MgtE is a dual-function protein in *Pseudomonas aeruginosa*

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The opportunistic pathogen *Pseudomonas aeruginosa* causes a wide range of infections, including chronic biofilm infections in the lungs of individuals with cystic fibrosis. We previously found that the inner-membrane protein MgtE can function both as a magnesium transporter and a virulence modulator, although the exact mechanism governing these activities is unclear. To address this issue, we carried out an experimental characterization of *P. aeruginosa* MgtE and generated a computer-rendered model. Our *in silico* analysis demonstrated the structural similarity of *P. aeruginosa* MgtE to that of the crystal structure of MgtE in *Thermus thermophilus*. Experimentally, we verified that MgtE is not essential for growth and found that it may not be involved directly in biofilm formation, even under low-magnesium conditions. We demonstrated both magnesium transport and cytotoxicity-regulating functions, and showed that magnesium-binding sites in the connecting helix region of MgtE are vital in coupling these two functions. Furthermore, limiting magnesium environments stimulated mgtE transcriptional responses. Our results suggested that MgtE might play an important role in linking magnesium availability to *P. aeruginosa* pathogenesis.

**INTRODUCTION**

The Gram-negative bacterium *Pseudomonas aeruginosa* is able to persist in a variety of environments, often through the formation of biofilms, which are antibiotic-resistant and immune-resistant aggregates. *P. aeruginosa* causes nosocomial infections and is the most common cause of life-threatening lung infections for individuals with the genetic disease cystic fibrosis (CF) (Parkins et al., 2012). In the characteristic mucus-filled lungs of an individual with CF, *P. aeruginosa* thrives long-term by forming biofilms, thus exacerbating the inflammatory environment and contributing to respiratory tissue destruction. The virulence factors which enable *P. aeruginosa* to cause chronic infection, including biofilm formation, mucoidy, motility, type III secretion and many others, are well-studied, but the mechanisms by which this bacterium dominates the CF lung environment are not completely understood.

It is thought currently that inhibition of the type III secretion system (T3SS) is an important step in the establishment of chronic *P. aeruginosa* infection in the CF lung (Jain et al., 2004). T3SS is a well-studied protein complex and a major host-cell-contact-dependent virulence factor (Diaz et al., 2011; Yahr & Wolfgang, 2006). CF isolates accumulate mutations in the T3SS and studies have suggested that T3SS activity is reduced in *P. aeruginosa* biofilms (Dötsch et al., 2012; Jain et al., 2004). Additionally, expression of T3SS in *P. aeruginosa* biofilms alters the normal biofilm architecture (Kuchma et al., 2005). These factors suggested that elaboration of T3SS effectors could be detrimental to *P. aeruginosa* biofilm persistence in the CF lung.

We have demonstrated recently that a lesser-known protein, MgtE, can influence the expression of the T3SS of *P. aeruginosa* (Anderson et al., 2010). MgtE is one of several magnesium transporters in *P. aeruginosa*. The importance of the mgtE gene in *P. aeruginosa* was first identified in a microarray study as a factor with increased expression in biofilms treated with the antibiotic tobramycin (Anderson et al., 2008). MgtE localized to the inner membrane and we demonstrated that, in addition to transporting magnesium, MgtE could participate in antibiotic-induced cytotoxicity repression in biofilms by inhibiting the T3SS. Through these studies, we found evidence that cytotoxicity modulation and magnesium transport functions of MgtE could be separated (Anderson et al., 2010).

To understand how MgtE can carry out these distinct functions, we set forth to examine how mutation of key portions of the protein affects the phenotype. Such an analysis is vitally important for the development of therapeutics targeting virulence and biofilm formation. Using...
the Thermus thermophilus crystal structure as a model (Hattori et al., 2007, 2009; Tanaka et al., 2007), we performed a structure-function analysis to examine the dual-function nature of P. aeruginosa MgtE and we generated a 3D homology model of the protein. In the work presented here, we illustrated the similarities between the functional domains of MgtE in P. aeruginosa and T. thermophilus, and we demonstrated dual functionality with two mgtE mutant constructs, which show that the magnesium transport and cytotoxicity-regulating functions are separable. Additionally, we investigated correlations between magnesium and cytotoxicity. Together, our results supported the hypothesis of P. aeruginosa MgtE as a dual-function protein and suggested a virulence regulation mechanism for further study.

METHODS

Micro-organisms and culture. Bacterial and yeast strains used in this study are listed in Table 1. Bacterial cultures were grown overnight in LB at 37 °C with shaking. Antibiotics were added to the media as necessary to maintain selectivity for the desired transformants. Gentamicin was added at a concentration of 50 μg ml⁻¹ for P. aeruginosa, and 10 μg ml⁻¹ for Escherichia coli and Salmonella. Cultures of Salmonella enterica strain MM281 without plasmids were supplemented with 100 mM MgSO₄ as required for growth of this magnesium transport-deficient strain. Saccharomyces cerevisiae was grown overnight in YEPD (yeast extract peptone dextrose) at 30 °C on a rolling drum. For bacterial growth assays, overnight cultures were diluted 1:100 in fresh medium and aliquoted into a 96-well microtitre plate, which was then maintained at 37 °C in a SpectraMax M2 spectrophotometer. OD₆₀₀ was measured every 30 min, and was shaken for 5 s before each reading.

