nature chemical biology

ARTICLE

PUBLISHED ONLINE: 24 APRIL 2011 | DOI: 10.1038/NCHEMBIO.560

Antitoxin MqsA helps mediate the bacterial general stress response

Xiaoxue Wang[1], Younghoon Kim[1], Seok Hoon Hong[1], Qun Ma[1], Breann L Brown[2], Mingming Pu[1], Aaron M Tarone[3], Michael J Benedik[4], Wolfgang Peti[2], Rebecca Page[5] & Thomas K Wood[1,4]*

Although it is well recognized that bacteria respond to environmental stress through global networks, the mechanism by which stress is relayed to the interior of the cell is poorly understood. Here we show that enigmatic toxin-antitoxin systems are vital in mediating the environmental stress response. Specifically, the antitoxin MqsA represses *rpoS*, which encodes the master regulator of stress. Repression of *rpoS* by MqsA reduces the concentration of the internal messenger 3,5-cyclic diguanylic acid, leading to increased motility and decreased biofilm formation. Furthermore, the repression of *rpoS* by MqsA decreases oxidative stress resistance via catalase activity. Upon oxidative stress, MqsA is rapidly degraded by Lon protease, resulting in induction of *rpoS*. Hence, we show that external stress alters gene regulation controlled by toxin-antitoxin systems, such that the degradation of antitoxins during stress leads to a switch from the planktonic state (high motility) to the biofilm state (low motility).

Although toxin-antitoxin (T-A) systems are ubiquitous in bacterial chromosomes[1], their role in cell physiology is controversial. It has been argued that they have no impact on the cell[2] or that they may have as many as nine roles[3] including addictive genomic debris, stabilization of genomic parasites, selfish alleles, gene regulation, growth control, persister cell formation (persisters are bacteria that are resistant to antibiotics without genetic change[4]), programmed cell arrest, programmed cell death and anti-phage measures[5]. They have also been linked to biofilm formation[6]. A T-A system typically consists of two genes located in an operon encoding a stable toxin that disrupts an essential cellular process and a labile antitoxin that can bind and form a tight complex with the toxin and neutralize its activity[1]. Although T-A systems were first thought to be related to cell death, there have been few clear examples of toxin-mediated cell death in a physiologically relevant situation[3]; hence, the primary role of these systems has been enigmatic.

Many chromosomal T-A systems have been characterized in *Escherichia coli* (16 so far[7]) including (listed as toxin-antitoxin) MqsR-MqsA[8], MazF-MazE[9], RelE-RelB[10], ChpB-ChpS[11], YoeB-YefM[12], YafQ-DinJ[13] and YhaV-PrlF[14]. Although the mechanism of toxicity at the molecular level is slightly different, MqsR[8], MazF[1], RelE[1], ChpB[1], YoeB[12] and YhaV[14] prevent translation by cleaving RNAs; the mode of translation inhibition by YafQ is unclear[2].

Of these redundant T-A systems, toxin MqsR (which stands for motility quorum sensing regulator) (YgiU, B3022)[15,16] and antitoxin MqsA (YgiT, B3021)[8] are particularly significant, as the genes that encode them make up the first locus that upon deletion decreases the formation of persister cells[17], and *mqsR* is also the most highly induced gene in persister cells as compared to nonpersisters[4]. MqsR-MqsA is also the first T-A system found to be induced in biofilms[16], the first to be related to quorum sensing[15], the first to be related to cell motility[15] and the first to be related to biofilm formation[15,16]. Furthermore, MqsA is the first antitoxin shown to regulate more than its own transcription as it binds the *mqsRA*, *cspD*, *mcbR* and *spy* promoters[8,18]. The three-dimensional structure of MqsR-MqsA[8] revealed that MqsR is an RNase similar to RelE and YoeB and that MqsA binds DNA via its helix-turn-helix motif in the C-terminal domain and binds the toxin via its N-terminal zinc-binding domain. MqsR cleaves mRNA at GCU sites[7]. MqsR-MqsA is also conserved in 40 eubacteria[15].

As the T-A pair MqsR-MqsA has been linked to both motility and biofilm formation[15], it appears intimately related to how *E. coli* switches between motile and sessile (that is, biofilm) growth. The switch between these two fundamental lifestyles is based on the antagonistic regulation of the master regulator of motility, FlhDC, and the master regulator of the stress response, RpoS[19], which controls up to 500 genes in *E. coli*[20]. Underlying both lifestyles is regulation of the concentration of the second messenger 3,5-cyclic diguanylic acid (c-di-GMP), which controls the switch from motility (low c-di-GMP) to sessility (high c-di-GMP)[19] through a variety of actions, such as binding YcgR and acting as a molecular flagellar brake[21]. c-di-GMP concentrations are modulated directly via *de novo* synthesis by diguanylate cyclases (proteins with GGDEF motifs) and via degradation by phosphodiesterases (proteins with EAL or HD-GYP motifs)[22]. Herein we show how extracellular stress is conveyed to FlhDC and RpoS, which was previously not understood[19].

Using a strain deficient in six major T-A systems, Δ6 (MazF-MazE, RelE-RelB, ChpB, YoeB-YefM, YafQ-DinJ and MqsR-MqsA), we provide insights into extracellular stress and both the general stress response and the switch from planktonic growth to biofilm formation. We show that the antitoxin MqsA regulates the RNA polymerase sigma factor σ^S, which is encoded by *rpoS*. Specifically, during stress, Lon protease degrades MqsA, which leads to induction of *rpoS*, which in turn increases c-di-GMP, inhibits motility and increases cell adhesion and biofilm formation.

[1]Department of Chemical Engineering, Texas A&M University, College Station, Texas, USA. [2]Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, Rhode Island, USA. [3]Department of Entomology, Texas A&M University, College Station, Texas, USA. [4]Department of Biology, Texas A&M University, College Station, Texas, USA. [5]Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island, USA. *e-mail: Thomas.Wood@chemail.tamu.edu

RESULTS

MqsA is a negative regulator of *rpoS*

Our previous whole-transcriptome analysis, in which the impact of the MqsR toxin was investigated, showed that *rpoS* was induced substantially by the RNase activity of MqsR[18]. To explore further the relationship between the MqsR-MqsA T-A system and the regulation of *rpoS* under stress conditions, we cultured cells under oxidative stress conditions in which RpoS is crucial for cell survival[23,24] by regulating antioxidant activities such as those of catalase and superoxide dismutase[25]. We used a genetic background devoid of the major *E. coli* T-A pairs by using the Δ5 strain[2], which lacks the MazF-MazE, RelE-RelB, ChpB, YoeB-YefM and YafQ-DinJ T-A systems (**Supplementary Results**, **Supplementary Table 1**), and the Δ6 strain, which also lacks MqsR-MqsA (Δ5 *mqsRA*). In this way, the impact of antitoxin MqsA could be studied largely in the absence of other antitoxins.

Our hypothesis is that the antitoxin MqsA is essential in mediating the RpoS stress response. To test this hypothesis, we analyzed *rpoS* transcripts during oxidative stress to observe the effect of MqsA. Under these oxidative stress conditions (20 mM H_2O_2 for 10 min), because of the complexity of the regulation of *rpoS* transcription and post-transcriptional modifications of *rpoS* mRNA upon stress[20], a consistent increase (~two-fold) in *rpoS* mRNA in wild-type cells was detected by quantitative real-time reverse-transcription PCR (qRT-PCR) (see **Supplementary Table 2** for all of the qRT-PCR data). When the Δ6 cells were exposed to this oxidative stress in the presence of plasmid-expressed MqsA, *rpoS* mRNA was reduced by 4 ± 1–fold (via qRT-PCR) compared to the empty plasmid control with oxidative stress. Corroborating this result, deleting *mqsRA* resulted in a 4.5 ± 0.4–fold increase in *rpoS* mRNA after 30 s with 20 mM H_2O_2 (Δ6 versus the MG1655 wild-type strain); similar results were seen upon deleting *mqsRA* in the related *E. coli* strain BW25113. Hence, MqsA directly or indirectly controls *rpoS* transcription. It was not possible to test directly the impact of deleting the antitoxin gene *mqsA* on *rpoS* transcription, as deleting *mqsA* is lethal[4,26], owing to the toxicity of MqsR; similar results have been seen with other antitoxins including MazE, ChpS and YefM[26]. The production of the antitoxin MqsA is not toxic[8].

