

# The MqsR/MqsA toxin/antitoxin system protects *Escherichia coli* during bile acid stress

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## Summary

**Toxin/antitoxin (TA) systems are ubiquitous within bacterial genomes, and the mechanisms of many TA systems are well characterized. As such, several roles for TA systems have been proposed, such as phage inhibition, gene regulation and persister cell formation. However, the significance of these roles is nebulous due to the subtle influence from individual TA systems. For example, a single TA system has only a minor contribution to persister cell formation. Hence, there is a lack of defining physiological roles for individual TA systems. In this study, phenotype assays were used to determine that the MqsR/MqsA type II TA system of *Escherichia coli* is important for cell growth and tolerance during stress from the bile salt deoxycholate. Using transcriptomics and purified MqsR, we determined that endoribonuclease toxin MqsR degrades YgiS mRNA, which encodes a periplasmic protein that promotes deoxycholate uptake and reduces tolerance to deoxycholate exposure. The importance of reducing YgiS mRNA by MqsR is evidenced by improved growth, reduced cell death and reduced membrane damage when cells without *ygiS* are stressed with deoxycholate. Therefore, we propose that MqsR/MqsA is physiologically**

**important for *E. coli* to thrive in the gallbladder and upper intestinal tract, where high bile concentrations are prominent.**

## Introduction

Most bacterial chromosomes contain numerous genetic elements encoding multi-component toxin/antitoxin (TA) systems (Makarova *et al.*, 2009; Fozo *et al.*, 2010; Blower *et al.*, 2012). TA systems typically consist of a stable protein toxin paired with a labile RNA or protein antitoxin (Schuster and Bertram, 2013). These toxins disrupt essential cellular processes (e.g. translation via specific mRNA degradation), and the toxic activity is prevented when a sufficient amount of antitoxin is present (Schuster and Bertram, 2013). Depending on the interaction between the toxin and antitoxin elements, TA systems are classified as type I through type V, which represent known mechanisms. Type I TA systems consist of an RNA antitoxin that inhibits translation of the toxin via antisense RNA binding (Schuster and Bertram, 2013). In type II and type III TA systems, activity of the protein toxin is inhibited by direct binding from a protein antitoxin or RNA antitoxin respectively (Schuster and Bertram, 2013). Protein antitoxins in type IV systems prevent toxicity by protecting the target from the toxin (Masuda *et al.*, 2012), and in type V systems, protein antitoxins prevent toxicity by specifically cleaving mRNA of the toxin (Wang *et al.*, 2012).

TA systems were first discovered as plasmid addiction modules in which the antitoxin and a toxin are encoded on a plasmid, eliminating plasmid-free cells (Ogura and Hiraga, 1983). Roles for TA systems have since been proposed as selfish alleles, gene regulation, growth control, persister cell formation, programmed cell arrest, programmed cell death and anti-phage measures (Magnuson, 2007). The role of TA systems in the formation of multidrug tolerant persister cells has been of particular interest in recent years due to relevance within bacterial infection models. TA systems appear to be a highly redundant mechanism for persister formation, with only a few TA systems affecting persistence upon deletion from the chromosome (Dörr *et al.*, 2010; Kim and Wood, 2010; Luidalepp *et al.*, 2011) and a requirement of several simultaneous TA system deletions to more significantly

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impact persistence (Maisonneuve *et al.*, 2011). Many TA systems are autoregulatory (Schuster and Bertram, 2013), and due to the regulatory nature of TA elements, some antitoxins exhibit a gene-specific regulatory effect via promoter binding of loci distinct from the TA loci (Kim *et al.*, 2010; Wang and Wood, 2011; Wang *et al.*, 2011; Soo and Wood, 2013) and some toxins exhibit a general regulatory effect via post-transcriptional differential mRNA decay (González Barrios *et al.*, 2006; Amitai *et al.*, 2009; Wang and Wood, 2011; Wang *et al.*, 2013). Hence, TA systems regulate global transcriptional networks [e.g. the general stress response (Wang and Wood, 2011; Wang *et al.*, 2011; Hu *et al.*, 2012)] and are also controlled within transcriptional regulons triggered by specific environmental cues [e.g. the SOS response (Singletary *et al.*, 2009; Dörr *et al.*, 2010; Tripathi *et al.*, 2012) and the stringent response (Aizenman *et al.*, 1996; Christensen *et al.*, 2001; Korch *et al.*, 2003; Lemos *et al.*, 2005; Jørgensen *et al.*, 2009; Christensen-Dalsgaard *et al.*, 2010)].

In *Escherichia coli*, there are at least 39 TA systems (Tan *et al.*, 2011; Wang *et al.*, 2012; Guo *et al.*, 2014) including MqsR/MqsA, a type II TA system in which toxin MqsR is an mRNA endonuclease and antitoxin MqsA binds MqsR to prevent toxicity (Brown *et al.*, 2009). MqsR was first characterized as a quorum sensing-related regulator of biofilm formation (Ren *et al.*, 2004a; González Barrios *et al.*, 2006). MqsR specifically cleaves mRNA at primarily 5'-GCU sites (Christensen-Dalsgaard *et al.*, 2010), and through this specificity, MqsR was found to regulate another TA system, the GhoT/GhoS type V TA system (Wang *et al.*, 2012; 2013) which shows that there is a hierarchy in TA systems. MqsA contains a helix–turn–helix DNA binding domain that allows MqsA to autoregulate expression of the *mqsRA* operon (Brown *et al.*, 2009; 2011). Through this DNA binding domain, MqsA also regulates the general stress response through repression of the stationary phase sigma factor RpoS (Wang and Wood, 2011; Wang *et al.*, 2011) and regulates biofilm formation through repression of biofilm regulator CsgD (Soo and Wood, 2013); thus, MqsA is a regulator of other cell regulators. Therefore, it is clear that the MqsR/MqsA system has regulatory roles to increase the stress response (which includes reducing metabolism through mRNA decay and activating toxin GhoT and RpoS) and to increase biofilm formation during oxidative stress. However, since all but 14 of the *E. coli* transcripts have 5'-GCU sites (Wang *et al.*, 2012), other regulatory roles of the MqsR/MqsA TA system are also possible, including regulating the cell's inhabitation of the GI tract.

In humans, bile is secreted after ingestion of food and contains bile acids, which are amphipathic molecules that aid in solubilizing lipids to digest fats (Hofmann, 1999a). Bile acids are synthesized in the liver from cholesterol and

the majority of bile is stored in the gallbladder (Begley *et al.*, 2005). During digestion, the gallbladder secretes bile into the duodenum (i.e. immediately after the stomach in the digestive tract) (Begley *et al.*, 2005) where the pH typically ranges from 5.3 to 6.3 (Rune and Viskum, 1969), and the majority of bile is recycled to the liver in the ileum (i.e. end of the small intestine) (Martínez-Augustin and Sánchez de Medina, 2008). Bile contains both primary bile acids (e.g. cholic acid and chenodeoxycholic acid) and secondary bile acids derived from primary bile acids by intestinal bacteria (e.g. lithocholic acid and deoxycholic acid) (Begley *et al.*, 2005). In order to increase water solubility, bile acids are often conjugated with glycine or taurine (Martínez-Augustin and Sánchez de Medina, 2008). Human bile typically contains ~40 mM (~2%) bile salts at a pH of 7.5 to 8.0, and bile is 5–10-fold concentrated in the gallbladder (Begley *et al.*, 2005), reaching concentrations as high as 300 mM (~15%) (Hofmann, 1999a). In contrast, bile acids are diluted to sub-millimolar concentrations within the lower intestine (Hofmann, 1999b).