Homology modelling. The amino acid sequence of MgtE from P. aeruginosa strain PA14 was retrieved from the Pseudomonas Genome Database (www.pseudomonas.com; Winsor et al., 2011). A PSI-BLAST (Altschul et al., 1997) search was carried out against the Protein Data Bank (PDB) (Bernstein et al., 1977) with default parameters to identify a suitable template based on high sequence identity and fewer gaps. The sequence alignment between template and target sequence was generated using CLUSTAL Omega (Sievers et al., 2011) with default parameters. The homology models were generated using MODELLER 9v11 (Eswar et al., 2006), which implements comparative protein structure modelling by satisfaction of spatial restraints. The discrete optimized protein energy (DOPE) score (Shen & Sali, 2006) profile plots for both the template and model structure were generated with Gnuplot graphing utility (http://gnuplot.info) (Janert, 2010). We generated a series of 20 models using standard ‘automodel’ class in MODELLER and from among the 20 models generated, the model with the best DOPE score was selected as representative of MgtE from P. aeruginosa (Fig. 1a). Plotted DOPE scores indicate a good model quality due to similar profiles of the T. thermophilus template (PDB ID: 2ZY9) and modelled P. aeruginosa MgtE structures (Fig. S1, available in the online Supplementary Material).

The quality of our modelled structure was validated with PROCHECK (Laskowski et al., 1996), ERRAT (Colovos & Yeates, 1993) and ProSa (https://prosa.services.came.sbg.ac.at/prosa.php) analyses. A PROCHECK Ramachandran plot shows the values of the phi (Φ) and psi (Ψ) torsion angles of all non-terminal amino acid residues within the predicted protein model to assess overall and residue-by-residue geometry. Generally, models are considered good and reliable if 90% of the residues are present in the allowed region in the Ramachandran plot (Laskowski, 2003). These analyses revealed a high degree of amino acids in allowed regions of the Ramachandran plot (94.3% of the residues are in core regions, 4.6% in allowed regions and 1.1% in generously allowed regions) (Fig. S2a). Additionally, none of the residues were in disallowed regions of the Ramachandran plot and the overall G factor value was −0.03, indicating that the geometry of the model structure corresponds to a high-probability conformation. The calculated ProSa z-score of −7.58 for our MgtE model structure is within the range of scores typically found for native proteins of similar size (Fig. S2b).

ERRAT analyses the non-bonded interactions between different atom types and our calculated score of 82.98% is well within the range of a high-quality model (Fig. S2c). Molecular visualization programs VMD (Humphrey et al., 1996) and Chimera (Pettersen et al., 2004) were used for visualization and imaging of 3D structures (http://www.ks.uiuc.edu/Research/vmd/). We superimposed the template and model structures, and calculated a root-mean-square deviation value of 0.67 Å over a total of 383 aligned residues (Fig. 1b), indicating a high degree of similarity between structures (Carugo & Pongor, 2001).

Construction of mgtE plasmids by yeast transformation. Plasmids used in this study are listed in Table 2 and illustrated diagrammatically in Fig. 2. Primers are listed in Table S1. Full-length and mutant mgtE constructs were ligated into expression vector pMQ72 (Shanks et al., 2006), which includes a gentamicin resistance gene to allow for selection of the desired transformants. Domain deletion and point mutant constructs of mgtE were created from an N-terminally His₆-tagged mgtE gene located on plasmid pSMC293 (Anderson et al., 2010). This plasmid requires carbenicillin to maintain selection, and we wanted to perform our studies using plasmids with gentamicin resistance because these plasmids are easier to maintain and work with in P. aeruginosa. His₆-tagged mgtE was PCR-amplified from pSMC293 using primers p729 and p730. Plasmid pMQ72 was digested with EcoRI and then digested plasmid and PCR fragments were recombined to create plasmid pMgtE using a one-step

Table 1. Experimental organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>Description or purpose</th>
<th>Source</th>
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<tbody>
<tr>
<td>Escherichia coli S17-1</td>
<td>Laboratory cloning strain</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa PAO1</td>
<td>WT, wound isolate</td>
<td>Holloway et al. (1955)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa PA14</td>
<td>WT, clinical isolate</td>
<td>Rahme et al. (1995)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa GGA52</td>
<td>PA14 ΔmgtE</td>
<td>Anderson et al. (2010)</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae InvSc1</td>
<td>ura3-52/ura3-52; creation of plasmids by homologous recombination</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Salmonella enterica sv. Typhimurium MM281</td>
<td>corA45::MudJ mgtA21::MudJ mgtB10::MudJ testing magnesium transport complementation</td>
<td>Hmeil et al. (1989)</td>
</tr>
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</table>

http://mic.sgmjournals.org
homologous recombination procedure involving *Saccharomyces cerevisiae*, as described previously (Shanks et al., 2006, 2009). For generation of all other plasmids, except for pDgkA, we recombined PCR fragments into HindIII-digested pMQ72, using similar methods. For C-terminal truncations, we PCR-amplified His6-tagged mgtE from pmgtE using primers 913Nleftfor and MgtETM1–5Left (pTmΔ1–5), MgtETM2–5Left (pTmΔ2–5), MgtETM3–5Left (pTmΔ3–5), MgtETM4–5Left (pTmΔ4–5) or MgtETM5Left (pTmΔ5). N-terminal truncations were constructed similarly using two PCR fragments, created with the following primer sets: 913Nleftfor/913Nleftrev and 913Nrightfor/p729 (pN-111), 913Nleftfor/MgtNDomDelLrev and MgtNDomDelRfor/p729 (pN-303), and 913Nleftfor/MgtNCBSDelLfor and MgtNCBSDelRfor/p729 (pN-324). Point mutations were also created with two PCR fragments using: p730/MgSite1left and MgSite1right/p729 (pMg1), p730/MgSite2/3left and MgSite2/3right/p729 (pMg23), p730/MgSite4left and MgSite4right/p729 (pMg4), p730/MgSite5left and MgSite5right/p729 (pMg5), and p730/MgSite6left and MgSite6right/p729 (pMg6). Plasmid pDgkA carried a construct encoding a fusion of the cytosolic domain of MgtE with the heterologous transmembrane protein DgkA, a diacylglycerol kinase found in *P. aeruginosa*. The plasmid was created by PCR amplification of dgkA from PA14 using primers DgkAfusfwd and DgkAfusrev. This PCR fragment was then recombined with HindIII-digested pmgtE, as described above, generating pDgkA. The DgkAfusfwd primer contained 40 bp homology to the 40 bp immediately upstream of the C-terminus-encoding portion of mgtE, as well as homology to the first 20 bp of the

