Toxin GhoT of the GhoT/GhoS toxin/antitoxin system damages the cell membrane to reduce adenosine triphosphate and to reduce growth under stress

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Summary
Toxin/antitoxin (TA) systems perhaps enable cells to reduce their metabolism to weather environmental challenges although there is little evidence to support this hypothesis. *Escherichia coli* GhoT/GhoS is a TA system in which toxin GhoT expression is reduced by cleavage of its messenger RNA (mRNA) by antitoxin GhoS, and TA system MqsR/MqsA controls GhoT/GhoS through differential mRNA decay. However, the physiological role of GhoT has not been determined. We show here through transmission electron microscopy, confocal microscopy and fluorescent stains that GhoT reduces metabolism by damaging the membrane and that toxin MqsR (a 5′-GCU-specific endoribonuclease) causes membrane damage in a GhoT-dependent manner. This membrane damage results in reduced cellular levels of ATP and the disruption of proton motive force (PMF). Normally, GhoT is localized to the pole and does not cause cell lysis under physiological conditions. Introduction of an F38R substitution results in loss of GhoT toxicity, ghost cell production and membrane damage while retaining the pole localization. Also, deletion of *ghoST* or *ghoT* results in significantly greater initial growth in the presence of antimicrobials. Collectively, these results demonstrate that GhoT reduces metabolism by reducing ATP and PMF and that this reduction in metabolism is important for growth with various antimicrobials.

Introduction
In contrast to laboratory conditions, bacteria usually grow in close association (Kolter, 2010) and with nutritional limitations (Nguyen et al., 2011); in fact, nutritional stress could be perceived as the universal stress (Khakimova et al., 2013). Therefore, distinct mechanisms have evolved to acquire fitness advantages over competitors since resources are limited; for example, cells may activate cellular functions that are normally silent under laboratory conditions, such as the *bgl* operon in *Escherichia coli* for the uptake and metabolism of the β-glucosides salicin and arbutin (Prasad and Schaeffer, 1974), or even kill competitors via bacteriocins. Furthermore, it has been hypothesized that cells may slow growth to acquire a competitive advantage; for example, to become more efficient in translation at times of reduced resources (Dethlefsen and Schmidt, 2007).

Bacteria can also weather unfavourable conditions by entering a dormant state known as the persister state where non-dividing cells can survive antibiotic treatments (Lewis, 2007). The most likely means for slowing growth during stress (in the extreme creating dormancy) is through toxin/antitoxin (TA) systems (Kim and Wood, 2010; Kwan et al., 2013). TA systems are prevalent in free-living prokaryotes (at least 37 in *E. coli*) (Tan et al., 2011), including many pathogenic bacteria (Gerdes et al., 2005). Typically, a protein-based TA system consists of a stable toxin to disrupt cellular processes and an unstable antitoxin to counteract the effect of the toxin and to impose physiological regulation of the toxin activity (Jayaraman, 2008).

Besides dormancy, TA systems may play other important roles in cell physiology such as biofilm formation (Ren et al., 2004; Kim et al., 2008), phage inhibition (Pecota and Wood, 1996) and the general stress response (Wang and Wood, 2011; Wang et al., 2011; Hu et al., 2012). Toxins with their messenger RNA (mRNA) endoribonuclease activity activated by stress, such as MqsR and MazF, have a global impact on gene expression by enriching some transcripts, and thus they are becoming recognized as global regulators through this property of differential mRNA decay (Wang and Wood, 2011). In fact, the MqsR/MqsA TA system regulates
another TA system, GhoT/GhoS (Wang et al., 2013). Anti-
toxins such as MqsA and DinJ also have a global impact 
through their inhibition of stationary stress response 
sigma factor RpoS (Wang and Wood, 2011; Wang et al.,  
2011; Hu et al., 2012); this inhibition is relieved upon 
stress that results in antitoxin degradation.

Although TA systems are present in the bacterial 
genome, their impact on cell physiology is often not promi-
nent, and there is usually no phenotype when a single 
system is deleted (Maisonuneuve et al., 2011); however, 
an effect is usually observed with plasmid-based over-
expression strategies or under various stress conditions. 
For example, several TA systems have been shown to be 
activated via amino acid or glucose starvation, includ-
ing RelB/RelE (Christensen et al., 2001), HigB/HigA 
(Christensen-Dalsgaard and Gerdes, 2006) and YafN/ 
YafO (Christensen-Dalsgaard et al., 2010); however, 
ectopic overexpression is usually required to exert their 
effect on bacterial growth. For the MazF/Maze TA system, 
an effect of mazEF deletion on bacterial growth is seen 
upon the introduction of severe environmental stresses, 
such as high temperature (50°C) and hydrogen peroxide 
(up to 50 mM) (Kolodkin-Gal and Engelberg-Kulka, 2006). 
Similarly, antibiotic stress (to create persisters cells) is 
required to see the effect of the mqsR mutation (mqsR 
encodes toxin MsqR), and this was the first time the 
absence of a toxin was shown to affect persistence (Kim 
and Wood, 2010). Deletion of tisB encoding the toxin TisB 
of the TisB/TisR-1 TA system also reduces persistence 
(Dörr et al., 2010), but deletion of most toxin genes has no 
effect on persistence even with severe antibiotic stress 
(Keren et al., 2004).

