microbial biotechnology

Microbial Biotechnology (2012) **5**(4), 509–522 doi:10.1111/j.1751-7915.2011.00327.x

# Bacterial persistence increases as environmental fitness decreases

**Seok Hoon Hong,[1] Xiaoxue Wang,[1] Hazel F. O'Connor,[1] Michael J. Benedik[2] and Thomas K. Wood[1,2]***
*Departments of [1]Chemical Engineering and [2]Biology, Texas A&M University, College Station, TX 77843-3122, USA.*

## Summary

**Since persister cells cause chronic infections and since *Escherichia coli* toxin MqsR increases persisters, we used protein engineering to increase the toxicity of MqsR to gain insights into persister cell formation. Through two amino acid replacements that increased the stability of MqsR, toxicity and persistence were increased. A whole-transcriptome study revealed that the MqsR variant increased persistence by repressing genes for acid resistance, multidrug resistance and osmotic resistance. Corroborating these microarray results, deletion of *rpoS*, as well as the genes that the master stress response regulator RpoS controls, increased persister formation dramatically to the extent that nearly the whole population became persistent. Furthermore, wild-type cells stressed by prior treatment to acid or hydrogen peroxide increased persistence 12 000-fold. Whole-transcriptome analyses of persister cells generated by two different methods (wild-type cells pretreated with hydrogen peroxide and the *rpoS* deletion) corroborated the importance of suppressing RpoS in persister cell formation. Therefore, the more toxic MqsR *increases* persistence by *decreasing* the ability of the cell to respond to antibiotic stress through its RpoS-based regulation of acid resistance, multidrug resistance and osmotic resistance systems.**

## Introduction

Persisters comprise a subpopulation of bacteria that become highly tolerant to antibiotics and reach this state without undergoing genetic change (Lewis, 2010). Persister cells in biofilms appear to be responsible for the recalcitrance of chronic infections since antibiotics kill the majority of cells but persisters remain viable and repopulate biofilms when the level of antibiotics drops (Lewis, 2010). Persisters are thought to be less sensitive to antibiotics since the cells are not undergoing cellular activities that antibiotics corrupt, which results in tolerance (i.e. no growth and slow death). In contrast, resistance mechanisms arise from genetic changes that block antibiotic activity which results in resistance; i.e. cells grow in the presence of antibiotics when they are resistant whereas persister cells do not grow and are dormant (Lewis, 2007). Therefore, understanding persister cell formation is important to derive strategies for controlling bacterial infections. However, the genetic mechanism of persister cell formation is not fully understood.

Bacterial toxin/antitoxin (TA) systems appear to be the most likely genetic basis of persister cell formation, since the expression of TA modules are frequently linked to the dormant state (Lewis, 2008; Kim and Wood, 2010; Maisonneuve *et al.*, 2011; Wang and Wood, 2011). TA systems are diverse and abundant in prokaryotic cells (Leplae *et al.*, 2011), and typically consist of two genes in one operon encoding a stable toxin that disrupts an essential cellular process and a labile antitoxin that neutralizes toxicity by binding to the protein or to the mRNA of the toxin (Wang and Wood, 2011). Since the role of TA systems in cell physiology is not well understood, nine possible roles have been proposed (Magnuson, 2007): addictive genomic debris, stabilization of genomic parasites, selfish alleles, gene regulation, growth control, persister cell formation, programmed cell arrest, programmed cell death and anti-phage measures. Recently, three new roles of TA modules in cell physiology have been discovered, including influencing biofilm formation (González Barrios *et al.*, 2006; Kim *et al.*, 2009), mediating the general stress response by controlling the stationary-phase sigma factor RpoS (Wang *et al.*, 2011), and regulating gene expression at a post-transcriptional level by differential mRNA decay (DMD) (González Barrios *et al.*, 2006; Amitai *et al.*, 2009; Wang and Wood, 2011).

Many bacterial chromosomes have several TA systems; for example, *Escherichia coli* has at least 37 TA systems (Tan *et al.*, 2011), and *Mycobacterium tuberculosis* has at least 88 TA systems (Ramage *et al.*, 2009). The various

Received 6 November, 2011; accepted 7 December, 2011. *For correspondence. E-mail twood@engr.psu.edu; Tel. (+1) 814 863 4811; Fax (+1) 814 865 7846.
Re-use of this article is permitted in accordance with the Terms and Conditions set out at http://wileyonlinelibrary.com/onlineopen#OnlineOpen_Terms

© 2012 The Authors
Microbial Biotechnology © 2012 Society for Applied Microbiology and Blackwell Publishing Ltd

TA systems may allow the cell to respond to a specific stress or groups of stresses in a highly regulated, elegant fashion (Wang and Wood, 2011). Hence, there are many TA systems involved in diverse regulatory pathways, but our understanding of these regulatory systems is far from complete.

Among *E. coli* TA loci, the MqsR/MqsA TA pair was the first to be shown to have a definitive role in persistence; deletion of the *mqsRA* locus or deletion of *mqsR* alone decreases persister cell formation and production of MqsR increases the number of persisters (Kim and Wood, 2010). Furthermore, *mqsR* is the most-induced gene in persister cells compared with non-persisters (Shah *et al*., 2006). *mqsR* was first identified in a whole-transcriptome study of biofilms cells as an induced gene (Ren *et al*., 2004a), and MqsR/MqsA was linked to biofilm formation and motility via the autoinducer-2 quorum sensing system (González Barrios *et al*., 2006). The three dimensional structure of MqsR/MqsA revealed that MqsR is an RNase similar to RelE and YoeB (Brown *et al*., 2009) that cleaves mRNA at GCU sites (Yamaguchi *et al*., 2009), and MqsR toxicity requires protease Lon and ClpXP (Kim *et al*., 2010; Wang *et al*., 2011). The MqsR/MqsA TA system was the first TA system to affect persistence upon deletion (Kim and Wood, 2010). Similarly, the TisAB/IstR-1 TA system of *E. coli* decreases persistence to ciprofloxacin upon deletion (Dörr *et al*., 2010). Thus, MqsR toxicity is directly associated with persister formation.

As a specific RNase, toxin MqsR is a global regulator that controls protein expression by enriching specific mRNAs that either lack the GCU cleavage sites or are protected from degradation by secondary structure or bound proteins (Wang and Wood, 2011). This specific RNase activity may dictate whether the cell responds to the stress by being able to rapidly form new proteins to cope with the stress while forming a biofilm or whether the cell becomes dormant (i.e. a persister cell) in a biofilm (Wang *et al*., 2011). Previously, we identified eight proteins (CspD, ClpX, ClpP, Lon, YfjZ, RelB, RelE and HokA) that are involved in MqsR DMD where three of these are related to the degradation of the antitoxin MqsA (ClpX, ClpP and Lon) (Kim *et al*., 2010). In addition, deletion of *mqsR* induces 76 genes, and MqsR production induces 132 genes (Kim *et al*., 2010); these initial results indicated that stress-associated proteins CstA, CspD, RpoS, ClpP, ClpB and Dps are produced when MqsR is activated which linked MqsR to the stress response although the mechanism was not clear. Similarly, toxin MazF is a specific RNase whose activity results in the production of a pool of small proteins that are necessary both for toxicity and for survival (Amitai *et al*., 2009).

In this study, we explored how the toxicity of MqsR is related to persistence by using protein engineering (Wood *et al*., 2011) to increase the toxicity of MqsR; we reasoned that the genetic basis for persistence would be easier to study if a toxin related to persistence had enhanced stability without a change in catalytic function. Utilizing a system biology approach (transcriptome analysis) along with quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and cell survival studies, we found that MqsR increases persister cell formation by repressing acid resistance, multidrug resistance and osmotic resistance, and that the general stress response master regulator RpoS is important for persister cell formation. Together with the result that wild-type cells that are stressed prior to antibiotic treatment increase 12 000-fold in persistence, we concluded that stressed cells become more persistent than those that are not stressed.

## Results

### *Random mutagenesis of MqsR for increased toxicity*

Previously, we demonstrated that producing MqsR is toxic (Zhang *et al*., 2008) and that MqsR forms a toxin/antitoxin pair with antitoxin MqsA (Brown *et al*., 2009; Kim *et al*., 2010). To explore how toxicity affects persister cell formation, we altered MqsR for increased toxicity. Using error prone PCR (epPCR) of *mqsR* with a 0.8% error rate confirmed by sequencing three random *mqsR* genes, a MqsR variant library was constructed, and a total of 2160 colonies were screened for reduced colony growth on Luria–Bertani (LB) agar plates. The inherent MqsR toxicity was decreased by eliminating *mqsR* expression prior to screening by using glucose and by using a strain with a chromosomal copy of the antitoxin gene. The MqsR variants of interest were confirmed by reduced cell growth in LB culture in shake flasks. Two MqsR variants were identified, which caused up to fourfold reduced cell growth compared with native MqsR (Fig. 1A), indicating that these MqsR variants are more toxic than native MqsR. As a control, producing native MqsR repressed cell growth compared with empty pBS(Kan) (Fig. 1A) as expected (Zhang *et al*., 2008). MqsR variant 2-1 had two amino acid replacements at K3N and N31Y, and MqsR 20-14 had three replacements at R9C, L35F and V70I; there were no changes to the promoter or ribosome binding site regions. Hence, MqsR was altered successfully to increase toxicity.