In bacteria, bile acids act as a detergent to cause membrane damage, and susceptibility of cells to bile acids is dependent on the protein and fatty acid composition of the membrane (Begley *et al.*, 2005). Antimicrobial activity of bile serves to limit bacterial cell growth in the small intestine (Hofmann and Eckmann, 2006), which regulates the bacterial terrain. Gram-negative bacteria are significantly more resistant to bile acid stress than Gram-positive bacteria, and *E. coli*, a very resistant species, is often isolated from the gallbladder (i.e. highest bile acid concentration) in humans (Begley *et al.*, 2005). In *E. coli*, lipopolysaccharide structure (*rfa* locus) and efflux pumps (AcrAB, EmrAB and MdtABCD) are important for bile acid resistance, and porins (OmpF and OmpC) affect bile acid susceptibility (Begley *et al.*, 2005). Additionally, promoters for some oxidative stress genes (*micF* and *osmY*) and a DNA damage protection gene (*dinD*) are induced upon exposure of *E. coli* to bile acids, indicating that the oxidative stress response may be triggered by bile acid stress (Bernstein *et al.*, 1999). MicF is a small RNA that represses *ompF* at high osmolarity (Aiba *et al.*, 1987), which restricts uptake of large solutes (e.g. bile salts) across the outer membrane due to the smaller pore size of OmpC compared with OmpF (1.08 nm versus 1.16 nm) (Nikaido and Vaara, 1987). After crossing the outer membrane, unconjugated bile acids are largely protonated due to the acidic pH of the periplasm, thus allowing permeation across the inner membrane (Thanassi *et al.*, 1997).

In this study, we discovered that the MqsR/MqsA TA system is important for managing the stress related to bile acids. Based on transcriptomics, we determined that MqsR reduces YgiS (an uncharacterized, predicted

periplasmic binding protein) through mRNA cleavage in the presence of bile, and that YgiS reduces growth with bile by promoting its uptake. Therefore, we have determined another physiological role for the MqsR/MqsA TA system. We posit that *E. coli* uses MqsR/MqsA in order to improve growth and survival with high bile acid concentrations that are relevant to cells living in the gut and/or gallbladder, an ecological role for TA systems that has not been previously described.

## Results

### *MqsR/MqsA decreases metabolism during deoxycholate stress*

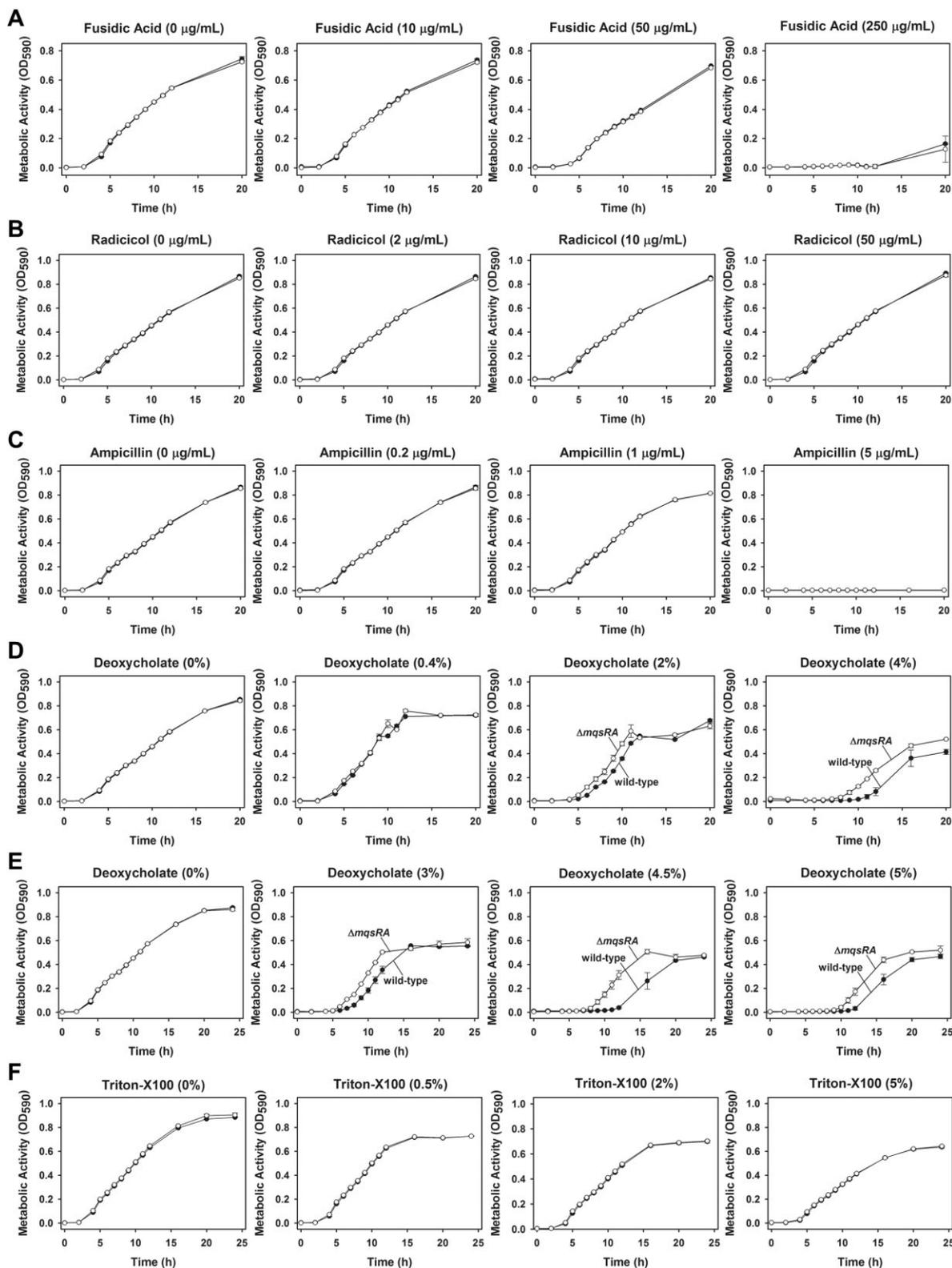
The PortEco phenotypic database (Nichols *et al.*, 2011) provides scores for growth phenotypes from screening a library of single gene mutants in *E. coli* against a panel of 324 different chemical treatments. Hence, we searched the PortEco database for significant positive (increased growth) or negative (reduced growth) scores involving mutants for *mqsR* and *mqsA* in order to determine chemical stresses which may be influenced by the MqsR/MqsA TA system. The database indicated that a strain lacking *mqsR* had reduced growth with fusidic acid (score:  $-3.11$  with  $50 \mu\text{g ml}^{-1}$  and  $-2.32$  with  $20 \mu\text{g ml}^{-1}$ ) and radicicol (score:  $-2.25$  with  $10 \mu\text{M}$  and  $-2.15$  with  $5 \mu\text{M}$ ) (Nichols *et al.*, 2011). Fusidic acid is a fusidane class antibiotic that inhibits bacterial protein synthesis via inhibition of elongation factor G, with bacteriostatic activity at low concentrations and bactericidal activity at high concentrations (Verbist, 1990). Radicicol is a resorcylic acid lactone mycotoxin that exhibits antibiotic activity and is a potent inhibitor of the HSP90 family of proteins (Winssinger and Barluenga, 2007), including HtpG in *E. coli* (Schulte *et al.*, 1999). Additionally, a strain with a hypomorphic mutation in *mqsA* (i.e. reduced activity) was indicated to have increased growth with ampicillin (score:  $3.07$  with  $1 \mu\text{g ml}^{-1}$ ) and reduced growth with deoxycholate (score:  $-2.50$  with 2%) (Nichols *et al.*, 2011). Ampicillin is a  $\beta$ -lactam antibiotic that inhibits peptidoglycan synthesis, preventing cell wall repair and causing cell lysis during division (Fisher *et al.*, 2005). Deoxycholate (also known as deoxycholic acid) is a bile acid that is formed as a metabolic by-product of intestinal bacteria (Begley *et al.*, 2005).