Of the five types identified so far, TA systems have been 
classified as type I if the antitoxin RNA prevents the trans-
lation of toxin RNA, type II if the antitoxin protein binds 
and inhibits the toxin protein, and type III if the antitoxin RNA 
binds and inhibits the protein toxin (Hayes and Van 
Melderen, 2011). In the type IV TA system, the protein 
antitoxin interferes with binding of the toxin to its target 
rather than through a direct antitoxin/toxin binding 
(Masuda et al., 2012). In the recently identified type V TA 
system, the antitoxin GhoS is not labile during stress nor 
doest it bind to DNA to regulate transcription; instead, it 
exhibits sequence-specific endoribonuclease activity to 
degrade ghoT mRNA, preventing its translation (Wang 
et al., 2012). Toxin GhoT in this system produces ghost 
cells when overproduced, but it has not been completely 
characterized in that it has not been shown to be a protein, 
it has not been determined whether it directly damages the 
membrane, nor has it been determined how it damages the 
membrane. We determine here the mechanism by which 
GhoT slows growth, by damaging the membrane at the cell 
poles, depleting cellular energy and disrupting the proton 
motive force (PMF). We also demonstrate that one physi-
ological role of the GhoT/GhoS TA system is to reduce 
metabolism in the presence of several antimicrobials 
(carbenicillin, cefoxitin, 5,7-dichloro-8-hydroxyquinoline 
and 2-phenyphenol); hence, there is a distinct phenotype 
upon deletion of the ghoST locus.

Results

GhoT causes membrane damage

GhoT is predicted to be a small (57 aa), highly hydropho-
bic polypeptide and has been shown to cause cell lysis 
when overproduced (Wang et al., 2012). Here we used 
transmission electron microscopy (TEM) to confirm 
cellular membrane rupture upon GhoT overproduction. 
Three phenomena were observed for E. coli cells 
overexpressing GhoT (Fig. 1A, right): (i) nucleoid condensa-
tion and leakage into the extracellular space, (ii) 
enlargement of the periplasmic space and (iii) cell break-
age. These observations are indicative of cell decay. As a 
negative control, cells producing GhoS (a cytoplasmic 
endonuclease) were examined, and no membrane 
damage was observed (Fig. 1A, left).

To investigate further how GhoT exerts its effect on the 
bacterial membrane, the cell morphology upon GhoT pro-
duction was compared with the treatment of nisin, a 
channel-forming toxin that causes cell lysis (Lamsa et al., 
2012), as well as the antibacterial compound carbonyl 
cyanoide m-chlorophenyl hydradze (CCCP), which disrupts 
membrane potential and ΔpH, the components of PMF (Liu

et al., 2010). Because these compounds are normally only 
effective in Gram-positive bacteria (Lamsa et al., 2012), 
the E. coli strain NR698, deficient in lptD that encodes a 
protein required for outer membrane biogenesis, was uti-
lized to assess the effect of the treatments (Sampson et al., 
1989). As shown in Fig. 1B, no significant change in mor-
phology was found for the negative control, NR698/ 
pCA24N cells treated with dimethyl sulfoxide (DMSO), 
whereas cells producing GhoT (NR698/pCA24N-ghoT) 
showed signs of compromised membranes as evidenced 
by hyper-permeability to the nucleic acid stains, 4',6-
diamidino-2-phenylindole (DAPI) and SYTOX Green (Life 
Technologies, Grand Island, NY, USA; 42%, 63 out of 
150 cells counted). For the two positive controls, the cells 
became hyper-permeable when treated with nisin and had 
significant membrane damage when challenged with 
CCCP, as evidenced by faint FM4–64 signals, which was 
likely due to the inability of FM4–64 to bind to the highly 
compromised membranes.

Deletion of ghoT reduces the persistence of an MqsR-
producing strain (Wang et al., 2012), and MqsR activates 
GhoT through differential mRNA decay (Wang et al., 
2013). To see if MqsR production leads to membrane 
damage through GhoT, we compared the cell morphology 
of BW25113/pCA24N-mqsR and ΔghoT/pCA24N-mqsR
with isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. As shown in Fig. 1C, a portion of cells producing MqsR became hyper-permeable to the nucleic acid stains (13%, 200 out of ∼1500 cells counted) and showed condensed poles characteristic of ghost cells; in contrast, this was not observed in the ΔghoT mutant (0.3% compromised cells, 8 out of ∼2600 cells counted). The results show that the production of the 5′-GCU-specific RNase MqsR causes membrane damage in a GhoT-dependent manner, supporting our previous conclusion that the type II TA MqsR/MqsA controls the type V TA GhoT/GhoS (Wang et al., 2013).

**GhoT does not lyse cells under physiological conditions**

Because it was unclear whether GhoT causes membrane damage and cell lysis without ectopic expression, we first investigated membrane integrity by microscopic analysis of BW25113 and the ΔghoT/pCA24N-mqsR cells induced with 1 mM IPTG for 1 h. The overlay (FM 4–64, DAPI and SYTOX Green), FM 4–64 only and the phase-contrast microscope images are shown. Scale bars represent 1 μm.
medium (turbidity of 0.8 to 1.0) (Fig. 2); for BW25113, 0 out of ~500 cells showed a compromised membrane, and for the ΔghoT mutant, 0 out of ~1200 cells showed a compromised membrane. To provide a more quantitative comparison, cell lysis was investigated by determining the extent to which β-galactosidase is released into the supernatant compared to intracellular β-galactosidase. During exponential growth in M9-Glu medium, we did not observe significant cell lysis upon deleting ghoT: for BW25113/pCA24N-lacZ, there was 0.018 ± 0.006% lysis, and for ΔghoT/pCA24N-lacZ, there was 0.022 ± 0.014% lysis. Similarly, during nutrient starvation imposed by overnight growth in M9-Glu medium, we did not observe significant cell lysis using the β-galactosidase assay (0.096 ± 0.042% for BW25113 versus 0.05 ± 0.01% for the ΔghoT mutant). As a control, we observed 5.4 ± 0.6-fold more cell lysis from GhoT overproduction (MG1655/pCA24N-ghoT versus MG1655/pCA24N). Therefore, together, our results suggest that, without ectopic expression, GhoT does not cause cell lysis.

