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Type II toxin/antitoxin MqsR/MqsA controls type V toxin/antitoxin GhoT/GhoS

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Summary

Toxin endoribonucleases of toxin/antitoxin (TA) systems regulate protein production by selectively degrading mRNAs but have never been shown to control other TA systems. Here we demonstrate that toxin MqsR of the MqsR/MqsA system enriches toxin *ghoT* mRNA *in vivo* and *in vitro*, since this transcript lacks the primary MqsR cleavage site 5′-GCU. GhoT is a membrane toxin that causes the ghost cell phenotype, and is part of a type V TA system with antitoxin GhoS that cleaves specifically *ghoT* mRNA. Introduction of MqsR primary 5′-GCU cleavage sites into *ghoT* mRNA reduces ghost cell production and cell death likely due to increased degradation of the altered *ghoT* mRNA by MqsR. GhoT also prevents cell elongation upon the addition of low levels of ampicillin. Therefore, during stress, antitoxin GhoS mRNA is degraded by toxin MqsR allowing *ghoT* mRNA translation to yield another free toxin that forms ghost cells and increases persistence. Hence, we show that GhoT/GhoS is the first TA system regulated by another TA system.

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Introduction

Bacterial toxin/antitoxin (TA) systems play prominent roles in cell physiology including altruistic behaviour such as phage inhibition (Pecota and Wood, 1996), global gene regulation (Wang and Wood, 2011) and tolerance to antibiotics (Lewis, 2010). For antitoxins, both MqsA (Wang *et al.*, 2011) and DinJ (Hu *et al.*, 2012) have been shown to repress the stationary-phase sigma factor RpoS and thereby influence the response to stress and biofilm formation as global regulators. Toxins in TA systems also play a role in gene regulation in that mRNA endoribonuclease toxins cleave specific mRNAs and thereby cause differential mRNA decay (Wang and Wood, 2011). For example, upon antibiotic stress, toxin MazF degrades most mRNAs with ACA sequences, but its activity also results in the preferential synthesis of a subset of small proteins whose mRNAs are not degraded (Amitai *et al.*, 2009). These enriched proteins are necessary both for toxicity and for survival (Amitai *et al.*, 2009). Therefore, both toxins and antitoxins may be considered global regulators.

The endoribonuclease toxin MqsR (motility quorum sensing regulator) (Ren *et al.*, 2004; González Barrios *et al.*, 2006; Brown *et al.*, 2011) of the MqsR/MqsA TA system is also a global regulator (González Barrios *et al.*, 2006; Kim *et al.*, 2010). It cleaves specific mRNA primarily at 5′-GCU sites (Yamaguchi *et al.*, 2009; Christensen-Dalsgaard *et al.*, 2010) and leads to enrichment of mRNAs that code for stress-associated proteins CstA, CspD, RpoS, Dps and HokD (Kim *et al.*, 2010). The binding of MqsR to its antitoxin MqsA potently inhibits MqsR toxicity (Brown *et al.*, 2011).

MqsR/MqsA is a type II TA system (protein toxin inhibited by the binding of a protein antitoxin), and type II TA systems are one of the most widespread forms of TA systems (Hayes and Van Melderen, 2011). In type I TA systems (RNA–RNA), the antitoxin RNA prevents the translation of toxin RNA through complementary base pairing (Hayes and Van Melderen, 2011), in type III TA systems (RNA–protein), the antitoxin RNA inhibits the protein toxin (Hayes and Van Melderen, 2011), in type IV TA systems (protein–protein), the protein antitoxin interferes with binding of the toxin to its target (Masuda *et al.*, 2012), and in type V systems (protein–RNA), the antitoxin is a specific RNase that cleaves the toxin mRNA (Wang *et al.*, 2012).

There are 14 *Escherichia coli* mRNA transcripts that do not contain the MqsR-preferred 5′-GCU cleavage site, and six of these (*pheL*, *tnaC*, *trpL*, *yciG*, *ygaQ* and *ralR*) are differentially regulated in biofilms (Domka *et al*., 2007). One of these 14 transcripts that lacks 5′-GCU sites is *ghoT* [*yjdO*, ghost cells (Wang *et al*., 2012)]. GhoT is a membrane toxin that produces the ghost-cell phenotype (lysed cells with damaged membranes) (Wang *et al*., 2012). The antitoxin for GhoT, GhoS, cleaves specifically *ghoT* mRNA thereby halting *ghoT* expression; hence, this TA system is the first type V system in that an enzyme antitoxin inactivates the mRNA of the toxin (Wang *et al*., 2012). The structure of antitoxin GhoS revealed it is related to the CRISPR-associated-2 (CAS2) sequence-specific endoribonuclease, and a whole-transcriptome study showed the specificity of GhoS as a ribonuclease (Wang *et al*., 2012). Although *mqsR* is more prevalent than *ghoT*, *ghoT*-related genes are found in *Escherichia, Shigella, Salmonella, Citrobacter* and *Proteus* spp.

Persister cells are a small fraction of bacteria that exhibit tolerance to antibiotics without genetic change (Lewis, 2007); it is believed they survive antibiotic treatment by becoming metabolically dormant through TA systems (Lewis, 2010). The evidence relating TA systems to persistence includes the finding that when MqsR is inactivated, persister cell formation is reduced (Kim and Wood, 2010); production of MqsR also increases persistence. Similarly, deletion of the TisB toxin of the TisAB/IstR-1 type I TA system decreases persistence (Dörr *et al*., 2010). Subsequent studies have also demonstrated the importance of TA systems for persistence by showing that cells lacking 10 mRNA endoribonuclease toxins including MqsR form less persister cells (Maisonneuve *et al*., 2012). In persister cells, *mqsR* is also the most induced gene (Shah *et al*., 2006).

Our previous studies showed that the antitoxin MqsA is rapidly degraded by Lon protease which frees toxin MqsR upon stress (Wang *et al*., 2011). The importance of Lon for persister cell formation has been demonstrated by showing both that cells lacking *lon* have reduced persister cell formation and that removal of the 10 toxins causes the *lon* mutant to no longer affect persistence (Maisonneuve *et al*., 2012).

We hypothesized that since *ghoT* mRNA lacks the primary MqsR 5′-GCU sites, it would be enriched under conditions when MqsR is produced. Here we demonstrate that MqsR does enrich *ghoT* transcripts and thereby activates expression of this membrane toxin which is involved in persister cell formation.

