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The *Pseudomonas aeruginosa* Magnesium Transporter MgtE Inhibits Transcription of the Type III Secretion System▽†

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***Pseudomonas aeruginosa* is an opportunistic pathogen that causes life-long pneumonia in individuals with cystic fibrosis (CF). These long-term infections are maintained by bacterial biofilm formation in the CF lung. We have recently developed a model of *P. aeruginosa* biofilm formation on cultured CF airway epithelial cells. Using this model, we discovered that mutation of a putative magnesium transporter gene, called *mgtE*, led to increased cytotoxicity of *P. aeruginosa* toward epithelial cells. This altered toxicity appeared to be dependent upon expression of the type III secretion system (T3SS). In this study, we found that mutation of *mgtE* results in increased T3SS gene transcription. Through epistasis analyses, we discovered that MgtE influences the ExsE-ExsC-ExsD-ExsA gene regulatory system of T3SS by either directly or indirectly inhibiting ExsA activity. While variations in calcium levels modulate T3SS gene expression in *P. aeruginosa*, we found that addition of exogenous magnesium did not inhibit T3SS activity. Furthermore, *mgtE* variants that were defective for magnesium transport could still complement the cytotoxicity effect. Thus, the magnesium transport function of MgtE does not fully explain the regulatory effects of MgtE on cytotoxicity. Overall, our results indicate that MgtE modulates expression of T3SS genes.**

Pseudomonas aeruginosa is an opportunistic pathogen that can cause a wide range of infections in humans (28). One of the most devastating illnesses that it can produce is chronic infection in individuals with cystic fibrosis (CF) (14, 39). CF is a common genetic disorder resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), which is a chloride channel (3, 24). CFTR mutation leads to increased inflammation in the lungs and thickened mucus secretions that plug the airways (3). Within these mucus plugs, *P. aeruginosa* can form biofilms that are highly resistant to antibiotic killing and immune clearance mechanisms (19, 29, 44). Growing as a biofilm, *P. aeruginosa* can persist in the CF lung for the life of the patient, and this chronic bacterial colonization is a major factor leading to morbidity and mortality in these individuals (39). Strategies aimed at disrupting the formation or maintenance of these biofilms would greatly enhance the quality of life for CF patients.

Recent evidence has revealed that *P. aeruginosa* inversely regulates biofilm formation and virulence attributes associated with acute infections (13, 15). One factor that appears to be regulated in this manner is the type III secretion system (T3SS), a host cell contact-dependent virulence determinant involved in host cell toxicity (51). The T3SS is a needle-like complex with a base structure that spans both the inner and outer membranes of the bacteria. The tip of this complex can insert into the cytoplasmic membrane of a host cell, leading to injection of specific toxin molecules from the bacterial cytoplasm directly into the host cell cytoplasm (6, 7).

The central regulatory pathway controlling T3SS gene expression in *P. aeruginosa* is carried out by ExsE, ExsC, ExsD, and ExsA, which modulate the availability of the master activator ExsA (51) (see Fig. S1 in the supplemental material). All known inputs that affect T3SS transcription in *P. aeruginosa* exert their effects through ExsA, and in the absence of ExsA, T3SS gene transcription is negligible. Control of ExsA activity is maintained by alternative sets of complexes, where ExsD binds and sequesters ExsA (ExsD-ExsA), and ExsC can alternatively bind to ExsD or ExsE (ExsC-ExsD or ExsE-ExsC) (51). ExsE, which sits at the top of this cascade, is exported through the T3SS apparatus upon EGTA stimulation or mammalian cell contact, thus freeing ExsC (11, 51). ExsC then binds to ExsD (ExsC-ExsD), which in turn liberates ExsA. As such, mutation of either *exsD* or *exsE* hyperactivates T3SS transcription by deregulating the Exs network (and freeing ExsA) (50). On the other hand, mutation of *exsC* inhibits transcription because ExsD will bind and sequester ExsA.

Expression of the T3SS during *P. aeruginosa* infection is associated with increased bacterial virulence (39). T3SS is important for killing of a variety of eukaryotic cell types, including epithelial cells, fibroblasts, and macrophages (21, 41). In the context of CF lung infection, it is thought that the T3SS is involved in initial tissue destruction and protection from phagocytes (29). However, over time, the number of T3SS-expressing *P. aeruginosa* isolates from an individual CF patient decreases, likely as the result of mutation of T3SS genes (21, 45). Still, mutation of T3SS genes is not universal, and many CF isolates retain T3SS functionality (9). In these T3SS-positive strains, gene expression is likely repressed by regulatory mechanisms involved in preventing virulence factor production

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during the biofilm mode of growth. Thus, control of T3SS gene expression appears to be an important factor in the establishment and maintenance of chronic CF lung infection with *P. aeruginosa*.

In a recent study describing *P. aeruginosa* biofilm formation on cultured human airway epithelial cells, we identified a number of genes that impact bacterial cytotoxicity toward the cultured cells (1). One of these genes encoded a putative member of the MgtE family of magnesium transporters. The *mgtE* gene was originally discovered during screening of plasmid libraries in *Bacillus firmus* as clones that complemented a magnesium transport-deficient strain (47, 49). Since then, members of the MgtE transporter family have been identified in Gram-negative and Gram-positive bacteria, as well as in archaea and eukaryotes, including humans (23, 30, 40). MgtE family members are unique among known magnesium transporters in that they do not resemble any other protein class or superfamily of transporters (23, 30). Intriguingly, mutation of *mgtE* in *Campylobacter jejuni* and *Aeromonas hydrophila* leads to defects in virulence properties, including colonization of host sites, biofilm formation, and swarming (22, 33). In *P. aeruginosa*, we found that mutation of *mgtE* resulted in increased cytotoxicity of the biofilms grown on epithelial cells in culture, and it was discovered that this effect required a functional T3SS (1). Transcription of T3SS genes was only slightly enhanced in the *mgtE* deletion mutant. Mutation of other putative magnesium transporter genes in *P. aeruginosa* failed to show a similar cytotoxic effect, suggesting a specific effect of MgtE on cytotoxicity. Importantly, biofilm formation on both epithelial cells as well as plastic wells remained unaffected by *mgtE* deletion. We also found that protease and lipase production in the Δ*mgtE* strain appeared to be similar to production in the wild type (1). Thus, mutating *mgtE* does not cause a general increase in virulence factor expression.

In this study, we demonstrate that mutation of *mgtE* leads to increased T3SS gene expression. We found small but significant increases in T3SS gene transcription in the *mgtE* mutant, and epistasis analysis with the *exsE-exsC-exsD-exsA* (*exsE-C-D-A*) regulatory cascade genes revealed that *mgtE* expression modulates expression of T3SS genes, likely via inhibition of ExsA-dependent transcription. We also show that regulatory functions of MgtE in *P. aeruginosa* can be separated from the magnesium transport functions of this protein. Thus, it appears that *P. aeruginosa* can utilize this membrane protein for two different roles: magnesium transport and regulation of gene expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Appropriate antibiotics were used to maintain plasmid selection (see below). Growth curves were performed in a SpectraMax M2 (Molecular Devices, Sunnyvale, CA). Bacteria were diluted 1/100 in 200 μl of medium with appropriate antibiotics in standard sterile 96-well flat-bottom microtiter plates, with four wells for each strain. Growth was assayed at 37°C by measuring the optical density at 600 nm (OD_{600}) every 30 min for 11 h, with shaking for 5 s before each reading.