**GhoT functions as a protein and Phe38 is important for its toxicity**

The toxin MqsR cleaves ghoS mRNA preferentially over ghoT mRNA in vitro and in vivo, which likely increases the relative level of free GhoT (Wang et al., 2013). Although the ghoT transcript does not harbour 5′-GCU, the primary MqsR cleavage recognition site (Yamaguchi et al., 2009), the transcript contains three of the less favourable 5′-GCA recognition sites. To investigate if any of these sites would affect GhoT toxicity in vivo, we introduced nucleotide substitutions to alter each of these 5′-GCA sites without changing the encoded amino acids. Three plasmids were generated: pCA24N-ghoTGCA1 with GCA (Ala) changed to GCC (Ala) at aa position 2, pCA24N-ghoTGCA2 with GCA (Ala) changed to GCC (Ala) at aa position 37, and pCA24N-ghoTGCA3 with GCA (Ala) changed to GCC (Ala) at aa position 51. In addition, to confirm that GhoT functions as a protein, mutations were introduced to pCA24N-ghoT to generate plasmid pCA24N-ghoTATG, which harbours a nucleotide substitution where A(TG) (Met) was changed to ACG (Thr) at GhoT aa position 1. Toxicity was then determined with a ΔghoS mutant carrying each pCA24N derivative, grown on IPTG-containing media. As shown in Fig. 3A, ΔghoS/pCA24N-ghoT did not grow on IPTG-containing media; however, ΔghoS/pCA24N-ghoTATG grew normally, showing that GhoT is a toxic protein because the removal of its start codon abolished toxicity. In addition, the MqsR secondary recognition 5′-GCA sites in ghoT are not important for toxicity because none of the ΔghoS strains harbouroing pCA24N derivatives with mutated 5′-GCA sites had enhanced GhoT toxicity on IPTG-containing media. Hence, ghoT mRNA does not appear to be processed by MqsR in vivo.

To identify residues important for GhoT activity, we modelled the GhoT structure using PHyre2 (Kelley and Sternberg, 2009). The modelled GhoT monomeric structure harbours two helices that appear to be transmembrane spanning regions (Fig. 3C). The termini of both transmembrane helices appear to be stabilized by a putative hydrophobic interaction formed between Ile21 and Phe38 at close proximity (interatomic distance less than 3 Å). To investigate if either of these residues helps stabilize GhoT in the inner membrane to confer its toxicity, we disrupted this hydrophobic interaction by separately changing Ile21 and Phe38 to Arg, with a bulky positively charged side chain, via plasmids pCA24N-ghoTI21R and pCA24N-ghoT38R respectively. As shown in Fig. 3B and D, ΔghoS carrying pCA24N-ghoS and pCA24N-ghoT38R grew normally with IPTG induction in both solid and liquid media, while the strains producing GhoT and GhoTI21R were unable to grow. Because overproduction of GhoT led to cell lysis (Wang et al., 2012), which caused complications in detection of the recombinant protein, the loss of toxicity of GhoTF38R provided a chance to detect and identify its cellular localization. As expected, GhoTF38R was detected in the initial cell pellet and in the membrane fraction via Western
GhoT damages the membrane to increase resistance

GhoT localizes at the cell pole

To investigate the cellular localization of GhoT, we examined BW25113 producing GhoT with a GFP fusion (via pCA24N-ghoT-GFP) using confocal microscopy and determined that GhoT-GFP proteins localize at the poles (Fig. 4A, upper panel). In order to verify if the loss of toxicity with GhoTF38R was due to altered cellular localization, the plasmid pCA24N-ghoT38R-GFP was constructed, and we determined that GhoT38R-GFP also localizes at the poles (Fig. 4A, lower panel). Using nucleic acid stains, DAPI and SYTOX Green, we compared the membrane damage caused by overproduction of wild-type GhoT and GhoT38R. We found that GhoT producing cells became more permeable (61%, 76 out of 124 cells became permeable) (Fig. 4B, overlay, upper panel) while most of the GhoT38R producing cells remained intact (3.52%, 60 out of ~1700 cells became permeable) (Fig. 4B, overlay, lower panel). In addition, using phase-contrast microscopy, we found that overproduction of GhoT38R reduced ghost cell formation compared with native GhoT production (Fig. 4B, phase-contrast). The polar localization of GhoT was not due to the accumulation of cellular proteins at the poles (which is the outcome of ghost cell formation) as we observed an even distribution of GFP in the control experiment in which cells produced GhoT and GFP separately (Fig. 4C). These results demonstrate that GhoT is a pole-localized protein and that introduction of the F38R substitution to GhoT renders it unable to cause membrane damage while maintaining the pole localization.