As *rpoS* is repressed by MqsA, the genes controlled by RpoS should also be repressed. We found that *adrA*, which encodes a diguanylate cyclase that controls cellulose synthesis and which is positively regulated by RpoS[27], was repressed by 6 ± 1–fold upon producing MqsA from a plasmid in cells under oxidative stress when directly compared to the identical experiment with cells with an empty plasmid and oxidative stress. Similarly, repression of *rpoS* by MqsA should further decrease c-di-GMP levels by inhibiting three other genes that encode diguanylate cyclases (*ydaM, yegE* and *yedQ*), all of which are upregulated by RpoS[19]. As expected, *ydaM* (3 ± 1–fold), *yegE* (3 ± 1–fold) and *yedQ* (2 ± 1–fold) were repressed by MqsA with oxidative stress compared to the empty plasmid with oxidative stress. In addition, *csgD*, which encodes the regulator for curli and cellulose, should also be repressed by repressing *rpoS* with MqsA[19]. As expected, *csgD* was repressed (3 ± 1–fold) when MqsA was produced from a plasmid in the presence of oxidative stress. Note that bioinformatics analysis showed that the *csgD* promoter also contains an *mqsRA*-like palindrome (5′-AACCT TA AGGTT) 78 basepairs (bp) upstream of the transcription initiation site that is used by MqsA to regulate transcription[7,28] (**Supplementary Fig. 1** and **Supplementary Table 3**). Therefore, MqsA potentially reduces curli and cellulose production through two pathways: indirectly via *rpoS* repression and directly by binding the *csgD* promoter.

In addition, as RpoS is a positive regulator of catalase activity via *katG* and *katE*[25], repression of *rpoS* by MqsA should lead to a reduction in transcription of these genes. As expected, transcription of *katE* (6 ± 1–fold) and *katG* (4 ± 1–fold) were both repressed when the Δ6 cells were exposed to oxidative stress (20 mM H_2O_2 for 10 min) in the presence of elevated MqsA, compared to oxidative stress with an empty plasmid. Corroborating this result, deleting *mqsRA* resulted in a 3.5 ± 0.4–fold increase in *katE* mRNA after 30 s with 20 mM H_2O_2 (Δ6 versus the MG1655 wild-type strain); similar results were seen upon deleting *mqsRA* in BW25113. Therefore, MqsA represses *rpoS*, either directly or indirectly, resulting in repression of the genes positively regulated by RpoS.

MqsA binds the *rpoS* promoter

There are two palindromes in the promoter of the *mqsRA* operon (5′-TAACCT TTT AGGTTA and 5′-ACCT TTT AGGT), and MqsA as well as the MqsR–MqsA complex binds to the two palindromic sequences to negatively regulate *mqsRA*[7,28]. Each MqsA of the dimer

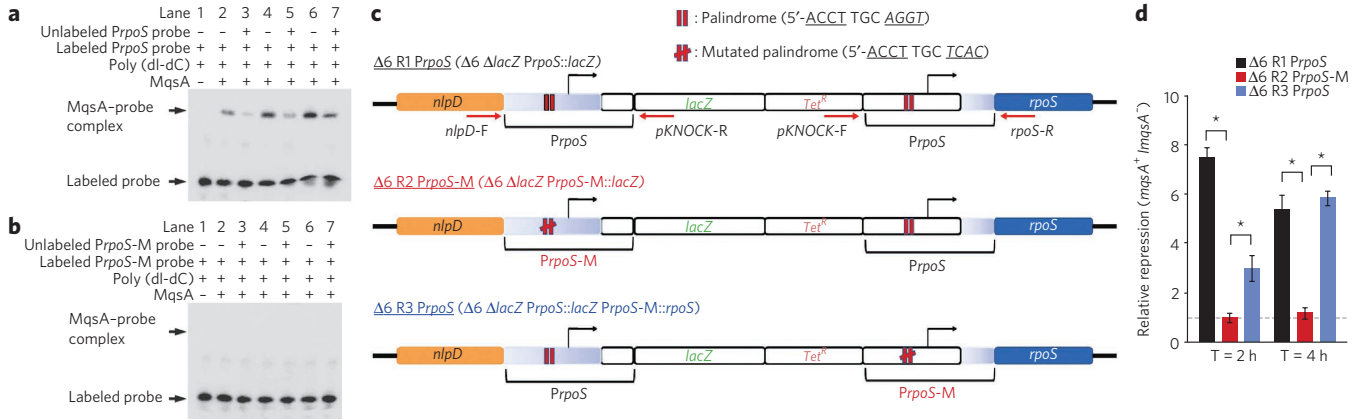

Figure 1 | MqsA binds to wild-type P*rpoS* but not to mutated P*rpoS*. (**a**) Biotin-labeled DNA corresponding to the *rpoS* promoter (P*rpoS*) was incubated with either a ten-fold (lanes 2 and 3), a 25-fold (lanes 4 and 5) or a 50-fold (lanes 6 and 7) excess of MqsA. At all protein concentrations, MqsA is able to bind and shift the labeled P*rpoS* DNA (lanes 2, 4, 6). Addition of 100-fold excess of unlabeled P*rpoS* DNA demonstrates specificity of binding (lanes 3, 5, 7). Biotin-labeled P*rpoS* in the absence of MqsA was used as a control (lane 1). (**b**) Biotin-labeled DNA corresponding to a mutated *rpoS* promoter (P*rpoS*-M) was incubated with MqsA as indicated in **a**. MqsA binding to the mutated P*rpoS* is dramatically reduced at all concentrations tested. (**c**) Reporter strain Δ6 R1 P*rpoS* was constructed by conjugating pKNOCK-P*rpoS* with the wild-type P*rpoS* into Δ6 Δ*lacZ*, whereas reporter strains Δ6 R2 P*rpoS*-M and Δ6 R3 P*rpoS* were constructed by conjugating pKNOCK-P*rpoS*-M with mutated P*rpoS* into Δ6 Δ*lacZ*. Transcription start sites of *rpoS* are indicated by black arrows. Primers used for strain verification by DNA sequencing are indicated by red arrows. (**d**) Relative repression of β-galactosidase activity in the three Δ6 reporter strains with MqsA (produced via pCA24N-*mqsA*) versus without MqsA (pCA24N). β-galactosidase activity was measured 2 h and 4 h after adding 0.1 mM IPTG when the turbidity (600 nm) was ~1. Error bars indicate s.e.m. (n = 3). Significant changes are marked with an asterisk for $P < 0.05$.

binds to this palindrome via residues Asn97 and Arg101, which make base-specific interactions with eight nucleotides including four from one strand (5′-TAAC) and four from the antiparallel strand (5′-AGGT)[28]. In addition, the intervening TTT does not seem to have any effect on MqsA binding affinity[28]. As with *csgD*, we identified a similar *mqsRA*-like palindrome in the promoter region of *rpoS* (**Supplementary Fig. 2**), 5′-ACCT TGC AGGT, and thus, by direct extension of our work with *mqsRA*, we hypothesized that MqsA binds directly to the *rpoS* promoter (P*rpoS*) and thus controls the transcription of the sigma factor that is necessary for control of the general stress response. To demonstrate direct binding of MqsA to the regulatory sequences of *rpoS*, electrophoretic mobility shift assays (EMSA) were used to detect the binding of MqsA to the 25-bp fragment of P*rpoS* that includes this palindrome (from residue –161 to residue –137, **Supplementary Fig. 2**). The EMSA showed that MqsA binds and shifts P*rpoS* with 10-, 25- and 50-fold molar excesses of MqsA (**Fig. 1a**). However, upon mutating the palindrome in P*rpoS*, 5′-ACCT TGC AGGT, to 5′-ACCT TGC TCAC in P*rpoS*-M, binding of MqsA to P*rpoS* was abolished (**Fig. 1b**). As expected, at the same concentrations, no shifts were observed for the promoter of the negative control *gadA* (P*gadA*) (**Supplementary Fig. 3a**). Furthermore, replacing the key residues of the base-specific interactions of MqsA (N97A and R101A) prevents MqsA from binding the *rpoS* promoter (**Supplementary Fig. 3b**). Therefore, MqsA binds directly and specifically to the *mqsRA*-like palindrome sequence of the *rpoS* promoter.

