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Escherichia coli toxin/antitoxin pair MqsR/MqsA regulate toxin CspD

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Summary

Previously we identified that the *Escherichia coli* protein MqsR (YgiU) functions as a toxin and that it is involved in the regulation of motility by quorum sensing signal autoinducer-2 (AI-2). Furthermore, MqsR is directly associated with biofilm development and is linked to the development of persister cells. Here we show that MqsR and MqsA (YgiT) are a toxin/antitoxin (TA) pair, which, in significant difference to other TA pairs, regulates additional loci besides its own. We have recently identified that MqsR functions as an RNase. However, using three sets of whole-transcriptome studies and two nickel-enrichment DNA binding microarrays coupled with cell survival studies in which MqsR was overproduced in isogenic mutants, we identified eight genes (*cspD*, *clpX*, *clpP*, *lon*, *yfjZ*, *relB*, *relE* and *hokA*) that are involved in a mode of MqsR toxicity in addition to its RNase activity. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) showed that (i) the MqsR/MqsA complex (and MqsA alone) represses the toxin gene *cspD*, (ii) MqsR overproduction induces *cspD*, (iii) stress induces *cspD*, and (iv) stress fails to induce *cspD* when MqsR/MqsA are overproduced or when *mqsRA* is deleted. Electrophoretic mobility shift assays show that the MqsA/MqsR complex binds the promoter of *cspD*. In addition, proteases Lon and ClpXP are necessary for MqsR toxicity. Together, these results indicate the MqsR/MqsA complex represses *cspD* which may be derepressed by titrating MqsA with MqsR or by degrading MqsA via stress conditions through proteases Lon and ClpXP. Hence, we demonstrate that the MqsR/MqsA TA system controls cell physiology via its own toxicity as well as through its regulation of another toxin, CspD.

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Introduction

Toxin/antitoxin (TA) systems are ubiquitous (Gerdes *et al.*, 2005) in bacterial chromosomes and in low-copy-number plasmids where they appear to stabilize these systems (Engelberg-Kulka *et al.*, 2006). TA systems typically consist of pairs of genes located in one operon, which encode two components, a stable toxin that can cause cell death by disrupting an essential cellular process and a labile antitoxin that can bind and form a tight complex with the toxin and neutralize its activity (Brown and Shaw, 2003). Influencing these TA systems may provide new antimicrobial agents; for example, agents that bind the antitoxin may free the toxin to cause bacteriostatic or bacteriocidal effects (Nieto *et al.*, 2007). Many chromosomal TA systems have been characterized in *Escherichia coli* including (listed as toxin/antitoxin) MazF/MazE (Aizenman *et al.*, 1996), RelE/RelB (Gotfredsen and Gerdes, 1998), ChpB/ChpS (Masuda *et al.*, 1993), YoeB/YefM (Cherny and Gazit, 2004), YafQ/DinJ (Motiejūnaitė *et al.*, 2007) and YhaV/PrlF (Schmidt *et al.*, 2007). Although the mechanism of toxicity at the molecular level is slightly different, MazF (Gerdes *et al.*, 2005), RelE (Gerdes *et al.*, 2005), ChpB (Gerdes *et al.*, 2005), YoeB (Christensen *et al.*, 2004) and YhaV (Schmidt *et al.*, 2007) prevent translation by cleaving RNAs; the mode of translation inhibition by YafQ is currently still unclear (Tsilibaris *et al.*, 2007). CcdB of the F plasmid inhibits gyrase while antitoxin CcdA actively dissociates the CcdB:gyrase complex in a process termed rejuvenation (De Jonge *et al.*, 2009).

The role of TA systems in cell physiology is not well understood, and indeed nine hypothetical biological functions of TA systems have been proposed (Magnuson, 2007) (the last suggested by us): addictive genomic debris, stabilization of genomic parasites, selfish alleles, gene regulation, growth control, persister formation, programmed cell arrest, programmed cell death and antiphage measures (Pecota and Wood, 1996). In regard to programmed cell death, there have been few clear

examples of toxin-mediated cell death in a physiologically relevant situation (Pandey and Gerdes, 2005; Magnuson, 2007). Our hypothesis is that TA system toxicity in bacteria is linked to biofilm formation, dispersal and quorum sensing (QS). For example, we found recently that the deletion of five *E. coli* TA systems influences biofilm development through fimbriae and dispersal (Kim *et al*., 2009). Also, we identified that the toxin Hha controls cell death and biofilm dispersal via its activation of prophage lytic genes (e.g. *rzpD*, *yfjZ*, *appY* and *alpA*) and several proteases (e.g. Lon and ClpXP) which may, in turn, activate toxins by degrading antitoxins (García-Contreras *et al*., 2008). Similarly, the putative holin Cid and anti-holin Lrg system of *Staphylococcus aureus* links cell death and lysis to biofilm development (Bayles, 2007). Hence, TA systems are directly related to biofilm formation.

The benefits of cell death for biofilm dispersal have been shown clearly using prophage. Autolysis via prophage Pf4 allows *Pseudomonas aeruginosa* cells to disperse from the biofilm matrix (Webb *et al*., 2003) and undergo phenotypic variation (Webb *et al*., 2004). Also, in the case of *Pseudoalteromonas tunicata,* it uses the autolytic protein AlpP for autolysis for biofilm dispersal (Mai-Prochnow *et al*., 2006). Furthermore, prophage control biofilm architecture, dispersal and virulence (Rice *et al*., 2009). In *E. coli*, we have shown that cryptic prophage CP4-57 affects motility, metabolism and biofilm formation (Wang *et al*., 2009). Hence, cell death via prophage has been linked to biofilm formation and may be necessary for dispersal. Thus, we propose a similar role for TA systems in that they may slow metabolism to influence biofilm formation.

Gerdes (2000) proposed that a realistic suicide hypothesis for a TA system must include a regulatory network system for sensing cell density such as QS signals. However, until now, a QS system has not been related previously to a TA pair. We discovered that MqsR (formerly YgiU) is induced in biofilms (Ren *et al*., 2004a) and that MqsR with antitoxin MqsA (YgiT) regulates directly or indirectly motility related to QS (González Barrios *et al*., 2006). This QS is directly associated with biofilm development as it mediates the ability of autoinducer 2 (AI-2) to increase *E. coli* biofilm formation (González Barrios *et al*., 2006). MqsR sequences are conserved in many genera including *Yersinia pseudotuberculosis*, *Y. pestis*, *Bordetella bronchiseptica* and *Pseudomonas fluorescens*. MqsR/MqsA influence biofilm formation via *qseBC*, which controls motility through *flhDC* (González Barrios *et al*., 2006). In addition, *mqsR* is the most highly induced gene in persister cells as compared with non-persisters (Shah *et al*., 2006). Furthermore, MqsR was hypothesized to be a toxin in conjunction with antitoxin MqsA, since deletion of *mqsA* is lethal (Baba *et al*., 2006; Shah *et al*., 2006). Previously, we showed that MqsR is a toxin (Zhang *et al*., 2008), and its three-dimensional structure revealed it is an RNase similar to RelE and YoeB (Brown *et al*., 2009). Furthermore, MqsA binds DNA via its helix–turn–helix (HTH) C-terminal domain (Brown *et al*., 2009). Unlike most TA pairs including RelE/RelB, YoeB/YefM and MazF/MazE, the MqsR toxin gene precedes the antitoxin gene in the bicistronic operon (Shah *et al*., 2006). Furthermore, the MqsR/MqsA TA pair is unique (Brown *et al*., 2009) in that (i) the antitoxin is larger than the toxin, (ii) both toxin and antitoxin are basic (typically the toxin is basic while the antitoxin is acidic), (iii) the *mqsRA* sequences are not homologous to any member of a recognized TA system, (iv) the antitoxin binds the toxin at its N-terminus and requires a metal, zinc, for structural stability, (v) the antitoxin is structured throughout its entire sequence, and (vi) the antitoxin binds more than its own promoter (e.g. *mqsRA*, *mcbR* and *spy*) via its C-terminal domain. Also, MqsR cleaves mRNA at GCU sites (Yamaguchi *et al*., 2009). Interestingly, six of the 14 MqsR-resistant mRNAs that lack MqsR-specific GCU sequences (*pheL*, *tnaC*, *trpL*, *yciG*, *ygaQ* and *ralR*) (Yamaguchi *et al*., 2009) have been shown by us to be regulated in biofilms (Domka *et al*., 2007).

Here, using whole-transcriptome analyses, regulator/DNA binding assays and isogenic mutants, we show eight proteins are involved in MqsR toxicity including toxin CspD; i.e. the MqsR/MqsA complex regulates *cspD* transcription. We also present evidence that MqsR toxicity is dependent on Lon and ClpXP protease activity.

Results

MqsR is toxic, MqsA reduces toxicity and MqsR/MqsA influence biofilm formation

We have shown previously that overproduction of MqsR is toxic (Zhang *et al*., 2008) and that MqsA diminishes the toxicity of MqsR in both BW25113 and MG1655 strains (Brown *et al*., 2009). More clearly, to explore the impact of MqsR toxicity on cell physiology, we constructed a *mqsR mqsA* double mutant and tested cell survival with or without MqsR production. Note that it is not possible to delete solely the gene that encodes the antitoxin, *mqsA* (Baba *et al*., 2006; Shah *et al*., 2006); similar results have been found with other antitoxins such as HigA of *Vibrio cholerae* (Budde *et al*., 2007). As expected, production of MqsR in the *mqsR mqsA* double mutant that lacks the antitoxin was much more toxic compared with production of MqsR in the single *mqsR* mutant (Fig. 1A). Also, overproduction of MqsR is not toxic when MqsA is also overproduced (Fig. 1A).

Since *mqsR* was discovered as an induced gene during biofilm formation (eightfold) (Ren *et al*., 2004a), we also tested whether MqsR/MqsA are linked to biofilm development using two K-12 strains, BW25113 and MG1655.