Fig. 3. GhoT is a toxic protein and the F38R substitution inactivates GhoT.
A and B. Growth of ΔghoS transformants carrying pCA24N derivatives with ghoT mutations on LB plates with kanamycin (50 μg ml⁻¹) and chloramphenicol (30 μg ml⁻¹) without or with 1 mM IPTG. Vector, pCA24N; GhoT, pCA24N-ghoT; GhoTATG, pCA24N-ghoTATG encoding GhoT with the start codon changed to Thr; GhoTGCA1, pCA24N-ghoTGCA1 encoding a silent change of the MqsR secondary cleavage site to GCC; GhoTGCA2, pCA24N-ghoTGCA2 encoding a silent change of the MqsR secondary cleavage site to GCC; GhoTGCA3, pCA24N-ghoTGCA3 encoding a silent change of the MqsR secondary cleavage site to GCC; GhoS, pCA24N-ghoS; GhoTI21R, pCA24N-ghoTI21R encoding GhoT with the I21R substitution; GhoTF38R, pCA24N-ghoTF38R encoding GhoT with the F38R substitution. Three independent cultures were evaluated. Scale bars represent 1 cm.
C. GhoT structure modelled by PHYRE2 (Kelley and Sternberg, 2009). Transmembrane spanning regions are annotated by UniProt (The UniProt Consortium, 2012) and are shown as gray and orange regions. The predicted positions of Ile21 and Phe38 are indicated.
D. Growth of cells carrying different plasmids in LB medium with chloramphenicol (30 μg ml⁻¹) and 1 mM IPTG. Three independent cultures were evaluated. Error bars, s.e.m. (n = 3).
E. Western blot analysis of GhoT38R. T, IPTG-induced total intracellular cell protein; I, insoluble fraction after ultracentrifugation and extraction with 6 N urea; C, cytoplasmic fraction after ultracentrifugation and extraction with 6 N urea; M, membrane fraction after ultracentrifugation.
GhoT reduces intracellular ATP levels and dissipates PMF

Because GhoT overproduction compromises membrane integrity, we hypothesized that the PMF would be disrupted, and subsequently the ATP concentration would be reduced from the leakage of nucleotides. To investigate the intracellular ATP concentrations quantitatively, a luciferase-based assay was performed using cell extracts prepared from IPTG-induced cultures. As shown in Fig. 5A, an 88-fold reduction in cellular ATP was found in BW25113/pCA24N-ghoT cells compared with BW25113/pCA24N after 1 mM IPTG induction for 2 h. In contrast, overproduction of GhoTF38R did not reduce intracellular ATP significantly (1.8-fold reduction compared with BW25113/pCA24N). Therefore, GhoT production depletes the cellular pool of ATP.

If GhoT disrupts PMF, it is expected that a portion of the cells should become polarized or depolarized because they have lost the ability to maintain the membrane potential. Hence, we used flow cytometry to detect the fluorescence released by DiBAC_4(3), a potential sensitive dye that enters depolarized cells and binds to membranes and proteins (Berney et al., 2006). As negative controls, NR698/pCA24N (with a compromised outer membrane) and BW25113/pCA24N cells were treated with DMSO, and for a positive control, cells were treated with CCCP (Wu et al., 2010). As shown in Fig. 5B, CCCP treatment rendered NR698/pCA24N highly depolarized, as reflected by the increased fluorescence signal, compared with the strain treated with 0.5% DMSO. The effect of CCCP on BW25113/pCA24N cells was less pronounced, probably because of its intact outer membrane structure. However, both BW25113/pCA24N-ghoT and NR698/pCA24N-ghoT cells became highly depolarized with IPTG induction (Fig. 5C). The effect of GhoT production in BW25113 cells was not as pronounced because not all the cells became depolarized as seen with NR698/pCA24N-ghoT cells. The membrane potential in both hosts remained unaffected with GhoTF38R overproduction because no significant increase in the fluorescence signal was observed. These results show that GhoT accumulation either depletes or...
leaks cellular ATP, that GhoT dissipates the membrane potential and that the F38R substitution abolishes these toxic effects.

**GhoT/GhoS protect the cell from antimicrobials**

To ascertain the physiological importance of the GhoT/GhoS TA locus, we conducted phenotype arrays to simultaneously test 1920 different growth conditions for the wild-type versus the \( \Delta ghoST \Delta \text{Kan} \) mutant. Note that it was imperative to utilize the scarless mutant (i.e. no antibiotic marker) so that the only genotypic difference was the deletion of the \( ghoST \) locus to avoid studying an effect that is an artifact of a residual antibiotic marker. These battery of tests investigate differences in metabolism between the two strains for basic cellular nutritional pathways (i.e. for C, N, P and S metabolism), for osmotic and pH sensitivity and for sensitivity to chemical agents. The measured metabolism assays cell respiration (reduced nicotinamide adenine dinucleotide production) through a redox indicator (Bochner et al., 2001): as the cells respire, a tetrazolium dye is reduced to formazan to form a strong purple colour so more colour indicates more rapid metabolism. No differences were found for the nutritional pathways and for osmotic and pH sensitivity; however, the \( \Delta ghoST \Delta \text{Kan} \) mutant had more metabolism with four antimicrobials (carbenicillin, cefoxitin, 5,7-dichloro-8-hydroxyquinoline and 2-phenyphenol) (Fig. 6A). 5,7-dichloro-8-hydroxyquinoline is an antibacterial drug used in infectious diarrhoea, 2-phenyphenol is a general disinfectant, cefoxitin is a second-generation cephalosporin that inhibits cell wall synthesis and carbenicillin is a bacteriolytic antibiotic similar to penicillin.

We then focused on carbenicillin and cefoxitin and found that the \( \Delta ghoST \Delta \text{Kan} \) mutant consistently had more metabolism initially with carbenicillin (3.5 \( \mu \text{g m}^{-1} \)) and cefoxitin (2.0 \( \mu \text{g m}^{-1} \)), but the wild-type strain ultimately achieved greater metabolism (Fig. 6B). Similarly, the \( \Delta ghoT \Delta \text{Kan} \) mutant had as much as 86 ± 1-fold faster growth.

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**Fig. 5.** GhoT reduces intracellular ATP levels and disrupts the membrane potential.