Genetic manipulations. (i) Construction of deletion mutants. Isogenic deletion mutants were created as previously described (1, 42). Briefly, gene-flanking regions were PCR amplified and joined to the suicide vector pMQ30 via homologous recombination in *Saccharomyces cerevisiae*. These knockout vectors were transformed into the cloning strain *Escherichia coli* S17 and verified by restriction digest. *P. aeruginosa* PA14 or PA103 was conjugated with these S17 transformants, and exconjugants were selected on LB agar with 50 μg/ml gentamicin and 20 μg/ml nalidixic acid; cells were grown overnight and plated on 10% sucrose LB agar plates. Mutations were confirmed by PCR and sequencing. Mutation of *mgtE* was carried out using plasmid pSMC233 (Table 1). To delete *exsA*, we created plasmid pSMC297 using primers ExsALfor, ExsALrev, ExsARfor, and ExsARrev, and primers ExsAfor and ExsArev were used to confirm mutation (Table 2). To delete *exsC*, we created plasmid pSMC298 using primers ExsCLfor, ExsCLrev, ExsCRfor, and ExsCRrev, and primers ExsCfor and ExsCrev were used to confirm mutation. To delete *exsD*, we created plasmid pSMC296 using primers ExsDLfor, ExsDLrev1, ExsDRfor1, and ExsDRrev, and primers ExsDfor and ExsDrev were used to confirm mutation. To delete *exsE*, we created plasmid pSMC299 using primers ExsELfor, ExsELrev, ExsERfor, and ExsERrev, and primers ExsEfor and ExsErev were used to confirm mutation.

(ii) Creation of complementing plasmids. To create a plasmid carrying *mgtE*, we PCR amplified *mgtE* from strain PA14 using primers 913forRBS (EcoRI site) and 913revHindIII (HindIII site) (Table 2). This fragment was ligated into EcoRI/HindIII-digested pMQ70, creating pSMC291. To create pSMC292 (C-terminally six-histidine tagged *mgtE*), we PCR amplified *mgtE* from PA14 using primers 913forRBS (EcoRI site) and 913revHindHIS (HindIII site). This fragment was ligated into EcoRI/HindIII-digested pMQ70. Plasmid pSMC293 (N-terminally six-histidine tagged *mgtE*) was created by PCR amplification of *mgtE* from pSMC291 using primers MgtNHISfor and 913Nrightrev. This fragment was then joined with HindIII-digested pMQ70 via homologous recombination in *S. cerevisiae*, as described previously (42). Plasmid pSMC295 (*mgtE* with mutations in Mg^{2+} binding sites 2 and 3) was created by PCR amplification of *mgtE* from pSMC293 in two pieces using primer pairs R-Pbad–MgSite2/

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
P. aeruginosa strains		
PA14	Wild type	38
PA103	Wild type	27
SMC3604	PA14 Δ*mgtE*	1
SMC4333	PA14 Δ*exsA*	This study
SMC4331	PA14 Δ*exsC*	This study
SMC4335	PA14 Δ*exsE*	This study
SMC4334	PA14 Δ*mgtE* Δ*exsA*	This study
SMC4332	PA14 Δ*mgtE* Δ*exsC*	This study
SMC4336	PA14 Δ*exsE* Δ*exsD*	This study
SMC4404	PA103 Δ*mgtE*	This study
SMC4405	PA14:$P_{exsD\text{-}lacZ}$	This study
SMC4406	SMC3604:P_{exsD}-lacZ	This study
SMC4407	PA103:$P_{exsD\text{-}lacZ}$	31
SMC4408	SMC4404: $P_{exsD\text{-}lacZ}$	This study
E. coli strain		
S17-1	Laboratory strain for cloning	43
S. cerevisiae strain		
InvSc1	*In vivo* cloning; *ura3-52*/*ura3-52*	Invitrogen
S. Typhimurium strain		
MM281	*corA45*::MudJ *mgtA21*::MudJ *mgtB10*::MudJ	Salmonella Genetic Stock Centre
Plasmids		
pMQ30	Allelic replacement vector; yeast cloning	42
pMQ70	P_{BAD} expression vector	42
pSMC233	Deletion of *mgtE*; pMQ30 backbone	1
pSMC297	Deletion of *exsA*	This study
pSMC298	Deletion of *exsC*	This study
pSMC296	Deletion of *exsD*	This study
pSMC299	Deletion of *exsE*	This study
pSMC291	pMQ70-*mgtE*	This study
pSMC292	pMQ70-*mgtE*-C-His	This study
pSMC293	pMQ70-*mgtE*-N-His	This study
pSMC295	pMQ70-*mgtE*-BS2/3 (Mg^{2+} binding sites 2 and 3 changed to alanine)	This study
Mini-CTX-$P_{exsD\text{-}lacZ}$	*lacZ* expression from *exsD* promoter	31

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)
913forRBS	AAGAATTCAGGAGGAGCGCGCTATGACCGAAGTAG
913revHindIII	AAAAGCTTGCTACATCAGGAAGATCGTC
913revHindHIS	AAAAGCTTGCTAATGATGATGATGATGATGCATCAGGAAGATCGTC
MgtNHISfor	CGTTTTTTTGGGCTAGCGAATTCAGGAGGAGCGCGCTATGCATCATCATCATCATCATACC GAAGTAGAAGCCAAGAAG
913Nrightrev	CAGACCGCTTCTGCGTTCTG
MgSite2/3Left	ACCGGCCATGTTGAGGACCGCCGCTTCGCTCGCCTCACGGATCAG
MgSite2/3Right	CTGATCCGTGAGGCGAGCGAAGCGGCGGTCCTCAACATGGCCGGT
F-t1t2	CAGACCGCTTCTGCGTTCTG
R-Pbad	GCAACTCTCTACTGTTTCTCC
PA1713F	GCTGATGCTCTTCGCGTTCAGTCC
PA1713R	TGGGCATAGAGGATTCTCCGCTCG
ExoURTfor	AATTGCGCGAGCAAACCGTTG
ExoURTrev	TTCTGTTGAGCAACACTGGTGAGC
PopNRTfor	ATGGACATCCTCCAGAGTTCCTC
PopNRTrev	TGCAGCATGGCCTGGACTTC
1693for	ATGATCCAGTTGCCCGACGAG
1693rev	TCACGTACAGGCTGAGGATG
PA5110for	CCTACCTGTTGGTCTTCGACCCG
PA5110rev	GCTGATGTTGTCGTGGGTGAGG
ExsALfor	TCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCAGGGTTTGAGCGGTTCCATG
ExsALrev	CGATTCTACTCATGCAGCCGCCCAACACTTCCCGTCGTAC
ExsARfor	GTACGACGGGAAGTGTTGGGCGGCTGCATGAGTAGAATCG
ExsARrev	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGATCCATGATTCCTCGGTAG
ExsAfor	TGCAAGTCTCGTTCGTTGTC
ExsArev	CGGGAGTACTGCTTATCGTC
ExsCLfor	TCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCGCGATCTGAGGAGACGTCAC
ExsCLrev	GATTTTCATGGAACCGCTCACTTGCTCGTTAAATCCATGG
ExsCRfor	CCATGGATTTAACGAGCAAGTGAGCGGTTCCATGAAAATC
ExsCRrev	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTCTGATTCGCAGAGCGAGTCG
ExsCfor	AAGGTCGAGGACCAGATGAG
ExsCrev	GCTCGGCAATCGTTTGCATC
ExsDLfor	TCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCTCAAGGTACGACGGGAAGTG
ExsDLrev1	CTCAGCTCTGCCAGTAGAAGTCTCTGCCTTGGCTTCCTCAC
ExsDRfor1	GTGAGGAAGCCAAGGCAGAGACTTCTACTGGCAGAGCTGAG
ExsDRrev	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTTACTCGATCTTGCGGTCCTC
ExsDfor	GCGACATGAGCATCGTCGAC
ExsDrev	CAGCAACAGGACGCTCTGTC
ExsELfor	TCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCAGGAGAAGACCCTGCAGAAG
ExsELrev	CAACAGCATCCAGCACCTCAGGAACCGCTCAAACCCTCATG
ExsERfor	CATGAGGGTTTGAGCGGTTCCTGAGGTGCTGGATGCTGTTG
ExsERrev	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTAACGCTCGACTTCGCTCAAC
ExsEfor	TGCTGTTCGACGAACAGGTG
ExsErev	ATCGTTTGCATCCCTCCCTG

3Left and MgSite2/3Right-913Nrightrev. MgSite2/3Left and MgSite2/3Right contained base changes relative to wild-type *mgtE* sequence such that glutamines encoded at amino acid positions 289 and 293 were changed to alanines, and serine 292 was changed to alanine. These PCR fragments were joined with HindIII-digested pMQ70 via homologous recombination in *S. cerevisiae*.