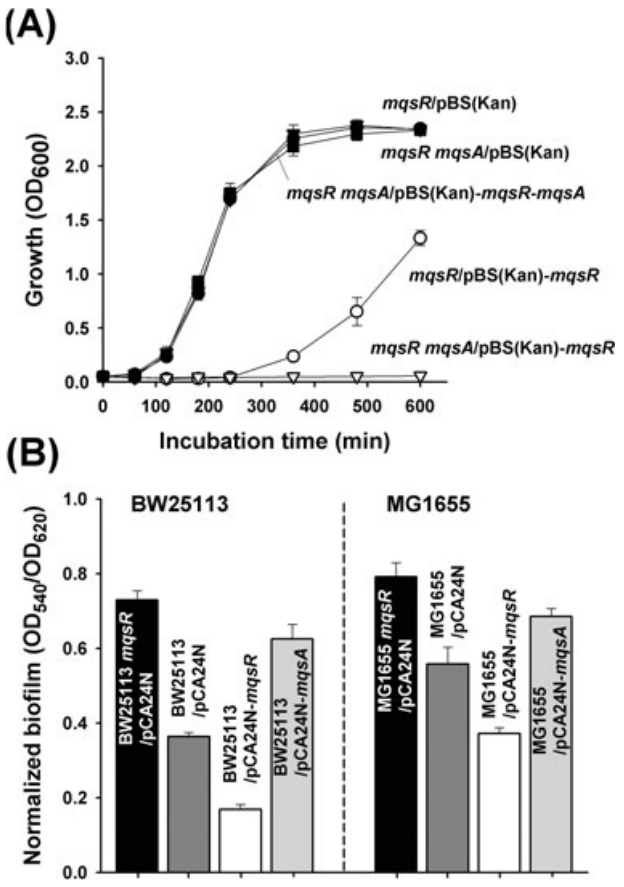

Fig. 1. Growth curves for BW25113 *mqsR* and BW25113 *mqsR mqsA* containing pBS(Kan) (empty plasmid), pBS(Kan)-*mqsR* and pBS(Kan)-*mqsR-mqsA* at 37°C in LB with 1 mM IPTG induction (A). Normalized biofilm formation (total biofilm/growth) with 96-well polystyrene plates at 37°C in LB with 1 mM IPTG induction for 24 h for *E. coli* BW25113 *mqsR*, BW25113, MG1655 *mqsR* and MG1655 containing pCA24N, pCA24N-*mqsA*, pCA24N-*mqsR* (B). Growth data are the average of two independent cultures, biofilm data are the average of 10 replicate wells from two independent cultures, and one standard deviation is shown.

Consistently, producing the antitoxin MqsA increased normalized biofilm formation (biofilm/growth) whereas overproducing MqsR reduced biofilm formation (Fig. 1B). Corroborating these effects, deleting *mqsR* increased biofilm formation in both strains (Fig. 1B). Taken together, these results confirm that MqsR is a toxin, that MqsA is the essential antitoxin for reducing MqsR toxicity, and that altering the ratio of MqsR/MqsA influences biofilm formation.

Identification of genes related to MqsR toxicity via whole-transcriptome analysis

We investigated the genes controlled by MqsR using whole-transcriptome analyses to investigate further its mode of toxicity beyond that as an RNase. We reasoned that the MqsR/MqsA complex may alter cell physiology in two ways when a stress is encountered: (i) unbound MqsR (after degradation of labile MqsA) decreases protein production via its degradation of mRNAs, and (ii) specific loci may be repressed/derepressed if MqsR/MqsA or MqsA alone serves to stimulate/repress transcription via the DNA-binding function of MqsA. In addition, we showed previously that MqsA regulates more than its own operon (Brown *et al*., 2009) as it also binds the promoters of *mcbR* and *spy*. To take advantage of the isogenic Keio library of single-gene knockouts (Baba *et al*., 2006), the remainder of the experiments were performed with BW25113.

Using planktonic cells, an initial whole-transcriptome study showed that upon *mqsR* deletion (BW25113 *mqsR* versus BW25113 at a turbidity of 0.5 at 600 nm), 239 genes are repressed by more than twofold while 76 genes are induced (Table 1). Among these, motility-related genes, including 16 flagella genes, six curli genes and six chemotaxis genes, were repressed by deleting *mqsR* (Table 1). These results were consistent with our initial report that AI-2 signalling and motility/curli are strongly influenced by MqsR/MqsA (González barrios *et al*., 2006) and suggest (i) that MqsA, in the absence of MqsR, represses motility genes directly, (ii) that MqsA represses a positive regulator of motility, or (iii) that MqsR degrades RNA of a positive regulator for motility. Significantly, *mqsR* deletion repressed *cspD*, which encodes a stress-induced DNA replication inhibitor (Yamanaka *et al*., 2001), and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) corroborated that *cspD* is repressed upon inactivating *mqsR* (3 ± 1-fold). This suggests MqsA represses *cspD* in the absence of MqsR. To confirm the hypothesis that MqsA represses *cspD* in the absence of MqsR, we quantified the transcript levels of *cspD* in BW25113 *mqsR mqsA* overproducing MqsA using qRT-PCR. As expected, *cspD* was repressed (2 ± 1-fold) by the overproduction of MqsA in the absence of MqsR. Therefore, MqsA appears to regulate *cspD* directly.

In addition, *hokA* and *hokE*, encoding small toxic polypeptides that damage the membrane (Pedersen and Gerdes, 1999), were repressed by deleting *mqsR*; hence, it is possible that HokA and HokE are also repressed by MqsA. Also repressed upon *mqsR* deletion were *gltL*, *gltK*, *gltJ* and *gltI* (encoding the glutamate/aspartate transport system), *cstA* (encoding the carbon starvation protein) (Schultz and Matin, 1991), *bssR* (encoding a repressor of biofilm formation involved in indole transport) (Domka *et al*., 2006), and *umuC* (encoding a protein involved in SOS mutagenesis and repair) (Shinagawa *et al*., 1983). Lastly, a number of genes encoding chaperone network proteins including *ibpA* (fivefold), *ibpB* (sixfold), *hslU* (twofold), *hslV* (threefold), *dnaK* (twofold) and *dnaJ* (threefold) were induced upon the deletion of *mqsR*.

Table 1. Partial list of differentially expressed genes for (i) planktonic BW25113 *mqsR* versus BW25113 at a turbidity of 0.5 at 600 nm, (ii) planktonic BW25113/pCA24N-*mqsR* versus BW25113/pCA24N with 2 mM IPTG added at a turbidity of 0.5 (grown for 3 h), and (iii) planktonic BW25113 *mqsR*/pCA24N-*mqsR* versus BW25113 *mqsR*/pCA24N grown to a turbidity of 0.5 and then 2 mM IPTG was added for 15 min.

		Fold change[a]			
Group and gene	b number	*mqsR* vs. WT	pCA24N-*mqsR* vs. pCA24N (3 h)	pCA24N-*mqsR* vs. pCA24N (15 min)	Description of encoded protein
Stress response related					
dnaK	b0014	**2.1**	1.5	**2.3**	Chaperone Hsp70, DNA biosynthesis, autoregulated heat shock proteins
dnaJ	b0015	**2.6**	1.6	**2.6**	Chaperone with DnaK, heat shock protein
clpP	b0437	1.5	2.3	1.9	ATP-dependent proteolytic subunit of ClpA-ClpP serine protease
lon	b0439	1.7	2.0	**2.8**	DNA-binding, ATP-dependent protease LA, *lon* deletion mutants form long cells
htpG	b0473	**2.5**	2.0	**3.7**	Heat shock chaperone, HSP90 family, has ATPase activity
cstA	b0598	**–2.0**	**4.3**	1.2	Carbon starvation protein
dps	b0812	1.6	**3.5**	1.0	Global regulator, starvation conditions
cspD	b0880	**–2.1**	**6.5**	–1.3	Stress-induced DNA replication inhibitor
umuC	b1184	**–2.0**	–1.8	1.0	SOS mutagenesis and repair
cspC	b1823	1.1	**5.7**	1.1	Cold shock protein
htpX	b1829	1.5	**5.3**	1.9	Heat shock protein, integral membrane protein
clpB	b2592	**2.8**	1.8	**4.6**	ATP-dependent protease and chaperone, protein disaggregation chaperone
rpoS	b2741	1.1	**3.7**	1.2	RNA polymerase, sigma S (sigma38) factor
sspA	b3229	1.1	**3.0**	1.2	Regulator of transcription, stringent starvation protein A
yrfH	b3400	**2.6**	1.4	**3.7**	Hsp15, DNA/RNA-binding heat shock protein
yrfI	b3401	**2.6**	1.0	**3.5**	Hsp33, redox-regulated chaperone
ibpB	b3686	**5.7**	1.6	**7.5**	Heat shock protein
ibpA	b3687	**4.9**	2.1	**8.6**	Heat shock protein
hslU	b3931	**2.1**	1.6	**3.2**	Heat shock protein HslVU, ATPase subunit, homologous to chaperones
hslV	b3932	**2.5**	1.6	**3.5**	Heat shock protein HslVU, proteasome-related peptidase subunit
osmY	b4376	**4.6**	1.7	–1.4	Hyperosmotically inducible periplasmic protein
Transcription, translation and regulation related					
fur	b0683	1.1	**3.2**	1.3	Ferric iron uptake global transcriptional repressor
bssR	b0836	**–3.3**	–1.3	1.2	Repressor of biofilm formation by indole transport regulation
bssS	b1060	1.2	**3.0**	**2.5**	Repressor of biofilm formation by indole transport regulation; global regulator, e.g. of AI-2 transport and motility genes
mqsA	b3021	**3.5**	**3.7**	**2.5**	Antitoxin part in MqsR-MqsA TA system
relE	b1563	–1.2	2.8	1.2	Toxin part in RelE-RelB TA system
relB	b1564	–1.2	2.8	1.2	Antitoxin part in RelE-RelB TA system
yhdM	b3292	1.5	1.7	**2.0**	Zn-responsive activator of *zntA* transcription
rpmF	b1089	–1.1	**4.0**	1.2	50S ribosomal subunit protein L32
rpsV	b1480	1.1	**5.3**	1.1	30S ribosomal subunit protein S22
rpsI	b3230	1.1	**3.2**	1.2	30S ribosomal subunit protein S9
rplN	b3310	1.1	1.9	**2.1**	50S ribosomal subunit protein L14
rplB	b3317	–1.3	1.9	**2.1**	50S ribosomal subunit protein L2, binds Zn (II)
rplW	b3318	1.1	1.7	**2.3**	50S ribosomal subunit protein L23
rplD	b3319	1.0	1.6	**2.0**	50S ribosomal subunit protein L4, erythromycin sensitivity
rplC	b3320	1.2	1.6	**2.1**	50S ribosomal subunit protein L3
rpsG	b3341	1.1	1.9	**2.0**	30S ribosomal subunit protein S7
Cell wall/membrane and motility/chemotaxis					
csgG	b1037	**–2.1**	–2.1	1.1	Curli production assembly/transport component, 2nd curli operons
csgF	b1038	**–2.5**	–2.1	1.1	Curli production assembly/transport component, 2nd curli operons
csgE	b1039	**–2.6**	–2.1	1.0	Curli production assembly/transport component, 2nd curli operons
csgD	b1040	**–2.0**	–1.6	1.2	Putative 2-component transcriptional regulator for 2nd curli operons
csgB	b1041	**–2.5**	–2.0	1.0	Minor curlin subunit precursor, similar to CsgA
csgA	b1042	**–2.0**	–1.6	1.0	Curlin major subunit, coiled surface structures, cryptic
csgC	b1043	**–2.1**	–1.6	1.0	Putative curli production protein
flgN	b1070	**–2.5**	–1.5	1.1	Protein of flagellar biosynthesis
flgM	b1071	**–2.3**	–1.3	1.0	Anti-FliA (anti-sigma) factor, also known as RflB protein
flgA	b1072	**–2.5**	–1.5	–1.2	Flagellar biosynthesis, assembly of basal-body periplasmic P ring
flgB	b1073	**–2.1**	–2.0	–1.5	Flagellar biosynthesis, cell-proximal portion of basal-body rod
flgC	b1074	**–2.0**	–1.8	–1.5	Flagellar biosynthesis, cell-proximal portion of basal-body rod
flgD	b1075	**–2.1**	–2.1	–1.3	Flagellar biosynthesis, initiation of hook assembly
flgF	b1077	**–2.1**	–2.8	–1.4	Flagellar biosynthesis, cell-proximal portion of basal-body rod
flgI	b1080	**–2.5**	–2.4	1.0	Homologue of *Salmonella* P-ring of flagella basal body
flgK	b1082	**–2.3**	–1.8	–1.1	Flagellar biosynthesis, hook–filament junction protein 1

