Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in *Escherichia coli*

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A B S T R A C T

Persisters are cells which evade stresses like antibiotics and which are characterized by reduced metabolism and a lack of genetic alterations required to achieve this state. We showed previously that MqsR and MqsA of *Escherichia coli* are a toxin–antitoxin pair that influence cell physiology (e.g., biofilm formation and motility) via RNase activity as well as through regulation of toxin CspD. Here, we show that deletion of the *mqsRA* locus decreases persister cell formation and, consistent with this result, over production of MqsR increases persister cell formation. Furthermore, toxins Hha, CspD, and HokA increase persister cell formation. In addition, by overproducing MqsR in a series of isogenic mutants, we show that Hha and CspD are necessary for persister cell formation via MqsR overexpression. Surprisingly, Hfq, a small RNA chaperone, decreases persistence. A whole-transcriptome study shows that Hfq induces transport-related genes (*opp* genes and *dppA*), outer membrane protein-related genes (*ybfM* and *ybfN*), toxins (*hha*), and proteases (*clpX*, *clpP*, and *lon*). Taken together, these results indicate that toxins CspD, Hha, and HokA influence persister cell formation via MqsR and that Hfq plays an important role in the regulation of persister cell formation via regulation of transport or outer membrane proteins OppA and YbfM.

Introduction

Persister cells are a small fraction of bacteria that demonstrate resistance to antibiotics without genetic change [1]. The number of persister cells may reach 1% of stationary-phase cells or biofilm cells for a variety of microorganisms including *Pseudomonas aeruginosa* [2], *Escherichia coli* [1], and *Candida albicans* [3]. However, the genetic mechanism of persister cell formation is still unclear.

Toxin/antitoxin (TA) systems are ubiquitous [4] in bacterial chromosomes and in low copy number plasmids where they appear to stabilize these systems [5]. TA systems typically consist of pairs of genes in an operon which encode a stable toxin that can cause cell death by disrupting an essential cellular process and a labile antitoxin that can bind and form a tight complex with the toxin and neutralize its activity [6]. A number of TA systems have been identified in *E. coli* including (listed as toxin/antitoxin) MqsR/MqsA [7–10], MazF/MazE [11], RelE/RelB [12], ChpB/ChpS [13], YoeB/YefM [14], YafQ/DinJ [15], and HipA/HipB [16], and several of these have been linked to biofilm formation [17,18].

TA systems have also been linked to persister cells [19,20]. In a whole-transcriptome study, *MqsRA* of *E. coli* was found to be the most induced locus in persister cells [20]. Although deletion of nearly all TA loci has no effect on persister cell formation due to

the redundancy of these systems [19,20] overexpression of HipA toxin increased persistence 10-fold whereas deletion of *hipBA* decreased persistence by 10–100-fold in stationary and biofilm conditions [19]; hence, *hipA* is the first verified persister gene.

Previously, we discovered that MqsR is induced in biofilms [21] and that MqsR is a regulator of motility and quorum sensing that is directly associated with biofilm development as it mediates the ability of autoinducer 2 to increase *E. coli* biofilm formation [22]. In addition, we found MqsR, in the absence of the antitoxin, MqsA, is much more toxic [10], and the three-dimensional structure of MqsA revealed it is an RNase similar to RelE and YoeB [9]. MqsR degrades RNA with a GCU motif [7]. Furthermore, MqsA binds DNA via a helix-turn-helix (HTH) C-terminal domain [9]. Unlike most TA pairs including RelE/RelB, YoeB/YefM, and MazF/MazE, the MqsR/MqsA TA pair is unique [9] in that (i) the antitoxin is larger than the toxin, (ii) both toxin and antitoxin are basic, (iii) the *mqsRA* sequences are not homologous to any member of a recognized TA system, and (iv) the antitoxin binds the toxin at its amino terminus and requires a zinc metal for structural stability. Most importantly, the antitoxin MqsA regulates more than its own synthesis as it is the first antitoxin that has been shown to bind directly the *mqsRA*, *spy*, *mcbR*, and *cspD* promoters as well as to regulate transcription of these loci [10].