To confirm that MqsA directly influences transcription of *rpoS*, a chromosomal *lacZ* transcriptional fusion was constructed. A 943-bp region upstream of the *rpoS* translation initiation site, including the 565-bp mRNA leader sequence and 378 bp upstream of the leader sequence (**Supplementary Fig. 2**), was fused to translationally independent *lacZ* and inserted into the suicide vector pKNOCK-Tc[29]. The constructed vector was conjugated into Δ6 Δ*lacZ* (**Supplementary Table 1**), generating an integrated *lacZ* reporter driven by the *rpoS* promoter and an intact copy of *rpoS* with its native promoter (**Fig. 1c**); this reporter strain (Δ6 Δ*lacZ* P*rpoS*::*lacZ*), named Δ6 R1 P*rpoS*, was used to probe the influence of MqsA on *rpoS* transcription. As expected, when compared to an empty plasmid, there was a 7.5 ± 0.5–fold repression of β-galactosidase activity with *mqsA* when induced from pCA24N-*mqsA* with 0.1 mM isopropyl-thio-2-*D*-galactopyranoside (IPTG) for 2 h, and a 5.4 ± 0.6–fold repression for 4 h (**Fig. 1d**). Corroborating this result, deleting *mqsRA* resulted in a 4.2 ± 0.7–fold increase in *rpoS* transcription in the stationary phase as determined by comparing β-galactosidase activity in reporter strains Δ6 R1 P*rpoS* versus Δ5 R1 P*rpoS* (**Supplementary Table 1**).

To confirm that MqsA regulates *rpoS* expression through the identified *mqsRA*-like palindrome in *rpoS*, the four nucleotides in the *rpoS* palindrome that are used for direct base-specific interactions[28] were mutated from 5′-AGGT to 5′-TCAC (**Supplementary Fig. 2**) in pKNOCK-P*rpoS*-M and integrated into Δ6 Δ*lacZ* to form Δ6 R2 P*rpoS*-M (**Fig. 1c**). Without the palindrome, MqsA no longer repressed *lacZ* transcription, as similar β-galactosidase activities were found for cells with or without production of MqsA (**Fig. 1d**). Moreover, the control strain, reporter Δ6 R3 P*rpoS*, with wild-type P*rpoS* fused to *lacZ* but with the mutated palindrome in front of the *rpoS* gene (**Fig. 1c**), had 3.0 ± 0.5–fold (2 h) and 5.8 ± 0.3–fold (4 h) reduced β-galactosidase activity upon production of MqsA (**Fig. 1d**). Therefore, MqsA directly represses *rpoS* transcription, and the repression is dependent on the palindrome in the promoter of *rpoS*.

MqsA reduces c-di-GMP

As MqsA represses *rpoS*, which, in turn, results in the repression of the genes related to synthesizing c-di-GMP, it is expected that c-di-GMP concentrations should decrease in the presence of MqsA. As expected, upon overexpression of *mqsA* from a plasmid in stationary-phase cells, the intracellular c-di-GMP concentration decreased by 1.7 ± 0.4–fold (**Fig. 2a**). Corroborating this result, the deletion of *mqsRA* increased c-di-GMP concentration by 3.3 ± 0.6–fold (Δ6/pCA24N versus Δ5/pCA24N) (**Fig. 2a**). Hence, MqsA reduces c-di-GMP concentrations.

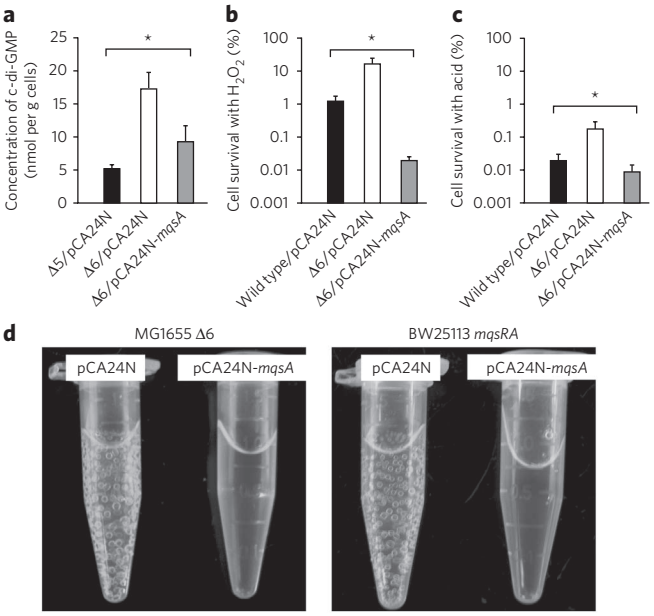

Figure 2 | MqsA decreases c-di-GMP and resistance to stress. (**a**) c-di-GMP concentrations in stationary-phase cultures (starving cells) after 15 h at 37 °C. (**b**) Percentage of cells that survive the oxidative stress induced by 20 mM H_2O_2 for 10 min. (**c**) Percentage of cells that survive the acid stress induced by pH 2.5 for 10 min. Error bars indicate standard error of mean (n = 3). Significant changes are marked with an asterisk for $P < 0.05$. (**d**) Images of MG1655 Δ6 and BW25113 *mqsRA* cultures (turbidity of 1) 10 min after adding 20 mM H_2O_2. Bubbles are oxygen produced by the decomposition of hydrogen peroxide by catalase: 2 H_2O_2 to 2 H_2O + O_2. *mqsA* was induced from pCA24N-*mqsA* via 0.5 mM IPTG for the c-di-GMP assay and via 1 mM IPTG for the stress assays.

MqsA reduces stress resistance

As MqsA represses *rpoS*, we reasoned that the deletion of the six T-A systems should increase cell survival under stress, as RpoS would be constitutively induced and the cells would be preconditioned for a stress response. As expected, Δ6 had over ten-fold greater resistance to both oxidative stress (20 mM H_2O_2 for 10 min) (**Fig. 2b**) and acid stress (pH 2.5 for 10 min) (**Fig. 2c**) compared to MG1655 (parent strain). Corroborating this result, overexpression of MqsA reduced cell survivability under oxidative stress by 850-fold compared to Δ6 with the empty plasmid (**Fig. 2b**) and reduced cell survivability under acid stress by 22-fold (**Fig. 2c**). This decrease in the resistance to oxidative stress is explained by a 19 ± 2–fold reduction in catalase activity when MqsA is produced in the Δ6 strain versus the empty plasmid (only trace catalase activity was seen in the MqsA-producing strain). Similarly, there was a 1.8 ± 0.3–fold decrease in catalase activity for Δ5 versus Δ6. Catalase converts H_2O_2 to H_2O and O_2; hence, the reduced ability of the cells to decompose H_2O_2 when MqsA represses *rpoS* was demonstrated by a dramatic reduction in oxygen bubbles upon addition of H_2O_2 to Δ6 (**Fig. 2d**). Moreover, overproduction of MqsA in the strain that only lacks *mqsRA* also had greatly reduced oxygen bubbles upon addition of H_2O_2 (**Fig. 2d**). Together, these nine sets of results (including the qRT-PCR results with *katE* and *katG*) convincingly show that the MqsRA T-A system and specifically the antitoxin MqsA regulate resistance to H_2O_2. Therefore, the MqsRA T-A system directly affects the ability of the cell to withstand external stress.