Cell culture. For bacterial-epithelial cell coculture, we used CFBE41o− parental (CFBE) cells, which are immortalized human CF-derived airway epithelial cells (4, 8, 48). CFBE cells were maintained in minimal essential medium (MEM) (10-010-CV; Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were incubated at 37°C in 5% CO_2 and fed fresh medium every 2 to 3 days.

Cytotoxicity assays. Cytotoxicity was assessed using the CytoTox96 Nonradioactive Cytotoxicity Assay kit (TB163; Promega, Madison, WI). CFBE cells were seeded at 2×10^5 cells per well in 24-well tissue culture plates with 0.5 ml of MEM, and these cells were grown for 7 to 10 days at 37°C in 5% CO_2 before use. Medium was replaced every 2 to 3 days. Confluent epithelial monolayers were inoculated with bacteria at 2×10^7 CFU/ml in each well (multiplicity of infection [MOI] of ~30:1), as previously described (1). Fresh MEM (0.5 ml) with 0.4% arginine was added at 1 h postinoculation, and the sample was incubated for an additional 5 h at 37°C in 5% CO_2. After this incubation, 400 μl of the supernatant was harvested, and cytotoxicity was assessed according to the manufacturer's instructions, as previously described (1). In experiments testing wild-type and mutant strains, bacteria were concurrently inoculated into separate wells, and supernatants were collected at the same time points (6 h postinoculation). Previous experiments have shown that this method results in an equal number of CFU of wild-type and Δ*mgtE* strains attached to the epithelial cells (1). As a positive control for the cytotoxicity assays, we added 1% Triton X-100 to a separate set of wells containing uninoculated CFBE cell monolayers. This resulted in a complete lysis of the epithelial cells. This control provided a measure of 100% cytotoxicity because it represents a maximum release of lactate dehydrogenase (LDH) from these cells. A value of 0% cytotoxicity represents the average value of the supernatant from a separate set of uninoculated wells in the absence of Triton X-100. This control provides a measure of the spontaneous release of LDH from the CFBE cells, and values were normalized to this level. Percent cytotoxicity was determined by the following calculation: 100 × (experimental value − spontaneous release value)/maximum release. We performed each assay in triplicate, and presented data are representative of at least three experiments.

Transcriptional assays. (i) qRT-PCR. Transcriptional levels of T3SS genes in CFBE-*P. aeruginosa* coculture were determined by quantitative real-time PCR (qRT-PCR), as previously described (1, 26). We used primer pairs PA1713F-PA1713R (*exsA*), ExoURTfor-ExoURTrev, PopNRTfor-PopNRTrev, and 1693for-1693rev (*pscR*) (Table 2). Expression values for each sample were normalized to *fbp* transcript levels (PA5110for-PA5110rev), which remain constant regardless of experimental conditions (1, 26).

(ii) β-Galactosidase assays. The $P_{exsD\text{-}lacZ}$ reporter strains were created using the mini-CTX-$P_{exsD\text{-}lacZ}$ construct, and β-galactosidase assays for planktonically grown cells were performed as previously reported (11, 31). T3SS was stimulated by chelation of calcium with 1 mM EGTA. To test the effect of magnesium on T3SS transcription, we added 20 mM $MgCl_2$. The reported values are the average of three independent experiments. β-Galactosidase assays in coculture biofilms were performed as above, with slight modifications. After a 6-h development of coculture biofilms, six replicate wells of each strain were washed two times with 1 ml of phosphate-buffered saline (PBS). Three wells were harvested in 1 ml of 0.1% Triton X-100 in PBS and plated for CFU determination. Three wells were harvested in 0.5 ml of 0.1% Triton X-100 in PBS for β-galactosidase activity. To this second set of samples, we added 0.5 ml of Z buffer (16.1 g/liter $Na_2HPO_4 \cdot 7H_2O$, 5.5 g/liter $NaH_2PO_4 \cdot 4H_2O$, 0.75 g/liter KCl, 0.246 g/liter $mgSO_4 \cdot 7H_2O$, 2.7 ml β-mercaptoethanol [pH 7.0]), 40 μl of chloroform, and 10 μl of 0.1% SDS. This solution was vortexed for 10 s and incubated at 30°C for 5 min. A total of 200 μl of 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was added for 90 min at 30°C, and then 0.5 ml of 1 M Na_2CO_3 was added to stop the reaction. $P_{exsD\text{-}lacZ}$ expression was determined by the following calculation: $\{[OD_{420} - (1.75 \times OD_{550})]/\text{number of CFU}\} \times 10^9$. This calculation represents a modification of the standard Miller unit equation in that data were normalized to the number of CFU rather than OD_{600} value of the bacteria due to difficulty in taking optical density measurements specifically of the bacteria in coculture experiments. Thus, on graphs displaying results from these experiments, we have labeled the *y* axis $P_{exsD\text{-}lacZ}$ expression rather than Miller units. Three replicate wells containing only CFBE cells were similarly processed, and OD_{420} and OD_{550} values for these CFBE-only samples were subtracted from the respective sample values above as a baseline. Data are representative of three independent experiments.

Western immunoblotting. For Western blotting of membrane fractions, bacterial cultures were grown overnight, and bacteria (1.2 ml of overnight culture) were resuspended in 200 mM Tris-HCl (pH 7.5), 20 mM EDTA, and 1× protease inhibitors and lysed on ice by six 10-s bursts with a tip sonicator (Vibra-Cell, Sonics and Materials, Inc., Newtown, CT) set at 30% power. Unlysed bacteria were removed by brief centrifugation in a microcentrifuge, and 1.1 ml of supernatant was centrifuged at 100,000 × *g* at 4°C for 1 h to pellet membranes. Membrane pellets were resuspended in 1 ml of 20 mM Tris-HCl, pH 7.6. Supernatant fractions were prepared by sedimenting 1×10^9 cells (centrifugation for 4 min at 12,500 × *g* at 4°C) and then transferring 1 ml of the cell-free supernatant to a fresh tube containing 350 μl of 50% trichloroacetic acid. Samples were incubated on ice for 30 min, and precipitated proteins were collected by centrifugation (15 min at 12,500 × *g* at 4°C), washed with acetone, and suspended in SDS-PAGE sample buffer. Western blotting was carried out according to standard molecular biology protocols using anti-histidine (Qiagen, Valencia, CA), anti-ExoU, and anti-PcrV primary antibodies and goat anti-rabbit or anti-mouse IgG-horseradish peroxidase (HRP) secondary antibodies (Bio-Rad, Hercules, CA). We used Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Boston, MA). Detection of SadB and SecY was performed as cytoplasmic and membrane fraction controls (5, 34). The presence of nonspecific bands in SadB lanes (see Fig. 7) as well as the apparent cytoplasmic presence of SecY has been reported previously (5, 34).