Here, we show direct evidence that MqsR influences persister formation since deletion of *mqsRA* reduces persister cell formation and MqsR overexpression increases persister cell formation. Using

whole-transcriptome analysis, quantitative real time-polymerase chain reaction (qRT-PCR), and cell survival studies in which selected genes were over expressed in isogenic mutants, we explored the mechanism by which MqsR influences persister cell formation and found that MqsR positively regulates persister cell production via Hha and CspD and that the small RNA chaperone Hfq represses persister production.

Materials and methods

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. For isogenic mutants and overexpression of specific genes, we used the Keio collection [23] and ASKA library [24], respectively, from the Genome Analysis Project in Japan. All experiments were conducted in Luria–Bertani (LB) medium [25] at 37 °C. Kanamycin (50 µg/mL) was used for pre-culturing isogenic knockout mutants and for maintaining pBS(Kan)-based plasmids [26], whereas chloramphenicol (30 µg/mL) was used for maintaining pCA24N-based plasmids [24].

Persister assay. Persister cell formation was performed as described [27] with slight modifications. To determine the number of persister cells with the knockout mutants, cells were inoculated in LB medium and grown to a turbidity of 0.5 at 600 nm. After collecting the cells, the concentration was adjusted to a turbidity of 1 (to obtain 1 to 3×10^8 CFU/mL of viable cells), and these cultures were exposed to 100 µg/mL ampicillin for 2 h. Cells were diluted by 10^2–10^7 via 10-fold serial dilution steps in 0.85% NaCl solution and applied as 10 µL drops on LB agar with kanamycin or chloramphenicol to determine persister cell viability [28]. For producing proteins using pBS(Kan)- or pCA24N-based plasmids, when the cell density reached a turbidity of 0.5, 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added for 2 h, the turbidity was adjusted to 1, and ampicillin was added as above. Two independent cultures were used for each strain.

RNA isolation and whole-transcriptome analysis. The whole-transcriptome analysis was performed using planktonic cells of BW25113 *hfq* vs. BW25113 by growing cells to a turbidity of 0.5 at 600 nm in LB medium at 37 °C, adjusting the turbidity to 1, and exposing the cells to 100 µg/mL ampicillin for 2 h. Total RNA was isolated from cells as described previously [21] using RNALater buffer® (Applied Biosystems, Foster City, CA). cDNA synthesis, fragmentation, and hybridizations were as described previously [22]. The *E. coli* GeneChip Genome 2.0 array (Affymetrix, Santa Clara, CA; P/N 511302) was used. Corroborating the deletion of *hfq*, the microarray signals of *hfq* were very low. For the binary microarray comparisons to determine differential genes expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 11 probe pairs (*P*-value less than 0.05), these genes were not used. A gene was considered differentially expressed when the *P*-value for comparing two chips was lower than 0.05 (to ensure that the change in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher than the standard deviation (3.5-fold) for all K-12 genes of the microarrays [29].

Quantitative real time-PCR (qRT-PCR). qRT-PCR was performed using the StepOne™ Real-Time PCR System (Applied Biosystems). The preparation of RNA samples were performed as described previously [21]. For the qRT-PCR experiments, we used RNALater buffer for RNA stabilization and protection during the RNA preparation steps. 50 ng of total RNA was used for the qRT-PCR reaction using the *Power* SYBR® Green RNA-to-C$_T$™ *1-Step* Kit (Applied Biosystems). Primers were designed using Primer3Input Software (v0.4.0) and are listed in Supplementary Table S1. The housekeeping gene *rrsG* [30] was used to normalize the gene expression data.