Table 1. *cont.*

Group and gene	b number	Fold change[a] *mqsR* vs. WT	Fold change[a] pCA24N-*mqsR* vs. pCA24N (3 h)	Fold change[a] pCA24N-*mqsR* vs. pCA24N (15 min)	Description of encoded protein
flgL	b1083	**–2.5**	–2.0	1.0	Flagellar biosynthesis; hook–filament junction protein
flhE	b1878	**–2.0**	–2.2	–1.2	Flagellar protein
flhB	b1880	**–2.0**	–2.6	1.0	Putative part of export apparatus for flagellar proteins
cheZ	b1881	**–2.3**	–1.8	1.0	Chemotactic response, CheY protein phophatase
cheY	b1882	**–2.6**	–2.2	–1.1	Chemotaxis regulator transmits chemoreceptor signals to flagellar motor components
cheB	b1883	**–2.6**	–2.2	–1.1	Response regulator for chemotaxis (CheA sensor), protein methylesterase
cheR	b1884	**–2.3**	–1.5	–1.1	Response regulator for chemotaxis, protein glutamate methyltransferase
cheW	b1887	**–3.3**	–2.4	–1.1	Positive regulator of CheA protein activity
cheA	b1888	**–3.3**	–2.0	1.0	Sensory transducer kinase between CheB and CheY
fliC	b1923	**–4.9**	1.1	–1.1	Flagellar biosynthesis, flagellin, filament structural protein
fliD	b1924	**–2.8**	–1.7	1.0	Flagellar biosynthesis, filament capping protein; enables filament assembly
fliS	b1925	**–2.8**	–2.1	–1.2	Flagellar biosynthesis, repressor of class 3a and 3b operons (RflA activity)
fliT	b1926	**–2.5**	–1.7	1.0	Flagellar biosynthesis, repressor of class 3a and 3b operons (RflA activity)
hokE	b4415	**–2.0**	**–3.7**	1.2	Small toxic membrane polypeptide
hokA	b4455	**–2.1**	**–4.9**	–1.1	Small toxic membrane polypeptide
hokD	b1562	–1.5	**9.8**	1.0	Polypeptide destructive to membrane potential
Metabolism related					
gltL	b0652	**–4.0**	–1.1	1.1	ATP-binding protein of glutamate/aspartate transport system
gltK	b0653	**–5.3**	1.1	–1.1	Glutamate/aspartate transport system permease
gltJ	b0654	**–6.5**	1.1	1.0	Glutamate/aspartate transport system permease
gltI	b0655	**–4.9**	2.0	1.2	Putative periplasmic binding transport protein
putA	b1014	**–2.0**	**6.1**	1.2	Proline dehydrogenase, P5C dehydrogenase
putP	b1015	**–4.3**	**6.1**	1.4	Major sodiumproline symporter
add	b1623	**3.5**	2.4	–1.3	Adenosine deaminase
katE	b1732	**2.0**	–1.3	–1.3	Catalase, hydroperoxidase HPII(III)
yeiN	b2165	**–6.5**	–1.2	–1.2	Hypothetical protein
yeiC	b2166	**–6.5**	–1.1	–1.2	Putative kinase
glpT	b2240	–1.7	**–4.0**	–1.4	sn-glycerol-3-phosphate permease
glpA	b2241	–1.4	**–4.3**	–1.5	Anaerobic glycerol-3-phosphate dehydrogenase subunit A
glpB	b2242	–1.3	**–4.9**	–1.7	sn-glycerol-3-phosphate dehydrogenase (anaerobic)
glpC	b2243	–1.4	**–7.5**	–1.8	sn-glycerol-3-phosphate dehydrogenase (anaerobic), K-small subunit
glpD	b3426	1.1	**–10.6**	1.2	sn-glycerol-3-phosphate dehydrogenase (aerobic)
glpK	b3926	1.1	**–4.0**	–1.3	Glycerol kinase
glpF	b3927	1.0	**–5.3**	1.1	Facilitated diffusion of glycerol
dsdA	b2366	**4.0**	–1.1	**–2.0**	D-serine dehydratase (deaminase)
cysK	b2414	1.3	**7.0**	–1.1	Cysteine synthase A, *o*-acetylserine sulfhydrolase A
cysA	b2422	–1.5	**5.7**	–1.1	ATP-binding component of sulfate permease A protein
eutG	b2453	–1.6	**–4.6**	–1.1	Ethanolamine, similar to iron-containing alcohol dehydrogenase
pheL	b2598	–1.9	**5.7**	–1.2	Leader peptide of chorismate mutase-P-prephenate dehydratase
relA	b2784	1.0	1.5	–1.1	(p)ppGpp synthetase I (GTP pyrophosphokinase); regulation of RNA synthesis, stringent factor
agaY	b3137	–1.3	**–5.3**	1.0	Tagatose-bisphosphate aldolase 2
glvB	b3682	–1.8	**–4.0**	–1.1	PTS system, arbutin-like IIB component
tnaC	b3707	–1.1	**13.9**	**–3.0**	Tryptophanase leader peptide
tnaA	b3708	1.3	**5.3**	–1.3	Tryptophanase
rbsA	b3749	**–3.7**	–1.3	1.3	ATP-binding component of D-ribose high-affinity transport system
rbsC	b3750	**–3.0**	1.0	1.3	D-ribose high-affinity transport system
ptsA	b3947	–1.6	**–4.0**	1.2	PEP-protein phosphotransferase system enzyme I
phnM	b4095	–1.5	**–4.6**	–1.6	Phosphonate metabolism
phnL	b4096	–1.8	**–4.0**	–1.6	ATP-binding component of phosphonate transport
phnH	b4100	–1.6	**–4.6**	–1.4	Phosphonate metabolism
phnE	b4104	–1.4	**–4.3**	–1.4	Membrane channel protein component of Pn transporter
Transport related					
ydgF	b1600	–1.2	–1.4	**–2.1**	Multidrug resistance efflux protein, with MdtI
tdcC	b3116	–1.8	–1.5	**–2.1**	Serine/threonine:H^+ symport permease
malP	b3417	1.7	–1.1	**–2.6**	Maltodextrin phosphorylase

Table 1. *cont.*

Group and gene	b number	Fold change[a] *mqsR* vs. WT	Fold change[a] pCA24N-*mqsR* vs. pCA24N (3 h)	Fold change[a] pCA24N-*mqsR* vs. pCA24N (15 min)	Description of encoded protein
malG	b4032	1.6	–1.5	**–2.0**	Maltose transport complex, inner membrane-spanning subunit
malF	b4033	1.3	1.0	**–2.0**	Maltose transport complex, inner membrane-spanning subunit
malE	b4034	1.5	1.1	**–3.5**	Maltose-binding protein, periplasmic, substrate recognition for active transport of and chemotaxis toward maltose and maltodextrin
malK	b4035	1.1	1.1	**–3.2**	Maltose transport complex, ATP-binding subunit
malL	b4036	1.9	1.2	**–4.3**	Maltoporin, maltose high-affinity uptake system
malM	b4037	1.6	1.3	**–3.0**	Periplasmic protein in maltose transport system, function unknown
cadB	b4132	–1.6	**–3.0**	**–3.0**	Lysine-cadaverine antiporter
frdC	b4152	–1.4	–1.4	**–3.0**	Fumarate reductase membrane anchor polypeptide
frdA	b4154	–1.3	1.8	**–2.3**	Fumarate reductase flavoprotein subunit
yjfF	b4231	–1.8	**–3.0**	**–2.1**	Putative ABC transporter permease protein, function unknown
fecE	b4287	**2.5**	–2.1	**–2.5**	ATP-binding component of citrate-dependent iron(III) transport protein
fecD	b4288	**2.1**	**–3.2**	**–2.3**	Citrate-dependent iron transport, membrane-bound protein
fecC	b4289	**2.5**	–2.5	**–2.5**	Citrate-dependent iron(III) transport protein, cytosolic
fecB	b4290	**2.3**	–2.6	**–3.0**	Citrate-dependent iron transport, periplasmic protein
fecA	b4291	1.9	–2.1	**–2.0**	Outer membrane ferric citrate receptor, ferric citrate uptake
Bacteriophage related					
rzpD	b0556	**–2.1**	**–4.6**	–1.2	Putative bacteriophage lambda murein endopeptidase
Small RNA related					
ffs	b0455	–1.1	**5.3**	1.7	4.5S RNA, component of ribonucleoprotein particle
rtT	b4425	1.1	**5.7**	1.6	rtT RNA, may modulate the stringent response
rye	b4438	–1.1	**4.0**	–1.1	Novel sRNA, function unknown

a. Genes considered differentially expressed based on the standard deviation for expression ratio for all genes are shown in boldface. All experiments were performed with LB medium at 37°C.

