Functional determinants of a small protein repressor controlling a broadly conserved bacterial sensor kinase

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Abstract

The PhoQ/PhoP two-component system plays a vital role in the regulation of Mg^{2+} homeostasis, resistance to acid stress, cationic antimicrobial peptides, and virulence in *E. coli*, Salmonella and related bacteria. Previous studies have shown that MgrB, a 47 amino acid membrane protein that is part of the PhoQ/PhoP regulon, inhibits the histidine kinase PhoQ. Thus, MgrB is part of a negative feedback loop modulating this two-component system, disruption of which is strongly associated with acquired colistin resistance in clinical isolates of *Klebsiella pneumoniae*. MgrB may also be important for preventing hyperactivation of PhoQ and possibly act as an input for alternate signals in the PhoQ/PhoP pathway. To explore the mechanism of action of MgrB, we have analyzed the effects of point mutations, C-terminal truncations and domain swaps on MgrB activity. In contrast with two other small membrane protein regulators of histidine kinases in *E. coli,* we find that the MgrB transmembrane (TM) domain is necessary for PhoQ inhibition. Bacterial two-hybrid assays suggest the TM domain mediates interactions with itself as well as with PhoQ. Additionally, alanine scanning mutagenesis of the periplasmic domain of MgrB indicates that, with the exception of a few highly conserved residues, most residues are not essential for MgrB's function as a PhoQ inhibitor. Our results indicate that the regulatory function of the small protein MgrB is derived via distinct contributions from multiple residues spread across domains. Interestingly, the TM domain also appears to interact with other non-cognate histidine kinases in a bacterial two-hybrid assay, suggesting a potential route for evolving new small protein modulators of histidine kinases.

Introduction

Bacteria thrive in a multitude of niches, often under challenging physicochemical conditions. They have therefore evolved stress response systems to monitor environmental cues and modulate their physiology accordingly. Many of these stress responses are controlled by two-component signaling systems, one of the primary modes of signal transduction in bacteria (1). The PhoQ/PhoP system in *Escherichia coli*, Salmonella, and related Gram-negative bacteria is a well-studied example of a two-component system that is critical for virulence and facilitates adaptation to conditions of low Mg^{2+} or Ca^{2+}, low pH, and the presence of cationic antimicrobial peptides (2-5). In response to input signal, the sensor histidine kinase PhoQ

autophosphorylates and modulates the phosphorylation state of the response regulator PhoP, which functions as a transcription factor (2, 6).

Previous work has shown that a 47-amino acid small inner-membrane protein MgrB is regulated by the PhoQ/PhoP pathway and inhibits the PhoQ sensor kinase, thus constituting a negative feedback loop in this signaling circuit (7). MgrB homologs are found in numerous enterobacteria and PhoQ repression by MgrB appears to be conserved in several Gram-negative genera. MgrB could serve as a point of control for additional input signals modulate PhoQ. For example, MgrB activity is affected by the redox state of the periplasm via the protein's two conserved periplasmic cysteines (8). MgrB is not essential and appears to mainly act in a regulatory role, however it can have a significant impact on bacterial physiology. In *E. coli*, recent work has shown that strong stimulation of the PhoQ/PhoP system hinders cell division (9). Cells grown in low levels of Mg^{2+} in the absence of MgrB form filaments due to strong activation of PhoQ in this condition. MgrB-mediated feedback inhibition thus appears to be important to appropriately limit PhoQ activity and prevent hyperactivation of the system. In at least some contexts, loss of *mgrB* can also confer a fitness advantage: mutation of *mgrB* has emerged as one of the primary mechanisms of acquired resistance to the last resort antibiotic colistin in clinical isolates of *Klebsiella pneumoniae* (10, 11).

MgrB is a member of a growing list of small proteins (12, 13) that have been shown to be expressed in bacteria. Most of these proteins were initially overlooked due to arbitrary size constraints for defining open reading frames. As a result, they are largely understudied and most have yet to be functionally characterized. However, it is clear that small proteins can play key roles in diverse cellular processes (13). Modulation of histidine kinases, transporters and other integral membrane proteins by small bitopic membrane proteins is one emerging theme. For example, in addition to MgrB, the PhoQ histidine kinase is modulated by another small membrane protein, SafA (14). SafA, which is present exclusively in *E. coli*, and acts as a connector between the EvgS/EvgA and PhoQ/PhoP two-component systems. In some *E. coli* isolates, this connection is required for PhoQ activation in response to acid stress (15, 16). Another example is the 127-amino acid long MzrA (17), which activates EnvZ and functions as a connector between the CpxA/CpxR and EnvZ/OmpR two component systems.

MgrB acts by inhibiting the kinase activity (18), however, the mechanism by which this small protein inhibits a protein that is roughly ten times larger than itself in size, and the sequence determinants responsible for this action are largely unknown. In this work, we examine the interactions between PhoQ and MgrB, including the role of TM domain. By swapping domains, we show that the TM domain of MgrB is required for the protein to repress PhoQ, in contrast with SafA and MzrA, which do not require their TM domains for their activity (19, 20). Using site-directed mutagenesis, we identified key residues in the MgrB TM and periplasmic domains that are important for PhoQ repression. We find that a large number of amino acids in the periplasmic domain can be replaced with minimal effect on MgrB function. We further show that while the TM domain of MgrB alone is insufficient to confer activity, it interacts with PhoQ and itself in a bacterial two-hybrid assay. Intriguingly, we also uncovered interactions of MgrB with other histidine kinases. Together our findings suggest that PhoQ inhibition by MgrB depends on coordinated interactions from amino acid residues spanning different domains.

Results

Role of MgrB transmembrane domain in PhoQ inhibition

As first step towards understanding how MgrB inhibits PhoQ at a structural level, we looked at the role of the predicted MgrB transmembrane (TM) domain. Based on previous work, MgrB is believed to contain a single 18-amino acid long TM domain oriented so that the N-terminus is in the cytoplasm (7). We performed a domain swapping experiment (Figure 1A), wherein the putative TM domain of MgrB was replaced with either (i) a 20-amino acid long TM from the *E. coli* maltose transporter MalF or (ii) a 21-amino acid long human glycophorin A (GpA) TM (Figure 1B). Note that the GpA TM used in our study is a variant that has a lower propensity to dimerize than the wild-type GpA TM sequence (21). An N-terminally-tagged GFP fusion of MgrB (GFP-MgrB) was previously shown to be functional and localized to the membrane (7). All of the variants (or mutants) of MgrB described henceforth are derivatives of this construct, enabling us to confirm expression and membrane localization by fluorescence microscopy. Both MgrB variants with swapped TM domains localized to the cell membrane (Figure S1A) and their expression levels were comparable to that of WT MgrB (Figure S3). Domain swap experiments of two previously studied histidine kinase regulators that also have a single TM domain –SafA and MzrA indicate that the transmembrane sequence is dispensable for their function (19, 20). Indeed, our control experiments where we replaced the TM sequences of SafA and MzrA with that of GpA confirmed these results. Thus, for SafA and MzrA, the TM domain appears to primarily act as an anchor to direct the regulatory domain of these proteins to the membrane (Figure S2). To determine if MgrB behaves in the same way, we monitored the effect of MgrB and its TM swap variants on PhoQ activity using a PhoQ/PhoP-regulated transcriptional reporter (P_{mgtA}-*lacZ*). Control cells expressing no MgrB have a high level of β-galactosidase (β-gal) activity, indicating uninhibited PhoQ, whereas cells expressing WT MgrB have minimal activity, indicating PhoQ inhibition by WT MgrB, as expected. In contrast with the behavior of WT MgrB, the MgrB TM swap variants MgrB-MalF TM or MgrB-GpA TM both show high β-gal activities that are about 80% of activity of no-MgrB control, indicating that the TM swaps unable to repress PhoQ efficiently. These results indicate that the MgrB TM domain does not simply function as a membrane anchor but is also critical for MgrB's activity as a PhoQ inhibitor.

Given that the MgrB TM sequence is necessary for its function, we wanted to further identify specific residues that might contribute to repression of PhoQ. The predicted TM sequence consists of residues 7-24 (underlined sequence shown in Figure 2), based on results from the TMHMM2 transmembrane domain prediction server (http://www.cbs.dtu.dk/services/TMHMM/). Hydrophobic residues (V, L, A) are often involved in surface interactions facing the hydrophobic interior of the membrane. Prior work suggests that the conserved cysteine within the TM domain (C16) may be less important for MgrB function relative to the two other conserved periplasmic Cys residues (8). Aromatic residues are known to preferentially occur in the lipid-water interface regions (22, 23) and could be crucial for membrane anchoring as well as surface interactions. Polar amino acids such as N and Q can facilitate TM domain interactions by forming stable hydrogen bonds (24). Based on these considerations, we chose to focus on residues W20, Q22, F24 of the TM domain and tested the effects of single alanine substitutions. All three mutant proteins localized to the cell membrane (Figure S1B) and their expression levels were comparable to that of WT (Figure S4). The W20A mutant greatly affected MgrB function and showed a high β-gal activity (~30-fold higher than WT MgrB) (Figure 2). Mutants Q22A and F24A displayed modest effects on reporter gene expression (~5- and 8-fold higher β-gal activity compared to WT MgrB, respectively). Recent bioinformatic analyses have found that a 'polar-xx-polar' motif (where polar = S/T/D/E/Q/N) is highly conserved in bacterial TM domains and drives

protein-protein interactions (24). We wondered if the motif Q^{22}-x-x-N^{25} could be relevant, and created a double alanine mutant MgrB Q22A/N25A. This mutant showed a slight increase in reporter gene expression relative to Q22A mutant alone suggesting that this motif could be partly important to MgrB activity.

The MgrB TM domain is necessary but not sufficient for its function

As swapping the TM domain sequence in MgrB severely affected its activity, we tested whether an MgrB mutant lacking the periplasmic domain retained an ability to repress PhoQ. This variant —MgrB-24, in which N25 is replaced with a stop codon—showed proper membrane localization (Figure S1C), but could not repress reporter gene expression, indicating that this variant composed of MgrB TM domain and the short N-terminal cytoplasmic sequence is not functional. To assess the minimum length of MgrB needed for efficient repression of PhoQ/PhoP-regulated reporter gene expression, we tested other C-terminal truncations of MgrB: MgrB-29, MgrB-34, MgrB-39, MgrB-44, corresponding to replacing the codons for Q30, F35, A40 and I45 with a stop codon, respectively. These mutants displayed similar membrane localization to that of WT MgrB (Figure S1). Of these four truncations, MgrB-29 and -34 did not show any detectable repression, MgrB-39 showed slight repression, and MgrB-44, which lacks only the last two residues, showed the strongest repression, although last variant was still compromised in activity compared with WT (Figure S5). Evidently, amino acid interactions in both the TM and the small periplasmic region of MgrB influence the protein's ability to inhibit PhoQ.