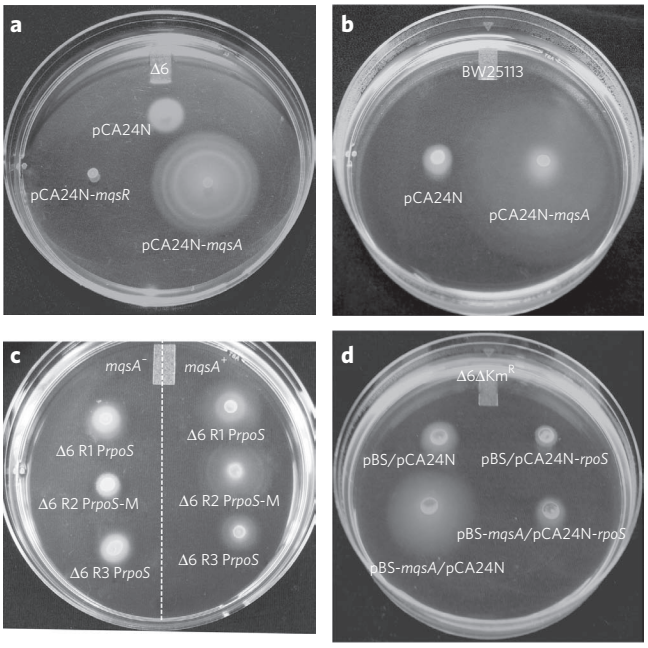

Figure 3 | MqsA increases motility by regulating *rpoS* transcription. (**a**) Swimming motility after 9 h of growth at 37 °C for Δ6 cells overexpressing *mqsA* or *mqsR*. (**b**) Swimming motility after 12 h of growth at 37 °C for BW25113 cells overexpressing *mqsA*. (**c**) Swimming motility after 12 h of growth at 37 °C for the three Δ6 reporter strains with MqsA (right side) and without MqsA (left side). For **a**–**c**, chloramphenicol (30 μg ml^{-1}) was used for plasmid maintenance, and kanamycin (50 μg ml^{-1}) and tetracycline (5 μg ml^{-1}) were used to select the host strains, where appropriate. (**d**) Swimming motility after 12 h of growth at 37 °C for Δ6 ΔKm^{R} cells overexpressing *mqsA* was from pBS(Kan)-*mqsA* (pBS-*mqsA*) and *rpoS* from pCA24N-*rpoS* via 0.1 mM IPTG. Control where *rpoS* was induced in the absence of MqsA was included to show the direct effect of RpoS on motility. Chloramphenicol (30 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) were used to maintain pCA24N-based and pBS(Kan)-based plasmids, respectively. Representative images are shown. Petri dishes (8.3-cm diameter) were used for all motility tests. MqsA, MqsR and RpoS were produced using 0.1 mM IPTG.

MqsA increases motility

As c-di-GMP levels are reduced in the presence of antitoxin MqsA, motility should increase, owing to lower RpoS levels that no longer inhibit expression of *flhD*, the master regulator of motility[19]. As expected, production of MqsA in Δ6 increased motility by 3.2 ± 0.3–fold (**Fig. 3a**). Similarly, it was expected that motility should decrease in the presence of MqsR, as elevated toxin levels should increase stress levels in the cell, leading to elevated RpoS and repression of *flhD*. As expected, production of MqsR abolished swimming motility (**Fig. 3a**), as the viable cells were nonmotile (with 0.1 mM IPTG, production of MqsR was not completely toxic—the cell number increased from 2.1 × 10^{4} to 8.5 × 10^{6}). In addition, production of MqsA in BW25113 increased cell motility by 4 ± 1–fold (**Fig. 3b**); therefore, the increase in motility via MqsA is not an artifact of the Δ6 strain.

The increase in motility by MqsA was also tested in an *rpoS*-deletion background, and, as expected, MqsA only increased motility slightly (1.2 ± 0.2–fold). This result confirms that MqsA works primarily through RpoS. Critically, the increase in motility upon producing MqsA was also tested in the *lacZ* reporter strains, where it was found that MqsA no longer increases motility in reporter Δ6 R3 P*rpoS* (**Fig. 3c**) in which the mutated palindrome lies in the promoter of the chromosomal *rpoS* gene (**Fig. 1c**). The other two reporter strains, Δ6 R1 P*rpoS* and Δ6 R2 P*rpoS*-M, which both have the wild-type P*rpoS* for *rpoS*, have P*rpoS* repressed by MqsA and became more motile (**Fig. 3c**). These results demonstrate that MqsA regulates *rpoS* transcription and further confirm that MqsA binds the *mqsRA*-like palindrome in the promoter of *rpoS*.

Moreover, when RpoS is produced from the expression vector pCA24N-*rpoS* such that *rpoS* lacks its native promoter and leader region, production of MqsA no longer increases cell motility (**Fig. 3d**). This result indicates that MqsA exerts its control directly on *rpoS* expression and requires its native upstream sequence. This result also eliminates the possibility that RpoS is regulated by MqsA at a post-translational level, as MqsA no longer increases motility once *rpoS* mRNA is translated.

MqsA decreases curli and cellulose

Additional phenotypes related to the reduced c-di-GMP levels should also be influenced by MqsA and MqsR. Our hypothesis predicts that if MqsA represses *rpoS*, both curli and cellulose production should likewise be repressed, as RpoS induces *csgD*[19]. Using Congo red, a dye that binds to both cellulose and curli[30], we found, as expected, that producing MqsA in the presence of oxidative stress (2 mM H_2O_2 for 180 min at 30 °C) decreased curli and cellulose production by 13 ± 2–fold (**Fig. 4a**). Furthermore, producing MqsR increased curli and cellulose production by 1.4 ± 0.3–fold compared to the empty plasmid control and by 20 ± 3–fold compared to production of MqsA (**Fig. 4a**). Moreover, deleting *mqsR* and *mqsA* leads to a 2.8 ± 0.3–fold increase in curli and cellulose (Δ6 versus Δ5) under the same oxidative stress conditions. In contrast, without H_2O_2, there was only a small reduction in curli and cellulose production when MqsA was produced (2.1 ± 0.6–fold) and no change in this phenotype for MqsR production.

MqsA decreases biofilm formation

As MqsA represses *rpoS*, which leads to reduced c-di-GMP levels and increased motility, deletion of *mqsA* should also increase biofilm formation, owing to the increase in c-di-GMP. As expected, the Δ6 strain with *mqsRA* deleted had 1.6 ± 0.2–fold more biofilm formation than the Δ5 strain after 24 h in minimal medium (**Fig. 4b**). Corroborating this result, production of MqsA in Δ6 (which reduces c-di-GMP) decreased biofilm formation 2.0 ± 0.4–fold after 24 h; this result also shows that producing MqsA in Δ6 reduces biofilm formation to the level of Δ5 so that the increase in biofilm formation seen with Δ6 was complemented (**Fig. 4b**). Taken together, these results show that MqsA decreases biofilm formation through its reduction of c-di-GMP.

MqsA is degraded by Lon under oxidative stress

To show that oxidative stress regulates proteolysis of MqsA, we performed a western blot analysis to monitor MqsA levels upon

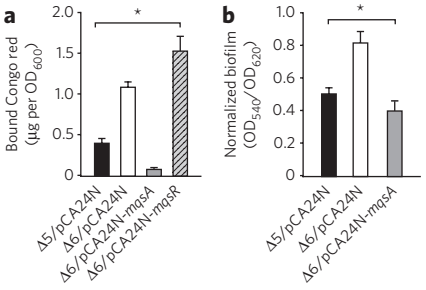

Figure 4 | MqsA decreases cellulose and curli and biofilm formation. (**a**) Cellulose and curli formation after 180 min at 30 °C with oxidative stress (2 mM H_2O_2). (**b**) Biofilm formation after 24 h in M9 glucose (0.2% (w/v)). *mqsA* and *mqsR* were induced from pCA24N-based plasmids via 0.1 mM IPTG for the cellulose and curli assays and via 1 mM IPTG for the biofilm assays. Error bars indicate standard error of mean (n = 3). Significant changes are marked with an asterisk for $P < 0.05$.