Table 2. Description of plasmids.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>pDgkA</td>
<td>Transmembrane domain replacement</td>
<td>This study</td>
</tr>
<tr>
<td>pN-111</td>
<td>N-terminal deletion (~111 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pN-303</td>
<td>N-terminal deletion (~303 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pN-324</td>
<td>N-terminal deletion (~324 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pmgtE</td>
<td>Full-length mgtE</td>
<td>This study</td>
</tr>
<tr>
<td>pTmΔ1-5</td>
<td>C-terminal deletion, α-helices 1–5</td>
<td>This study</td>
</tr>
<tr>
<td>pTmΔ2-5</td>
<td>C-terminal deletion, α-helices 2–5</td>
<td>This study</td>
</tr>
<tr>
<td>pTmΔ3-5</td>
<td>C-terminal deletion, α-helices 3–5</td>
<td>This study</td>
</tr>
<tr>
<td>pTmΔ4-5</td>
<td>C-terminal deletion, α-helices 4–5</td>
<td>This study</td>
</tr>
<tr>
<td>pTmΔ5</td>
<td>C-terminal deletion, α-helix 5</td>
<td>This study</td>
</tr>
<tr>
<td>pMg1</td>
<td>Mg2+–binding site 1 (pore), point mutation (D466A)</td>
<td>This study</td>
</tr>
<tr>
<td>pMg23</td>
<td>Mg2+–binding sites 2/3 (connecting helix), point mutations (E288A, E292A)</td>
<td>This study</td>
</tr>
<tr>
<td>pMg4</td>
<td>Mg2+–binding site 4 (cytosol), point mutation (E280A)</td>
<td>This study</td>
</tr>
<tr>
<td>pMg5</td>
<td>Mg2+–binding site 5 (cytosol), point mutation (D259A)</td>
<td>This study</td>
</tr>
<tr>
<td>pMg6</td>
<td>Mg2+–binding site 6 (cytosol), point mutation (D131A)</td>
<td>This study</td>
</tr>
<tr>
<td>pMQ72</td>
<td>Control empty expression vector with gentamicin-resistance gene</td>
<td>Shanks et al. (2009)</td>
</tr>
</tbody>
</table>
C-terminus-encoding portion of *dkgA*. Thus, recombination led to replacement of the 3′ end of *mgtE* (encoding the transmembrane helices) with the 3′ end of *dkgA* (encoding the transmembrane region of *DgkA*) (Shanks *et al.*, 2009). All plasmid constructs were verified by sequencing using the p729 and p730 primers.

**Tissue culture.** Tissue culture of human-derived CF bronchial epithelial (CFBE) cells (Cozens *et al.*, 1994) was maintained in 750 ml polystyrene culture flasks at 37 °C in 5% CO₂ and fed with essential medium (1 × MEM; cellgro Minimal Essential Medium Eagle; Mediatech) plus 10% FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹, every 2–3 days. The 24-well tissue culture plates were seeded at a concentration of 2 × 10⁵ cells per well and fed every 2–3 days for 7–10 days before experiments, as described previously (Anderson *et al.*, 2008). Tissue culture was performed in a sterile hood using aseptic techniques.

**Co-culture model system and cytotoxicity assay.** The co-culture model system was developed in order to provide a means to study *P. aeruginosa* biofilm interactions with CF airway epithelial cells (Anderson *et al.*, 2008). We measured cytotoxicity of co-culture biofilms using the Promega CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (G1780; Promega), as described previously (Anderson *et al.*, 2010). This colorimetric assay detects lactate dehydrogenase (LDH), a cytosolic protein released upon membrane damage of CFBE cells. Briefly, CFBE cells were grown to confluence, washed with PBS (cellgro Dulbecco’s PBS without calcium and magnesium; Mediatech) and given fresh medium containing MEM without phenol red, plus 2 mM glutamine. Next, aliquots of 3 ml bacterial culture from an overnight culture were added to each well and the co-culture was incubated at 37 °C for 1 h, at which point the media were replaced with the same media with the addition of 0.4% arginine. Arginine, for an unknown reason, was found to inhibit bacterially mediated destruction of CFBE cells long enough for establishment of biofilms (Anderson *et al.*, 2010) and is added to abiotic biofilms to enhance biofilm formation (Müskens *et al.*, 2010). At the indicated time points, 300 μl samples of supernatant were taken and processed according to kit instructions. Data were normalized to LDH levels in uninoculated CFBE monolayers as a measure of maximum LDH release (100% cytotoxicity).

**Magnesium transport bioassay.** We used *Salmonella enterica* strain MM281 as a surrogate for testing the ability of *P. aeruginosa mgtE*
mutant constructs to transport magnesium (Anderson et al., 2010). *Salmonella* MM281 contains mutations in all of its magnesium transporters and grows only if supplemented with 100 mM magnesium or if transformed with a gene for a functional magnesium transporter (Townsend et al., 1995). Numerous studies, including our own, have used *Salmonella* MM281 to demonstrate magnesium transport function encoded within a particular gene (Hicks et al., 2003; Kolisek et al., 2008; Li et al., 2001). After transforming *Salmonella* MM281 with our plasmids containing *P. aeruginosa* mgtE mutant constructs, we plated culture samples onto N minimal medium without added magnesium (Miao et al., 2002). Aliquots of 10 μl bacterial culture were spotted on these plates and allowed to dry. Then the plates were incubated at 37 °C for 24 h. Only transformants with functional magnesium transporters were able to grow.

