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Toxin YafQ increases persister cell formation by reducing indole signalling

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Summary

Persister cells survive antibiotic and other environmental stresses by slowing metabolism. Since toxins of toxin/antitoxin (TA) systems have been postulated to be responsible for persister cell formation, we investigated the influence of toxin YafQ of the YafQ/DinJ *Escherichia coli* TA system on persister cell formation. Under stress, YafQ alters metabolism by cleaving transcripts with in-frame 5′-AAA-G/A-3′ sites. Production of YafQ increased persister cell formation with multiple antibiotics, and by investigating changes in protein expression, we found that YafQ reduced tryptophanase levels (TnaA mRNA has 16 putative YafQ cleavage sites). Consistently, TnaA mRNA levels were also reduced by YafQ. Tryptophanase is activated in the stationary phase by the stationary-phase sigma factor RpoS, which was also reduced dramatically upon production of YafQ. Tryptophanase converts tryptophan into indole, and as expected, indole levels were reduced by the production of YafQ. Corroborating the effect of YafQ on persistence, addition of indole reduced persistence. Furthermore, persistence increased upon deleting *tnaA*, and persistence decreased upon adding tryptophan to the medium to increase indole levels. Also, YafQ production had a much smaller effect on persistence in a strain unable to produce indole. Therefore, YafQ increases persistence by reducing indole, and TA systems are related to cell signalling.

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Introduction

Persisters are predominantly dormant cells (Bigger, 1944; Kwan *et al*., 2013) that are highly tolerant to antibiotics without undergoing a genetic change (Keren *et al*., 2004a). Though persister cell numbers are low [they are absent in exponentially growing cultures (Keren *et al*., 2004a)] and reach a maximum of about 1% in the stationary phase and in biofilms (Lewis, 2007; 2008), these antibiotic-tolerant bacterial subpopulations have been implicated as the culprits for recurrent infections (Lewis, 2010). Unlike resistant cells which grow in the presence of antibiotics due to genetic changes, persisters survive antibiotic treatments since these cells are not undergoing the metabolic activities that antibiotics inhibit (Lewis, 2010). Therefore, understanding the mechanism of persister cell formation is important to derive strategies for controlling bacterial infections. However, the molecular mechanisms involved in the formation and waking of persister cells are not understood well.

Bacterial toxin/antitoxin (TA) systems appear to constitute the primary mechanism of persister cell formation, as they may be used to induce a state of dormancy (Lewis, 2008; Wang and Wood, 2011). There are well-established links between the frequencies of persister cells in *Escherichia coli* populations and the chromosomal TA gene modules HipA/HipB and RelE/RelB (Korch *et al*., 2003; Keren *et al*., 2004b). For example, the first gene linked to persisters (Moyed and Bertrand, 1983), HipA, is a toxin that was named for its high persistence (*hip*) mutants (Korch *et al*., 2003), and its ectopic expression causes multidrug tolerance (Keren *et al*., 2004b). Additional evidence that TA systems are related to persister cell formation was found by deleting the gene that encodes toxin MqsR of the MqsR/MqsA TA system and showing a reduction in persistence (Kim and Wood, 2010). Subsequently, inactivation of 10 TA systems that utilize endoribonuclease toxins in *E. coli* was also shown to reduce persistence (Maisonneuve *et al*., 2011).

Similarly, deletion of the gene encoding the YafQ toxin of the YafQ/DinJ TA pair reduced the persistence of biofilm cells but not that of planktonic cells (Harrison *et al*., 2009). Moreover, transcriptional profiling of persisters isolated from planktonic cell cultures indicates that *dinJ-yafQ* is induced (Keren *et al*., 2004b); hence, the YafQ/DinJ TA

system, like other TA systems, is linked to persister cell formation. Toxin YafQ is a specific endoribonuclease that blocks translation elongation through mRNA cleavage at in-frame 5′-AAA-G/A-3′ sequences via its association with the 50S ribosomal subunit (Prysak *et al*., 2009).

Although some persister cells arise stochastically (Balaban *et al*., 2004), stress appears to activate persister cell formation since cells that are less fit to deal with stress have increased persistence (Hong *et al*., 2012). Furthermore, persistence increases as a result of stress from ciprofloxacin (Dörr *et al*., 2010), rifampicin (Kwan *et al*., 2013), tetracycline (Kwan *et al*., 2013) and carbonyl cyanide *m*-chlorophenyl hydrazone (Kwan *et al*., 2013). Therefore, cell stress, and thus the general stress response master regulator RpoS (Hengge-Aronis, 2002), are important for persister cell formation.

One of the compounds regulated by RpoS is the cell signal indole (Lacour and Landini, 2004). Indole is produced by a large number of Gram-positive and Gram-negative bacterial species, including *E. coli* (Lee and Lee, 2010). Indole acts as a signal in *E. coli* by activating genes such as *gabT* and *astD* (Wang *et al*., 2001); this signal is primarily active at low temperatures whereas autoinducer-2 is the primary signal in the gastrointestinal tract (Lee *et al*., 2008). Indole also acts as an intercellular signal by reducing the pathogenicity of cells that do not synthesize it (Lee *et al*., 2009b) and by influencing the biofilm of other cells (Lee *et al*., 2007a), and acts as an interkingdom signal by tightening epithelial cell junctions (Bansal *et al*., 2010). Moreover, indole was shown initially to increase antibiotic resistance by activating efflux pumps (Hirakawa *et al*., 2005; Kobayashi *et al*., 2006), and later to increase the antibiotic resistance of neighboring cells (Lee *et al*., 2010). Therefore, indole is intimately related to RpoS and antibiotic resistance.

Like indole, TA systems have also been related to RpoS. Toxin MqsR enriches RpoS transcripts through differential mRNA decay (Kim *et al*., 2010), and antitoxin MqsA represses *rpoS* transcription and influences the response to oxidative stress by binding to a palindrome in the *rpoS* promoter (Wang *et al*., 2011). Hence, during non-stress conditions, antitoxin MqsA serves to limit RpoS, while during stress, toxin MqsR serves to increase RpoS. Similarly, antitoxin DinJ influences the general stress response by indirectly regulating the translation of RpoS transcripts via direct repression of *cspE* (Hu *et al*., 2012); cold-shock protein CspE enhances translation of RpoS mRNA. Therefore, TA systems are intimately related to RpoS and the stress response.