The annealing temperature was 60 °C for all the genes in this study. For the level of expression of the *hha*, *cspD*, *hokA*, and *hfq* transcripts, overnight cultures of BW25113/pBS(Kan) and BW25113/pBS(Kan)-*mqsR* were inoculated into LB medium and grown to a turbidity of 1 at 600 nm, and 1 mM IPTG was added for 1 h to induce *mqsR*. To explore whether *mqsR*, *hha*, *cspD*, and *hfq* transcription was affected by antibiotic stress, overnight cultures of BW25113 were grown to a turbidity of 0.5, cells were adjusted to a turbidity of 1 and were exposed with or without 100 µg/mL ampicillin for 2 h.

Microarray accession number. The differential gene expression data have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through accession number GSE18852.

Results

MqsR strongly influences persister production

Previously, we found that overexpression of MqsR is toxic [8], whereas overexpression of MqsA diminished MqsR toxicity [9,10]. Using *E. coli* MG1655, MqsR production increased persister cell formation 10,000–100,000-fold against a number of antibiotics including ofloxacin and cefotaxime while deletion of *mqsR* did not reduce the number of persisters [20]. In the current study, we used *E. coli* BW25113 so that we could take advantage of the single gene knockout Keio library [23] and used ampicillin (100 µg/mL) as a representative antibiotic since non-persister cells die within 15 min at this concentration [19]. Unlike the results with MG1655, deletion of *mqsR* from BW25113 significantly repressed persister production (6-fold) compared to the wild-type strain (Fig. 1A). Corroborating this result, deletion of *mqsR* and *mqsA* also repressed persister production (7-fold) compared to the wild-type strain (Fig. 1A). In addition, producing MqsR (using two different expression vectors) increased persister cell formation (12–16-fold), but producing MqsA or MqsR/MqsA did not affect persister cell formation (Fig. 1B). Taken together, these results show the MqsR/MqsA TA pair has an important role in persister cell formation as it becomes only the second locus that upon deletion reduces persistence.

Hha, CspD, and HokA increase persistence

Previously, using whole-transcriptome studies and nickel-enrichment DNA binding microarrays coupled with cell survival experiments, we identified eight proteins (CspD, HokA, ClpX, ClpP, Lon, YfjZ, RelB, and RelE) that are involved in MqsR toxicity [10]. We also identified that the toxin Hha controls cell death and biofilm dispersal [18]. Since the RelE toxin in the RelE/RelB pair is already linked to persister cell formation [19], we explored the impact of seven of these nine proteins in persister cell formation by performing the persister formation assay while inducing the genes that encode these eight proteins in hosts that lack each of these genes (Fig. 2A).

Among the seven proteins, Hha, CspD, and HokA increased persister cell formation by 6–85-fold (Fig. 2A), whereas ClpX, ClpP, and Lon only slightly increased the number of persisters (2–3-fold) (Supplementary Fig. S1). Corroborating these studies, the deletion of *cspD* (2-fold), *hokA* (3-fold), and *hha* (4-fold) significantly repressed the production of persister cells (Fig. 2A); hence, these three toxins are directly involved in persister production.