To determine whether MqsR and/or MqsA do in fact affect cell growth during stress with these four compounds, we performed a metabolic activity assay that utilizes tetrazolium dye. Tetrazolium dye is reduced to the purple compound formazan due to NADH produced during cellular respiration (Berridge *et al.*, 2005). A BW25113 strain lacking both *mqsR* and *mqsA* and without any antibiotic selection marker (Kim *et al.*, 2010) (hereafter referred to as  $\Delta mqsRA$ ) was grown alongside

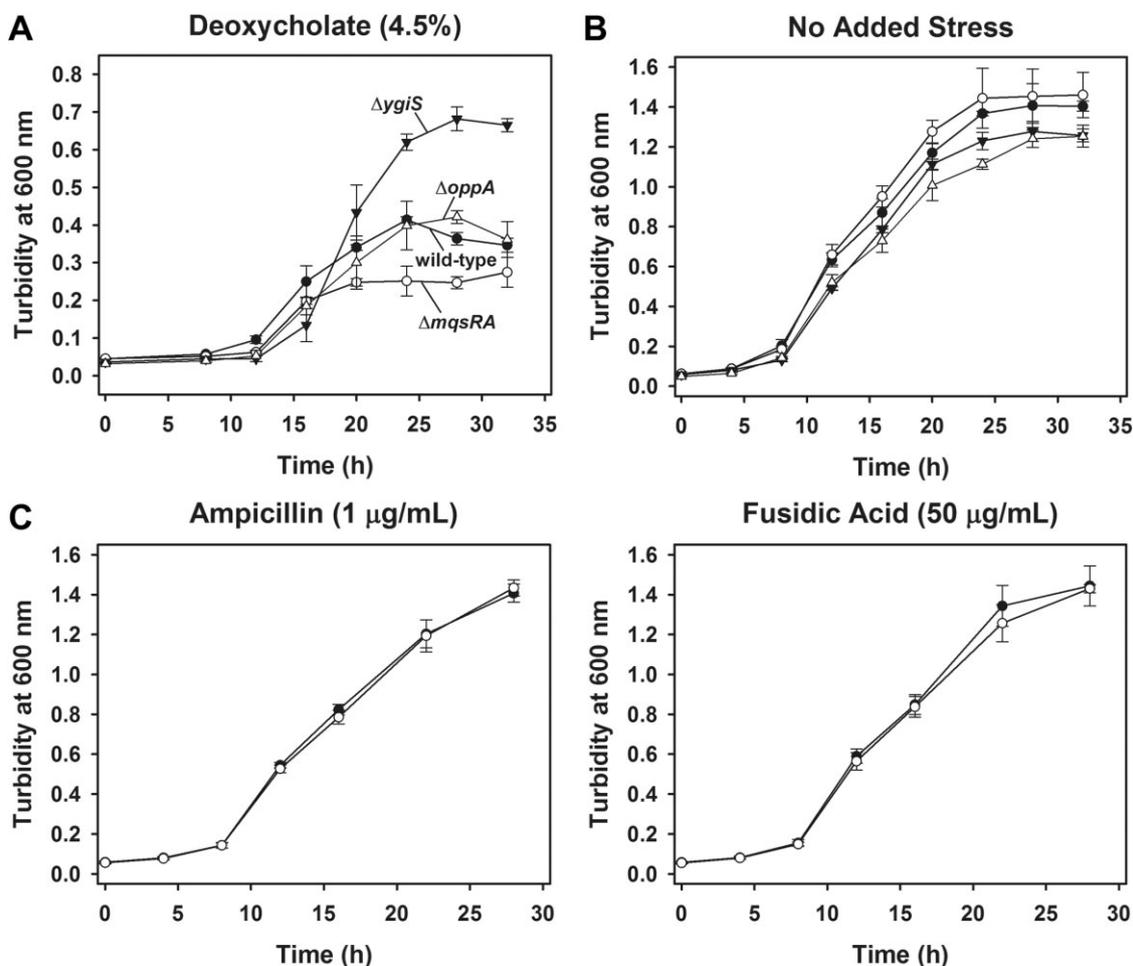
the wild type in media containing fusidic acid (0, 10, 50 and  $250 \mu\text{g ml}^{-1}$ ) (Fig. 1A), radicicol (0, 2, 10 and  $50 \mu\text{M}$ ) (Fig. 1B), ampicillin (0, 0.2, 1.0 and  $5.0 \mu\text{g ml}^{-1}$ ) (Fig. 1C) or deoxycholate (0%, 0.4%, 2.0% and 4.0%) (Fig. 1D). Cultures were monitored spectrophotometrically at 590 nm, gauging overall metabolism based on both cell division (i.e. cell density) and cellular respiration (i.e. reduced tetrazolium dye). Treatments with fusidic acid, radicicol and ampicillin did not show any significant difference in metabolism; however, the  $\Delta mqsRA$  mutant displayed higher metabolic activity than the wild type when grown with both 2% and 4% deoxycholate. We performed the assay using additional concentrations of deoxycholate (0%, 3.0%, 4.5% and 5.0%) to verify our initial result and check for concentration dependence, and we obtained consistent results with the  $\Delta mqsRA$  mutant displaying the most significant increase in metabolic activity during stress with 4.5% deoxycholate (Fig. 1E). Additionally, upon performing the assay with the detergent Triton-X100 (0%, 0.5%, 2.0% and 5.0%), we observed no differences in metabolism (Fig. 1F), demonstrating that the phenotype was not due to the detergent activity of deoxycholate. These results indicate that in wild-type *E. coli*, MqsR/MqsA reduces metabolic activity during bile acid stress from deoxycholate.

### *MqsR/MqsA improves growth during deoxycholate stress*

Since the metabolic activity assay is a combined measurement of cell division and cellular respiration, we sought to distinguish the two metabolic characteristics by monitoring growth based solely on cell density (i.e. no tetrazolium dye). Deoxycholate gelation is a known issue in bacteriological media (Sobotka and Czechowiczka, 1958), and as such we developed a growth medium (referred to here as HEPES-glucose) in which deoxycholate did not undergo gelation (to concentrations above 20%). Using this medium, we performed a growth curve comparing the  $\Delta mqsRA$  mutant with the wild type in the presence of 4.5% deoxycholate and found that the  $\Delta mqsRA$  mutant consistently grew worse than the wild-type (Fig. 2A), suggesting that MqsR/MqsA improves growth during deoxycholate stress. Additionally, there was only a very small difference in growth for the  $\Delta mqsRA$  mutant grown in the HEPES-glucose medium without any stress (Fig. 2B) or with either ampicillin ( $1 \mu\text{g ml}^{-1}$ ) or fusidic acid ( $50 \mu\text{g ml}^{-1}$ ) (Fig. 2C). These results confirmed that the reduced growth of the  $\Delta mqsRA$  mutant was specific to deoxycholate stress. The previously observed increased metabolic activity (i.e. cell division and respiration) of the  $\Delta mqsRA$  mutant with deoxycholate (Fig. 1D and 1E) despite reduced



**Fig. 1.** MqsR/MqsA reduces metabolic activity specifically during deoxycholate stress. Comparison of metabolic activity and growth measured in the presence of tetrazolium dye by optical density at 590 nm for BW25113  $\Delta mqsRA$  ( $\circ$ ) and wild-type ( $\bullet$ ) when exposed to (A) fusidic acid (0, 10, 50 and 250  $\mu\text{g ml}^{-1}$ ), (B) radicolol (0, 2, 10 and 50  $\mu\text{g ml}^{-1}$ ), (C) ampicillin (0, 0.2, 1.0 and 5.0  $\mu\text{g ml}^{-1}$ ), (D and E) deoxycholate (0%, 0.4%, 2.0%, 3.0%, 4.0%, 4.5% and 5.0%) or (F) Triton-X100 (0%, 0.5%, 2.0% and 5.0%). Data are averaged from two independent cultures and one standard deviation is shown.