### *MqsR 2-1 is more stable than native MqsR*

Amino acid residues K56, Q68, Y81 and K96 of MqsR are key residues for catalysis in MqsR-mediated toxicity as identified by alanine-scanning mutagenesis of evolutionarily and structurally conserved residues (Brown *et al*., 2009). Since the two amino acid substitutions K3N and

© 2012 The Authors

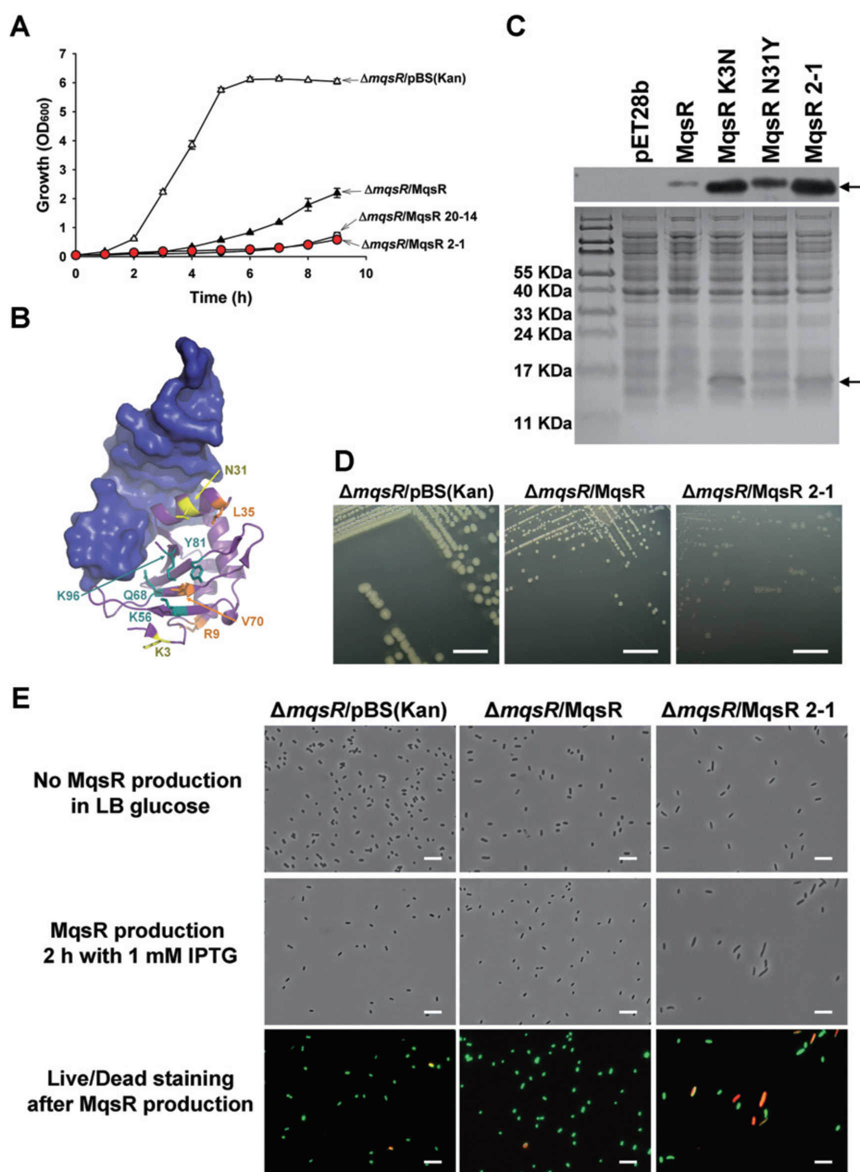


**Fig. 1.** Growth, stability and colony morphology of MqsR variants.

A. Growth curves in LB medium with 1 mM IPTG induction at 37°C for BW25113 Δ*mqsR* strains containing pBS(Kan) (empty plasmid control), pBS(Kan)-*mqsR* (MqsR), pBS(Kan)-*mqsR* 2-1 (MqsR 2-1) and pBS(Kan)-*mqsR* 20-14 (MqsR 20-14). Error bars indicate standard deviation ($n = 3$).

B. Structure of MqsR in ribbon representation with MqsA in surface representation (based on Protein Data Bank accession code 3HI2). Cyan indicates MqsR active site residues (K56, Q68, Y81 and K96), yellow indicates the residues (K3 and N31) where the substitutions occur in MqsR 2-1 (K3N and N31Y), and orange indicates the residues (R9, L35 and V70) where the substitutions occur in MqsR 20-14 (R9C, L35F and V70I).

C. Protein stability of native MqsR and the MqsR variants (MqsR K3N, MqsR N31Y and MqsR 2-1). Western blot (upper panel) and SDS-PAGE show the protein levels of His-MqsR and variants detected by a His-tagged antibody. MqsR and the variants were induced from pET28a-based plasmids in *E. coli* BL21 (DE3) via 1 mM IPTG. Arrows indicate the MqsR proteins.

D. Colony morphology of BW25113 Δ*mqsR* strains containing pBS(Kan) (empty plasmid), pBS(Kan)-*mqsR* and pBS(Kan)-*mqsR* 2-1 grown on LB agar plates at 37°C after 24 h. Scale bars indicate 1 cm. Representative images are shown.

E. Observation of BW25113 Δ*mqsR* strains containing pBS(Kan) (empty plasmid control), pBS(Kan)-*mqsR* and pBS(Kan)-*mqsR* 2-1. IPTG was added to LB for 2 h to produce native MqsR and MqsR 2-1 (middle row), while glucose (0.2%) was added to LB to repress MqsR production (upper row). Lower row shows the result of Live/Dead staining after producing MqsR and MqsR 2-1. Live cells are stained in green, and dead cells are stained in red. Scale bars indicate 10 μm. Representative images are shown.

© 2012 The Authors
Microbial Biotechnology © 2012 Society for Applied Microbiology and Blackwell Publishing Ltd, 

N31Y in MqsR 2-1 are distant from the active site residues and residues involved in binding MqsA (Fig. 1B), these substitutions probably do not affect catalytic activity of MqsR (Brown *et al*., 2009). It is also possible that the MqsR 2-1 aa substitutions may affect MqsR binding with MqsA, which might change MqsA-mediated gene regulation based on its palindrome recognition site (Wang *et al*., 2011).

To investigate this further, two amino acid substitutions K3N and N31Y in MqsR 2-1 were introduced into plasmid pET28a-*mqsR*, which has a N-terminal His-tag and is controlled by a T7 promoter, using site-directed mutagenesis, to generate the pET28a-*mqsR* K3N, pET28a-*mqsR* N31Y and pET28a-*mqsR* 2-1 (K3N and N31Y) plasmids. Western blot analysis and SDS-PAGE were used (Fig. 1C); these results showed that MqsR 2-1 is more than an order of magnitude more stable than native MqsR; hence, the increase in activity of MqsR 2-1 is due to its increased stability. Furthermore, since each amino acid substitution (K3N and N31Y) of MqsR 2-1 increased the stability of MqsR (eightfold and fourfold respectively) compared with native MqsR, the combination of both amino acid replacements of MqsR 2-1 increase the protein level of MqsR 2-1. Therefore, the increase in toxicity of MqsR 2-1 is due to the dramatic increase in its stability, as it is easier to increase protein stability while maintaining high function than to change function while maintaining high stability (Bloom *et al*., 2004).

### *MqsR 2-1 reduces colony size and enhances cell death*

The increased toxicity of MqsR 2-1 was also visible by the changes in colony morphology. Cells producing MqsR 2-1 formed colonies that were non-uniform, translucent and thin compared with those of cells producing native MqsR (Fig. 1D); similar colony morphology was observed with MqsR 20-14. Furthermore, producing MqsR 2-1 made the cell elongate 2.7-fold (Fig. 1E) and lyse (Fig. 1E) compared with native MqsR. After 2 h of induction, 17% of the cells producing MqsR 2-1 were dead (Fig. 1E); most dead cells were the elongated, thus the increase in cell size by MqsR 2-1 is indicative of cell death. For comparison, only 3% of cells died upon producing native MqsR. Taken together, MqsR 2-1 induces cell death and lysis via its increased toxicity when it is produced from a high copy number plasmid.

### *MqsR 2-1 increased toxicity induces persister cell formation*

We investigated whether the MqsR variants with enhanced toxicity enhance persister cell formation with ampicillin. MqsR increases persistence while deletion of *mqsR* reduces persistence (Kim and Wood, 2010). Consistent with these previous results, producing native MqsR in a cell which lacks a chromosomal copy of *mqsR* increased cell survival more than 200-fold under antibiotic stress compared with the empty plasmid control (Fig. 2). Producing MqsR 2-1 increased persister cell formation another 73-fold compared with producing native MqsR, and so overall a 14 000-fold increase in persistence was obtained by MqsR 2-1 compared with no MqsR (Fig. 2). Similar results were observed with two other concentrations of ampicillin. Therefore, the increased toxicity of MqsR dramatically enhances persister cell formation.

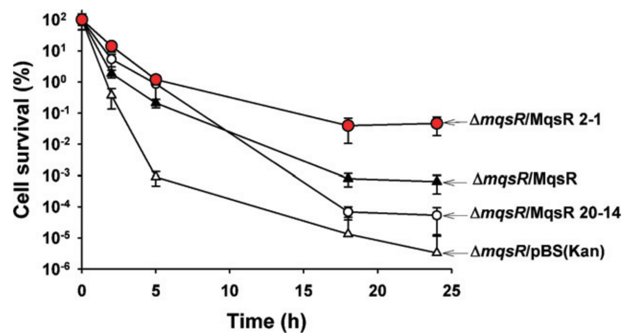


**Fig. 2.** Persister cell formation of MqsR variants. BW25113 Δ*mqsR* strains containing pBS(Kan) (empty plasmid control), pBS(Kan)-*mqsR*, pBS(Kan)-*mqsR* 2-1 and pBS(Kan)-*mqsR* 20-14 were grown to a turbidity of 0.5 at 600 nm in LB with 1 mM IPTG at 37°C, adjusted to a turbidity of 1, and exposed to 26 μg ml$^{-1}$ ampicillin with 1 mM IPTG for 24 h. Error bars indicate the standard deviation ($n = 3$).