Given that the YafQ/DinJ TA system actively participates in the general stress response regulated by RpoS (Hu *et al*., 2012) and that persister cell formation is triggered by stress (Dörr *et al*., 2010; Kwan *et al*., 2013), we focused here on how YafQ influences persister cell formation. We found that YafQ significantly reduces both RpoS and TnaA which results in reduced levels of indole. We also show that the reduction in indole leads to increased persistence. Additionally, the effect of YafQ on persistence is reduced in a strain unable to synthesize indole. Hence, toxin YafQ increases persister cell formation by reducing levels of the extracellular signal indole.

Results

YafQ increases persister cell formation

Since the production of toxins such as MqsR (Kim and Wood, 2010) and RelE (Keren *et al*., 2004b) increase persister cell formation, we investigated the possible impact of YafQ, a homologue of RelE, on persistence to see if this toxin also increases persister cell formation. We used a Δ*yafQ* host so that YafQ could be studied in a host that does not produce background levels of YafQ, and we used ampicillin (100 μg ml^{-1}) and ciprofloxacin (5 μg ml^{-1}) as representative antibiotics of two different classes, β-lactams and fluoroquinolones respectively. As expected, YafQ produced from pCA24N-*yafQ* increased persister cell formation to both ampicillin (980-fold) and ciprofloxacin (43-fold) (Fig. 1A), indicating that YafQ has an important role in persister cell formation.

YafQ reduces TnaA

Toxins have been shown to control metabolism by differential mRNA decay (González Barrios *et al*., 2006; Amitai *et al*., 2009) and YafQ regulates gene expression at the post-transcriptional level through its endoribonuclease activity (Prysak *et al*., 2009), so we hypothesized that production of YafQ should alter global protein expression. Hence, we investigated the impact of producing toxin YafQ via a proteome analysis so that changes in mRNA cleavage could be studied in terms of how they impact final protein levels. We identified 23 proteins with levels altered by producing YafQ (Table 1 and representative two dimensional electrophoresis (2DE) gel image in Fig. S2). All of the excised proteins spots resulted in a positive identification with all Mascot Molecular Weight Search (MOWSE) protein scores (Pappin *et al*., 1993) over 100 (Table 1), indicating significant matches (Baldwin, 2004). Of these, some ribosomal subunit proteins (RplQ and RpsF) and outer membrane proteins (OmpA and OmpX) were increased by producing YafQ, while several stress-related proteins (CspC, CspE, DnaK, HupA, HdeB, AhpC and SodA) were reduced by producing YafQ. Critically, the levels of tryptophanase (TnaA), which produces indole during the stationary phase (Newton and Snell, 1962; Ren *et al*., 2004b), and MdtE, an indole derivative exporter (Zhang *et al*., 2011), were reduced when YafQ was produced.

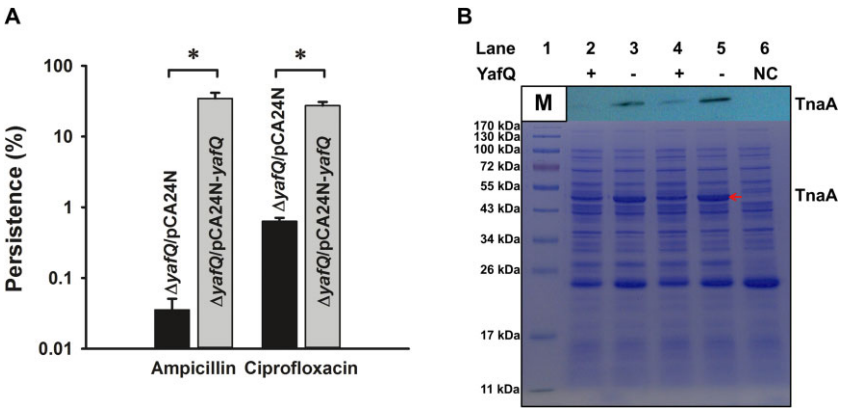

Fig. 1. YafQ increases persister cell formation and reduces TnaA.
A. Persister cell formation for BW25113 Δ*yafQ*/pCA24N-*yafQ* and BW25113 Δ*yafQ*/pCA24N after inducing YafQ production via 1 mM IPTG for 2 h and after treating with 100 μg ml^{-1} of ampicillin or 5 μg ml^{-1} of ciprofloxacin for 3 h. Persister data are the average of two independent cultures, and one standard deviation is shown. The asterisk indicates statistical significance as determined using Student's *t*-test ($P < 0.05$).
B. The upper panel (Western blot) shows TnaA levels as detected by a His-tag antibody for BW25113 Δ*tnaA*/pCA24N-*tnaA*/pBS(Kan)-*yafQ* (YafQ+, lanes 2 and 4) and BW25113 Δ*tnaA*/pCA24N-*tnaA*/pBS(Kan) (YafQ–, lanes 3 and 5). The corresponding whole cell lysates were visualized by SDS-PAGE (lower panel). Both *yafQ* and *tnaA* were induced by 1 mM IPTG for 2 h. Two independent cultures were used for each strain and both replicates are shown. 'M' indicates the protein ladder with protein standards covering a molecular weight range from 11 to 170 kDa. Lane 6 is the negative control (NC) where TnaA is absent (BW25113 Δ*tnaA*/pCA24N). The red arrow indicates the protein band for TnaA.

Table 1. Summary of proteins with levels changed by producing YafQ.

