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RESEARCH ARTICLE

Pseudomonas aeruginosa Magnesium Transporter MgtE Inhibits Type III Secretion System Gene Expression by Stimulating *rsmYZ* Transcription

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ABSTRACT *Pseudomonas aeruginosa* causes numerous acute and chronic opportunistic infections in humans. One of its most formidable weapons is a type III secretion system (T3SS), which injects powerful toxins directly into host cells. The toxins lead to cell dysfunction and, ultimately, cell death. Identification of regulatory pathways that control T3SS gene expression may lead to the discovery of novel therapeutics to treat *P. aeruginosa* infections. In a previous study, we found that expression of the magnesium transporter gene *mgtE* inhibits T3SS gene transcription. MgtE-dependent inhibition appeared to interfere with the synthesis or function of the master T3SS transcriptional activator ExsA, although the exact mechanism was unclear. We now demonstrate that *mgtE* expression acts through the GacAS two-component system to activate *rsmY* and *rsmZ* transcription. This event ultimately leads to inhibition of *exsA* translation. This inhibitory effect is specific to *exsA* as translation of other genes in the *exsCEBA* operon is not inhibited by *mgtE*. Moreover, our data reveal that MgtE acts solely through this pathway to regulate T3SS gene transcription. Our study reveals an important mechanism that may allow *P. aeruginosa* to fine-tune T3SS activity in response to certain environmental stimuli.

IMPORTANCE The type III secretion system (T3SS) is a critical virulence factor utilized by numerous Gram-negative bacteria, including *Pseudomonas aeruginosa*, to intoxicate and kill host cells. Elucidating T3SS regulatory mechanisms may uncover targets for novel anti-*P. aeruginosa* therapeutics and provide deeper understanding of bacterial pathogenesis. We previously found that the magnesium transporter MgtE inhibits T3SS gene transcription in *P. aeruginosa*. In this study, we describe the mechanism of MgtE-dependent inhibition of the T3SS. Our report also illustrates how MgtE might respond to environmental cues, such as magnesium levels, to fine-tune T3SS gene expression.

KEYWORDS ExsA, GacAS, MgtE, *Pseudomonas aeruginosa*, RsmA, gene regulation, magnesium, posttranscriptional, type III secretion

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The Gram-negative bacterium *Pseudomonas aeruginosa* is implicated in a wide range of opportunistic infections in humans (1, 2). A major virulence factor used by *P. aeruginosa* to initiate acute infections is a type III secretion system (T3SS) (3, 4). This macromolecular apparatus spans the bacterial cell envelope and acts like a syringe, injecting several toxins directly into host cells (5). This leads to actin cytoskeleton rearrangement, host cell rounding, and cell death (5, 6). These actions promote tissue damage and decrease phagocytic clearance (7–9). In addition to acute infections, *P. aeruginosa* is also able to establish chronic infections through formation of biofilms, most notably in the airways of cystic fibrosis (CF) patients (9, 10). During *P. aeruginosa*

biofilm formation, altered gene regulation typically leads to a reduction in T3SS gene expression (9, 11–14). Additionally, isolates from chronically colonized CF patients usually contain mutations that decrease T3SS production (9, 15). Thus, during both acute and chronic infections, *P. aeruginosa* appears to tightly regulate T3SS gene expression in response to environmental conditions.

P. aeruginosa T3SS gene expression is controlled by the master transcription factor ExsA, which is responsible for activating transcription of all T3SS genes, including *exsA* itself (16). Under noninducing conditions, ExsA is bound by the antiactivator protein ExsD and is unable to bind to its target promoters to initiate gene transcription. Two other proteins important for T3SS regulation, ExsC and ExsE, form a separate complex. Under inducing conditions (contact of *P. aeruginosa* with host cells, the presence of serum, or low-Ca^{2+} conditions), ExsE is secreted through the T3SS apparatus, thus permitting ExsC to sequester ExsD. ExsA, released from ExsD, subsequently activates the T3SS regulon. This mechanism has been referred to as "intrinsic regulation" (16).

In addition to the ExsDCE network, several other pathways also control *exsA* expression and/or synthesis (9, 16). These pathways work concurrently but distinctly from "intrinsic regulation" to further control T3SS gene expression and are referred to as "extrinsic regulation." One example of extrinsic regulation is the RsmA/RsmY/RsmZ signaling cascade. RsmA is an RNA binding protein belonging to the CsrA family (17). CsrA family members regulate gene expression at the posttranscriptional level by binding to target mRNAs at conserved sequence motifs and impacting their stability and/or translation (17). RsmA appears to control T3SS gene expression by increasing *exsA* translation through an undetermined mechanism (18). This activity depends upon the concentration of free RsmA in the cell and is controlled by two noncoding RNAs, RsmY and RsmZ (18, 19). RsmY and RsmZ function by directly sequestering RsmA from target mRNA (18, 20–22) and are thus negative regulators of ExsA synthesis. Transcription of *rsmYZ* is directly controlled by the GacAS two-component system (TCS) (17, 18, 23, 24). The environmental signals governing RsmY and RsmZ expression are poorly understood but include two additional sensor kinases, LadS and RetS. Both GacS and LadS are able to phosphorylate the GacA response regulator to enhance *rsmY* and *rsmZ* transcription (25, 26). In contrast, RetS inhibits GacA-mediated *rsmY* and *rsmZ* transcription by forming a heterodimer with GacS and preventing GacA phosphorylation (27). Though this pathway controls production of ExsA, the availability of ExsA to regulate T3SS gene expression is still dependent on the intrinsic regulation described above.