***Salmonella* magnesium bioassay.** Magnesium transport was assessed indirectly using *Salmonella enterica* serovar Typhimurium MM281, as previously described (47, 49). This strain contains mutations in all of its known magnesium transporters and thus will not grow without supplementation with 100 mM magnesium. Growth of this strain in minimal medium without added magnesium requires the bacterium to be transformed with a plasmid containing a magnesium transporter gene. This assay has been used as a surrogate for magnesium uptake assays by serving as a sensitive test for functional complementation of the deficient magnesium transport in MM281 (47, 49). Thus, the ability of MgtE and derivative constructs to transport magnesium was determined by electroporating MM281 with plasmid p*mgtE*, p*mgtE* with a C-terminal His tag (p*mgtE*-C-His, or pCHIS), p*mgtE* with an N-terminal His tag (p*mgtE*-N-His, or pNHIS), or pBS2/3 (Table 1). Transformants were selected on LB plates supplemented with 100 mM $MgSO_4$ and 50 μg/ml carbenicillin (to maintain plasmid selection), and proper transformation was confirmed by plasmid isolation and restriction digest. These clones were then grown overnight at 37°C in LB medium with 100 mM $MgSO_4$ and 50 μg/ml carbenicillin, and 20 μl of this culture was streaked out on N minimal medium agar plates [5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 0.1 M Tris-HCl, 0.1% Casamino Acids, 38 mM glycerol](35). The ability to transport magnesium was assessed as growth overnight on N plates at 37°C.

Statistical analysis. Statistical significance was determined as a *P* value of <0.05 using a Student's *t* test.

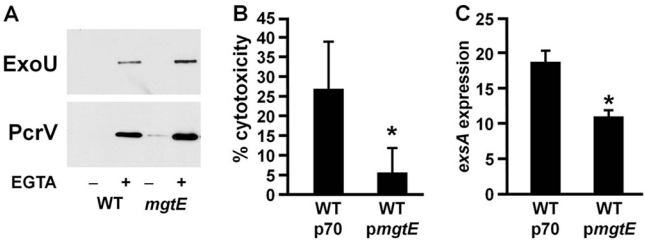

FIG. 1. Mutation of *mgtE* leads to increased effector molecule secretion through T3SS. (A) Western immunoblotting of supernatants from planktonic cultures of strain PA14 (WT) and its isogenic *mgtE* deletion mutant. Supernatants were probed with antibodies to ExoU and PcrV in the presence and absence of the calcium chelator EGTA. (B) Cytotoxicity of wild-type PA14 (WT), with vector control (p70, or pMQ70) plasmid or with a plasmid carrying *mgtE* (p*mgtE*, or pSMC291), toward CFBE cells at 6 h postinoculation. The value of 100% cytotoxicity represents a maximum release from the CFBE cells by Triton X-100 lysis. ∗, $P < 0.05$. (C) Relative *exsA* expression by qRT-PCR in 6-h coculture biofilms formed by the wild type (WT) with vector control (p70) or the wild type expressing *mgtE* from plasmid p*mgtE*. *fbp* transcript levels were used as a normalization control. ∗, $P < 0.05$.

RESULTS

MgtE affects T3SS gene expression. Recent studies have found that *P. aeruginosa* forms biofilms upon cultured airway epithelial cells within 6 to 8 h after inoculation (1). Using this biofilm coculture system, we discovered that deletion of the *mgtE* gene resulted in increased cytotoxicity of *P. aeruginosa* biofilms grown on cultured CFBE cells (1). Because this increased cytotoxicity required a functional T3SS, we reasoned that the Δ*mgtE* deletion mutant might exhibit increased secretion of effector molecules through the T3SS apparatus. To test this hypothesis, we performed Western immunoblotting on bacterial supernatants prepared from planktonic bacteria grown in the presence or absence of EGTA to chelate extracellular calcium. Low-calcium conditions are known to stimulate type III secretory activity, which then results in an increase in T3SS gene expression. Supernatant fractions were probed for the T3SS-secreted proteins ExoU and PcrV (12, 51). As expected, EGTA treatment resulted in greater amounts of ExoU and PcrV in the supernatant fraction (Fig. 1A). In addition, we found an increase in both ExoU and PcrV in supernatants of the Δ*mgtE* mutant compared to wild-type *P. aeruginosa* strain PA14 (Fig. 1A). Quantification of band intensity revealed this increase to be approximately 2-fold. Similar to results for PA14, we found an approximate 2-fold increase in the amount of ExoU and PcrV secreted into the supernatant in planktonic cultures of the Δ*mgtE* mutant compared to wild-type PA103 (see Fig. S2A in the supplemental material).

These data suggest that one activity of MgtE might be to inhibit T3SS gene expression. In support of this hypothesis, we grew CFBE-*P. aeruginosa* coculture biofilms of wild-type PA14 transformed with a multicopy plasmid carrying *mgtE* (PA14 p*mgtE*), and we monitored cytotoxicity toward the CFBE cells. We discovered a significant decrease in the cytotoxicity of PA14 p*mgtE* compared to PA14 carrying a vector control (Fig. 1B). We also tested gene expression of T3SS by qRT-PCR in biofilms of these same strains grown on epithelial cells. As expected, we found significantly decreased transcript levels of

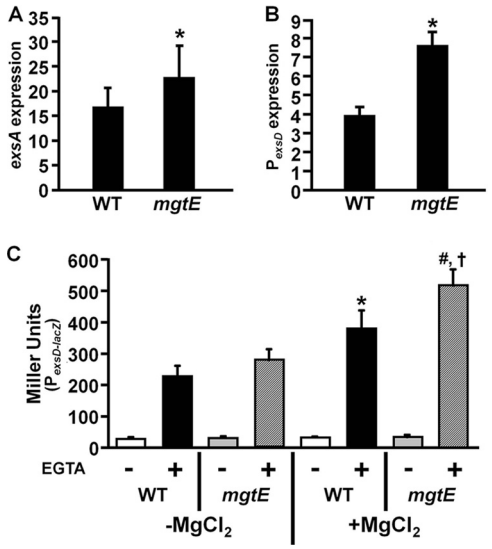

FIG. 2. Mutation of *mgtE* results in small increases in T3SS transcription. (A) Transcription of T3SS was measured as *exsA* transcript levels by qRT-PCR in wild-type PA14 (WT) and the Δ*mgtE* strain grown as biofilms in coculture with CFBE cells. The *y* axis measures relative *exsA* transcript levels, using *fbp* transcript levels as a normalization control (see Materials and Methods). Data represent the average of three independent experiments, each containing triplicate or quadruplicate samples. Each of these samples was assayed in triplicate by qRT-PCR. ∗, $P < 0.05$. (B) β-Galactosidase activity was measured from the $P_{exsD\text{-lacZ}}$ construct in wild-type PA14 (WT) and the Δ*mgtE* strain grown as biofilms in coculture with CFBE cells. The *y* axis represents relative β-galactosidase activity from the $P_{exsD\text{-lacZ}}$ transcriptional fusion construct, normalized to the number of CFU (see Materials and Methods). Data are representative of three independent experiments. ∗, $P < 0.05$. (C) Transcription of T3SS was also measured in broth cultures as β-galactosidase activity (Miller units) using the $P_{exsD\text{-lacZ}}$ fusion construct in wild-type PA14 (WT) and the isogenic *mgtE* deletion mutant. Transcriptional activity was assessed in the presence and absence of 1 mM EGTA, as indicated. Replicate samples were additionally treated with 20 mM $MgCl_2$, as indicated. ∗, $P < 0.05$, compared to EGTA-stimulated WT cells without magnesium; #, $P < 0.05$, compared to EGTA-stimulated Δ*mgtE* cells without magnesium; †, $P < 0.05$ compared to EGTA-stimulated WT cells with magnesium.

the T3SS gene *exsA* in the wild-type strain carrying *mgtE* on a plasmid compared to vector control (Fig. 1C). Thus, *mgtE* expression inhibits bacterial cytotoxicity, and this effect most likely occurs through inhibition of T3SS gene expression.