Western blotting. To determine whether mutant MgtE proteins were present in the cells, we performed Western blots on all transformants using an anti-His antibody. Cells were first harvested by centrifugation for 15 min at 6000 r.p.m. The pellet was washed in PBS and resuspended in Buffer A (200 mM Tris/HCl, 20 mM EDTA, 1 × protease inhibitors). The suspension was sonicated on ice at 30% power, alternating 10 s on/10 s off for six cycles. After cell lysis, samples were run on 4–15% Tris/HCl Bio-Rad Ready Gels and electrophoretically transferred to nitrocellulose membranes in a Bio-Rad Mini-PROTEAN system. Blocking and subsequent detection was then performed according to procedures described for HisProbe-HRP (Thermo Scientific). This probe was selected because our *P. aeruginosa* mgtE plasmids include an N-terminal His tag. One limitation of this method was that it did not allow us to test the N-terminal truncations.

Real-time quantitative PCR (RT-qPCR). For transcriptional analysis of mgtE, *P. aeruginosa* PA14 was grown overnight in N minimal medium (Miao et al., 2002) with varying levels of MgSO4 (0.1, 0.5, 1.0 and 2.0 mM). Cells were lysed, and RNA was isolated and purified using an RNeasy kit (74104; Qiagen). Purified RNA was used to prepare cDNA with a SuperScript III First-Strand cDNA Synthesis kit (Invitrogen). We ran RT-qPCR in an Applied Biosystems 7300 Real-Time PCR System using primer pairs MgtERTforNewest/MgtErevNewest (for mgtE) and PA5110for/PA5110rev (for normalization control fbp) (Anderson et al., 2008). Primer sequences are given in Table S1.

DNA sequencing. DNA sequencing was performed at the Indiana University School of Medicine DNA Sequencing Core Facility.

Biofilm assay. Microtitre (96-well) plate biofilm assays were adapted from the method described previously (O'Toole & Kolter, 1998; O'Toole, 2011). Bacterial cultures were grown overnight in low-magnesium minimal medium (0.1 mM MgSO4) and then diluted 1:100 into fresh low-magnesium medium supplemented with varying levels of MgSO4, as indicated. Aliquots of 100 μl were added to U-bottom 96-well polystyrene plates (Serocluster; Corning Costar). Samples were incubated at 37 °C for 24–48 h and then the plates were rinsed in water to remove planktonic cells. The remaining surface-adhered cells were stained with 0.1% crystal violet, solubilized with 30% acetic acid and quantified by spectrophotometry at 590 nm.

**RESULTS**

Homology model of MgtE in *P. aeruginosa* reveals structural similarity to the crystal structure of MgtE in *T. thermophilus*

The validated 3D homology model of *P. aeruginosa* MgtE is shown in Fig. 1(a). We selected the improved crystal structure of the magnesium transporter MgtE from *T. thermophilus* (PDB ID: 2ZY9, resolution 2.94 Å) (Hattori et al., 2009) as a template for building this model, based on sequence identity and functional residue conservation. Sequence analysis revealed that MgtE from *P. aeruginosa* contains an extra 37 N-terminal amino acids compared with the primary amino acid sequence of *T. thermophilus* (Fig. S3). In addition, the PDB ID: 2ZY9 chain A (T. *thermophilus*) structure model excludes the first 22 aa of MgtE from *P. aeruginosa*. As a result, the first 57 aa were omitted from our homology model because no suitable template structure could be found. Typically, generation of a reliable structure requires >25% identity (Kopp & Schwede, 2004) and our template–target alignment showed 30.62 and 55.53% sequence identity and sequence similarity, respectively.

We analysed our modelled structure using PROCHECK to assess stereochemical quality by the Ramachandran plot, using ProSA to evaluate the overall model quality, and with ERRAT to calculate an additional ‘overall quality factor’, as described in Methods (Figs S1 and S2). The results of the homology modelling validation experiments are summarized in Table S2. Taken together, the data from PROCHECK, ERRAT and ProSA analyses validate our model as a likely representation of the *P. aeruginosa* MgtE structure.

Finally, we superimposed our *P. aeruginosa* MgtE structure on the *T. thermophilus* structure. In particular, *P. aeruginosa* MgtE contains both the N-terminal globular domain and the C-terminal helices, joined by a connecting helix, similar to *T. thermophilus* MgtE (Fig. 1). Subsequent sequence alignment from a structural match shows that the functional residues are well conserved between our model and template structures (Fig. S3). Together, these data support the use of structural information from *T. thermophilus* MgtE as a valid source for functional analysis of *P. aeruginosa* MgtE. Based on the modelled structure, we hypothesized that successive truncations of the five transmembrane α-helices (TM1–TM5, see Figs 2a and S3) of *P. aeruginosa* MgtE may abolish all or part of the Mg2+-conducting ability, and point mutations of the Mg2+ -binding sites could affect protein function without necessarily abolishing magnesium transport capacity. We also predicted that certain deletions may not result in an expressed protein.