A. Nucleotides from cultures induced with 1 mM IPTG for 2 h were extracted by TCA, and a luciferase-based assay was performed to determine ATP concentrations. The ATP levels were expressed as RLU/OD600 (relative light units/optical density at 600 nm). The fold reduction relative to the vector control strain is indicated. Representative results from two of the three independent trials are shown and depicted as the average ± standard deviation.

B. Flow cytometry assay using DiBAC\(_4\)(3) stained NR698/pCA24N (left) or BW25113/pCA24N (right) treated with 0.05% DMSO (black, dotted line, negative control) or 500 \( \mu \text{M} \) CCCP (green) for 1 h. Two independent cultures were evaluated.

C. Flow cytometry assay using DiBAC\(_4\)(3) stained NR698 (left) or BW25113 (right) carrying pCA24N (black, dotted line), pCA24N-ghoT (blue) or pCA24N-ghoT\(_{F38R} \) (red) induced with 1 mM IPTG for 1 h. Two independent cultures were evaluated.

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metabolism at 6 h with carbenicillin (Fig. 6C) and 21 ± 7-fold faster metabolism at 6 h with cefoxitin; however, the ΔghoT ΔKan mutant always had greater metabolism than the wild type in the presence of these two antibiotics, which indicates that the antitoxin is required to regulate the toxin to slow the cellular metabolism, which is sometimes beneficial (e.g. persister cell formation). Critically, there was no difference in metabolism at all times in the absence of stress, so the strains were inoculated with the same number of active cells and grew identically (Fig. 6B and C, first panels). Although speculative, our results also suggest that under stressful conditions, while the toxin GhoT suppresses bacterial growth at the initial stage, the antitoxin GhoS eventually counteracts the effect to confer greater growth in the wild-type system. Therefore, the GhoT/GhoS locus is used by the cell to fine-tune growth during stress.

**Discussion**

In this study, we demonstrate that GhoT is responsible for causing membrane damage upon MqsR-producing conditions, which is crucial for cells to enter the dormant state to become persisters. GhoT acts as a pole-localized membrane toxin that reduces cellular metabolism by causing membrane damage, by reducing cellular ATP levels and by disrupting membrane potential. Collectively, we determine how GhoT slows cell growth and then show that this reduction in metabolism is important for growth in the presence of several antimicrobials under physiological conditions.

To date, the phenomenon of bacterial programmed cell death (PCD) is based on the study of the *E. coli* MazF/MazE TA system in which toxin MazF performs the dual role of either reversible stasis or cell death (Amitai *et al.*, 1997).
GhoT damages the membrane to increase resistance

2009). MazF/MazE triggers cell death in response to culture density; i.e. MazF/MazE-mediated death is population dependent so that the death of the bulk cell population provides nutrients for survivor cells to resume growth as the conditions become propitious (Engelberg-Kulka et al., 2006). Compared with the MazF/MazE system, GhoT/GhoS-mediated metabolic slowdown is distinct from bacterial PCD in that there were no significant signs of cell lysis from activation of chromosomal GhoT; in addition, the reduced growth under stress conditions is likely the outcome of energy depletion from continuous membrane damage by GhoT.

Similar to the SOS-induced inner membrane toxin TisB (Unoson and Wagner, 2008), GhoT damages the inner membrane and reduces ATP production and reduces the PMF. The cause of the loss of toxicity for GhoTF38R remains to be discerned. In the absence of empirical structural data, we assumed that F38R and I21R would yield non-toxic GhoT, as the charged arginines would damage the putative interaction held by Phe38 and Ile21. This subsequently perhaps creates a structural bottleneck where either GhoT oligomerization, interaction with other binding partners or its insertion into cell membrane is disrupted. However, we propose that Phe38 has a functional role instead of a structural role, based on two lines of evidence: (i) in contrast to GhoTF38R, GhoTI21R is toxic (Fig 3B and D); and (ii) similar to GhoT, GhoTF38R remains localized at the cell poles (Fig. 4A and B). Therefore, the non-toxicity of GhoTF38R appears to be due to the mutation itself, rather than a failure in oligomerization, abolished interaction or membrane insertion. We further speculate that Phe38 might form a ‘phenylalanine clamp’ that aligns the polypeptide in pores formed by GhoT oligomers. For example, a ring of seven phenylalanines forms a hydrophobic core in the anthrax toxin, and substrate recognition is facilitated by hydrophobic effect, π-π interaction and cation-π interaction (Krantz et al., 2005; Janowiak et al., 2010). By analogy, the charged arginines in GhoTF38R may ‘seal off’ the pores and, thus, limit leakage of cellular materials to the outer environment. Our results from confocal microscopy, the ATP assay and PMF flow cytometry support the notion that material leakage is minimal in GhoTF38R-expressing cells.

Protein localization has several important impacts on cell physiology, including chemotaxis (Sourjik and Berg, 2000), osmoregulation (Romantsov et al., 2007), cell division (Wu and Errington, 2003), phage attack (Edgar et al., 2008) and elimination of non-functional protein aggregates (Winkler et al., 2010). The pole localization of GhoT-GFP and GhoTF38R-GFP (Fig. 4) provides indirect evidence for the production of both proteins, and to our knowledge, this is the first demonstration of cellular localization of a bacterial toxin of a TA pair (here, at the poles). It is currently unknown, though, if such pole localization is regulated by cellular systems such as MinCDE, which oscillate from pole to pole to ensure the assembly of the FtsZ pre-division ring at mid-cell (Li and Young, 2012).