Previous studies found that the *P. aeruginosa* inner membrane magnesium transporter MgtE inhibits T3SS gene expression (28). Whereas an *mgtE* mutant demonstrates enhanced T3SS gene expression, *mgtE* overexpression inhibits the T3SS (28). The mechanism by which *mgtE* inhibits the T3SS was not elucidated in these prior studies, although the effect of MgtE on T3SS gene expression is distinct from its role as an Mg^{2+} transporter in *P. aeruginosa* (28). Additionally, deletion of both *mgtE* and *exsA* results in negligible T3SS activity (28), indicating that MgtE acts through ExsA to regulate T3SS gene expression. In the present study, we show that *mgtE* expression inhibits ExsA translation by increasing *rsmY* and *rsmZ* transcription. We also demonstrate that *mgtE* acts exclusively through the RsmA/RsmY/RsmZ signaling pathway to inhibit ExsA-mediated T3SS gene transcription. Because *mgtE* transcription is significantly upregulated by growth under low-Mg^{2+} conditions and in the presence of some antibiotics (29, 30), this pathway may provide a mechanism for *P. aeruginosa* to modulate T3SS gene expression in response to signals encountered during infections.

RESULTS

MgtE inhibits T3SS gene expression at the posttranscriptional level. Previous studies found that *mgtE* expression inhibits T3SS gene transcription by acting through ExsA (28). We considered three possibilities to account for the inhibitory effect of *mgtE* expression: (i) reduced *exsA* transcription, (ii) reduced ExsA synthesis, and/or (iii) impaired ExsA function. Because *exsA* regulates its own transcription (by acting at the

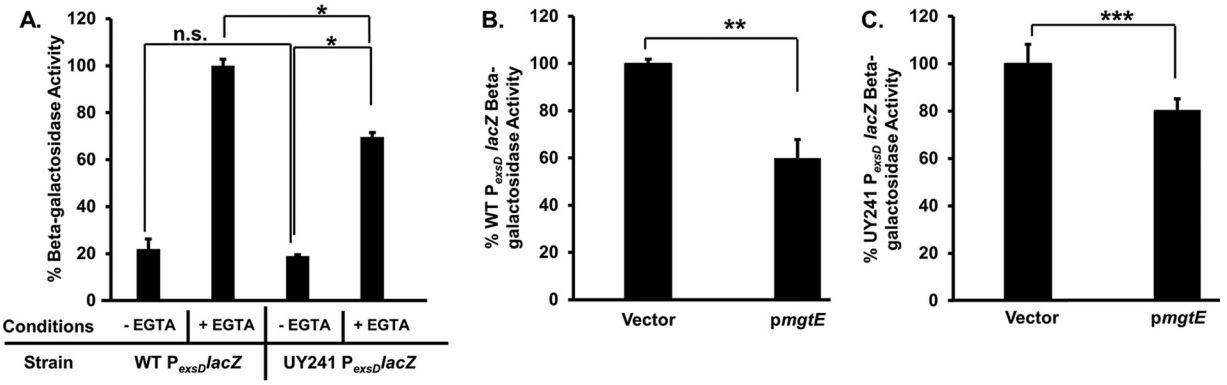

FIG 1 *mgtE* inhibits T3SS gene expression at the posttranscriptional level. (A) PA103 P_{exsD}-*lacZ* (WT) and UY241 were assayed under either T3SS-noninducing (–EGTA) or -inducing (+EGTA) conditions and assayed for β-galactosidase activity from the P_{exsD}-*lacZ* reporter construct. The percentage of activity was calculated considering the P_{exsD}-*lacZ* activity in EGTA-treated WT as 100%. *, $P < 0.0005$; n.s., not significant. (B) PA103 P_{exsD}-*lacZ* (WT) with either the vector control or p*mgtE* was assayed under T3SS-inducing conditions, and β-galactosidase activity from the P_{exsD}-*lacZ* construct was measured. The percentage of activity was calculated considering the P_{exsD}-*lacZ* activity in the WT with blank vector as 100%. **, $P < 0.05$. (C) Strain UY241 with either the vector control or p*mgtE* was assayed under T3SS-inducing conditions, and β-galactosidase activity from the P_{exsD}-*lacZ* construct was measured. The percentage of activity was calculated considering the P_{exsD}-*lacZ* activity in UY241 with blank vector as 100%. ***, $P < 0.005$.