In a second whole-transcriptome study in which MqsR was produced for 3 h rather than deleted [BW25113/pCA24N-*mqsR* versus BW25113/pCA24N at a turbidity of 0.5 at 600 nm with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) induction], MqsR induced 132 genes and repressed 299 genes by more than threefold (Table 1). *tnaA* (encodes a tryptophanase) and *tnaC* (encodes a tryptophanase leader peptide), both linked to indole synthesis, were induced by 5- and 14-fold respectively. Consistent with the *mqsR* deletion microarray results, *cspD* was induced significantly (sevenfold) upon overexpressing *mqsR*. Hence, these results corroborate that CspD plays an important role in MqsR-mediated toxicity. Also, the RelE (threefold) and RelB (threefold) TA systems (Gotfredsen and Gerdes, 1998) along with HokD (10-fold) (Gerdes *et al*., 1986) were induced (Table 1). In addition, genes encoding nutrient starvation-related proteins including *cstA* (fourfold) (Schultz and Matin, 1991), *rpoS* (encoding the stress and stationary-phase sigma S factor, fourfold) (Lange and Hengge-Aronis, 1991), and *dps* (stress response DNA-binding protein; fourfold) (Almirón *et al*., 1992) were strongly induced (Table 1) upon overexpressing *mqsR* as a result of its toxicity. *mqsA* was also induced by fourfold. Induction of these genes during MqsR production was confirmed using qRT-PCR. Consistent with the microarray data, transcription of *relB*, *relE*, *hokD*, *cstA*, *rpoS*, *dps* and *mqsA* were induced by 3 ± 1-, 4 ± 1-, 5 ± 1-, 4 ± 1-, 4 ± 1-, 3 ± 1- and 8 ± 1-fold respectively. Hence, overexpressing *mqsR* for 3 h dramatically and consistently induces the transcription of starvation- and stress-related genes as well as *mqsA,* the gene that encodes its own antitoxin. Also, the transcription of *bssS* (threefold), the regulator of biofilm formation through signal secretion (Domka *et al*., 2006), was induced by MqsR production for 3 h.

Overexpressing *mqsR* repressed significantly *glpA* (fourfold), *glpB* (fivefold), *glpC* (eightfold), *glpD* (11-fold), *glpF* (fivefold), *glpK* (fourfold) and *glpT* (fourfold) (Table 1); these genes are related to glycerol-3-phosphate (G3P) metabolism via G3P dehydrogenase. This result further supports that MqsR regulates AI-2 uptake systems in *E. coli* (González Barrios *et al*., 2006) by affecting glycerol and G3P metabolism. Among these genes, *glpD* encodes a component of the multi-drug tolerance mechanism for persister formation (Spoering *et al*., 2006), which is one of the hypothetical functions of TA systems (Magnuson, 2007). GlpD overproduction induced tolerance to ampicillin and ofloxacin, whereas the deletion of *glpD* repressed the production of persister cells (Spoering *et al*., 2006); hence, MqsR may be linked to persister formation through GlpD.

To identify additional genes that are influenced by MqsR, we performed a third whole-transcriptome analysis

for MqsR in which *mqsR* was induced for only for 15 min (BW25113 *mqsR*/pCA24N-*mqsR* versus BW25113 *mqsR*/pCA24N grown to a turbidity of 0.5 at 600 nm and 2 mM IPTG added for 15 min). This type of array, a pulsed expression of the regulatory gene which may destabilize mRNA, was used recently to find changes due to overproduction of regulatory protein CsrA, which was found to influence mRNA stability and led to its association to cyclic-di-GMP (Jonas *et al*., 2008). By producing MqsR for a short period, 46 genes were induced and 48 genes were repressed by twofold (Table 1). As expected, *mqsR* was induced (955-fold). Consistent with the 3 h *mqsR* overexpression microarray data*, mqsA* was also induced threefold. Therefore, it appears that MqsR, in association with MqsA, induces *mqsRA* and acts as a positive regulator of its own transcription.

Genes influenced by production of MqsR in the pulsed expression microarray study included genes that are related to stress-associated chaperones and heat shock proteins including *ibpA*, *ibpB*, *clpB, yrfH, yrfI, htpG*, *dnaJ*, *dnaK*, *hslV* and *hslU* (Table 1). In contrast to the *mqsR* deletion microarray data, these stress-related, protein-encoding genes were induced by MqsR after the short induction period (15 min). Thus, these genes are most likely affected due to MqsR RNase activity. *lon* (threefold) and *clpP* (twofold), which encode antitoxin-degrading proteases (Christensen *et al*., 2004), were also induced by the overexpression of *mqsR* for 15 min. In contrast, transport-related gene clusters including those for the maltose transport system (*malE*, *malF*, *malG*, *malK*, *malL* and *malM*) and ferric citrate transport system genes (*fecA*, *fecB*, *fecC*, *fecD* and *fecE*) were repressed significantly by overexpressing *mqsR* for 15 min. Hence, this short-term experiment linked MqsR to Lon and ClpP proteases.

MqsR and MqsA autoregulate their transcription as positive regulators

To corroborate that MqsR stimulates its own transcription as predicted by the whole-transcriptome analysis (Table 1) and to investigate whether MqsA stimulates transcription of the *mqsRA* operon, we confirmed the enhanced transcription of *mqsR* and *mqsA* via qRT-PCR respectively. As expected, a dramatic induction was observed not only for transcripts of *mqsA* by producing MqsR (37 $\pm$ 2-fold), but also for transcripts of *mqsR* by producing MqsA (57 $\pm$ 1-fold). Also, the deletion of *mqsR* resulted in a sevenfold decrease in the transcription of *mqsA*. A similar induction in transcription of a TA operon also occurs upon producing the toxin RelE (toxin part of the RelE/RelB TA pair) (Overgaard *et al*., 2008). Also, we showed previously that the MqsR/MqsA complex and MqsA alone bind directly the *mqsRA* promoter (Brown *et al*., 2009). Together taken, the availability of MqsR or MqsA alters the balance of free and bound MqsR and MqsA respectively. This disproportional ratio between MqsR and MqsA may affect to the binding affinity of MqsR/MqsA complex and positively stimulates its own transcription. Typically, most toxin/antitoxin complexes bind their own promoters and act as autorepressors (Jensen and Gerdes, 1995).

Putative MqsR/MqsA DNA-binding regions

Previously, we found that deletion of *mqsR* affects the expression of many genes as well as affects biofilm formation through motility and QS (González Barrios *et al*., 2006); hence, based on the interaction between MqsR and MqsA (Brown *et al*., 2009), it is evident that direct DNA binding is achieved via MqsA. To identify MqsR/MqsA-binding sequences regions under biofilm conditions, we performed a nickel-enrichment DNA microarray in which we cross-linked the MqsR/MqsA complex to DNA using formaldehyde and purified MqsR/MqsA with the bound DNA via a His_6-affinity tag on MqsR using biofilms cells (*E. coli* AG1/pCA24N-*mqsR* versus *E. coli* AG1/pCA24N with 2 mM IPTG induction for 24 h). This analysis identified 37 regions of DNA (10 intergenic regions and 27 genes) bound to MqsR/MqsA including *cspD* (which agrees with two of the whole-transcriptome analyses and qRT-PCR), *mcbR*, *spy*, *bssR*, and other genes encoding transport proteins such as *ykfD* and *feoA* (Table S1).

Among the identified sequences, MqsR/MqsA appears to bind *bssR in vivo* (Table S1). BssR is repressor of biofilm formation and regulates uptake and export of the stationary-phase signal indole (Domka *et al*., 2006). This gene was also repressed by deleting *mqsR* (deletion microarray, Table 1) which should increase MqsA; hence, it is possible that MqsR/MqsA represses *bssR* transcription. This observation may be associated with the reduction of biofilm formation via MqsR overproduction (Fig. 1B); i.e. overproducing toxin MqsR should result in the degradation of MqsA which would derepress *bssR* and thereby reduce biofilm formation.

Previously, we showed that McbR (formerly YncC) stimulates biofilm formation by reducing mucoidy via colanic acid production and that *mcbR* is dramatically repressed by the deletion of *mqsR* and inactivation of *mqsA* (González Barrios *et al*., 2006; Zhang *et al*., 2008). In addition, we showed that the MqsR/MqsA complex or MqsA alone binds tightly the promoter region of *mcbR* (Brown *et al*., 2009). Therefore, it appears MqsA may induce transcription of *mcbR* which likely explains the increased biofilm formation detected upon overproducing MqsA.

To verify that MqsA alone (not bound to MqsR) regulates these genes, we performed a second nickel-enrichment DNA microarray in which we purified MqsA

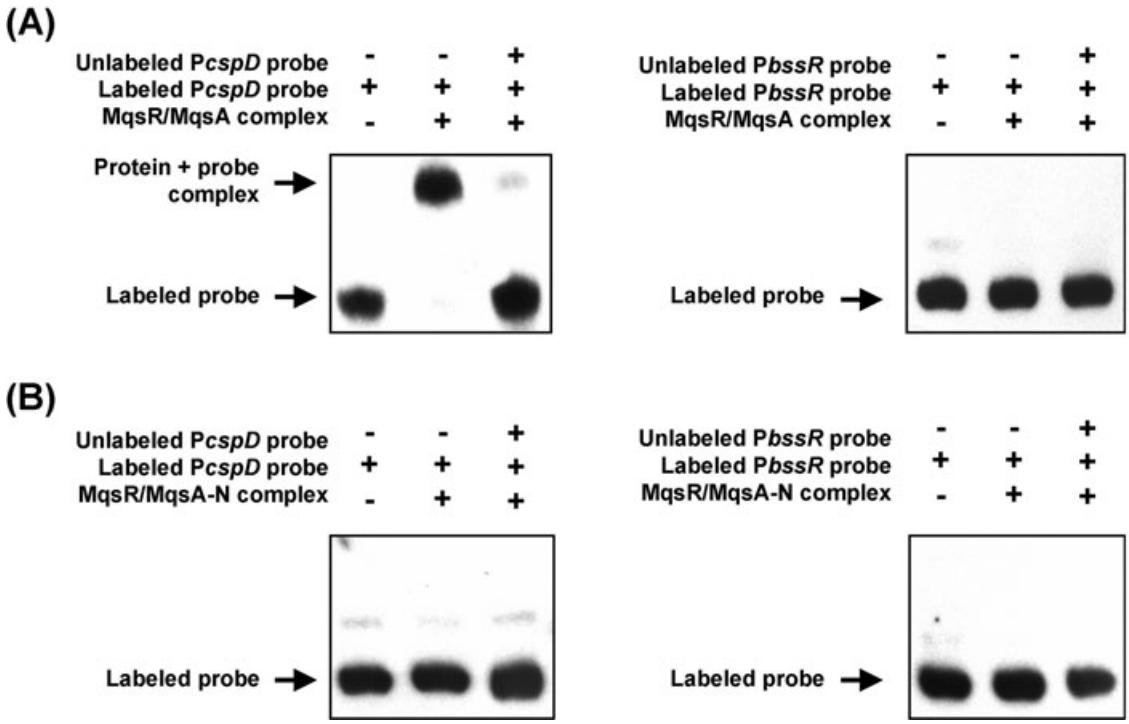

Fig. 2. Electrophoretic mobility shift assay (EMSA) to confirm the specific DNA binding of the MqsR/MqsA complex. MqsR/MqsA (full length of MqsA) (A) and MqsR/MqsA-N (N-terminal of MqsA) (B) were tested for binding to the promoter regions of *cspD* (P*cspD*) and *bssR* (P*bssR*). Lane 1: labelled promoter regions (6 ng), lane 2: protein complex (200 ng) and labelled promoter regions (6 ng), and lane 3: protein complex (200 ng) with labelled (6 ng) and unlabelled (specific competitor) promoter regions (600 ng). The unspecific competitor poly(dI-dC) was used for all samples.