**Fig. 2.** MqsR/MqsA improves growth and YgiS reduces growth specifically during deoxycholate stress. Comparison of growth in HEPES-glucose medium measured by turbidity at 600 nm for BW25113  $\Delta mqsRA$  (○),  $\Delta ygiS$  (▼),  $\Delta oppA$  (△) and wild-type (●) when exposed to (A) 4.5% deoxycholate, (B) no added stress, (C) 1  $\mu\text{g ml}^{-1}$  ampicillin (left panel) or 50  $\mu\text{g ml}^{-1}$  fusidic acid (right panel). Data are averaged from three independent cultures and one standard deviation is shown.

growth (i.e. cell division) (Fig. 2A) indicates that cells without MqsR/MqsA exhibit significantly elevated cellular respiration during deoxycholate stress. Therefore, we hypothesized that MqsR/MqsA may reduce the susceptibility to deoxycholate by reducing cellular metabolism, which results in improved overall population growth.

#### MqsR decreases *YgiS* mRNA

MqsR regulates the GhoT/GhoS TA system via directed endonuclease activity against mRNA of antitoxin GhoS (Wang *et al.*, 2013) and MqsA is a global regulator, controlling the general stress response via RpoS (Wang and Wood, 2011; Wang *et al.*, 2011) and biofilm physiology via CsgD (Soo and Wood, 2013) by binding specific DNA palindromes. Additionally, regulation of several genes via RpoS affects deoxycholate tolerance in *Salmonella*

*enterica*, demonstrating a multiplicity of bile stress-related regulatory targets (Hernández *et al.*, 2012). Therefore, the MqsR/MqsA system likely affects deoxycholate tolerance through regulatory activity of either the toxin MqsR or the antitoxin MqsA. To test this, we performed a DNA microarray study comparing gene expression in the  $\Delta mqsRA$  mutant compared with the wild type during 4.5% deoxycholate stress. We found that in the  $\Delta mqsRA$  mutant, *ygiS* was the most significantly induced gene (14-fold), while few other genes had significantly altered expression (Table S2). The microarray data were confirmed through quantitative real-time reverse-transcription PCR (qRT-PCR), showing increased expression of *ygiS* ( $3.6 \pm 0.4$ -fold) and relatively little difference in expression of two control genes (*katE*:  $1.4 \pm 0.7$ -fold and *osmY*:  $2.0 \pm 1.6$ -fold) (Table S3). YgiS is an uncharacterized protein, and *ygiS* is located directly downstream of the *mqsRA* operon. BLAST

analysis shows that YgiS is most closely related to OppA, an oligopeptide ABC transporter periplasmic binding protein, although the identity is only 44%. Critically, the YgiS transcript contains 43 5'-GCU sites. Migration of mRNAs (Montero Llopis *et al.*, 2010) and proteins (Kuhlman and Cox, 2012) away from the chromosomal gene location is limited; therefore, the close proximity to the *mqsRA* operon (i.e. high local concentration of MqsR) and the high amount of MqsR-specific endonuclease cleavage sites means that *ygiS* mRNA is a prime substrate for MqsR. Therefore, it is likely that MqsR reduces YgiS by degrading YgiS mRNA.

#### *MqsR degrades YgiS mRNA*

As confirmation that YgiS mRNA is degraded by MqsR endonuclease activity, we performed an *in vitro* cleavage assay. The 5'-GCU selectivity of MqsR was previously demonstrated via lack of degradation of GhoT mRNA, with no cleavage sites, and degradation of GhoS mRNA and mutated GhoT mRNA, both containing 5'-GCU cleavage sites (Wang *et al.*, 2013). Here, we synthesized *in vitro* a 192 nt segment of the YgiS mRNA transcript containing six 5'-GCU sites; this size fragment was more convenient to synthesize than the whole-length transcript. We found that purified MqsR readily degraded the YgiS mRNA similarly to the positive controls, OmpA and GhoS mRNAs (each containing three cleavage sites). As a negative control, GhoT mRNA lacking cleavage sites had almost no degradation (Fig. S1). Thus, YgiS mRNA is a substrate for MqsR-specific cleavage, which is likely well regulated due to the chromosomal proximity of *ygiS* and *mqsR*.

#### *YgiS reduces growth during deoxycholate stress*

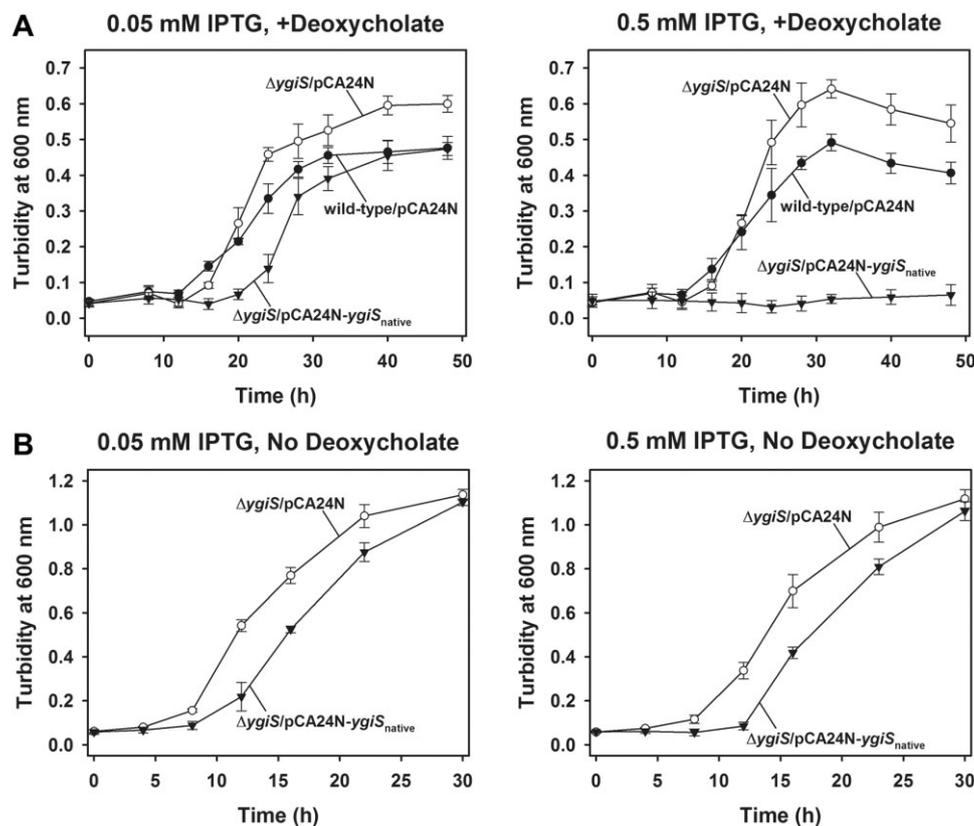
In order to test whether regulation of *ygiS* is involved in the deoxycholate growth phenotype, we removed the antibiotic selection marker in the BW25113  $\Delta ygiS$  strain (used for all subsequent experiments) from the Keio single-gene knock-out collection (Baba *et al.*, 2006), eliminating any confounding effects of the antibiotic resistance cassette. We then compared growth of the  $\Delta ygiS$  mutant to the wild type in the presence of 4.5% deoxycholate. Critically, the  $\Delta ygiS$  mutant consistently grew better than the wild type (Fig. 2A). Due to the sequence homology with *oppA*, we also included a  $\Delta oppA$  mutant that we found grew similarly to the wild type, thus confirming that the growth phenotype was specific to *ygiS*. Additionally, we confirmed that this improved growth is specific to deoxycholate stress, since the  $\Delta ygiS$  mutant grew the same as the wild type in media lacking deoxycholate (Fig. 2B). This suggests that YgiS reduces the ability of cells to grow in the presence of deoxycholate stress.