Mutation of *mgtE* leads to small but significant increases in T3SS gene transcription. In our previous study, we used qRT-PCR to investigate transcriptional activity of T3SS genes in wild-type PA14 and a Δ*mgtE* strain (1). While we saw no change in *pcrV* or *exoT* transcript levels between wild-type PA14 and the Δ*mgtE* strain at the time point that we examined, we did find a small but significant increase in expression of *pcrH* in the Δ*mgtE* mutant. These results suggested that mutation of *mgtE* might lead to small changes in T3SS gene transcription. To explore this effect further, we assayed *exsA* transcript levels by qRT-PCR in CFBE coculture biofilms formed by either wild-type or Δ*mgtE* mutant strains. In contrast to the inhibition of *exsA* transcription seen by overexpressing *mgtE* in wild-type *P. aeruginosa* (Fig. 1C), we observed a significant increase ($P < 0.05$) in relative *exsA* expression in the Δ*mgtE* strain over the wild-type strain (Fig. 2A). Combining data from multiple experiments, we also observed a significant increase in relative *exoU* transcript levels in the Δ*mgtE* strain (data not shown) although occasionally we found no difference between wild-type and Δ*mgtE* strains in individual experiments (see below and Fig. S3 in the supplemental material). We further assayed T3SS gene expression in these strains using a chromosomally integrated construct that expresses the *lacZ* gene from the *exsD* promoter ($P_{exsD\text{-lacZ}}$) (see Materials and Methods). *exsD* is the first gene in the *exsD-pscL* operon, and the $P_{exsD\text{-lacZ}}$ construct has been used to monitor activation of T3SS gene transcription (11). Wild-type and Δ*mgtE* strains were grown as biofilms in coculture with CFBE cells, and $P_{exsD\text{-lacZ}}$ activity was determined as described in Materials and Methods (providing a relative $P_{exsD\text{-lacZ}}$ expression activity rather than Miller units). This experiment also revealed a significantly greater T3SS promoter activity in the Δ*mgtE* strain than in the wild type (Fig. 2B). In planktonic, broth-grown cultures stimulated with EGTA, the Δ*mgtE* strain carrying the $P_{exsD\text{-lacZ}}$ reporter exhibited a similar trend toward increased β-galactosidase activity over the wild-type strain although this effect was not significant (Fig. 2C).

As mentioned above, T3SS gene expression is induced under low-calcium conditions due to a stimulation of ExsA-mediated gene transcription (12, 51). MgtE has been described as a magnesium transporter in several microorganisms, and it is formally possible that the *P. aeruginosa mgtE* mutant has reduced intracellular magnesium levels that similarly affect T3SS gene expression. Because magnesium, when present in high concentrations, has been shown to diffuse across bacterial membranes in the absence of transporters (45, 46), we reasoned that supplementation with magnesium might overcome any potential magnesium transport deficiency and inhibit T3SS gene expression. To test this hypothesis, wild-type and Δ*mgtE* strains carrying the $P_{exsD\text{-lacZ}}$ reporter were cultured in medium containing 20 mM $MgCl_2$ in the presence or absence of EGTA and then assayed for β-galactosidase activity. In the absence of EGTA, $P_{exsD\text{-lacZ}}$ reporter activity in wild-type PA14 was similar to that of the Δ*mgtE* strain regardless of magnesium (Fig. 2C). Intriguingly, EGTA-dependent expression of the $P_{exsD\text{-lacZ}}$ reporter was slightly increased (<2-fold) for both the wild-type and Δ*mgtE* strains when cells were grown in the presence of magnesium (Fig. 2C). β-Galactosidase activity was also significantly enhanced in the Δ*mgtE* strain versus the wild type under magnesium stimulation.

We also tested T3SS activity in *P. aeruginosa* strain PA103, which displays a much higher level of T3SS transcription and secretion than strain PA14 (25, 36). We reasoned that this relative increase in T3SS gene expression might accentuate any differences in T3SS transcription between wild-type PA103 and Δ*mgtE*. Using the $P_{exsD\text{-lacZ}}$ construct in PA103 and its isogenic Δ*mgtE* strain, we found a significant increase (~2-fold) in β-galactosidase activity in planktonic cultures of the Δ*mgtE* strain under both high- and low-calcium conditions compared with levels in wild-type PA103 (see Fig. S2B in the supplemental material). These data correlate well with the ~2-fold increase in secretion and transcription seen with the PA14 Δ*mgtE* strain, as shown above (Fig. 1A and 2B). Culturing PA103 and its isogenic Δ*mgtE* strain in the presence of 20 mM $MgCl_2$ resulted in no differences between magnesium-treated versus untreated samples although we did observe a

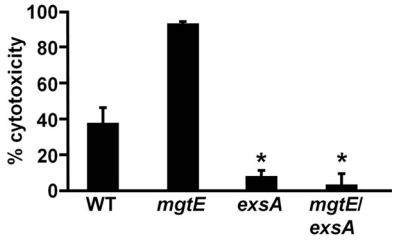

FIG. 3. Cytotoxicity effects of MgtE require ExsA-mediated gene transcription. Wild-type PA14 (WT), the *mgtE* deletion mutant, the *exsA* deletion mutant, and the double deletion strain Δ*mgtE* Δ*exsA* were tested for cytotoxicity toward CFBE cells at 6 h. The value of 100% cytotoxicity represents a maximum release from the CFBE cells by Triton X-100 lysis. *, $P < 0.05$, compared to the *mgtE* mutant.

significant increase in Δ*mgtE* samples versus wild-type samples (see Fig. S2B). Thus, although this effect is small, it is clear that mutation of *mgtE* increases T3SS gene transcription. This consequence appears to be independent of magnesium levels (see below).

MgtE influences the ExsE-C-D-A regulatory cascade. As mentioned above, transcriptional regulation of T3SS gene expression relies on the availability of the master activator protein ExsA. We deleted *exsA* in PA14 and in the Δ*mgtE* background (see Fig. S1B in the supplemental material) and tested for cytotoxicity toward CFBE cells. As expected, the Δ*exsA* mutant exhibited very little cytotoxicity toward the epithelial cells (Fig. 3). We also found that the Δ*mgtE* Δ*exsA* double mutant had very little cytotoxicity. This result demonstrates that Δ*mgtE* requires ExsA-mediated transcriptional activation to achieve increased toxicity toward cultured cells, a result that is consistent with our previous data showing that *mgtE* mutation requires a functional T3SS (1). This experiment is also consistent with our hypothesis that MgtE affects T3SS transcription.

To investigate the transcriptional effects of MgtE on T3SS gene expression further, we performed an epistasis analysis between *mgtE* and the *exs* regulatory genes. As mentioned above, control of ExsA activity is maintained by alternate sets of complexes formed by the ExsE, ExsD, ExsC, and ExsA proteins. Mutation of the *exs* genes affects the availability of ExsA and, thus, the transcriptional state of T3SS genes. We first deleted *exsC* in the wild type as well as the Δ*mgtE* background. As expected, the *exsC* mutant showed very little cytotoxicity (Fig. 4A). In this situation, ExsD is free to bind to ExsA, which would inhibit T3SS transcription (see Fig. S1C in the supplemental material). When we tested the Δ*mgtE* Δ*exsC* double mutant, we found that this strain also exhibited very little cytotoxicity (Fig. 4A). We also examined transcript levels of *exsA* in coculture biofilms by qRT-PCR in these same backgrounds; *exsA* is autoregulatory. We found ~3-fold lower relative levels of *exsA* transcription in both the Δ*exsC* and Δ*mgtE* Δ*exsC* strains than in the wild-type and Δ*mgtE* strains (Fig. 4B). Similar patterns were also observed when we assayed transcript levels of *pscR*, *popN*, and *exoU* (see Fig. S3A to C), which all map to different T3SS operons. These data support the hypothesis that MgtE-mediated T3SS inhibition functions via ExsC although it is still possible that MgtE acts directly on ExsA (see below).