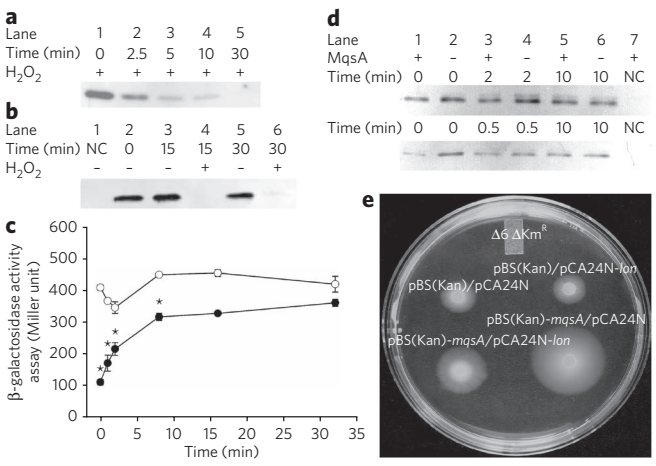

Figure 5 | MqsA is degraded under oxidative stress by Lon, and MqsA decreases RpoS levels. (**a**) Lanes 1–5 (western, short time) show the degradation of His-MqsA detected by a His tag antibody with 20 mM H_2O_2 (Δ6/pCA24N-*mqsA*). (**b**) Lanes 1–6 (western, longer time) show the degradation of His-MqsA detected by a His tag antibody with and without 20 mM H_2O_2 (Δ6/pCA24N-*mqsA*). Lane 1 is the negative control (NC) where His-MqsA is absent (Δ6/pCA24N). *mqsA* was induced from pCA24N-*mqsA* via 0.5 mM IPTG. (**c**) β-galactosidase activity for Δ5 R1 P*rpoS* (contains MqsA, closed circles) versus Δ6 R1 P*rpoS* (lacks MqsA, open circles) with 20 mM H_2O_2 at a turbidity of ~3. Error bars indicate standard error of mean ($n = 3$). Significant changes are marked with an asterisk for $P < 0.05$. (**d**) Lanes 1–6 (western) show RpoS levels as detected by an anti-RpoS antibody with 20 mM H_2O_2 (upper blot shows BW25113 (active MqsA) versus *mqsRA* (no MqsA), lower blot shows MG1655 (active MqsA) versus Δ6 (no MqsA)). Lane 7 is the negative control (NC) where RpoS is absent (BW25113 *rpoS*). **Supplementary Figure 5** shows full SDS-PAGE and western blots. (**e**) Swimming motility after 12 h of growth at 37 °C for Δ6 ΔKm^R cells overexpressing *mqsA* from pBS(Kan)-*mqsA* and *lon* from pCA24N-*lon* via 0.1 mM IPTG. Chloramphenicol (30 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) were used for maintaining the pCA24N-based and pBS(Kan)-based plasmids, respectively. Control where *lon* was induced in the absence of MqsA shows the effect of Lon on motility. Representative image is shown.

addition of oxidative stress. Rifampin was added to the cultures to stall RNA polymerase to block transcription of the *mqsA* mRNA[31]. As expected, MqsA was degraded rapidly upon the addition of H_2O_2 (20 mM) (**Fig. 5a**). Within 10 min, the amount of MqsA was greatly reduced and barely detectable (**Fig. 5a**), thus the half-life of MqsA under oxidative stress is approximately 1.25 min. However, MqsA was stable without oxidative stress up to 60 min (30 min time point shown in **Fig. 5b**).

Consistent with these MqsA degradation results, after prolonged (10 min) contact with 20 mM H_2O_2, there was no difference in *rpoS* and *katE* mRNA levels, in contrast to an induction with short contact (30 s) for Δ6 versus MG1655, as MqsA is rapidly degraded under these conditions and is no longer available to repress *rpoS*; similar results were seen upon deleting *mqsRA* in BW25113. The P*rpoS::lacZ* reporter strains also exhibited this behavior with higher initial promoter activity in Δ6 R1 P*rpoS* compared to Δ5 R1 P*rpoS* with H_2O_2, but this difference gradually disappeared (**Fig. 5c**) because of degradation of MqsA with longer H_2O_2 contact time. Western blotting also showed three-fold higher RpoS levels upon deleting *mqsRA* for short H_2O_2 contact time (0 to 0.5 min), but after long incubations (for example, 10 min), there was no difference between strains with and without MqsA, because of the degradation of MqsA (**Fig. 5d**). The relatively small changes in RpoS protein (**Fig. 5d**) are expected because of the translational regulation of RpoS synthesis via OxyR-induced OxyS RNA under oxidative stress[32], which may offset somewhat-increased *rpoS* transcription. Therefore, both P*rpoS* activity and RpoS levels are repressed by MqsA, but upon oxidative stress, MqsA is degraded and *rpoS* is derepressed.

We previously determined that the three genes encoding the proteases Lon, ClpX and ClpP are all induced upon oxidative stress (8 ± 1–fold, 6 ± 1–fold and 6 ± 1–fold, respectively)[18]. To further explore which protease degrades MqsA, the effect of Lon, ClpP and ClpX on MqsA in a motility assay was examined. Producing Lon and MqsA simultaneously significantly reduced the ability of MqsA to increase motility (**Fig. 5e**). As a control, producing Lon did not affect motility (**Fig. 5e**). However, in the presence of ClpP, MqsA increased motility. Furthermore, producing ClpX abolished motility completely, making it impossible to observe the effect of MqsA. Therefore, the protease Lon degrades MqsA, as shown for antitoxin CcdA[33] and antitoxin RelE[34], under oxidative stress conditions where *lon* is induced.

DISCUSSION

Previously, it was reported that five T-A systems had no influence on the general stress response[2]; however, the most important T-A system, MqsR-MqsA, was not considered in this analysis, nor was biofilm formation considered. Subsequently, we have shown that T-A systems are important for biofilm formation using MqsR-MqsA[15,16] and the *E. coli* Δ5 strain[6], which lacks five T-A systems. In addition, two other groups have confirmed our initial result of the importance of T-A systems in biofilm formation[35,36]. Clearly, the role for T-A systems is expanding rapidly. In addition, we have found stress increases *E. coli* biofilm formation (for example, oxidative, acid, low-temperature and heavy-metal stress)[37], and this phenomenon appears to hold for other bacteria as well. In the current study, we link T-A systems to both biofilm formation and the general stress response by showing that the antitoxin MqsA represses *rpoS*, which, in turn, alters the level of the secondary messenger c-di-GMP, thereby regulating motility and cell adhesins. Hence, for the first time, we link T-A systems to the secondary messenger c-di-GMP and to RpoS. This constitutes a novel role for antitoxins: direct regulation of the general stress response.

RpoS is important for biofilm formation, as its inactivation prevents mature biofilm formation by inducing motility-related genes and by repressing colanic acid synthesis (a biofilm exopolysaccharide)[38]. Many RpoS-regulated genes are also expressed in a temporal manner in biofilms[39]. Furthermore, RpoS enhances resistance to antibiotics[40]. Both of these earlier results can now be explained by the direct interaction we have discovered between MqsA and *rpoS*: increasing MqsA represses *rpoS*, which reduces the ability of the cells to withstand stresses like antibiotics, oxidative stress and acid stress. In addition, the repression of *rpoS* by MqsA also decreases c-di-GMP and increases motility and reduces biofilm formation. Moreover, for mature biofilms, *rpoS* expression was greater for cells in the outer regions of the biofilm where *mqsA* was repressed[41]; this provides another line of evidence that MqsA and *rpoS* are related. Our work also serves to discern the mechanism by which the MqsR-MqsA T-A pair was first related to motility and *flhD*[15]; MqsR-MqsA are related indirectly to motility via RpoS and c-di-GMP rather than via the direct control of *flhD* transcription by MqsA.