We sought to further study the role of YgiS during growth with deoxycholate by producing YgiS from an IPTG-inducible plasmid. Based on the primary protein sequence, YgiS is predicted to be a periplasmic protein, and the widely used ASKA collection of single-gene expression vectors includes additional N-terminal amino acid residues (e.g. 6 $\times$  His-tag) (Kitagawa *et al.*, 2005) which likely affects protein translocation via the altered signal peptide. Therefore, we cloned the chromosomal copy of *ygiS* from BW25113 (including the signal sequence) into the pCA24N IPTG-inducible expression vector under the control of the  $P_{T5-lac}$  promoter with the strong, synthetic ribosome binding site used to make the ASKA clones (Kitagawa *et al.*, 2005) to form pCA24N-*ygiS*<sub>native</sub> that produces full-length YgiS including its signal peptide.

By using our native *ygiS* expression vector in a  $\Delta ygiS$  host, we found that production of YgiS complemented the improved deoxycholate growth phenotype of the  $\Delta ygiS$  mutant, reducing growth at low induction (0.05 mM IPTG) and completely inhibiting growth at high induction (0.5 mM IPTG) (Fig. 3A). We also measured growth without deoxycholate and observed that YgiS production caused a slight reduction in growth (Fig. 3B); however, the growth effect was consistent between 0.05 mM and 0.5 mM IPTG induction, suggesting that the slight growth reduction is not due to YgiS production. Additionally, this effect was minor in comparison to the significantly reduced growth with observed for YgiS production with deoxycholate stress. These results demonstrate that there is a clear growth inhibitory phenotype for producing YgiS specifically in conjunction with deoxycholate stress, indicating that YgiS reduces cell fitness against deoxycholate.

#### *YgiS is a periplasmic protein that increases deoxycholate uptake*

Since bioinformatics analysis of the primary sequence suggests that YgiS is a periplasmic protein with a structure somewhat similar to that of OppA, a periplasmic-binding protein, we produced YgiS via our native *ygiS* expression vector, visualized the separate protein fractions using SDS-PAGE and identified protein bands via mass spectrometry to confirm that YgiS is in fact a periplasmic protein (Fig. S2). Since OppA is an ABC membrane transporter, we investigated the effect of YgiS on deoxycholate transport. We grew the  $\Delta mqsRA$  mutant (i.e. increased YgiS) and the  $\Delta ygiS$  mutant (i.e. abolished YgiS) in the presence of 4.5% deoxycholate and isolated the cell lysate in order to quantify intracellular deoxycholate via HPLC analysis. The  $\Delta mqsRA$  mutant accumulated  $37 \pm 12\%$  more deoxycholate and the  $\Delta ygiS$  mutant accumulated  $21 \pm 6\%$  less deoxycholate in



**Fig. 3.** Complementation of the  $\Delta ygiS$  growth phenotype. Comparison of growth in HEPES-glucose medium measured by turbidity at 600 nm for BW25113  $\Delta ygiS/pCA24N$  (○), BW25113  $\Delta ygiS/pCA24N-ygiS_{native}$  (▼) and BW25113/pCA24N (●) with 0.05 mM and 0.5 mM IPTG induction during exposure to (A) 4.5% deoxycholate or (B) no deoxycholate. Data are averaged from three independent cultures and one standard deviation is shown.

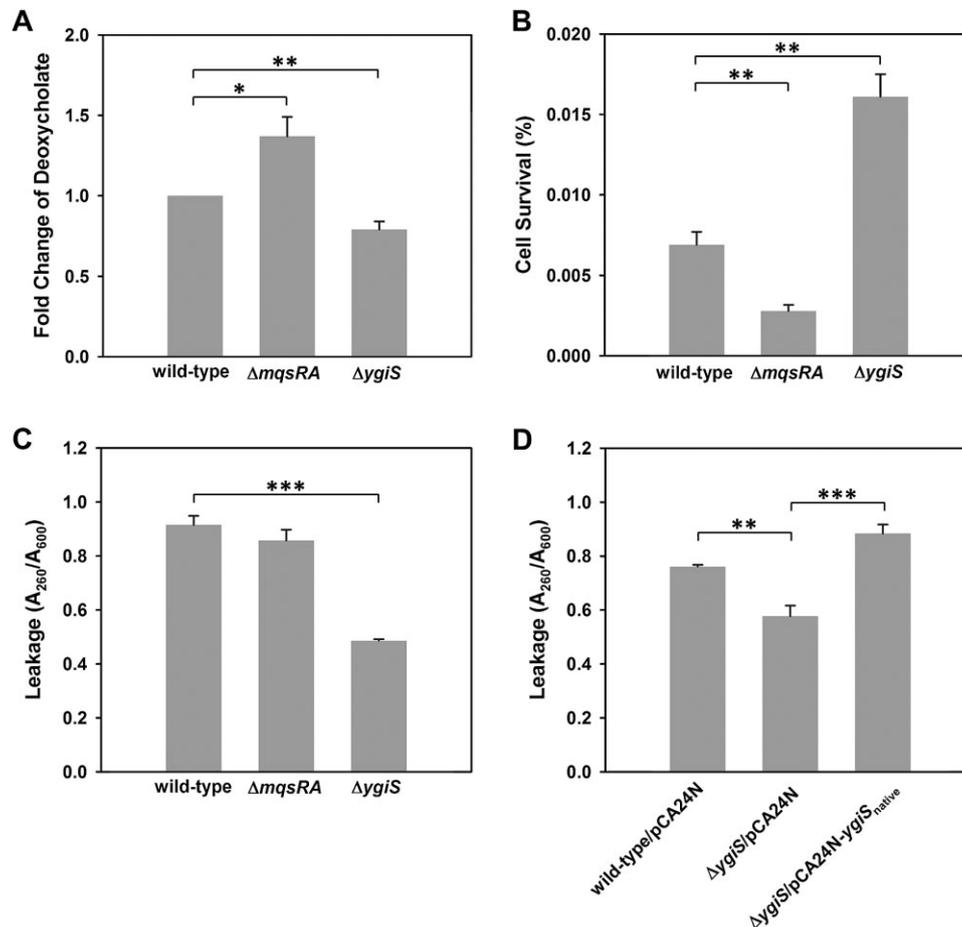
comparison to the wild type (Fig. 4A). This indicates the MqsR/MqsA TA system reduces intracellular deoxycholate and that YgiS increases intracellular deoxycholate concentrations either by increasing import or by decreasing export. For comparison, a similar magnitude change was reported for the deletion of two bile acid efflux pumps (AcrAB and EmrAB), which caused cells to accumulate 68% more chenodeoxycholate, another bile acid (Thanassi *et al.*, 1997). Therefore, YgiS increases intracellular deoxycholate concentrations, which likely increases cell stress.

#### MqsR and YgiS influence deoxycholate tolerance

Since YgiS affects deoxycholate transport, we hypothesized that the altered intracellular deoxycholate concentrations should affect the ability of *E. coli* to tolerate high concentrations and maintain membrane integrity. We exposed cells to 20% deoxycholate and consistently found that the  $\Delta mqsRA$  mutant had fewer survivors ( $-2.5 \pm 0.3$ -fold reduced survival) while the  $\Delta ygiS$  mutant had more survivors ( $2.3 \pm 0.2$ -fold increased survival) in

comparison to the wild type (Fig. 4B). Thus, the MqsR/MqsA TA system increases tolerance to deoxycholate and YgiS reduces tolerance of *E. coli* to deoxycholate, mediated by an adverse effect on transport.