C-terminal truncation mutants fail to transport magnesium and regulate cytotoxicity

To begin to dissect the dual functions of *P. aeruginosa* MgtE, we created and tested six C-terminal mutations, as described in Methods (Figs 2 and S3). The C-terminal transmembrane domain consists of five α-helices (Fig. S4). In the homodimer, MgtE forms a 10-helix bundle, which constitutes the expected magnesium-conducting pore, and we predicted that residual activity might still be present after deletion of one or more helices. Alternatively, all helices might be vital for function. We created five
mutations by successive truncations of the five transmembrane \( \alpha \)-helices (Fig. 2). A sixth mutant was created by fusing the MgtE N-terminal cytosolic region with the transmembrane protein DgkA. DgkA has no known transport functions and its transmembrane domain is located in the C-terminal region, similar to the structure of MgtE (Winsor et al., 2011); thus, this fusion serves as a non-magnesium-transporting control. We anticipated that C-terminal mutations would be unlikely to form a functional protein due to elimination of \( \alpha \)-helices that form the transmembrane pore. The results of magnesium transport assays and growth curve analyses (using Salmonella MM281 transformed with \( P. \) aeruginosa mgtE; see Methods) supported our predictions, indicating that all six of the mgtE C-terminal mutants failed to transport magnesium (Fig. 3). Furthermore, the deletion MgtE proteins failed to be detected by Western blot analysis of whole-cell bacteria (Fig. S5).

For the construct pTmΔ3-5, which is a truncation of three transmembrane domains, we observed unusual punctate growth on agar plates (Fig. 3a), with replicable results. To eliminate suspicion of a contaminated or mixed culture, or perhaps a spontaneous mutation in the lab stock culture, we subcultured and replated several generations. Punctate growth persisted through subcultures of the original stock. However, when individual spot colonies were selected from a plate, and either streaked for isolation or grown overnight in liquid medium and then plated, solid growth occurred. Additionally, three samples (one laboratory stock sample and two samples from magnesium transport assays) were sent for DNA sequencing, which indicated no spontaneous mutations in the mgtE TmΔ3-5 construct. The mechanisms behind the punctate growth are unclear at this time. Although we were unable to determine the reason for the anomalous growth of this mutant, it is unlikely that a functional transmembrane pore could be formed from this construct and indeed the protein was absent in Western blots of whole-cell bacteria (Fig. S5). Taken together, our data on C-terminal truncations suggested that the full C-terminal domain, with all five transmembrane \( \alpha \)-helices intact, is required for magnesium transport.

To measure the effect of expression of mgtE mutant constructs in \( P. \) aeruginosa, we transformed an isogenic mgtE deletion mutant (strain GGA52) with our plasmids. We have shown previously that this ΔmgtE strain displays increased cytotoxicity through the T3SS, compared with WT, in biofilms formed on cultured CFBE cells, and that expression of WT mgtE inhibited this cytotoxicity (Anderson et al., 2010). In cytotoxicity assays of \( P. \) aeruginosa biofilms growing on CFBE cells, we observed C-terminal mgtE mutants to induce levels of cytotoxicity in a range similar to or greater than the ΔmgtE strain with empty vector (pMQ72) (Figs 3c and S6). Importantly, all of these clones lost the ability to inhibit cytotoxicity and were significantly different from the expression of full-length mgtE (Student’s \( t \)-test \( P<0.05 \)). It was presumed, based on our previously published study (Anderson et al., 2010), that \( P. \) aeruginosa cytotoxicity was a function of the T3SS.

N-terminal mutant regulates cytotoxicity, but only weakly transports magnesium

In their study of \( T. \) thermophilus MgtE magnesium binding, Hattori et al. (2009) stated that the cytosolic domain of MgtE functions to sense intracellular magnesium levels and regulate the opening and closing of the transmembrane pore. Thus, we predicted that N-terminal truncations would impair or eliminate magnesium transport function. Additionally, the N-terminal cytosolic region contains cystathionine \( \beta \)-synthase (CBS) domains, which are globular structures with various functions (such as signalling) found in diverse eukaryotic and prokaryotic proteins (Bateman, 1997; Estevez et al., 2004; Ignoul & Eggermont, 2005; Kushwaha et al., 2009). These residues might be involved in protein–protein interactions or signalling processes. Our experiments revealed unique phenotypes for each of the three N-terminal truncations. Overall, the entire N-terminal intracellular domain of \( P. \) aeruginosa MgtE is essential for full complementation of the magnesium transport when transformed into Salmonella MM281. On agar plates and in planktonic culture, the shortest N-terminal truncation (pN-111) of \( P. \) aeruginosa mgtE resulted in weak growth of Salmonella MM281 transformants compared with full-length mgtE. No growth was seen with longer N-terminal truncations which included the CBS and globular domains (pN-303 and pN-324), indicating that these mutations were unable to transport magnesium (Fig. 4a, b).

In seven independent experiments of N-terminal mgtE mutants transformed in \( P. \) aeruginosa ΔmgtE strain GGA52, it was observed that progressive truncations resulted in progressive loss of regulation of cytotoxicity (Fig. 4c), which was presumed to be a function of the T3SS, based on our previously published study (Anderson et al., 2010). The shortest truncation (pN-111) demonstrated regulation of cytotoxicity comparable to overexpression of mgtE (pmsgE). The 37 N-terminal amino acids truncated in this mutant were unique to \( P. \) aeruginosa MgtE and were not found in the \( T. \) thermophilus peptide sequence (Fig. S3). As mentioned above, the magnesium transport function was diminished in this mutant, making pN-111 a construct that displayed a separation of functions: it regulated cytotoxicity, but had impaired magnesium transport function.

Magnesium-binding site mutations in the connecting helix region cause uncoupling of magnesium transport and cytotoxicity regulation

Magnesium-binding sites in \( P. \) aeruginosa MgtE were identified based on homology with \( T. \) thermophilus MgtE (Fig. S3). According to the \( T. \) thermophilus MgtE model, there is one magnesium-binding site in the transmembrane domain and six in the cytosolic domain (Hattori et al., 2007, 2009). The cytosolic magnesium-binding sites are predicted to work cooperatively and therefore we expected that mutation in one of these sites would not result in complete dysfunction of the protein.
Results of our magnesium transport assays, in which the magnesium transporter-deficient Salmonella MM281 was transformed with P. aeruginosa mgtE mutants, demonstrated that magnesium-binding site 1 in the transmembrane pore region (plasmid pMg1) is essential for magnesium transport (Fig. 5a, b). The Asp residue at this site is also the only Asp residue located within the lipid bilayer of the transmembrane domain of MgtE (Fig. S7).

