THE INFLUENCE OF ENVIRONMENTAL STRESSES ON ESCHERICHIA COLI TOLERANCE TO ANTIMICROBIALS

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ABSTRACT OF DISSERTATION

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ABSTRACT

Persisters are bacterial cells that survive antibiotic treatment without acquiring resistance-confering mutations. Upon antibiotic removal, they form a population identical to the parental one, complicating treatment of infectious diseases. Persisters are multidrug-tolerant and form in response to stresses. Persisters can form in response to fluoroquinolone (FQ) treatment through the induction of the SOS response. FQ treatment produces DNA double strand breaks (DSBs), which induce the SOS response. TisB, part of a toxin-antitoxin (TA) module, is induced by the SOS response and causes persister formation by decreasing the proton motive force and ATP levels. Some persisters also form prior to antibiotic challenge. For example, the number of persisters increases with the rise of cell density during normal growth, probably due to the stress of nutrient limitation.

We wonder if non-antibiotic environmental stresses cause persister formation. In this thesis, we investigated the influence of oxidative stress and heat shock on bacterial drug tolerance, respectively.

Bacterial pathogens are routinely exposed to reactive oxygen species (ROS) including hydrogen peroxide (H₂O₂), superoxide (O₂⁻) and hydroxyl radial (OH⁻) produced by the host immune system. Bactericidal antibiotics have been reported to generate ROS. These oxidants cause damage to cellular macromolecules and induce the oxidative stress response. By pretreating a growing Escherichia coli population with superoxide-producing paraquat, we found that tolerance to FQs increased significantly. This increase in tolerance is mainly due to the induction of the efflux pump AcrAB-TolC, a part of the soxRS regulon. The efflux pump AcrAB-TolC decreases the intracellular concentration of FQs, reducing the amount of DSBs. Consequently, more cells can survive by repairing the damage or reducing the ATP level by the
expression of TisB through the induction of the SOS response. The effect of superoxide also contributes to the increased tolerance to a lesser extent. This finding demonstrates synergy between drug resistance and drug tolerance, thus improving bacterial survival against antibiotic treatment.

Bacteria endure temperature shifts upon host immune response like inflammation and fever, or during food processing. They respond to sharp increases in temperature by inducing over 100 heat shock proteins (HSPs). We found that heat shock response increased *E. coli* tolerance to ofloxacin. The screening of a single-gene knockout collection of HSPs resulted in the identification of potential candidate genes important for tolerance. These mutants, when pretreated with heat shock, demonstrated significantly less tolerance to ofloxacin compared to the wild type, indicating the role of these genes in drug tolerance.
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CHAPTER 1 Introduction

The history of persister research

In 1944, Joseph Bigger found that penicillin lysed a growing population of *Staphylococcus aureus* but left a small number of cells alive. These cells were phenotypic variants rather than genetic mutants. In fresh antibiotic-free medium they formed a new population with the same susceptibility as the parental population. Bigger named these cells “persisters” (12). Similar experiments were conducted approximately 60 years later and the same results were observed (55). The level of persisters is represented by the plateau in the bacterial killing curve shown in Figure 1.1. If the surviving cells are drug resistant mutants, then they would grow in the presence of antibiotics as shown by the dashed black line. The survival of persisters from the antibiotic treatment is due to their tolerance to the lethal molecules, apparently by entering into an inactive state (9).

![Figure 1.1. The representative biphasic killing curve of bacteria by antibiotics.](image-url)
Around 40 years later, Harris Moyed and co-workers revisited the topic of persisters (13, 14, 80, 81). By repeatedly lysing a growing population of *Escherichia coli* (*E. coli*) with ampicillin for several cycles, they selected a mutant *hipA7* with a significantly higher persistence but unchanged minimum inhibitory concentrations (MIC) to different antibiotics. The mutation was mapped to the *hipBA* locus, encoding a toxin-antitoxin (TA) module. The overexpression of *hipA* resulted in a higher level of persistence compared to the wild type, but the deletion of *hipA* failed in reducing the persister level, raising the questions about its role in persister formation (26, 36, 62, 70). Approximately two decades later, HipA was found to be a kinase which phosphorylates the translation factor EF-Tu, resulting in the cell growth arrest and persister formation (26, 104).

Bacterial biofilms have been associated with many chronic infections such as dental disease, endocarditis, cystitis, urinary tract infections, indwelling device and catheter infections, otitis media (86) and the incurable disease of cystic fibrosis (68). Biofilms had long been regarded as highly resistant to antimicrobials until 2001 when they were found to be no more resistant than the stationary phase cells, and their high tolerance to antimicrobials was then suggested to be dependent on the presence of persister cells (112). As shown in Figure 1.2, both regular cells and persisters in the biofilm shed off the biofilm matrix. With antibiotic treatment, regular cells within or outside the matrix are rapidly killed by antibiotics. Persisters outside the biofilm matrix are eliminated by host immunity factors. However, the persisters within the matrix are shielded from the immunity factors. When the level of antibiotics drop, the persisters in the biofilm resuscitate, causing a relapse of the infection (68).
Persisters exist in all bacterial populations tested so far, and also in the biofilm of eukaryotic microorganism *Candida albicans* (63). Various pathogenic high persister (*hip*) mutants, without increased MICs, have been isolated from patients periodically exposed to high doses of antibiotics. For example, the late isolate of a clonal pair of early/late isolates of *Pseudomonas aeruginosa* from a single cystic fibrosis (CF) patient showed a 100-fold increase in persister levels (82). *hip* mutants of *C. albicans* were also isolated from cancer patients who had long-term oral carriage of *Candida* and who had been treated with topical chlorhexidine once a day (64). These findings suggest that persister cells are clinically relevant, and that antimicrobial therapy selects for *hip* strains *in vivo*.