To provide additional evidence of the effect of increased deoxycholate uptake on cell physiology, we took cells grown with 4.5% deoxycholate, resuspended them in a buffered saline solution, and monitored leakage of 260 nm-absorbing material as an indicator of membrane integrity (i.e. more leakage means more membrane damage). Although both the  $\Delta mqsRA$  mutant and the wild type had similar membrane integrity, the  $\Delta ygiS$  mutant exhibited significantly less leakage of 260 nm-absorbing material ( $47 \pm 1\%$  reduced) (Fig. 4C), suggesting higher membrane integrity. Additionally, producing YgiS in the  $\Delta ygiS$  mutant complemented the membrane integrity phenotype by increasing leakage of 260 nm-absorbing material ( $53 \pm 6\%$  increased) in comparison to the  $\Delta ygiS$  mutant with the empty vector (Fig. 4D). Therefore, YgiS causes cells to sustain more membrane damage from deoxycholate stress. This corroborates the reduced growth phenotype observed in the  $\Delta mqsRA$  mutants which are unable to regulate *ygiS* expression (Fig. 2A).



**Fig. 4.** MqsR/MqsA increases deoxycholate tolerance via YgiS.

A. Fold change of normalized intracellular deoxycholate concentrations for BW25113  $\Delta mqsRA$ ,  $\Delta ygiS$  and wild-type grown in HEPES-glucose medium with 4.5% deoxycholate to a turbidity of  $\sim 0.2$ – $0.5$  at 600 nm.  
 B. Cell survival for BW25113  $\Delta mqsRA$ ,  $\Delta ygiS$  and wild-type grown in LB medium to a turbidity of 1.0 at 600 nm and resuspended in HEPES-glucose medium with 20% deoxycholate for 30 min.  
 C. Leakage of 260 nm-absorbing material from BW25113  $\Delta mqsRA$ ,  $\Delta ygiS$  and wild-type grown in HEPES-glucose with 4.5% deoxycholate to a turbidity of  $\sim 0.2$  to 0.6 at 600 nm and incubated in HEPES-buffered saline for 2 h.  
 D. Leakage of 260 nm-absorbing material from BW25113  $\Delta ygiS/pCA24N$ , BW25113  $\Delta ygiS/pCA24N-ygiS_{native}$  and BW25113/pCA24N grown in HEPES-glucose with 4.5% deoxycholate to a turbidity of  $\sim 0.2$  to 0.6 at 600 nm, induced with 0.5 mM IPTG for 4 h, and incubated in HEPES-buffered saline for 2 h. Data are averaged from three independent cultures and one standard deviation is shown. The asterisk indicates statistical significance as determined using a Student's *t*-test (\* $P < 0.05$ , \*\* $P < 0.025$ , \*\*\* $P < 0.01$ ).

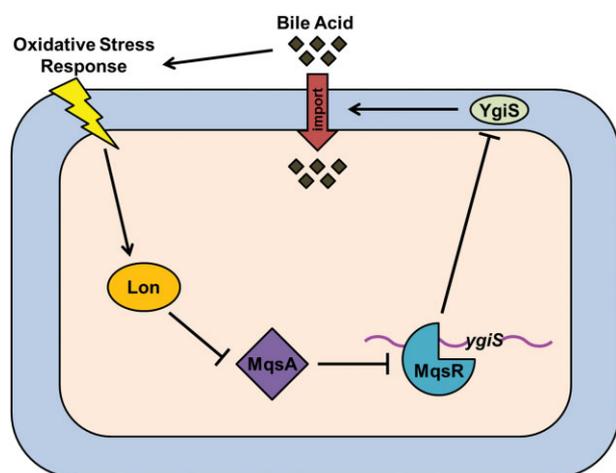
Therefore, MqsR reduces production of YgiS to improve growth during deoxycholate stress.

## Discussion

Here we demonstrated that the MqsR/MqsA type II TA system is physiologically important for the growth of *E. coli* during exposure to deoxycholate stress. We found that MqsR degrades YgiS mRNA, and YgiS is a periplasmic protein that increases the uptake of deoxycholate. High intracellular concentrations of the detergent deoxycholate cause the cells to sustain more membrane damage, thus reducing growth and tolerance.

Therefore, MqsR/MqsA mediates cell growth with deoxycholate stress by reducing YgiS; a schematic of our understanding of the mechanism is shown in Fig. 5.

Bile is stored in the gallbladder and secreted into the duodenum (i.e. upper small intestine) during the digestive process, and contains multiple bile salts that are highly similar in structure, including deoxycholate (Begley *et al.*, 2005). Deoxycholate is by far the most active bile salt, demonstrated in mammalian studies (Delzenne *et al.*, 1992); hence, it was the focus of our study. The physiological relevance of the 4.5% deoxycholate concentration used throughout the majority of this study is substantiated by previous work which demonstrated that



**Fig. 5.** Schematic for regulation of bile acid tolerance by MqsR/MqsA. Bile acid stress induces an oxidative stress response, activating Lon protease which degrades antitoxin MqsA. MqsA binds to toxin MqsR, preventing its endonuclease activity towards mRNA containing 5'-GCU sites (e.g. YgiS mRNA). YgiS is a periplasmic protein that facilitates bile acid uptake. → indicates induction and ⊥ indicates inhibition.

bile typically contains ~2% bile salts (Begley *et al.*, 2005) and is concentrated to ~15% in the gallbladder (Hofmann, 1999a). Since *E. coli* is an enteric bacterial species that also resides in the gallbladder (Begley *et al.*, 2005), our experimental conditions are consistent with the environmental conditions which could be encountered in the human gut. Therefore, our findings suggest that MqsR/MqsA mediates growth and survival of *E. coli*, particularly located near the upper intestinal tract.

Bile salt stress causes lipid peroxidation (Delzenne *et al.*, 1992) and induces an oxidative stress response, based on activation of oxidative stress genes and DNA damage-related genes in both *E. coli* (Bernstein *et al.*, 1999) and mammalian cells (Payne *et al.*, 1998). Importantly, our lab demonstrated that MqsA is degraded by Lon protease in the presence of oxidative stress (Wang and Wood, 2011; Wang *et al.*, 2011; 2012). Therefore, MqsA is likely degraded by bile salt stress to activate MqsR, thus improving bile salt tolerance. While there are instances in which inducing the chromosomal copy of a TA system reduces viability [e.g. reduced translation (Christensen *et al.*, 2001) or increased cell death (Aizenman *et al.*, 1996)], here we found a case in which the TA system is beneficial; i.e. inactivation of MqsR/MqsA leads to a clear phenotype (decrease in growth) under physiological conditions (presence of bile acid). Hence, our results elucidate the importance of this oxidative stress response for *E. coli*, which typically resides under highly anaerobic conditions in the GI tract (i.e. an environment with few reactive oxygen species).

Bile salts also induce bacterial adhesion and biofilm formation (de Jesus *et al.*, 2005; Pumbwe *et al.*, 2007;

Begley *et al.*, 2009; Ambalam *et al.*, 2012), and MqsA inhibits biofilm formation (Wang and Wood, 2011; Wang *et al.*, 2011) via CsgD regulation (Soo and Wood, 2013). Therefore, deactivation of MqsA from bile stress should increase biofilm formation in the GI tract, corroborating that bile salts induce biofilm formation. We posit that bile causes degradation of MqsA via oxidative stress, activating both MqsR and CsgD. Activation of MqsR improves growth and tolerance to bile salts, while simultaneously activation of CsgD promotes adhesion to establish a population and promotes biofilm formation to protect cells against stress from a fluctuating environment (e.g. temporal excretion of bile). Therefore, the type II TA system MqsR/MqsA is a multi-faceted regulator that facilitates growth of *E. coli* populations residing in the gut during exposure to bile stress. Since bile plays an important role as an interkingdom signal in the GI tract (Joyce *et al.*, 2014), our results also illustrate how a TA system can play an important role in host–microbe interactions by ensuring the survival of a commensal bacterium.