Site-directed mutagenesis analysis of MgrB periplasmic domain

To explore the relative importance of different residues in the MgrB periplasmic domain, we performed alanine scanning mutagenesis and analyzed the effects of these mutations on PhoQ inhibition. The majority of the alanine substitutions did not affect PhoQ/PhoP reporter gene expression, but six displayed a notable increase in β-gal activity relative to WT (Figure 3). Mutants C28A, C39A showed the highest effects (80-90 fold higher β-gal activity than WT MgrB), consistent with previous data indicating that these two cysteine residues are important for MgrB activity (8). In addition, mutants G37A D31A, F34A and W47A displayed a significant increase in reporter gene expression – approximately 30-, 8-, 10-, and 10-fold relative to WT MgrB, respectively. These residues may be important for structural stability of the periplasmic domain or for inter-protein interactions. We confirmed that these mutants localized properly to the cell membrane (Figure S1D) and that the protein levels were not considerably different from that of the WT (Figure S6).

MgrB lacking periplasmic domain is capable of physical interaction with PhoQ

Although MgrB-24 (N-terminus+TM domain) does not have the ability to inhibit PhoQ, it could still play a role in establishing physical contact with PhoQ. To explore this possibility, we used a bacterial two-hybrid (BACTH) system based on split adenylyl cyclase (25, 26). To assay interactions of MgrB N-terminus+TM domain with PhoQ, we prepared N-terminal fusions of MgrB-24 (MgrB lacking periplasmic domain) and PhoQ to T18 and T25 fragments, respectively. As controls, we tested interactions between MgrB–PhoQ, MgrB–*Vc*ToxR (*Vibrio cholerae* inner membrane protein ToxR) as well as MgrB–T25 fragment alone. In this spot assay, reconstitution of the T18 and T25 fragments restores the activity of adenylate cyclase (CyaA), which leads to an increase in cAMP levels. This increased cAMP in turn enables efficient induction of the genes required for maltose catabolism, resulting in pink colonies on maltose MacConkey plates. Cells expressing T18-MgrB-24 and T25-PhoQ showed a dark pink colony after 48 h at 30 °C,

whereas cells expressing T18-MgrB-24 and T25 fragment alone remained colorless (Figure 4). In addition, cells expressing T18-MgrB-24 and either T25-MgrB or T25-MgrB-24 were also pink. These results suggest that the MgrB N-terminus+TM domain physically interacts with itself and also with PhoQ. Control cells expressing T18-MgrB and T25-PhoQ or T25-MgrB showed a dark pink spot, consistent with previous work showing an interaction between MgrB with itself and with PhoQ (7) and cells expressing either T18-MgrB or T18-MgrB-24 with either T25- *Vc*ToxR or T25 fragment alone remained colorless. Together, these results suggest that the TM domain and N-terminus of MgrB are important for the interaction between MgrB and PhoQ as well as for the interaction between MgrB and itself.

MgrB interaction with non-cognate histidine kinases

To test if MgrB is capable of physically interacting with histidine kinases other than PhoQ, we made constructs of *E. coli* EnvZ, AtoS, CpxA, and PhoR fused to the T25 fragment of adenylyl cyclase and performed BACTH analysis by spot assays as well as measuring β-galactosidase activities of cells grown in liquid cultures. Intriguingly, cells expressing T18-MgrB and either T25-AtoS, -EnvZ, or -PhoR showed a high level of activity although less than the β-activity of cells expressing T18-MgrB and T25-PhoQ (Figure 5). Cells expressing T18-MgrB and T25-CpxA displayed a level of β-activity only marginally higher than the negative controls. These results suggest that MgrB is capable of physically interacting with EnvZ, AtoS, and PhoR, at least in the context of the bacterial two-hybrid assay. To test whether MgrB affects the activity of these histidine kinases, we measured gene expression from promoters regulated by these histidine kinases, in the presence or absence of plasmid-driven MgrB expression. However, we did not see any effect of MgrB on gene expression controlled by any of these histidine kinases (data not shown).

Discussion

In this study, we have explored the regions of MgrB that are important for its activity. Using a functional GFP-tagged derivative of MgrB to assay membrane localization and expression level, we determined the amino acid determinants in MgrB TM and periplasmic domains that are important for MgrB regulation of PhoQ. Previous work using a PhoQ chimera indicated that the periplasmic domain of MgrB, and in particular two conserved cysteines in this domain, are critical for PhoQ inhibition (7, 8), however little was known about the role of the MgrB TM domain or other residues in the periplasmic domain. Studies of two other bitopic membrane proteins that regulate histidine kinases determined that the transmembrane domains were not required for activity, suggesting the domain may serve primarily as a membrane anchor (19, 20). In contrast, our results show a striking loss of function when the MgrB TM is replaced with unrelated TM sequences. Bacterial two-hybrid experiments further indicate that the TM domain alone interacts with PhoQ, as well as with MgrB, although the TM domain alone does not show any inhibitory interaction. We also identified specific residues at the C-terminus of the TM domain that are particularly important for MgrB activity. Taken together, these results indicate that that the specific TM domain sequence is critical for MgrB interactions with PhoQ as well as with itself.

By making a series of C-terminal truncations in MgrB, we found that virtually all of the periplasmic domain is required for full activity. To identify which periplasmic residues in MgrB are required for PhoQ repression activity, we analyzed individual alanine substitutions across the entire periplasmic domain. Remarkably, despite the small size of MgrB (47 amino acids in total with 22 periplasmic residues), substitutions at most positions had little effect if any on MgrB

activity. Of the 22 residues in the periplasmic domain, only six were individually essential for full activity: the two conserved cysteines (C28 and C39), as previously noted (8), as well as G37, D31, F34 and W47. Furthermore, substitutions at these latter four residues lowered but did not eliminate MgrB activity. Interestingly, mutations for each of these positions are also associated with colistin-resistant Klebsiella isolates (10, 11, 27-32). Taken together, our findings show that the sequence determinants for MgrB activity are not restricted to either one of the domains – periplasmic or TM, and that functionality is mediated by collective interactions of key amino acid residues scattered across the polypeptide.

Unexpectedly, we also found that the MgrB lacking its periplasmic domain, as well as full-length MgrB, interact with several other histidine kinases in addition to PhoQ in a bacterial two-hybrid assay. However, we did not observe a significant effect of MgrB on gene expression regulated by these histidine kinases. The TM domain in many histidine kinases such as PhoQ, EnvZ etc. are believed to form a helical bundle consisting of four helices (33). Our results may reflect an interaction between the MgrB TM dimers and a conserved transmembrane conformation shared by multiple histidine kinases. The periplasmic domain of MgrB likely provides additional interactions facilitating specific regulation of PhoQ. It will be interesting to determine if there are other small membrane proteins that have evolved to interact with other histidine kinases using structural features similar to those of the MgrB TM domain. In addition, the results from our study of MgrB, as well as studies of other small membrane proteins, may provide a platform for reengineering these proteins to regulate new targets through rational design and directed evolution.

Materials and Methods

Strains, plasmids and cloning. See Supplementary Materials for tables of strains (Table S1), plasmids (Table S2), and primers (Table S3) used in this study. All strains were derived from *E. coli* K-12 MG1655. Gene deletions and reporter constructs were transferred between strains using transduction with $P1_{vir}$. Deletions in the Keio collection (34) that were used for strain construction were confirmed by PCR using primers flanking the targeted gene. Kanamycin resistance markers were excised, when required, using the FLP-recombinase-expressing plasmid pCP20 (35). Strain SAM72 was constructed by first excising the kanamycin resistance cassette from the *ompC-mCherry* transcriptional reporter strain AFS256 to give SAM71. Kanamycin resistance from the *mzrA* deletion strain JW3067 was then transduced into SAM71 by P1 transduction. A deletion of *safA* (SAM73) was constructed as described in (15, 35). SAM73 was then used to construct SAM76 by transducing the *mgtA-lacZ* transcriptional reporter from TIM199. Strain SAM85, which is a *lacI*^{q} containing version of BTH101 was made by conjugation of the bacterial two-hybrid host strain BTH101 (F−) with XL1-Blue (F+). This strain was used instead of BTH101 as it gave reduced background in our MacConkey plate assays. Plasmids expressing *gfpA206K* fusions to *mgrB* mutants (pSY3-9, pSY72-75, pJC1-7, pTG1-15) were created by either QuikChange site-directed mutagenesis (Stratagene) or Inverse PCR (36) using pAL38 as template. Plasmids expressing *gfpA206K* fusions of *mzrA* (pSY29) and *safA* (pSY31) were constructed by Gibson assembly (37). Plasmids pSY32 (MzrA TM swap) and pSY33 (SafA TM swap) were made by Inverse PCR using pSY29 and pSY31 as templates, respectively. Plasmid pSY34 expressing T18-MgrB-24 only was created using pAL33 as template by Inverse PCR. For all the plasmids mentioned above, see Table S3 for specific primer sequences used for each construction. Plasmids pSY61, pSY64 and pSY68 were constructed as follows: *atoS*, *cpxA*, and *phoR* were PCR-amplified off of the MG1655 genome using the following primer pairs – atoS-xbaI-U1/atoS-kpnI-L1, cpxA-xbaI-U1/cpxA-kpnI-L1 and

phoR-xbaI-U2/phoR-kpnI-L1, respectively, digested with XbaI/KpnI restriction enzymes and then cloned into pKT25 at XbaI/KpnI sites. All constructs were confirmed by DNA sequencing.

Media and growth conditions. Liquid cultures were grown at 37 °C with aeration, unless otherwise indicated, in either LB Miller medium (Fisher Scientific) or in minimal A medium (38) containing 0.2% glucose, 0.1% casamino acids and 1 mM $MgSO_4$. For routine growth on solid medium, LB or minimal medium containing bacteriological grade agar (Fisher Scientific) was used. The antibiotics ampicillin, kanamycin and chloramphenicol were used at concentrations 50–100, 25 and 20–25 μg mL^{-1}, respectively. The *lac* and *trc* promoters were induced with isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM when indicated. When IPTG was not mentioned in the description of the culture conditions, the basal transcription from the *trc* promoter was used to drive expression. For bacterial two-hybrid screening, MacConkey/maltose indicator plates were prepared as described (25) using MacConkey agar base powder (Difco™), 100 μg mL^{-1} ampicillin, 25 μg mL^{-1} kanamycin, 0.5 mM IPTG and 1% maltose.