Hha and CspD are essential for persister cell production via MqsR

Confirming the relationship between Hha and CspD with MqsR and persister cells, deleting *hha* and *cspD* abolished persister cell
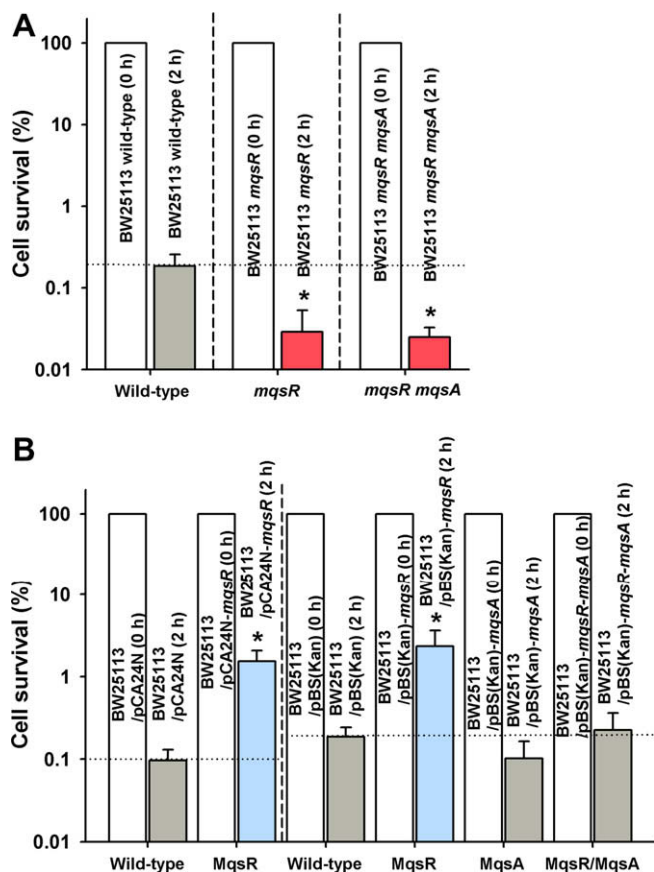
Fig. 1. Effect of *mqsR* and *mqsA* mutations on persister cell formation. BW25113 wild-type, BW25113 *mqsR*, and BW25113 *mqsR mqsA* were grown to a turbidity of 0.5 at 600 nm in LB medium at 37 °C, adjusted on a turbidity of 1, and exposed to 100 μg/mL ampicillin for 2 h (A). Persister cell formation of BW25113/pCA24N, BW25113/pCA24N-*mqsR*, BW25113/pBS(Kan), BW25113/pBS(Kan)-*mqsA*, BW25113/pBS(Kan)-*mqsR*, and BW25113/pBS(Kan)-*mqsR-mqsA* grown to a turbidity of 0.5 in LB medium at 37 °C, contacted with 1 mM IPTG for 2 h for induction, adjusted to a turbidity of 1, and exposed to 100 μg/mL ampicillin for 2 h in LB (B). Persister data are the average of two independent cultures, and one standard deviation is shown. The asterisk indicates statistical significance determined using Student's *t*-test ($P < 0.05$).

Fig. 2. Effect of isogenic mutations on persister cell production. BW25113 *hha*/pCA24N-*hha*, BW25113 *cspD*/pCA24N-*cspD*, BW25113 *hokA*/pCA24N-*hokA*, and BW25113 *hfq*/pCA24N-*hfq* were grown to a turbidity of 0.5 in LB medium at 37 °C, contacted with 1 mM IPTG for 2 h for induction, adjusted to a turbidity of 1, and exposed to 100 μg/mL ampicillin for 2 h in LB (A). Effect of *hha*, *cspD*, *hokA*, and *hfq* isogenic deletions on persister cell formation with MqsR via pCA24N-*mqsR* (B). Persister data are the average of two independent cultures, and one standard deviation is shown. The asterisk indicates statistical significance determined using Student's *t*-test ($P < 0.05$).

formation upon producing MqsR (Fig. 2B). In contrast, deleting *hokA* only slightly reduced the ability of MqsR to increase persistence (Fig. 2B). Therefore, toxins Hha and CspD are most directly involved in persister cell formation with MqsR.

Antibiotic stress induces Hha and CspD via MqsR

Using qRT-PCR and BW25113/pBS(Kan)-*mqsR*, we found that *hha* (10 ± 1-fold) and *cspD* (13 ± 1-fold) are induced by overproducing MqsR. Also, under antibiotics stress (100 μg/mL ampicillin for 2 h), *mqsR* was induced (5 ± 1-fold) in wild-type BW25113. Under these conditions, *hha* (4 ± 1-fold) and *cspD* (3 ± 1-fold) were also induced. These results show that persister cells produced by antibiotic stress induce *mqsR*, and its induction influences production of both the Hha and CspD toxins.