together with bound DNA via a His_6-affinity tag on MqsA (BW25113/pBAD-*mqsA* versus BW25113/pBAD-*Myc*-His C with 0.5% L-arabinose to induce *mqsA* added at a turbidity of 0.8 at 600 nm and cells grown for 24 h). Consistent with the DNA sequences bound by the MqsR/MqsA complex in the initial nickel-enrichment DNA microarray, we identified that seven sequences, including *cspD*, *mcbR* and *spy,* are also enriched using MqsA alone (Table S1). Corroborating these results, using qRT-PCR, we found that MqsA overproduction dramatically induced the transcriptions of *mcbR* and *spy* by 20 ± 1- and 15 ± 1-fold respectively. Therefore, MqsA alone or in a complex with MqsR induces transcription of *mcbR* and *spy* and represses transcription of *cspD* by binding their promoter regions directly.

MqsR/MqsA binds the cspD *promoter directly via MqsA*

Since the DNA microarrays, nickel-enrichment DNA microarray and qRT-PCR results indicated the MqsR/MqsA complex is related to the expression of *cspD*, we investigated if the MqsR/MqsA complex (via the C-terminus of MqsA) binds directly to the promoter of *cspD* using electrophoretic mobility shift assays (EMSA). Previously, we confirmed that the MqsR/MqsA complex or MqsA alone tightly bind to the promoter regions of *mcbR* and *spy* as well as its own promoter (Brown *et al*., 2009). Consistently, in this study, we found direct binding of the MqsR/MqsA complex to the promoter regions for *cspD* (Fig. 2A). However, MqsR/MqsA does not bind to the *bssR* promoter region (Fig. 2A). As a negative control, we show that the N-terminal domain of MqsA does not bind to *cspD* (Fig. 2B); hence, the C-terminal domain of MqsA is necessary and sufficient to bind the promoter of *cspD*. This result agrees with earlier results showing that MqsR alone (Yamaguchi *et al*., 2009) and MqsR associated with the N-terminus of MqsA (Brown *et al*., 2009) do not bind DNA, and that the C-terminal domain of MqsA in the MqsR/MqsA complex or MqsA alone is responsible for promoter binding (Brown *et al*., 2009). Taken together, transcription of *cspD* is be controlled by the direct binding of the MqsR/MqsA complex or MqsA via the MqsA C-terminal domain.

*MqsR/MqsA regulates P*cspD *under stress conditions*

Since TA pairs are induced under stress (Engelberg-Kulka *et al*., 2006) and since MqsA binds the promoter region of *cspD* to repress its transcription and this repression is derepressed by overexpressing *mqsR*, we investigated

further the impact of the MqsR/MqsA complex and stress regulation on *cspD*. Using qRT-PCR and a BW25113 *mqsR mqsA* mutant overproducing MqsR/MqsA, we found, similar to overproduction of MqsA alone, that *cspD* was repressed by overproducing MqsR/MqsA (3 ± 1-fold). Also, under conditions of oxidative stress (30 mM H_2O_2) (Lee *et al*., 2007), *cspD* was strongly induced (5 ± 1-fold) in wild-type BW25113. Under these conditions, *clpX* (6 ± 1-fold), *clpP* (6 ± 1-fold), *lon* (8 ± 1-fold) and *mqsR* (4 ± 1-fold) were also induced. These results show that environmental stress not only induces proteases that degrade antitoxins (ClpXP and Lon), but it also induces production of both the MqsR and CspD toxins. Critically, *cspD* was not induced by oxidative stress in the BW25113 *mqsR mqsA* mutant.

We also reasoned that since MqsA represses *cspD*, if there is excess MqsA, then *cspD* would remain repressed even under stress conditions where *cspD* is induced strongly. We found *cspD* was repressed by the overproduction of MqsR/MqsA under stress conditions [4 ± 1-fold by qRT-PCR, using BW25113 *mqsR mqsA*/pBS(Kan)-*mqsR-mqsA* versus BW25113 *mqsR mqsA*/pBS(Kan) in LB with 1 mM IPTG at a turbidity of 1 at 600 nm and 30 mM H_2O_2 for 15 min]. Hence, the significant induction of *cspD* by H_2O_2 stress is eliminated when the MqsR/MqsA complex or MqsA alone is overproduced. Therefore, *cspD* transcription is directly and strongly linked to the MqsR/MqsA TA system.

CspD and HokA are toxic

To investigate whether MqsR/MqsA induction of CspD and HokA leads to toxicity, growth and cell viability were assayed by producing these proteins in strains that lacked the genes that encode these two toxins. As expected, cell growth and viability were dramatically arrested for CspD and HokA production (Fig. 3 and Fig. S1). Therefore, stimulation of the production of CspD and HokA via MqsR/MqsA is toxic.

Taken together, our results indicate that under normal growth, *cspD* is repressed by the MqsR/MqsA TA complex. Under stress, MqsA is degraded by proteases (ClpXP and Lon), and *cspD* transcription is derepressed by the breakdown of the MqsA. The combination of both toxins CspD and MqsR, the latter functioning as an RNase, subsequently leads to a reduction in metabolism.

Confirmation of proteins linked to MqsR toxicity

In an effort to identify proteins other than CspD that may be related to MqsR toxicity as well as to demonstrate MqsR toxicity is associated with CspD, we examined four other toxin-related proteins (RelB, RelE, HokA and HokD) and six stress response-related proteins (CstA, HslU, IbpA, IbpB, UmuC and UmuD) based on the three sets of whole-transcriptome data and the two nickel-enrichment DNA microarray data. In addition, based on the toxin-associated genes we identified to be associated with toxin Hha and its antitoxin TomB (García-Contreras *et al*., 2008), we also examined ClpX, ClpP, Lon, RzpD and YfjZ. *clpX* encodes the AAA+ ATPase that binds the serine protease ClpP, *clpP* encodes a serine protease and *lon* encodes an ATP-dependent protease. *rzpD* encodes a putative murein endopeptidase, *yfjZ* encodes the antitoxin component of the putative TA pair YpjF/YfjZ, *cspD* encodes a DNA replication inhibitor, *relB* encodes the antitoxin for the RelE/RelB TA systems, *relE* encodes the toxin for RelE/RelB, and *hokA* and *hokD* encode small toxic membrane polypeptides. *cstA* encodes a carbon starvation protein, *umuC* and *umuD* encode a SOS mutagenesis protein, and *ibpA*, *ibpB* and *hslU* encode heat shock proteins.

Among these 16 genes, deletion of eight genes (*cspD*, *clpX*, *clpP*, *lon*, *yfjZ*, *relB*, *relE* and *hokA*) prevented MqsR from inhibiting cell growth (*clpX*, *clpP*, *lon*, *yfjZ* and *cspD*) or reduced its ability to inhibit growth (*relB*, *relE* and *hokA*) (Fig. 4A), as well as abolished the ability of MqsR to reduce biofilm formation (Fig. 4B); hence, the eight proteins encoded by these genes may be directly involved in MqsR toxicity as well as biofilm formation. If this hypothesis is correct, then overproduction of the toxin MqsR should induce these genes. We tested this by overexpressing *mqsR* and measuring transcription via qRT-PCR (using RNA samples from BW25113/pCA24N-*mqsR* grown to a turbidity of 0.5 with 2 mM IPTG for induction in LB for 3 h); we found that all eight genes were induced significantly: *clpX* (2 ± 1-fold), *clpP* (3 ± 1-fold), *lon* (4 ± 1-fold), *yfjZ* (3 ± 1-fold), *hokA* (3 ± 1-fold), *cspD* (5 ± 1-fold), *relB* (3 ± 1-fold) and *relE* (4 ± 1-fold). Therefore, MqsR, via MqsA, must influence the transcription of these genes.