### *MqsR 2-1 reduces production of stress response proteins*

To discern how the increase in toxicity of MqsR 2-1 increases persister production via ampicillin treatment, we performed a whole-transcriptome analysis of cells producing MqsR 2-1 versus native MqsR. Unlike a traditional DNA microarray, apparent induction of gene expression in this experiment may be due to both greater degradation of other transcripts by MqsR 2-1, i.e. enrichment, as well as due to induction of gene expression.

Under ampicillin antibiotic stress, MqsR 2-1 led to the enrichment of 78 transcripts by more than fourfold (Table S1). MqsR 2-1 production enriched the master motility regulator transcripts (*flhDC*), which was confirmed by the higher swimming motility of cells producing MqsR 2-1 relative to native MqsR, which had no motility (halo diameter 0.8 ± 0.1 cm for MqsR 2-1 versus 0.0 ± 0.0 cm for native MqsR, Fig. S1). Also, *tnaC* encoding the tryptophanase leader peptide was enriched by MqsR 2-1. *tnaC* is one of the 14 MqsR-resistant mRNAs that lack the MqsR-specific GCU sequences (Yamaguchi *et al*., 2009). Carbon starvation (*cstA*), glycerol-3-phosphate metabolism (*glpQT*), galactitol metabolism (*gatABCDYZ*), ribose

© 2012 The Authors

transport (*rbsACD*) and glucitol/sorbitol metabolism (*srlABDE*) transcripts were also enriched by MqsR 2-1. Enrichment of these transcripts during MqsR 2-1 production was confirmed by qRT-PCR using independent cultures. Corroborating the DNA microarray data, MqsR 2-1 enriched transcripts of *tnaC*, *cstA*, *glpT*, *rbsA* and *srlA* by three- to fivefold (Table S2). Glycerol-3-phosphate metabolism might be related to MqsR toxicity and persister formation (Kim *et al*., 2010) since producing GlpD increased tolerance to ampicillin and ofloxacin, while deletion of *glpD* repressed persister production (Spoering *et al*., 2006). Moreover, enrichment of transcripts related to galactitol metabolism may also be linked to persister cell formation as the genes for galactitol metabolism are highly regulated in biofilms (Domka *et al*., 2007) in which persister frequencies are elevated (Lewis, 2010).

Critically, MqsR 2-1 also differentially degraded 94 transcripts by more than fourfold compared with native MqsR (Table S1). Primarily, MqsR 2-1 degraded transcripts related to acid resistance (*gadABCEWX* and *hdeABD*), multidrug resistance (*mdtEF*), osmotic resistance (*osmEY*) and oxidative stress (*yggE*, *ygiW* and *yodD*). Corroborating the whole-transcriptome results, qRT-PCR confirmed that MqsR 2-1 differentially degraded transcripts of *gadA*, *gadB*, *gadE*, *gadW*, *gadX*, *hdeA*, *hdeB*, *mdtE*, *mdtF* and *osmY* by threefold to 89-fold (Table S2). Corroborating these results, *hdeAB* and *osmE* are repressed in *E. coli* persister cells isolated using a flow cytometer compared with non-persisters (Shah *et al*., 2006). Hence, the increased MqsR toxicity during ampicillin antibiotic stress degraded transcripts of multiple stress response loci including acid resistance, osmotic resistance, oxidative stress resistance, and multidrug resistance. These results suggest that the stress response transcripts that are degraded by MqsR 2-1 are closely connected to persister cell production.

### *Deletion of* gadB*,* gadX*,* mdtF *and* osmY *increase persistence*

To investigate whether the stress-related genes that were identified in the whole-transcriptome experiment are important for persister cell formation, cell survival of isogenic deletion strains of each stress gene was measured under antibiotic stress. Deletion of *gadB* significantly increased persister production (43 000-fold) compared with BW25113 wild-type to the extent that 55% of the cells were persistent (Fig. 3A); by comparison, the

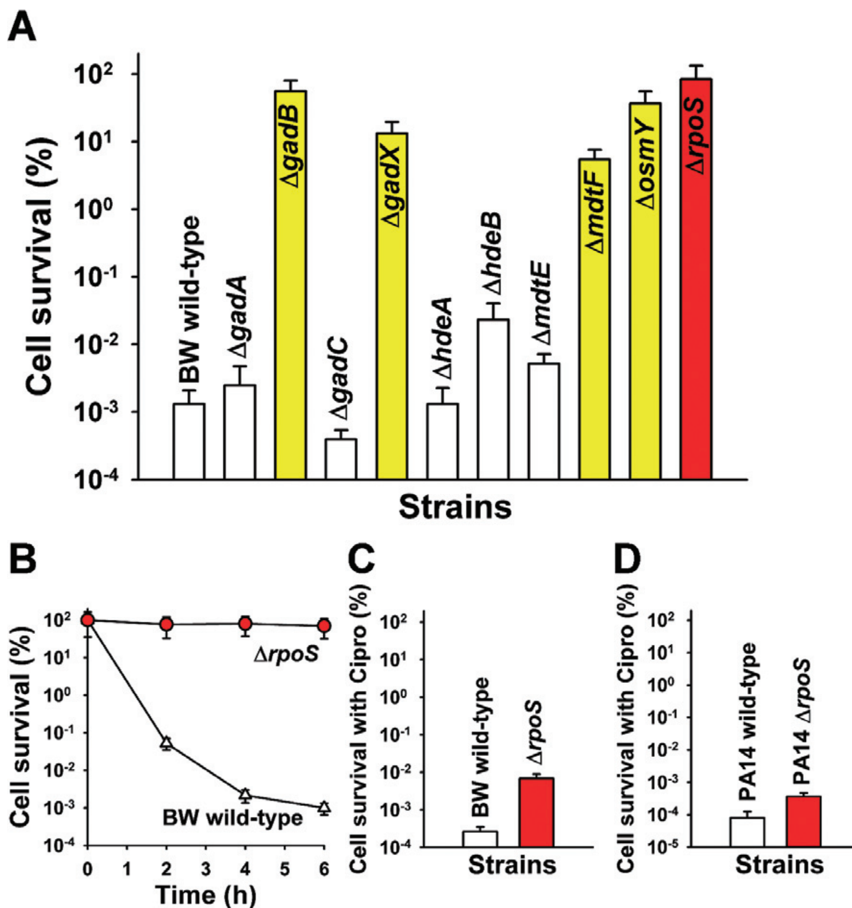


**Fig. 3.** Effect of isogenic mutations on persister cell formation. BW25113 and its isogenic mutants were grown to a turbidity of 1 in LB at 37°C and exposed to 20 μg ml$^{-1}$ ampicillin for 5 h (A). Time-course of persister formation of BW25113 Δ*rpoS* exposed to 20 μg ml$^{-1}$ ampicillin for 6 h (B). Persister formation of BW25113 Δ*rpoS* exposed to 1 μg ml$^{-1}$ ciprofloxacin (Cipro) for 5 h (C). Persister formation of PA14 Δ*rpoS* exposed to 1 μg ml$^{-1}$ Cipro for 5 h (D). Error bars indicate the standard deviation ($n = 3$).

© 2012 The Authors
Microbial Biotechnology © 2012 Society for Applied Microbiology and Blackwell Publishing Ltd, 

wild-type had only 0.001% persister cells. *gadB* encodes glutamate decarboxylase and confers resistance to extreme acid conditions (Waterman and Small, 2003). *gadB* expression is also increased in ampicillin-resistant *E. coli* (Adam *et al.*, 2008), and the cell appears to use GadB to thwart the action of ampicillin, since glutamate decarboxylase may remove the carboxyl group from ampicillin, thereby inactivating it (Adam *et al.*, 2008). Therefore, deletion of *gadB* dramatically increases persistence by making the cell less fit.

Along with the increased persistence of *gadB* deletion, the *gadX* deletion also resulted in a large increase in persisters (10 000-fold increase and 13% persist) (Fig. 3A). GadX is a transcriptional activator of the *gadBC* operon (Tramonti *et al.*, 2002). Also, deletion of *mdtF* encoding a component of the multidrug efflux pump MdtEF (Nishino and Yamaguchi, 2001) increased persistence by 4000-fold and increased the percentage of persisters to 5% (Fig. 3A). Deletion of *osmY* encoding hyperosmotic resistance protein (Yim and Villarejo, 1992) enhanced persistence 28 000-fold and increased the percentage of persisters to 37% (Fig. 3A). This change in persistence could be complemented since overproducing OsmY reduced the persistence level to that of wild-type strain (Fig. S2). Hence, MqsR 2-1 reduces acid resistance, multidrug resistance and osmotic resistance systems, which injure the cells and make them enter a dormant state.

### *RpoS is important for persister formation*

Since the general stress response master regulator RpoS (Hengge-Aronis, 1996) positively regulates *gadB* (Waterman and Small, 2003), *gadX* (Tramonti *et al.*, 2002), *osmY* (Vijayakumar *et al.*, 2004) and *mdtF* (Nishino *et al.*, 2008), directly or indirectly, we hypothesized that MqsR 2-1 reduces *rpoS* transcripts and thereby controls all of these stress-response genes. Hence, cells less able to respond to stress more readily become persisters. Using qRT-PCR, we found that MqsR 2-1 degraded *rpoS* transcripts by 2.9-fold (Table S2) compared with native MqsR. The *rpoS* transcript has 26 GCU sites in 1558 nt for possible cleavage by MqsR. Although *E. coli* K-12 BP792, the parent strain of BW25113, has an amber mutation in *rpoS*, this mutation was removed from BW25113 (Baba *et al.*, 2006), and we confirmed that there is no amber mutation in *rpoS* gene by sequencing the *rpoS* coding region. Hence, RpoS from BW25113 strain is active, and deletion of *rpoS* makes cells more sensitive to stress.