**The mechanisms of persister formation**

In earlier time of persister research it was thought that persisters represent a certain stage in a cell cycle (101), which was ruled out later since in early exponential phase cells go through the entire cell cycle but do not produce persisters (55). Persisters start to form at early-mid exponential phase, increase sharply from mid-exponential phase, and reach a plateau in stationary phase (55), as shown in Figure 1.3. The persisters at the lag phase and early
exponential phase were leftover from the inoculum and can be diluted out by repeated inoculation. The authors then speculated that persisters are not produced by antibiotic treatment because no persisters are produced in the early exponential phase. Based on the pattern of persister formation shown in Figure 1.3, the authors predicted that persister formation is regulated by the growth stage of the population.

**Figure 1.3.** Growth-stage dependence of persister formation in *E. coli* (A) HM21 and (B) HM22 (55).

The first persister gene *hipA* in the TA module *hipBA* encodes a toxin, the overexpression of which results in multidrug tolerance of cells (26, 36, 62, 70). The toxin is usually stable and inhibits important cellular functions. It forms an inactive complex with the unstable antitoxin. TA modules were first found on plasmids as a mechanism for maintaining the plasmid (43, 44, 49). In the cytoplasm of a daughter cell without the plasmid, the antitoxin degrades quickly and sets the toxin free to kill the cell. Later TA modules were found also common on chromosomes. Ectopic expression of the toxins in some TA modules, such as MazF in MazEF and RelE in RelBE, increased the drug tolerance of the cells, which can be counteracted by the antitoxin (23,
The deletion of these loci, however did not have an altered phenotype (56). During bacterial growth, if some cells stochastically overexpress the toxins of TA modules, persisters may form. The argument against this speculation is that the stochastic overexpression of some toxins is random so it also occurs in very early exponential phase but during which no persisters form (68).

To find specific genes accounting for persister formation, researchers screened libraries to search for altered persistence phenotypes. The screening of an *E. coli* library of transposon insertion mutants showed nine candidates with reduced tolerance to kanamycin. The mutant most sensitive to kanamycin had reduced tolerance to other antibiotics as well. This mutant had a mutation in an intergenic region, indicating that this region may regulate the multidrug tolerance of cells (52). The screening of another *E. coli* transposon mutant library identified *phoU*, encoding a global negative regulator, as a switch for the tolerance to multiple drugs and stresses such as starvation, acids, heat, peroxide, and energy inhibitors, especially in stationary phase (71). The screening of an expression library of *E. coli* identified two persister genes: *glpD* and *plsB*. The overexpression of GlpD, sn-glycerol-3-phosphate dehydrogenase, increased the drug tolerance and its deletion reduced the level of tolerance. PlsB, the glycerol-3-phosphate acetyltransferase, is an essential protein involved in the G3P metabolism. The mutant *plsB26* with reduced PIsB function produced significantly fewer persisters than the wild type (113). The screening of the *E. coli* gene knockout library (KEIO library) generated a list of interesting genes: *dnaJ* and *dnaK* (chaperones), *apaH* (diadenosine tetraphosphatase), *surA* (peptidyl-prolyl-cis-trans isomerase), *fis* and *hns* (global regulators), *hnr* (response regulator of RpoS), *dksA* (transcriptional regulator of rRNA transcription), *ygfA* (5-formyl-tetrahydrofolate cyclo-ligase), and *yigB* (flavin mononucleotide [FMN] phosphatase). Most of these are global regulators and related to stress responses (47). The deletion of any gene identified by the above screenings
did not eliminate persisters. In addition, the functions of these gene products differ greatly. These observations indicate that persisters may form through multiple mechanisms.

The redundancy of persister formation mechanisms is further supported by the transcriptomes of persisters. The transcriptome of persisters isolated by ampicillin lysis showed that many genes are upregulated, including the SOS stress response genes *recA*, *sulA*, *uvrBA*, and *umuDC*; the phage-shock genes *pspABCDE*; heat and cold shock genes *cspH*, *htrA*, *ibpAB*, *htpX*, and *clpB*; TA modules *dinJ/yafQ*, *relBE*, and *mazEF* and some genes inhibiting macromolecular synthesis, such as *rmf* (56). Since the ampicillin lysis introduces lethal stress to the cell population, the influence of ampicillin may be included in the transcriptome of persisters. To avoid introducing lethal stresses, persisters were isolated using fluorescence-activated cell sorting (FACS) which will be described later in this chapter. Although the persister transcriptome acquired by FACS differs to some degree from that obtained by ampicillin lysis (107), both have upregulated genes relevant to phage shock, heat shock and TA modules.