2D Spot ID#	Fold change	Protein	Gene	MW (aa)	MOWSE score	pI	Description of protein
1	5.0	RpsF	*rpsF*	15 704 (135aa)	276	4.9	30S ribosomal subunit protein S6
2	2.1	RplQ	*rplQ*	14 365 (127aa)	267	4.9	50S ribosomal subunit protein L17
3	7.0	YahO	*yahO*	9 895 (91aa)	760	5.8	Periplasmic protein, YhcN family, function unknown.
4	4.0	OmpA	*ompA*	37 201 (346aa)	466	5.7	Outer membrane protein A
5	3.5	OmpX	*ompX*	18 603 (171aa)	466	5.0	Outer membrane protein X; role in inducing RNAP-sigma E production
6	–5.0	OmpF	*ompF*	39 333 (362aa)	1870	4.8	Outer membrane porin F
7	–5.8	AceA	*aceA*	47 522 (434aa)	950	5.2	Isocitrate lyase, acetate utilization, glyoxylate shunt; tetrameric
8	–3.0	AhpC	*ahpC*	20 761 (187aa)	970	5.0	Alkyl hydroperoxide reductase, subunit C
9	–3.0	Ppa	*ppa*	19 704 (176aa)	248	5.1	Inorganic pyrophosphatase; binds Zn(II)
10	–1.5	CspC	*cspC*	7 402 (69aa)	140	6.5	Cold shock protein homolog constitutively expressed at 37°C; affects *rpoS* expression
11	–1.7	CspE	*cspE*	7 464 (69aa)	362	8.1	Binds and melts RNA, antitermination protein; affects *rpoS* expression
12	–2.8	DnaK	*dnaK*	69 115 (638aa)	2540	4.8	Hsp70 molecular chaperone, heat-inducible
13	–1.8	Frr	*frr*	20 639 (185aa)	478	6.5	Ribosome recycling factor (RRF)
14	–2.0	GlnH	*glnH*	27 190 (248aa)	1010	8.4	Glutamine-binding protein, periplasmic
15	–2.2	GroL	*groL*	57 329 (548aa)	1440	4.9	Chaperonin Cpn60; binds and regulates *rpoH*
16	–5.0	HupA	*hupA*	9 535 (90aa)	441	9.6	Histone-like protein HU-alpha, HU-2
17	–2.1	HdeB	*hdeB*	12 043 (108aa)	193	5.7	Periplasmic chaperone of acid-denatured proteins; H-NS repressed
18	–3.6	RibE	*ribE*	16 157 (156aa)	320	5.2	Lumazine synthase; penultimate step in the biosynthesis of riboflavin
19	–2.0	SodA	*sodA*	23 097 (206aa)	791	6.6	Superoxide dismutase, Mn
20	–4.5	TufB	*tufB*	43 314 (394aa)	783	5.3	EF-Tu, Elongation Factor-Translation, unstable
21	–4.5	TufA	*tufA*	43 284 (394aa)	730	5.3	EF-Tu, Elongation Factor-Translation, unstable
22	–10.0	TnaA	*tnaA*	52 774 (471aa)	1100	5.9	Tryptophanase, interconverts tryptophan and indole
23	–2.0	MdtE	*mdtE*	41 191 (385aa)	149	5.7	Anaerobic multidrug efflux transporter MdtEF-TolC; toxic nitrosated indole efflux

Changes in protein levels for BW25113 Δ*yafQ*/pCA24N-*yafQ* versus BW25113 Δ*yafQ*/pCA24N after growth to a turbidity of 1.0 and production of YafQ for 1 h via 1 mM IPTG.

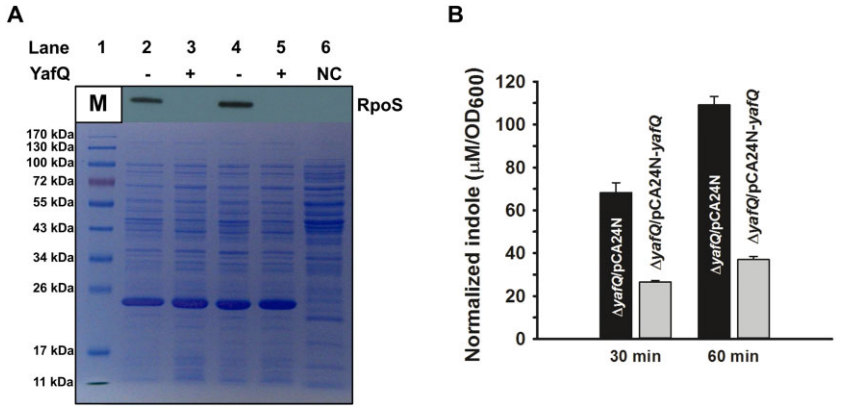

Fig. 2. YafQ reduces RpoS and indole.
A. The upper panel (Western blot) shows RpoS levels as detected by an anti-RpoS antibody for BW25113 Δ*yafQ*/pCA24N-*yafQ* (YafQ+, lanes 2 and 4), and BW25113 Δ*yafQ*/pCA24N (YafQ–, lanes 3 and 5). The corresponding whole cell lysates were visualized by SDS-PAGE (lower panel). *yafQ* was induced from pCA24N-*yafQ* via 1 mM IPTG for 2 h. Two independent cultures were used for each strain and both replicates are shown. 'M' indicates the protein ladder with protein standards covering a molecular weight range from 11 to 170 kDa. Lane 6 is the negative control (NC) where RpoS is absent (BW25113 Δ*rpoS*).
B. Indole production of BW25113 Δ*yafQ*/pCA24N-*yafQ* and BW25113 Δ*yafQ*/pCA24N strain after 30 and 60 min induction of *yafQ* by 1 mM IPTG. Error bars indicate standard error of mean ($n = 2$).

To verify the proteomic analysis, we investigated TnaA levels during YafQ production using a Western blot. Consistent with the proteomic results, TnaA was decreased by YafQ (Fig. 1B). This reduction in TnaA was significant enough to be visible in the SDS-PAGE (Fig. 1B). These results are reasonable since there are 16 putative in-frame YafQ-cleavage cites (5′-AAA-G/A-3′) in the coding region of *tnaA* (Fig. S1), which suggests that YafQ production leads to TnaA mRNA decay. These results also suggest that the reduction in TnaA by YafQ is direct and independent of RpoS since expression of *tnaA* was via a non-native promoter for the Western experiment.

To determine directly if YafQ reduces the *tnaA* transcript levels, we quantified *tnaA* mRNA using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) after producing YafQ (i.e. for BW25113 Δ*yafQ*/pCA24N-*yafQ* versus BW25113 Δ*yafQ*/pCA24N) and found that YafQ reduced *tnaA* transcripts significantly (10.6 ± 0.2-fold). Therefore, YafQ cleaves *tnaA* mRNA, which results in a reduction of TnaA.