Consistent with our model, cells that lack *rpoS* dramatically increased persister production by 64 000-fold (84% of cell survival after 5 h) compared with BW25113 wild-type cells with ampicillin stress (Fig. 3A). Furthermore, the *rpoS* deletion strain maintained high persistence during ampicillin treatment (2, 4 and 6 h) while the viability of the wild-type strain continuously decreased (Fig. 3B). Hence, the high cell survival was not due to ampicillin resistance as there was no growth, only persistence. Since we found *rpoS* in pCA24N from the ASKA library (Kitagawa *et al.*, 2005) lacks 117 nt encoding the N-terminal 39 aa of RpoS, we constructed a plasmid which has the full *rpoS* sequence to investigate *rpoS* complementation in persistence. The change in persistence based on *rpoS* could be complemented since overproducing RpoS reduced the persistence level to that of wild-type strain (Fig. S2). Furthermore, since growth of the *rpoS* deletion strain in rich medium was only 15% less than that of the wild-type strain ($1.30 \pm 0.01\ h^{-1}$ for $\Delta$*rpoS* versus $1.52 \pm 0.01\ h^{-1}$ for BW25113), the dramatic increase in persistence of the *rpoS* mutant is not due to persister cell formation prior to antibiotic addition. Hence, the antibiotic treatment itself leads to the persister cells by killing those cells, which do not obtain the persister state and by causing cells, which are less fit to fight the effects of the antibiotic to enter a state of persistence.

To test our hypothesis further that cells less able to respond to stress generate greater numbers of persister cells, we tested persistence with another antibiotic, ciprofloxacin. Ciprofloxacin inhibits DNA replication by interfering DNA gyrase, while ampicillin inhibits cell wall synthesis by binding to penicillin-binding proteins (Kohanski *et al.*, 2010). As expected, deletion of *rpoS* increased persister cell formation by 26-fold compared with the wild-type strain in the presence of ciprofloxacin (Fig. 3C).

To investigate further whether *rpoS* mutation influences persistence in other bacteria rather than in *E. coli*, we tested the effect of *rpoS* mutation in persister formation of *Pseudomonas aeruginosa*. Ciprofloxacin was used for testing *P. aeruginosa* persistence rather than ampicillin, since *P. aeruginosa* is naturally ampicillin resistant. RpoS regulates virulence factors as well as the stress response in *P. aeruginosa* (Suh *et al.*, 1999). Mutation of *rpoS* in *P. aeruginosa* PA14 increased persister formation by five-fold compared with the wild-type strain in the presence of ciprofloxacin (Fig. 3D). Therefore, persister cell formation is controlled by RpoS levels that regulate many stress response genes.

### *Persistence increases when cells are damaged by oxidative and acid stresses*

Since deletion of *rpoS* as well as the other stress-resistance genes (*gadB*, *gadX*, *mdtF* and *osmY*) dramatically increased persister cell formation (Fig. 3A), we hypothesized that cells damaged by stress have increased persister cell formation compared with the cells without stress. To test this hypothesis, we first measured cell survival after oxidative ($H_2O_2$) and acid (pH 2.5) stress

© 2012 The Authors

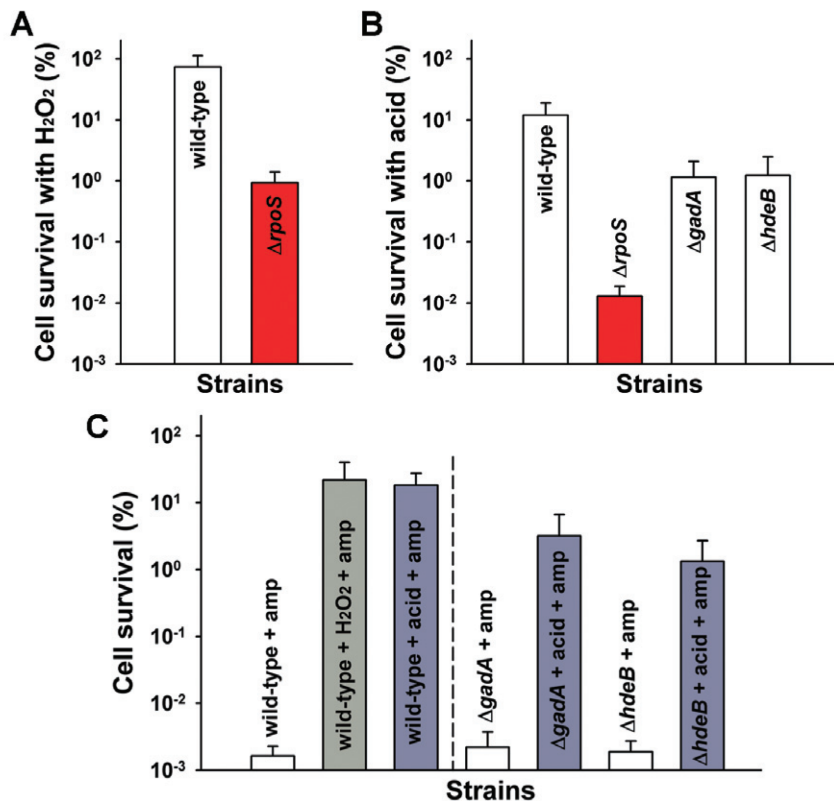


**Fig. 4.** Persister formation after oxidative and acid stresses. Cell survival (%) with oxidative stress (20 mM $H_2O_2$) for 10 min (A) and with acid stress (pH 2.5) for 2 min (B). BW25113 and its isogenic mutants were grown to a turbidity of 1 in LB at 37°C and exposed to $H_2O_2$ or pH 2.5. C. Persister cell formation of BW25113 and its isogenic mutants exposed to 20 μg ml$^{-1}$ ampicillin for 5 h after oxidative or acid stress. Error bars indicate the standard deviation ($n = 3$).

in the *rpoS* deletion strain to ensure that the cells were less viable. As expected, cells that lacked the ability to respond to stress through RpoS were more sensitive to oxidative stress (80-fold less survival than wild-type) (Fig. 4A) as well as acid stress (930-fold less survival than wild-type) (Fig. 4B). Similarly, deleting *gadA* and *hdeB* decreased viability 10-fold compared with the wild-type strain (Fig. 4B). Hence, cells lacking stress response-genes are more sensitive to non-antibiotic stress. Then we investigated persister cell formation after stress in order to test whether the damaged cells become more persistent than intact cells. As deletion of *rpoS* already causes high persistence (Fig. 3A and B), we measured persister formation of the wild-type strain after exposing the cells to $H_2O_2$ prior to antibiotic treatment. We found that wild-type cells that received oxidative stress prior to antibiotic treatment had a 11 000-fold increase in persister cell formation compared with the cells with no stress (Fig. 4C). This increase is not just a percentage increase in that the total number of wild-type cells that persist was $9.8 \times 10^7$ cells ml$^{-1}$ when cells were stressed first with hydrogen peroxide versus $9.8 \times 10^3$ cells ml$^{-1}$ that persist without hydrogen peroxide (both samples originally had roughly $5 \times 10^8$ cells ml$^{-1}$). Hence, a 25% reduction in viability leads to an extraordinary increase in persistence. Similarly, persister cell formation of the wild-type strain increased 13 000-fold with acid stress (Fig. 4C). Furthermore, both *gadA* and *hdeB* knockout strains, which did not increase persister cell formation (Fig. 3A), also increased persister cell formation 1400- and 700-fold with acid stress, respectively, compared with no stress condition (Fig. 4C). Taken together, persister cell formation increases dramatically if cells are stressed prior to antibiotic addition.

### *Whole-transcriptome analyses of persister cells*

Utilizing our ability to increase the number of persister cells dramatically, we investigated gene regulation in persister cells to gain further insights into how persister cells are formed. Whole-transcriptome studies were performed using persister cells generated two different ways: (i) wild-type cells were pretreated with oxidative stress prior to ampicillin treatment and (ii) the *rpoS* deletion strain was used with ampicillin. Since ampicillin exposure was for 5 h, non-persister cells were lysed and did not contribute to the RNA pool (Keren *et al*., 2004). The cells were compared with stationary-phase wild-type cells treated with a fourfold lower concentration of ampicillin with the rationale that these cells would be stressed but not in the persister state (Table S3). There was significant agreement for gene expression in the two data sets of persister cells (Table S3), and many of the same genes that were identified as differentially degraded/repressed by producing MqsR 2-1 (Table S1) are in common among the repressed genes in both persister data sets. Furthermore,

© 2012 The Authors

42 of the most-significantly repressed genes in the two sets of persister cells (Table S4) are RpoS-regulated genes (Patten *et al.*, 2004; Weber *et al.*, 2005). These results support that reduced RpoS activity may be the main mechanism for induction of persistence with ampicillin as discovered using MqsR 2-1.

**Discussion**

Persister cell formation appears to be one of the main physiological roles of TA systems (Lewis, 2008; Kim and Wood, 2010; Maisonneuve *et al.*, 2011; Wang and Wood, 2011), as TA modules induce dormancy (Lewis, 2008). In this study, we showed that toxin MqsR may be altered for enhanced toxicity (Fig. 1) and that this increased toxicity leads to increased persister cell formation (Fig. 2). The increase in persistence resulted from a reduction in transcripts for the stress response cascades, including acid resistance, multidrug resistance and osmotic resistance systems, all regulated by RpoS, as shown in whole-transcriptome results and by the dramatic increase in persistence upon deleting *gadB*, *gadX*, *mdtF*, *osmY* and *rpoS* (Fig. 3A). Hence, we identified part of the genetic basis of persister cell formation by using the tools of protein engineering and systems biology. This is the first report that differential degradation of *rpoS* transcripts leads to persister cell formation by downregulating stress responsive genes. This result was corroborated by showing that a reduction in transcripts of genes controlled by RpoS also led to an increase in persistence via *gadB*, *gadX*, *mdtF* and *osmY*, and this is the first report of the influence of these acid resistance, antibiotic resistance and osmolarity resistance on persister cell formation. This is also the first report of exceptionally high levels of persister cells; normally they are no more than 1% of stationary-phase cultures (Lewis, 2007) but their concentration reaches 80% here (Fig. 3A). Hence, we have determined a new mechanism for increasing persistence. Furthermore, since we can enhance persister cell formation dramatically using knockout strains of stress resistance genes or pre-exposing cells to stresses prior to antibiotic treatment, isolating persister cells may be achieved far easier than in previous efforts where persister cells were isolated using a flow cytometer with a GFP-tagged *E. coli*; dormant cells are dim whereas active cells glow green (Shah *et al.*, 2006).