We next deleted *exsE* in PA14, which has the predicted effect of dramatically increasing T3SS activation (see Fig. S1D in the supplemental material). In fact, we observed nearly 100% cytotoxicity toward CFBE cells at the time point we investigated (Fig. 4C). We decided to complement the Δ*exsE* strain with

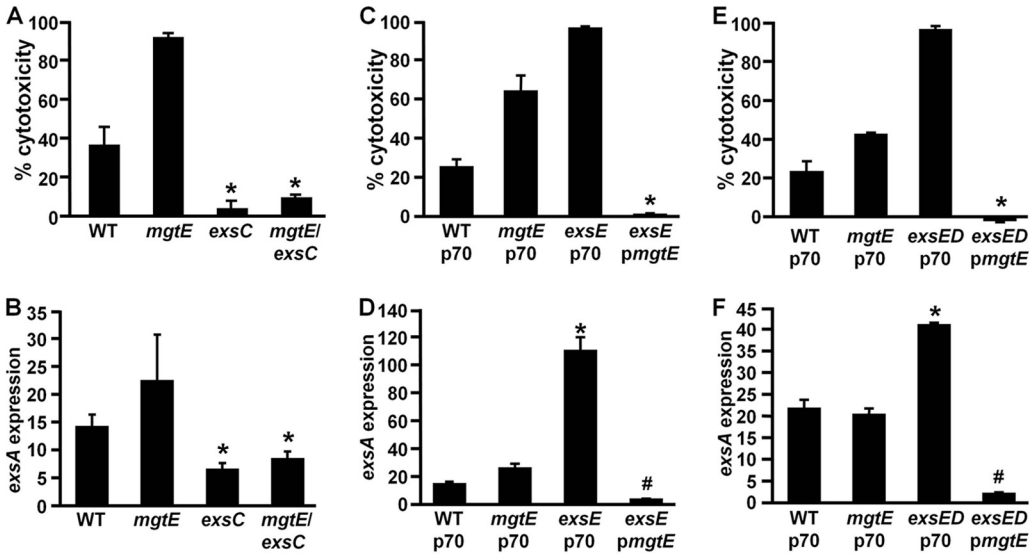

FIG. 4. MgtE expression impacts the Exs regulatory system. Cytotoxicity assays (A, C, and E) and expression levels of *exsA* (B, D, and F) were examined in the *exsC* mutant and the Δ*mgtE* Δ*exsC* double mutant (A and B), the *exsE* mutant complemented with vector control (p70) or p*mgtE* (C and D), and the *exsED* double mutant complemented with vector control or p*mgtE* (E and F). Results were compared to wild-type PA14 (WT) and the isogenic *mgtE* deletion mutant. Relative *exsA* expression was determined by qRT-PCR of coculture biofilms at 6 h postinoculation, as described in Materials and Methods. A value of 100% cytotoxicity represents a maximum release from the CFBE cells by Triton X-100 lysis. *, $P < 0.05$, compared to WT and *mgtE* strains (A and B), *exsE* p70 (C), *exsED* p70 (E), and WT p70 and *mgtE* p70 (D and F); #, $P < 0.05$, compared to *exsE* p70 (D) or *exsED* p70 (F).

plasmid p*mgtE* to determine whether *mgtE* expression can override the hyperactivated Exs cascade to inhibit cytotoxicity, similar to the effect of *mgtE* expression in wild-type PA14 (Fig. 1B). We found that complementation with p*mgtE* severely abrogated cytotoxicity in the Δ*exsE* strain (Fig. 4C).

While it is likely that MgtE is inhibiting transcription of T3SS genes, the above data are also consistent with the hypothesis that expression of MgtE might instead inhibit secretion of T3SS exoproteins, possibly as the result of interaction with the T3SS structure. In other words, MgtE might be directly or indirectly blocking the secretion pore. If this hypothesis is correct, then expression of *mgtE* in the Δ*exsE* strain would result in decreased cytotoxicity (because of decreased secretion of T3SS exotoxins) while transcription of T3SS genes should be unaffected compared to the Δ*exsE* strain without p*mgtE*. However, it is also possible that MgtE production specifically affects T3SS transcription rather than secretion. In this scenario, both cytotoxicity and transcription should be decreased by expression of *mgtE* in the Δ*exsE* strain. To test these hypotheses, we performed qRT-PCR on *exsA*, *pscR*, *popN*, and *exoU* (Fig. 4D; see also Fig. S3D to F in the supplemental material). As shown, transcription of T3SS genes is dramatically lower in the Δ*exsE* strain complemented with p*mgtE* than in the vector control, which is consistent with the hypothesis that MgtE inhibits T3SS gene transcription. Thus, our data suggest that MgtE inhibits cytotoxicity by decreasing T3SS gene transcription rather than by blocking the secretion pore.

The above data suggest that MgtE acts through either ExsC or ExsA to impact T3SS gene expression. To differentiate between these possibilities, we constructed the double deletion mutant strain Δ*exsE* Δ*exsD*, which is predicted to display an increased cytotoxicity phenotype due to release of ExsD-mediated inhibition of ExsA (see Fig. S1E). We favored this double mutant over the single Δ*exsD* construct because we felt it was important to examine T3SS transcription in the absence of any Exs inhibitory protein. If MgtE acts at the level of ExsC, then we would expect that complementation of the Δ*exsE* Δ*exsD* mutant with p*mgtE* would have no effect on cytotoxicity or transcription because ExsA would still be free to bind to T3SS promoters and activate transcription. Intriguingly, we found that complementation of this double mutant with p*mgtE* abrogated cytotoxicity (Fig. 4E) as well as T3SS gene transcription (Fig. 4F). Thus, MgtE appears to act directly or indirectly on ExsA.

Western blotting using Δ*exsE* and Δ*exsED* mutant bacteria grown in broth cultures showed further that complementation with p*mgtE* resulted in decreased levels of ExoU and PcrV secreted into the supernatant compared to levels with the vector control (Fig. 5). The wild type complemented with p*mgtE* also exhibited decreased PcrV secretion, but, unexpectedly, ExoU levels changed little or perhaps increased slightly. This result suggests that there is some physiological difference in MgtE-mediated T3SS triggering between EGTA-stimulated planktonic bacteria and coculture biofilms, the basis of which we do not fully understand at this time. While it is also unclear why there appeared to be more secretion in the Δ*exsE* strain than in the Δ*exsED* mutant, it is important to note that expression of *mgtE* led to diminished ExoU and PcrV secretion in both strains.

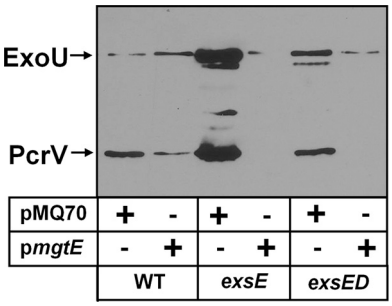

FIG. 5. p*mgtE* inhibits ExoU and PcrV secretion. Western blots of supernatants from wild-type (WT), Δ*exsE*, and Δ*exsED* strains grown planktonically and complemented with empty vector (pMQ70) or p*mgtE*. Cultures were grown in broth and treated with 1 mM EGTA to stimulate T3SS, as described in Materials and Methods.

Separation of cytotoxicity and magnesium transport functions. Considering that magnesium is not known to alter T3SS activity and considering our previous observations that mutation of other magnesium transporters does not affect cytotoxicity (1), the data presented above (Fig. 2; see also Fig. S2 in the supplemental material) support the hypothesis that magnesium treatment does not inhibit T3SS activity. If the regulatory effects of MgtE on cytotoxicity are different than the magnesium transport functions, then it should be possible to separate these functions. This is an important question because of our data in Fig. 2 showing no role for increased magnesium concentration in MgtE-mediated inhibition of T3SS transcription. We felt that this observation made it necessary to explicitly address whether magnesium uptake, or another function of MgtE, is important for the observed phenotypes caused by the *mgtE* mutation. Toward this end, we explored whether it was possible to create *mgtE* mutants that could complement cytotoxicity (as performed above) but that were defective with respect to magnesium transport.