YafQ reduces RpoS

Since antitoxin MqsA of the MqsR/MqsA TA system (Wang *et al*., 2011) and antitoxin DinJ of the YafQ/DinJ system (Hu *et al*., 2012) have been found to influence the response of the cell to oxidative stress via the stationary-phase sigma factor RpoS, we investigated the possible role of toxin YafQ on *rpoS* transcription and RpoS protein levels to see if this toxin influences RpoS. Using qRT-PCR to investigate whether YafQ affects *rpoS*, we found that *rpoS* transcript levels were not changed upon YafQ production. In contrast, production of YafQ dramatically reduced RpoS levels (Fig. 2A). The reduction in RpoS levels is expected since both CspC and CspE were found via the proteomics analysis to be reduced upon YafQ production (Table 1) and both positively regulate RpoS levels (Phadtare and Inouye, 2001). Therefore, YafQ reduces RpoS post-transcriptionally, likely by reducing CspC and CspE.

YafQ reduces indole

Given that RpoS stimulates indole production (Lacour and Landini, 2004) and YafQ reduces both RpoS and TnaA, we investigated whether YafQ reduces indole levels. As expected, YafQ reduced indole production (Fig. 2B). In comparison to YafQ, a smaller reduction in indole levels was found upon producing another endonuclease, toxin MqsR, for 60 min (-1.9 ± 0.3-fold for MqsR versus -2.9 ± 0.1-fold for YafQ) despite a higher presence of cleavage sites within TnaA mRNA (18 MqsR cleavage sites). This confirms that YafQ is important for regulating indole, and that degradation of TnaA mRNA is not simply due to random occurrence of cleavage sites. Therefore, YafQ reduces indole due to its reduction of TnaA and RpoS. Note that indole regulation of persistence is independent of YafQ toxicity as addition of 1 mM indole did not alter the reduction in growth seen upon producing YafQ.

Indole decreases persister cell formation

Indole acts as an intercellular and interkingdom signal, influencing multiple aspects of bacterial physiology and has proven to be an important factor in the transition to the

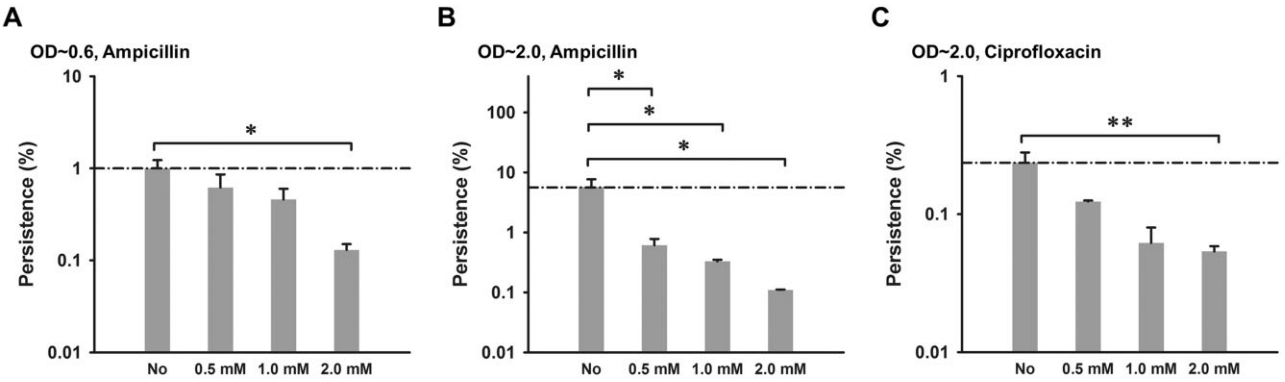

Fig. 3. Indole reduces persister cell formation. (A) Persister cell formation of BW25113 Δ*tnaA* grown to a turbidity of 0.6 (exponential stage) in LB medium, contacted with indole at 0, 0.5, 1 and 2 mM for 2 h, adjusted to a turbidity of 1.0 and exposed to 100 μg ml^{-1} ampicillin for 2 h. (B and C) Persister cell formation of BW25113 Δ*tnaA* grown to a turbidity of 2.0 (stationary phase) in LB medium, contacted with indole at 0, 0.5, 1 and 2 mM for 2 h, adjusted to a turbidity of 1.0 and exposed to (B) 100 μg ml^{-1} ampicillin or (C) 5 μg ml^{-1} ciprofloxacin for 2 h. For both panels, the data are the average of two independent cultures, and one standard deviation is shown. The asterisk indicates statistical significance as determined using Student's *t*-test (*$P < 0.05$; **$P < 0.055$).

stationary phase. Since YafQ reduced both TnaA and its product indole, we investigated whether indole affects persister cell formation. We found that persister cell formation against multiple antibiotics is repressed upon addition of 0.5 to 2 mM indole in both the exponential (Fig. 3A) and the stationary phase (Fig. 3B and C). Note that intracellular levels of indole as high as 60 mM have been reported (Gaimster *et al.*, 2014) as well as extracellular levels of 0.6 mM (Domka *et al.*, 2006); hence, the concentrations of indole tested are physiologically relevant.

We reasoned that since indole reduces persistence, then persistence should increase in a *tnaA* knockout strain that lacks indole production, compared with the wild-type strain. As expected, we found that persistence increases 6.4 ± 0.2-fold upon deleting *tnaA* (BW25113 Δ*tnaA* versus BW25113). Furthermore, production of indole via TnaA should decrease persistence and a 2.4 ± 0.3-fold decrease in persistence was found when TnaA was produced (i.e. for BW25113 Δ*tnaA*/pCA24N-*tnaA* versus BW25113 Δ*tnaA*/pCA24N).

We also reasoned that since the addition of tryptophan to the medium increases indole levels (Li and Young, 2013), persistence should decrease for the wild-type strain upon the addition of tryptophan. As expected, addition of 3.5 mM tryptophan to lysogeny broth (LB) medium resulted in both a 3.3-fold increase in extracellular indole (0.69 ± 0.001 mM to 2.1 ± 0.04 mM) and a 19 ± 3.6-fold decrease in persistence for BW25113 (Fig. 4A). These four sets of experiments demonstrate clearly that indole reduces persister cell formation.