We utilized our novel approach to increase persister cells for whole-transcriptome studies by generating persister cells two different ways (in addition to producing toxin MqsR 2-1): (i) wild-type cells were exposed to oxidative stress prior to ampicillin treatment and (ii) *rpoS* deletion cells were used with ampicillin treatment. Remarkably, the two data sets were consistent with each other and with the data from producing MqsR 2-1 which confirms persister cells were studied. Comparing the transcriptomes of the two persister cell populations to the transcriptome of stationary-phase wild-type cells stressed by ampicillin (Table S3), we confirmed that repression of genes controlled by *rpoS* is the main mechanism for persister cell formation with this antibiotic (as suggested first by producing MqsR 2-1). However, we recognize that there may be more than one way to generate persister cells; i.e. persistence may be a group of non-growth states with different genetic elements required to enter these states. Using our new tool for increasing persistence, further studies will be facilitated for determining what proteins are expressed during persistence and what proteins are required to wake these sleeping cells (these experiments are underway).

In most cases, when genes are deleted, mutant cells become more sensitive to antibiotics, as shown in antibiotic hypersensitivity screening of nearly 4000 single-gene-knockouts in *E. coli* (Tamae *et al.*, 2008). Thus, it is interesting that deletion of stress resistance genes increases persister cell formation, which indicates the persistence mechanism is quite different from the resistant mechanisms. Previously, labs had searched (Hu and Coates, 2005; Hansen *et al.*, 2008) for deletions that would make cells less persistent when in fact key deletions in the stress response make the cell more persistent (Fig. 3). Sigma factor RpoS regulation is one of the main stress-resistant mechanisms in the cell since RpoS regulates many stress-responsive genes (Vijayakumar *et al.*, 2004) that allow the cell to survive various stresses, including UV exposure, acid shock, heat shock, oxidative stress and starvation (Dong and Schellhorn, 2009). Antitoxin MqsA represses *rpoS*, while upon stress, RpoS is induced via degradation of MqsA by Lon protease (Wang *et al.*, 2011). Thus, deletion of *rpoS* makes cells more sensitive to non-antibiotic stresses (Fig. 4A and B), but the damaged cells become dramatically more persistent to the antibiotic (Figs 3A and 4C).

Hence, our results show cells behave somewhat counter intuitively: in terms of persistence, it is better for cells to have less fitness for *extreme* environmental stress; i.e. it is better for persistence for the cell to be more sensitive to stress which likely overstimulates toxins. Therefore, it appears cells may have two different survival mechanisms upon external stresses (Fig. 5): stress turns on the stress-responsive gene cascades, and most cells combat the stress while keeping cellular metabolism relatively active. Alternatively, a low percentage of cells may induce Lon-mediated degradation of antitoxins (Maisonneuve *et al.*, 2011; Wang *et al.*, 2011), and the activated toxins then degrade most mRNA transcripts (Wang and Wood, 2011), including stress responsive genes and make the cell dormant. Therefore, our results indicate persister cells are generated in response to stresses

© 2012 The Authors

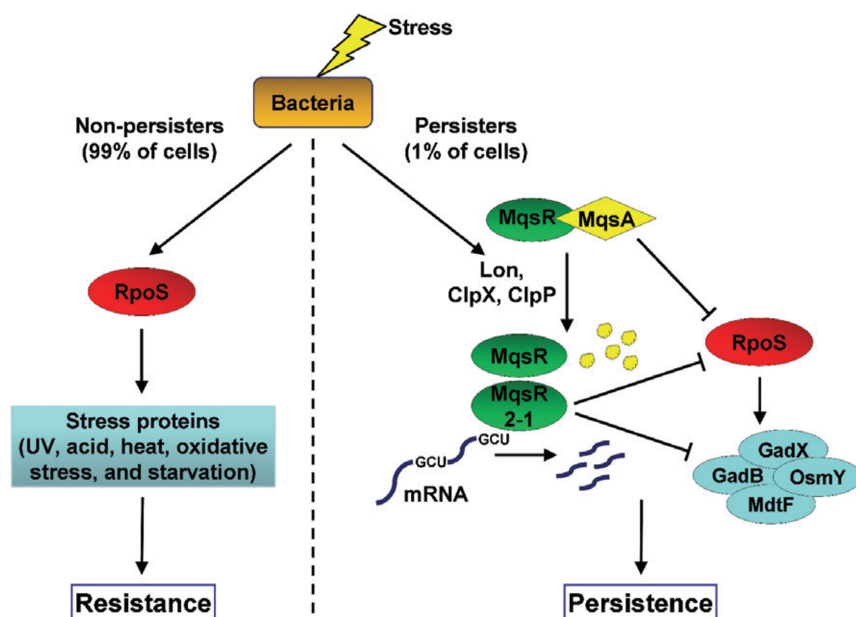


**Fig. 5.** Schematic of persistence and resistance with *rpoS*. Upon stresses such as UV exposure, acid shock, heat shock, oxidative stress and starvation, most cells induce resistance by RpoS (left panel). For a small percentage of cells, stresses also increase persister formation (right panel). MqsA is degraded by proteases (ClpXP and Lon), and MqsR 2-1 with enhanced toxicity degrades mRNA transcripts including stress responsive mRNAs. The degradation of *rpoS* mRNA by the toxin leads to downregulation of the acid (*gadB*, *gadX*), osmotic (*osmY*), and multidrug (*mdtF*) resistance systems. Then, upon antibiotic treatment, cells enter a dormant state. '→' indicates induction, and '⊥' indicates repression or differential mRNA decay.

rather than pre-existing heterogeneity in bacterial populations (Balaban *et al*., 2004). The decision whether the cell actively combats the stress or becomes persistent may depend on intensity and type of stresses.

Deletion of *mqsR* decreases persister cell formation using *E. coli* BW25113 (Kim and Wood, 2010), and this result was verified by an independent group (Luidalepp *et al*., 2011). Using different strains (e.g. *E. coli* MG1655) and different conditions, others have found that the *mqsR* deletion does not affect persistence (Shah *et al*., 2006; Maisonneuve *et al*., 2011) unless multiple TA loci are deleted (Maisonneuve *et al*., 2011). In addition, the age of the inoculum strongly influences persister cell development in that the lower persister frequencies of cells such as *glpD*, *dnaJ* and *surA* knockout strains were elevated to the level of the wild-type when older inocula were used (Luidalepp *et al*., 2011). In contrast, persister cell formation by production of MqsR is consistent when MqsR is produced (Shah *et al*., 2006; Kim and Wood, 2010) because of its RNase activity. In this study, we altered MqsR for greater toxicity and persistence by two amino acid replacements (K3N and N31Y) that increase protein stability (Fig. 1C) probably without affecting binding affinity with MqsA and catalytic activity (Fig. 1B).

In summary, we demonstrate that increasing toxicity of MqsR increases persister cell formation and that cells enter the persistent state through repression of stress resistance modules. Hence, TA systems have a vital role in cell physiology especially in the formation of persister cells. Understanding protein targets for maintaining persisters is important for discovery of drugs for effectively treating chronic infections (Lewis, 2010), and here we provide some new targets and strategies for combating persister cells.

## Experimental procedures

### *Bacterial strains and growth conditions*

The bacterial strains and plasmids used in this study are listed in Table 1. We used the Keio collection (Baba *et al*., 2006) for isogenic mutants and pBS(Kan) (Canada *et al*., 2002) and pCA24N (Kitagawa *et al*., 2005) for overexpressing genes in *E. coli*. The gene deletions of *mqsR*, *gadB*, *gadX*, *mdtF*, *osmY* and *rpoS* were confirmed by PCR (all primers are shown in Table S5). All strains were initially streaked from –80°C glycerol stocks on LB (Sambrook *et al*., 1989) with glucose (0.2%) agar plates and were cultured at 37°C in LB. Kanamycin (50 μg $ml^{-1}$) was used for pre-culturing the isogenic knockout mutants and for maintaining the pBS(Kan)-based and pET28a-based plasmids, chloramphenicol (30 μg $ml^{-1}$) was used for maintaining the pCA24N-based plasmids, and gentamicin (15 μg $ml^{-1}$) was used for pre-culturing the PA14 Δ*rpoS* mutant. Genes were expressed by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St. Louis, MO, USA).