Discussion

We have shown previously that TA systems are important for biofilm formation using MqsR (Ren *et al*., 2004a; González Barrios *et al*., 2006; Zhang *et al*., 2008) and the *E. coli* Δ5 strain, which lacks five TA systems (listed as toxin/antitoxin: MazF/MazE, RelE/RelB, ChpB, YoeB/YefM and YafQ/DinJ) (Kim *et al*., 2009). In the current study, we confirm that MqsR is a toxin paired with its antitoxin MqsA and show that the toxicity of this system is related to its direct regulation of CspD as well as related to the activities ClpX, ClpP, Lon, YfjZ, RelB, RelE and HokA. In addition, our results begin to explain how the MqsR/MqsA complex acts as a global regulatory protein (Table 1 and Table S1) that controls more than its own transcription

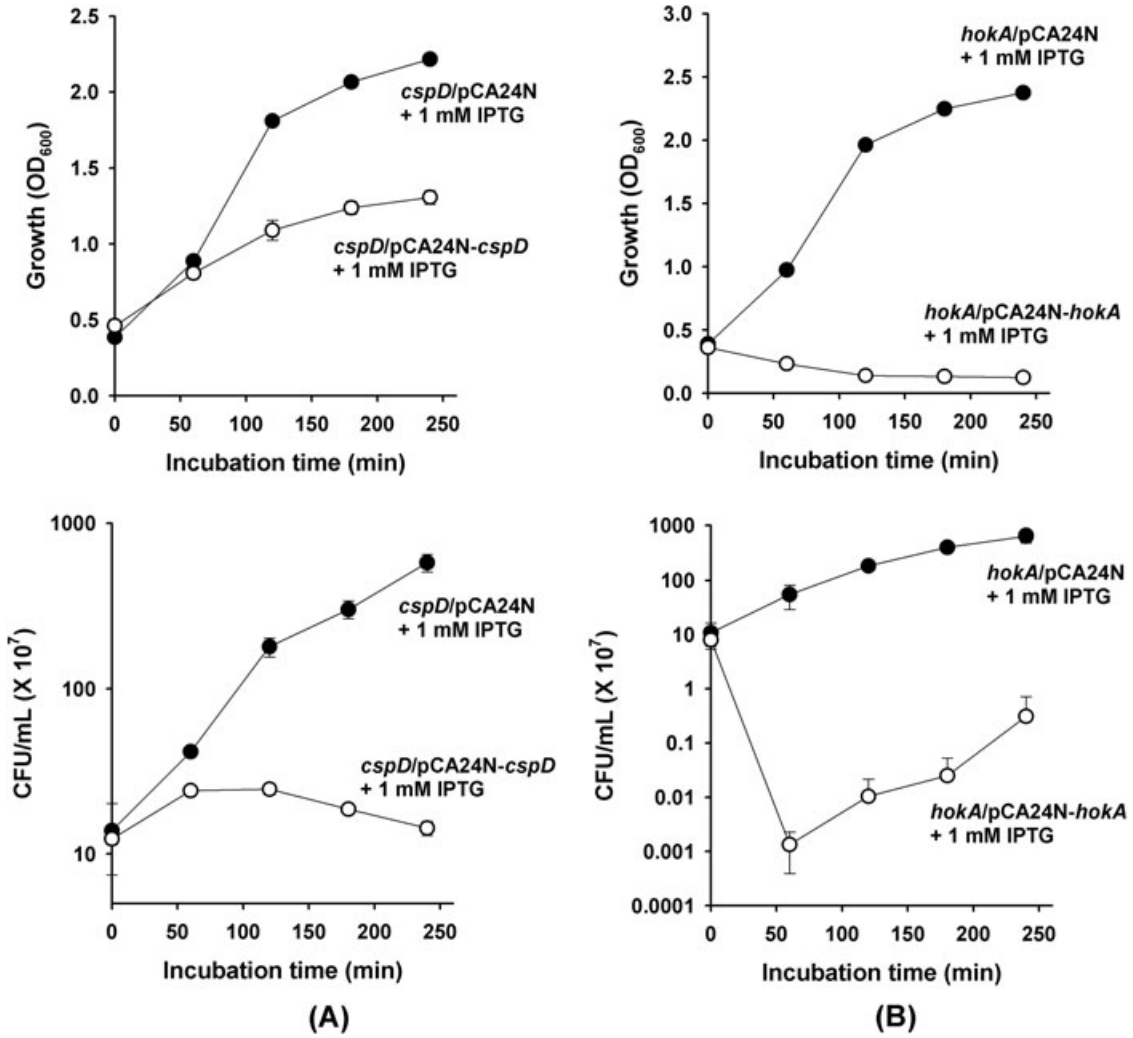

Fig. 3. Growth curves and cell viability (cfu ml^{-1}) for BW25113 *cspD*/pCA24N and BW25113 *cspD*/pCA24N-*cspD* (A) and for BW25113 *hokA*/pCA24N and BW25113 *hokA*/pCA24N-*hokA* (B). Cells were grown to a turbidity of 0.5 at 600 nm at 37°C in LB medium, then 1 mM IPTG was added to induce the pCA24N-based genes. Data are the average of two independent cultures, and one standard deviation is shown.

(González Barrios *et al*., 2006). We confirm that MqsR/MqsA binds specific promoter regions including *cspD* (Fig. 2), in addition to its own promoter (Brown *et al*., 2009). This direct interaction is mediated via the C-terminal HTH DNA-binding domain of MqsA, as we have shown via multiple gel shift assays. These results are consistent with our previous report that the MqsR/MqsA complex or MqsA alone binds to the promoter regions of *mcbR* and *spy* as well as to its own promoter (Brown *et al*., 2009). In addition, Yamaguchi and colleagues (2009) found that there are two palindrome sequences in the promoter region of the *mqsRA* locus and that the MqsR/MqsA complex or MqsA alone can bind to these two palindromic sequences. Moreover, we found here that the promoter regions *cspD*, *mcbR* and *spy,* that are controlled by the MqsR/MqsA TA pair as confirmed by the gel shift assay (Fig. 2 and Brown *et al*., 2009) and two nickel enrichment DNA microarray studies (Table S1), also have similar regions with T-rich palindromic spacers (TCAAATTTTTGA for *cspD*; CATCATTGTTCTGC TG for *mcbR*; and AGTGTTTTTTACACT for *spy*). Also, the 58 bp and 74 bp promoter fragments of *mcbR* and *spy*, respectively, containing these palindromes, are bound by MqsA in the gel shift assays (data not shown). Hence, these palindrome sequences are important for recognition of specific promoter regions by the C-terminal part of MqsA. Yamaguchi and colleagues (2009) also found that the binding activity of MqsA is enhanced in the presence of MqsR compared with MqsA alone. This result is supported by our qRT-PCR result that the overexpression of MqsR/MqsA repressed more *cspD* transcription compared with that of MqsA alone.

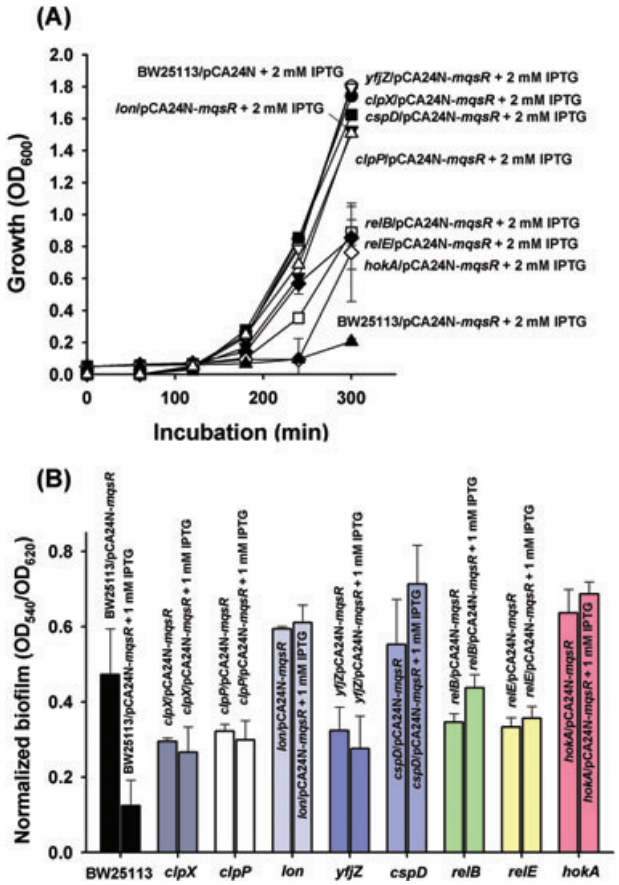

Fig. 4. Growth curves (A) and normalized biofilm formation (total biofilm/growth; B) of isogenic deletion strains (*clpX*, *clpP*, *lon*, *yfjZ*, *cspD*, *relB*, *relE* and *hokA*) with production of MqsR via pCA24N-*mqsR* and IPTG at 37°C in LB medium. Growth data are the average of two independent cultures, biofilm data are the average of 10 replicate wells from two independent cultures, and one standard deviation is shown.

Here, the lines of evidence that indicate the MqsR/MqsA TA pair directly regulate toxin CspD are (i) growth and biofilm results that show CspD is involved in MqsR-related toxicity (Fig. 4), (ii) MqsR/MqsA and MqsA regulate *cspD* transcription as shown by two sets of whole-transcriptome studies (Table 1) and qRT-PCR, (iii) the *cspD* promoter is bound directly by the MqsR/MqsA complex as well as by MqsA alone as shown by two sets of nickel-enrichment DNA microarray studies (Table S1) and by EMSA (Fig. 2), and (iv) qRT-PCR shows that induction of *cspD* by H_2O_2 stress is abolished by high levels of the MqsR/MqsA complex and by MqsA alone as well as by the absence of the *mqsRA* locus. Hence, the MqsR/MqsA TA system is a global regulator through its RNase activity (Brown *et al.*, 2009) and through its regulation of the CspD toxin.

CspD inhibits chromosomal replication in nutrient-depleted cells (Yamanaka *et al.*, 2001). As previously shown (Yamanaka *et al.*, 2001) and reconfirmed in this work (Fig. 3A), CspD production is toxic. Furthermore, we linked MqsR to the nutrient starvation genes *cstA*, *rpoS* and *dps* (Table 1); hence, it is likely that MqsR induces starvation-like responses. TA systems are induced by starvation including depletion of amino acids and glucose, and the TA systems coordinately reduce DNA replication and translation (Gerdes, 2000). Hence, MqsR toxicity should be linked, either directly or indirectly, to induction of other TA systems including RelE/RelB. Corroborating this hypothesis, we established that toxin Hha induces genes of other TA pairs, such as RelE (threefold)/RelB (threefold), YoeB (sevenfold)/YefM (sevenfold) and YafQ (twofold)/DinJ (fourfold) (García-Contreras *et al.*, 2008). Lastly, MqsR toxicity also involves the membrane toxin peptide HokA (Fig. 4A). Hence, the mechanism of MqsR toxicity is complex.