Results

MqsR requires GhoT to prevent cell elongation

Previously, we examined the 14 *E. coli* transcripts that lack 5′-GCU sites to determine if these transcripts were related to the ability of MqsR to increase persistence (Wang *et al*., 2012). Among them, deleting gene *ghoT* had one of the largest negative effects on MqsR-mediated persistence; corroborating this result, production of GhoT increased persistence 48-fold (Wang *et al*., 2012). We repeated these persister experiments by varying the ampicillin concentration and found similar results at both 35 μg ml^{-1} and 100 μg ml^{-1} ampicillin for MqsR production in the wild-type and in the *ghoT* mutant so the results are not influenced by the antibiotic concentration. In addition, we determined that the minimum inhibitory concentration for ampicillin was the same for both strains (approximately 5 μg ml^{-1}). Hence, we investigated further the relationship between MqsR and GhoT in *E. coli* to determine how GhoT influences persister cell formation when MqsR is produced.

To initially probe the relationship between MqsR and GhoT, we used microscopy. High concentrations of ampicillin (100 μg ml^{-1}) are commonly used to assess persister cell formation in *E. coli* (Balaban *et al*., 2004; Keren *et al*., 2004; Shah *et al*., 2006; Kim *et al*., 2010). When added at 100 μg ml^{-1}, ampicillin rapidly lyses non-persister cells, which makes it difficult to probe for changes in cell morphology; hence, here we used a lower ampicillin concentration (20 μg ml^{-1}), which causes cell elongation rather than lysis allowing for the observation of the response to β-lactam antibiotics (Rolinson, 1980). We reasoned that since MqsR increases persister cells (Kim and Wood, 2010), MqsR should prevent cells from elongating upon addition of ampicillin since these persisters would be tolerant of the antibiotic. As expected, we found that addition of 20 μg ml^{-1} ampicillin to wild-type cells (BW25113/pCA24N) caused cell elongation of most cells (23 ± 3 μm) after 4 h by inhibiting cell division (Donachie and Begg, 1970; Staugaard *et al*., 1976), and bulges or collars were observed at the division site (red arrows in Fig. 1A) as reported by others (Donachie and Begg, 1970; Rolinson, 1980). After 10 h, the majority of the cells lysed (Fig. 1A, left panel). In contrast, the majority of cells producing MqsR (BW25113/pCA24N-*mqsR*) were not elongated (1.5 ± 0.6 μm) after 4 h with ampicillin, and the cells remained intact with sizes increased moderately after 10 h (3.5 ± 0.9 μm) (Fig. 1A, middle panel). This dramatic increase in persister cells is due to production of the toxin MqsR. For cells producing MqsR in the absence of *ghoT* (Δ*ghoT*/pCA24N-*mqsR*), a few cells were significantly elongated after 4 h with ampicillin, and the majority of cells were significantly elongated after 10 h (38 ± 7 μm) (Fig. 1A, right panel). Hence, MqsR prevents cell elongation upon the addition of ampicillin primarily via its control of GhoT.

We also investigated how cells, with or without GhoT, respond to ampicillin challenge. After 4 h with 20 μg ml^{-1} ampicillin, cells producing GhoT (Δ*ghoT*/pCA24N-*ghoT*)