P_{exsC} promoter to control transcription of the *exsCEBA* operon) (31), it was necessary to uncouple *exsA* transcription from its own control to analyze potential *mgtE* effects on *exsA* transcription. To this end, we used the previously described *P. aeruginosa* UY241 strain (32), in which the ExsA-dependent P_{exsC} promoter has been replaced with a constitutive variant of the P_{lacUV5} promoter (P_{con}). Removal of the native promoter should uncouple *exsCEBA* transcription from activity of regulatory molecules that naturally bind to the P_{exsC} promoter. Indeed, UY241 has been shown to display constitutive *exsA* transcription (32). As a control, P_{exsD}-*lacZ* reporter activity (as a marker for ExsA-dependent transcription) was measured in wild-type (WT) PA103 and the UY241 strain following growth under noninducing (high-calcium [–EGTA]) and inducing (low-calcium [+EGTA]) conditions for T3SS gene expression (33–35). Whereas ExsA is sequestered by ExsD in the WT strain under noninducing conditions and P_{exsD}-*lacZ* reporter activity is low, EGTA stimulation results in the release of ExsA from ExsD and induction of P_{exsD}-*lacZ* reporter activity (Fig. 1A) (36). Strain UY241 also demonstrates EGTA-dependent induction of P_{exsD}-*lacZ* reporter activity, but the overall level of activity is reduced due to the lack of ExsA autoregulation at the P_{exsC} promoter (Fig. 1A). We next expressed *mgtE* in the WT (Fig. 1B) and UY241 strains (Fig. 1C) and measured P_{exsD}-*lacZ* reporter activity. In both the WT and UY241 strains, *mgtE* expression resulted in a significant reduction in P_{exsD}-*lacZ* reporter activity. Because native transcriptional control has been lost in the UY241 strain, these data suggest that MgtE inhibits T3SS gene expression by acting on ExsA at a posttranscriptional level. *mgtE* expression appears to lead to less T3SS inhibition in UY241 compared to wild-type PA103 (Fig. 1B and C). We attribute this to ExsA autoregulation in the wild type; uninhibited ExsA levels are higher in the wild type than in UY241 (leading to the higher T3SS gene expression seen in Fig. 1A), and so *mgtE*-mediated inhibition has an apparent greater effect. Importantly, we found that *mgtE* expression in PA103 resulted in an approximately 100-fold increase in *mgtE* transcript abundance compared to the vector control (see Fig. S1A in the supplemental material). This increase is concordant with transcript levels seen in physiologically relevant concentrations of antibiotics and magnesium (29, 30). Similar results were obtained with *P. aeruginosa* strain PA14 (Fig. S1B).

ExsA translation is repressed by *mgtE* expression. We next tested the hypothesis that *mgtE* expression inhibits ExsA translation. We introduced the *mgtE* expression vector (p*mgtE*) into a panel of WT PA103 strains carrying *lacZ* translational reporters within the *exsCEBA* operon, wherein *lacZ* is fused at *exsC* codon 15 (*exsC′-′lacZ*), *exsE* codon 15 (*exsCE′-′lacZ*), *exsB* codon 2 (*exsCEB′-′lacZ*), or *exsA* codon 77 (*exsCEBA′-′lacZ*)

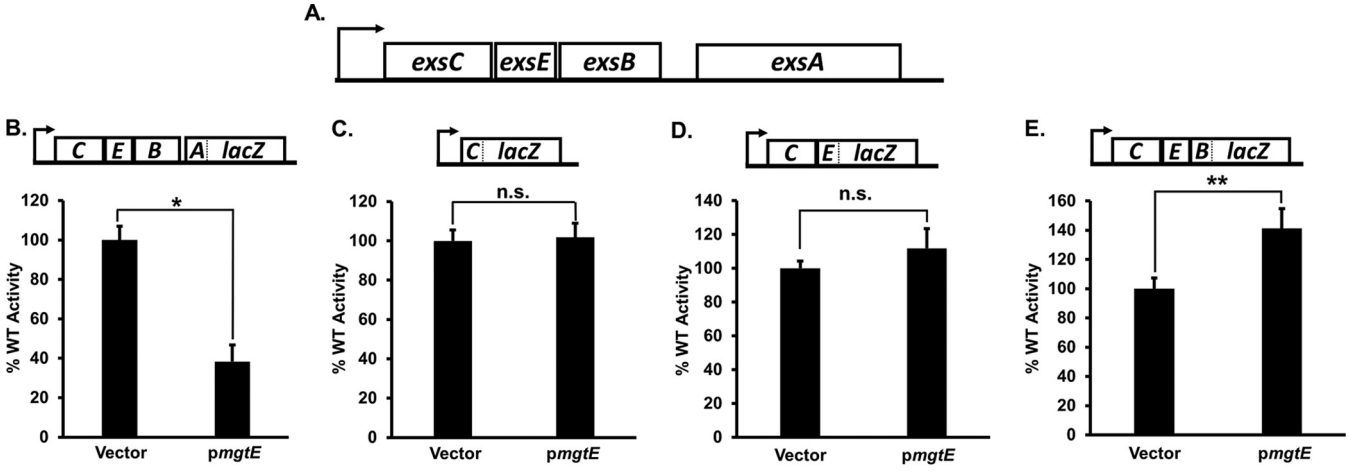

FIG 2 *mgtE* expression specifically represses *exsA* translation. (A) Diagram of the *exsCEBA* operon. (B to E) Translational *exsA* (*exsCEBA′-′lacZ*) (B), *exsC* (*exsC′-′lacZ*) (C), *exsE* (*exsCE′-′lacZ*) (D), and *exsB* (*exsCEB′-′lacZ*) (E) reporter strains, with either the vector control or p*mgtE*, were assayed under T3SS-inducing (+EGTA) conditions for β-galactosidase activity. The percentage of activity was calculated considering the *lacZ* activity from the respective strains with the blank vector as 100%. The reporter constructs were transcribed from a constitutive P_{lacUV5} promoter. The particular translational fusion tested in each panel is diagrammed above each graph. *, $P < 0.005$; **, $P < 0.05$; n.s., not significant.

(18); for reference, the full *exsCEBA* operon is diagrammed in Fig. 2A. Each reporter is integrated in single copy on the chromosome at the CTX phage attachment site and transcribed from a constitutive P_{lacUV5} promoter. Whereas plasmid-expressed *mgtE* reduced *exsCEBA′-′lacZ* translational reporter activity by almost 3-fold (Fig. 2B), *mgtE* had no negative impact on the *exsC′-′lacZ* (Fig. 2C), *exsCE′-′lacZ* (Fig. 2D), or *exsCEB′-′lacZ* (Fig. 2E) reporter activities. We found a similar decrease in *exsCEBA′-′lacZ* reporter activity in *P. aeruginosa* strain PA14 (see Fig. S2 in the supplemental material). These data suggest that *mgtE* expression inhibits ExsA translation and that this activity is specific to *exsA* in the *exsCEBA* operon. For reasons that are unclear, the *exsCEB′-′lacZ* reporter showed a significant increase upon *mgtE* expression.