TA modules and genes of stress responses appeared in both transcriptomes. In addition, Fluoroquinolone antibiotics (FQs) make double strand breaks (DSBs) in DNA, subsequently inducing the SOS response, which in turn induces several TA genes in *E. coli*: *symER*, *yafN/yafO*, and *tisAB/istR*. The focus on such observations and bacterial drug tolerance resulted in the first discovery of one mechanism of persister formation. FQs make DSBs in DNA, subsequently activates RecA. The activated RecA stimulates the autocleavage of LexA, the repressor of the SOS response. Consequently SOS genes are sequentially induced, mostly for DNA repair which contributes to the formation of persisters. When the amount of DSBs is high, the strong induction of SOS induces the TA module *tisAB/istR*. The toxin TisB is a pore-forming membrane peptide depleting the Proton Motive Force (PMF) and the ATP level, causing the
formation of the majority persisters in response to a high dosage of FQs (31, 32). Whether classes of antibiotics other than quinolones induce persister formation has yet to be revealed.

**Stresses and persistence**

Figure 1.3 clearly illustrates that persisters form in a manner dependent on the growth stage. In the early exponential phase cells are in a state of steady growth where cells grow at a constant rate, without the change in cellular parameters, *e.g.*, cell size, cell density and intracellular macromolecular composition, and culture parameters, *e.g.*, the composition and pH of the medium. The steady-state growth of *E. coli* stops at around $OD_{600} 0.3$ (around $5 \times 10^7$ cells/ml) in Luria-Bertani broth (LBB) (105), or even earlier (3). After that the average cell mass starts to decrease rapidly in the range of $OD_{600} 0.3 \sim 1.0$, which corresponds to the range when persister level increases sharply (55). This indicates that persister formation happens during non-optimal growth. Noteworthy is the fact that TA modules can be activated by stresses from non-optimal growth (24, 25), and ppGpp, the alarmone for stringent stress response upon amino acid starvation, was reported to be involved in persister formation (61). The persister transcriptomes consistently showed that genes governing stress responses are upregulated. All of the observations mentioned above strongly indicate that spontaneously formed persisters are the products of stresses.

The production of reactive oxygen species (ROS) is inevitable during aerobic metabolism. Oxidizing agents are commonly used for sterilization in households, public facilities, and hospitals. In the host environment, when macrophages encounter bacteria, they generate superoxide ($O_2^-$), nitric oxide (NO), and many other reactive species (87). Furthermore, it has been reported that bactericidal antibiotics generate ROS, which contribute to the killing by the antibiotics (60). ROS damage biological macromolecules such as lipids, proteins, and nucleic
acids. Bacteria respond to oxidative stress by inducing a global response that eliminates ROS, repairs oxidative damage, and bypasses damaged functions (15, 116). Heat shock occurs to bacteria frequently in the natural environment. For instance, in summer under strong sunlight the soil surface can reach temperature above 50°C. In the host environment, local temperatures can rise much above 37°C during inflammation or when the host gets a fever. Heat shock proteins are well conserved in different organisms, suggesting that heat shock is a universal stress. Specific to persisters, the heat shock genes *clpB*, *dnaK* and *dnaJ* were upregulated in two transcriptomes of persisters (56, 107). Also, the heat shock deletion mutants *dnaK* and *dnaJ* conferred lower persistence than the wild type (47). Therefore, in this work we aim to investigate the possible links between oxidative stress response / heat shock response and bacterial drug tolerance.

**Native Persister Isolation**

So far we have found one mechanism of persister formation which occurs in response to FQ treatment. However, some persisters form prior to the addition of antibiotics. In this thesis, they are called native persisters. It is very important to isolate native persisters so as to acquire their transcriptome, proteome, and metabolome for the mechanisms of persister formation. In most bacterial populations persisters account for $10^{-6}$ to $10^{-4}$ of a growing population and around $10^{-2}$ of a stationary population (55). In addition, persisters are easily resuscitated by the removal of antibiotics. These characteristics of persisters make their isolation very challenging. As mentioned before, two isolation methods have been developed. One isolates persisters by ampicillin lysis: the antibiotic lyses actively growing cells and leaves inactive persisters for analysis (56). The other one is through FACS: an unstable GFP was put under a growth-rate dependent promoter such that fast growing cells are bright and persisters are dim (107). The cell lysis method introduced lethal stress to the population before obtaining persisters. It is
possible that the introduced lethal stress will influence the persister transcriptome. Comparison of the transcriptome obtained by ampicillin lysis with that obtained by cell sorting reveals that genes of some stress responses, such as the SOS response, are upregulated in the ampicillin lysis transcriptome but not in the FACS transcriptome, and vice versa, metabolism genes are missing from the list of the ampicillin lysis transcriptome. These differences may result from the difference in isolation method. The cell sorting method avoided the lethal stress on the whole population but was limited by efficiency (106). Furthermore, this method requires suspending cells in PBS buffer and the suspension sits for hours during the sorting, which may also affect the persister transcriptome. In this work, some effort was made to develop an efficient method to physically isolate native persisters.
CHAPTER 2 Role of Oxidative Stress in *Escherichia coli*

**Tolerance to Fluoroquinolone Antibiotics**

**Introduction**

Persisters are antibiotic tolerant cells that have been found in all bacteria tested and in the eukaryote *Candida albicans* (63). They are phenotypic variants rather than genetic mutants. Upon the drug removal they reform a population that is as susceptible as the parental one, presenting a challenge for treating chronic infections. Persisters have been implicated in failure to eradicate biofilms by various antimicrobials (17, 54-56, 63, 69, 70, 112). Biofilms have been associated with many chronic infections such as dental disease, endocarditis, urinary tract infections, otitis media (86), cystic fibrosis (68) as well as with indwelling devices. High persister (*hip*) mutants of different pathogens with unaffected minimum inhibitory concentrations (MICs) have been isolated from patients who have been periodically exposed to high doses of antibiotics, *e.g.* *hip* mutants of *Pseudomonas aeruginosa* from cystic fibrosis patients (82) and those of *C. albicans* from cancer patients (64). These findings suggest that persister cells are clinically relevant, and antimicrobial therapy may select for *hip* strains *in vivo*.