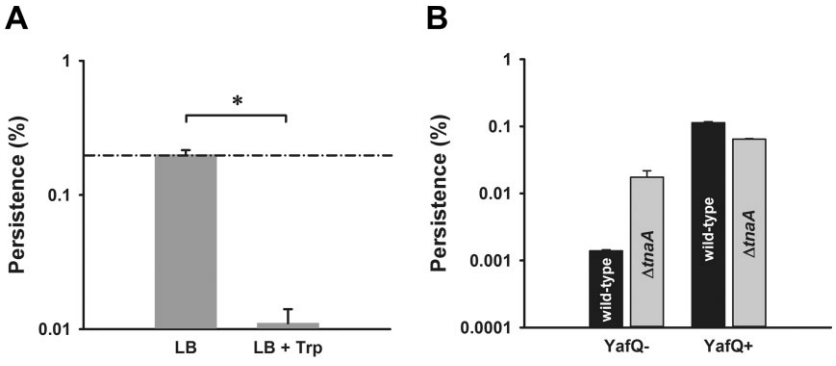

Fig. 4. YafQ-derived persistence decreases in the presence of indole from tryptophan and is dependent on indole.
A. Persister cell formation of BW25113 wild-type grown to stationary phase in LB medium with 0 ('LB') and 3.5 mM additional L-tryptophan ('LB + Trp') to increase indole concentrations, adjusted to a turbidity of 1.0 and exposed to 100 μg ml^{-1} ampicillin for 2 h. The asterisk indicates statistical significance as determined using Student's *t*-test ($P < 0.05$).
B. Persister cell formation for production of YafQ in the BW25113 wild-type strain and for production of YafQ in the BW25113 Δ*tnaA* host. Cultures were grown to early stationary phase in LB medium with 3.5 mM additional L-tryptophan, exposed to 1 mM IPTG for 3 h to induce YafQ production, adjusted to a turbidity of 1.0 and exposed to 100 μg ml^{-1} ampicillin for 2 h. Persister data are the average of two independent cultures, and one standard deviation is shown. 'YafQ–' indicates strains with the empty plasmid pCA24N and 'YafQ+' indicates strains with pCA24N-*yafQ*.

YafQ persistence is dependent on indole regulation

Through independent experiments, we have shown that YafQ increases persistence, YafQ reduces indole, and indole negatively regulates persistence. However, we sought to determine the significance of indole regulation with respect to YafQ mediated persistence. To test this, persistence from production of YafQ was determined using the wild-type and Δ*tnaA* (i.e. unable to synthesize indole) hosts under conditions in which indole is normally produced (i.e. stationary phase). As expected, we found that the effect of YafQ production on persistence was decreased by 21 ± 5-fold in the Δ*tnaA* host compared with the wild-type strain (Fig. 4B), demonstrating that the increased persistence from producing YafQ is dependent on the downregulation of indole synthesis. This experiment also confirms the earlier result that deletion of *tnaA* increases persistence by showing an increase of 12 ± 3-fold (i.e. for BW25113 Δ*tnaA*/pCA24N versus BW25113/pCA24N) (Fig. 4B). Note that this dependence on indole was specific for toxin YafQ since the reduction in persistence by toxin MqsR was not affected by deleting *tnaA* (5 ± 1% persistence for BW25113/pCA24N-*mqsR* versus 3.9 ± 0.2% persistence for BW25113 Δ*tnaA*/pCA24N-*mqsR*). Furthermore, simultaneous deletion of both *rpoS* and *tnaA*, in comparison to sole deletion of *tnaA*, did not appreciably change persistence when YafQ was produced, so the effect of RpoS on persistence is via TnaA (3.8 ± 0.7-fold increased persistence from YafQ production for BW25113 Δ*rpoS* Δ*tnaA*/pCA24N-*yafQ* versus BW25113 Δ*rpoS* Δ*tnaA*/pCA24N compared with a 3.7 ± 0.1-fold increased persistence from YafQ production for BW25113 Δ*tnaA*/pCA24N-*yafQ* versus BW25113 Δ*tnaA*/pCA24N).

Discussion

In this study, we show clearly that production of toxin YafQ increases persister cell formation (Fig. 1A) and the mechanism is through a dramatic reduction of TnaA (Fig. 1B and qRT-PCR results) that leads to reduced indole levels (Fig. 2B). Furthermore, YafQ also reduces RpoS (Fig. 2A); hence, the reduction in RpoS and TnaA protein levels by YafQ appear to work in concert to lower indole levels. However, the primary means by which YafQ increases persistence is via a reduction in indole, since deletion of both *rpoS* and *tnaA* did not affect persistence appreciably in comparison to deletion of *tnaA*. Furthermore, the regulation of *tnaA* by RpoS is complex (Dong *et al*., 2008), so other factors may be involved. For example, RpoS is important at the start of the stationary phase, but its levels decrease in this growth phase, so it is important for genes as a transient regulator (Schellhorn, 2014).

Together with the results that exogenous indole addition reduces persistence (Fig. 3), that persistence increases in a Δ*tnaA* strain (Fig. 4B), that TnaA reduces persistence and that tryptophan addition reduces persistence (Fig. 4A), we also demonstrate that persistence and indole levels are inversely proportional. Additionally, persistence from YafQ production is reduced in a host that is unable to synthesize indole (Δ*tnaA*) (Fig. 4B), confirming the importance of indole regulation to the YafQ persister mechanism. A schematic of our current understanding of how toxin YafQ influences persister cell formation is shown in Fig. 5.