### *epPCR for random mutagenesis*

*mqsR* from plasmid pBS(Kan)-*mqsR* under the control of the *lac* promoter was mutated by epPCR as described previously (Fishman *et al*., 2004) using primers ep-pBS(Kan)-f and ep-pBS(Kan)-r. The epPCR product was cloned into pBS(Kan)-*mqsR* using BamHI and XbaI after treating the plasmid with Antarctic phosphatase (New England Biolabs, Beverly, MA, USA). The ligation mixture was electroporated into BW25113 *mqsR* competent cells using the Gene

© 2012 The Authors

**Table 1.** Strains and plasmids used in this study.

| Strains and plasmids | Genotype/relevant characteristics | Source |
|---|---|---|
| Strains | | |
| BL21 (DE3) | $F^-$ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* λ(DE3) Ω $P_{lacUV5}$::T7 polymerase | Novagen |
| BW25113 | *lacI*$^q$ *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR*514 Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ | Baba *et al.* (2006) |
| BW25113 Δ*mqsR* | BW25113 Δ*mqsR* | Kim *et al.* (2010) |
| BW25113 Δ*gadA* | BW25113 Δ*gadA* Ω $Km^R$ | Baba *et al.* (2006) |
| BW25113 Δ*gadB* | BW25113 Δ*gadB* Ω $Km^R$ | Baba *et al.* (2006) |
| BW25113 Δ*gadC* | BW25113 Δ*gadC* Ω $Km^R$ | Baba *et al.* (2006) |
| BW25113 Δ*gadX* | BW25113 Δ*gadX* Ω $Km^R$ | Baba *et al.* (2006) |
| BW25113 Δ*hdeA* | BW25113 Δ*hdeA* Ω $Km^R$ | Baba *et al.* (2006) |
| BW25113 Δ*hdeB* | BW25113 Δ*hdeB* Ω $Km^R$ | Baba *et al.* (2006) |
| BW25113 Δ*rpoS* | BW25113 Δ*rpoS* Ω $Km^R$ | Baba *et al.* (2006) |
| BW25113 Δ*mdtE* | BW25113 Δ*mdtE* Ω $Km^R$ | Baba *et al.* (2006) |
| BW25113 Δ*mdtF* | BW25113 Δ*mdtF* Ω $Km^R$ | Baba *et al.* (2006) |
| BW25113 Δ*osmY* | BW25113 Δ*osmY* Ω $Km^R$ | Baba *et al.* (2006) |
| PA14 | PA14 wild-type strain | Liberati *et al.* (2006) |
| PA14 Δ*rpoS* | PA14_17480 Ω *Mar2xT7*, $Gm^R$ | Liberati *et al.* (2006) |
| Plasmids | | |
| pBS(Kan) | $Km^R$, pBS(Kan) | Canada *et al.* (2002) |
| pBS(Kan)-*mqsR* | $Km^R$, pBS(Kan) $P_{lac}$::*mqsR*$^+$ | Kim *et al.* (2010) |
| pBS(Kan)-*mqsR* 2-1 | $Km^R$, pBS(Kan) $P_{lac}$::*mqsR* 2-1$^+$ (K3N, N31Y aa substitutions) | This study |
| pBS(Kan)-*mqsR* 20-14 | $Km^R$, pBS(Kan) $P_{lac}$::*mqsR* 20-14$^+$ (R9C, L35F and V70I aa substitutions) | This study |
| pET28b[a] | $Km^R$, pET28b | Novagen |
| pET28a-*mqsR* | $Km^R$, pET28a $P_{T7}$::*mqsR*$^+$ | Brown *et al.* (2009) |
| pET28a-*mqsR* K3N | $Km^R$, pET28a $P_{T7}$::*mqsR* K3N$^+$ (K3N aa substitution) | This study |
| pET28a-*mqsR* N31Y | $Km^R$, pET28a $P_{T7}$::*mqsR* N31Y$^+$ (N31Y aa substitution) | This study |
| pET28a-*mqsR* 2-1 | $Km^R$, pET28a $P_{T7}$::*mqsR* 2-1$^+$ (K3N, N31Y aa substitutions) | This study |
| pCA24N | $Cm^R$; *lacI*$^q$, pCA24N | Kitagawa *et al.* (2005) |
| pCA24N-*gadB* | $Cm^R$; *lacI*$^q$, pCA24N $P_{T5\text{-}lac}$::*gadB*$^+$ | Kitagawa *et al.* (2005) |
| pCA24N-*gadX* | $Cm^R$; *lacI*$^q$, pCA24N $P_{T5\text{-}lac}$::*gadX*$^+$ | Kitagawa *et al.* (2005) |
| pCA24N-*mdtF* | $Cm^R$; *lacI*$^q$, pCA24N $P_{T5\text{-}lac}$::*mdtF*$^+$ | Kitagawa *et al.* (2005) |
| pCA24N-*osmY* | $Cm^R$; *lacI*$^q$, pCA24N $P_{T5\text{-}lac}$::*osmY*$^+$ | Kitagawa *et al.* (2005) |
| pCA24N-full *rpoS* | $Cm^R$; *lacI*$^q$, pCA24N $P_{T5\text{-}lac}$::*rpoS*$^+$ | This study |

**a.** pET28b is identical to pET28a except for 1 bp that is deleted near BamHI in the multiple cloning site.
$Km^R$, $Gm^R$ and $Cm^R$ are kanamycin, gentamicin and chloramphenicol resistance respectively.

Pulser/Pulse Controller (Bio-Rad, Hercules, CA, USA) at 1.25 kV $cm^{-1}$, 25 μF and 200 Ω.

### *Toxicity screening of MqsR variants*

In order to screen MqsR variants for the increased toxicity, each colony of BW25113 Δ*mqsR* producing MqsR variants from pBS(Kan)-*mqsR* was transferred to fresh LB agar plates by touching with a toothpick and incubated for 24 h to select colonies with reduced cell growth (i.e. small colony). As controls, BW25113 Δ*mqsR* with empty pBS(Kan) and pBS(Kan)-*mqsR* (native MqsR) were used. Interesting *mqsR* alleles were re-analysed by measuring cell growth in LB cultures in shake flasks after re-electroporation of the plasmids; overnight cultures were diluted to a turbidity of 0.05 at 600 nm in LB medium, incubated in a shake flask for 9 h, and cell growth was measured at $OD_{600}$ every 1 h. Mutant *mqsR* alleles were sequenced using a primer ep-pBS(Kan)-f. Each data point was averaged from three independent cultures.

### *Site-directed mutagenesis*

Site-directed mutagenesis was performed at the codons corresponding to positions at K3 and N31 of MqsR using plasmid pET28a-*mqsR* as a template with site-directed mutagenesis primers. PCR was performed using *Pfu* DNA polymerase at 95°C for 1 min, with 20 cycles of 95°C for 1 min, 55°C for 50 s, and 68°C for 9 min, and a final extension of 68°C for 7 min. The constructed plasmids were electroporated into BL21 (DE3) after DpnI digestion of template plasmids. Mutations were confirmed by sequencing the constructed plasmids using the T7-f and T7-ter-r primers.

### *Cloning full* rpoS *into pCA24N*

Since *rpoS* in pCA24N from the ASKA library (Kitagawa *et al.*, 2005) lacks 117 nt encoding the N-terminal 39 aa, we constructed a plasmid with a full *rpoS* sequence. *rpoS* was amplified from *E. coli* BW25113 using rpoS-BseRI-f and rpoS-NS-r primers and then a second PCR was performed on the first PCR with rpoS-BseRI-f2 and rpoS-NS-r primers to obtain an N-terminal His tag and BseRI restriction site with *rpoS*, which was cloned into pCA24N using the BseRI and SalI restriction sites. Full *rpoS* insertion was confirmed by sequencing using the pCA24N-seq-f, pCA24N-seq-r and rpoS-RT-f primers.

### *Persister assay*

Persister levels were determined by counting the number of colonies grown on solid media after washing and serially

© 2012 The Authors

diluting the cells after exposure to antibiotic (Dörr *et al*., 2009). To determine the number of persister cells with MqsR variants, cells were inoculated in LB medium and grown to a turbidity of 0.5 (to obtain 3–7 × $10^8$ cfu $ml^{-1}$ of viable cells) at 600 nm with 1 mM IPTG. Cells were washed with the same amount of 0.85% NaCl solution, adjusted to a turbidity of 1, and were exposed to 20, 26 or 40 μg $ml^{-1}$ ampicillin with 1 mM IPTG for 24 h. Cells were washed and diluted by $10^2$ to $10^7$ via 10-fold serial dilution steps in 0.85% NaCl solution and applied as 10 μl drops on LB agar with kanamycin to determine persister cell viability (Donegan *et al*., 1991). For isogenic mutants, cells were grown to a turbidity of 1 at 600 nm and exposed to 20 μg $ml^{-1}$ ampicillin or 1 μg $ml^{-1}$ ciprofloxacin for 5 h. For complementation strains, 1 mM IPTG was added at a turbidity of 0.5 at 600 nm to produce proteins from pCA24N-based plasmids, and cells at a turbidity of 1 were exposed to 20 μg $ml^{-1}$ ampicillin with 1 mM IPTG for 5 h.

### *RNA isolation and whole-transcriptome studies*

For the whole-transcriptome study of BW25113 Δ*mqsR*/pBS(Kan)-*mqsR* 2-1 versus BW25113 Δ*mqsR*/pBS(Kan)-*mqsR*, planktonic cells were grown to a turbidity of 0.5 at 600 nm in LB medium with 1 mM IPTG at 37°C, adjusted to a turbidity to 1, and exposed to 20 μg $ml^{-1}$ ampicillin with 1 mM IPTG for 1 h. Cells were isolated by centrifuging at 0°C, and RNA*Later*® buffer (Applied Biosystems, Foster City, CA, USA) was added to stabilize RNA during the RNA preparation steps. Total RNA was isolated from cell pellets as described previously (Ren *et al*., 2004a) using a bead beater (Biospec, Bartlesville, OK, USA). cDNA synthesis, fragmentation and hybridizations to the *E. coli* GeneChip Genome 2.0 array (Affymetrix, Santa Clara, CA, USA; P/N 511302) were described previously (González Barrios *et al*., 2006). Genes were identified as differentially expressed if the *P*-value for comparing two chips was less than 0.05 and if the expression ratio was higher than the standard deviation (1.3-fold) (Ren *et al*., 2004b); since the standard deviation was low, a fourfold cut-off for the DNA microarrays was used. The whole-transcriptome data have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through accession number GSE31054.

