a mechanism is predicated on the assumption that the concentration of free RsmA in wt cells is near the equilibrium binding constant for RsmYZ. Another mechanism that could reduce the level of free RsmA in the *mucA* mutant is increased expression of one or more transcripts that are targets for RsmA-mediated regulation. Increased expression of those targets could have a sequestering effect similar to that of RsmYZ, thereby resulting in a net decrease in free RsmA. A recent study found that fimbrial gene expression in *Salmonella enterica* serovar Typhimurium is controlled through a similar mechanism (60).

Several studies have shown that RsmA inhibits gene expression

through direct interactions with target mRNAs (28, 32). The prototypical member of the family, CsrA, can also function as a direct activator of gene expression (34). Although it is unclear whether the *exsA* mRNA is a direct RsmA target, there are several potential mechanisms that might account for our observation that the *exsA* translational reporter responds to smaller changes in RsmA availability than *tssA1*. The simplest is that the binding affinity of RsmA for the relevant T3SS target is lower than it is for *tssA1*. Under this scenario, the intracellular pool of free RsmA would have to be near the binding constant such that small fluctuations in free RsmA alter expression of low-affinity RsmA targets. Conversely, larger fluctuations in RsmA levels would be required to significantly alter the expression of high-affinity targets. Another potential explanation for the differential sensitivity of RsmA targets may be the inherent properties of the gene being controlled. For instance, the gene for a transcriptional regulator such as ExsA that participates in a positive feedback loop to autoregulate its own expression might be far more sensitive to changes in RsmA levels than a gene encoding a structural protein lacking a regulatory function.

To the best of our knowledge, all studies conducted to date have examined the output of the RsmA regulon at the extremes of the potential spectrum, i.e., essentially all (*gacAS*, *ladS*, or *rsmYZ* mutants) or nothing (*retS* or *rsmA* mutants). Whereas deletion of *rsmYZ* or their activators (*gacAS* and *ladS*) results in high levels of free RsmA, deletion of *rsmA* or *retS* results in complete loss of RsmA. While such extremes have shed insight into the RsmA regulon, it is more likely that RsmA availability fluctuates over a much narrower range to fine-tune gene expression. Such an interpretation is consistent with the fact that GacS, LadS, and RetS are sensing proteins and presumably modulate gene expression in response to environmental signals to control the output of the RsmA regulon as a gradient as opposed to an on/off binary switch. Our finding that RsmA availability in the *mucA* mutant is only modestly reduced without resulting in a global shift in expression of the RsmA regulon provides a physiologically relevant example of fluctuation occurring toward the middle of the potential spectrum.

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REFERENCES

1. **Sadikot RT, Blackwell TS, Christman JW, Prince AS.** 2005. Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. Am. J. Respir. Crit. Care Med. **171:**1209–1223. http://dx.doi.org/10.1164/rccm.200408-1044SO.
2. **Hoiby N, Johansen HK.** 2007. Isolation measures for prevention of infection with respiratory pathogens in cystic fibrosis: a systematic review? J. Hosp. Infect. **65:**374–375. http://dx.doi.org/10.1016/j.jhin.2006.11.012.
3. **Garau J, Gomez L.** 2003. *Pseudomonas aeruginosa* pneumonia. Curr. Opin. Infect. Dis. **16:**135–143. http://dx.doi.org/10.1097/00001432-200304000-00010.
4. **Hauser AR, Cobb E, Bodi M, Mariscal D, Valles J, Engel JN, Rello J.** 2002. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. Crit. Care. Med. **30:**521–528. http://dx.doi.org/10.1097/00003246-200203000-00005.
5. **Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J, Sawa T, Frank DW, Wiener-Kronish JP.** 2001. Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. J. Infect. Dis. **183:**1767–1774. http://dx.doi.org/10.1086/320737.
6. **Barbieri JT, Sun J.** 2004. *Pseudomonas aeruginosa* ExoS and ExoT. Rev. Physiol. Biochem. Pharmacol. **152:**79–92. http://dx.doi.org/10.1007/s10254-004-0031-7.
7. **Hauser AR.** 2009. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. Nat. Rev. Microbiol. **7:**654–665. http://dx.doi.org/10.1038/nrmicro2199.
8. **Sato H, Frank DW.** 2004. ExoU is a potent intracellular phospholipase. Mol. Microbiol. **53:**1279–1290. http://dx.doi.org/10.1111/j.1365-2958.2004.04194.x.
9. **Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP.** 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature **407:**762–764. http://dx.doi.org/10.1038/35037627.