Protein aggregation resulting from failure of quality control is not uncommon under stressful conditions. In E. coli, these aggregates are known to be delivered to the poles to form a large polar aggregate (LPA), and the formation, delivery and disaggregation of LPA are all energy dependent (Rokney et al., 2009). This implies that the disruption of PMF and the reduction of ATP levels by GhoT production may have a negative effect on breaking down the aggregated proteins at the poles. Based on this assumption, it can also be inferred that the striking GhoT-induced ghost cell phenotype may be the result of LPA formation at the poles while GhoT may exert its effect through a global impact on energy supply or through a direct interaction with chaperone proteins (such as DnaK and DnaJ) or proteases (such as ClpB) to prevent the removal of LPAs. The latter hypothesis is supported by the result that GhoT localizes at the poles (Fig. 4) and that these proteins are recruited to the poles to dissolve LPAs in conjunction (Acebrón et al., 2008; Doyle and Wickner, 2009). It should be noted that GhoTF38R, which is non-toxic, also localizes at the poles; therefore, it is also likely that the F38R substitution abolishes the interaction between GhoT and its partners, if any.

It was hypothesized that lower levels of GhoT increases bacterial persistence by inducing a dormant state while the cell lysis is an ultimate outcome of overexpression (Wang et al., 2012). Here we show that cell lysis does not occur without ectopic expression of ghoT (Fig. 2), and thus GhoT does not seem to play a role in releasing cellular components, such as DNA or nutrients, for the rest of the population under physiological conditions. Therefore, when GhoT is active, the majority of cells have reduced metabolism; however, for those cells in which GhoT production may have a negative effect on breaking down the aggregated proteins at the poles. Based on this assumption, it can also be inferred that the striking GhoT-induced ghost cell phenotype may be the result of LPA formation at the poles while GhoT may exert its effect through a global impact on energy supply or through a direct interaction with chaperone proteins (such as DnaK and DnaJ) or proteases (such as ClpB) to prevent the removal of LPAs. The latter hypothesis is supported by the result that GhoT localizes at the poles (Fig. 4) and that these proteins are recruited to the poles to dissolve LPAs in conjunction (Acebrón et al., 2008; Doyle and Wickner, 2009). It should be noted that GhoTF38R, which is non-toxic, also localizes at the poles; therefore, it is also likely that the F38R substitution abolishes the interaction between GhoT and its partners, if any.

Although the exact mechanism of how GhoT damages the membrane remains to be elucidated, it is clear that one of the physiological roles of this bacterial TA system is a component of the stress response. Because the GhoT/GhoS TA system significantly decreases growth in the presence of four antimicrobials with diverse bacterial modes of action (e.g. cell wall synthesis, disinfectant and bacteriostatic agent), this TA system plays an integral part in adaptation of growth under unfavourable conditions. Also, it appears that the cell chooses to reduce metabolic activity (i.e. to keep the TA system) so ultimately it achieves greater metabolism during stress. Therefore, these results serve to cement the importance of TA systems for growth under stress.
Table 1. Bacterial strains and plasmids used in this study. The antibiotics used are ampicillin (100 µg mL⁻¹), chloramphenicol (30 µg mL⁻¹), erythromycin (300 µg mL⁻¹) and kanamycin (50 µg mL⁻¹).

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<tr>
<td>TG1</td>
<td>supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM5), (rC mC), F [traD36 proAB lacI::Zac2 M15]</td>
</tr>
<tr>
<td>NR698</td>
<td>MC4100 pD213</td>
</tr>
<tr>
<td>MG1655</td>
<td>F' ilvG rfb-50 rps-1</td>
</tr>
<tr>
<td>BW25113</td>
<td>lacI ΔlacZΔM15 hsdR514ΔaraBAD600ΔraBAD408</td>
</tr>
<tr>
<td>BW25113 ΔghoT</td>
<td>BW25113 ΔghoT Ω KmR</td>
</tr>
<tr>
<td>BW25113 ΔghoS</td>
<td>BW25113 ΔghoS Ω KmR</td>
</tr>
<tr>
<td>BW25113 ΔghoST</td>
<td>BW25113 ΔghoST Ω KmR</td>
</tr>
<tr>
<td>BW25113 ΔghoT ΔKan</td>
<td>BW25113 ΔghoT</td>
</tr>
<tr>
<td>BW25113 ΔghoST ΔKan</td>
<td>BW25113 ΔghoST</td>
</tr>
</tbody>
</table>

Bacterial strains/plasmids

Table 1. Bacterial strains and plasmids used in this study. The antibiotics used are ampicillin (100 µg mL⁻¹), chloramphenicol (30 µg mL⁻¹), erythromycin (300 µg mL⁻¹) and kanamycin (50 µg mL⁻¹).

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used are listed in Table 1. Experiments were conducted at 37°C in Luria-Bertani (LB) medium unless indicated otherwise. The ΔghoST mutant strain was constructed via the λ Red method (Datsenko and Wanner, 2000) using polymerase chain reaction (PCR) primers DghoST1wd and DghoST1rev (Table 2) and verified by PCR using primers CghoST1wd2 and CghoSTrev2 (Table 2) as well as by sequencing. The kanamycin resistance cassette was removed from ΔghoST and ΔghoT using pCP20 (Datsenko and Wanner, 2000), and verified by PCR using primers CghoST1wd2 and CghoSTrev2 (Table 2) and by sequencing.

Site-directed mutagenesis

PCR was performed using pCA24N-ghoT or pCA24N-ghoTGFP (Table 1) as the template with different sets of primers (Table 2). The PCR products were recovered, subjected to DpnI digestion and transformed into E. coli TG1 (Table 1). The plasmids prepared from the resulting transforms were confirmed by sequencing and designated as pCA24N-ghoTATG, pCA24N-ghoTGCA1, pCA24N-ghoTGCA2, pCA24N-ghoTCA3, pCA24N-ghoT121R, pCA24N-ghoT38R and pCA24N-ghoTF38R (Table 1).