Fluorescence microscopy. For each microscopy experiment, strains were grown in minimal medium containing with antibiotics as needed and single-cell measurements were performed essentially as described previously (9). For MgrB localization experiments, saturated overnight cultures of AML67 strains carrying *gfpA206K* fusions of either wild-type *mgrB* (pAL38), *mgrB* mutants (pSY3-9, pSY72-75, pJC1-7, pTG1-15) or *gfpA206K*-only (pAL39) were diluted 1:1,000 into fresh medium containing 100 μg mL^{-1} ampicillin and grown at 37 °C with aeration to an OD_{600} ~0.2–0.3 (~4-5 h) before visualization. For single-cell fluorescence measurements from transcriptional reporter strains, cultures were grown as outlined above, then rapidly cooled in an ice-water slurry and streptomycin was added to a final concentration of 250 μg mL^{-1} to stop protein synthesis. Microscope slides were prepared with 1% agarose pads and cell fluorescence was measured by imaging and quantified with in-house software as described previously (9, 39).

β-galactosidase reporter gene assay. Cultures of strain AML67 carrying *gfpA206K* fusions of either wild-type *mgrB* (pAL38), *mgrB* mutants (pSY3-9, pSY72-75, pJC1-7, pTG1-15) or *gfpA206K*-only (pAL39) plasmids were grown overnight in LB with 50 μg mL^{-1} ampicillin. The next day, they were diluted 1:1000 into the same medium and grown to OD_{600} ~0.3-0.4 at 37 °C. Cells were permeabilized with chloroform/SDS and assayed as in (38).

Bacterial two-hybrid assays. For the colorimetric spot assay, multiple clones from the transformation plate were inoculated in 3 mL of LB containing 100 μg mL^{-1} ampicillin and 25 μg mL^{-1} kanamycin. (Several clones were picked in order to reduce heterogeneity (25)) Cultures were grown overnight at 30 °C with shaking. The next day, 3 μL of each culture was spotted on MacConkey/maltose indicator plates and plates were then incubated at 30 °C. For each pair of plasmids, triplicate experiments were performed. Color change on the indicator plates was recorded after 48 h of incubation. Alternatively, *β*-gal assays were performed on the overnight liquid cultures prepared as described for the colorimetric assay.

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Author contributions

S.S.Y., J.N.C. and M.G. designed the experiments. S.S.Y., T.G. and J.N.C. performed the experiments. S.S.Y., T.G., J.N.C. and M.G. analyzed the data. S.S.Y. and M.G. wrote the manuscript.

Figures

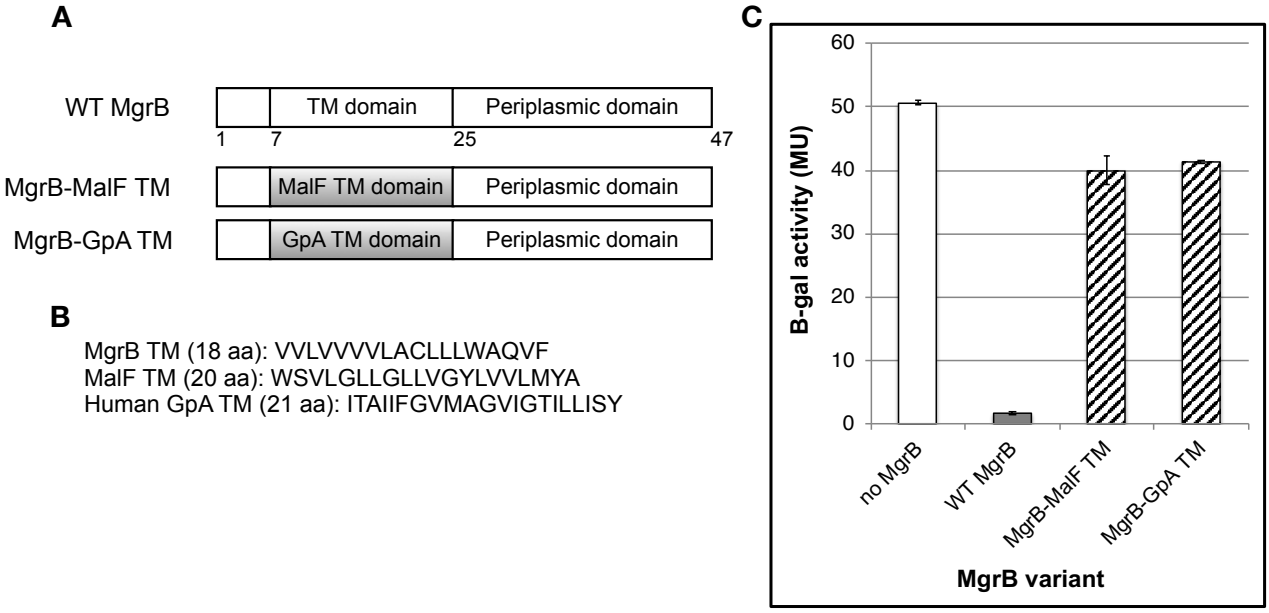

Figure 1. The MgrB transmembrane domain is essential for its function as a PhoQ repressor. A, Schematic representation of transmembrane (TM) domain swap constructs to replace the MgrB TM sequence with TM sequences derived from either the protein MalF or human glycophorin A, GpA. **B**, Sequences of transmembrane domains of MalF and GpA used in the study together with that of MgrB TM. **C**, β-galactosidase activities of wild-type MgrB (pAL38), its variants MgrB-MalF TM (pSY3), MgrB-GpA TM (pSY4) and no MgrB control (pAL39) in a Δ*mgrB* strain containing a PhoQ/PhoP-regulated transcriptional reporter P_{mgtA}-*lacZ* (AML67). Inducer was not added as the leaky expression from the *trc* promoter resulted in sufficient levels for strong repression by WT MgrB. Data represent averages and ranges for two independent experiments. MU = Miller Units.

MgrB: MKKFRWVVLVVVVLACLLLWAQVFNMMCDQDVQFFSGICAINQFIPW

putative TM domain

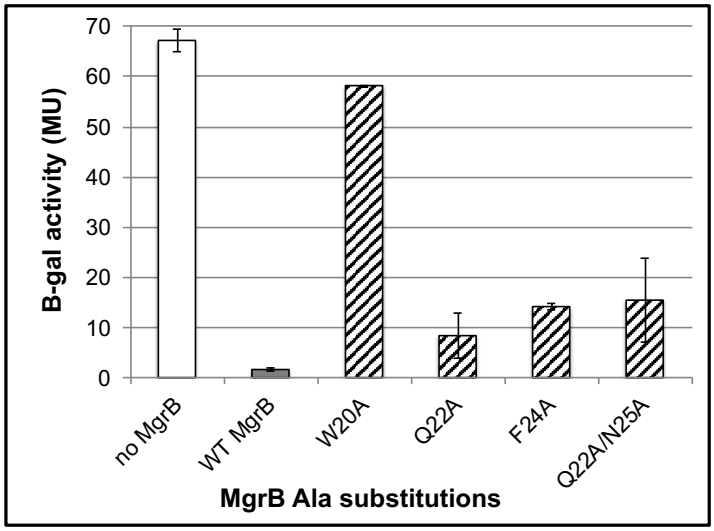

Figure 2. Effect of alanine substitutions in the MgrB transmembrane domain on PhoQ activity. β-galactosidase levels were measured in a Δ*mgrB* strain containing the PhoQ/PhoP-regulated transcriptional reporter P_{mgtA}-*lacZ* (AML67) and also containing either an empty vector (pAL39), wild-type MgrB (pAL38), or the indicated mutants MgrB W20A (pSY72), Q22A (pSY73), F24A (pSY74) and Q22A/N25A (pSY75). Inducer (IPTG) was not added as the leaky expression from the *trc* promoter resulted in sufficient levels of protein. Data represent averages and ranges for two independent experiments. MU = Miller Units.

MgrB: MKKFRWVVLVVVVLACLLLWAQVFNMMCDQDVQFFSGICAINQFIPW

putative TM domain periplasmic domain

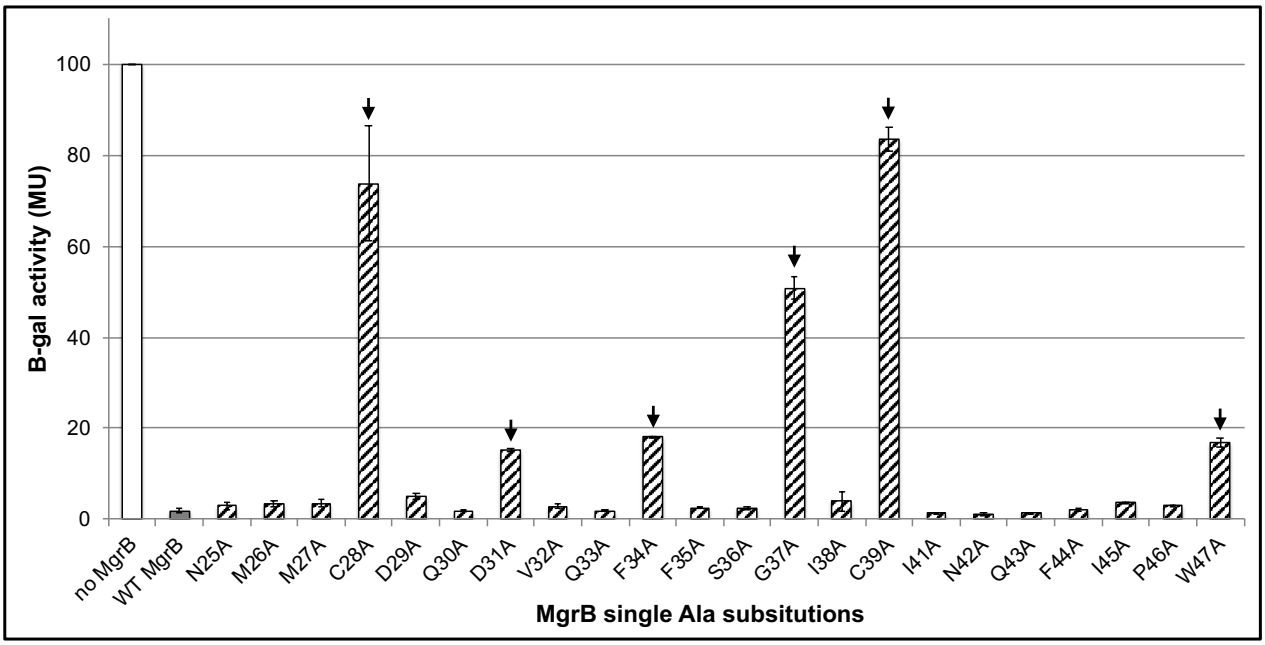

Figure 3. Effects of site-directed alanine substitutions of MgrB periplasmic residues on PhoQ activity. β-galactosidase levels were measured in a Δ*mgrB* strain containing the PhoQ/PhoP-regulated transcriptional reporter P_{mgtA}-*lacZ* (AML67) and also containing either an empty vector (pAL39), wild-type MgrB (pAL38), or the indicated mutants (pJC1 through pJC7 and pTG1 through pTG15; please see Table S2 for details). Inducer (IPTG) was not added as the leaky expression from the *trc* promoter resulted in sufficient levels of protein. Data for wild-type MgrB and each mutant was normalized to the no-MgrB control. Averages and standard deviations for three independent experiments are shown. MU = Miller Units.