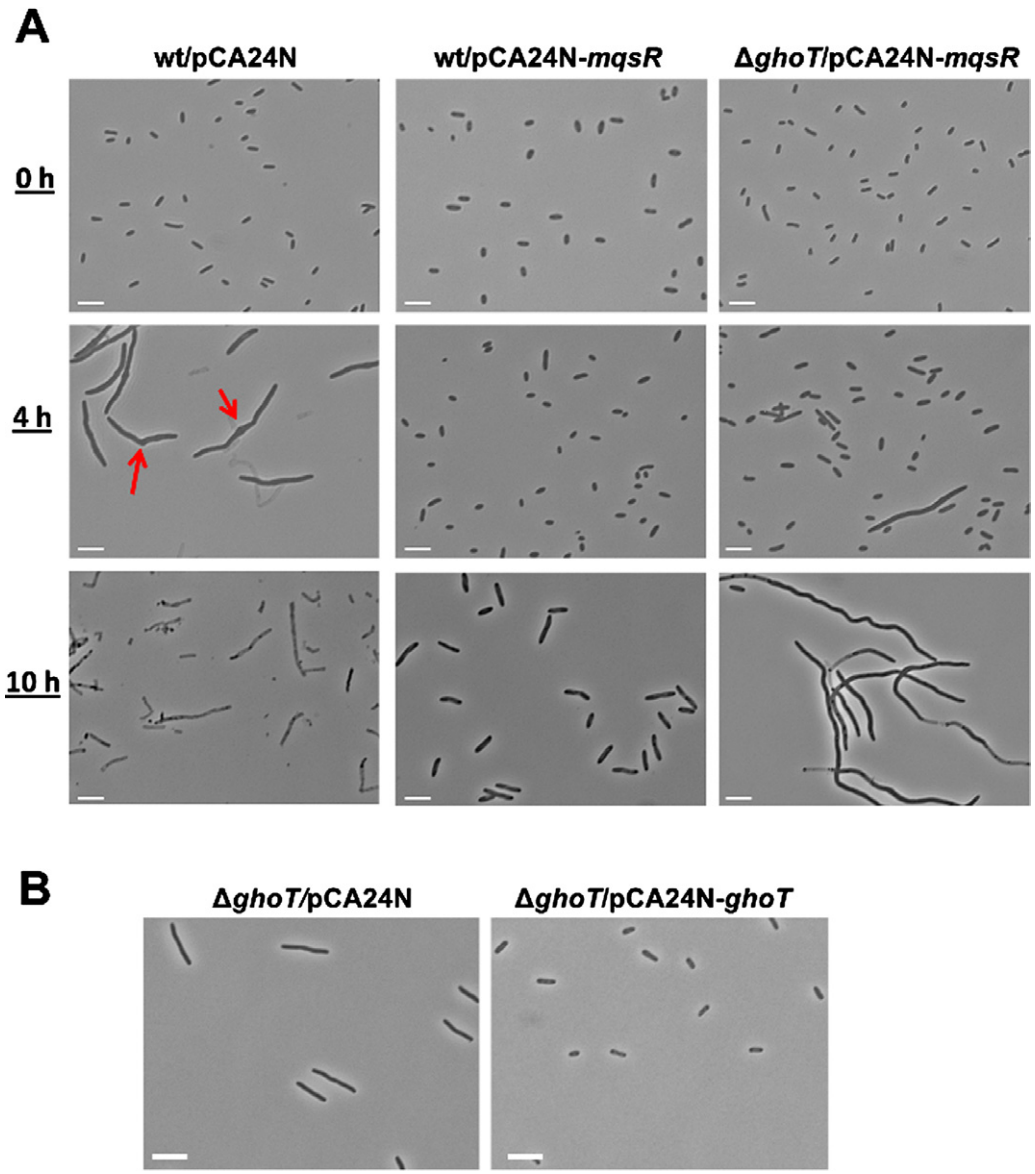

Fig. 1. GhoT decreases cell size in the presence of ampicillin.
A. Cell morphology after 0 h, 4 h and 10 h with ampicillin (20 μg ml^{-1}) for cells producing MqsR (wt/pCA24N-*mqsR*) and cells producing MqsR but without *ghoT* (Δ*ghoT*/pCA24N-*mqsR*). Red arrows point to the bulges or collars with ampicillin.
B. Cell morphology after 4 h with ampicillin (20 μg ml^{-1}) for cells without GhoT (Δ*ghoT*/pCA24N) and cells with GhoT (Δ*ghoT*/pCA24N-*ghoT*). Two independent cultures of each strain were evaluated and representative images were shown. Scale bars indicate 5 μm.

were not elongated (1.7 ± 0.5 μm) whereas those that lacked GhoT (Δ*ghoT*/pCA24N) were elongated (5 ± 1 μm) (Fig. 1B). Note that normal cells prior to antibiotic treatment were about 1.8 μm. This result corroborates that GhoT prevents cell elongation with ampicillin stress. Since persister cells are not dividing and are metabolically dormant with smaller sizes than non-persister cells when exposed to bactericidal antibiotics including ampicillin (Lewis, 2010), these micrographs provide additional evidence that MqsR helps to maintain a dormant state in the presence of ampicillin through GhoT action and that deletion of *ghoT* reduces the ability of MqsR to maintain persistence.

MqsR regulates production of GhoT in vivo *through differential ghoS mRNA decay*

Given the link between MqsR and GhoT, we investigated *in vivo* whether MqsR can regulate *ghoST* mRNA stability at a post-transcriptional level by using quantitative real-

Table 1. Oligonucleotides used for RT-PCR, qRT-PCR, mutant verification, site-directed mutagenesis (target mutated nucleotides are underlined) and *in vitro* transcription (for the forward primers, the T7 promoter sequence is underlined and the bases of the promoter sequence incorporated into RNA during transcription are in bold).

Purpose/name	Sequence (5′ to 3′)
RT-PCR and qRT-PCR	
ghoS-f	CCATCATCTTATTCCTCAGTGTTCT
ghoS-r	TAAGTCTAAGCATTTGAGCCTGATT
ghoT-f	TGGTGTGAACATATCCTTTGTCA
ghoT-r	TAATGCCACAGGCAGACTCA
ralR-f	AAACCATGTCCATTTTGTGGT
ralR-r	TCCAGTGGTTCGTTTATTCCA
ompA-f	CACTGGCTGGTTTCGCTACCG
ompA-r	ACCCATTTCAAAGCCAACATC
ompF-f	AAGCGCAATATTCTGGCAGT
ompF-r	TGCCACCGTAACTGTTTTCA
yciG-f	ACATCGTGGTGGTTCAGGAA
yciG-r	TACCACCGCTTTGTTGACCG
Deletion mutant verification	
GhoS-veri-f	CTCCTATATGAGAATCATCAATCGGGG
GhoS-veri-r	GACAAAGGATATGTTCACACCAATCAC
GhoT-veri-f	GACCTCAACATTATGACAGTTGATGAC
GhoT-veri-r	GCTTCGTTCATCGTTCCGCAAATCCAG
Site-directed mutagenesis	
GhoT-GCU-1-f	GTGATTGGTGTGAACATAAGCTTTGTCATTATCTGGTTTATCTC
GhoT-GCU-1-r	GAGATAAACCAGATAATGACAAAGCTTATGTTCACACCAATCAC
GhoT-GCU-2-f	CACATATTCGTTTACTTAGTGCTTTCCTGGTCGGAATAACCTGG
GhoT-GCU-2-r	CCAGGTTATTCCGACCAGGAAAGCACTAAGTAAACGAATATGTG
T7 *in vitro* transcription	
PT7-GhoT-f	TAATACGACTCACTATA**GGGAGA**ATGGCACTATTCTCTAAAATATTAATTTTT
PT7-GhoT-r	CTAAAAGAGAGAAAAAAGTAATGC
PT7-GhoS-f	TAATACGACTCACTATA**GGGAGA**ATGGAAGGTAAAAACAAGTTCAATACTTAT
PT7-GhoS-r	TCATCAACTGTCATAATGTTGAG

f indicates forward primer and r indicates reverse primer.

time reverse transcription PCR (qRT-PCR) using a primer pair within the coding portion of each gene (Table 1). We found that when MqsR was produced [BW25113/pBS(Kan)-*mqsR* versus BW25113/pBS(Kan)], there was a 5 ± 1-fold increase in the 3′ *ghoT* portion of the *ghoST* mRNA compared with the 5′ *ghoS* portion, which suggests that the *ghoST* transcript is preferentially degraded at the 5′ end when MqsR is active (Table 2 lists the qRT-PCR

Table 2. Summary of the qRT-PCR results.