The small intergenic region between *exsB* and *exsA* contains a minor, Vfr-dependent promoter (P_{exsA}) that controls *exsA* transcription (37). Because the P_{exsA} promoter is present in the *exsCEBA′-′lacZ* translational reporter, we considered the possibility that the observed reduction in *exsCEBA′-′lacZ* activity (Fig. 2A) resulted from MgtE transcriptional inhibition of P_{exsA} promoter activity. To investigate this hypothesis, we monitored the effect of *mgtE* expression on a cyclic AMP (cAMP)-Vfr signaling (CVS) reporter (38). The CVS reporter consists of *lacZ* fused to the cAMP- and CRP/Vfr-dependent *lac*P1 promoter from *Escherichia coli*. As shown in Fig. S3A in the supplemental material, *mgtE* expression had no significant effect on CVS reporter activity. This result is further supported by the finding that *mgtE* expression in a *vfr* mutant still inhibits P_{exsD}-*lacZ* activity (Fig. S3B). We infer from these data that *mgtE* does not alter transcription from the P_{exsA} proximal promoter, which is consistent with the conclusion that *mgtE* affects ExsA at a posttranscriptional level.

It is also possible that *mgtE* decreases *exsA* mRNA stability rather than specifically inhibiting *exsA* translation. To distinguish between these two possibilities, we isolated mRNA from UY241, carrying either a vector control or p*mgtE*, and performed quantitative reverse transcription-PCR (qRT-PCR) using primer pairs designed to amplify a central region of *exsA* (see Materials and Methods). Intriguingly, *mgtE* expression increased *exsA* transcript levels (Fig. 3). The same effect was also observed in the strain carrying the *exsCEBA′-′lacZ* translational reporter used for the experiment in Fig. 2 (see Fig. S4 in the supplemental material). These findings suggest that *mgtE* might have positive effects on *exsA* mRNA stability, but more importantly, they strongly suggest that the posttranscriptional inhibition of ExsA synthesis by *mgtE* is due to repression of ExsA translation as opposed to impaired *exsA* mRNA stability.

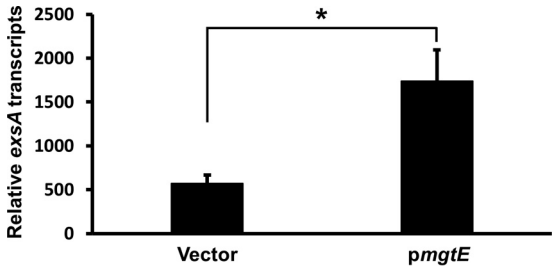

FIG 3 *exsA* mRNA remains stable upon *mgtE* expression. mRNA was isolated from *P. aeruginosa* UY241 containing either the vector control or p*mgtE*, and *exsA* transcript stability was analyzed by qRT-PCR. *exsA* transcript abundance was normalized to that of the control transcript *fbp*. *, $P < 0.05$.

***mgtE* upregulates *rsmY* and *rsmZ* transcription in a GacAS dependent manner.** We next examined possible mechanisms for the *mgtE*-mediated *exsA* inhibition. We reasoned that since *mgtE* is an inner membrane protein (28), its translation-repressive effects on *exsA* were likely indirect—possibly by stimulating one of the established signaling pathways that control ExsA translation. For this reason, we investigated whether *mgtE* affected RsmA/RsmY/RsmZ signaling (18). To explore this hypothesis, we measured P_{rsmY}-*lacZ*, P_{rsmZ}-*lacZ*, and P_{rsmA}-*lacZ* transcriptional reporter activity (18) and found that *mgtE* expression significantly upregulates *rsmY* and *rsmZ* transcription by approximately 2.5-fold and 2-fold, respectively (Fig. 4A and B). In PA14 strains containing these constructs, *mgtE* expression also significantly enhanced *rsmY* and *rsmZ* transcription (see Fig. S5 in the supplemental material). Interestingly, *mgtE* expression also stimulated *rsmA* transcription (Fig. 4C), but to a smaller degree than measured for *rsmY* and *rsmZ*. Because the GacAS two-component system is essential for *rsmY* and *rsmZ* transcription (17, 18, 23, 24), we hypothesized that the *mgtE* effect requires GacAS. As evident from Fig. 4A and B, *mgtE* expression failed to stimulate P_{rsmY}-*lacZ* and P_{rsmZ}-*lacZ* reporter activities in the absence of either *gacA* or *gacS*, thus supporting a role for GacAS in *mgtE*-mediated RsmYZ regulation.