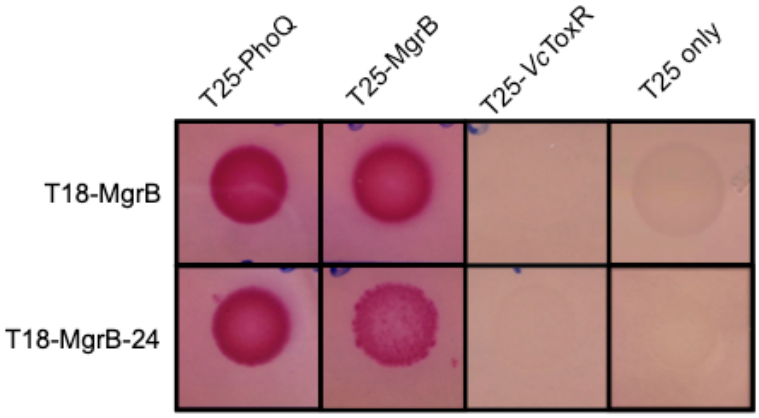

Figure 4. The MgrB transmembrane (TM) domain interacts with full length MgrB and with PhoQ. Bacterial two-hybrid (BACTH) colorimetric spot assays with cells expressing protein fusions to the T18 and T25 subunits of *B. pertussis* adenylyl cyclase. Representative images showing interactions between MgrB-24 (T18-MgrB-24) and partner proteins PhoQ (T25-PhoQ) and MgrB (T25-MgrB). Interactions between MgrB-PhoQ, MgrB-MgrB, MgrB-T25 only and (MgrB-24)-T25 only were included as positive and negative controls. As an additional control, interactions were tested between MgrB-24 and *Vibrio cholerae* membrane protein ToxR (*Vc*ToxR), and also between MgrB and *Vc*ToxR . Each pair of interactions was assayed in at least three independent trials in a *cyaA*$^-$ BACTH host strain containing *lacI*^{q} (SAM85), on maltose MacConkey indicator plates (results from one representative experiment are shown). The plates were incubated at 30°C for 48 h before images of plates were acquired. The pink color indicates reconstitution of adenylyl cyclase activity.

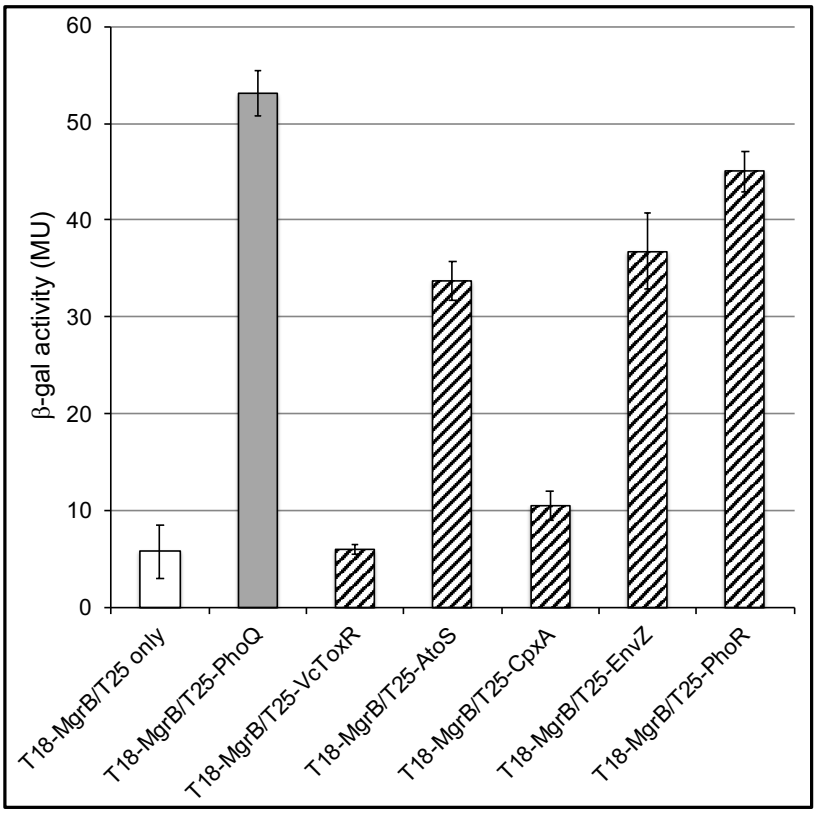

Figure 5. MgrB interacts with other histidine kinases. Bacterial two-hybrid (BACTH) assays with cells expressing protein fusions to the T18 and T25 subunits of *B. pertussis* adenylyl cyclase. β-gal expression from the *lac* promoter was monitored as a measure of interactions between T18-MgrB and T25 fusion with either PhoQ, AtoS, CpxA, EnvZ or PhoR. The T25 fragment alone and T25-*Vc*ToxR (*Vibrio cholerae* ToxR) serve as negative controls. β-gal assays were carried out in a *cyaA*$^-$ BACTH host strain containing *lacI*^{q} (SAM85). Data represent averages and ranges for two independent experiments. MU = Miller Units.

Supplementary Materials

Supplementary Tables

Table S1. Strains

Strain	Genotype	Source or Reference
AFS256	MG1655 λ_{att}::(P_{ompC}-*mCherry FRT-kan-FRT*)	Goulian lab collection
AML67	MG1655 Δ*mgrB*::(*FRT-kan-FRT*) Δ*lacZ* λ_{att}::(P_{mgtA}-*lacZ cat*)	(7)
BTH101	F-, *cya-99 araD139 galE15 galK16 rpsL1* (Str^r) *hsdR2 mcrA1 mcrB1*	Euromedex
BW25113	*rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1*	(40)
JW3067	BW25113 Δ*mzrA*::(*FRT-kan-FRT*)	KEIO collection (34)

MG1655	F^- λ^- ilvG- rfb-50 rph-1	*E. coli* Genetic Stock Center, CGSC# 7740
SAM71	MG1655 λ_{att}::(P_{ompC}-*mCherry*)	This study
SAM72	MG1655 Δ*mzrA*::(*FRT-kan-FRT*) λ_{att}::(P_{ompC}-*mCherry*)	This study
SAM73	MG1655 Δ*safA*::(*FRT-kan-FRT*)	This study
SAM76	MG1655 Δ*safA*::(*FRT-kan-FRT*) Δ*lacZ* λ_{att}::(P_{mgtA}-*lacZ cat*)	This study
SAM85	F+ *lacI*^{q}ZΔ*M15* Tn*10* (Tet^r), *cya-99 araD139 galE15 galK16 rpsL1* (Str^r) *hsdR2 mcrA1 mcrB1*. Strain prepared by conjugation: BTH101 (F-) x XL1-Blue (F+).	This study
TIM199	MG1655 Δ*lacZ* λ_{att}::(P_{mgtA}-*lacZ cat*)	(7)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^r)]	(41)

Table S2. Plasmids

Plasmid	**Relevant genotype**	**Source or Reference**
pAL25	pKT25 P_{lac}-*cyaA*$_{T25}$-*mgrB*, Kan^R	(7)
pAL27	pKT25 P_{lac}-*cyaA*$_{T25}$-*phoQ*, Kan^R	(7)
pAL30	pBAD33 P_{araBAD}-*phoQ(his)*$_6$, Cam^R	Goulian lab collection
pAL33	pUT18C P_{lac}-*cyaA*$_{T18}$-*mgrB*, Amp^R	(7)
pAL38	pEB52 P_{trc}-*gfpA206K-mgrB*, Amp^R	(7)
pAL39	pEB52 P_{trc}-*gfpA206K*, Amp^R	(7)
pBAD33	*ori*$_{p15A}$ P_{araBAD}-MCS *araC*, Cam^R	ATCC 87402
pCP20	*λcI857(ts) repA101(ts) oriR101 bla cat λpR-FLP*, Amp^R Cam^R	(42)
pEB52	pTrc99a with the NcoI site removed, Amp^R	Goulian lab collection
pJC1	pEB52 P_{trc}-*gfpA206K-mgrB Q30A*, Amp^R	This study
pJC2	pEB52 P_{trc}-*gfpA206K-mgrB D29A*, Amp^R	This study
pJC3	pEB52 P_{trc}-*gfpA206K-mgrB F34A*, Amp^R	This study
pJC4	pEB52 P_{trc}-*gfpA206K-mgrB G37A*, Amp^R	This study
pJC5	pEB52 P_{trc}-*gfpA206K-mgrB I38A*, Amp^R	This study
pJC6	pEB52 P_{trc}-*gfpA206K-mgrB P46A*, Amp^R	This study
pJC7	pEB52 P_{trc}-*gfpA206K-mgrB W47A*, Amp^R	This study
pKD13	*oriR*$_{\gamma R6k}$ *bla FRT::kan::FRT*, Kan^R	(35)
pKD46	*repA101(ts) oriR101 bla ParaB-(gam bet exo)*, Amp^R	(35)
pKK14	pKT25 P_{lac}-*cyaA*$_{T25}$-*envZ*, Kan^R	Goulian lab collection
pKT25	*ori*$_{p15A}$ P_{lac}-*cyaA*$_{T25}$-MCS, Kan^R	Euromedex
pKT25-toxR	pKT25 P_{lac}-*cyaA*$_{T25}$-*Vibrio cholerae toxR*, Kan^R	(43)
pSY3	pEB52 P_{trc}-*gfpA206K-mgrB-malF TM swap*, Amp^R	This study
pSY4	pEB52 P_{trc}-*gfpA206K-mgrB-gpA TM swap*, Amp^R	This study
pSY5	pEB52 P_{trc}-*gfpA206K-mgrB-24 (N25->stop)* truncation, Amp^R	This study
pSY6	pEB52 P_{trc}-*gfpA206K-mgrB-29 (Q30->stop)* truncation, Amp^R	This study
pSY7	pEB52 P_{trc}-*gfpA206K-mgrB-34 (F35->stop)* truncation, Amp^R	This study
pSY8	pEB52 P_{trc}-*gfpA206K-mgrB-39 (A40->stop)* truncation, Amp^R	This study
pSY9	pEB52 P_{trc}-*gfpA206K-mgrB-44 (I45->stop)* truncation, Amp^R	This study