Conditions	Target mRNA	Fold change	5′-GCU site
BW25113/pBS(Kan)-*mqsR* versus BW25113/pBS(Kan)	*ghoT*	40 ± 1	0
1 mM IPTG for 60 min	*ghoS*	8 ± 1	3
	ompA	–4.5 ± 1	27
	ompT	3 ± 2	14
	ompF	–2 ± 1	20
	kilR	10.8 ± 0.4	0
	ralR	12 ± 1	0
	yciG	11± 1	0
BW25113 wild-type	*mqsR*	18 ± 2	1
2 μg ml^{-1} nalidixic acid for 90 min versus no nalidixic acid	*ghoT*	40 ± 1	0
	ompA	1 ± 1	27
BW25113 wild-type	*mqsR*	20 ± 2	1
10 μg ml^{-1} azlocillin for 90 min versus no azlocillin	*ghoT*	48 ± 2	0
	ompA	–1 ± 1	27
BW25113 wild-type	*mqsR*	4.6 ± 0.6	1
20 mM H_2O_2 for 5 min versus no H_2O_2	*ghoT*	6 ± 2	0

Experimental conditions and the target mRNA are indicated along with the number of 5′-GCU sites in the target mRNA. The fold changes indicate the changes in enrichment (not necessarily changes in transcription) after differential mRNA decay by RNase MqsR and were calculated as described earlier (Pfaffl, 2001) relative to the housekeeping gene (*rrsG*), which was used to normalize the data. Values less than one are indicated as negative fold changes (i.e. mRNA levels were reduced relative to other mRNA). The specificity of the qRT-PCR products was verified by a melting curve analysis.

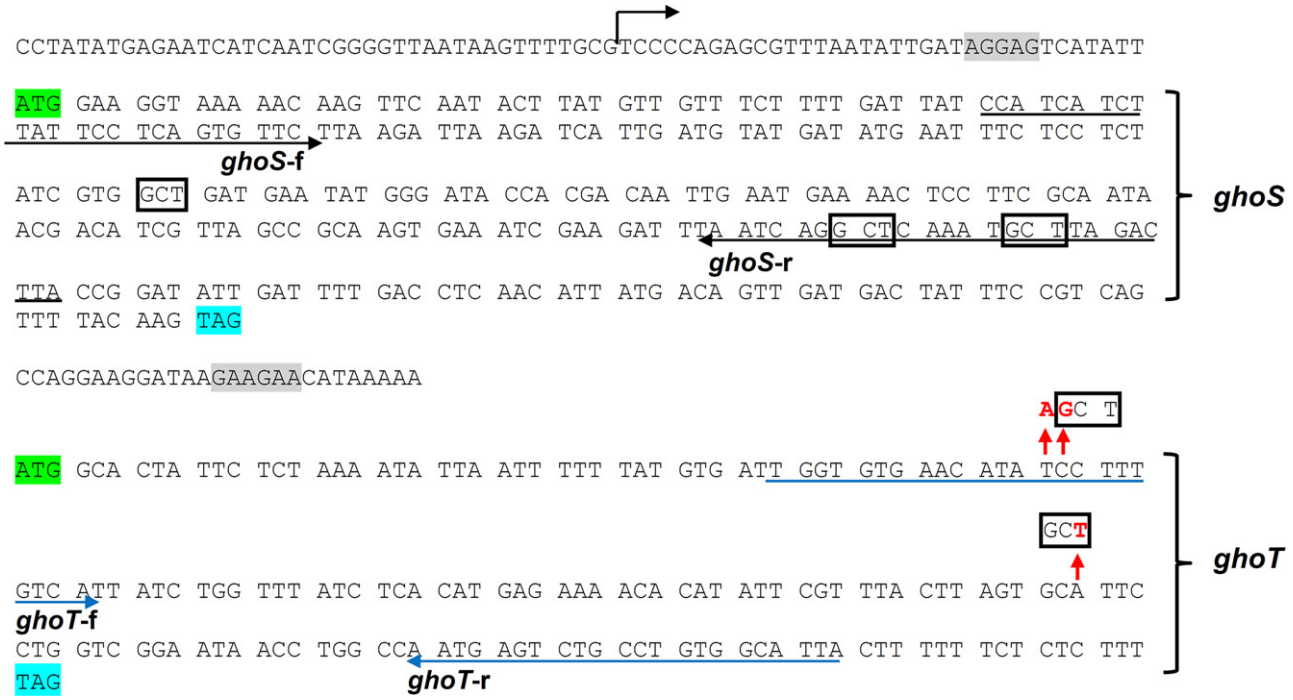

Fig. 2. DNA sequence of *ghoST*. The positions with introduced mutations in *ghoT* are indicated by red arrows with the changed nucleotide in red font. Native 5′-GCT sites in *ghoS* are boxed in black. Start codons are highlighted in green and stop codons are highlighted in blue. The putative transcription start site of the *ghoST* operon is indicated by a black arrow, and the putative RBS sites are highlighted in grey. Primers from *ghoS* (*ghoS*-f and *ghoS*-r) and the primers from *ghoT* (*ghoT*-f and *ghoT*-r) are underlined with their direction indicated.

results). This result is not unexpected since the region of the *ghoST* transcript that codes for *ghoS* (5′ end) contains three of the primary MqsR 5′-GCU cleavage sites, whereas the region that codes for *ghoT* (3′ end) does not contain any 5′-GCU cleavage sites (Fig. 2). Therefore, one mechanism by which MqsR regulates GhoT activity is by selectively degrading the *ghoST* transcript in a manner that leaves the portion of the transcript for toxin GhoT intact, while that of GhoS is degraded.

If MqsR degrades other transcripts in addition to that of *ghoS*, but not *ghoT*, *ghoT* mRNA should be enriched by comparison with these other transcripts. We investigated this using qRT-PCR (Table 2) and found that production of MqsR enriches *ghoT* mRNA 40 ± 1-fold compared with 5′-GCU-containing transcripts *ompA* (27 5′-GCU sites) and *ompF* (20 5′-GCU sites), which have been shown to be degraded by MqsR by *in vivo* and *in vitro* (Yamaguchi *et al*., 2009), as well as *ompT* (14 5′-GCU sites). As expected, we found that three other mRNAs that lack 5′-GCU sites were enriched during production of MqsR: *kilR* by 10.8 ± 0.4-fold, *ralR* by 12 ± 1-fold and *yciG* by 11 ± 1-fold. Also, ribosomal RNA *rrsG* was not affected by production of MqsR (confirmed by an intact band of 16S rRNA with agarose gel electrophoresis) since this RNA should be protected by its incorporation into ribosomes.