To assay for magnesium transport, we used *S. enterica* serovar Typhimurium MM281 because this strain contains mutations in all of its known magnesium transport genes, and it requires supplementation with 100 mM magnesium in order to grow in minimal medium (46, 49). This strain is a tool that has been used to identify magnesium transporters by transformation with plasmids containing putative magnesium transport genes (46, 49). If the gene encodes a viable magnesium transporter, the strain will grow on minimal medium plates without added magnesium, whereas strains with an empty vector will not grow. Importantly, when we transformed MM281 with a plasmid carrying *mgtE* (p*mgtE*), we observed growth on minimal medium plates while MM281 with empty vector (p70) did not grow (Fig. 6A).

In order to create *mgtE* mutations with altered magnesium transport capabilities, we first added a six-histidine epitope tag to the C terminus of MgtE (see Materials and Methods) so that we could use Western immunoblotting to monitor whether our *mgtE* mutants were stably expressed. After transformation into MM281, we discovered that this p*mgtE*-C-His (pCHIS) construct failed to support growth on minimal medium plates (Fig. 6A). This effect might be due to the fact that His_6 tags bind to metals quite readily, and perhaps our tag is binding to magnesium and preventing transport. Furthermore, the His_6 tag that

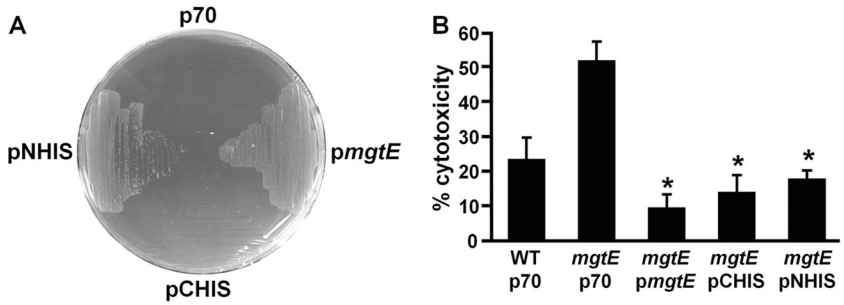

FIG. 6. Magnesium transport and cytotoxicity can be separated. (A) Growth of *S.* Typhimurium MM281 on N minimal agar plates. MM281 was complemented with vector control (p70), p*mgtE*, and plasmids pCHIS and pNHIS. (B) Cytotoxicity at 6 h of strain PA14 and the isogenic *mgtE* deletion mutant complemented with the plasmids in panel A. A value of 100% cytotoxicity represents a maximum release from the CFBE cells by Triton X-100 lysis. *, $P < 0.05$, compared to *mgtE* p70.

we added appears to be positioned directly over the periplasmic opening of the magnesium pore of MgtE, as predicted by the recent crystal structure of MgtE from *Thermus thermophilus* (18). Intriguingly, we found that the pCHIS construct fully complemented the cytotoxicity phenotype of the Δ*mgtE* strain (Fig. 6B). Thus, by simply adding a His_6 tag to the C terminus of MgtE, we demonstrated that the magnesium transport and cytotoxicity functions of MgtE could be separated.

To resolve the transport issues associated with the C-terminal His tag, we created an N-terminally His_6-tagged MgtE (pNHIS). This construct complemented both magnesium transport and cytotoxicity (Fig. 6A and B). These data support the hypothesis that the regulatory effects of MgtE on cytotoxicity are independent of the magnesium transport functions of MgtE.

However, it is possible that pCHIS permits a small amount of magnesium to be transported, and this very small amount might be enough to affect T3SS gene expression. We tested this hypothesis by performing minimal medium growth curves of MM281 complemented with the vector control, p*mgtE*, pCHIS, or pNHIS. We found that pNHIS permitted robust growth of MM281 while MM281 pCHIS did not grow at all and was similar to the strain carrying the vector control (see Fig. S4 in the supplemental material). MM281 strains carrying the untagged *mgtE* on a plasmid or expressing the N-terminally tagged variant grew equally well. These data indicate that the pCHIS construct of MgtE is nonfunctional for magnesium transport.

The above results encouraged us to look for point mutants of *mgtE* that could still inhibit cytotoxicity but could no longer transport magnesium. Using the pNHIS plasmid as a background, we mutated glutamine at position 289, serine at 292, and glutamine at 293 to alanines (E289A, S292A, and E293A, respectively). These three residues form two different predicted binding magnesium binding sites in MgtE (18). MgtE is predicted to contain six magnesium binding sites that appear to be important for effecting proper subunit positioning during conformational changes that occur as the magnesium pore opens and closes (20). The E289A/S292A/E293A mutations affected amino acid residues in predicted binding sites 2 and 3. This plasmid construct (pBS2/3) failed to complement magnesium transport in *S.* Typhimurium (Fig. 7A) but fully complemented cytotoxicity in PA14 (Fig. 7B). Western immunoblotting revealed that MgtE-N-His, MgtE-BS2/3, and MgtE-C-His were expressed in the membrane of the *P. aeruginosa* Δ*mgtE* strain (Fig. 7C). Each of these constructs was detected as a faint doublet on the blot. The faint band intensity has been replicated several times, suggesting that MgtE might be a low-abundance protein in the membrane of *P. aeruginosa*. The results of these experiments demonstrate that we could separate magnesium transport and regulation of cytotoxicity by changing just three amino acids.

DISCUSSION

In this study, we have demonstrated that expression of MgtE downregulates T3SS gene expression in *P. aeruginosa*. T3SS is a potent virulence factor responsible for epithelial cell damage in a number of different disease states. Modulation of T3SS activity could potentially mitigate host damage during infection, and MgtE may be one factor modulating activity of this virulence system. Our observation that deletion of *mgtE* results in greater secretion of T3SS exoproteins suggests that *P. aeruginosa* utilizes MgtE to carry out such modulation (Fig. 1A). In fact, we found that transformation of the wild-type strain with p*mgtE* led to decreased cytotoxicity toward cultured epithelial cells (Fig. 1B) and decreased transcription of T3SS genes (Fig. 1C). Moreover, deletion of *mgtE* led to an increase in cytotoxicity and T3SS transcription (Fig. 2 and 4). While these changes were small, it has been shown previously that small increases in ExoU secretion lead to large changes in cytotoxicity (11, 31). Thus, adjusting the level of MgtE expression or activity during an infection might impact the ability of *P. aeruginosa* to secrete T3SS toxins. In support of this hypothesis, we previously found that tobramycin treatment of *P. aeruginosa* biofilms on CFBE cells led to upregulation of *mgtE* expression but downregulation of several T3SS genes (1). Unexpectedly, we found that *mgtE* expression in Δ*exsE* and Δ*exsE* Δ*exsD* strains inhibited T3SS transcription to a greater extent than *mgtE* expression in the wild type (Fig. 1C and 4). We do not fully understand the basis of this effect although we speculate that it might be the result of competition between MgtE and the intact T3SS regulatory network in the wild type.