Based on growth curves (Fig. 5b) and Western blot analysis (Fig. S5), there may be a small amount of this mutant that formed a completed protein, but it was insufficient to sustain long-term growth or regulate cytotoxicity (Fig. 5b, c). Binding sites 4–6 (cytosolic region) were not individually essential for magnesium transport or growth of Salmonella MM281 in minimal medium (Fig. 5a, b), although growth was somewhat delayed. These mutants also regulated P. aeruginosa cytotoxicity in a manner similar to WT (Fig. 5c). Each of the protein products was expressed in Salmonella MM281 membranes (Fig. S5).

Mutations of magnesium-binding sites 2 and 3, located in the connecting helix region, were combined in one construct (pMg23) due to their proximity to each other.

Fig. 3. Effects of C-terminal truncations of mgtE. (a) Ability of P. aeruginosa mgtE C-terminal truncation mutants to complement magnesium transport in the magnesium transporter-deficient strain Salmonella MM281 was assessed by growth on minimal medium agar plates. WT MM281 was complemented with empty vector control (pMQ72), full-length mgtE (pmgtE) or truncation plasmids, as indicated. (b) Growth of MM281 complemented with the indicated plasmids was assessed. (c) Cytotoxicity of P. aeruginosa ΔmgtE mutant (52) complemented with empty vector, or full-length or mutant mgtE plasmids, was assessed. Cytotoxicity was normalized as a percentage of maximum LDH release by CFBE cells following treatment with Triton X-100. Controls are shown as white bars. C-terminal truncations demonstrate significant loss of cytotoxicity regulation compared with expression of full-length mgtE (pmgtE). Data are representative of five or more independent experiments. *P<0.05, Student’s t-test, compared to pmgtE.
Plasmid pMg23 restored growth of Salmonella MM281 (Fig. 5a, b), whilst demonstrating levels of cytotoxicity similar to the mgtE deletion mutant GGA52 (Fig. 5c). This indicated that mutation of the connecting helix region of MgtE did not impair magnesium transport function but did eliminate the cytotoxicity-regulating function. Importantly, the protein product from the pMg23 construct was present in the Salmonella MM281 membrane (Fig. S5). This was the only mutant we tested which restored magnesium transport in Salmonella MM281 whilst losing regulation of cytotoxicity in P. aeruginosa.

**mgtE transcription increases under low-magnesium conditions**

Deletion of mgtE does not affect growth in rich medium (Anderson et al., 2008), suggesting to us that MgtE might not serve as a major magnesium transporter for P. aeruginosa and thus magnesium transport through MgtE might not be a major signal affecting phenotype. P. aeruginosa expresses several other magnesium transporters that could maintain intracellular magnesium homeostasis. However, cytotoxicity differences might be related to the mere presence of the protein or by an alternative function of MgtE, which could be modulated by magnesium-triggered transcriptional changes. To explore this question, we wanted to determine whether extracellular magnesium levels would affect mgtE transcription. We grew PA14 WT cultures under varying magnesium levels and then analysed mgtE transcription by RT-qPCR. We chose magnesium concentrations within a physiologically relevant range (0.1, 0.5, 1.0 and 2.0 mM MgSO₄) such as may be encountered by bacteria during infection of the CF lung (Guerrero-Romero et al., 2011; Gupta et al., 2007; Palmer et al., 2007;

**Fig. 4.** Effects of N-terminal truncations of mgtE. (a) Ability of P. aeruginosa mgtE N-terminal truncation mutants to complement magnesium transport in the magnesium transporter-deficient strain Salmonella MM281 was assessed by growth on minimal medium agar plates. WT MM281 was complemented with empty vector control (pMQ72), full-length mgtE (pmgtE) or truncation plasmids, as indicated. (b) Growth of MM281 complemented with the indicated plasmids was assessed. (c) Cytotoxicity of P. aeruginosa ΔmgtE mutant (52) complemented with empty vector, or full-length or mutant mgtE plasmids, was assessed. Cytotoxicity was normalized as a percentage of maximum LDH release by CFBE cells following treatment with Triton X-100. Controls are shown as white bars. Increasing truncations of the N-terminal region of mgtE result in increasing loss of cytotoxicity regulation. Data are representative of five or more independent experiments. *P<0.05, Student’s t-test, compared to pmgtE.
Within this range of magnesium, the ΔmgtE mutant strain GGA52 exhibited growth similar to PA14 WT (Fig. 6a). However, RT-qPCR analysis revealed a significant increase in mgtE transcription at the lowest magnesium level (0.1 mM), compared with other magnesium concentrations (0.5, 1.0 and 2.0 mM) and LB (Fig. 6b). These results suggested that mgtE exerts little effect on overall bacterial physiology, but might be adapted to carry out specific functions (like cytotoxicity modulation) under magnesium limitation. Intriguingly, we found that a majority of bacteria with T3SSs also have an MgtE homologue (Table S3).