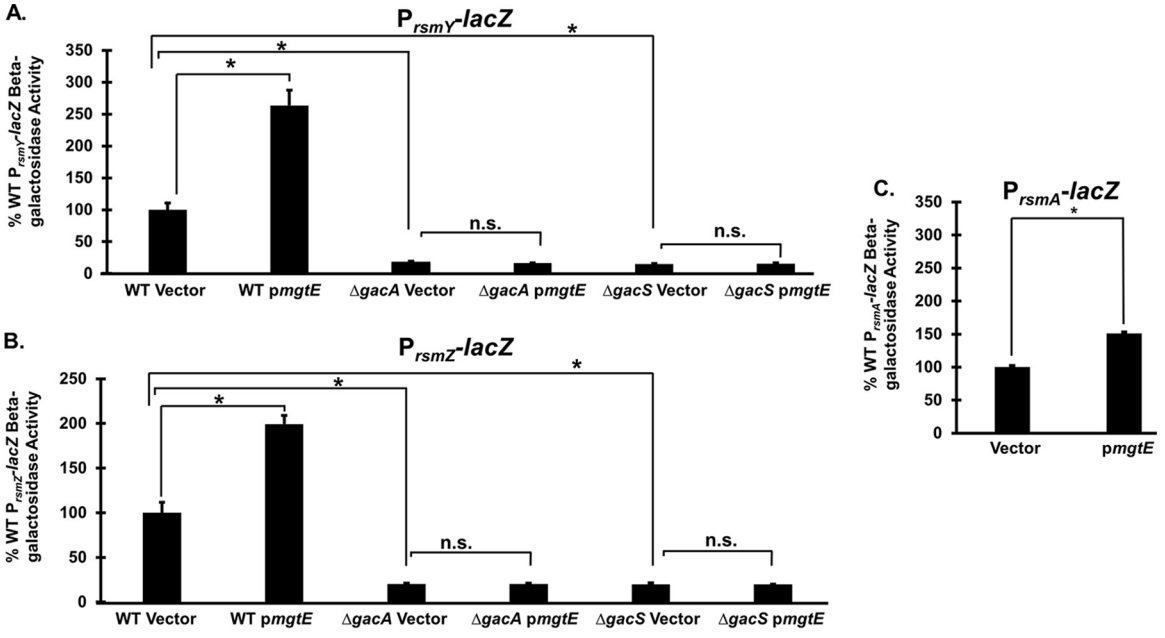

FIG 4 *mgtE* expression affects Rsm signaling by a GacAS-dependent mechanism. Transcriptional *rsmY* (P_{rsmY}-*lacZ*) (A), *rsmZ* (P_{rsmZ}-*lacZ*) (B), and *rsmA* (P_{rsmA}-*lacZ*) (C) reporter strains in the WT, Δ*gacA*, and Δ*gacS* backgrounds with either the vector control or p*mgtE* were assayed under T3SS-inducing (+EGTA) conditions for β-galactosidase activity. The percentage of activity was calculated considering the *lacZ* activity from the respective WT reporter strains with the blank vector as 100%. *, $P < 0.01$; n.s., not significant (ANOVA).

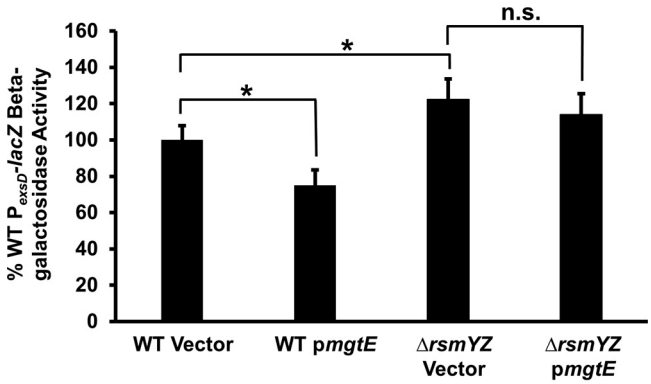

FIG 5 RsmYZ are required for MgtE-mediated inhibition of T3SS gene transcription. PA103 P_{exsD}-*lacZ* and Δ*rsmYZ* P_{exsD}-*lacZ* strains with either the vector control or p*mgtE* were assayed under T3SS-inducing (+EGTA) conditions for β-galactosidase activity from the P_{exsD}-*lacZ* reporter construct. The percentage of activity was calculated considering the P_{exsD}-*lacZ* activity in WT with blank vector as 100%. *, $P < 0.01$; n.s., not significant (ANOVA).

***mgtE* expression in an *rsmY rsmZ* mutant fails to inhibit T3SS gene expression.** Since our data suggest that *mgtE* affects RsmA/RsmY/RsmZ signaling to inhibit *exsA* translation, we next tested whether the effect of MgtE functions solely through RsmA/RsmY/RsmZ signaling to inhibit ExsA-dependent transcription by expressing *mgtE* in an *rsmY rsmZ* double mutant. Consistent with a previous report (16), the *rsmY rsmZ* mutant demonstrates increased P_{exsD}-*lacZ* reporter activity compared to the WT (Fig. 5). Whereas *mgtE* expression significantly inhibited P_{exsD}-*lacZ* reporter activity in the WT background, reporter activity was unaffected in the *rsmY rsmZ* mutant (Fig. 5). These data suggest that *mgtE* works solely through the RsmA/RsmY/RsmZ signaling cascade to inhibit *exsA* translation.

Previous studies identified an interaction between *mgtE* and *algR* during T3SS transcriptional regulation, though the nature of this interaction was unclear (30). Among other activities, the AlgZR two-component system inhibits T3SS gene expression (30, 39); one mechanism by which this occurs is by enhancing *rsmY* and *rsmZ* transcription, thus inhibiting ExsA translation (18). Therefore, we considered the possibility that MgtE could additionally stimulate *rsmY* and *rsmZ* transcription through AlgZR. However, *mgtE* expression inhibited P_{exsD}-*lacZ* reporter activity, even in an *algZR* deletion mutant (Fig. 6), suggesting that *mgtE*-mediated T3SS repression does not work directly through AlgZR.