Similarly, we investigated *ghoT* mRNA levels under external stress conditions, where MqsR was induced at its normal level from the chromosomal locus. Upon addition of sublethal concentrations of nalidixic acid or azlocillin to wild-type cells, *mqsR* mRNA was induced 18 ± 2-fold and 20 ± 2-fold respectively (Table 2). Under these conditions, *ghoT* mRNA was enriched by 40 ± 1-fold and 48 ± 2-fold, respectively, whereas 5′-GCU-containing *ompA* mRNA was not enriched. Upon oxidative stress (20 mM hydrogen peroxide for 5 min), due to the rapid degradation of MqsA by Lon (Wang *et al*., 2012), *mqsR* transcription was induced 4.5 ± 0.6-fold, which, due to the increase in MqsR protein, enabled *ghoT* mRNA to increase 6 ± 2-fold. Therefore, these three stress conditions which induce *mqsR* transcription result in concomitant enrichment of *ghoT* mRNA relative to cellular mRNA.

MqsR preferentially cleaves ghoS mRNA in vitro

Using purified toxin MqsR, we also found that MqsR cleaves *ghoS* mRNA far more efficiently than *ghoT* mRNA (Fig. 3). The relatively small amount of degradation of native *ghoT* mRNA (Fig. 3, lane 5) appears to be related to purified MqsR cleavage at the three less favourable GCA sites based on the sizes of the fragments generated.

To further demonstrate that the lack of *ghoT* mRNA degradation was due to the absence of MqsR cleavage sites, we added two of the primary MqsR 5′-GCU cleavage sites to *ghoT* mRNA and tested whether the mutated

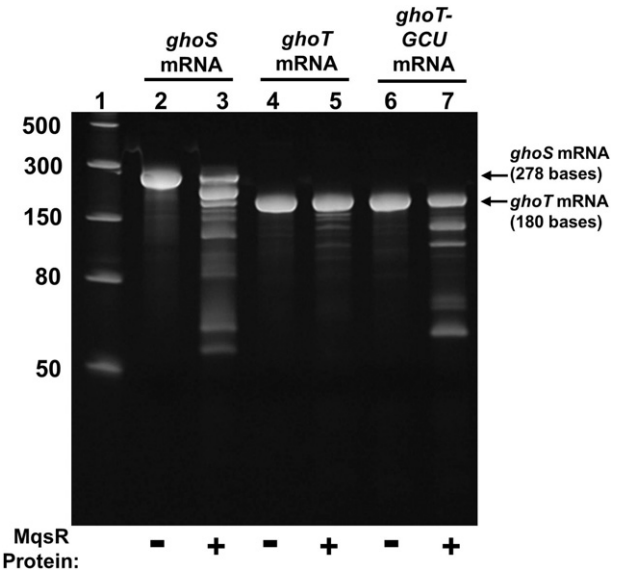

Fig. 3. MqsR cleaves *ghoS* mRNA more efficiently than *ghoT* mRNA *in vitro*. Two micrograms of *in vitro* synthesized *ghoS* mRNA (278 nt, lanes 2, 3), *ghoT* mRNA (180 nt, lanes 4, 5) and mutated *ghoT-GCU* mRNA with two GCU sites (180 nt, lanes 6, 7) were incubated either without (–) or with (+) 30 ng of MqsR protein for 15 min at 37°C. The arrows indicate the uncleaved *ghoS* and *ghoT* transcripts. Lane 1, low-range ssRNA ladder (sizes indicated in nt).

ghoT-GCU mRNA was degraded by MqsR. As it is currently unknown whether MqsR cleaves mRNA at specific bases of a codon similar to RelE, which preferentially cleaves after the second or third base of a codon (Hurley *et al*., 2011), we introduced both a non-codon 5′-GCU and a codon 5′-GCU site into *ghoT* mRNA. Both changes are silent: at aa position 18, TCC (Ser) was changed to AGC (Ser) to create the non-codon 5′-GCU site and at aa position 37, GCA (Ala) was changed to GCT (Ala) to create a codon 5′-GCU site (Fig. 2; pCA24N-*ghoT-GCU*). Using purified toxin MqsR, we found that MqsR cleaves the mutated *ghoT* mRNA with two 5′-GCU sites (Fig. 3). Compared with the wild-type *ghoT* mRNA that lacks GCU sites, multiple cleavage products were clearly observed when the *ghoT-GCU* mRNA was used as a template. Furthermore, we also found that the sizes of the dominant cleavage products agree well with those that are predicted when the *ghoT-GCU* mRNA is cleaved at 5′-GCU sites (56, 59, 65, 115 and 121 nt) (Fig. 3, lane 7). These *in vitro* results demonstrate that MqsR preferentially cleaves 5′-GCU sequences, and that it functions without ribosomes (Yamaguchi *et al*., 2009).

Furthermore, using a *ghoT* deletion strain (BW25113 Δ*ghoT*) to ensure that no endogenous *ghoT* transcript was produced, we found that producing MqsR resulted in threefold fewer ghost cells for those cells transformed with pCA24N-*ghoT-GCU* versus those with pCA24N-*ghoT* (Fig. 4). Live/Dead staining was also used to differentiate between cells with intact membranes (live) and cells with damaged membranes (dead). A fourfold increase in the number of cells with intact membranes was observed in cells transformed with mutant *ghoT* DNA with two GCT (5′-GCU in the transcribed mRNA) sites (Fig. 4) as compared with wild-type *ghoT* which confirms that adding the two 5′-GCU sites decreased production of ghost cells. Therefore, both *in vitro* and *in vivo* studies demonstrate that MqsR regulates GhoT activity by GCU-dependent cleavage of *ghoS* in the *ghoST* mRNA transcript upon stress.