**Biofilm formation is unaffected by mgtE**

To further parse out the effects of mgtE on overall *P. aeruginosa* physiology, we performed biofilm assays. Previously published data show that mgtE deletion mutants form biofilms similar to PA14 WT in a standard biofilm assay (Anderson et al., 2008). We modified the magnesium content of the assay medium to investigate the possibility that varying magnesium levels might produce different biofilm phenotypes in PA14 WT and ΔmgtE strains. Biofilm assays comparing PA14 WT, ΔmgtE strain GGA52 and expression of mgtE (GGA52 pmgtE) showed...
comparable trends in all three strains (Fig. 7). Cultures were grown in both LB and low-magnesium minimal medium (0.1 mM MgSO$_4$, the level which corresponded to an increase in $mgtE$ transcription), and were assayed over a range of magnesium concentrations from 0.1 to 2.0 mM MgSO$_4$. These concentrations were above those which are known to support biofilm formation (Mulcahy & Lewenza, 2011). In general, we observed no differences in biofilm level at individual magnesium concentrations, although PA14 WT displayed significantly more robust biofilm growth than the mutant strains at 0.5 and 1.0 mM MgSO$_4$ (ANOVA, $P<0.01$). This may be the result of more robust planktonic growth by the WT strain. It has been reported that low magnesium conditions stimulate $P. aeruginosa$ biofilm formation (Mulcahy & Lewenza, 2011), and our data supported this conclusion by showing that there was a statistically significant increase in biofilm at 0.5 mM magnesium compared with 2.0 mM magnesium in all three strains (ANOVA, $P<0.01$) (Fig. 7).

**DISCUSSION**

MgtE is a member of a unique class of proteins unrelated to any other protein family. In this study, we present an experimental characterization and 3D homology model of MgtE in $P. aeruginosa$, and we provide evidence for its role as a dual-function protein. Following the identification of MgtE in 1995 (Smith et al., 1995), very few studies have included experimental characterization of this protein in prokaryotes (Dann et al., 2007; Merino et al., 2001; O’Connor et al., 2009). Our structure–function analysis includes three groups of $mgtE$ mutants – C-terminal truncations, N-terminal truncations and magnesium-binding site point mutations – which were tested for their ability to complement magnesium transport in $Salmonella$ MM281 and regulate cytotoxicity in $P. aeruginosa$. Our validated

**Fig. 6.** Growth in varying magnesium concentrations. (a) Both the WT and mutant displayed similar growth patterns over all magnesium concentrations tested. N minimal medium was supplemented with MgSO$_4$ (SO$_4$) or MgCl$_2$ (Cl$_2$). (b) Low magnesium (0.1 mM MgSO$_4$) stimulates $mgtE$ transcription. *$P<0.0001$, Student’s $t$-test, compared to all other groups. Error bars are present but extremely small.

**Fig. 7.** Biofilm formation under varying magnesium levels. WT, GGA52 and GGA52 pmgtE were assayed for biofilm formation, as described in Methods, in either LB or N minimal medium supplemented with a range of magnesium concentrations, from 0.1 to 2.0 mM MgSO$_4$. Significance was calculated using ANOVA, with asterisks indicating significance ($P<0.01$) between low and high magnesium (0.5 versus 2.0 mM) for all three strains. Representative data from four independent experiments. Significant differences were also seen between WT and GGA52 at 0.5 and 1.0 mM MgSO$_4$ (ANOVA, $P<0.01$).
homology model provides a structurally reliable depiction of the 3D conformation of \textit{P. aeruginosa} MgtE (Figs 1 and S1–S3, Table S2).

In support of our hypothesis of MgtE as a dual-function protein, we found that a point mutation in the magnesium-binding site located in the connecting helix region of MgtE (expressed on plasmid pMg23) resulted in the uncoupling of magnesium transport and cytotoxicity-regulating functions. This \textit{P. aeruginosa} mgtE mutant was able to transport magnesium and restore growth similar to WT \textit{mgtE} (as assessed by transformation into magnesium-transporter-deficient strain \textit{Salmonella} MM281); however, it did not regulate cytotoxicity in \textit{P. aeruginosa} (Fig. 5). We also found a second mutant, the N-terminal truncation of 37 aa (expressed on plasmid pN-111), which failed to fully complement magnesium transport but regulated cytotoxicity at a level similar to overexpression of WT \textit{mgtE} (Fig. 4). Although MgtE is generally identified as a magnesium transporter, this is unlikely to be its primary function. Our transcriptional assay (RT-qPCR) reveals that the cell calls upon MgtE for additional magnesium transport only in the most dire of nutritional circumstances (Fig. 6). Notably, \textit{mgtE} transcription was low in LB compared with magnesium-limiting conditions, suggesting that MgtE may be a key protein in linking nutrient availability to modulation of virulence factors. It is currently thought that two important virulence factors – T3SS and biofilm formation – are regulated inversely. We speculate that \textit{P. aeruginosa} might encounter low magnesium conditions in the CF lung environment (Gupta et al., 2007; Palmer et al., 2007; Sanders et al., 2006), which may provide an environmental signal for increased MgtE expression. If this is the case, then it is possible that MgtE plays an important role in \textit{P. aeruginosa} pathogenicity in the CF lung as the infection progresses from an acute state toward a chronic state characterized by antibiotic-resistant biofilms, lung tissue destruction and increased patient morbidity (Bruce et al., 1985; Folkesson et al., 2012; Rosenbluth et al., 2004). More specifically, low magnesium conditions may trigger an increase in MgtE expression, leading to decreased T3SS. Our experiments did not implicate \textit{mgtE} in the regulation of biofilm formation, nor did we find MgtE to be required for growth in low magnesium conditions (Figs 6a and 7). We did observe increased growth of the WT at 0.5 and 1.0 mM MgSO$_4$, which might indicate that \textit{mgtE} is involved in biofilm formation under certain circumstances. Moreover, it is unclear why complementation failed to restore biofilm formation to WT levels in moderate magnesium concentrations (0.5–1.0 mM). Differences in planktonic growth between the WT and mutant strains or changes in ionic balance which are unfavourable to plasmid expression may contribute to this effect. This does not necessarily indicate a failure of the T3SS and biofilm formation to be inversely regulated; rather, it underscores the complexity of the \textit{P. aeruginosa} regulatory network and highlights the metabolic flexibility that enables this organims to survive in a wide range of environments. Overall, our data suggest that the primary activity of MgtE in \textit{P. aeruginosa} is modulation of cytotoxicity.