ClpXP is an important protease system for stress-induced environments and degrades RpoS (Zhou *et al.*, 2001) and Dps (Stephani *et al.*, 2003); hence, ClpXP induction by MqsR may lead to induction of stress-induced protection proteins regulated by RpoS and Dps which leads to toxicity. Moreover, other groups reported that labile antitoxins including MazE (Aizenman *et al.*, 1996) and RelB (Christensen *et al.*, 2001) are degraded by the ATP-dependent ClpP and Lon proteases respectively. Therefore, we propose that ClpXP might be involved in MqsR toxicity via MqsA-specific degradation. This is based on the observations that *clpP* is induced by *mqsR* overexpression for 15 min (the whole-transcriptome study; Table 1) and that the deletion of *clpP* abolishes MqsR toxicity (Fig. 4A). In addition, many genes encoding chaperones such as *ibpA*, *ibpB*, *hslU*, *hslV*, *dnaK* and *dnaJ* were directly influenced by MqsR; therefore, MqsR may prevent access of cells to key chaperone proteins. RpoS expression in *E. coli* is stimulated by a secreted extracellular signal (Holland and Rather, 2008); hence, the specific relationship among RpoS, ClpXP and AI-2 may be considered as a key factor for AI-2-related MqsR toxicity.

We showed previously that the balance between toxins and antitoxins has an important role in biofilm development (Kim *et al.*, 2009). When bacteria encounter nutrient deficient or stressful conditions, toxins are activated by specific degradation of their antitoxins through cellular proteases (Gerdes, 2000), and it is this altered ratio between toxins and antitoxins that can influence biofilm formation (Kim *et al.*, 2009). Consistent with this finding, production of MqsA increased biofilm formation whereas that of MqsR reduced biofilm formation (Fig. 1B); hence, we found additional evidence that the balance between the toxin and antitoxin influences biofilm formation. Similarly, for the RelE/RelB TA pair, excess of free RelE displaces or breaks the interaction between a C-terminal part of RelB and the binding site of RelE under environmental change, and thereby derepresses its own *relBE* transcrip-

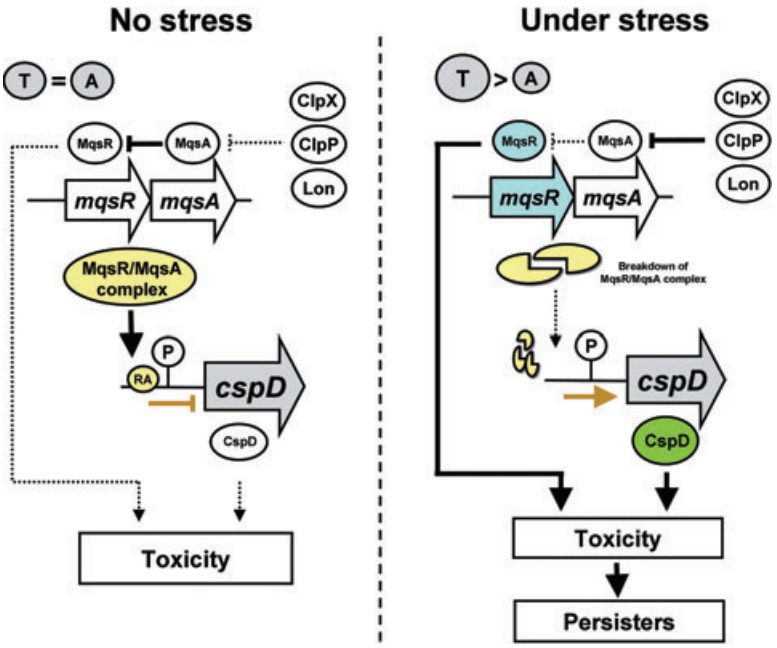

Fig. 5. Schematic of MqsR toxicity via CspD. *cspD* is repressed by the MqsR/MqsA complex. Under stress, MqsA is degraded by proteases (ClpXP and Lon), and *cspD* transcription is derepressed by the breakdown of the MqsR/MqsA complex. Stress also elevates the level of free-MqsR toxin. → indicates induction, ⊥ indicates repression, and dotted lines indicate regulatory pathways that are not active whereas solid lines indicate active regulatory pathways.

tion (Overgaard *et al*., 2008). Analogously, *cspD* is regulated by the ratio of MqsR and MqsA and involves MqsA decay through proteases Lon and ClpXP as a result of stress conditions. Hence, CspD production may be related to toxicity and biofilm formation via MqsR.

The induction of Hok-like proteins including HokA is very toxic to *E. coli* (Fig. 3B), resulting in loss of membrane potential, cell respiration arrest and efflux of small molecules (Gerdes *et al*., 1997). After inducing cell death via HokA, cell viability (cfu ml^{-1}) and growth may be restored (Fig. 3B); a similar phenomenon occurs after induction of MqsR (Brown *et al*., 2009). Hence, our data suggest that MqsR potentially increases persister cell formation (Shah *et al*., 2006) via HokA. In addition, since *mqsR* transcription is strongly induced by several stresses including hydrogen peroxide (H_2O_2) (our qRT-PCR result and Zheng *et al*., 2001) and heat (50°C) (Richmond *et al*., 1999) and since *mqsR* is the most highly induced gene under persistence conditions (Shah *et al*., 2006), it appears that *mqsRA* transcription may be activated under stress conditions to increase persistence. Recently, we found that the deletion of the *mqsRA* locus repressed persister formation, that overproduction of MqsR/MqsA increases persistence, and that CspD also influences MqsR-related persister production (Kim and Wood, 2010). Taken together, these observations suggest that MqsR toxicity is due to both its RNase activity (Brown *et al*., 2009) and its interaction with MqsA to form a complex that acts as a DNA regulator which regulates at least other toxins like CspD (Fig. 5). Moreover, the CspD toxin is associated with persister production through MqsR (Kim and Wood, 2010).

To our knowledge, this is first report that a TA system regulates the transcription of other toxins as well as its own, via direct binding to the promoter region. Given that MqsR is associated with MqsA as a specific regulator of QS that is directly associated with biofilm formation (González Barrios *et al*., 2006), our results support the idea that TA systems may form an important part of a regulatory network that senses cell density (Gerdes, 2000) and controls cell physiology.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. For isogenic mutants and overexpression of specific genes, we used the Keio collection (Baba *et al*., 2006) and ASKA library (Kitagawa *et al*., 2005), respectively, from the Genome Analysis Project in Japan. Strains BW25113 *mqsR* and MG1655 *mqsR* were verified via PCR, and the rest of the Keio knockouts were verified by Baba and colleagues (2006).

All experiments were conducted in Luria–Bertani (LB) medium (Sambrook *et al*., 1989) at 37°C with the exception of LB supplemented with 0.5% glucose (LB glu) which was used to silence *lac* promoter transcription of toxin gene *mqsR* with pBS(Kan)-*mqsR,* and lower temperatures were used to overproduce proteins for the *in vitro* work. Kanamycin (50 μg ml^{-1}) was used for pre-culturing isogenic knockout mutants and for maintaining the pBS(Kan)-based plasmids. Chloramphenicol (30 μg ml^{-1}) was used for maintaining pCA24N-based plasmids.

Table 2. Bacterial strains and plasmids used in this study.

Strains and plasmids	Genotype/relevant characteristics	Source
E. coli K-12 strains		
TG1	*supE thi*-1 Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5 (r_k^- m_k^-) [F' *traD*36 *proAB* $lacI^qZ\Delta M15$]	Gibson (1984)
AG1	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 (r_k^- m_k^-) *supE*44 *relA*1	Kitagawa *et al.* (2005)
MG1655	F^- λ^- *ilvG rfb*-50 *rph*-1	Blattner *et al.* (1997)
BW25113	$lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR*514 $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$	Baba *et al.* (2006)
MG1655 *mqsR*	MG1655 Δ*mqsR* Ω Tn*5*Kan-2	Kang *et al.* (2004)
BW25113 *mqsR*	BW25113 Δ*mqsR* Ω Km^R	Baba *et al.* (2006)
BW25113 *mqsR mqsA*	BW25113 Δ*mqsR* Δ*mqsA* Ω Km^R	This study
BW25113 *clpX*	BW25113 Δ*clpX* Ω Km^R	Baba *et al.* (2006)
BW25113 *clpP*	BW25113 Δ*clpP* Ω Km^R	Baba *et al.* (2006)
BW25113 *lon*	BW25113 Δ*lon* Ω Km^R	Baba *et al.* (2006)
BW25113 *yfjZ*	BW25113 Δ*yfjZ* Ω Km^R	Baba *et al.* (2006)
BW25113 *cspD*	BW25113 Δ*cspD* Ω Km^R	Baba *et al.* (2006)
BW25113 *relB*	BW25113 Δ*relB* Ω Km^R	Baba *et al.* (2006)
BW25113 *relE*	BW25113 Δ*relE* Ω Km^R	Baba *et al.* (2006)
BW25113 *hokA*	BW25113 Δ*hokA* Ω Km^R	Baba *et al.* (2006)
Plasmids		
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-*mqsA*	Cm^R; $lacI^q$, pCA24N $P_{T5\text{-}lac}$::*mqsA*$^+$	Kitagawa *et al.* (2005)
pCA24N-*cspD*	Cm^R; $lacI^q$, pCA24N $P_{T5\text{-}lac}$::*cspD*$^+$	Kitagawa *et al.* (2005)
pCA24N-*hokA*	Cm^R; $lacI^q$, pCA24N $P_{T5\text{-}lac}$::*hokA*$^+$	Kitagawa *et al.* (2005)
pBS(Kan)	Km^R; cloning vector	Canada *et al.* (2002)
pBS(Kan)-*mqsR*	Km^R; pBS(Kan) P_{lac}::*mqsR*$^+$	This study
pBS(Kan)-*mqsA*	Km^R; pBS(Kan) P_{lac}::*mqsA*$^+$	This study
pBS(Kan)-*mqsR-mqsA*	Km^R; pBS(Kan) P_{lac}::*mqsR-mqsA*$^+$	This study
pET28a(+)-*mqsR*	Km^R; pET28a(+) P_{T7}::*mqsR*$^+$	This study
pCA21a-*mqsA*	Cm^R; pCA21a P_{T7}::*mqsA*$^+$	This study
pBAD-*Myc*-His C	Amp^R; L-arabinose inducible expression vector	Invitrogen
pBAD-*mqsA*	Amp^R; P_{BAD}::*mqsA*$^+$ in pBAD-*Myc*-His C	This study

Km^R, Cm^R and Amp^R are kanamycin, chloramphenicol and ampicillin resistance, respectively.