10. **Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV.** 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc. Natl. Acad. Sci. U. S. A. **103:**8487–8492. http://dx.doi.org/10.1073/pnas.0602138103.
11. **Mena A, Smith EE, Burns JL, Speert DP, Moskowitz SM, Perez JL, Oliver A.** 2008. Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. J. Bacteriol. **190:**7910–7917. http://dx.doi.org/10.1128/JB.01147-08.
12. **Jain M, Bar-Meir M, McColley S, Cullina J, Potter E, Powers C, Prickett M, Seshadri R, Jovanovic B, Petrocheilou A, King JD, Hauser AR.** 2008. Evolution of *Pseudomonas aeruginosa* type III secretion in cystic fibrosis: a paradigm of chronic infection. Transl. Res. **152:**257–264. http://dx.doi.org/10.1016/j.trsl.2008.10.003.
13. **Jain M, Ramirez D, Seshadri R, Cullina JF, Powers CA, Schulert GS, Bar-Meir M, Sullivan CL, McColley SA, Hauser AR.** 2004. Type III secretion phenotypes of *Pseudomonas aeruginosa* strains change during infection of individuals with cystic fibrosis. J. Clin. Microbiol. **42:**5229–5237. http://dx.doi.org/10.1128/JCM.42.11.5229-5237.2004.
14. **Mathee K, McPherson CJ, Ohman DE.** 1997. Posttranslational control of the *algT* (*algU*)-encoded sigma22 for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its antagonist proteins MucA and MucB (AlgN). J. Bacteriol. **179:**3711–3720.
15. **Firoved AM, Deretic V.** 2003. Microarray analysis of global gene expression in mucoid *Pseudomonas aeruginosa*. J. Bacteriol. **185:**1071–1081. http://dx.doi.org/10.1128/JB.185.3.1071-1081.2003.
16. **Wood LF, Ohman DE.** 2009. Use of cell wall stress to characterize sigma 22 (AlgT/U) activation by regulated proteolysis and its regulon in *Pseudomonas aeruginosa*. Mol. Microbiol. **72:**183–201. http://dx.doi.org/10.1111/j.1365-2958.2009.06635.x.
17. **Boucher JC, Yu H, Mudd MH, Deretic V.** 1997. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of *muc* mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. Infect. Immun. **65:**3838–3846.
18. **Ohman DE, Chakrabarty AM.** 1981. Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. Infect. Immun. **33:**142–148.
19. **Schurr MJ, Yu H, Martinez-Salazar JM, Boucher JC, Deretic V.** 1996. Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. J. Bacteriol. **178:**4997–5004.
20. **Li Z, Kosorok MR, Farrell PM, Laxova A, West SE, Green CG, Collins J, Rock MJ, Splaingard ML.** 2005. Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease progression in children with cystic fibrosis. JAMA **293:**581–588. http://dx.doi.org/10.1001/jama.293.5.581.
21. **Pedersen SS, Hoiby N, Espersen F, Koch C.** 1992. Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. Thorax **47:**6–13. http://dx.doi.org/10.1136/thx.47.1.6.
22. **Wozniak DJ, Ohman DE.** 1994. Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. J. Bacteriol. **176:**6007–6014.
23. **Martin DW, Schurr MJ, Mudd MH, Govan JR, Holloway BW, Deretic V.** 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc. Natl. Acad. Sci. U. S. A. **90:**8377–8381. http://dx.doi.org/10.1073/pnas.90.18.8377.
24. **Mohr CD, Leveau JH, Krieg DP, Hibler NS, Deretic V.** 1992. AlgR-binding sites within the *algD* promoter make up a set of inverted repeats separated by a large intervening segment of DNA. J. Bacteriol. **174:**6624–6633.
25. **Wu W, Badrane H, Arora S, Baker HV, Jin S.** 2004. MucA-mediated coordination of type III secretion and alginate synthesis in *Pseudomonas*

aeruginosa. J. Bacteriol. **186:**7575–7585. http://dx.doi.org/10.1128/JB.186.22.7575-7585.2004.
26. **Jones AK, Fulcher NB, Balzer GJ, Urbanowski ML, Pritchett CL, Schurr MJ, Yahr TL, Wolfgang MC.** 2010. Activation of the *Pseudomonas aeruginosa* AlgU regulon through *mucA* mutation inhibits cyclic AMP/Vfr signaling. J. Bacteriol. **192:**5709–5717. http://dx.doi.org/10.1128/JB.00526-10.
27. **Wolfgang MC, Lee VT, Gilmore ME, Lory S.** 2003. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. Dev. Cell **4:**253–263. http://dx.doi.org/10.1016/S1534-5807(03)00019-4.