Persistor formation depends on the growth stage, as shown by the accumulation of persisters during growth of *Escherichia coli*, *P. aeruginosa* and *Staphylococcus aureus* (55). Persisters do not form during early exponential phase. The number of persisters starts to increase at the early-mid exponential phase. A shoot up in the persister level continues and reaches a plateau upon entry into stationary phase. In the early exponential phase cells are in a state of steady-state growth where cells grow at a constant rate, without the change in cellular (cell size, cell density and intracellular macromolecular composition) and culture parameters (composition and pH of the medium). The steady-state growth of *E. coli* stops at around OD$_{600}$ 0.3 (around 5x10$^7$ cells mL$^{-1}$).
cells/ml) in Luria-Bertani broth (LBB) (105), or even earlier (3). After that the average cell mass starts to decrease rapidly in the $OD_{600}$ range of 0.3 ~1.0, which corresponds to the range when persister level increases sharply (55). This indicates that persister formation happens during non-optimal growth.

The transcriptome of persisters isolated by ampicillin lysis showed that many upregulated genes are those involved in different stresses such as cold shock, heat shock, phage shock, SOS response and toxin/antitoxin (TA) modules (55, 107). The SOS induction and a TA module, $tisAB/istR$, were shown to be required for the formation of the majority of persisters to fluoroquinolone antibiotics (FQs) during a treatment with a high concentration of the antibiotic (31, 32). FQs block the ligase activity of DNA gyrase or topoisomerase IV, resulting in double strand breaks (DSBs) in DNA. During the subsequent events, RecA is activated and promotes the autocleavage of LexA, the repressor of the SOS response, activating a number of genes of DNA repair and TA modules including $tisAB/istR$. TisB is a pore-forming membrane peptide which collapses the Proton Motive Force (PMF), leading to persister formation.

Bacteria frequently encounter oxidative damages in the environment. The production of reactive oxygen species (ROS) is inevitable during the aerobic metabolism. Oxidizing agents are commonly used for sterilization in households, public facilities and hospitals. In the host environment, when macrophages encounter bacteria, they generate superoxide ($O_2^-$), nitric oxide (NO) and many other reactive species (87). Furthermore, it has been reported that bactericidal antibiotics generate ROS, which contribute to the killing of bacterial cells (60). ROS damage biological macromolecules such as lipids, proteins and nucleic acids. Bacteria respond to oxidative stress by inducing a global response which eliminates ROS, repairs oxidative damage and bypasses damaged functions (15, 116). We hypothesize that the response to oxidative stress influences the formation of persisters.
The sensor-regulator systems in *E. coli* that respond to oxidative stress are the OxyR and SoxRS systems, which regulate the responses to hydrogen peroxide and superoxide, respectively (92, 132). Compounds that produce intracellular superoxide include viologens such as paraquat (PQ), and quinones such as naphthoquinone menadione (NM) and plumbagin (PB), a natural antimicrobial and a derivative of naphthoquinone (NQ). These compounds transfer electrons from NADPH or NADH to O$_2$ to generate O$_2^-$, hence they are commonly referred to as redox-cycling agents. PQ was used in this study due to its high solubility and stability in water.

The transcriptional and metabolic cascade induced by PQ is shown in Figure 2.1. The _soxRS_ regulon is induced through a two-stage process. The repressor SoxR contains a [2Fe-2S] cluster, which is inactive in the reduced form. When the [2Fe-2S] cluster is oxidized, it induces _soxS_ expression and SoxS in turn activates the transcription of over 100 genes in the regulon by binding to the _sox-box_ in their promoters (15, 30, 41). Recent studies demonstrated that SoxR is activated directly by PQ rather than superoxide (45) which had long been accepted as the signal for SoxR. SoxR can also be activated when its [2Fe-2S] cluster is nitrosylated by nitric oxide (NO) (29). Superoxide is degraded to H$_2$O$_2$ by superoxide dismutases SodA (Mn), SodB (Fe) and SodC (Cu, Zn). H$_2$O$_2$ is further degraded to water and oxygen by enzymes KatG and AhpCF, or it can be converted to hydroxyl radical through Fenton reaction (FR) (60, 123).