For the whole-transcriptome studies with persister cells, two conditions were utilized to generate persister cells: (i) BW25113 wild-type cells were pretreated with oxidative stress prior to ampicillin treatment and (ii) *rpoS* mutant cells were treated with ampicillin. Gene expression profiles were compared with stationary-phase BW25113 wild-type cells. BW25113 wild-type cells were grown to a turbidity of 1 at 600 nm in LB medium at 37°C, centrifuged, resuspended in LB and exposed to 20 mM $H_2O_2$ for 10 min. After $H_2O_2$ treatment, cells were washed with 0.85% NaCl and then exposed to 20 μg $ml^{-1}$ ampicillin for 5 h. *rpoS* mutant cells were exposed to ampicillin for 5 h at a turbidity of 1. BW25113 wild-type cells in the stationary phase (turbidity of 3) were exposed to 5 μg $ml^{-1}$ ampicillin for 30 min. The whole-transcriptome data of these persister cells are accessible through Accession No. GSE34028.

### *qRT-PCR*

To corroborate the DNA microarray data, qRT-PCR was used to quantify relative RNA concentrations using 100 ng as a template using the Power *SYBR Green RNA-to-$C_T$*™ 1-Step Kit (Applied Biosystems, Foster City, CA, USA). The reaction and analysis was carried out by the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The housekeeping gene *rrsG* was used to normalize the gene expression data. The annealing temperature was 60°C for all the genes in this study.

### *Western blot analysis and SDS-PAGE*

To investigate MqsR protein levels, Western blot and SDS-PAGE were performed. BL21 (DE3) strains containing pET28b, pET28a-*mqsR*, pET28a-*mqsR* K3N, pET28a-*mqsR* N31Y and pET28a-*mqsR* 2-1 were grown to a turbidity of 0.2, then 1 mM ITPG was added to produce MqsR and the MqsR variants. When the turbidity reached 0.5, cells were washed with TE buffer, and protease inhibitor cocktail (Sigma-Aldrich, USA) was added to protect proteins. Samples were sonicated using a 60 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, USA) at level 4 for 30 s twice. Total protein was quantified using a Pierce BCA Protein Assay kit (Fisher Scientific, Pittsburgh, PA, USA), 2× SDS sample buffer was added, and protein was denatured at 95°C for 5 min. The Western blot was performed using 2.5 μg protein of each sample with primary antibodies raised against a His tag (Cell Signaling Technology, Danvers, MA, USA) and horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Millipore, Billerica, MA, USA). CL-Xposure film (Thermo Scientific, Rockford, IL, USA) was used after a 30 s exposure. We confirmed that the same amount of total cell protein was loaded for each sample by over-exposing the Western blot for 2 min. For SDS-PAGE, 25 μg of protein of each sample was loaded, and the gel was stained with Coomassie blue.

### *Oxidative and acid stress assays*

Overnight cultures were diluted to a turbidity of 0.05 and grown to a turbidity of 1 at 600 nm. Cells were centrifuged and resuspended in LB and exposed to either 20 mM $H_2O_2$ for 10 min or pH 2.5 for 2 min.

### *Live/dead staining*

The ratio of live and dead cells was quantified using a Live/Dead *Bac*Light® kit (Invitrogen, Carlsbad, CA, USA). Cells were grown to a turbidity of 0.5 with 2 h induction of MqsR 2-1 and native MqsR by adding 1 mM IPTG, diluted 10 times, and washed with 0.85% NaCl. Live/Dead staining dye mixture (3 μl of a 1:1 mix of Component A and B) was added to 1 ml of washed culture and incubated at room temperature in a dark room for 15 min. Stained culture (1 μl) was observed using a 40×/0.75 Plan-NEOFLUAR dry objective with an Axiovert 200 M microscope (Carl Zeiss, Berlin, Germany). A FITC filter (excitation 490 nm and emission 525 nm) was used to observe cells stained green (live cells), and a Nile Red filter (excitation 515~530 nm and emission 525~605 nm) was used to observe cells stained red (dead cells). Three images were taken for each sample to quantify the average cell size (40 cells) and percentage of dead cells (more than 300 cells).

© 2012 The Authors
Microbial Biotechnology © 2012 Society for Applied Microbiology and Blackwell Publishing Ltd, 

### *Swimming motility assay*

Swimming motility of BW25113 Δ*mqsR* strains containing pBS(Kan), pBS(Kan)-*mqsR*, pBS(Kan)-*mqsR* 2-1 and pBS(Kan)-*mqsR* 20-14 was examined on motility agar plates (1% tryptone, 0.25% NaCl and 0.3% agar). Kanamycin (50 μg ml$^{-1}$) was added to maintain the plasmids. Overnight cultures (2 μl) were used to inoculate the motility plates, and the swimming halo was measured after 20 h at 37°C.

## Acknowledgements

This work was supported by the National Institutes of Health (R01 GM089999). We are grateful for the Keio and ASKA strains provided by the National Institute of Genetics of Japan as well as for the help with the MqsR structural images provided by Rebecca Page.

## References

Adam, M., Murali, B., Glenn, N.O., and Potter, S.S. (2008) Epigenetic inheritance based evolution of antibiotic resistance in bacteria. *BMC Evol Biol* **8:** 52.

Amitai, S., Kolodkin-Gal, I., Hananya-Meltabashi, M., Sacher, A., and Engelberg-Kulka, H. (2009) *Escherichia coli* MazF leads to the simultaneous selective synthesis of both 'death proteins' and 'survival proteins'. *PLoS Genet* **5:** e1000390.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., *et al*. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2:** 2006.0008.

Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L., and Leibler, S. (2004) Bacterial persistence as a phenotypic switch. *Science* **305:** 1622–1625.

Bloom, J.D., Wilke, C.O., Arnold, F.H., and Adami, C. (2004) Stability and the evolvability of function in a model protein. *Biophys J* **86:** 2758–2764.

Brown, B.L., Grigoriu, S., Kim, Y., Arruda, J.M., Davenport, A., Wood, T.K., *et al*. (2009) Three dimensional structure of the MqsR:MqsA complex: a novel TA pair comprised of a toxin homologous to RelE and an antitoxin with unique properties. *PLoS Pathog* **5:** e1000706.

Canada, K.A., Iwashita, S., Shim, H., and Wood, T.K. (2002) Directed evolution of toluene *ortho*-monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation. *J Bacteriol* **184:** 344–349.

Domka, J., Lee, J., Bansal, T., and Wood, T.K. (2007) Temporal gene-expression in *Escherichia coli* K-12 biofilms. *Environ Microbiol* **9:** 332–346.

Donegan, K., Matyac, C., Seidler, R., and Porteous, A. (1991) Evaluation of methods for sampling, recovery, and enumeration of bacteria applied to the phylloplane. *Appl Environ Microbiol* **57:** 51–56.

Dong, T., and Schellhorn, H. (2009) Global effect of RpoS on gene expression in pathogenic *Escherichia coli* O157:H7 strain EDL933. *BMC Genomics* **10:** 349.

Dörr, T., Lewis, K., and Vulić, M. (2009) SOS response induces persistence to fluoroquinolones in *Escherichia coli*. *PLoS Genet* **5:** e1000760.

Dörr, T., Vulić, M., and Lewis, K. (2010) Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. *PLoS Biol* **8:** e1000317.

Fishman, A., Tao, Y., Bentley, W.E., and Wood, T.K. (2004) Protein engineering of toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 for synthesizing 4-nitrocatechol from nitrobenzene. *Biotechnol Bioeng* **87:** 779–790.

González Barrios, A.F., Zuo, R., Hashimoto, Y., Yang, L., Bentley, W.E., and Wood, T.K. (2006) Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). *J Bacteriol* **188:** 305–306.

Hansen, S., Lewis, K., and Vulić, M. (2008) Role of global regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli*. *Antimicrob Agents Chemother* **52:** 2718–2726.

Hengge-Aronis, R. (1996) Back to log phase: $\sigma^S$ as a global regulator in the osmotic control of gene expression in *Escherichia coli*. *Mol Microbiol* **21:** 887–893.

Hu, Y., and Coates, A.R.M. (2005) Transposon mutagenesis identifies genes which control antimicrobial drug tolerance in stationary-phase *Escherichia coli*. *FEMS Microbiol Lett* **243:** 117–124.

Keren, I., Shah, D., Spoering, A., Kaldalu, N., and Lewis, K. (2004) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J Bacteriol* **186:** 8172–8180.

Kim, Y., and Wood, T.K. (2010) Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in *Escherichia coli*. *Biochem Biophys Res Commun* **391:** 209–213.

Kim, Y., Wang, X., Ma, Q., Zhang, X.-S., and Wood, T.K. (2009) Toxin-antitoxin systems in *Escherichia coli* influence biofilm formation through YjgK (TabA) and fimbriae. *J Bacteriol* **191:** 1258–1267.

Kim, Y., Wang, X., Zhang, X.-S., Grigoriu, S., Page, R., Peti, W., and Wood, T.K. (2010) *Escherichia coli* toxin/antitoxin pair MqsR/MqsA regulate toxin CspD. *Environ Microbiol* **12:** 1105–1121.

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H., and Mori, H. (2005) Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. *DNA Res* **12:** 291–299.

Kohanski, M.A., Dwyer, D.J., and Collins, J.J. (2010) How antibiotics kill bacteria: from targets to networks. *Nat Rev Microbiol* **8:** 423–435.

Leplae, R., Geeraerts, D., Hallez, R., Guglielmini, J., Drèze, P., and Van Melderen, L. (2011) Diversity of bacterial type II toxin–antitoxin systems: a comprehensive search and functional analysis of novel families. *Nucleic Acids Res* **39:** 5513–5525.

Lewis, K. (2007) Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* **5:** 48–56.