As the production of proteins from plasmids should be interpreted with caution, we corroborated many of the results obtained from expressing MqsA from a plasmid by also observing the effects of deleting *mqsRA*. c-di-GMP levels, response to oxidative stress and acid stress, curli and cellulose production, biofilm formation, *rpoS* mRNA levels and *katE* mRNA levels were all shown to be altered by the deletion of *mqsRA*. In addition, we used a strain that has a single copy of *lacZ* transcribed from the *rpoS* promoter in the chromosome as a reporter to investigate the regulatory effects of MqsA on

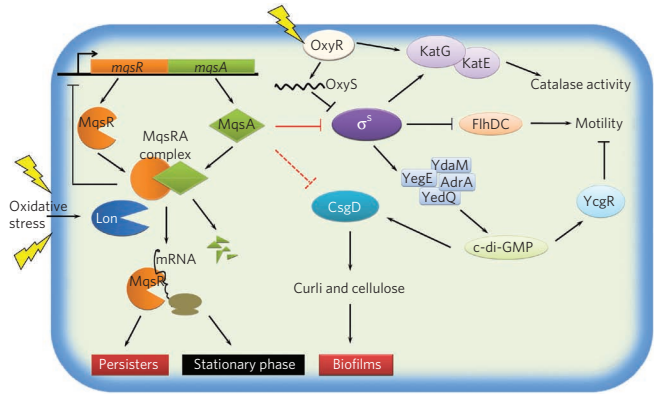

Figure 6 | Schematic of the interaction of antitoxin MqsA with *rpoS* and its impact on the stress response. Transcription of *rpoS* is repressed by MqsA through direct binding. Under oxidative stress, MqsA is degraded by protease Lon and *rpoS* transcription is derepressed. The increase in *rpoS* transcription leads to an increase in σ^S activity, which induces genes encoding catalase, c-di-GMP and curli and cellulose production and represses the genes encoding the master regulator of motility, FlhDC. Upon oxidative stress, OxyR induces *katG* and the regulatory RNA OxyS, which inhibits translation of the *rpoS* message. In addition, the RNase activity of MqsR may serve to rapidly direct the cell toward the translation of newly transcribed, stress-related transcripts and leads to the formation of persister cells. The lightning bolt indicates oxidative stress, → indicates induction and ⊥ indicates repression. Moreover, MqsA represses *csgD*, probably via the *mqsRA*-like palindrome in the promoter region as indicated by a dotted line (direct binding studies were not performed here).

rpoS transcription (while leaving the wild-type *rpoS* system intact) and found that MqsA binds directly to the *rpoS* promoter region and represses *rpoS* transcription (**Fig. 1**). When the *rpoS* palindrome was mutated to disrupt the sequence-specific DNA binding, MqsA binding to the promoter is abolished (**Fig. 1b**) and no longer represses *rpoS* transcription (**Fig. 1d**). The dependence of MqsA on this palindrome was also demonstrated by the inability of MqsA to increase motility when the palindrome in the native *rpoS* promoter was mutated (**Fig. 3c**). As the palindrome lies upstream (144 bp) of the major transcription start site of *rpoS*, further investigation is needed to elucidate whether the repression of MqsA involves cooperative binding (two smaller *mqsRA*-type palindromes are also present in P*rpoS*).

A schematic of our understanding of how the antitoxin MqsA is involved in mediating the general stress response and biofilm formation is shown in **Figure 6**. Note regulation of RpoS is complex and includes regulation at the level of transcription, translation and protein stability and activity[20]; hence, **Figure 6** is a simplification and shows primarily the features important for MqsA. Our results show clearly that MqsA is degraded in the presence of oxidative stress (**Fig. 5a**,**b**); this degradation leads to derepression of *rpoS* transcription (**Fig. 5c**) and an increase in *rpoS* mRNA, and that production of Lon prevents MqsA from increasing motility (**Fig. 5e**). Hence, the protease Lon degrades MqsA upon stress. These results are consistent with our previous results indicating MqsR is not toxic in a *lon*-deletion background[18]. Hence, upon oxidative stress, Lon is induced and degrades MqsA like other antitoxins, *rpoS* is derepressed, and the cell directs transcription toward stress-related genes, which includes increasing c-di-GMP concentrations and catalase activity. It appears that MqsA may also regulate directly other stress-related genes such as *csgD*, *rnc* and *sspA* as identified by the palindrome search (**Supplementary Table 3**). In addition, upon oxidative stress, transcriptional dual regulator OxyR induces *katG*[42] and the regulatory RNA OxyS[32], which inhibits translation of the *rpoS* message[43]. Thus, RpoS must be tightly controlled, as cells that are stress resistant grow more slowly than those not using RpoS because resources are directed away from σ^{70}-related genes that are used for growth[44]. RpoS controls over 70 genes important for resistance to oxidative stress, UV-radiation, heat shock, hyperosmolarity, low pH and ethanol[44], so the impact of T-A systems may be involved in diverse stresses.

Although speculative, our model has some other implications for cell physiology. As the antitoxin MqsA is degraded rapidly upon stress (**Fig. 5**) and *mqsRA* is induced upon stress[18], it is expected that the RNase activity of MqsR would be increased during stress; this enhanced MqsR activity may serve to rapidly direct the cell toward the translation of newly transcribed, stress-related transcripts by degrading the older σ^{70}-related mRNA. The duration of MqsR RNase activity, from a burst to sustained RNase activity, would dictate whether the cell responds to the stress by rapidly producing new proteins necessary to withstand stress while forming a biofilm (higher RpoS activity during stress would elevate c-di-GMP levels and increase biofilm formation) or whether the cell becomes dormant (that is, a persister cell) in a biofilm and avoids the stress by not metabolizing. Hence, our model suggests that persister cell formation may be viewed in part as an extreme example of the general stress response mediated by the MqsR-MqsA T-A system. In support of this model, dormant cells have been generated by inducing the general stress response after DNA damage[45], and persister cell formation for fluoroquinolone antibiotics is dependent on the general stress response[46]. In addition, *mqsR* is induced in biofilms[16] and is the most induced gene in persister cells[4], and the number of persister cells decreases upon deletion of *mqsR*[17].

Our current results indicate that the formerly ambiguous T-A systems, especially MqsR-MqsA, are key regulators of gene activity by controlling in part the induction of the stress response, which, in turn, leads to enhanced biofilm formation and reduced motility. Therefore, T-A systems have a broad and an important impact on cell physiology by influencing such developmental cascades as the switch from planktonic cells to biofilm cells.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids are listed in **Supplementary Table 1**. LB medium at 37 °C was used except where indicated.

Construction of Δ6, Δ5 Δ*lacZ* and Δ6 Δ*lacZ*. P1 transduction was used to transfer the Δ*mqsRA* Kan^R mutation from BW25113 *mqsRA*[17] to Δ5 (ref. 2) to form Δ6. Removal of *mqsRA*, *relBE*, *dinJ*, *yefM*, *mazEF* and *chpB* in Δ6 was verified by PCR using primers shown in **Supplementary Table 4**. P1 transduction was also used to remove *lacZYA* from Δ5 and from Δ6 using BW25993 DE(*lacZYA*)*514* (ref. 47).

***rpoS* promoter activity.** The 943 bp upstream of the *rpoS* start codon were amplified using primers P*rpoS*-*Xho*I and P*rpoS*-*Bam*HI. To form pKNOCK-P*rpoS* (**Supplementary Fig. 4**), the PCR product was cloned into the suicide vector pKNOCK-Tc[29] at the *Xho*I and *Bam*HI sites upstream of a *lacZ* cassette that includes the native *lacZ* translational initiation region cloned into the *Not*I site. The P*rpoS*::*lacZ* reporter was integrated as a single copy into the chromosome of Δ5 Δ*lacZ* and Δ6 Δ*lacZ*, leaving an intact copy of *rpoS* with its native promoter. Δ5 R1 P*rpoS* and Δ6 R1 P*rpoS* (**Supplementary Fig. 4**), were verified by PCR (**Fig. 1c**, *nlpD*-F/pKNOCK-R and pKNOCK-F/*rpoS*-R) and by DNA sequencing.