TEM

BW25113/pCA24N-ghoS and BW25113/pCA24N-ghoT were grown in LB-chloramphenicol to a turbidity at 600 nm of 0.5, and 1 mM of IPTG was added to induce protein production from plasmids. After 4 h of induction, cells were fixed with 2% formaldehyde and 1.5% glutaraldehyde in 100 mM of sodium cacodylate buffer (pH 7.4) at 4°C for 12 h, followed by three washes with buffer alone for 5 min each wash. Cells were then fixed with 1% osmium tetroxide for 1 h in dark, followed by three washes with buffer. After treating the cells with 2% uranyl acetate in the dark for 1 h, cells were sequentially dehydrated in a graded ethanol series (50%, 70%, 85%, 95% and three times with 100% ethanol, for 5 min each), and three times with 100% acetone (5 min each). Dehydrated cells were embedded into epoxy resins for at least 12 h, and then sectioned into thin specimens (70 nm thick) using a ultramicrotome (UC6, Leica, Buffalo Grove, IL, USA). Specimens were stained with uranyl acetate and lead citrate, and
then examined on a FEI Tecnai G2 Spirit BioTwin TEM (Penn State Microscopy and Cytometry Facility, University Park, PA, USA) at an accelerating voltage of 120 kV.

Confocal fluorescence microscopy

Samples for fluorescence microscopy were prepared as described previously (Lamsa et al., 2012) with minor modifications. In brief, BW25113 and the ΔghoT mutant were grown in LB medium overnight, washed twice with M9-Glu medium, and resuspended in 100 μl of a stain mix containing 5 μl of 0.5% DMSO or 1.18 nmol g–1 cell) is around one third of the value reported in exponential-phased E. coli MG1655 (Buckstein et al., 2008) [25.5 nmol l–1, or 85 nmol g–1 cell given that 1 OD600 = 0.3 gl–1].

Table 2. Oligonucleotides used for site-directed mutagenesis (target mutated nucleotides are underlined), mutant construction and verification of kanamycin cassette insertion/removal (the homologous regions for the ghoST locus are double underlined).

<table>
<thead>
<tr>
<th>Purpose/name</th>
<th>Sequence (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site-directed mutagenesis</td>
<td>ghoT-ATG-f AAAGAGGAGAAATTAACTACGAGGATCTCACCAT</td>
</tr>
<tr>
<td>ghoT-ATG-r ATGGTGAGACCTCCTCGTAGTTAATTTCCTCTCTT</td>
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</tr>
<tr>
<td>ghoT-GCA1-f GATCCGGCCCTGAGGCGCCGCTATTCTCTTTAATTT</td>
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</tr>
<tr>
<td>ghoT-GCA1-r AATATATTAGAAGATGCGCCGCTCAGGGCGGATC</td>
<td></td>
</tr>
<tr>
<td>ghoT-GCA2-f TCGTTTTACTATGTCAGGCTCGTTGCGGAAATA</td>
<td></td>
</tr>
<tr>
<td>ghoT-GCA2-r TATTCCGAGCCAAGAAAATAGGCGCCACAGGACCT</td>
<td></td>
</tr>
<tr>
<td>ghoT-GCA3-f AATGAGTCTGCGTGCCCTGCTTGTGG</td>
<td></td>
</tr>
<tr>
<td>ghoT-GCA3-r CCAGAAAGAGAAAAGTTGAGGGCCACAGGACCTT</td>
<td></td>
</tr>
<tr>
<td>ghoT-I21R-f ATTCGTTTACTTAGTGCCTTCCTGGTCGGAATA</td>
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</tr>
<tr>
<td>ghoT-I21R-r CCAGGTATTCCGACCCAGGCCTCGACTAATAAAAGCAGGAT</td>
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</tr>
<tr>
<td>ghoT-R38F-f ATTCGTTTTACTTAGTACCTCGGAAATACCTGG</td>
<td></td>
</tr>
<tr>
<td>ghoT-R38F-r CCAGGTATTCCGACCCAGGAATGCACTAATGAAAGCAGGAT</td>
<td></td>
</tr>
<tr>
<td>Mutant construction</td>
<td>DghoStwd GAGTACAGAGTCCAGATATTTACCTTCTCTCAGGATGTTGAGCTGGAGCTGTC</td>
</tr>
<tr>
<td>DghoStrev GACGCTTCGTTTACTTAGTGCCTTCCTGGTCGGAATA</td>
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<tr>
<td>Verification of Kan insertion/removal</td>
<td>CghoStwd2 AAGCGCTGAAAGGGCAAGGGATGGTAAAC</td>
</tr>
<tr>
<td>CghoStrev2 GTCAAGGGACACTGCTCGTTTCATCGTTC</td>
<td></td>
</tr>
</tbody>
</table>

f indicates forward primer, and r indicates reverse primer.