pSY29	pEB52 P_{trc}-*gfpA206K-mzrA*, Amp^R	This study
pSY31	pEB52 P_{trc}-*gfpA206K-safA*, Amp^R	This study
pSY32	pEB52 P_{trc}-*gfpA206K-mzrA-gpA TM swap*, Amp^R	This study
pSY33	pEB52 P_{trc}-*gfpA206K-safA-gpA TM swap*, Amp^R	This study
pSY34	pUT18C P_{lac}-*cyaA*$_{T18}$-*mgrB-24* (MgrB truncation mutant lacking periplasmic domain), Amp^R	This study
pSY61	pKT25 P_{lac}-*cyaA*$_{T25}$-*atoS*, Kan^R	This study
pSY64	pKT25 P_{lac}-*cyaA*$_{T25}$-*cpxA*, Kan^R	This study
pSY68	pKT25 P_{lac}-*cyaA*$_{T25}$-*phoR*, Kan^R	This study
pSY72	pEB52 P_{trc}-*gfpA206K-mgrB W20A*, Amp^R	This study
pSY73	pEB52 P_{trc}-*gfpA206K-mgrB Q22A*, Amp^R	This study
pSY74	pEB52 P_{trc}-*gfpA206K-mgrB F24A*, Amp^R	This study
pSY75	pEB52 P_{trc}-*gfpA206K-mgrB Q22A N25A*, Amp^R	This study
pTG1	pEB52 P_{trc}-*gfpA206K-mgrB N25A*, Amp^R	This study
pTG2	pEB52 P_{trc}-*gfpA206K-mgrB M26A*, Amp^R	This study
pTG3	pEB52 P_{trc}-*gfpA206K-mgrB I45A*, Amp^R	This study
pTG4	pEB52 P_{trc}-*gfpA206K-mgrB C28A*, Amp^R	This study
pTG5	pEB52 P_{trc}-*gfpA206K-mgrB M27A*, Amp^R	This study
pTG6	pEB52 P_{trc}-*gfpA206K-mgrB D31A*, Amp^R	This study
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pTG8	pEB52 P_{trc}-*gfpA206K-mgrB Q33A*, Amp^R	This study
pTG9	pEB52 P_{trc}-*gfpA206K-mgrB F35A*, Amp^R	This study
pTG10	pEB52 P_{trc}-*gfpA206K-mgrB S36A*, Amp^R	This study
pTG11	pEB52 P_{trc}-*gfpA206K-mgrB C39A*, Amp^R	This study
pTG12	pEB52 P_{trc}-*gfpA206K-mgrB I41A*, Amp^R	This study
pTG13	pEB52 P_{trc}-*gfpA206K-mgrB N42A*, Amp^R	This study
pTG14	pEB52 P_{trc}-*gfpA206K-mgrB Q43A*, Amp^R	This study
pTG15	pEB52 P_{trc}-*gfpA206K-mgrB F44A*, Amp^R	This study
pTrc99a	*lacI*^{q}, P_{trc}-MCS, Amp^R	(44)
pUT18C	pUC19 P_{lac}-*cyaA*$_{T18}$-MCS, Amp^R	Euromedex

Table S3. Primers

Primer	**Sequence (5' -> 3')**	**Use**
atoS-xbaI-U1	ACTCTAGAGATGCATTATATGAAGTGGATTTATC	Cloning of *cyaA*$_{T25}$-*atoS*
atoS-kpnI-L1	CATGGTACCCAGTCATACAGTCTGATTTCCCTG	Cloning of *cyaA*$_{T25}$-*atoS*
b1500-red-u1	ACTGATTAACGATTTTTAACGTTATCCGCTAAATAAACATATTTGAAATGATTCCGGGGATCCGTCGACC	Generation of Δ*safA*::(*FRT-kan-FRT*)
b1500-red-l1	ATTTCATATTTATAATTTGCTGTTTGTTTTCAGCCTTGCAAACTATTGATTGTAGGCTGGAGCTGCTTCG	Generation of Δ*safA*::(*FRT-kan-FRT*)
cpxA-xbaI-U1	ACTCTAGAGATGATAGGCAGCTTAACCG	Cloning of *cyaA*$_{T25}$-*cpxA*
cpxA-kpnI-L1	CATGGTACCCCTGCAAATGCGAAGTTTAACTC	Cloning of *cyaA*$_{T25}$-*cpxA*

gfp-Cterm-L1	TTTGTATAGTTCATCCATGCCATGTGTAATCCCAG	Cloning of *gfpA206K-mzrA, -safA*
gfp-mzrA-U1	ACATGGCATGGATGAACTATACAAAATGCAAATACCTCGCATGTCGCTTC	Cloning of *gfpA206K-mzrA*
gfp-safA-U1	ACATGGCATGGATGAACTATACAAAATGCATGCGACCACAGTGAAAAACAAAATC	Cloning of *gfpA206K-safA*
GpA-TM-U1	GTGATTGGCACCATTCTGCTGATTAGCTATAACATGATGTGCGATCAGGATG	Cloning of *gfpA206K-mgrB-gpA TM swap*
GpA-TM-L1	GCCCGCCATCACGCCAAAAATAATCGCGGTAATCCATCGAAACTTTTTCATTTTGTATAG	Cloning of *gfpA206K-mgrB-gpA TM swap*
MalF-TM-U1	GTGGGTTACCTTGTTGTTTTAATGTACGCAAACATGATGTGCGATCAGGATG	Cloning of *gfpA206K-mgrB-malF TM swap*
MalF-TM-L1	CAGCAGGCCGAGCAGACCTAGCACTGACCACCATCGAAACTTTTTCATTTTGTATAG	Cloning of *gfpA206K-mgrB-malF TM swap*
mgrB_stop-pAL38-U1	TGAACTAGAGTCGACCTGCAG	Cloning of *gfpA206K-mgrB-24*, *mgrB-29*, *mgrB-34*, *mgrB-39 and mgrB-44*
mgrB-N25stop-L1	GAATACCTGCGCCCAAAGC	Cloning of *gfpA206K-mgrB-24*, and T18-*mgrB*
mgrB-Q30stop-L1	ATCGCACATCATGTTGAATACCTG	Cloning of *gfpA206K-mgrB-29*
mgrB-F35stop-L1	AAATTGTACATCCTGATCGCACATC	Cloning of *gfpA206K-mgrB-34*
mgrB-A40stop-L1	ACAAATTCCGCTGAAAAATTGTACATCC	Cloning of *gfpA206K-mgrB-39*
mgrB-I45stop-L1	AAACTGGTTAATGGCACAAATTCCGC	Cloning of *gfpA206K-mgrB-44*
mgrB_stop-pAL33-U1	TGAATAAGTAGGGTACCGAGCTC	Cloning of T18-*mgrB*
mgrB W20A-U1	GCGCAGGTATTCAACATGATGTGC	Cloning of *gfpA206K-mgrB W20A*
mgrB W20A-L1	CGCAAGCAGCAAGCAAGCCAACAC	Cloning of *gfpA206K-mgrB W20A*
mgrB Q22A-U1	GTATTCAACATGATGTGCGATCAGGATG	Cloning of *gfpA206K-mgrB Q22A*
mgrB Q22A-L1	CGCCGCCCAAAGCAGCAAGCAAGC	Cloning of *gfpA206K-mgrB Q22A*
mgrB F24A-U1	AACATGATGTGCGATCAGGATGTAC	Cloning of *gfpA206K-mgrB F24A*
mgrB F24A-L1	CGCTACCTGCGCCCAAAGCAGCAAG	Cloning of *gfpA206K-mgrB F24A*
mgrB Q22A N25A-U1	CTTGCTGCTTTGGGCGGCGGTATTCGCGATGATGTGCGATCAG	Cloning of *gfpA206K-mgrB Q22A N25A*
mgrB Q22A N25A-L1	CTGATCGCACATCATCGCGAATACCGCCGCCCAAAGCAGCAAG	Cloning of *gfpA206K-mgrB Q22A N25A*
mgrB-N25A-U2	ATGATGTGCGATCAGGATGTAC	Cloning of *gfpA206K-mgrB N25A*

mgrB-N25A-L2	CGCGAATACCTGCGCCCAAAGC	Cloning of *gfpA206K-mgrB N25A*
mgrB_M26A_U1	GCGCAGGTATTCAACGCGATGTGCGATCAGGATG	Cloning of *gfpA206K-mgrB M26A*
mgrB_M26A_L1	CATCCTGATCGCACATCGCGTTGAATACCTGCGC	Cloning of *gfpA206K-mgrB M26A*
mgrB_M27A_U1	GCAGGTATTCAACATGGCGTGCGATCAGGATGTAC	Cloning of *gfpA206K-mgrB M27A*
mgrB_M27A_L1	GTACATCCTGATCGCACGCCATGTTGAATACCTG	Cloning of *gfpA206K-mgrB M27A*
mgrB_C28A_U1	AGGTATTCAACATGATGGCCGATCAGGATGTACAATTTTTC	Cloning of *gfpA206K-mgrB C28A*
mgrB_C28A_L1	AATTGTACATCCTGATCGGCCATCATGTTGAATACCT	Cloning of *gfpA206K-mgrB C28A*
mgrB_D29A	AGGTATTCAACATGATGTGCGCTCAGGATGTACAATTTTTCAG	Cloning of *gfpA206K-mgrB D29A*
mgrB_D29A_as	CTGAAAAATTGTACATCCTGAGCGCACATCATGTTGAATACCT	Cloning of *gfpA206K-mgrB D29A*
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mgrB_F34A_as	GGCACAAATTCCGCTGAAAGCTTGTACATCCTGATCGCAC	Cloning of *gfpA206K-mgrB F34A*
mgrB_F35A_U1	TCAGGATGTACAATTTGCCAGCGGAATTTGTGCC	Cloning of *gfpA206K-mgrB F35A*
mgrB_F35A_L1	TGGCACAAATTCCGCTGGCAAATTGTACATCCTG	Cloning of *gfpA206K-mgrB F35A*
mgrB_S36A_U1	GATGTACAATTTTTCGCCGGAATTTGTGCCATTAAC	Cloning of *gfpA206K-mgrB S36A*
mgrB_S36A_L1	AATGGCACAAATTCCGGCGAAAAATTGTACATCC	Cloning of *gfpA206K-mgrB S36A*
mgrB_G37A	GGATGTACAATTTTTCAGCGCAATTTGTGCCATTAACCAGT	Cloning of *gfpA206K-mgrB G37A*
mgrB_G37A_as	ACTGGTTAATGGCACAAATTGCGCTGAAAAATTGTACATCC	Cloning of *gfpA206K-mgrB G37A*