Discussion

Toxin MqsR regulates toxin GhoT via post-transcriptional differential mRNA cleavage resulting in a regulatory hierarchy where one TA system controls another. The nine lines of evidence that demonstrates this are that (i) when MqsR is produced [BW25113/pBS(Kan)-*mqsR* versus BW25113/pBS(Kan)], there is a 5 ± 1-fold increase in the 3′ *ghoT* portion of the *ghoST* mRNA compared with the 5′ *ghoS* portion, (ii) when MqsR is produced, *ghoT* mRNA is enriched 40 ± 1-fold compared with 5′-GCU-containing transcripts *ompA* and *ompF*, which have been shown to be degraded by MqsR by *in vivo* and *in vitro* (Yamaguchi *et al*., 2009), (iii) the addition of sublethal concentrations of nalidixic acid induces *mqsR* mRNA 18 ± 2-fold, which results in concomitant enrichment of *ghoT* mRNA 40 ± 1-fold while there is no enrichment of *ompA* mRNA, (iv) the addition of sublethal concentrations of azlocillin induces *mqsR* mRNA 20 ± 2-fold, which results in the concomitant enrichment of *ghoT* mRNA 48 ± 2-fold while there is no enrichment of the *ompA* mRNA, (v) upon oxidative stress, *mqsR* transcription was induced 4.5 ± 0.6-fold, which, in turn, enabled *ghoT* mRNA to increase 6 ± 2-fold, (vi) upon addition of two 5′-GCU sites to *ghoT* mRNA, the number of ghost cells and dead cells were reduced due to the presence of the primary cleavage sites for MqsR, (vii) MqsR cleaves more readily mutated *ghoT* mRNA with two introduced 5′-GCU sites *in vitro*, (viii) MqsR preferentially cleaves *ghoS* mRNA more efficiently than *ghoT* mRNA *in vitro*, and (ix) MqsR-derived persistence depends on GhoT activity. Although it is possible that other endoribonuclease toxins might also impact the stability of *ghoST* mRNA under stressed conditions *in vivo*, the *in vitro* studies demonstrate the importance of toxin MqsR on the stability of *ghoST* mRNA. Therefore, MqsR is the first identified toxin that regulates a second TA system, GhoT/GhoS, illustrating how MqsR, along with MqsA (Wang *et al*., 2011), function as global regulators of environmental stress in *E. coli*. These results also illustrate how cells have interwoven TA systems into their genetic fabric to control cell physiology.

Our model of the response of *E. coli* to oxidative stress, as mediated by the MqsR/MqsA and GhoT/GhoS TA

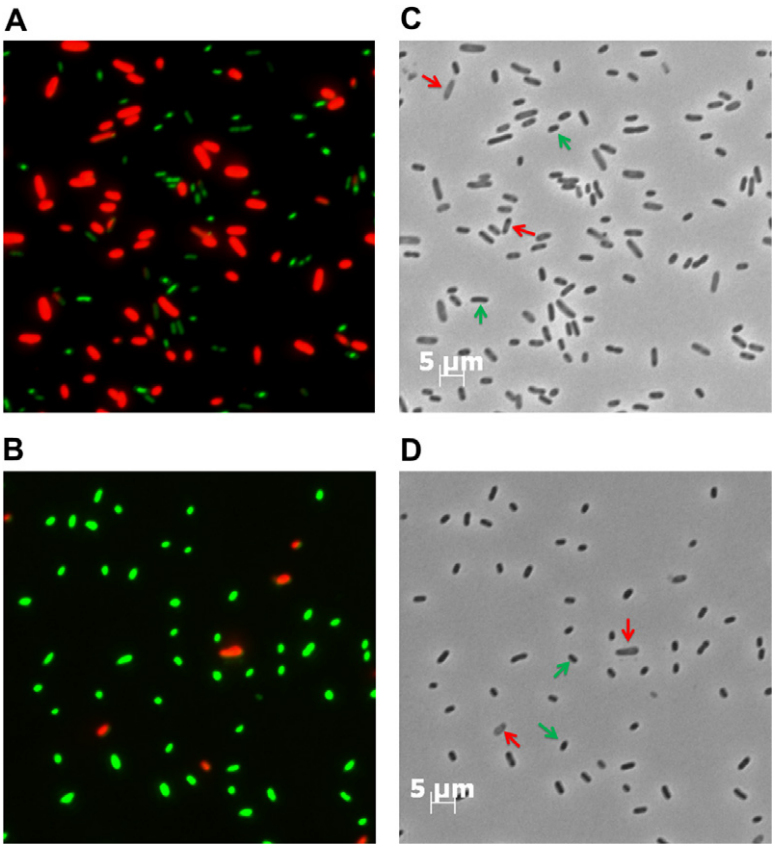

Fig. 4. Addition of 5′-GCU to *ghoT* mRNA reduces MqsR toxicity. Live/dead staining via fluorescence microscopy showing that the production of wild-type GhoT (A) causes ghost cell morphology and leads to cell death (red), while production of mutant *ghoT* mRNA (containing two introduced 5′-GCU sites) (B) has less ghost cells and less cell death (live cells are green). Panels (C) and (D) show the corresponding phase-contrast microscopy images. Red arrows indicate representative ghost cells, and green arrows indicate representative live cells. Images were taken 3 h after the addition of 0.5 mM IPTG to cultures of Δ*ghoT* ΔKm/pBS(Kan)-*mqsR*/pCA24N-*ghoT* (A and C) and Δ*ghoT* ΔKm/pBS(Kan)-*mqsR*/pCA24N-*ghoT-GCU* (B and D) when the turbidity reached 0.5, which allows production of MqsR through its leaky P_{lac} promoter followed by the production of GhoT through its $P_{T5\text{-}lac}$ promoter. Two independent cultures of each strain were evaluated and representative images were shown. Scale bars on the right column indicate 5 μm which is the same for all four panels.

systems, is shown in Fig. 5. In the absence of stress, the antitoxin MqsA binds to the toxin MqsR to block its RNase activity. Furthermore, MqsA also binds to the *rpoS* promoter region via a palindrome to inhibit its transcription (Wang *et al*., 2011). In the absence of stress, the antitoxin GhoS is produced which prevents the expression of *ghoT* by cleaving its transcript, and the translation of both proteins is coupled. Under stress, Lon protease degrades MqsA which frees the toxin MqsR (Wang *et al*., 2011) and further induces *mqsR* expression. MqsR then degrades unprotected mRNAs primarily at 5′-GCU sites, such as *ompA* and the 5′ end of *ghoST* mRNA (i.e. the coding region of *ghoS*, which contains three 5′-GCU sites). As *ghoST* is more efficiently degraded in the *ghoS* coding region, while the *ghoT* coding region stays intact, GhoT protein levels increase. This allows GhoT to exert its effects on the cell membrane, ultimately increasing persistence.