We found that PQ increases the level of tolerance to fluoroquinolones (FQs) but not to β-lactams and aminoglycosides. This increase in tolerance is mediated primarily by the induction of the major multidrug-efflux pump AcrAB-TolC. The effect of superoxide also contributes to the increased tolerance to a lesser extent. This finding demonstrates the synergy between drug resistance and drug tolerance.
**Material and methods**

**Strains and growth conditions.** The genotypes of all strains used in this work are listed in Table 2.1. The medium used for culture was Luria-Bertani Broth (10 g Bacto-tryptone, 5 g yeast extract and 10 g NaCl/liter), buffered by 0.1 M HEPES/KOH pH 7.2, and supplemented with appropriate antibiotic in strains carrying antibiotic markers. Under anaerobic condition, the medium was supplemented with 50 mM KNO₃. Cultures were inoculated in 3 ml buffered LB broth in 17- by 100-mm polypropylene tubes (Fisherbrand), and incubated for 16-20 hours at 37°C with shaking at 220 rpm.
**Strain Construction.** Wild type *E. coli* K-12 BW25113 is the parent strain for the KEIO *E.coli* single-gene knockout collection (8). Wild type *E.coli* K-12 MG1655 is the parent for the *E. coli* promoter collection, a library of transcriptional fusions of GFP to each of about 2,000 different promoters on a low- copy-number plasmid (128). To construct mutant Δ*sodAB*, the kanamycin resistant cassette from KEIO strain Δ*sodA::kan* was first cured according to protocol described by Datsenko *et al.*(27), and deletion sodB was introduced by P1 transduction from the corresponding KEIO strain. Δ*sodAB::kan* accumulates suppressors under aerobic conditions, therefore, the selection of Δ*sodAB::kan* transductants was carried out anaerobically (anaerobic chamber, Coy Laboratory Products, INC, USA) as well as transduction of Δ*acrB::cam* into the resulting strain. Mutant Δ*acrB::cam* was made by knocking out *acrB* from MG1655 by method described by Datsenko *et al.* (27). The SOS mutant lexA3(Ind') was constructed by first transducing Δ*malF::kan* allele from the strain from KEIO library and then transducing lexA3 allele from MV2057 into the resulting strain and selecting for UV-sensitive transductants on minimal medium (MOPS) plates supplemented with 0.2% maltose as the sole carbon source.

**Measurements of Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentration (MBC).** The MIC was measured using 96-well plates (COSTAR®, Corning Inc., USA). All wells in a row of the 96-well plate were filled with 50 µl of the medium except the first one which was filled with 100 µl of medium, serving as a negative control. 50 µl medium with the antibiotics at the concentration over 20-fold of the expected MIC (5) were added to the second well and the serial 2-fold dilutions were made from the second to the 11th well. The last well contained no antibiotics, serving as the positive control. The overnight culture of the strain to be tested was diluted at 1:10 into 3-ml fresh medium in a 17- by 100-mm polypropylene tube, which was incubated at 37°C with shaking for 1 hour. The cultures then were diluted 1:2000 to the fresh medium. 50 µl of the cultures were added to wells 2 to 12 resulting in 10^5 CFU/ml. The 96-
well plate was then capped and incubated at 37°C with shaking for 16-20 hours before it was read by the microtiter plate reader (Synergy Mx Monochromator-based Multi-Mode microplate reader, BioTeK). The lowest concentration at which no growth was observed was taken as MIC for the tested antibiotics for each strain. Each measurement was performed in four replicates. From the “MIC well” and three neighboring wells on each side, 10 µl samples were taken for plating to measure the surviving fraction. The antibiotic concentration that caused 1000-fold killing was taken as MBC. For the measurement under anaerobic condition, all liquid media and the LB agar plates were put into the anaerobic chamber at least one day before use.

**Drug tolerance assays.** The overnight cultures of the strains were diluted 1:100 into buffered LB Broth, supplemented with appropriate antibiotics, and incubated at 37°C for 1 hour (OD$_{600nm}$ was ~ 0.1). The culture was divided into aliquots of 5 ml per polypropylene tube (17 mm x100 mm). The tubes were further grouped for control and PQ (paraquat dichloride, Sigma) or sodium salicylate (Sigma) treatment, respectively. PQ or salicylate was added to the culture for 30 min (OD$_{600nm}$ = 0.2 ~ 0.3) prior to the addition of ofloxacin, ampicillin, kanamycin, or tobramycin (all from Sigma) for further 24 hours. The concentration of antibiotics was 10x MIC, unless indicated otherwise. Each treatment was done in triplicate. At designated time points, 1 ml samples were taken out and washed, and resuspended in 1ml 1% NaCl solution. The suspension was then serially diluted and plated on LB agar plates supplemented with 20 mM MgSO$_4$ and 2 mg/ml sodium pyruvate, an antioxidant improving the plating efficiency (16). In some experiments the entire 1 ml sample was washed and plated to increase the limit of detection.

$\Delta$sod$AB$ and $\Delta$sod$AB\Delta$acr$B$ easily accumulate suppressors under aerobic condition, therefore those strains as well as wild type control were grown overnight in the anaerobic chamber in the medium supplemented with nitrate. The anaerobic overnight cultures were then diluted 1:100
into buffered, aerobic LB medium pre-warmed at 37°C. The culture was then incubated aerobically at 37°C, with shaking for two hours before challenge with antibiotics for 24 hours.