Hfq reduces persistence

Hfq was selected for study here because of its impact on persistence since it is an RNA-binding protein that is important for small RNA–mRNA transactions [31] and because MqsR is an RNase. We found that inducing *hfq* reduced persister cell formation by 2-fold compared to the empty plasmid (Fig. 2A). More importantly, deleting *hfq* increased persister production 11-fold (BW25113 *hfq*/
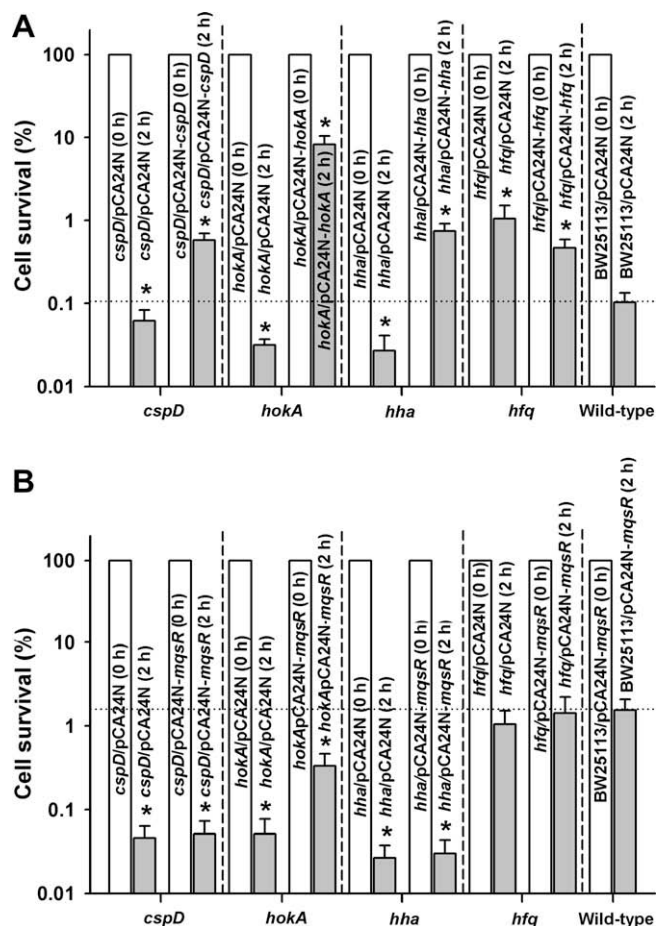
pCA24N vs. BW25113/pCA24N for 2 h) (Fig. 2A). Similar phenotypes were observed for the persister assay with strains BW25113 *hfq* and BW25113 in the absence of plasmids (data not shown). Also, *hfq* was not induced by stress (qRT-PCR). Hence, Hfq reduces persister cell formation.