It is interesting that *mgtE* mutation and expression affected T3SS activity in biofilms as well as in the planktonic state. T3SS transcriptional activation can be stimulated by decreasing calcium concentration and by eukaryotic cell contact, such as might be found during coculture biofilm formation (51). Our data show that MgtE can affect T3SS under both conditions

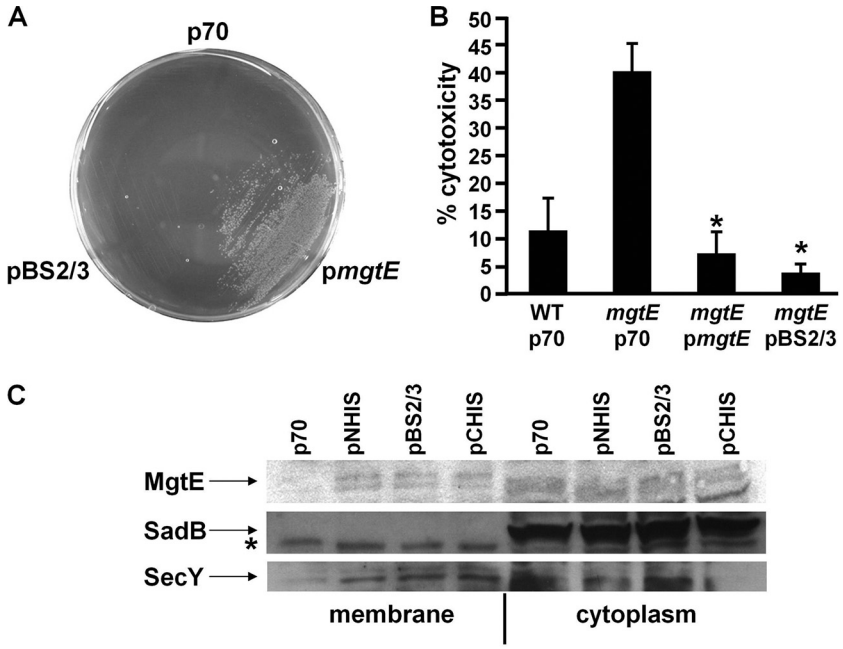

FIG. 7. Point mutations in the magnesium binding sites of MgtE disrupt magnesium transport but not cytotoxicity. (A) Growth of *S.* Typhimurium MM281 complemented with vector control (p70), *mgtE* plasmid (p*mgtE*), or the *mgtE* plasmid containing point mutations in magnesium binding sites 2 and 3 (pBS2/3). Strains were grown overnight on N minimal medium agar plates. (B) Cytotoxicity at 6 h of PA14 and the *mgtE* deletion mutant complemented with the plasmids in panel A. A value of 100% cytotoxicity represents a maximum release from the CFBE cells by Triton X-100 lysis. *, $P < 0.05$, compared to *mgtE* p70. (C) Western blot of total membrane fractions (left side) and cytoplasmic fractions (right side) from Δ*mgtE* transformed with vector control (p70), pNHIS, pBS2/3, and pCHIS. Fractions were incubated with anti-six-histidine (HIS) primary antibodies as well as anti-SadB or anti-SecY primary antibodies to detect cytoplasmic and membrane control proteins, respectively. The presence of the lower, nonspecific bands in SadB lanes (marked by an asterisk) has been noted previously (5). Apparent cytoplasmic presence of SecY has also been previously reported (34). Importantly, there appears to be no cytoplasmic contamination of the membrane fraction.

(Fig. 1 and S2). As mentioned above, recent studies have suggested that T3SS expression is decreased in *P. aeruginosa* biofilms compared to planktonic bacteria (13, 15). However, in a previous study, we demonstrated that T3SS is expressed in the coculture biofilm because deletion of the T3SS secretion apparatus reduced cytotoxicity of the biofilm (1, 26). While it is currently unknown how basal T3SS expression levels in coculture biofilms compare to levels in planktonic bacteria, it is clear that T3SS expression in coculture biofilms can be affected by MgtE levels in a manner similar to planktonic bacteria.

There are a number of different regulators that alter T3SS transcription in *P. aeruginosa* although the ExsE-D-C-A cascade appears to be the major regulatory system (51). Furthermore, all known T3SS regulators studied to date exert their effects on ExsA (51). We have also found that the increased cytotoxicity of our Δ*mgtE* mutant requires *exsA*-mediated gene transcription (Fig. 3). Our observation that *mgtE* expression in the Δ*exsE* Δ*exsD* double mutant strain abrogated cytotoxicity and gene expression (Fig. 4) indicates that MgtE exerts its effects on the T3SS by either directly or indirectly impacting the activity of ExsA. Our data reflect multiple experimental replicates performed in two different *P. aeruginosa* strains, suggesting a specific effect of MgtE on T3SS rather than random metabolic fluctuations.

Other T3SS regulators also impact the activity of ExsA. For instance, the copper-regulated inhibitor PtrA behaves similarly to MgtE. Deletion of *ptrA* led to increased secretion of T3SS exoproteins, and overexpression of *ptrA* decreased T3SS gene transcription (17). It was also found that PtrA physically interacts with ExsA and thus acts as an antiactivator, sequestering ExsA to prevent T3SS transcription (17). Due to the similarity in phenotypes between PtrA and MgtE, it seems possible that MgtE might also be binding to ExsA to inhibit T3SS gene transcription. Alternatively, *mgtE* expression might lead to the upregulation of another factor that binds directly to ExsA. Future studies will be aimed at investigating the molecular mechanisms of MgtE-mediated T3SS inhibition in more detail.

One of the key findings of this report is that the *mgtE* gene in *P. aeruginosa* encodes a magnesium transporter. While members of the MgtE family of magnesium transporters have been studied in many different organisms (including humans), to our knowledge the *P. aeruginosa mgtE* homologue has never been characterized (23, 30, 40). It is unknown whether *P. aeruginosa* MgtE can transport calcium although calcium transport through MgtE would be intriguing, considering the effect of calcium on T3SS. While MgtE proteins from *B. firmus* and *Providencia stuartii* have been shown to transport magnesium and cobalt, they are not predicted to transport calcium (47, 49). Moreover, it has been shown that mammalian homologues of MgtE cannot transport calcium although these proteins can transport many other cations (23, 30, 40). Our data demonstrate that expression of *P. aeruginosa mgtE* can support magnesium transport (Fig. 6A) and that the protein product is present in the membrane (Fig. 7C).

On the other hand, we found that magnesium transport is not necessary for inhibition of cytotoxicity (Fig. 6 and 7). By

making three point mutations in putative magnesium binding sites, we have disrupted the ability of MgtE to transport magnesium (Fig. 7A). Structural analysis of MgtE under high-magnesium concentrations has identified at least six sites at which magnesium binds to MgtE under these conditions, thus holding the pore in a closed conformation (18). Further computer modeling has suggested a mechanism whereby sequential binding of magnesium to these sites leads to proper closing of the magnesium pore (20). By mutating two of these sites, we have potentially altered the ability of the protein to transition between open and closed conformations. However, these actions do not appear to be necessary for inhibition of cytotoxicity because our binding site 2/3 mutant can fully complement the *mgtE* mutant for its cytotoxicity phenotype (Fig. 7B). Our results with this binding site mutant suggest that inhibition of cytotoxicity is effected by some part of the MgtE structure rather than the act of transporting magnesium or switching between open and closed conformations. Recent studies of the CorA magnesium transporter in *S.* Typhimurium (strain SL1344) have reached a similar conclusion; CorA affected virulence despite inhibition of magnesium transport through the protein (37).

The data presented in this study suggest the hypothesis that *P. aeruginosa* has evolved dual functions for MgtE: magnesium transport and virulence regulation. While these activities appear to be independent from one another, it is still formally possible that magnesium can stimulate transcription of *mgtE* in *P. aeruginosa*, as is the case in other organisms (10). Indeed, a recent study found that *mgtE* transcription in *P. aeruginosa* can be activated by limiting magnesium concentrations and that this effect requires the magnesium-sensitive response regulator PmrA (32). Thus, changes in magnesium concentration might influence *mgtE* expression through PmrA and, thus, T3SS transcription. However, magnesium must exert additional effects on T3SS because magnesium treatment led to increased transcription in the absence of *mgtE* as well (Fig. 2B). One possible explanation comes from recent studies showing (i) that magnesium treatment can alter *P. aeruginosa* lipopolysaccharide structure and (ii) that alterations in lipopolysaccharide (LPS) can lead to changes in T3SS activity (2, 16). It will be interesting to investigate the potential role of magnesium in regulating bacterial toxicity.

It is likely that MgtE is only one part of an interconnected network of regulators involved in modulating cytotoxicity. Future studies will focus on determining the relationship between MgtE and other virulence-regulatory molecules.

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