Therefore, our data further demonstrate the complexity and interconnectivity of TA systems by showing one TA system (MqsR/MqsA) may control another (GhoT/GhoS). This interconnectivity is to be expected given both the prevalence of mRNA cleavage sites of toxin RNases and their differential mRNA decay; hence, production of some proteins is facilitated during stress and this now includes preferential production of toxins via enrichment of their mRNA. For GhoT, its production allows the cell to increase its persistence, probably by disrupting ATP generation. The physiological consequence of enrichments of other transcripts including *kilR*, *ralR* and *yciG* remains to be elucidated (these genes encode a cell division inhibitor, a protein that alleviates restriction of unmodified DNA and promotes methylation, and an unknown protein respectively). Nevertheless, it is also clear now that a hierarchy of TA systems exists in the cells (e.g. MqsR/MqsA controlling GhoT/GhoS). This hierarchy was predicted (Bukowski *et al*., 2011) and now has been demonstrated.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids are listed in Table 3. To remove the kanamycin marker for Δ*ghoS* and Δ*ghoT*, pCP20 was used to express the FLP recombinase (Datsenko and Wanner, 2000). To construct BW25113 Δ*ghoS* Δ*mqsRA*, P1 transduction (Maeda *et al*., 2008) was used to transfer the Δ*mqsRA* deletion to BW25113 Δ*ghoS*. Luria–Bertani (LB) at 37°C was used except where indicated. Kanamycin (50 μg ml^{-1}) was used for pre-culturing isogenic knockout mutants and for maintaining pBS(Kan)-based plasmids, chloramphenicol (30 μg ml^{-1}) was used for maintaining pCA24N-based plasmids, and ampicillin (100 μg ml^{-1}) was used to

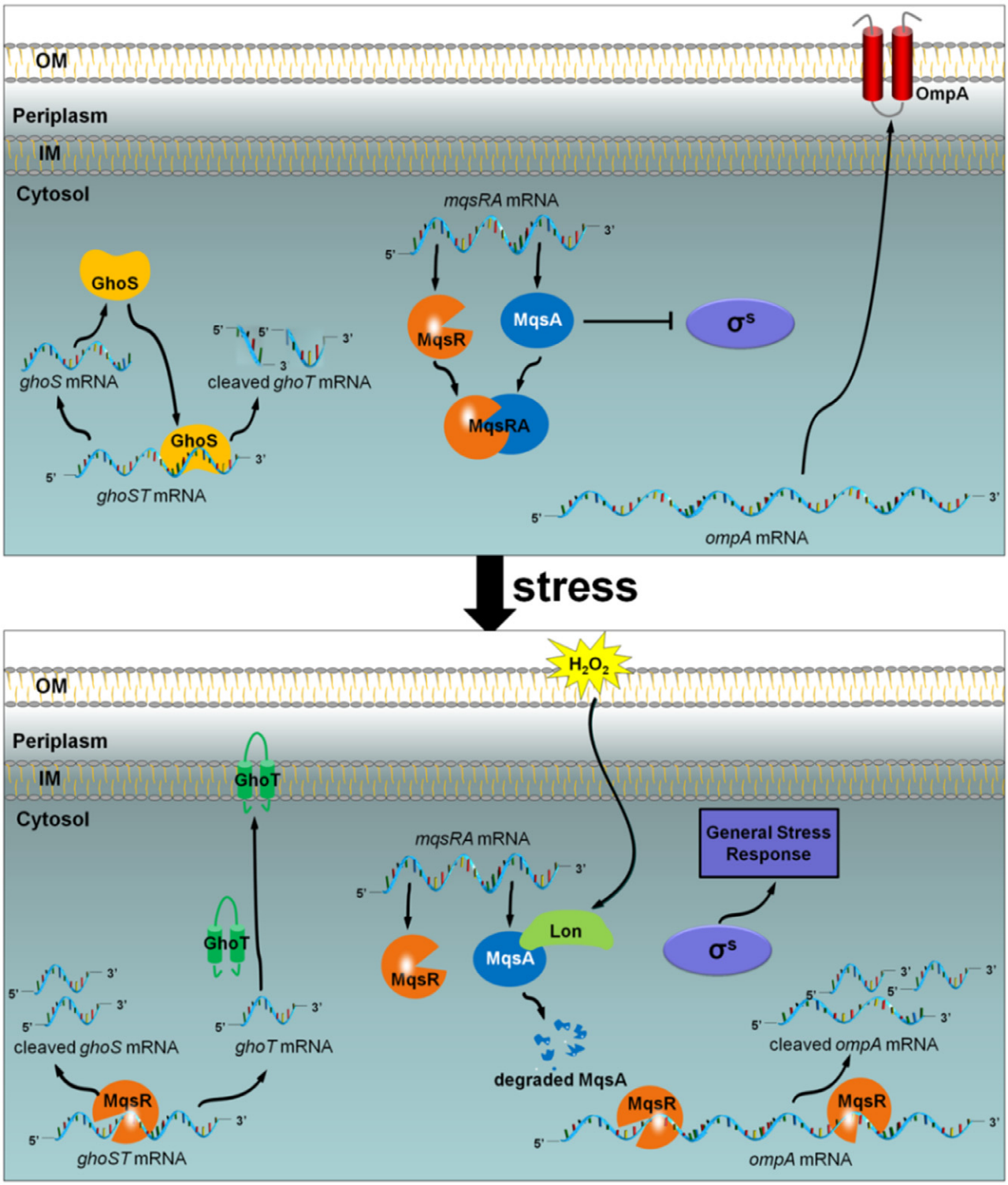

Fig. 5. Schematic of MqsR-mediated control of GhoT/GhoS. Without stress (upper panel), MqsR and MqsA bind and form a complex that prevents MqsR toxicity while GhoS cleaves *ghoT* mRNA to prevent GhoT toxicity. Upon oxidative stress (lower panel), MqsA is degraded by protease Lon, which derepresses *rpoS* and triggers the general stress response. The liberated MqsR functions as sequence-specific endoribonuclease to cleave *ghoS* mRNA, *ompA* mRNA, and other unprotected mRNAs primarily at 5′-GCU sites. As a result, MqsR rapidly enriches *ghoT* mRNAs which does not contain 5′-GCU sites as well as other stress-related transcripts and leads to the formation of persister cells.

Table 3. Bacterial strains and plasmids used in this study.