Hfq whole-transcriptome study

Since Hfq reduces persistence, we hypothesized that Hfq, as an RNA chaperone, may be strongly associated with persister-related RNA under antibiotic stress. Hence, we investigated the genes controlled by Hfq under antibiotic stress using a whole-transcriptome analysis. Using planktonic cells, *hfq* deletion under persister conditions repressed 124 genes by more than 3.5-fold and induced 22 genes (Supplementary Table S2). Primarily, the glycolate utilization *glc* cluster [32] was repressed upon deleting *hfq* including *glcB* (23-fold), *glcG* (21-fold), *glcF* (23-fold), *glcD* (15-fold), and *glcC* (29-fold). Also, motility-related genes including flagella, curli, and chemotaxis were repressed by deleting *hfq* along with *emrY* (encoding multi-drug resistance protein Y) and *micC* (encoding a MicC small RNA regulator of OmpC translation). Deletion of *hfq* induced *hha* as well as *clpP*, *clpX*, and *lon* which encode antitoxin-degrading proteases.
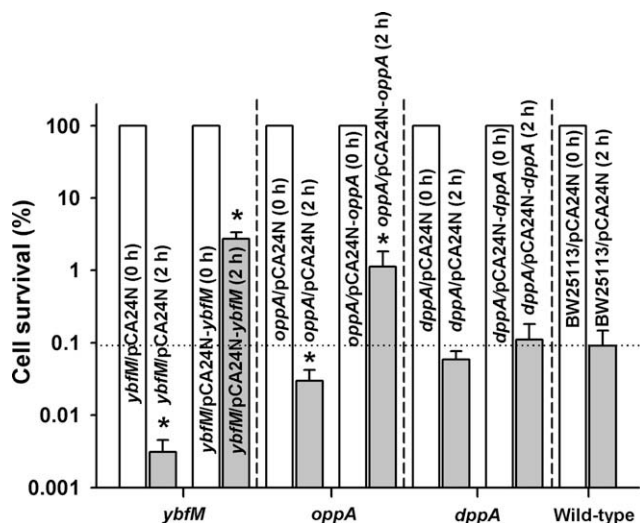
Fig. 3. Impact of YbfM, OppA, and DppA on the production of persister cells. BW25113 ybfM/pCA24N-ybfM, BW25113 oppA/pCA24N-oppA, and BW25113 dppA/pCA24N-dppA were grown to a turbidity of 0.5 in LB medium at 37 °C, contacted with 1 mM IPTG for 2 h for induction, adjusted to a turbidity of 1, and exposed to 100 µg/mL ampicillin for 2 h. Persister data are the average of two independent cultures, and one standard deviation is shown. The asterisk indicates statistical significance determined using a Student's t-test (P < 0.05).

Transport proteins encoded by dppA and the opp locus as well as genes encoding outer membrane proteins ybfM and ybfN were induced upon deleting hfq (Supplementary Table S2). Therefore, Hfq may decrease persistence by repressing specific transport- or membrane-related proteins. Corroborating these results, producing YbfM and OppA increased persistence by 28- and 12-fold, respectively, and deleting ybfM and oppA decreased persistence (Fig. 3).

Discussion

Understanding the genetic basis of persistence may lead to new concepts in anti-microbial therapy. To date, several E. coli proteins such as GlpD, PlsB, DnaJ, and PmrC have been linked to persistence [27,33]. Here, we linked for the first time Hha, CspD, HokA, Hfq, YbfM, and OppA to persistence as well as show that deletion of mqsRA decreases persistence making it only the second locus that upon deletion, affects persistence. A number of toxins in TA systems that inhibit translation via RNase activity are well-suited for initiating cell dormancy [20]. The role of TA systems on cell physiology is not clear, and nine hypothetical biological functions of TA systems have been proposed [34] including persister formation. Recently, we found one more function for TA systems, that of influencing biofilm formation, as shown with Hha/TomB [18], MqsR/MqsA [8,21,22], and the five TA systems of the E. coli Δ5 strain [17]. Among these toxin genes, here we focused on the function of MqsR in persisters.