DISCUSSION

The central role of ExsA as the primary regulator of *P. aeruginosa* T3SS makes it an attractive target for therapeutic development (16). Defining signaling networks that

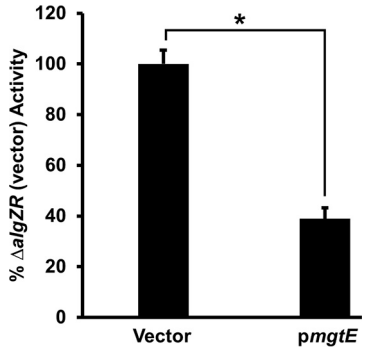

FIG 6 *mgtE* inhibits T3SS gene transcription in a Δ*algZR* background. Δ*algZR* strains with either the vector control or p*mgtE* were assayed under T3SS-inducing (+EGTA) conditions for β-galactosidase activity from the P_{exsD}-*lacZ* reporter construct. The percentage of activity was calculated considering the P_{exsD}-*lacZ* activity in the Δ*algZR* strain with blank vector as 100%. *, $P < 0.0005$.

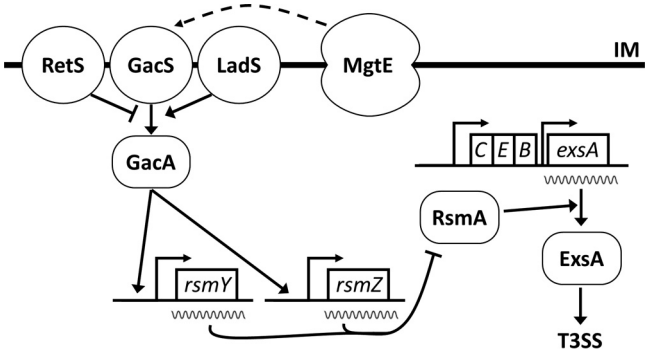

FIG 7 A model for *mgtE*-mediated control of T3SS. MgtE affects signaling through GacAS, by an unknown mechanism, to stimulate transcription of *rsmY* and *rsmZ*. Increased *rsmY* or *rsmZ* levels, in turn, sequester RsmA, preventing it from enhancing translation of *exsA*, thus inhibiting T3SS gene transcription. IM, inner membrane.

control *exsA* expression, synthesis, and activity is critical to realizing that goal. Previous work found that the MgtE magnesium transporter inhibits T3SS gene expression (28); *mgtE* gene expression inhibits, while *mgtE* deletion enhances, T3SS. In this study, we demonstrate that MgtE accomplishes this activity by inhibiting *exsA* translation (Fig. 7). Considering the function of MgtE, it is intriguing to speculate that magnesium concentration fluctuations could mediate toxicity changes. However, as magnesium has pleiotropic effects on *P. aeruginosa* (40), this signaling is likely complex.

MgtE-mediated inhibition of *exsA* translation appears to occur through the RsmA/RsmY/RsmZ pathway (Fig. 4 and 5). Increased RsmY and RsmZ levels sequester RsmA, resulting in decreased *exsA* translation. Importantly, we found that *mgtE* expression does not decrease *exsA* transcript levels (Fig. 3), further supporting the conclusion of a specific effect on translation. Additionally, lack of involvement of Vfr in MgtE-mediated T3SS gene transcription inhibition strongly indicates that *exsA* translation inhibition is not the result of decreased transcription activity from the P_{exsA} promoter located on translational fusion constructs (see Fig. S3 in the supplemental material). The fact that MgtE does not inhibit other genes in the *exsCEBA* operon (Fig. 2) suggests that there may be regulatory sequences specific to *exsA* translational control. However, it is also formally possible that the lack of additional coding sequences for these other genes masks any potential effects MgtE exerts on downstream motifs. There does seem to be a stronger inhibition by *mgtE* in the Δ*vfr* strain compared to *vfr*-competent strains (Fig. 1 versus Fig. S3B). The reasons for this effect are unclear, but it could be the result of competing effects on T3SS expression through the Vfr/CVS and Rsm pathways, an as-yet-unidentified Vfr pathway, or simply stochastic variation.

Overexpression of *mgtE* also enhances *rsmA* transcription (Fig. 4C), although to a much lesser degree than *rsmY* and *rsmZ* (Fig. 4A and B). As was proposed in another study, it is plausible that the net result from increased *rsmA*, *rsmY*, and *rsmZ* transcription is reduced RsmA availability (18). First, RsmA positively regulates *rsmY* and *rsmZ* transcription, which likely plays a role in maintaining homeostasis (18, 41). Second, RsmA binds to its own mRNA to repress translation (42). Therefore, the *mgtE*-dependent increase in *rsmA*, *rsmY*, and *rsmZ* expression could result in reduced RsmA availability and decreased *exsA* translation (Fig. 2).

MgtE lacks helix-turn-helix or other DNA binding motifs (43, 44), which would be needed to directly impact transcription. Therefore, it seems that the positive effect of MgtE on *rsmY* and *rsmZ* transcription is indirect. This is indeed supported by our data that *mgtE* expression fails to affect *rsmY* and *rsmZ* transcription in both a *gacA* mutant and a *gacS* mutant (Fig. 4A and B). These results also establish that MgtE influences *rsmY* and *rsmZ* transcription through GacAS. As a membrane protein, MgtE could be involved in direct or indirect binding interactions with GacS, LadS, RetS, or a novel membrane protein that affects signaling through GacS. Because MgtE signals through

GacA and GacS, it would be interesting to investigate whether MgtE expression leads to higher phosphorylated states of GacA. It is important to note that RetS was found to regulate biofilm formation in response to magnesium limitation (45), a condition that also enhances *mgtE* transcription (29). Additionally, a recent report showed that AlgR influences *rsmY* and *rsmZ* activity through an unknown mechanism (18). However, we found that *mgtE* expression still repressed T3SS transcription in a Δ*algZR* background (Fig. 6), indicating that MgtE affects RsmY and RsmZ levels solely through GacAS. Future work will investigate the mechanism by which *mgtE* affects GacAS signaling.