mgrB_I38A	AGGATGTACAATTTTTCAGCGGAGCTTGTGCCATTAACCAGTTTATCC	Cloning of *gfpA206K-mgrB I38A*
mgrB_I38A_as	GGATAAACTGGTTAATGGCACAAGCTCCGCTGAAAAATTGTACATCCT	Cloning of *gfpA206K-mgrB I38A*
mgrB_C39A_U1	AATTTTTCAGCGGAATTGCTGCCATTAACCAGTTTAT	Cloning of *gfpA206K-mgrB C39A*
mgrB_C39A_L1	ATAAACTGGTTAATGGCAGCAATTCCGCTGAAAAATTG	Cloning of *gfpA206K-mgrB C39A*
mgrB_I41A_U1	TCAGCGGAATTTGTGCCGCTAACCAGTTTATCCCG	Cloning of *gfpA206K-mgrB I41A*
mgrB_I41A_L1	ACGGGATAAACTGGTTAGCGGCACAAATTCCGCTG	Cloning of *gfpA206K-mgrB I41A*
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mgrB_N42A_L1	CCACGGGATAAACTGGGCAATGGCACAAATTCC	Cloning of *gfpA206K-mgrB N42A*
mgrB_Q43A_U1	GAATTTGTGCCATTAACGCGTTTATCCCGTGGTGAAC	Cloning of *gfpA206K-mgrB Q43A*
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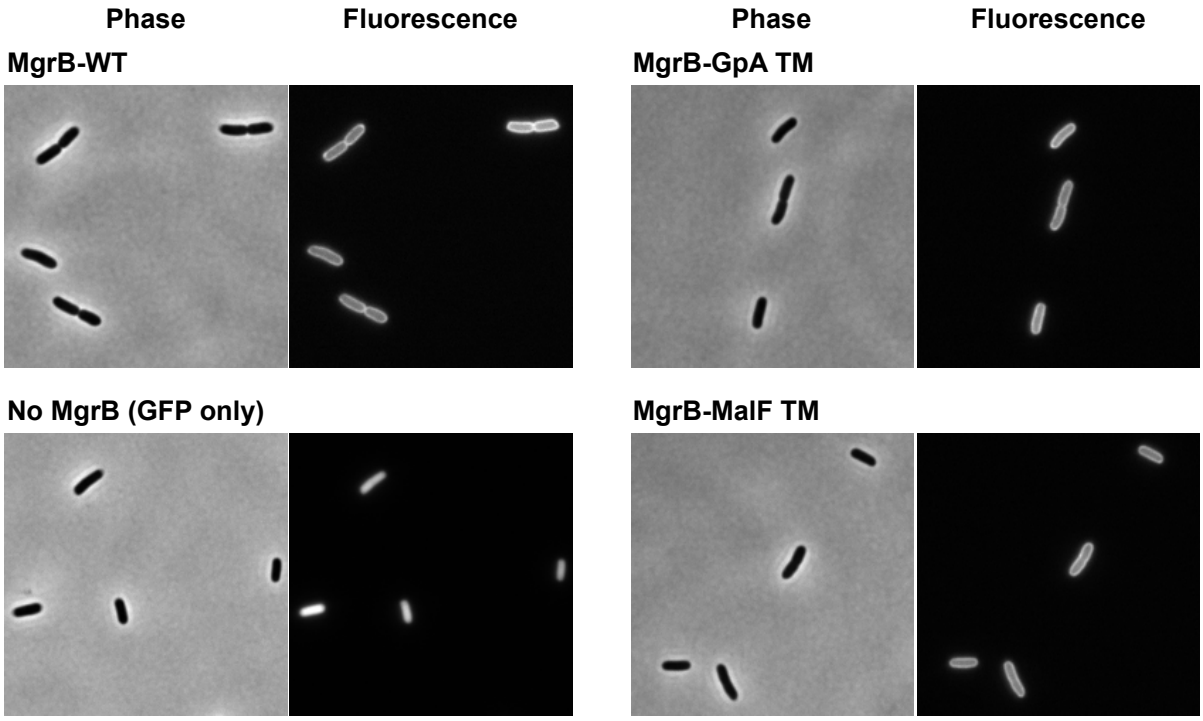
mgrB-I45A-L2	CGCAAACTGGTTAATGGCACAAATTCC	Cloning of *gfpA206K-mgrB I45A*
mgrB(pAL38)_P46A	GCCATTAACCAGTTTATCGCGTGGTGAACTAGAGTC	Cloning of *gfpA206K-mgrB P46A*
mgrB(pAL38)_P46A_as	GACTCTAGTTCACCACGCGATAAACTGGTTAATGGC	Cloning of *gfpA206K-mgrB P46A*
mgrB(pAL38)_W47A	CATTAACCAGTTTATCCCGGCGTGAACTAGAGTCGACCTG	Cloning of *gfpA206K-mgrB W47A*
mgrB(pAL38)_W47A_as	CAGGTCGACTCTAGTTCACGCCGGGATAAACTGGTTAATG	Cloning of *gfpA206K-mgrB W47A*
mzrA-GpA-TM-U1	GTGATTGGCACCATTCTGCTGATTAGCTATCGCCAGCAAGAGTCTACGCTG	Cloning of *gfpA206K-mzrA-gpA TM swap*
mzrA-GpA-TM-L1	GCCCGCCATCACGCCAAAAATAATCGCGGTAATCTGGCGAAGCGACATGCGAG	Cloning of *gfpA206K-mzrA-gpA TM swap*
pEB52-U1	ACTAGAGTCGACCTGCAGGCATGCAAG	Cloning of *gfpA206K-mzrA, -safA*
pEB52-L1	GGGTACCGAGCTCGAATTCTCTGTTTC	Cloning of *gfpA206K-mzrA, -safA*
pEB52-gfp-U1	AACAGAGAATTCGAGCTCGGTACCC	Cloning of *gfpA206K-mzrA, -safA*
pEB52-mzrA-L1	TGCATGCCTGCAGGTCGACTCTAGTTTATCCGAAGCGATGAGAGTTATCCC	Cloning of *gfpA206K-mzrA*
pEB52-safA-L1	TGCATGCCTGCAGGTCGACTCTAGTTCAGCCTTGCAAACTATTGATAATGAAATGTG	Cloning of *gfpA206K-safA*

phoR-xbaI-U2	ACTCTAGAGATGCTGGAACGGCTGTCGTG	Cloning of *cyaA*$_{T25}$-*phoR*
phoR-kpnI-L1	TGGCTGGTACCGATGACAAAGGCGGATTAATC	Cloning of *cyaA*$_{T25}$-*phoR*
safA-GpA-TM-U1	GTGATTGGCACCATTCTGCTGATTAGCTATATTGATCAGCTGAGTATTTCAGAAATGG	Cloning of *gfpA206K-safA-gpA TM swap*
safA-GpA-TM-L1	GCCCGCCATCACGCCAAAAATAATCGCGGTAATTTCTTTATAGTTGTCTCTTTGCGTGATTTTGTTTTTC	Cloning of *gfpA206K-safA-gpA TM swap*

Supplementary Figures

A



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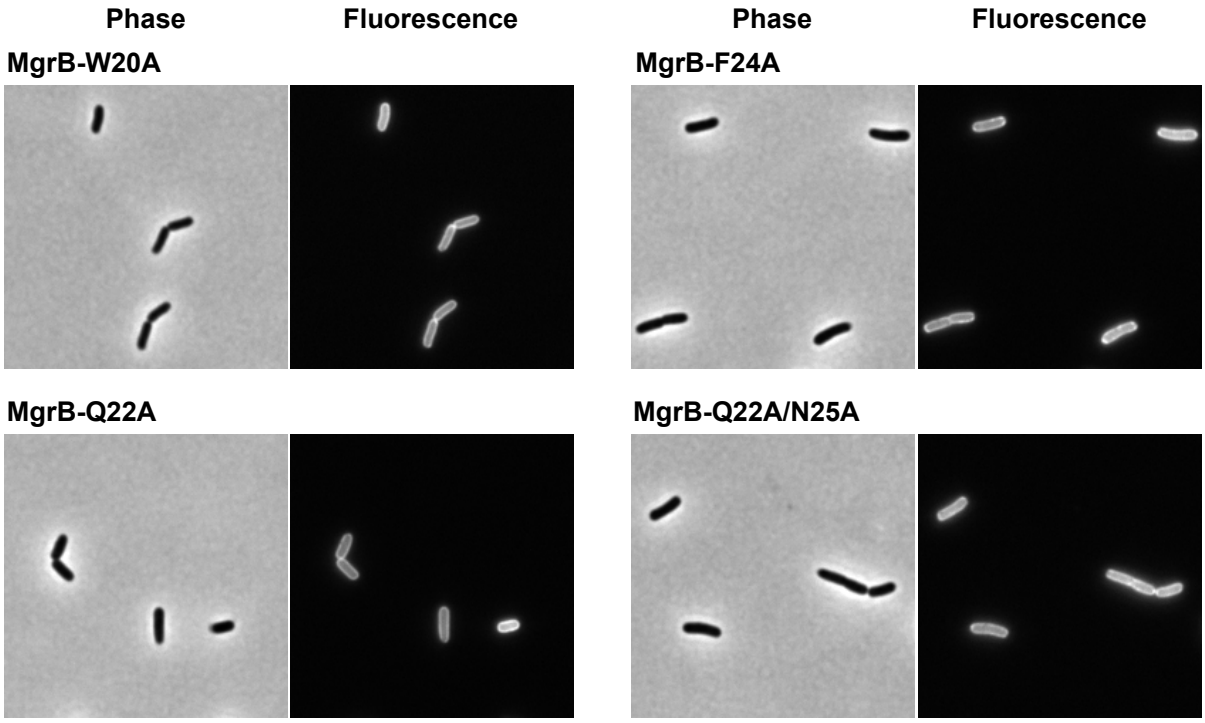
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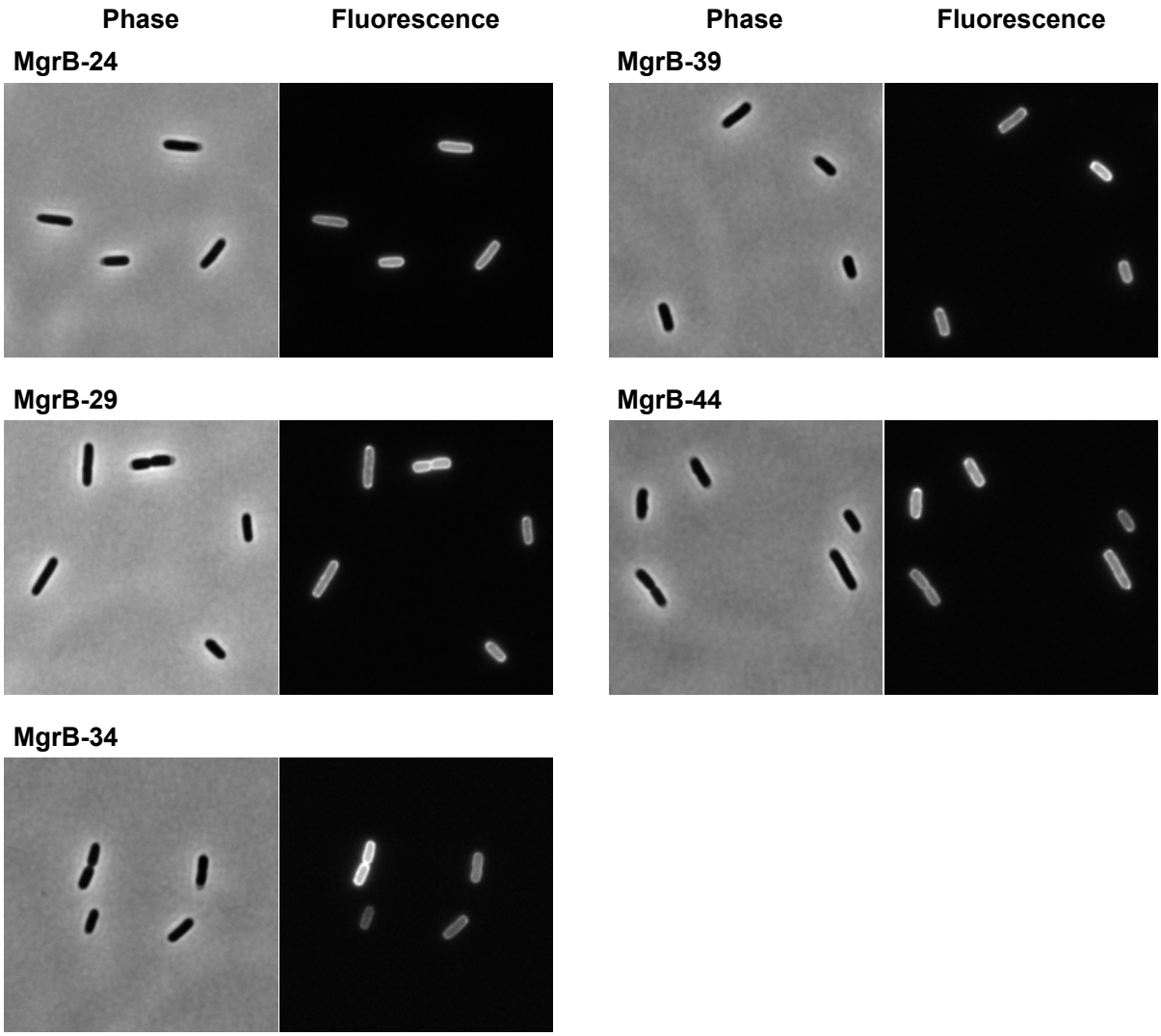
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D