**The accumulation of persisters during growth under anaerobic condition.** BW25113 was cultured overnight in an anaerobic chamber at 37°C in LB buffered with HEPES/KOH pH 7.0, with or without 50 mM KNO$_3$. The culture was then diluted 1:100 into the same medium in three 50 ml Falcon tubes and incubated on a bidirectional rotator (4630Q, thermo scientific). At every time point, 2 ml samples were taken, 10 µl from each sample was serially diluted plated for colony-forming units (CFU) counts. The rest of the sample was split into two aliquots, which were treated with 100 µg/ml ampicillin or 1 µg/ml ofloxacin for 3 hours, before plating for CFU counts. The plates were supplemented with pyruvate and KNO$_3$.

**Measurement of promoter activity.** The strains were grown at 37°C with shaking at 220 rpm to $OD_{600nm} \approx 0.2$, the cultures were then transferred into the wells of a black opaque 96-well plate (Costar), 200 µl per well. The wells were divided into six groups; control, control with antibiotics, PQ, PQ with antibiotics, salicylate and salicylate with antibiotics. 0.8 mM PQ, or 2.5 mM sodium salicylate were added into the wells for half an hour before the addition of 1 µg/ml ofloxacin or 50 µg/ml ampicillin. Immediately after the addition of antibiotics, the plate was put into the microtiter plate reader and incubated at 37°C. The intensity of fluorescence was measured at 485, 585 nm every 20 mins for 3 hours.

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Results

PQ pretreatment increases the persister level to fluoroquinolones, but not to β-lactams or aminoglycosides. Paraquat, an artificial herbicide, is highly toxic to many organisms. The MIC of PQ for *E. coli* BW25113 was 1.2 mM. To examine the influence of PQ on bacterial tolerance to antibiotic treatments, we exposed the population of *E. coli* to 0.8 mM or 2.4 mM of PQ, respectively, then treated the population with ofloxacin, ampicillin or kanamycin, for 24 hours. Both PQ concentrations had similar effect on the tolerance (data not shown), so 0.8 mM of PQ was used in all further experiments. As shown in Figure 2.2, PQ at this dosage does not affect the growth, but it significantly increases the persister level to ofloxacin (Figure 2.2 A), not to ampicillin (Figure 2.2 A) nor to kanamycin (Figure 2.2 B). Similar PQ protection against ofloxacin was also observed in MG1655, another wild type *E. coli* strain (data not shown). Ofloxacin is a FQ antibiotic that inhibits the bacterial gyrase and the topoisomerase IV, converting them into endonucleases. Ampicillin belongs to β-lactam antibiotic class which inhibits cell-wall synthesis. Kanamycin is an aminoglycoside antibiotic that interacts with the 30S ribosomal subunit inhibiting protein synthesis. To examine if similar PQ protection occurs to other FQs, we challenged BW25113 with ofloxacin, norfloxacin and ciprofloxacin (10x MIC each) for 24 hours after 30-min PQ pretreatment. As shown in Figure 2.3, PQ increased the tolerance by 100-, 1000- and 100,000-fold to norfloxacin, ofloxacin and ciprofloxacin, respectively. In all the killing curves shown in this work, the time “0” represents the time at which antibiotic was added.
Figure 2.2. The influence of 0.8 mM PQ on the tolerance of wild type *E. coli*, BW25113, to different antibiotics. (A) Ampicillin 50 µg/ml (8x MIC) and ofloxacin 1µg/ml (10x MIC); (B) kanamycin 50 µg/ml (4x MIC) and 100 µg/ml (8x MIC)

Figure 2.3. The influence of 0.8 mM PQ on the tolerance of wild type *E. coli*, BW25113, to different FQs (10x MIC): ofloxacin 1 µg/ml, norfloxacin 1 µg/ml and ciprofloxacin 0.15 µg/ml.
**PQ-induced persisters to FQs are multidrug tolerant.** To test whether PQ-induced persisters to FQ are tolerant to other classes of antibiotics, we first challenged the cells with or without PQ pretreatment with 1 µg/ml ofloxacin for 6 hours. At 6-hour time point, the cultures pretreated with PQ were divided into four groups: group 1 was a control with no extra addition of any antibiotic, group 2 was treated with additional 4 µg/ml ofloxacin (so the concentration of ofloxacin in medium was 5 µg/ml), group 3 was treated with 100 µg/ml ampicillin, and group 4 with 30 µg/ml tobramycin, for 18 hours. Further antibiotic challenge did not significantly reduce survival, as shown in Figure 2.4, indicating that PQ induced persisters are multidrug tolerant.

![Figure 2.4](image)

**Figure 2.4.** Multidrug tolerance of the persisters induced by PQ treatment. The growing population of BW25113 was treated by 0.8 mM PQ for 30 min before 1 µg/ml ofloxacin was introduced (the initial time point in the figure). Further antibiotic challenge was introduced to PQ-treated samples at 6-hour ofloxacin treatment.
**Efflux pump AcrAB-TolC plays the major role in the formation of PQ-induced persisters.**

