Bacterial persistence increases as environmental fitness decreases

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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). Persisters 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
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 (Dör 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. Similarily, 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 persistance, 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

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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 ΔmqsR 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 ΔmqsR 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 ΔmqsR 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.
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 persistance 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.

**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 (cst4), glycerol-3-phosphate metabolism (glpQT), galactitol metabolism (gatABCDYZ), ribose
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...
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 ± 0.01 h⁻¹ for ∆*rpoS* versus 1.52 ± 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 (H₂O₂) and acid (pH 2.5) stress...
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 H₂O₂ 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 × 10⁷ cells ml⁻¹ when cells were stressed first with hydrogen peroxide versus 9.8 × 10⁶ cells ml⁻¹ that persist without hydrogen peroxide (both samples originally had roughly 5 × 10⁸ 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,
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 showing that a reduction in transcripts of genes controlled by RpoS is the main mechanism for persistence via showing that a reduction in transcripts 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 (Tamai 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.
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 ΔrpoS 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)-1 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

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**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 (ClpP 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 (mdF) resistance systems. Then, upon antibiotic treatment, cells enter a dormant state. → indicates induction, and /H indicates repression or differential mRNA decay.
Table 1. Strains and plasmids used in this study.

| Strains and plasmids | Genotype/relevant characteristics | Source |
|----------------------|----------------------------------|--------|
| Strains              |                                  |        |
| BL21 (DE3)           | F'ompT hsdS2 (rK2 mcrA) gal dcm λ(DE3) | Novagen |
| BW25113 ΔmqsR        | ΔlacZ ΔgalD ΔaraBADΔrhaBADΔKmR | Baba et al. (2006) |
| BW25113 ΔmqsR         | ΔlacZ ΔgalD ΔaraBADΔrhaBAD ΔKmR | Kim et al. (2010) |
| BW25113 ΔmqsR         | ΔlacZ ΔgalD ΔaraBADΔrhaBAD ΔKmR | Baba et al. (2006) |
| BW25113 ΔmqsR         | ΔlacZ ΔgalD ΔaraBADΔrhaBAD ΔKmR | Baba et al. (2006) |
| BW25113 ΔmqsR         | ΔlacZ ΔgalD ΔaraBADΔrhaBAD ΔKmR | Baba et al. (2006) |
| PA14                 | pET28a-ΔmqsR                    | Liberati et al. (2006) |
| PA14                  | pET28a-ΔmqsR                    | Liberati et al. (2006) |
| pBS(Kan)             | KmR, pBS(Kan)                   | Canada et al. (2002) |
| pBS(Kan)-mqsR        | KmR, pBS(Kan) Pm::mqsR1         | Kim et al. (2010) |
| pBS(Kan)-mqsR 2-1     | KmR, pBS(Kan) Pm::mqsR 2-1 (K3N, N31Y aa substitutions) | This study |
| pBS(Kan)-mqsR 20-14   | KmR, pBS(Kan) Pm::mqsR 20-14 (R9C, L35F and V70I aa substitutions) | This study |
| pET28a β             | KmR, pET28b                     | Novagen |
| pET28a-mqsR           | KmR, pET28a Pm::mqsR            | Brown et al. (2009) |
| pET28a-mqsR K3N       | KmR, pET28a Pm::mqsR K3N (K3N aa substitution) | This study |
| pET28a-mqsR N31Y      | KmR, pET28a Pm::mqsR N31Y (N31Y aa substitution) | This study |
| pET28a-mqsR 2-1       | KmR, pET28a Pm::mqsR 2-1 (K3N, N31Y aa substitutions) | This study |
| pCA24N               | CmR, lacI, pCA24N               | Kitagawa et al. (2005) |
| pCA24N-gadB           | CmR, lacI, pCA24N Pm::gadB      | Kitagawa et al. (2005) |
| pCA24N-gadX           | CmR, lacI, pCA24N Pm::gadX      | Kitagawa et al. (2005) |
| pCA24N-mdtF           | CmR, lacI, pCA24N Pm::mdtF      | Kitagawa et al. (2005) |
| pCA24N-omsmY          | CmR, lacI, pCA24N Pm::omsmY     | Kitagawa et al. (2005) |
| pCA24N-full rpoS      | CmR, lacI, pCA24N Pm::rpoS      | This study |
| Plasmids              |                                  |        |

a. pET28bβ is identical to pET28a except for 1 bp that is deleted near BamHI in the multiple cloning site.

KmR, GmR and CmR are kanamycin, gentamicin and chloramphenicol resistance respectively.

Pulsar/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 ΔmqsR 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 ΔmqsR 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 OD₆₀₀ 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 electropropared 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. This was cloned into pCA24N using the BseRI and Sall restriction sites. Full rpoS insertion was confirmed by sequencing using the pCA24N-seq-f, pCA24N-seq-r and rpoS-RT-f primers.

Persistor assay

Persistor levels were determined by counting the number of colonies grown on solid media after washing and serially
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 exposed to 20, 26, or 40 μg ml^{-1} ampicillin with 1 mM IPTG for 24 h. Cells were washed and diluted by 10² to 10^³ 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 ΔmqsR/pBS(Kan)-mqsR 2-1 versus BW25113 ΔmqsR/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 of 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 RNALater® 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_{2}O_{2} for 10 min. 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_{2}O_{2} for 10 min or pH 2.5 for 2 min.

**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 IPTG 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 Cooomassie 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_{2}O_{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 BacLight® 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).
Swimming motility assay

Swimming motility of BW25113 ΔmqsR strains containing pBS(Kan), pBS(Kan)-ΔmqsR, 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.

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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*PpBS(Kan)-*mqsR*
2-1 versus BW25113 ΔmqsR/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 ΔmqsR/pBS(Kan)-mqsR 2-1 versus BW25113 ΔmqsR/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 ΔΔCt (n = 3). Ct 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 H₂O₂ (20 mM) pretreatment for 10 min and (ii) BW25113 ΔrpoS 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 ΔmqsR/pBS(Kan)-mqsR 2-1 versus BW25113 ΔmqsR/pBS(Kan)-mqsR) under ampicillin persistence conditions (Table S1).

Table S4. Summary of the transcriptome data for the (i) H₂O₂ 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.

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