Construction of overexpression plasmids

To construct plasmids for producing MqsR, MqsA and MqsR-MqsA from a *lac* promoter, fragments from genomic DNA were amplified by polymerase chain reaction (PCR) using primers containing BamHI and XbaI restriction sites (Table S2) and directionally cloned into the multiple cloning site in pBS(Kan) (Canada *et al*., 2002). *Escherichia coli* TG1 (Gibson, 1984) was used as the host for plasmid construction. The sizes of the amplified fragments were 376 bp for *mqsR*, 396 bp for *mqsA* and 768 bp for *mqsR-mqsA*. In addition, for the nickel-enrichment DNA microarray studies with MqsA, pBAD-*mqsA* was constructed with primers containing XhoI and HindIII restriction sites. This vector allows *mqsA* to be induced using the pBAD promoter. Cloned fragments were confirmed by DNA sequencing and restriction digestion with at least different three enzymes.

To overproduce MqsR for the *in vitro* studies, the *mqsR* sequence was PCR-amplified using primer set pE-*mqsR* (Table S2) and ligated into the NdeI and HindIII restriction enzyme sites of pET-28a(+) (Novagen, Madison, WI) to form pET-28a(+)-*mqsR* with MqsR expressed from a T7 promoter. pET-28a(+) has a pBR322 replication origin, is kanamycin resistant, and MqsR contains an N-terminal hexahistidine (His_6) purification tag that is cleavable by thrombin. To overproduce MqsA, the *mqsA* sequence was PCR-amplified using primer set pA-*mqsA* (Table S2) and ligated into the NdeI and HindIII restriction enzyme sites of vector pCA21a (Expression Technologies, San Diego, CA) to form pCA21a-MqsA with *mqsA* expressed from a T7 promoter. pCA21a has a pACYC replication origin, is chloramphenicol resistant and does not contain any expression, solubility or purification tags.

Construction of BW25113 mqsR mqsA

The whole region encoding the transcript of *mqsRA* except for the first 30 bp from the start codon of *mqsR* was deleted from the chromosome using the one-step inactivation procedure (Baba *et al*., 2006) with the *mqsR mqsA* primers (Table S2). After isolating positive colonies, deletion of the *mqsR mqsA* locus was verified by DNA sequencing from PCR fragments using the *mqsRA (detect)* primer set (Table S2).

Growth and survival assays

The toxicity of selected proteins was investigated using pBS(Kan)- and pCA24N-based expression plasmids with 1 or 2 mM IPTG added upon inoculation (for CspD and HokA, IPTG was also added at a turbidity of 0.5 at 600 nm; Fig. 3). The *mqsR mqsA* double mutant was cultured initially in LB glu to silence *mqsR* on pBS(Kan)-*mqsR*, then cells were grown in LB with 1 mM IPTG. Cells were diluted by 10^2–10^7 via 10-fold serial dilution steps into 0.85% NaCl solution and applied as 10 μl drops on LB agar with kanamycin or chloramphenicol to determine cell viability (Donegan *et al*., 1991). Two independent cultures were used for each strain.

Crystal violet biofilm assay

The biofilm formation assay was performed in 96-well polystyrene plates (Corning Costar, Cambridge, MA) (Pratt and Kolter, 1998). Briefly, each well was inoculated at an initial turbidity at 600 nm of 0.05, grown for 24 h without shaking, and the cell density (turbidity at 620 nm) and total biofilm (absorbance at 540 nm) were measured using 0.1% crystal violet staining. Normalized biofilm was calculated by dividing total biofilm by bacterial growth for each strain. Two independent cultures were used for each strain.

RNA isolation and whole-transcriptome analysis

Three sets of whole-transcriptome analyses were performed: (i) planktonic cells of BW25113 *mqsR* versus BW25113 at a turbidity of 0.5 at 600 nm, (ii) planktonic cells of BW25113/pCA24N-*mqsR* versus BW25113/pCA24N with 2 mM IPTG added at a turbidity of 0.5 (grown for 3 h), and (iii) planktonic cells of BW25113 *mqsR*/pCA24N-*mqsR* versus BW25113 *mqsR*/pCA24N grown to a turbidity of 0.5 and then 2 mM IPTG was added for 15 min (Jonas *et al*., 2008). Total RNA was isolated from cells as described previously (Ren *et al*., 2004a) using a mini bead beater (Biospec, Bartlesville, OK) and RNeasy Mini Kit (Qiagen). cDNA synthesis, fragmentation and hybridizations were as described previously (González Barrios *et al*., 2006). The *E. coli* GeneChip Genome 2.0 array (Affymetrix, Santa Clara, CA; P/N 511302) was used. Corroborating the deletion mutations and overexpression of genes, the microarray signals of the *mqsR* gene had very low (deletion microarray) and high (overexpression microarray) signals in their respective microarray experiments. In addition, expected signals including *araA* and *rhaA* based on the *E. coli* K-12 BW25113 genotype (Table 2) were also low. For the three sets of binary microarray comparisons to determine differential genes expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 11 probe pairs (*P*-value less than 0.05), these genes were not used. A gene was considered differentially expressed when the *P*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%). Since the standard deviation for expression ratio for all the genes was 1.4 for condition (i), 2.0 for condition (ii) and 1.2 for condition (iii), genes were considered differentially expressed if they had greater than twofold changes for condition (i), greater than threefold for condition (ii) and greater than twofold for condition (iii) (Ren *et al*., 2004b). Gene functions were obtained from the Ecogene database (http://www.ecogene.org/).

Nickel-enrichment DNA microarrays

We performed two sets of nickel-enrichment DNA microarray experiments to identify promoters bound by MqsA as described previously (Zhang *et al*., 2008). For the first set, His-tagged MqsR was overproduced and the MqsR/MqsA complex was isolated from cells in biofilms using AG1/pCA24N-*mqsR* and compared with AG1/pCA24N; *mqsR* was induced with 2 mM IPTG and cells were grown for 24 h in 250 ml of LB medium containing 10 g glass wool (Corning Glass Works, Corning, NY). For the second set, His-tagged MqsA was overproduced and isolated from planktonic cells using BW25113/pBAD-*mqsA* and compared with BW25113/pBAD-*Myc*-His C. L-arabinose (0.5%) was added at a turbidity of 0.8 at 600 nm in LB medium and grown for 24 h. Briefly, formaldehyde (1%) was added for 20 min with shaking at room temperature to promote cross-linking between His-tagged MqsR and its affiliated MqsA which co-purified with it and DNA or between His-tagged MqsA and the DNA to which it was associated. Ni-NTA agarose gel resin (Novagen) was used to bind His-tagged MqsR/MqsA–DNA or MqsA–DNA complexes after lysing cells with a French press. The DNA fragments were labelled and DNA microarrays were performed using an *E. coli* Genechip antisense genome array (P/N 4011592, Affymetrix) as indicated above. The analysis of the data were performed as described previously (Zhang *et al*., 2008).

Electrophoretic mobility shift assays (EMSA)

To confirm binding between the promoter regions and the MqsR/MqsA (full-length MqsA, isolated as described in *Supporting information*) and MqsR/MqsA-N (N-terminal region of MqsA containing 1–76 aa) (Brown *et al*., 2009), EMSA were performed as described previously (Zhang *et al*., 2008). The targeted promoter regions (region 150–250 bp upstream of the start codon using primers shown in Table S2) were amplified, purified, and then labelled with biotin using the Biotin 3′-end DNA Labelling Kit (Pierce Biotechnology, Rockford, IL). After binding the protein complex (200 ng) with the biotin-labelled target promoters (6 ng), electrophoresis was conducted at 100 V at 4°C using a 6% DNA retardation gel (Invitrogen). The bound protein/DNA mixtures were transferred to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA), and 3′-biotin-labelled DNA was detected with the Light-Shift Chemiluminescent EMSA kit (Pierce Biotechnology).

qRT-PCR

qRT-PCR was performed using the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA). After isolating RNA (Ren *et al*., 2004a) using RNA*later*™ (Ambion, Austin, TX), 50 ng of total RNA was used for the qRT-PCR reaction using the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen) or *Power* SYBR® Green RNA-to-C_T™ *1-Step* Kit (Applied Biosystems). Primers were designed using Primer3Input Software (v0.4.0) and are listed in Table S2. The housekeeping gene *rrsG* was used to normalize the genes expression data. The annealing temperature was 60°C for all the genes in this study. To investigate the regulation of promoters by MqsA, overnight cultures of BW25113/pBS(Kan)-*mqsR* and BW25113/pBS(Kan)-*mqsA* were cultured to a turbidity of 1 at 600 nm, then 1 mM IPTG was added for 1 h to induce *mqsR* and *mqsA*. In addition, *mqsA* transcript levels in the BW25113 *mqsR* mutant were investigated after growing to a turbidity of 0.5 at 600 nm in LB. For the MqsR/MqsA overproduction and *cspD* transcrip-

tion experiments, overnight cultures of BW25113 *mqsR mqsA*/pBS(Kan)-*mqsR-mqsA*, BW25113 *mqsR mqsA*/pBS(Kan)-*mqsA* and BW25113 *mqsR mqsA*/pBS(Kan) were grown to a turbidity of 0.5 at 600 nm, and then 1 mM IPTG was added for 1 h. For the H_2O_2 stress and *clpX*, *clpP*, *lon*, *cspD* and *mqsR* transcription experiments, overnight cultures of the BW25113 wild type and BW25113 *mqsR mqsA* were grown to a turbidity of 1.0 at 600 nm, and then exposed to 30 mM H_2O_2 for 15 min (Lee *et al.*, 2007). In addition, to examine whether MqsR/MqsA complex affects *cspD* transcript by H_2O_2 stress, overnight cultures of the BW25113 *mqsR mqsA*/pBS(Kan)-*mqsR-mqsA* and BW25113 *mqsR mqsA*/pBS(Kan) were inoculated into LB medium and grown to a turbidity of 1.0 at 600 nm with 1 mM IPTG for induction of the MqsR/MqsA complex, and then exposed to 30 mM H_2O_2 for 15 min.

Microarray accession numbers

The differential gene expression data have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through Accession No. GSE14203.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Growth curves and cell viability (cfu ml^{-1}) for BW25113 *cspD*/pCA24N and BW25113 *cspD*/pCA24N-*cspD* (A), and for BW25113 *hokA*/pCA24N and BW25113 *hokA*/pCA24N-*hokA* (B). pCA24N-based genes were induced with 1 mM IPTG at 0 min. Data are the average of two independent cultures, and one standard deviation is shown.

Table S1. DNA binding sites for MqsR/MqsA and MqsA identified *in vivo* using nickel-enrichment DNA microarrays. Enrichment indicates the ratio of the MqsR/MqsA and MqsA signal relative to the empty plasmid controls, pCA24N and pBAD, respectively.

Table S2. Oligonucleotides used in this study. f indicates forward primer and r indicates reverse primer.

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