Our results differ from a previous report indicating that indole increases persistence (Vega *et al*., 2012). However, different strains and antibiotics were used which may address the apparent discrepancy in the results. Furthermore, our result that the signal indole reduces persistence follows from previous findings that indole increases drug resistance through RpoS-dependent induction of drug efflux (Hirakawa *et al*., 2005; Kobayashi *et al*., 2006) (i.e. cells with less indole are less resistant to antibiotics), and cells that are less resistant to stress are more likely to become persisters (Hong *et al*., 2012). Moreover, the inverse relationship between indole and persistence is also reasonable since indole reduces biofilm formation in *E. coli* (Domka *et al*., 2006; 2007; Lee *et al*., 2007a,b; 2009a) and is reduced in biofilms (Domka *et al*., 2007). Since persister cells are more prevalent in biofilms (Lewis, 2008), cells should reduce indole levels in order to increase persistence in biofilms. Hence, our results provide insights into an important physiological role for TA systems: as cells are stressed in biofilms, toxins are activated and indole cell signalling is diminished to facilitate entry of cells into the persister state. Although speculative, our results further suggest that *E. coli* in biofilms may progressively use several TA systems to first activate RpoS (e.g. via MqsR/MqsA) and then deactivate RpoS (e.g. via YafQ/DinJ).

In summary, our results demonstrate how some toxins increase persistence, which is important because production of nearly all toxins increases persistence. In addition, our results demonstrate the importance of indole in biofilm cell physiology as a signal for controlling when cells become persistent in biofilms. Hence, our results cement the role of TA systems in biofilms (Ren *et al*., 2004a; González Barrios *et al*., 2006; García-Contreras *et al*., 2008; Kim *et al*., 2009) and link TA systems to cell signalling through indole. Clearly, since not all cells produce indole, there are indubitably additional mechanisms that contribute to persister cell formation.

Experimental procedures

Bacterial strains, P1 transduction, plasmids and growth conditions

The bacterial strains and plasmids are listed in Table 2. LB (Sambrook *et al*., 1989) at 37°C was used for all the

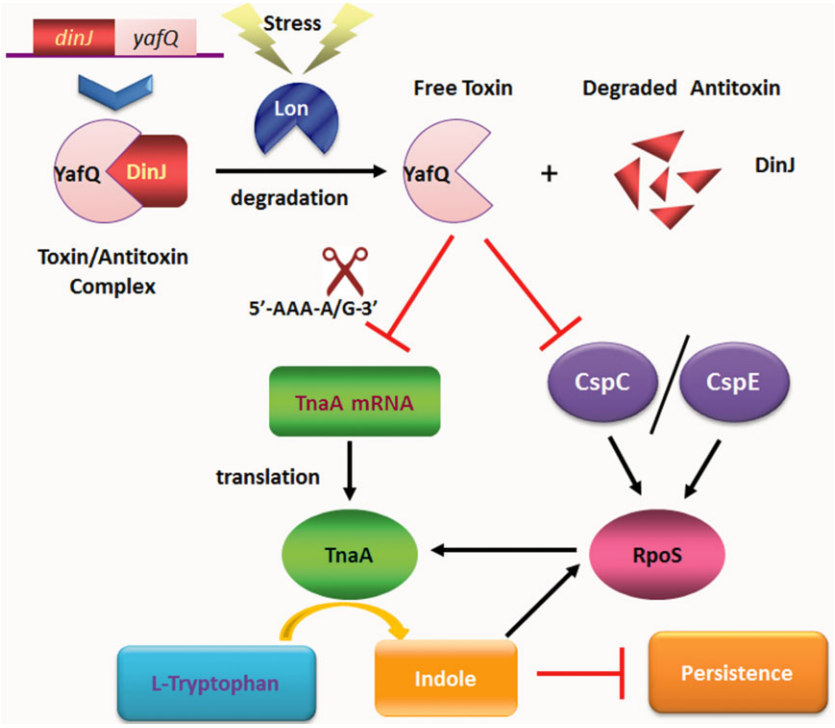

Fig. 5. Schematic of how YafQ increases persistence by reducing indole. Under antibiotic stress, free toxin, YafQ, is released due to the degradation of antitoxin DinJ by protease Lon, and YafQ cleaves *tnaA* mRNA at its 5′-AAA-G/A-3′ sites. RpoS levels are also reduced indirectly by YafQ (through a reduction in CspC and CspE), leading to further repression of *tnaA*. The decreased TnaA levels lead to a reduction in indole production, which increases persister formation. The lightning bolt indicates stress, → indicates induction and ⊥ indicates repression.

experiments. 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 expressing genes in *E. coli*. P1 transduction (Maeda *et al*., 2008) was used to create the double deletion strain, BW25113 Δ*rpoS* Δ*tnaA* (Table 2). Prior to P1 transduction, the kanamycin resistance cassette was removed from BW25113 Δ*rpoS* by using plasmid pCP20 (Cherepanov and Wackernagel, 1995; Datsenko and Wanner, 2000). After P1 transduction to add Δ*tnaA*, both gene deletions were verified by DNA sequencing using primers S*rpoS*-F/-R and S*tnaA*-F/-R (primers are shown in Table S1). For construction of pBS(Kan)-*yafQ*, *yafQ* was polymerase chain reaction (PCR)-amplified from *E. coli* MG1655 chromosomal DNA as a template, using primers *yafQ*-KpnI-F and *yafQ*-SacI-R (Table S1). The PCR product was cloned into the multiple cloning site of pBS(Kan) (Canada *et al*., 2002) after double digestion with restriction enzymes KpnI and SacI. The *yafQ* gene in pBS(Kan) is under the control of a *lac* promoter. The pBS(Kan)-*yafQ* plasmid was confirmed by DNA sequencing with primer pBS(Kan)-seq (Table S1). Cell growth was assayed using the turbidity at 600 nm. Kanamycin (50 μg ml^{-1}) and chloramphenicol

Table 2. *Escherichia coli* bacterial strains and plasmids used in this study.