**ATP assay**

The strains were grown as described above, induced with 1 mM of IPTG for 2 h and harvested by centrifugation. The cells were washed with 50 mM Tris-acetate (pH 7.75), and ATP was extracted by 1% trichloroacetic acid in 100 μl Tris-acetate buffer at 4°C for 10 min. One millilitre of Tris-acetate buffer was added to obtain a pH of 7.75, and cell debris was removed after centrifugation. Ten microlitres of the supernatant was then mixed with 100 μl of a reagent (EPLUS ATP assay, Promega, Madison, WI, USA) pre-warmed at room temperature. Luminescence values were determined using a 5 s delay time and a 10 s relative light unit (RLU) signal integration time on a Turner 20–20e luminometer (Turner Designs, Sunnyvale, CA, USA). The amount of extracted ATP was calculated based on values determined using serial dilutions of known amounts of ATP for a standard curve following the manufacturer’s recommendations. In each experimental sample, the RLU was divided by the turbidity at 600 nm to represent the levels of ATP in each culture. Values obtained from GhoT or GhoTF38R producing strains were divided by the value from BW25113/pCA24N to obtain the relative ATP levels. The ATP level in BW25113/pCA24N (25.13 ± 1.18 nmol g–1) is around one third of the value reported in exponential-phased E. coli MG1655 (Buckstein et al., 2008) [25.5 nmol l–1, or 85 nmol g–1 cell given that 1 OD600 = 0.3 g l–1] (Kim et al., 2012).**

**PMF**

The PMF was measured by flow cytometry as described previously (Lamsa et al., 2012). Strains were grown as described for fluorescence microscopy, and 6 μl of DMSO/CCCP-treated cells or the IPTG-induced cells was added to 1.5 μl of a stain mix containing 5 μl of DiBAC4(3) in 1 x PBS. The stained sample was added to 500 μl 1 x PBS, and 100 000 events were counted using a Fortessa LSR II flow cytometer.
flow cyrometer (BD, Franklin Lakes, NJ, USA). After the excitation by a 488 nm laser, the fluorescence data were collected using the 505 nm filter.

**Lysis assay**

To determine the level of cell lysis, β-galactosidase activity was measured and calculated as described previously (Ma and Wood, 2009). For checking cell lysis during the exponential phase, overnight cultures of BW25113 and the ΔghoT mutant carrying pCA24N-lacZ grown in LB medium with chloramphenicol were washed twice with M9-Glu medium, resuspended and diluted in M9-Glu medium to turbidity of 0.05. Cells were grown for 4 h, then induced with 0.5 mM IPTG for 4 h and collected by centrifugation. For checking cell lysis during the stationary phase, the same strains were grown in M9-Glu medium for 20 h. For the positive control in which cell lysis was caused by overproducing GhoT, overnight cultures of BW25113 and the ΔghoT mutant carrying pCA24N were diluted into LB medium with chloramphenicol to a turbidity of 0.05, grown to a turbidity of 0.5 and induced with 1 mM IPTG for 10.5 h. The β-galactosidase activities were determined from both supernatant and cell pellets (the cell pellets were sonicated to release all of the cytosolic β-galactosidase), and the level of cell lysis was calculated from the percentage of the supernatant β-galactosidase activity in both the supernatant and cell pellets. Two independent samples were investigated (n = 2).

**Western blot analysis**

Overnight cultures of *E. coli* BW25113/pCA24N-ghoT-F38R were diluted 500-fold into 11 of LB medium with chloramphenicol, grown to a turbidity of 1.0, induced with 0.1 mM of IPTG at room temperature overnight and collected by centrifugation at 8132 r.p.m. (for Beckman rotor JA-20) at 4°C. A 1 ml sample was taken out from the 1 l induced culture (to be used as a total cellular protein sample 'T'), the cell pellet, mixed with 1× sodium dodecyl sulfate polyacrylamide gel electrophoresis sample loading buffer, and boiled at 95°C for 20 min before loading to the gel. Cells were broken by passing through a French press twice and centrifuged at 12858 r.p.m. (for Beckman rotor JA-20) for 60 min at 4°C. The insoluble protein from 1 l culture was extracted with 25 ml purification buffer (20 mM Tris-HCl, pH 7.9 and 500 mM NaCl) containing 6 N urea, and a sample was taken from the remaining insoluble fraction (sample 'I'). The supernatant was subjected to ultracentrifugation at 31170 r.p.m. (for Beckman rotor type 70 Ti) for 60 min at 4°C to separate the cytosolic (supernatant, sample 'C') and membrane proteins (pellet, sample 'M'). Western blot was performed as described previously (Wang et al., 2012), with ~20 μg of each protein sample and probed with primary antibodies raised against a His tag (Cell Signaling Technology, Danvers, MA, USA) and horseradish peroxidase-conjugated goat antimouse secondary antibodies (Fisher Scientific, Pittsburgh, PA, USA).

**Phenotype microarrays and verification**

A full phenotype array analysis (PM plates 1–20) was conducted by BioLog (Hayward, CA, USA). Two independent cultures were used for the initial screen of 1197 conditions, including 960 different nutrient conditions and 237 toxins (at four concentrations) for differences in metabolic activity. Four antimicrobials were identified, including cefoxitin, carbencillin, 5,7-dichloro-8-hydroxyquinoline and 2-phenylphenol. The results were verified via six independent cultures using BioLog reagents by growing cells to a turbidity of 1.0, diluting to a turbidity of 0.07 in IF-10a (Cat. no. 72264) and then further diluting 200-fold into a reagent mixture containing IF-10a, BioLog Redox Dye D (Cat. no. 74224) and rich medium (2.0 g of tryptone, 1.0 g of yeast extract, and 1.0 g of NaCl per litre) to a final turbidity of 0.00035. This cell suspension (100 μl) was transferred into 96-well microtitre plates, incubated at 37°C, and the metabolic activity was monitored hourly by taking the absorbance (590 nm), which indicates the intracellular reducing state based on the generation of formazane (purple) from the tetrazolium dye.

**Acknowledgements**

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**References**


GhoT damages the membrane to increase resistance 1753