## Experimental procedures

### Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Overnight cultures were grown in lysogeny broth (LB) medium (Sambrook *et al.*, 1989) at 37°C unless otherwise indicated, washed and inoculated in HEPES-glucose medium (79.6 mM NaCl, 20.1 mM KCl, 20.3 mM NH<sub>4</sub>Cl, 3.0 mM Na<sub>2</sub>SO<sub>4</sub>, 100 mM HEPES, 1.0 mM MgCl<sub>2</sub>, 0.2 mM CaCl<sub>2</sub>, 0.66 mM K<sub>2</sub>HPO<sub>4</sub> and 0.4% glucose) at 37°C with shaking, unless otherwise indicated. Chloramphenicol (30 µg ml<sup>-1</sup>) was utilized to maintain the pCA24N-based plasmids (Kitagawa *et al.*, 2005). The kanamycin resistance cassette from BW25113  $\Delta ygiS$  and BW25113  $\Delta oppA$  was removed by expressing FLP recombinase from pCP20 (Datsenko and Wanner, 2000). Gene deletions and removal of kanamycin resistance cassettes were verified by sequencing (primers listed in Table S1). Strains with kanamycin resistance cassettes removed were used for all experiments of this study.

### Metabolic activity assay

Overnight cultures of BW25113  $\Delta mqsRA$  and BW25113 wild-type were inoculated in LB medium, grown to a turbidity of 1.0 at 600 nm and resuspended in IF-10 (BioLog, Hayward, CA, USA). Samples were diluted in IF-10 to a turbidity of 0.07 at 600 nm and further diluted 200-fold (turbidity of 0.00035 at 600 nm) into a medium containing IF-10, BioLog Redox Dye D (BioLog), rich medium (0.1% yeast extract, 0.2% tryptone and 0.1% NaCl), and either fusidic acid (0, 10, 50 and 250 µg ml<sup>-1</sup>), radicicol (0, 2, 10 and 50 µM), ampicillin (0, 0.2, 1.0 and 5.0 µg ml<sup>-1</sup>), deoxycholate (0%, 0.4%, 2.0%, 3.0%, 4.0%, 4.5% and 5.0%) or Triton-X100 (0%, 0.5%, 2.0% and 5.0%). Cultures were grown at 37°C in 96-well microtiter plates (100 µl per well) and metabolic activity was monitored

**Table 1.** Bacterial strains and plasmids used in this study.

	Genotype	Source
<b>Strain</b>		
BW25113	<i>rrmB3</i> $\Delta$ <i>lacZ4787</i> <i>hsdR514</i> $\Delta$ ( <i>araBAD</i> )567 $\Delta$ ( <i>rhaBAD</i> )568 <i>rph-1</i>	Baba <i>et al.</i> , 2006
BW25113 $\Delta$ <i>mqsRA</i> $\Delta$ Kan	BW25113 $\Delta$ <i>mqsRA</i> $\Delta$ Km <sup>R</sup>	Kim <i>et al.</i> , 2010
BW25113 $\Delta$ <i>ygiS</i>	BW25113 $\Delta$ <i>ygiS</i> $\Omega$ Km <sup>R</sup>	Baba <i>et al.</i> , 2006
BW25113 $\Delta$ <i>ygiS</i> $\Delta$ Kan	BW25113 $\Delta$ <i>ygiS</i> $\Delta$ Km <sup>R</sup>	This study
BW25113 $\Delta$ <i>oppA</i>	BW25113 $\Delta$ <i>oppA</i> $\Omega$ Km <sup>R</sup>	Baba <i>et al.</i> , 2006
BW25113 $\Delta$ <i>oppA</i> $\Delta$ Kan	BW25113 $\Delta$ <i>oppA</i> $\Delta$ Km <sup>R</sup>	This study
<b>Plasmid</b>		
pCA24N	Cm <sup>R</sup> ; <i>lacI</i> , pCA24N	Kitagawa <i>et al.</i> , 2005
pCA24N- <i>ygiS</i> <sub>native</sub>	Cm <sup>R</sup> ; <i>lacI</i> , pCA24N P <sub>T5-lac</sub> :: <i>ygiS</i> <sub>native</sub>	This study
pCA24N- <i>oxyR</i>	Cm <sup>R</sup> ; <i>lacI</i> , pCA24N P <sub>T5-lac</sub> :: <i>oxyR</i>	Kitagawa <i>et al.</i> , 2005
pET30a- <i>mqsR</i>	Km <sup>R</sup> ; pET30a(Kan) P <sub>T7</sub> :: <i>mqsR</i> <sup>+</sup>	Brown <i>et al.</i> , 2013
pCP20	Ap <sup>R</sup> , Cm <sup>R</sup> ; FLP <sup>+</sup> , $\lambda$ cI857 <sup>+</sup> , $\lambda$ p <sub>R</sub> Rep <sup>ts</sup>	Cherepanov and Wackernagel, 1995

by measuring optical density at 590 nm, indicating both reduction of tetrazolium dye to formazan (Berridge *et al.*, 2005) and sample turbidity. Experiments were performed with at least two independent cultures.

#### DNA microarrays and qRT-PCR

Overnight cultures of BW25113  $\Delta$ *mqsRA* and BW25113 wild-type were washed, inoculated in HEPES-glucose medium, grown to a turbidity of 0.6 at 600 nm and exposed to 4.5% deoxycholate for 30 min. Cell pellets were collected with RNALater buffer (Applied Biosystems, Foster City, CA, USA), to stabilize RNA, and rapidly frozen in ethanol/dry ice. Cells were lysed using 0.1 mm zirconia/silica beads and a bead beater (Biospec, Bartlesville, OK, USA) and total RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany) (Ren *et al.*, 2004a). cDNA synthesis, fragmentation and hybridization to *E. coli* GeneChip Genome 2.0 arrays (Affymetrix, Santa Clara, CA, USA) were performed as described previously (González Barrios *et al.*, 2006). Genes were identified as differentially expressed if the expression signal ratio was higher than the standard deviation (1.64) and the *P*-value for comparing two chips was less than 0.05 (Ren *et al.*, 2004b). The gene expression dataset is available through NCBI GEO Accession No. GSE59441.

qRT-PCR was performed to verify differential gene expression from the microarray study following the manufacturer's instructions for the Power SYBR Green RNA-to-C<sub>T</sub> 1-Step kit (Life Technologies, Carlsbad, CA, USA) using 100 ng of total RNA as the template. Primers were annealed at 60°C, and data were normalized against the housekeeping gene *rrsG* (Soo and Wood, 2013). The specificity of qRT-PCR primers (Table S1) was verified via standard PCR, and fold changes were calculated using the 2<sup>- $\Delta\Delta$ CT</sup> formula (Pfaffl, 2001).

#### MqsR endoribonuclease assay

MqsR was produced from pET30a-*mqsR* and purified/refolded as described previously (Brown *et al.*, 2013). PCR was performed on BW25113 chromosomal DNA using primers listed in Table S1, with the T7 RNA polymerase promoter sequence included in the forward primers. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and

0.5–1.0  $\mu$ g were used as DNA templates for *in vitro* transcription of *YgiS*, *OmpA*, *GhoS* and *GhoT* mRNAs with the AmpliScribe T7-Flash Transcription Kit (Epicentre, Madison, WI, USA). The MqsR endoribonuclease cleavage assay was performed in a reaction mixture (10  $\mu$ l) containing 2  $\mu$ g of mRNA, 100 mM KCl, 2.5 mM MgCl<sub>2</sub> and either 30 ng of purified MqsR in MqsR buffer (10 mM Tris pH 7.0, 250 mM NaCl and 0.5 mM TCEP) or an equivalent volume of MqsR buffer without MqsR. The reaction mixture was incubated at 37°C for 15 min and then quenched by addition of an equal volume of 2 $\times$  sample loading buffer (Invitrogen, Carlsbad, CA, USA), addition of RNase Inhibitor (New England Biolabs, Ipswich, MA, USA) to a concentration of 2.5% and heating at 65°C for 5 min. The reaction products were resolved via electrophoresis with a 15% TBE-Urea denaturing gel and stained with ethidium bromide.