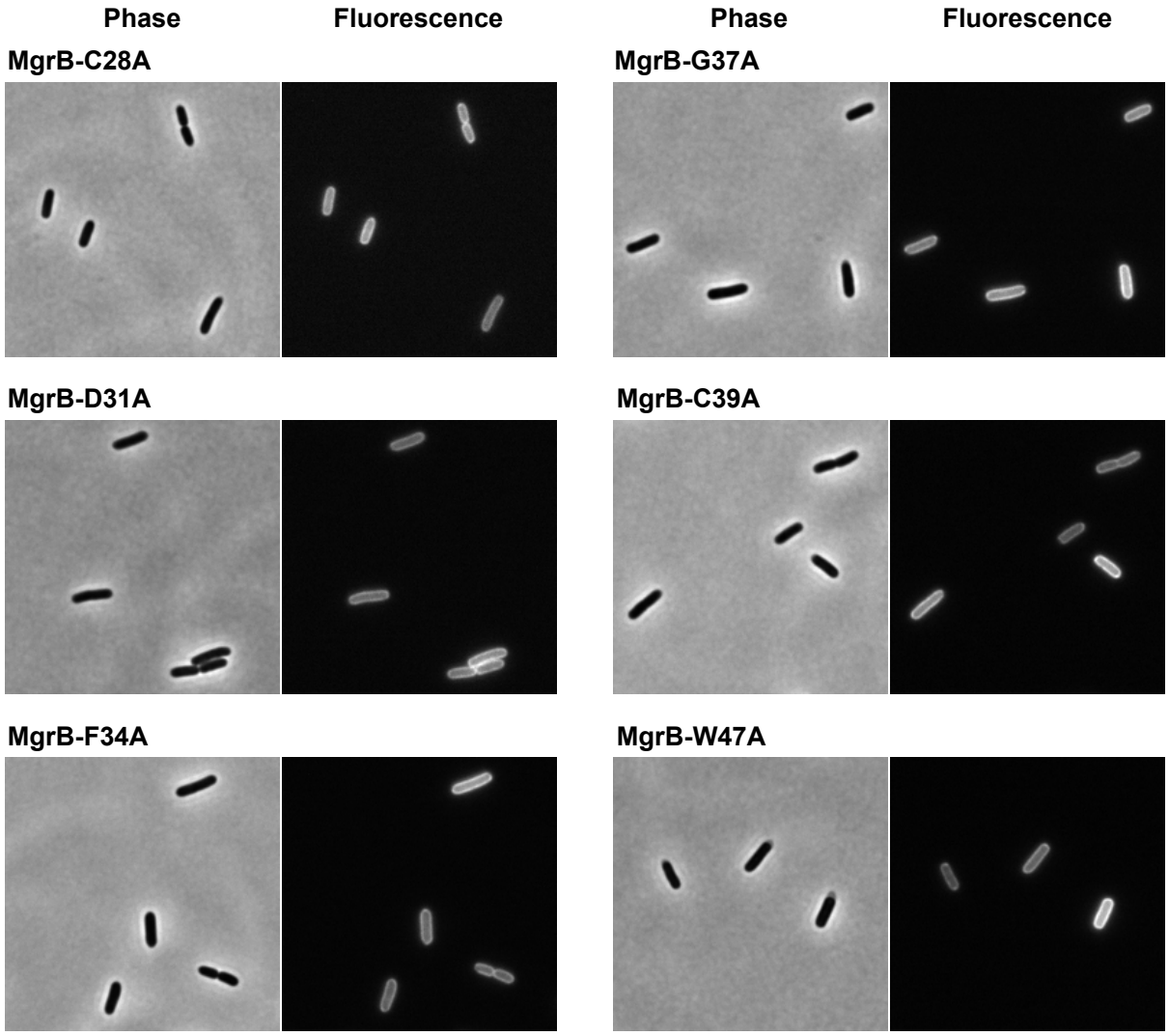

Figure S1. Visualization of cellular localization of MgrB and its variants by microscopy. **A**, Representative phase contrast and fluorescence microscopy images of cells expressing wild-type MgrB, GFP-only, and MgrB variants where the transmembrane region is swapped with transmembrane domain from either human GpA or MalF, respectively. **B**, Representative phase contrast and green fluorescence images of cells expressing TM domain single Ala mutants of MgrB – W20A, Q22A, F24A and Q22A/N25A. **C**, Representative phase contrast and green fluorescence images of cells expressing C-terminal truncations of MgrB – MgrB-24 (TM only construct, no periplasmic domain), MgrB-29, MgrB-34, MgrB-39 and MgrB-44. **D**, Representative phase contrast and green fluorescence images of cells expressing periplasmic single Ala mutants of MgrB – C28A, D31A, F34A, G37A, C39A and W47A. AML67 is the strain background in all cases. Inducer (IPTG) was not added as the leaky expression from the *trc* promoter resulted in sufficient levels of protein.

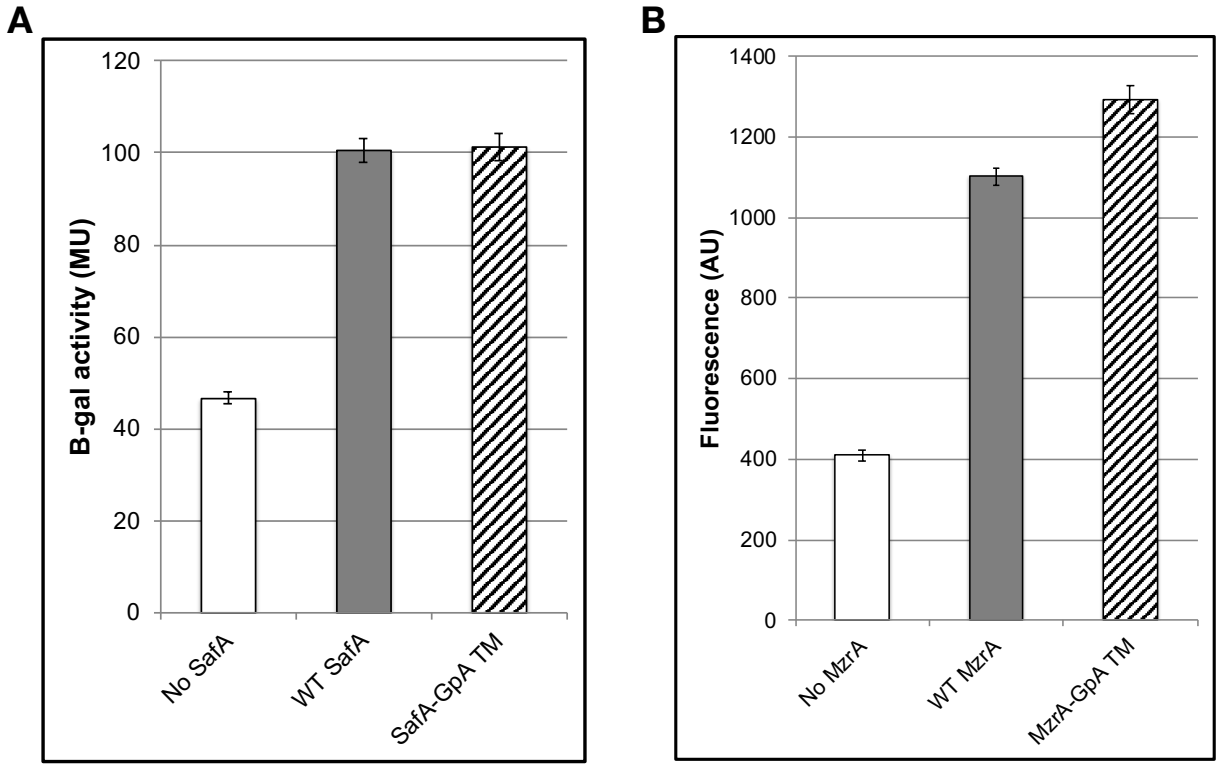

Figure S2. Transmembrane sequences of histidine kinase regulators – SafA and MzrA, are dispensable for their function. **A**, Activities of wild-type SafA and its variant where the transmembrane region is swapped with transmembrane domain from human glycophorin A (GpA) measured as a function of β-galactosidase levels in a Δ*safA* strain containing PhoQ/PhoP-regulated transcriptional reporter P_{mgtA}-*lacZ* (SAM76), MU = Miller Units. **B**, Activities of wild-type MzrA and its variant where the transmembrane region is swapped with transmembrane domain from human GpA measured as a function of red fluorescence levels in a Δ*mzrA* strain containing EnvZ/OmpR-regulated transcriptional reporter P_{ompC}-*mCherry* (SAM72), AU = Arbitrary Units. Inducer (IPTG) was not added as the leaky expression from the *trc* promoter resulted in sufficient levels of protein. Data represent averages and ranges for two independent experiments.