Strains	Genotype	Source
MG1655	F$^-$ λ$^-$ *ilvG rfb*-50 *rph*-1	(Blattner *et al*., 1997)
BW25113	*rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1*	(Baba *et al*., 2006)
BW25113 Δ*rpoS*	BW25113 Δ*rpoS* Ω Km^R	(Baba *et al*., 2006)
BW25113 Δ*tnaA*	BW25113 Δ*tnaA* Ω Km^R	(Baba *et al*., 2006)
BW25113 Δ*yafQ*	BW25113 Δ*yafQ* Ω Km^R	(Baba *et al*., 2006)
BW25113 Δ*mqsRA*	BW25113 Δ*mqsRA* Ω Km^R	(Kim *et al*., 2010)
BW25113 Δ*rpoS* Δ*tnaA*	BW25113 Δ*rpoS tnaA* Ω Km^R	This study
DH5α	*luxS supE44* Δ*lacU169*(Δ*80 lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	(Ren *et al*., 2004b)
Plasmids		
pCA24N	Cm^R; *lacI*^{q}, pCA24N	(Kitagawa *et al*., 2005)
pCA24N-*yafQ*	Cm^R; *lacI*^{q}, pCA24N $P_{T5\text{-}lac}$::*yafQ* $^+$	(Kitagawa *et al*., 2005)
pCA24N-*tnaA*	Cm^R; *lacI*^{q}, pCA24N $P_{T5\text{-}lac}$::*tnaA* $^+$	(Kitagawa *et al*., 2005)
pCA24N-*mqsR*	Cm^R; *lacI*^{q}, pCA24N $P_{T5\text{-}lac}$::*mqsR* $^+$	(Kitagawa *et al*., 2005)
pBS(Kan)	Km^R; pBS(Kan)	(Canada *et al*., 2002)
pBS(Kan)-*yafQ*	Km^R; pBS(Kan)P_{lac}::*yafQ*$^+$	This study
pCP20	Ap^R, Cm^R; FLP$^+$, λ *c*I857$^+$, λ p_R Rep^{ts}	(Cherepanov and Wackernagel, 1995)

Cm^R and Km^R are chloramphenicol and kanamycin resistance respectively.

(30 μg ml^{-1}) were used to maintain the pBS(Kan)-based and pCA24N-based plasmids (Kitagawa *et al*., 2005), and ampicillin (100 μg ml^{-1}) was used to maintain pCP20.

Persister assay

Persister levels were determined by counting the number of colonies that grew on solid media after washing and serially diluting the cells after exposure to the antibiotic (Dörr *et al*., 2009). To determine the number of persister cells from producing YafQ, overnight cultures were diluted to a turbidity of 0.05 and grown in LB medium with chloramphenicol (30 μg ml^{-1}) to a turbidity of 1.0, then 1 mM IPTG was used to induce *yafQ*. After 2 h, cells were washed, adjusted to a turbidity of 1.0 in LB and were exposed to 100 μg ml^{-1} ampicillin or 5 μg ml^{-1} ciprofloxacin with 1 mM IPTG for 3 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 to determine cell viability (Donegan *et al*., 1991). For BW25113 Δ*tnaA*, cells were grown to a turbidity of ~ 0.6 or ~ 2.0 at 600 nm, and indole was added at 0, 0.5, 1 and 2 mM (from 500 mM stock; dimethylformamide was used as negative control). After 2 h, cells were washed, adjusted to a turbidity of 1.0 in LB and were exposed to 100 μg ml^{-1} ampicillin or 5 μg ml^{-1} ciprofloxacin for 2 h. For YafQ persistence in Δ*tnaA* or Δ*rpoS* Δ*tnaA* hosts, cells were grown in LB supplemented with additional L-tryptophan (3.5 mM) to a turbidity of ~ 2.0 at 600 nm, and 1 mM IPTG was used to induce *yafQ* expression. After 3 h, cells were washed, adjusted to a turbidity of 1.0 in LB and exposed to 100 μg ml^{-1} ampicillin for 2 h.

Protein sample preparation and two-dimensional gel electrophoresis (2DE)

For proteomic analysis, BW25113 Δ*yafQ*/pCA24N-*yafQ* and BW25113 Δ*yafQ*/pCA24N were grown to a turbidity of 1.0, then 1 mM IPTG was added to express *yafQ*. After induction for 1 h, cells were washed with and resuspended in TE buffer with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) and then sonicated to lyse cells using a 60 sonic dismembrator (Fisher Scientific, Pittsburgh, PA, USA). After centrifugation at 17 000 × *g* for 10 min to remove debris, the supernatants were used to determine the protein concentration via the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Salt was removed from the protein samples via a Bio-Rad ReadyPrep 2D cleanup Kit. 2DE was performed using a Protean IEF cell and mini electrophoresis system (Bio-Rad). To ensure 2DE reproducibility and to prevent variations occurring due to the technique, all 2DE gels were performed under the same electrophoresis conditions, and each sample was run on duplicate 2D gels. Protein (200 μg) was mixed with rehydration buffer (Bio-Rad) and loaded onto an immobilized pH gradient (IPG) strip (7 cm pH 3–10, Bio-Rad). After an overnight rehydration, the IPG strip was focused by applying current for 15 min at 250 V followed by 2 h with voltage ramping linearly to 4000 V to reach 20 000 Vh and then the samples were frozen at –80°C. Prior to the second-dimension SDS-PAGE, the focused strip was equilibrated for 15 min with equilibration buffer (6 M urea, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 2% SDS and 0.002% bromophenol blue) containing 2% dithiothreitol and for another 15 min with same solution containing 2.5% iodoacetamide. For the second-dimension separation, the IPG strips were positioned on 12% polyacrylamide gels, and the proteins were separated at 100 V for 2 h at room temperature. Gels were washed in ultrapure water and fixed in 20% methanol and 10% acetic acid solution for 30 min. ImageJ software (http://rsb.info.nih.gov/ij/index.html) was used to quantify the change in the density of the spots on the 2D gel. Duplicate gels of each sample were analysed.

Mass spectrometry (MS)

2D gels were visualized with Coomassie brilliant blue G-250 to identify differentially expressed proteins. The protein spots of interest were manually excised from gels. The excised gel plugs were approximately 2 mm in diameter and 1.5 mm in thickness. Trypsin digestion was performed following standard protocols consisting of a series of washing and dehydrating steps using 25 mM ammonium bicarbonate and acetonitrile respectively. The gel spots then were digested with trypsin at 37°C for 18 h. Trypsin-digested samples were desalted, mixed with the matrix-assisted laser desorption/ionization (MALDI) matrix and spotted onto a MALDI target plate. Samples were cleaned with 70% acetonitrile and 0.1% formic acid using C18 Ziptips (Millipore, Billerica, MA, USA) prior to spotting.