A 2003 proteomic study by Guina et al. (2003) did not identify MgtE in \textit{P. aeruginosa} strain PAO1 among the proteins expressed differentially in low magnesium (8 µM low magnesium, versus 1 mM high magnesium as MgCl$_2$), although other magnesium transporters/regulators (PhoP, MgtA, MgtC) were identified. This suggests that even under low magnesium conditions, MgtE remains a relatively low-abundance protein and that there might be an optimal magnesium concentration range over which \textit{mgtE} transcription is stimulated.

Given the reliability of our \textit{P. aeruginosa} MgtE homology model and its similarity to the \textit{T. thermophilus} template, we expect regions of the protein to function in the same manner in both prokaryotic species. We have demonstrated that the magnesium-binding sites of the connecting helix region are essential for coupling the dual functions of MgtE in \textit{P. aeruginosa}. Our experimental results are consistent with crystallographic studies of MgtE in \textit{T. thermophilus}, which show that the magnesium-binding sites in the connecting helix region serve to maintain stability of the closed-pore conformation when MgtE is in a magnesium-bound state. In \textit{T. thermophilus}, closed-pore dimerization is facilitated by the N-terminal globular domains (Hattori et al., 2009) and our structure–function analysis suggests that \textit{P. aeruginosa} MgtE functions in a similar manner: Mutations in the connecting helix region (plasmid pMg23) presumably prevent magnesium binding, thus keeping the pore open and allowing ion transport. This mutant, whilst normal for magnesium transport, fails to regulate cytotoxicity (Fig. 5).

In a previous study, a connecting helix mutant (expressed from plasmid pSMC295) displayed the opposite effect: it failed to transport magnesium, but it regulated cytotoxicity (Anderson et al., 2010). In the same study, it was also demonstrated that full-length \textit{mgtE} with a C-terminal His$_6$ tag failed to transport magnesium, but was able to regulate cytotoxicity. It is interesting to note that mutations in the connecting helix region could produce inverse results. We compared the sequences of both plasmids with the \textit{mgtE} sequence from PA14. The pMg23 plasmid had the same sequence as PA14, but with point mutations around magnesium-binding sites 2 and 3. The pSMC295 plasmid also contained these point mutations, but had three additional point mutations, leading to amino acid changes A236T, R275H and I353V. These changes occur in the middle of the CBS domain, the end of the CBS domain and the beginning of transmembrane helix 2, respectively. The CBS mutations in particular could potentially affect rotation of these domains, leading to differences in activity. Future studies will examine this issue in greater detail. To speculate further on the mechanism behind this phenomenon, we refer to the Hattori et al. (2009) study, which explains that there is a substantial difference between the magnesium-bound and -unbound states of MgtE,
and the magnesium-binding sites work cooperatively to facilitate structural change and regulate MgtE function. According to Hattori et al. (2009), the overall conformation of the cytosolic domain largely depends on the presence of magnesium ions. There is a large shift between the CBS and N domains in the magnesium-bound state compared with the magnesium-unbound state, resulting in a regulation of the gating of the magnesium-transporting pore by sensing of the intracellular magnesium concentration. In other words, the cytosolic domain in MgtE might work as an intracellular magnesium sensor. In P. aeruginosa mgtE mutants, it is possible that the mutant from the Anderson et al. (2010) study freezes MgtE in a closed-pore state, whilst leaving the N-terminal cytosolic domain in a position to interact with other cytosolic proteins and thus regulate cytotoxicity. However, the connecting helix mutant in our current study (pMg23) may freeze MgtE in an open-pore state, whilst interfering with the positioning of the cytosolic domains in such a way that renders them unable to interact with other proteins in the virulence regulatory network. Together, these studies support the hypothesis of MgtE as a dual-function protein and demonstrate that the connecting helix region is essential for coupling these two functions (Anderson et al., 2010; Hattori et al., 2007, 2009).

The coupling of magnesium transport and cytotoxicity modulation in a single protein represents a novel regulation mechanism, and there is evidence that MgtE orthologues in other species may couple magnesium transport to other functions. Studies of MgtE in Enterococcus faecalis (Ragumani et al., 2010), Aeromonas hydrophila (Merino et al., 2001), Campylobacter jejuni (Kakuda & DiRita, 2006) and Bacillus subtilis (Dann et al., 2007; O’Connor et al., 2009) all reveal possible roles for this protein in addition to its magnesium transport function. It is interesting to note that, among common CF respiratory pathogens, P. aeruginosa is unique in its possession of both mgtE and the T3SS (Table S3). In speculating what connects MgtE to regulation of the T3SS, we propose that the N-terminal (cytosolic) domain interacts with a signalling molecule or is involved in a protein–protein interaction. This interaction would be dependent on magnesium levels and the open or closed state of the transmembrane pore, which affects the conformation of the N-terminal domain and the exposure of the CBS domain for interaction with other cytosolic molecules. This would be an expected function of a CBS domain, since these domains are known to be involved in a variety of signalling roles (Bateman, 1997; Estévez et al., 2004; Ignoul & Eggermont, 2005; Kushwaha et al., 2009). Future experiments will test this model with protein co-precipitation and two-hybrid assays.

Genomic studies using modern bioinformatics approaches continue to reveal the complexity of P. aeruginosa regulatory networks (Balasubramanian et al., 2013; Lee et al., 2006). Together, in silico analyses and experimental evidence will progress our understanding of P. aeruginosa virulence modulation. Our results indicate that MgtE might play an important role in adapting cytotoxicity responses to environmental conditions, and we anticipate that further investigation into the role of MgtE will elucidate the virulence mechanisms of P. aeruginosa especially as it relates to life-threatening lung infections for CF patients.

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