28. **Brencic A, Lory S.** 2009. Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. Mol. Microbiol. **72:**612–632. http://dx.doi.org/10.1111/j.1365-2958.2009.06670.x.
29. **Mulcahy H, O'Callaghan J, O'Grady EP, Adams C, O'Gara F.** 2006. The posttranscriptional regulator RsmA plays a role in the interaction between *Pseudomonas aeruginosa* and human airway epithelial cells by positively regulating the type III secretion system. Infect. Immun. **74:**3012–3015. http://dx.doi.org/10.1128/IAI.74.5.3012-3015.2006.
30. **Burrowes E, Baysse C, Adams C, O'Gara F.** 2006. Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis. Microbiology **152:**405–418. http://dx.doi.org/10.1099/mic.0.28324-0.
31. **Baker CS, Eory LA, Yakhnin H, Mercante J, Romeo T, Babitzke P.** 2007. CsrA inhibits translation initiation of *Escherichia coli hfq* by binding to a single site overlapping the Shine-Dalgarno sequence. J. Bacteriol. **189:**5472–5481. http://dx.doi.org/10.1128/JB.00529-07.
32. **Irie Y, Starkey M, Edwards AN, Wozniak DJ, Romeo T, Parsek MR.** 2010. *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. Mol. Microbiol. **78:**158–172. http://dx.doi.org/10.1111/j.1365-2958.2010.07320.x.
33. **Dubey AK, Baker CS, Romeo T, Babitzke P.** 2005. RNA sequence and secondary structure participate in high-affinity CsrA-RNA interaction. RNA **11:**1579–1587. http://dx.doi.org/10.1261/rna.2990205.
34. **Wei BL, Brun-Zinkernagel AM, Simecka JW, Pruss BM, Babitzke P, Romeo T.** 2001. Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. Mol. Microbiol. **40:**245–256. http://dx.doi.org/10.1046/j.1365-2958.2001.02380.x.
35. **Wang X, Dubey AK, Suzuki K, Baker CS, Babitzke P, Romeo T.** 2005. CsrA post-transcriptionally represses *pgaABCD*, responsible for synthesis of a biofilm polysaccharide adhesin of *Escherichia coli*. Mol. Microbiol. **56:**1648–1663. http://dx.doi.org/10.1111/j.1365-2958.2005.04648.x.
36. **Romeo T.** 1998. Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. Mol. Microbiol. **29:**1321–1330. http://dx.doi.org/10.1046/j.1365-2958.1998.01021.x.
37. **Patterson-Fortin LM, Vakulskas CA, Yakhnin H, Babitzke P, Romeo T.** 2013. Dual posttranscriptional regulation via a cofactor-responsive mRNA leader. J. Mol. Biol. **425:**3662–3677. http://dx.doi.org/10.1016/j.jmb.2012.12.010.
38. **Kay E, Humair B, Denervaud V, Riedel K, Spahr S, Eberl L, Valverde C, Haas D.** 2006. Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. J. Bacteriol. **188:**6026–6033. http://dx.doi.org/10.1128/JB.00409-06.
39. **Pessi G, Williams F, Hindle Z, Heurlier K, Holden MT, Camara M, Haas D, Williams P.** 2001. The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*. J. Bacteriol. **183:**6676–6683. http://dx.doi.org/10.1128/JB.183.22.6676-6683.2001.
40. **Sorger-Domenigg T, Sonnleitner E, Kaberdin VR, Blasi U.** 2007. Distinct and overlapping binding sites of *Pseudomonas aeruginosa* Hfq and RsmA proteins on the non-coding RNA RsmY. Biochem. Biophys. Res. Commun. **352:**769–773. http://dx.doi.org/10.1016/j.bbrc.2006.11.084.
41. **Heurlier K, Williams F, Heeb S, Dormond C, Pessi G, Singer D, Camara M, Williams P, Haas D.** 2004. Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **186:**2936–2945. http://dx.doi.org/10.1128/JB.186.10.2936-2945.2004.
42. **Brencic A, McFarland KA, McManus HR, Castang S, Mogno I, Dove SL, Lory S.** 2009. The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. Mol. Microbiol. **73:**434–445. http://dx.doi.org/10.1111/j.1365-2958.2009.06782.x.
43. **Burrowes E, Abbas A, O'Neill A, Adams C, O'Gara F.** 2005. Characterisation of the regulatory RNA RsmB from *Pseudomonas aeruginosa* PAO1. Res. Microbiol. **156:**7–16. http://dx.doi.org/10.1016/j.resmic.2004.07.004.