The lines of evidence that MqsR is important for persister production are (i) mqsR is induced 8-fold in biofilms [21], a mode of growth resistant to antibiotics and which generates persisters [1], (ii) MqsR is a toxin paired with antitoxin MqsA and its toxicity is due to both its RNase activity and regulation of the CspD toxin by interaction with MqsA [10], and toxins have been linked to persister cell formation [20], (iii) MqsR cleaves mRNA at GCU sites and 4212 open reading frames (99.6% population) of the 4226 genes of the E. coli genome contain a GCU sequence, hence MqsR is a potent toxin [7], (iv) mqsR is the most highly induced gene in persister cells [20], and (v) production of MqsR increases persistence and deletion of mqsR decreases persistence (Fig. 1). Fig. 4 summarizes our understanding of the genetic mechanism of MqsR in persister cell production.
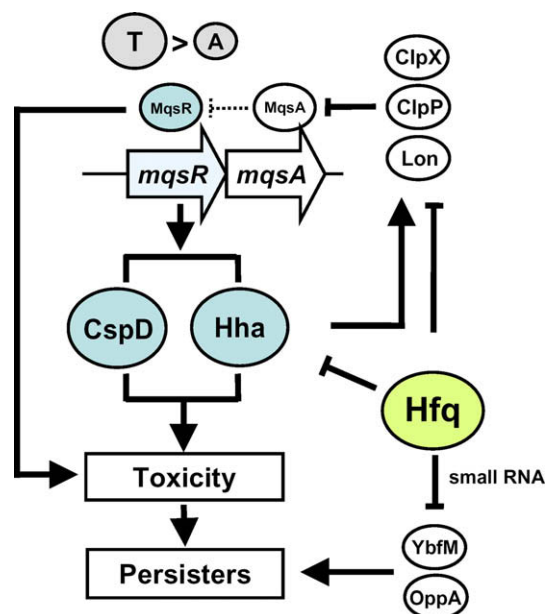

Fig. 4. Schematic of the mechanism of persister production via MqsR. CspD and Hha are directly involved in persister formation via MqsR, and Hfq negatively regulates transport and outer membrane proteins. → indicates induction, and ⊥ indicates repression.

Hha and CspD are also related to persister cell formation with MqsR. Hha, and CspD increase the production of persister cells, deletion of hha and cspD decreases persistence significantly, and deletion of hha and cspD diminish the ability of MqsR to cause persistence (Fig. 2A and B). Also, hha and cspD are induced by stress and by the production of MqsR. Hha also induces genes encoding toxins in other TA pairs, including RelE (3-fold), YoeB (7-fold), and YafQ (2-fold) and induces specific proteases including Lon (4-fold), ClpP (4-fold), and ClpX (4-fold) which may, in turn, activate toxins by degrading antitoxins [18].

We also show that Hfq reduces persistence (Fig. 2A); hence, this is the first protein found to have a negative role in persistence, and this is the first report of Hfq being related to persistence. Also, the whole-transcriptome analysis indicates that hfq deletion induces production of Hha and several proteases including ClpX, ClpP, and Lon (Supplementary Table S2); induction of these genes should increase persistence, and we show ClpP and Lon increase persistence (Supplementary Fig. S1). Therefore, it appears deletion of hfq increases persistence by up-regulating hha, clpX, clpP, and lon. Also, consistent with previous results, dnaJ, dnaK, fis, hns, and dksA were induced by the deletion of hfq under antibiotic conditions (Supplementary Table S2), and these genes have been identified as induced in persister cells using ofloxaxin with E. coli [35]. Also, ybfM and dppA/oppA are strongly regulated by small RNAs, micM [36], and gcvB [37], respectively, and are dependent on the RNA chaperone Hfq.

Since ybfM and oppA are induced by the deletion of hfq under persister conditions (Supplementary Table S2), we tested for their impact on persistence. YbfM is a putative outer membrane porin protein and is similar to OprD of P. aeruginosa (38% identity) [36]. The porin proteins are involved in the uptake of extracellular compounds, including antibiotics, and are related to the multi-drug resistance phenotype [38]. The link between YbfM and persistence was corroborated by a 30-fold change in survival upon deleting and overexpressing ybfM (Fig. 3). Also, OppA is a periplasmic oligopeptide-transport protein [39], and similar to YbfM, OppA also increased persistence by 12-fold upon overexpressing oppA (Fig. 3). Taken together, these results suggest that Hfq may indirectly influ-

ence persister cell formation under antibiotic stress by regulating mRNA encoding transport and outer membrane proteins including porins as well as by regulating *hha*, *clpX*, *clpP*, and *lon*.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.033.

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