As shown in Figure 2.1, PQ treatment induces soxRS regulon and rapidly produces superoxide, which has been reported to protect bacterial cells against FQs (20, 123). There are over 100-genes under the control of the two-component transcriptional regulators SoxRS, including efflux pump complex AcrAB-TolC (15, 91). The Resistance-Nodulation-Division (RND) family efflux pump AcrB has a very large periplasmic domain, and form tripartite complex with the outer membrane channel TolC, and the periplasmic adaptor protein AcrA (84), as shown in Figure 2.14. AcrA, AcrB and TolC are indispensible parts of the pump. Individual deletions render the pump inactive (84). The AcrAB-TolC pump is the major contributor to the intrinsic resistance of *E. coli* to solvents, dyes, detergents, and lipophilic antibiotics including FQs and β-lactams (84, 129). Compared to AcrAB-TolC, other drug exporters contribute negligibly to drug resistance (114). It has been found previously that FQs-induced persisters form in a manner dependent on the concentration of FQs (31), therefore it is reasonable to speculate that the AcrAB-TolC pump may play a role in the observed PQ protection by reducing the intracellular antibiotic concentration. To distinguish the possible contribution of the efflux pump from that of superoxide, we pretreated the growing culture of BW25113 and mutants ΔacrA and ΔacrB with sodium salicylate and PQ, respectively. Sodium salicylate induces the pump, but does not generate superoxide (91). 5 mM sodium salicylate was tested for its effect on the growth of BW25113 and mutants ΔacrA and ΔacrB. As shown in Figure 2.5 A, salicylate has slightly greater inhibiting effect on BW25113 than on the pump mutants. Overall, the inhibiting effect was negligible. 2.5 mM and 5 mM sodium salicylate were tested for protection against ofloxacin. Treatment with both concentrations resulted in similar persister level to FQs (Figure 2.5 B). No significant inhibiting effect of 0.8 mM PQ was observed on the growth of ΔacrA and ΔacrB, neither. Therefore, 2.5 mM sodium salicylate and 0.8 mM PQ were used to pretreat growing cultures of BW25113, ΔacrA and ΔacrB as well before the challenge with 1 μg/ml ofloxacin. Sodium salicylate treatment resulted in 10-fold more persisters compared to the untreated control.
(Figure 2.6 A). The protection was completely abolished in acrAB-tolC mutant (Figure 2.6 B-C), indicating that the AcrAB-TolC pump is fully responsible for the salicylate-induced increase in tolerance. PQ dramatically increased the persister level 10,000-fold to the wild type (Figure 2.6 A), but only 10-fold to the pump mutants (Figure 2.6 B-C), suggesting that the AcrAB-TolC pump plays a major role, rather than the only role, in the PQ protection. The PQ protection to pump mutants may come from superoxide. Colonies recovered after 24-hour killing from all cultures shown in Figure 2.6 were checked for their ofloxacin MIC and no change was observed, ruling out the selection of resistant mutants.
**Figure 2.5.** The effect of salicylate on the growth or protection of BW25113 and that of PQ on BW25113. (A) The effect of 5 mM salicylate on growth of BW25113 and the deletion mutants $\Delta$acrA and $\Delta$acrB. (B) The protection by 2.5 mM and 5 mM salicylate to BW25113 against the challenge with 1 $\mu$g/ml ofloxacin. (C) The effect of 0.8 mM PQ on the growth of BW25113 and the deletion mutants $\Delta$acrA and $\Delta$acrB.
**Figure 2.6.** The influence of 0.8 mM PQ of and 2.5 mM sodium salicylate on the tolerance of *E. coli* to ofloxacin (10x MIC) and its dependence on the MDR efflux pump AcrAB-TolC. (A) wild type *E. coli* BW25113; (B) and (C) the pump mutants ΔacrA and ΔacrB, respectively.

**PQ protects *E. coli* cells against FQs under anaerobic condition.** As mentioned above, PQ increases the persister level of AcrAB-TolC pump mutants, probably due to superoxide. The presence of oxygen is required to generate superoxide. If superoxide protects against FQs, then PQ should not protect pump mutants *acrA* and *acrB* under anaerobic conditions. Under aerobic...
conditions, *E. coli* obtains energy mainly through oxidative respiration with oxygen as the terminal electron acceptor in the electron transport chain. Under anaerobic conditions, exogenous electron acceptors are required in the medium for oxidative respiration. Otherwise, cells can only obtain energy through fermentation. Therefore, for antibiotic challenge under anaerobic conditions 50 mM KNO$_3$ was included in the medium as an electron acceptor. Because it has been reported that bactericidal antibiotics generate superoxide and hydroxyl radical which are the causes of cell death (60), we wondered if the bactericidal antibiotics kill cells with equivalent efficiency under anaerobic conditions. We measured the MIC and MBC of different classes of antibiotics under aerobic and anaerobic conditions with or without nitrate supplementation. MIC of ampicillin and ofloxacin do not change in the absence of oxygen (Figure 2.7). Aminoglycosides kanamycin and tobramycin have significant variation in the MIC and MBC between replicates. However, overall, they tend to have slightly lower inhibitory or killing efficiency under anaerobic conditions. This result indicates that the contribution of ROS to the killing effect of bactericidal antibiotics may not be significant.
Figure 2.7. MIC (A) and MBC (B) of ampicillin, ofloxacin, kanamycin and tobramycin to wild type E. coli BW25113 under aerobic condition and anaerobic condition with/without 50 mM nitrate supplementation in the medium. The secondary y-axis represents ofloxacin concentration.