Lewis, K. (2008) Multidrug tolerance of biofilms and persister cells. In *Bacterial Biofilms*. Romeo, T. (ed.). Berlin, Germany: Springer-Verlag, pp. 107–131.

Lewis, K. (2010) Persister cells. *Annu Rev Microbiol* **64:** 357–372.

© 2012 The Authors

Liberati, N.T., Urbach, J.M., Miyata, S., Lee, D.G., Drenkard, E., Wu, G., *et al*. (2006) An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci USA* **103:** 2833–2838.

Luidalepp, H., Jõers, A., Kaldalu, N., and Tenson, T. (2011) Age of inoculum strongly influences persister frequency and can mask effects of mutations implicated in altered persistence. *J Bacteriol* **193:** 3598–3605.

Magnuson, R.D. (2007) Hypothetical functions of toxin-antitoxin systems. *J Bacteriol* **189:** 6089–6092.

Maisonneuve, E., Shakespeare, L.J., Jørgensen, M.G., and Gerdes, K. (2011) Bacterial persistence by RNA endonucleases. *Proc Natl Acad Sci USA* **108:** 13206–13211.

Nishino, K., and Yamaguchi, A. (2001) Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. *J Bacteriol* **183:** 5803–5812.

Nishino, K., Senda, Y., and Yamaguchi, A. (2008) The AraC-family regulator GadX enhances multidrug resistance in *Escherichia coli* by activating expression of *mdtEF* multidrug efflux genes. *J Infect Chemother* **14:** 23–29.

Patten, C.L., Kirchhof, M.G., Schertzberg, M.R., Morton, R.A., and Schellhorn, H.E. (2004) Microarray analysis of RpoS-mediated gene expression in *Escherichia coli* K-12. *Mol Genet Genomics* **272:** 580–591.

Ramage, H.R., Connolly, L.E., and Cox, J.S. (2009) Comprehensive functional analysis of *Mycobacterium tuberculosis* toxin-antitoxin systems: implications for pathogenesis, stress responses, and evolution. *PLoS Genet* **5:** e1000767.

Ren, D., Bedzyk, L.A., Thomas, S.M., Ye, R.W., and Wood, T.K. (2004a) Gene expression in *Escherichia coli* biofilms. *Appl Microbiol Biotechnol* **64:** 515–524.

Ren, D., Bedzyk, L.A., Ye, R.W., Thomas, S.M., and Wood, T.K. (2004b) Differential gene expression shows natural brominated furanones interfere with the autoinducer-2 bacterial signaling system of *Escherichia coli*. *Biotechnol Bioeng* **88:** 630–642.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Shah, D., Zhang, Z., Khodursky, A.B., Kaldalu, N., Kurg, K., and Lewis, K. (2006) Persisters: a distinct physiological state of *E. coli*. *BMC Microbiol* **6:** 53.

Spoering, A.L., Vulić, M., and Lewis, K. (2006) GlpD and PlsB participate in persister cell formation in *Escherichia coli*. *J Bacteriol* **188:** 5136–5144.

Suh, S.-J., Silo-Suh, L., Woods, D.E., Hassett, D.J., West, S.E.H., and Ohman, D.E. (1999) Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J Bacteriol* **181:** 3890–3897.

Tamae, C., Liu, A., Kim, K., Sitz, D., Hong, J., Becket, E., *et al*. (2008) Determination of antibiotic hypersensitivity among 4,000 single-gene-knockout mutants of *Escherichia coli*. *J Bacteriol* **190:** 5981–5988.

Tan, Q., Awano, N., and Inouye, M. (2011) YeeV is an *Escherichia coli* toxin that inhibits cell division by targeting the cytoskeleton proteins, FtsZ and MreB. *Mol Microbiol* **79:** 109–118.

Tramonti, A., Visca, P., De Canio, M., Falconi, M., and De Biase, D. (2002) Functional characterization and regulation of *gadX*, a gene encoding an AraC/XylS-like transcriptional activator of the *Escherichia coli* glutamic acid decarboxylase system. *J Bacteriol* **184:** 2603–2613.

Vijayakumar, S.R.V., Kirchhof, M.G., Patten, C.L., and Schellhorn, H.E. (2004) RpoS-regulated genes of *Escherichia coli* identified by random *lacZ* fusion mutagenesis. *J Bacteriol* **186:** 8499–8507.

Wang, X., and Wood, T.K. (2011) Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. *Appl Environ Microbiol* **77:** 5577–5583.

Wang, X., Kim, Y., Hong, S.H., Ma, Q., Brown, B.L., Pu, M., *et al*. (2011) Antitoxin MqsA helps mediate the bacterial general stress response. *Nat Chem Biol* **7:** 359–366.

Waterman, S.R., and Small, P.L.C. (2003) Identification of the promoter regions and $\sigma^s$-dependent regulation of the *gadA* and *gadBC* genes associated with glutamate-dependent acid resistance in *Shigella flexneri*. *FEMS Microbiol Lett* **225:** 155–160.

Weber, H., Polen, T., Heuveling, J., Wendisch, V.F., and Hengge, R. (2005) Genome-wide analysis of the general stress response network in *Escherichia coli*: $\sigma^S$-dependent genes, promoters, and sigma factor selectivity. *J Bacteriol* **187:** 1591–1603.

Wood, T.K., Hong, S.H., and Ma, Q. (2011) Engineering biofilm formation and dispersal. *Trends Biotechnol* **29:** 87–94.

Yamaguchi, Y., Park, J.-H., and Inouye, M. (2009) MqsR, a crucial regulator for quorum sensing and biofilm formation, is a GCU-specific mRNA interferase in *Escherichia coli*. *J Biol Chem* **284:** 28746–28753.

Yim, H.H., and Villarejo, M. (1992) *osmY*, a new hyperosmotically inducible gene, encodes a periplasmic protein in *Escherichia coli*. *J Bacteriol* **174:** 3637–3644.

Zhang, X.-S., García-Contreras, R., and Wood, T.K. (2008) *Escherichia coli* transcription factor YncC (McbR) regulates colanic acid and biofilm formation by repressing expression of periplasmic protein YbiM (McbA). *ISME J* **2:** 615–631.

## Supporting information

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

**Fig. S1.** Swimming motility for MqsR variants. BW25113 Δ*mqsR* strains containing pBS(Kan) (empty plasmid), pBS(Kan)-*mqsR*, pBS(Kan)-*mqsR* 2-1 and pBS(Kan)-*mqsR* 20-14 were inoculated and incubated for 20 h on motility agar plates at 37°C. Two independent cultures were tested, and a representative image is shown.

**Fig. S2.** Persister cell formation in isogenic mutants with stress-related deletions (blue bars). Grey bars show persister cells after producing proteins from pCA24N-based plasmids. Strains were grown in LB at 37°C for 2 h, 1 mM IPTG was added (cells producing RpoS protein had 0.1 mM IPTG), cells were grown to a turbidity of 1 at 600 nm, and cells were exposed to 20 μg ml$^{-1}$ ampicillin for 5 h. Error bars indicate the standard deviation ($n = 2$).

**Table S1.** Partial list of differentially enriched and differentially degraded genes of BW25113 Δ*mqsR*/pBS(Kan)-*mqsR*

2-1 versus BW25113 Δ*mqsR*/pBS(Kan)-*mqsR* for cells exposed to ampicillin (20 μg $ml^{-1}$) in LB with 1 mM IPTG at 37°C for 1 h. Strains were grown to a turbidity of 0.5 at 600 nm in LB with 1 mM IPTG and adjusted to a turbidity of 1 before ampicillin treatment. Raw data for the two DNA microarrays are available using GEO series accession number GSE31054.

**Table S2.** qRT-PCR results of differentially enriched and differentially degraded genes of BW25113 Δ*mqsR*/pBS(Kan)-*mqsR* 2-1 versus BW25113 Δ*mqsR*/pBS(Kan)-*mqsR* for cells exposed to ampicillin (20 μg $ml^{-1}$) in LB with 1 mM IPTG at 37°C for 1 h. Standard deviation is shown for $\Delta\Delta C_T$ ($n = 3$). $C_T$ is the threshold cycle of the target genes.

**Table S3.** Summary of differentially expressed genes of (i) BW25113 wild-type cells with ampicillin (20 μg $ml^{-1}$) for 5 h after $H_2O_2$ (20 mM) pretreatment for 10 min and (ii) BW25113 Δ*rpoS* cells with ampicillin (20 μg $ml^{-1}$) for 5 h both relative to BW25113 wild-type stationary phase cells with ampicillin (5 μg $ml^{-1}$) for 30 min. Raw data for the three DNA microarrays are available using GEO series accession number GSE34028. Bold denotes those genes which are differentially regulated by MqsR 2-1 (BW25113 Δ*mqsR*/pBS(Kan)-*mqsR* 2-1 versus BW25113 Δ*mqsR*/pBS(Kan)-*mqsR*) under ampicillin persistence conditions (Table S1).

**Table S4.** Summary of the transcriptome data for the (i) $H_2O_2$ pretreated wild-type persister cells and (ii) *rpoS* deletion persister cells compared with data for *rpoS* deletion mutants to identify RpoS-controlled genes.

**Table S5.** Primers used for epPCR, site-directed mutagenesis, *rpoS* cloning, DNA sequencing, confirmation of the mutants and qRT-PCR. Underlined italic text indicates the site-directed mutation for the codon corresponding to amino acid replacement for K3N (5′-AAA to 5′-AAC) in mqsR-K3N-f and mqsR-K3N-r and for N31Y (5′-AAT to 5′-TAT) in mqsR-N31Y-f and mqsR-N31Y-r. Underlined bold text indicates the BseRI restriction site in rpoS-BseRI-f2 and SalI restriction site in rpoS NS-r.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

© 2012 The Authors