Palindrome mutagenesis. The *mqsRA*-like palindrome in the P*rpoS*::*lacZ* fusion was mutated using PCR with template pKNOCK-P*rpoS* and primers P*rpoS*-M-F and P*rpoS*-M-R to form pKNOCK-P*rpoS*-M (**Supplementary Fig. 4**). Conjugating this vector into Δ6 Δ*lacZ* led to two strains: Δ6 R2 P*rpoS*-M with mutated palindrome upstream of *lacZ* and the native promoter *rpoS* unchanged and Δ6 R3 P*rpoS* with mutated palindrome upstream of *rpoS* and the wild-type palindrome upstream of *lacZ* gene (**Fig. 1c**). Correct integration was verified by PCR (**Fig. 1c**, Set 1: *nlpD*-F and pKNOCK-R, and Set 2: pKNOCK-F and *rpoS*-R), then DNA sequencing at both the *lacZ* and *rpoS* promoter regions.

β-galactosidase activity assay. β-galactosidase activity[48] was determined with strains grown in low-salt LB medium (LB with 0.5 g l^{-1} NaCl) to a turbidity at 600 nm of ~1.0, then IPTG (0.1 mM) was added to induce production of MqsA via pCA24N-*mqsA*. For experiments with 20 mM H_2O_2, strains were grown to turbidity ~3.0.

Survival assays. Overnight cultures were diluted to a turbidity of 0.05 and grown to a turbidity of 0.5, then 1 mM IPTG was used to induce *mqsA* for 2 h. Cells were centrifuged and resuspended in LB to a turbidity of 1.0 and exposed to either 20 mM H_2O_2 for 10 min or pH 2.5 for 10 min.

c-di-GMP assay. c-di-GMP was quantified using HPLC[48]. Strains were grown for 2.5 h, then 0.5 mM IPTG was added to induce *mqsA* for 15 h. c-di-GMP (BIOLOG Life Science Institute) was used as a standard and to verify the c-di-GMP peak via spiking.

Swimming motility, curli and cellulose and catalase assays. Cell motility was examined on motility agar plates (1% tryptone, 0.25% NaCl and 0.3% agar (w/v)). Curli and cellulose production was quantified by the Congo red–binding assay performed at 30 °C[30] in the presence of oxidative stress (2 mM H_2O_2 for 180 min). IPTG (0.1 mM) was added in both assays to induce *mqsA* and *mqsR* via the pCA24N-based plasmids. Catalase activity was quantified by a colorimetric assay[49] using dicarboxidine and lactoperoxidase to detect the remaining H_2O_2.

Crystal violet biofilm assay. Biofilm formation was assayed without shaking using 0.1% crystal violet staining[16] in 96-well polystyrene plates after 24 h using M9 glucose (0.2% (w/v)) medium with 1 mM of IPTG to induce *mqsA* (initial turbidity of 0.05).

Quantitative real-time reverse-transcription PCR (qRT-PCR). After the isolation of RNA[17] using RNA*later* (Ambion), 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* was used to normalize the data. To investigate the regulation of promoters by MqsA under oxidative stress conditions, overnight cultures of Δ6/pCA24N-*mqsA* and Δ6/pCA24N were regrown to a turbidity of 0.5, then 1 mM IPTG was added for 2 h to induce *mqsA,* and after diluting to turbidity ~1.0, we exposed the cells to 20 mM H_2O_2 for 10 min. To investigate the *rpoS* and *katE* mRNA changes, overnight cultures were inoculated into LB low-salt medium with an initial turbidity of 0.2 and grown until a turbidity ~3.0. After being diluted to a turbidity ~1.0, cells were exposed to 20 mM H_2O_2 for 30 s and 10 min.

Electrophoretic mobility shift assay (EMSA). Complimentary oligos (25-mers) labeled with biotin at the 3′ end corresponding to the wild-type *mqsRA*-like palindrome and the corresponding mutated palindrome of the *rpoS* promoter (**Supplementary Table 4**) were purchased, solubilized and annealed as previously described[28]. For EMSA binding reactions, biotin-P*rpoS* was incubated with purified MqsA[8] either with or without unlabeled P*rpoS* DNA for 20 min at room temperature. Samples were run on a 6% DNA retardation gel (Invitrogen) at 100V in 0.5× TBE for 75 min. DNA was then transferred to a nylon membrane at 390 mA for 45 min then UV cross-linked at 302 nm. Chemiluminescence was performed with the LightShift Chemiluminescent EMSA Kit (Thermo Scientific), and samples were detected with a CCD imager (Typhoon 9410 Imager).

Western blot analysis. For MqsA, strains were grown to a turbidity of 0.1, then 0.5 mM ITPG was added to induce *mqsA*. When the turbidity reached 1, 200 μg ml^{-1} rifampin was added to inhibit transcription, and 20 mM H_2O_2 was added. After various times, samples were processed with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich), and the western blot was performed with primary antibodies raised against a His tag (Cell Signaling Technology) and horseradish peroxidase–conjugated goat anti-mouse secondary antibodies (Millipore). For RpoS, strains were grown until a turbidity ~3.0 and were exposed to 20 mM H_2O_2, and anti-RpoS monoclonal antibody (Neoclone) was used.

Palindrome search. The motifs 5′-ACCT $(N)_{2-4}$ AGGT were identified using the Biostrings and BSgenome libraries in the R statistical package (version 2.9.2) and Fuzznuc (EMBOSS)[50].

Statistical analysis. Data are presented as means ± s.e.m. of three or more independent cultures. Statistical significance was assessed using two-tailed unpaired Student's *t*-test.

Received 23 September 2010; accepted 10 February 2011; published online 24 April 2011