#### Construction of the *ygiS* expression vector with an intact signal sequence

To construct a plasmid for producing native *YgiS* including its signal sequence, genomic DNA from BW25113 was amplified for *ygiS* by PCR using primers containing *Sall* and *HindIII* restriction sites and the strong synthetic ribosome binding site used in constructing the ASKA collection (Kitagawa *et al.*, 2005). The amplified product was cloned into pCA24N under the control of the P<sub>T5-lac</sub> promoter to form pCA24N-*ygiS*<sub>native</sub> and the cloned vector was confirmed by DNA sequencing. Primer sequences are listed in Table S1.

#### Verification of *YgiS* in the periplasm

Cultures of BW25113  $\Delta$ *ygiS*/pCA24N-*ygiS*<sub>native</sub>, BW25113  $\Delta$ *ygiS*/pCA24N (empty vector control) and BW25113/pCA24N-*oxyR* (cytoplasmic protein control) were grown in LB medium to a turbidity of 0.5 at 600 nm and induced with 1 mM IPTG for 6 h. Cells were pelleted, resuspended in osmotic shock buffer (200 mM Tris, 20% sucrose and 1 mM EDTA at pH 7.5) and incubated at 30°C for 15 min. Cells were pelleted, resuspended in chilled deionized water and incubated on ice for 15 min. Cells were centrifuged and the supernatant was collected (i.e. periplasmic protein fraction). Cells were resuspended in lysis buffer (25 mM Tris at pH 8.0) and sonicated to lyse cells using a 60 Sonic Dismembrator

(Fisher Scientific, Hampton, NH, USA). Samples were pelleted and the supernatant was collected (i.e. cytoplasmic protein fraction). An EDTA-free protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) was used to stabilize protein fractions. This modified procedure was based on previous periplasmic protein isolation protocols (French *et al.*, 1996) (PeriPreps Periplasting kit; Epicentre). Samples were visualized by SDS-PAGE with Coomassie blue staining and the identity of protein bands was verified via mass spectrometry.

#### Intracellular deoxycholate assay

Overnight cultures were washed, inoculated into HEPES-glucose medium containing 4.5% deoxycholate and grown until reaching a turbidity of ~0.2–0.5 at 600 nm. Cells were pelleted, resuspended in HEPES-glucose medium, sonicated to lyse cells using a 60 Sonic Dismembrator (Fisher Scientific) and centrifuged to remove cell debris. Deoxycholate in the supernatant was purified with a BakerBond SPE reversed-phase (C18) disposable column (Avantor Performance Materials, Center Valley, PA, USA) (Girard *et al.*, 1994), using a MasterFlex peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA) to maintain a flow rate of 5 ml min<sup>-1</sup>. The column was washed with methanol and conditioned with deionized water, the supernatant was applied to the column, the column was washed with deionized water and the pump was run to remove remaining solvent from the column. Deoxycholate was eluted from the column in methanol and quantified by comparison to a standard curve via HPLC analysis. HPLC analysis was performed on a reversed-phase (C18) column (Waters Spherisorb ODS2, 5 µm, 4.6 mm × 250 mm; Waters, Milford, MA, USA) at a flow rate of 1 ml min<sup>-1</sup> with a mobile phase consisting of methanol: acetonitrile: 0.02 M sodium acetate (37.5:37.5:25) adjusted to pH 4.3 (Girard *et al.*, 1994) with HCl. Peak absorbance for deoxycholate was analysed at 210 nm with a retention time of ~14–16 min and the results were normalized against turbidity at 600 nm. Samples were spiked with deoxycholate solubilized in methanol to verify that the correct peak was analysed. Experiments were performed with at least three independent cultures.

#### Deoxycholate tolerance assay

Overnight cultures were inoculated into LB medium and grown until reaching a turbidity of 1.0 at 600 nm. Cells were pelleted, resuspended in HEPES-glucose medium with 20% deoxycholate and incubated for 30 min. To determine survival, cell viability was measured for samples taken before and after exposure to deoxycholate. Samples were serially diluted in 0.85% NaCl solution, plated on LB agar and grown overnight at 37°C to determine cfu ml<sup>-1</sup> (Donegan *et al.*, 1991). Experiments were performed with at least three independent cultures.

#### Leakage assay of 260 nm-absorbing material

Overnight cultures were washed, inoculated into HEPES-glucose containing 4.5% deoxycholate and grown until reach-

ing a turbidity of ~0.2 to 0.6 at 600 nm. For strains with expression vectors, cultures were incubated for an additional 4 h with 0.5 mM IPTG induction. Cultures were pelleted and resuspended in HEPES-buffered saline (140 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub> and 50 mM HEPES). Samples were taken after 2 h and centrifuged, the supernatant was filtered (0.2 µm pore size), the absorbance was measured at 260 nm (Carson *et al.*, 2002), and the results were normalized against turbidity at 600 nm. Experiments were performed with at least three independent cultures.

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### Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** MqsR cleaves YgiS mRNA *in vitro*. Two micrograms of *in vitro* synthesized OmpA mRNA (211 nt, three 5'-GCU

sites), YgiS mRNA (192 nt, six 5'-GCU sites), GhoT mRNA (180 nt, no 5'-GCU sites) and GhoS mRNA (278 nt, three 5'-GCU sites) were incubated at 37°C with (+) or without (-) 30 ng of purified MqsR for 15 min. M, low-range ssRNA ladder (sizes indicated in nt on the left).

**Fig. S2.** YgiS is localized in the periplasm. Cytoplasmic (lanes 2–4) and periplasmic (lanes 5–7) protein fractions from BW25113  $\Delta ygiS/pCA24N-ygiS_{native}$  (lanes 3 and 6), BW25113  $\Delta ygiS/pCA24N$  (empty vector control, lanes 2 and 5) and BW25113/pCA24N-*oxyR* (cytoplasmic protein control, lanes 4 and 7) were visualized by SDS-PAGE. Lane 1 is the protein ladder with standards covering a molecular weight range from 10 to 170 kDa. Arrows indicate YgiS (lane 6) and OxyR (lane 4).

**Fig. S3.** Comparison of deoxycholate uptake. (A, B and C) BW25113 wild-type, (D, E and F)  $\Delta mqsRA$  and (G, H and I)  $\Delta ygiS$  were grown with 4.5% deoxycholate and HPLC was used to analyse intracellular deoxycholate via a reversed-phase (C18) column and absorbance at 210 nm.

Deoxycholate levels were compared based on integration of the peak with a retention time of ~14.3 min, and this peak was verified by spiking samples.

**Table S1.** Oligonucleotides used for cloning *ygiS* expression vector, qRT-PCR, verification of Kan<sup>R</sup> excision and verification of plasmid construct. 'F' indicates forward primers and 'R' indicates reverse primers.

**Table S2.** Summary of the largest fold changes in gene expression for BW25113  $\Delta mqsRA$  versus BW25113 wild-type stressed with 4.5% deoxycholate.

**Table S3.** Summary of qRT-PCR results. The cycle number ( $C_i$ ) for each sample is indicated for the target genes (*ygiS*, *katE* and *osmY*) as well as for the housekeeping gene, *rrsG*, which was used to normalize the data. Fold changes in transcription were calculated using (Pfaffl, 2001):  $2^{-(C_{t \text{ target}, \Delta mqsRA} - C_{t \text{ rrsG}, \Delta mqsRA})} / 2^{-(C_{t \text{ target}, \text{wild-type}} - C_{t \text{ rrsG}, \text{wild-type}})}$ . The specificity of the qRT-PCR products were verified by melting curve analysis (Pfaffl, 2001). Means and standard deviations are indicated ( $n = 2$ ).