It is noteworthy that *mgtE* transcription is significantly upregulated under low-Mg^{2+} and high-antibiotic conditions (29, 30), which are commonly found during host infections like in the CF lung environment (46–52). We expressed *mgtE* from a plasmid to simulate the effects of high *mgtE* expression (Fig. S1), such as could occur during host infection. Thus, our study describes a mechanism that might allow *P. aeruginosa* to respond to the host environment and optimize T3SS gene expression. Additionally, because MgtE signals through GacAS, our results indicate two environmental signals encountered by *P. aeruginosa* during infection (low Mg^{2+} concentrations and high antibiotic concentrations during infection) that potentially affect the GacAS signaling pathway. Thus, it is possible that MgtE serves as a sensor, altering T3SS expression in response to changes in the extracellular environment (i.e., magnesium levels and antibiotics). Because the effects on *exsA* levels and T3SS gene expression are modest (Fig. 1, 2, and 5) (28), we propose that MgtE "fine-tunes" the T3SS response in accordance with the chemical environment, rather than acting as a binary on/off switch.

MgtE is important for the pathogenesis of other microorganisms, such as *Aeromonas hydrophila* and *Campylobacter jejuni* (53, 54). *A. hydrophila*, in particular, has both a T3SS and an RsmA homologue (55, 56). An intriguing avenue of future research will be to investigate whether MgtE homologs in other pathogens inhibit T3SS through a conserved mechanism of action (i.e., modulation of RsmA activity). Similar to MgtE in *P. aeruginosa*, the housekeeping Mg^{2+} transporter CorA, found in numerous bacteria, is reported to transport Mg^{2+} and modulate virulence as two distinct functions (57). Future work will investigate whether CorA signaling is similar to that of *P. aeruginosa* MgtE.

Taken together, the results of our present study describe the mechanism by which MgtE influences T3SS in *P. aeruginosa*: by inhibiting *exsA* translation, which, in turn, leads to downstream effects on ExsA-mediated T3SS gene transcription. This signaling cascade is one mechanism used by *P. aeruginosa* to respond to Mg^{2+} scarcity and high-antibiotic conditions. Additional characterization of upstream events of this signaling cascade (i.e., how MgtE affects GacAS signal transduction) would further increase our understanding of the mechanism used by *P. aeruginosa* to orchestrate signaling pathways in response to the host environment.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Expression of *mgtE* was accomplished by transforming the indicated strains with plasmid p*mgtE* (28), which contains the full-length *mgtE* gene immediately downstream from the P_{araBAD} promoter on vector pMQ72 (58). This promoter is leaky in *P. aeruginosa*, and we have previously found *mgtE* expression in the absence of arabinose induction (28). Plasmids were maintained in *Escherichia coli* S17 (28) cultured on LB agar plates or LB containing 10 μg/ml gentamicin; we used Miller's LB (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl). Plasmids were isolated from *E. coli* using a QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions and electroporated into the appropriate *P. aeruginosa* strains. Transformed *P. aeruginosa* cells were cultured in Vogel Bonner minimal (VBM) medium (18, 59) agar plates with 60 μg/ml gentamicin, and the presence of the respective plasmids was confirmed by PCR with primers p729 (5′-CAGACCGCTTCTGCGTTCTG-3′) and p730 (5′-GCAACTCTCTACT GTTTCTCC-3′) (30). These primers bind to sequences on vector pMQ72 that flank the *mgtE* insertion site. For β-galactosidase assays, *P. aeruginosa* strains were cultured overnight on VBM agar plates with gentamicin. Cells were subcultured the next day to a starting concentration at an optical density at 600 nm (OD_{600}) of 0.1 in Trypticase soy broth (TSB) supplemented with 100 mM monosodium glutamate and 1% glycerol (18). EGTA (2 mM) was added to the medium to activate T3SS gene expression, through induction of the intrinsic regulatory cascade (i.e., secretion of ExsE, leading to desequestration of the T3SS activator ExsA) (33, 60).