All MALDI-MS and MS-MS experiments were performed using a 4800 Proteomics Analyser (Applied Biosystems, Foster City, CA, USA). The MS data for the MALDI plates were acquired using the reflectron detector in positive mode (700–4500 Da, 1900 Da focus mass) using 800 laser shots (40 shots per subspectrum) with internal calibration. The collision gas was air at the medium pressure setting, with 1 kV of collision energy applied across the collision cell. All MS data were searched against the NCBInr database using the GPS Explorer V2.1 (Applied Biosystems) software. A Mascot MOWSE protein score of greater than 100 obtained with the Mascot search engine (Matrix Science, UK) was considered as a significant match (Baldwin, 2004). Two spots were analysed to confirm the generated MS data.

Indole assay

Extracellular indole was measured with *E. coli* BW25113 Δ*yafQ*/pCA24N-*yafQ* and BW25113 Δ*yafQ*/pCA24N as described previously (Domka *et al*., 2006). The strains were cultured overnight in LB with kanamycin (50 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}). Overnight cultures were then inoculated into LB medium containing chloramphenicol (30 μg ml^{-1}) with an initial turbidity of 0.05. When cells grew to a turbidity of 1.0, 1 mM IPTG was added to induce *yafQ* expression. Extracellular indole concentrations were measured after 30 min or 60 min induction of *yafQ*. For the effect of tryptophan addition, BW25113 was cultured in LB with and without tryptophan (720 μg ml^{-1}) for 6 h.

To measure extracellular indole, 1 ml of cell-free culture fluid was mixed for 2 min with 0.4 ml of Kovac's reagent (10 g of *p*-dimethylaminobenzaldehyde, 50 ml of HCl and 150 ml of

amyl alcohol), 100 μl of the reaction mixture was diluted in 900 μl of HCl-amyl alcohol solution (50 ml of HCl and 150 ml of amyl alcohol), and the absorbance at 540 nm was measured. Concentrations were calculated based on a calibration curve.

qRT-PCR

After isolating RNA using RNA*later* (Life Technologies, Carlsbad, CA, USA) and an ethanol-dry ice quick cooling method during cell harvest, 50 ng of total RNA was used for qRT-PCR using the *Power* SYBR® Green RNA-to-C_T *1-Step* Kit and the StepOne Real-Time PCR System (Applied Biosystems). Primers were designed using Primer3Input Software (v0.4.0) and are listed in Table S1. 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. To investigate the *rpoS* and *tnaA* mRNA changes by producing YafQ, overnight cultures of BW25113 Δ*yafQ*/pCA24N-*yafQ* and BW25113 Δ*yafQ*/pCA24N were inoculated into LB medium containing chloramphenicol (30 μg ml^{-1}) with an initial turbidity of 0.1 and grown to a turbidity of 2.0, then 1 mM IPTG was added for 2 h to induce *yafQ* until a turbidity ~ 3.0.

Western blot analysis

To investigate the influence of YafQ on RpoS protein levels, Western blots and SDS-PAGE were performed as described previously (Wang *et al*., 2011). To investigate how YafQ affects TnaA levels, BW25113 Δ*tnaA*/pCA24N-*tnaA*/pBS(Kan)-*yafQ* and BW25113 Δ*tnaA*/pCA24N-*tnaA*/pBS(Kan) were grown until a turbidity ~ 1.0, and 1 mM IPTG was added to induce both *tnaA* and *yafQ*. After inducing for 2 h, cells were washed and resuspended in TE buffer with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich). Samples were sonicated twice using a 60 sonic dismembrator (Fisher Scientific) at level 4 for 15 s. Soluble protein samples in supernatants were obtained by centrifuging the cell pellets at 17 000 × *g* for 4 min. Total protein was quantified using a Pierce BCA Protein Assay kit (Fisher Scientific), and 2 μg was loaded into each well of a 12% SDS-PAGE gel. The protein was transferred to a polyvinylidene difluoride membrane that was blocked with 4% BSA in TBST (10 mM Tris pH 7.5, 100 mM NaCl and 0.1% Tween 20) for 1 h at room temperature. The Western blots were probed with a 1:2000 dilution of primary antibodies against a His tag (Cell Signaling Technology, Beverly, MA, USA), and then with a 1:20 000 dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Millipore). For SDS-PAGE, 12 μg of each protein sample was loaded, and the gel was stained with Coomassie brilliant blue G-250. To investigate how YafQ affects RpoS levels, BW25113 Δ*yafQ*/pCA24N-*yafQ* and BW25113 Δ*yafQ*/pCA24N were grown to a turbidity of 2.0, then 1 mM IPTG was added to induce *yafQ* for 2 h. Cells were processed as described previously and a 1:2000 dilution of anti-RpoS monoclonal primary antibody (Neoclone, Madison, WI, USA) was used for the Western blot.

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Conflict of interest

The authors have no conflicts of interest.

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Supporting information

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

Fig. S1. The *tnaA* encoding region. Sequences 500 bp upstream of the translation start of *tnaA* are in blue font. The putative, in-frame YafQ-cleavage sites (5′-AAA-A/G-3′) are highlighted in green. The primer pair used in qRT-PCR to investigate *tnaA* mRNA levels is highlighted in yellow. The boxes indicate the −35 and −10 promoter regions. The Shine-Dalgarno sequence is in red.

Fig. S2. Representative 2DE gel images of soluble proteins from (A) BW 25113 Δ*yafQ*/pCA24N-*yafQ* and (B) BW25113 Δ*yafQ*/pCA24N after growth to a turbidity of 1.0 and production of YafQ for 1 h via 1 mM IPTG. The first dimension of separation was performed using 7 cm pH 3–10 IPG strips, followed by SDS-PAGE with 12% polyacrylamide gels in the second dimension and Coomassie brilliant blue G-250 staining. The identified proteins with more than twofold upregulation and downregulation are labeled with red and black circles respectively. The number near the circle refers to the protein ID in Table 1 (e.g. spot 22 is TnaA).

Table S1. Oligonucleotides (listed 5′ to 3′) used in this study for DNA sequencing and qRT-PCR.