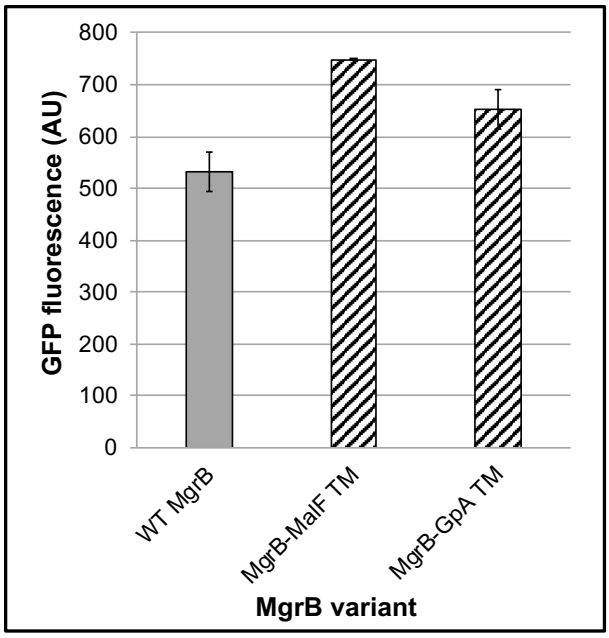

Figure S3. GFP-MgrB expression levels for the TM swap variants relative to wildtype. Cells expressing WT MgrB and variants were imaged by fluorescence microscopy and GFP fluorescence levels for quantified (see Methods for details). Data represent averages and ranges for two independent experiments comprised of at least 50 cells each, AU = Arbitrary Units.

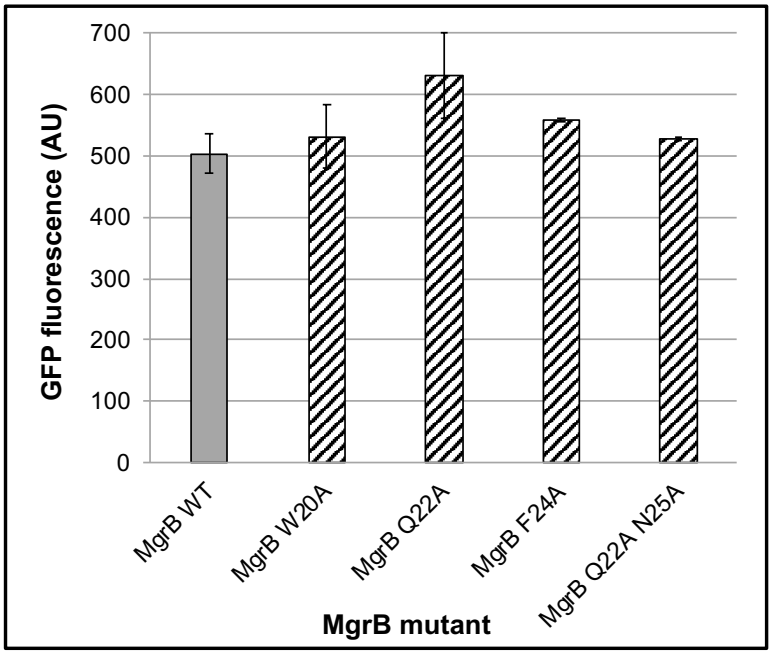

Figure S4. GFP-MgrB expression levels for the site-directed TM domain alanine mutants relative to wild-type. GFP fluorescence was quantified as described in the legend for Figure S3, AU = Arbitrary Units.

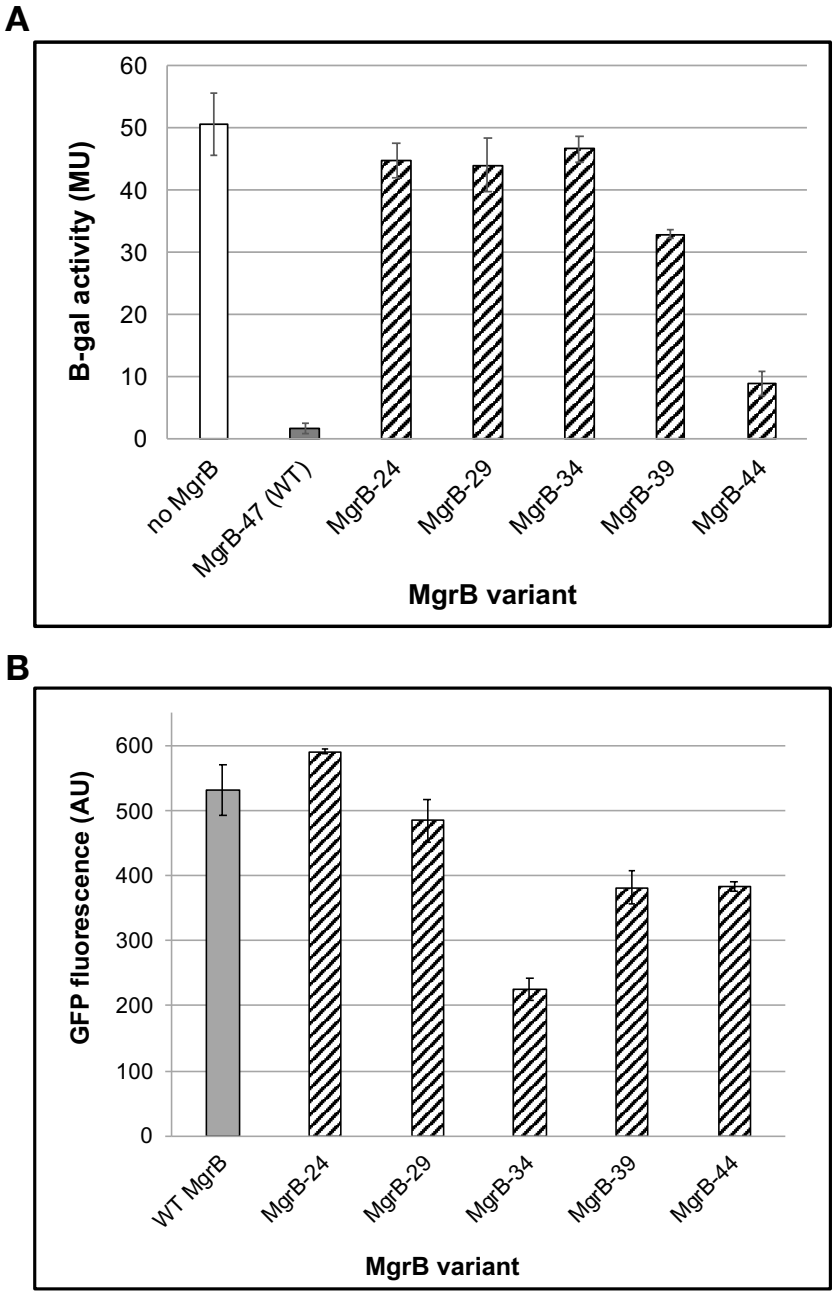

Figure S5. Effect of C-terminal truncations on MgrB activity. **A**, Effect of wild-type MgrB and the indicated mutants were measured in a Δ*mgrB* strain containing the PhoQ/PhoP-regulated transcriptional reporter P_{mgtA}-*lacZ* (AML67). Inducer (IPTG) was not added as the leaky expression from the *trc* promoter resulted in sufficient levels of protein. Data represent averages and ranges for two independent experiments. MU = Miller Units. **B**, GFP-MgrB expression levels for the MgrB truncation mutants relative to wild-type. GFP fluorescence was quantified as described in the legend for Figure S3, AU = Arbitrary Units.

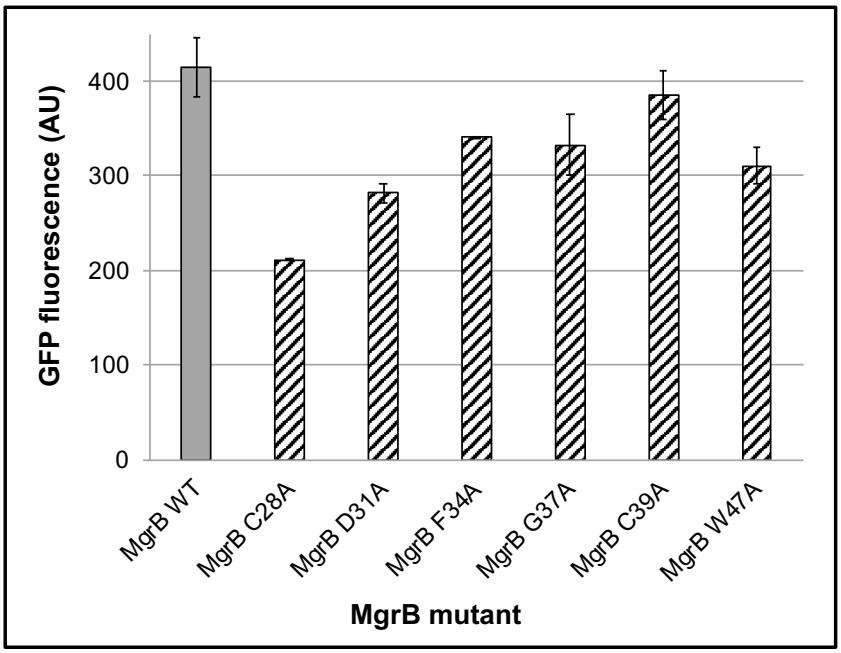

Figure S6. GFP-MgrB expression levels for the site-directed periplasmic single alanine mutants relative to wild-type. GFP fluorescence was quantified as described in the legend for Figure S3, AU = Arbitrary Units.

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