TABLE 1 Bacterial strains used in this study

Strain	Relevant characteristic(s)	Reference(s) or source
PA103 P_{exsD}-*lacZ*	WT strain with ExsA-dependent P_{exsD}-*lacZ* reporter chromosomally integrated at CTX site	4, 10, 18, 64
UY241	Constitutive transcription of *exsCEBA* in PA103 P_{exsD}-*lacZ* background	32
PA14	WT strain	65
PA103 P_{lacUV5}-*exsCEBA*′-′*lacZ*	P_{lacUV5}-driven *exsA* translational reporter integrated at CTX site	18
PA103 P_{lacUV5}-*exsCEB*′-′*lacZ*	P_{lacUV5}-driven *exsB* translational reporter integrated at CTX site	18
PA103 P_{lacUV5}-*exsCE*′-′*lacZ*	P_{lacUV5}-driven *exsE* translational reporter integrated at CTX site	18
PA103 P_{lacUV5}-*exsC*′-′*lacZ*	P_{lacUV5}-driven *exsC* translational reporter integrated at CTX site	18
PA14 P_{lacUV5}-*exsCEBA*′-′*lacZ*	P_{lacUV5}-driven *exsA* translational reporter integrated at CTX site	This study
PA103 CVS	P_{lacP1}-*lacZ* reporter for cAMP-Vfr signaling	38
PA103 P_{exsD}-*lacZ* Δ*vfr*	Isogenic deletion of *vfr* in PA103 P_{exsD}-*lacZ* background	18
PA103 P_{exsD}-*lacZ* Δ*algZR*	Isogenic deletions of *algZ* and *algR* in PA103 P_{exsD}-*lacZ* background	18
PA103 P_{exsD}-*lacZ* Δ*rsmYZ*	Isogenic deletions of *rsmY* and *rsmZ* in PA103 P_{exsD}-*lacZ* background	18
PA103 P_{rsmY}-*lacZ*	*rsmY* transcriptional reporter chromosomally integrated at CTX site	18
PA103 P_{rsmZ}-*lacZ*	*rsmZ* transcriptional reporter chromosomally integrated at CTX site	18
PA103 P_{rsmA}-*lacZ*	*rsmA* transcriptional reporter chromosomally integrated at CTX site	18, 66
PA103 P_{rsmY}-*lacZ* Δ*gacA*	Isogenic deletion of *gacA* in PA103 P_{rsmY}-*lacZ* background	18
PA103 P_{rsmY}-*lacZ* Δ*gacS*	Isogenic deletion of *gacS* in PA103 P_{rsmY}-*lacZ* background	18
PA103 P_{rsmZ}-*lacZ* Δ*gacA*	Isogenic deletion of *gacA* in PA103 P_{rsmZ}-*lacZ* background	18
PA103 P_{rsmZ}-*lacZ* Δ*gacS*	Isogenic deletion of *gacS* in PA103 P_{rsmZ}-*lacZ* background	18
PA14 P_{rsmY}-*lacZ*	*rsmY* transcriptional reporter chromosomally integrated at CTX site	This study
PA14 P_{rsmZ}-*lacZ*	*rsmZ* transcriptional reporter chromosomally integrated at CTX site	This study

Genetic manipulations. PA14 P_{lacUV5}-*exsCEBA*′-′*lacZ* was constructed by conjugation of plasmid p3UY51 (18) from *Escherichia coli* strain S17 into PA14. PA14 P_{rsmY}-*lacZ* and PA14 P_{rsmZ}-*lacZ* were constructed by conjugation of plasmids mini-CTX-P_{rsmY}-*lacZ* and mini-CTX-P_{rsmY}-*lacZ* (18), respectively, into PA14. Exconjugants were selected on VBM plates with tetracycline, as described above.

β-Galactosidase assays. *P. aeruginosa* was grown to an OD_{600} of 1.0, and β-galactosidase activity was measured as reported earlier (18). *ortho*-Nitrophenyl-β-D-galactopyranoside (ONPG) was used as the substrate for β-galactosidase in all the β-galactosidase assays involving transcriptional reporters; chlorophenol red–β-D-galactopyranoside (CPRG) was used as the substrate in assays involving translational reporters (18, 61). Plasmid p*mgtE* (28) and its empty backbone vector pMQ72 (58) were used to assess the effect of *mgtE* expression on transcription and translation.

RNA isolation and real-time qRT-PCR. *P. aeruginosa* strains were cultured as described above for β-galactosidase assays and harvested at an OD_{600} of 1.0, whereupon the pellet was washed with phosphate-buffered saline (PBS). This was followed by RNA isolation using the RNeasy Plus kit (Qiagen) according to the manufacturer's instructions. A few modifications were made to the protocol, as described earlier (30). Briefly, the RNA was subjected to on-column DNase digestion prior to elution. Additionally, after elution, a second DNase digestion was performed, followed by the RNA cleanup procedure. These digestions result in negligible DNA contamination of the final isolated RNA sample (30). cDNA was synthesized from the RNA using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen), according to the manufacturer's guidelines (30). DNA contamination of the RNA preparations was tested in control reactions by performing cDNA synthesis in the absence of reverse transcriptase. Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed as previously reported (30) using the following primers: *exsA*RTfor (5′-GCTGATGCTCTTCGCGTTCAGTCC-3′) and *exsA*RTrev (5′-TGGGCATAGAGGATTCTCCGCTCG-3′), which amplify *exsA* from nucleotides 436 to 676; *mgtE*RTforNewest (5′-AAGCAAGTGCTGGAAGTCATGG-3′) and *mgtE*RTrevNewest (5′-ATGTTGAGGACTTCGCTTTCGC-3′), which amplify *mgtE* from nucleotides 332 to 587; and *lacZ*RTfor (5′-CAACTGTTTACCTTGTGGAG-3′) and *lacZ*RTrev (5′-TATGAACGGTCTGGTCTTTG-3′), which amplify the *lacZ* transcript from nucleotides 2271 to 4800. Samples were normalized to the *fbp* transcript using primers PA5110for (5′-CCTACCTGTTGGTCTT CGACCCG-3′) and PA5110rev (5′-GCTGATGTTGTCGTGGGTGAGG-3′) (28, 30, 62, 63).

Statistical analyses. At least three independent experiments were performed for each assay. A two-sample Student *t* test was used to determine statistical significance ($P < 0.05$). For multiple comparisons, a one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) *post hoc* test was used to determine statistical significance ($P < 0.05$).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00268-17.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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