44. **Davies JA, Harrison JJ, Marques LL, Foglia GR, Stremick CA, Storey DG, Turner RJ, Olson ME, Ceri H.** 2007. The GacS sensor kinase controls phenotypic reversion of small colony variants isolated from biofilms of *Pseudomonas aeruginosa* PA14. FEMS Microbiol. Ecol. **59:**32–46. http://dx.doi.org/10.1111/j.1574-6941.2006.00196.x.
45. **Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS, Lory S.** 2004. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. Dev. Cell **7:**745–754. http://dx.doi.org/10.1016/j.devcel.2004.08.020.
46. **Laskowski MA, Osborn E, Kazmierczak BI.** 2004. A novel sensor kinase-response regulator hybrid regulates type III secretion and is required for virulence in *Pseudomonas aeruginosa*. Mol. Microbiol. **54:**1090–1103. http://dx.doi.org/10.1111/j.1365-2958.2004.04331.x.
47. **Ventre I, Goodman AL, Vallet-Gely I, Vasseur P, Soscia C, Molin S, Bleves S, Lazdunski A, Lory S, Filloux A.** 2006. Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. Proc. Natl. Acad. Sci. U. S. A. **103:**171–176. http://dx.doi.org/10.1073/pnas.0507407103.
48. **Goodman AL, Merighi M, Hyodo M, Ventre I, Filloux A, Lory S.** 2009. Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. Genes Dev. **23:**249–259. http://dx.doi.org/10.1101/gad.1739009.
49. **Vogel HJ, Bonner DM.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
50. **Dasgupta N, Lykken GL, Wolfgang MC, Yahr TL.** 2004. A novel anti-anti-activator mechanism regulates expression of the *Pseudomonas aeruginosa* type III secretion system. Mol. Microbiol. **53:**297–308. http://dx.doi.org/10.1111/j.1365-2958.2004.04128.x.
51. **Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86. http://dx.doi.org/10.1016/S0378-1119(98)00130-9.
52. **Hoang TT, Kutchma AJ, Becher A, Schweizer HP.** 2000. Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. Plasmid **43:**59–72. http://dx.doi.org/10.1006/plas.1999.1441.
53. **McCaw ML, Lykken GL, Singh PK, Yahr TL.** 2002. ExsD is a negative regulator of the *Pseudomonas aeruginosa* type III secretion regulon. Mol. Microbiol. **46:**1123–1133. http://dx.doi.org/10.1046/j.1365-2958.2002.03228.x.
54. **Diaz MR, King JM, Yahr TL.** 2011. Intrinsic and extrinsic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*. Front. Microbiol. **2:**89. http://dx.doi.org/10.3389/fmicb.2011.00089.
55. **Yahr TL, Frank DW.** 1994. Transcriptional organization of the trans-regulatory locus which controls exoenzyme S synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**3832–3838.
56. **Dasgupta N, Ashare A, Hunninghake GW, Yahr TL.** 2006. Transcriptional induction of the *Pseudomonas aeruginosa* type III secretion system by low Ca^{2+} and host cell contact proceeds through two distinct signaling pathways. Infect. Immun. **74:**3334–3341. http://dx.doi.org/10.1128/IAI.00090-06.
57. **Perez-Martinez I, Haas D.** 2011. Azithromycin inhibits expression of the GacA-dependent small RNAs RsmY and RsmZ in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **55:**3399–3405. http://dx.doi.org/10.1128/AAC.01801-10.
58. **Brutinel ED, Vakulskas CA, Yahr TL.** 2010. ExsD inhibits expression of the *Pseudomonas aeruginosa* type III secretion system by disrupting ExsA self-association and DNA binding activity. J. Bacteriol. **192:**1479–1486. http://dx.doi.org/10.1128/JB.01457-09.
59. **Marden JN, Diaz MR, Walton WG, Gode CJ, Betts L, Urbanowski ML, Redinbo MR, Yahr TL, Wolfgang MC.** 2013. An unusual CsrA family member operates in series with RsmA to amplify posttranscriptional responses in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **110:**15055–15060. http://dx.doi.org/10.1073/pnas.1307217110.
60. **Sterzenbach T, Nguyen KT, Nuccio SP, Winter MG, Vakulskas CA, Clegg S, Romeo T, Baumler AJ.** 2013. A novel CsrA titration mechanism regulates fimbrial gene expression in *Salmonella typhimurium*. EMBO J. **32:**2872–2883. http://dx.doi.org/10.1038/emboj.2013.206.