Bacterial strains/plasmids	Description	Source
E. coli K12 strains		
BW25113	*lacI*^{q}*rrnB*$_{T14}$Δ*lacZ*$_{WJ16}$ *hsdR514*Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Baba *et al.* (2006)
BW25113 Δ*ghoT*	BW25113 Δ*ghoT* Ω Km^R	Baba *et al.* (2006)
BW25113 Δ*ghoT* ΔKm	BW25113 Δ*ghoT*	This study
BW25113 Δ*ghoS*	BW25113 Δ*ghoS* Ω Km^R	Baba *et al.* (2006)
BW25113 Δ*ghoS* ΔKm	BW25113 Δ*ghoS*	This study
BW25113 Δ*mqsRA*	BW25113 Δ*mqsRA* Ω Km^R	Kim *et al.* (2010)
BW25113 Δ*ghoS* Δ*mqsRA*	BW25113 Δ*ghoS* Δ*mqsRA* Ω Km^R	This study
Plasmids		
pCP20	Amp^R & Cm^R; temperature-sensitive replication, thermal induction of FLP recombinase synthesis	Datsenko and Wanner (2000)
pCA24N	Cm^R; *lacI*^{q}, pCA24N	Kitagawa *et al.* (2005)
pCA24N-*mqsR*	Cm^R; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*mqsR*$^+$	Kitagawa *et al.* (2005)
pCA24N-*ghoT*	Cm^R; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*ghoT*$^+$	Kitagawa *et al.* (2005)
pCA24N-*ghoT-GCU*	Cm^R; *lacI*^{q}, pCA24N $P_{T5\text{-}lac}$::*ghoT*$^+$ with two 5′-GCU sites in *ghoT* mRNA	This study
pBS(Kan)	Km^R; pBS(Kan)	Canada *et al.* (2002)
pBS(Kan)-*mqsR*	Km^R; pBS(Kan) P$_{lac}$::*mqsR*$^+$	Kim *et al.* (2010)
pET30a-*mqsR*	Km^R; pET30a(Kan) P$_{T7}$::*mqsR*$^+$	Brown *et al.* (2012)

Chloramphenicol (30 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) were used to maintain pCA24N-based plasmids and for pBS(Kan)-based plasmids, respectively, and ampicillin (100 μg ml^{-1}) was used to maintain plasmid pCP20.
Amp^R, Cm^R and Km^R are ampicillin, chloramphenicol and kanamycin resistance respectively.

maintain plasmid pCP20. The persistence assay was conducted as described (Wang *et al.*, 2012) with 35 and 100 μg ml^{-1} ampicillin.

Microscopy

For the measurement of the cell size after exposure to ampicillin, overnight cultures of BW25113/pCA24N, BW25113/pCA24N-*mqsR* and Δ*ghoT*/pCA24N-*mqsR* were inoculated into LB with chloramphenicol (30 μg ml^{-1}) at a turbidity of 0.1, and 1 mM IPTG and ampicillin (20 μg ml^{-1}) were added. Cell samples were taken at 0 h, 4 h and 10 h, and washed with 0.85% NaCl. For the measurement of the cell size after exposure to ampicillin for GhoT overproduction, overnight cultures of Δ*ghoT*/pCA24N and Δ*ghoT*/pCA24N-*ghoT* were inoculated into LB with chloramphenicol at a turbidity of 0.1, and 1 mM IPTG was then added to induce *ghoT* expression for 2 h. Cells were washed and resuspended in LB, and ampicillin (20 μg ml^{-1}) was added to the cell suspension. Cell size was measured with a Zeiss Axiovert microscope with a 40 × dry objective.

To evaluate cell membrane integrity with GhoT, the Live/Dead *Bac*Light™ Bacterial Viability Kit (Molecular Probes) was used. The kit contains SYTO 9 and propidium iodide to differentiate between cells with intact membranes (live) and cells with damaged membranes (dead). After inducing *ghoT* and *mqsR* for 3 h with 0.5 mM IPTG, BW25113 Δ*ghoT* ΔKm/pBS(Kan)-*mqsR*/pCA24N-*ghoT* and BW25113 Δ*ghoT* ΔKm/pBS(Kan)-*mqsR*/pCA24N-*ghoT-GCU* were harvested by centrifugation (15 000 *g*, 10 min), washed, and resuspended in 0.85% NaCl to reach a turbidity of 0.3 at 600 nm. Cells were then stained with 0.15 mM propidium iodine and 0.025 mM SYTO 9 dye for 1 h at ambient temperature. An aliquot of each cell suspension was also treated with 70% isopropyl alcohol to use as a dead cell control. Bacterial cells were imaged using a fluorescence microscope (Zeiss Axiovert) with 40× dry objective.

qRT-PCR

For qRT-PCR, 50 ng of total RNA was used for qRT-PCR using the *Power* SYBR® Green RNA-to-C$_T$™ *1-Step* Kit and the StepOne™ Real-Time PCR System (Applied Biosystems). Primers were annealed at 60°C, and *rrsG* (Wang *et al.*, 2009) was used to normalize the data.

Site-directed mutagenesis

Site-directed mutagenesis (Wang *et al.*, 2011) was used to introduce two 5′-GCU sites into the coding region of *ghoT* in pCA24N-*ghoT* to create pCA24N-*ghoT-GCU* (Fig. 2) using two primer pairs, GhoT-GCU-1 and GhoT-GCU-2 respectively (Table 1). DNA sequencing using the BigDye Terminator Cycle Sequencing kit was performed to confirm the targeted mutations at these sites.

MqsR endoribonuclease assay

Refolding and purification of MqsR was performed as described (Brown *et al.*, 2012) after producing MqsR from pET30a-*mqsR*. For the synthesis of *ghoS*, *ghoT* and *ghoT-GCU* mRNAs, PCR products were obtained using the primers shown in Table 1 and were used as templates for *in vitro* transcription with T7 RNA polymerase. The T7 RNA polymerase promoter sequence was included in the forward primers. PCR products were purified using the PureLink PCR purification kit (Invitrogen), and 1 μg of the PCR product was used as the template for the *in vitro* RNA reaction with the AmpliScribe T7-Flash transcription kit (Epicentre). The reaction

mixture for the MqsR endoribonuclease cleavage assay (10 μl) contained 2 μg of mRNA, 100 mM KCl, 2.5 mM $MgCl_2$, and either 30 ng of purified MqsR protein in MqsR buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.5 mM TCEP) or an equivalent volume of MqsR buffer (for those reaction without MqsR protein). The reaction mixture was incubated at 37°C for 15 min and quenched by the addition of an equal volume of 2× sample loading buffer (Invitrogen). The reaction products were resolved by electrophoresis with RNA denaturing gels (15% polyacrylamide with 7 M urea, Invitrogen).

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