References

1. Gerdes, K., Christensen, S.K. & Løbner-Olesen, A. Prokaryotic toxin-antitoxin stress response loci. *Nat. Rev. Microbiol.* **3**, 371–382 (2005).
2. Tsilibaris, V., Maenhaut-Michel, G., Mine, N. & Van Melderen, L. What is the benefit to *Escherichia coli* of having multiple toxin-antitoxin systems in its genome? *J. Bacteriol.* **189**, 6101–6108 (2007).
3. Magnuson, R.D. Hypothetical functions of toxin-antitoxin systems. *J. Bacteriol.* **189**, 6089–6092 (2007).
4. Shah, D. *et al.* Persisters: A distinct physiological state of *E. coli*. *BMC Microbiol.* **6**, 53 (2006).
5. Pecota, D.C. & Wood, T.K. Exclusion of T4 phage by the *hok*/*sok* killer locus from plasmid R1. *J. Bacteriol.* **178**, 2044–2050 (1996).
6. Kim, Y., Wang, X., Ma, Q., Zhang, X.-S. & Wood, T.K. Toxin-antitoxin systems in *Escherichia coli* influence biofilm formation through YjgK (TabA) and fimbriae. *J. Bacteriol.* **191**, 1258–1267 (2009).
7. Yamaguchi, Y., Park, J.-H. & Inouye, M. MqsR, a crucial regulator for quorum sensing and biofilm formation, is a GCU-specific mRNA interferase in *Escherichia coli*. *J. Biol. Chem.* **284**, 28746–28753 (2009).
8. Brown, B.L. *et al.* Three dimensional structure of the MqsR:MqsA complex: A novel TA pair comprised of a toxin homologous to RelE and an antitoxin with unique properties. *PLoS Pathog.* **5**, e1000706 (2009).
9. Aizenman, E., Engelberg-Kulka, H. & Glaser, G. An *Escherichia coli* chromosomal "addiction module" regulated by 3′,5′-bispyrophosphate: A model for programmed bacterial cell death. *Proc. Natl. Acad. Sci. USA* **93**, 6059–6063 (1996).
10. Gotfredsen, M. & Gerdes, K. The *Escherichia coli relBE* genes belong to a new toxin-antitoxin gene family. *Mol. Microbiol.* **29**, 1065–1076 (1998).
11. Masuda, Y., Miyakawa, K., Nishimura, Y. & Ohtsubo, E. *chpA* and *chpB*, *Escherichia coli* chromosomal homologs of the *pem* locus responsible for stable maintenance of plasmid R100. *J. Bacteriol.* **175**, 6850–6856 (1993).
12. Cherny, I. & Gazit, E. The YefM antitoxin defines a family of natively unfolded proteins: Implications as a novel antibacterial target. *J. Biol. Chem.* **279**, 8252–8261 (2004).
13. Motiejūnaite, R., Armalytė, J., Markuckas, A. & Sužiedėlienė, E. *Escherichia coli dinJ-yafQ* genes act as a toxin-antitoxin module. *FEMS Microbiol. Lett.* **268**, 112–119 (2007).
14. Schmidt, O. *et al. prlF* and *yhaV* encode a new toxin-antitoxin system in *Escherichia coli*. *J. Mol. Biol.* **372**, 894–905 (2007).
15. González Barrios, A.F. *et al.* Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). *J. Bacteriol.* **188**, 305–316 (2006).
16. Ren, D., Bedzyk, L.A., Thomas, S.M., Ye, R.W. & Wood, T.K. Gene expression in *Escherichia coli* biofilms. *Appl. Microbiol. Biotechnol.* **64**, 515–524 (2004).
17. Kim, Y. & Wood, T.K. Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **391**, 209–213 (2010).
18. Kim, Y. *et al. Escherichia coli* toxin/antitoxin pair MqsR/MqsA regulate toxin CspD. *Environ. Microbiol.* **12**, 1105–1121 (2010).
19. Pesavento, C. *et al.* Inverse regulatory coordination of motility and curli-mediated adhesion in *Escherichia coli*. *Genes Dev.* **22**, 2434–2446 (2008).
20. Hengge, R. The two-component network and the general stress sigma factor RpoS (σ^S) in *Escherichia coli*. in *Bacterial Signal Transduction: Networks and Drug Targets* 40–53 (Springer, New York, 2008).
21. Boehm, A. *et al.* Second messenger-mediated adjustment of bacterial swimming velocity. *Cell* **141**, 107–116 (2010).
22. Pesavento, C. & Hengge, R. Bacterial nucleotide-based second messengers. *Curr. Opin. Microbiol.* **12**, 170–176 (2009).
23. Sammartano, L.J., Tuveson, R.W. & Davenport, R. Control of sensitivity to inactivation by H_2O_2 and broad-spectrum near-UV radiation by the *Escherichia coli katF* locus. *J. Bacteriol.* **168**, 13–21 (1986).
24. Storz, G. & Imlay, J.A. Oxidative stress. *Curr. Opin. Microbiol.* **2**, 188–194 (1999).
25. Lacour, S. & Landini, P. σ^S-dependent gene expression at the onset of stationary phase in *Escherichia coli*: Function of σ^S-dependent genes and identification of their promoter sequences. *J. Bacteriol.* **186**, 7186–7195 (2004).
26. Baba, T. *et al.* Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**, 2006.0008 (2006).
27. Landini, P. Cross-talk mechanisms in biofilm formation and responses to environmental and physiological stress in *Escherichia coli*. *Res. Microbiol.* **160**, 259–266 (2009).
28. Brown, B.L., Wood, T.K., Peti, W. & Page, R. Structure of the *Escherichia coli* antitoxin MqsA (YgiT/b3021) bound to its gene promoter reveals extensive domain rearrangements and the specificity of transcriptional regulation. *J. Biol. Chem.* **286**, 2285–2296 (2011).
29. Alexeyev, M.F. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. *Biotechniques* **26**, 824–828 (1999).
30. Ma, Q. & Wood, T.K. OmpA influences *Escherichia coli* biofilm formation by repressing cellulose production through the CpxAR two-component system. *Environ. Microbiol.* **11**, 2735–2746 (2009).
31. Donegan, N.P., Thompson, E.T., Fu, Z. & Cheung, A.L. Proteolytic regulation of toxin-antitoxin systems by ClpPC in *Staphylococcus aureus*. *J. Bacteriol.* **192**, 1416–1422 (2010).
32. Altuvia, S., Weinstein-Fischer, D., Zhang, A., Postow, L. & Storz, G. A small, stable RNA induced by oxidative stress: Role as a pleiotropic regulator and antimutator. *Cell* **90**, 43–53 (1997).

33. Van Melderen, L., Bernard, P. & Couturier, M. Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria. *Mol. Microbiol.* **11**, 1151–1157 (1994).
34. Christensen, S.K., Mikkelsen, M., Pedersen, K. & Gerdes, K. RelE, a global inhibitor of translation, is activated during nutritional stress. *Proc. Natl. Acad. Sci. USA* **98**, 14328–14333 (2001).
35. Kasari, V., Kurg, K., Margus, T., Tenson, T. & Kaldalu, N. The *Escherichia coli mqsR* and *ygiT* genes encode a new toxin-antitoxin pair. *J. Bacteriol.* **192**, 2908–2919 (2010).
36. Kolodkin-Gal, I., Verdiger, R., Shlosberg-Fedida, A. & Engelberg-Kulka, H. A differential effect of *E. coli* toxin-antitoxin systems on cell death in liquid media and biofilm formation. *PLoS ONE* **4**, e6785 (2009).
37. Zhang, X.-S., García-Contreras, R. & Wood, T.K. YcfR (BhsA) influences *Escherichia coli* biofilm formation through stress response and surface hydrophobicity. *J. Bacteriol.* **189**, 3051–3062 (2007).
38. Ito, A., May, T., Kawata, K. & Okabe, S. Significance of *rpoS* during maturation of *Escherichia coli* biofilms. *Biotechnol. Bioeng.* **99**, 1462–1471 (2008).
39. Domka, J., Lee, J., Bansal, T. & Wood, T.K. Temporal gene-expression in *Escherichia coli* K-12 biofilms. *Environ. Microbiol.* **9**, 332–346 (2007).
40. Ito, A., Taniuchi, A., May, T., Kawata, K. & Okabe, S. Increased antibiotic resistance of *Escherichia coli* in mature biofilms. *Appl. Environ. Microbiol.* **75**, 4093–4100 (2009).
41. Ito, A., May, T., Taniuchi, A., Kawata, K. & Okabe, S. Localized expression profiles of *rpoS* in *Escherichia coli* biofilms. *Biotechnol. Bioeng.* **103**, 975–983 (2009).
42. Zheng, M. *et al.* DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J. Bacteriol.* **183**, 4562–4570 (2001).
43. Zhang, A. *et al.* The OxyS regulatory RNA represses *rpoS* translation and binds the Hfq (HF-I) protein. *EMBO J.* **17**, 6061–6068 (1998).
44. Gruber, T.M. & Gross, C.A. Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu. Rev. Microbiol.* **57**, 441–466 (2003).
45. Pennington, J.M. & Rosenberg, S.M. Spontaneous DNA breakage in single living *Escherichia coli* cells. *Nat. Genet.* **39**, 797–802 (2007).
46. Dörr, T., Lewis, K. & Vulić, M. SOS response induces persistence to fluoroquinolones in *Escherichia coli*. *PLoS Genet.* **5**, e1000760 (2009).
47. Datsenko, K.A. & Wanner, B.L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**, 6640–6645 (2000).
48. Ueda, A. & Wood, T.K. Connecting quorum sensing, c-di-GMP, Pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). *PLoS Pathog.* **5**, e1000483 (2009).
49. Macvanin, M. & Hughes, D. Assays of sensitivity of antibiotic-resistant bacteria to hydrogen peroxide and measurement of catalase activity. in *Antibiotic Resistance Protocols: Second Edition, Methods in Molecular Biology* Vol. 642 (Gillespie, S.H. & McHugh, T.D., eds), 95–103 (Humana Press, 2010).
50. Rice, P., Longden, I. & Bleasby, A. EMBOSS: The European Molecular Biology Open Software Suite (2000). *Trends Genet.* **16**, 276–277 (2000).

Acknowledgments

This work was supported by grants from the US National Institutes of Health to T.K.W. and W.P. and an F31 fellowship to B.L.B. and by a US National Science Foundation CAREER award to R.P. We are grateful for the Keio and ASKA strains provided by the Genome Analysis Project in Japan.

Author contributions

T.K.W. conceived the project, T.K.W., X.W., R.P., W.P. and M.J.B. designed the experiments. X.W., S.H.H., Q.M., B.L.B., M.P. and Y.K. performed the experiments. M.J.B. and A.M.T. performed the bioinformatics. All authors discussed the results and commented on the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available online at http://www.nature.com/naturechemicalbiology/. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to T.K.W.