Beside the killing efficiency of antibiotics, cell metabolism can also affect drug tolerance under aerobic and anaerobic conditions. Therefore, we measured the persister accumulation under anaerobic condition with and without the nitrate supplementation. As shown in Figure 2.8, the persister level is low during lag and early exponential phase, and starts to increase from the mid-exponential phase, then reaches plateau in stationary phase. The similar antibiotic killing efficiency and the similar pattern of persister accumulation under aerobic and anaerobic conditions suggest that it is plausible to compare the anaerobic killing curve results with the aerobic ones. We challenged BW25113 and pump mutants ∆acrA and ∆acrB with ofloxacin (10x MIC) anaerobically in the medium supplemented with 50 mM KNO₃. As shown in Figure 2.9, PQ still confers higher tolerance to the wild type strain, but not to the AcrAB-TolC pump mutants. This demonstrates that under the anaerobic condition with nitrate supplementation, PQ-induced tolerance is entirely dependent on the AcrAB-TolC pump. SoxR is directly activated
by the oxidized form of PQ, rather than superoxide (45). Under anaerobic condition, the expression of SoxS is upregulated by PQ if a terminal electron acceptor such as nitrate is present in the medium. The level of SoxS expression increases with the concentration of PQ (45). When nitrate is present, nitric oxide, which forms as a by-product of nitrate reduction, also activates SoxR (110, 111). Therefore, the PQ protection shown in Figure 2.9 is conferred by the AcrAB-TolC pump through the SoxRS response. Comparison between Figure 2.9 and Figure 2.6 shows that superoxide contributes to the PQ-induced tolerance under aerobic condition.

\[ \text{Figure 2.8. The accumulation of persister during population growth under anaerobic condition without (A) and with (B) nitrate supplementation. Antibiotics used were ampicillin of 100 } \mu\text{g/ml and ofloxacin 1 } \mu\text{g/ml.} \]
Figure 2.9. The influence of 0.8 mM PQ on the tolerance of BW25113 to 1 µg/ml ofloxacin under anaerobic condition with 50 mM KNO₃ supplementation.

Superoxide increases tolerance of *E. coli* to FQs independently of the AcrAB-TolC efflux pump. The results presented so far suggest that superoxide partially contributes to the increased tolerance by PQ. Superoxide has been reported to be protective against a 90-minute challenge with norfloxacin, ampicillin and kanamycin (123) and against bleomycin lethality in a 12-min challenge (20). Similar to ofloxacin, norfloxacin and bleomycin kill cells by making double strand breaks in DNA. However, the challenge durations in these studies (20, 123) were not sufficient to establish the level of tolerance. Superoxide is generated naturally during aerobic growth. Its concentration is maintained at a very low level by superoxide dismutases SodA, SodB and SodC (Figure 2.1). In the presence of oxygen, superoxide accumulates in mutant ΔsodAB, affecting the growth and resulting in smaller colonies than the wild type. As shown in Figure 2.10 A, the built-up of superoxide increased the tolerance to ofloxacin independently of AcrB. This result is consistent with the previous report on the protective effect of superoxide (20, 123). In our study, superoxide did not increase tolerance to ampicillin or tobramycin, as in Figure 2.10 B. At 1-hour time point, ampicillin killed more ΔsodAB cells than the wild type cells,
contrary to the results of Wang and Zhao (123). However, the level of tolerance cannot be predicted from the rate of killing to the bulk, especially during an incubation of less than two hours. The result shown in Figure 2.10 is consistent with the PQ protection observed in this study: no effect on tolerance to β-lactam and aminoglycoside antibiotics, increased tolerance to FQs, and the protection to the pump mutants under aerobic conditions.

**Figure 2.10.** (A) The 24-hour killing of ofloxacin of 10x MIC to BW25113 (1 µg/ml) and double deletion mutant ΔsodAB (1 µg/ml) as well as triple deletion ΔsodAB ΔacrB (0.2 µg/ml). (B) The 24-hour killing with 50 µg/ml ampicillin (4x MIC) and 30 µg/ml tobramycin (3x MIC) of BW25113 and the mutant ΔsodAB.
The efflux pump AcrAB-TolC increases the level of tolerance to FQs by reducing the damage to DNA. Results in Figure 2.6 and Figure 2.9 demonstrate that the major contributor to PQ protection against FQs is the AcrAB-TolC pump. By the mechanism of FQ-induced persister formation (31): (1) FQs cause DSBs, leading to the induction of the SOS response which is indispensible for the formation of the majority of FQ-induced persisters. The inability to induce SOS greatly reduces the level of tolerance to FQs. (2) Genes in the SOS response are induced sequentially. The SOS genes induced early are mostly for DNA repair. \textit{tisB} in TA module \textit{tisAB/istR} is induced at later stage of SOS. (3) Increase in DNA damage results in stronger SOS induction. Lower concentration of FQ causes lower level of DNA damage and induces moderate level of the SOS response. DNA repair plays a major role in the persister formation. With a higher FQ concentration, the SOS induction is stronger. TisB then plays a major role in persister formation by forming a pore in membrane which depletes PMF. However, if the cellular damage is beyond a critical level, even strong induction of SOS would not rescue the cells. Indeed, around 90% of the persisters after 3-hour ciprofloxacin treatment did not induce SOS strongly (31, 32).

The treatment with PQ or salicylate induces the efflux pump AcrAB-TolC. The pump reduces the intracellular concentration of antibiotics which could underlie the increase in tolerance. To test this, we challenged different SOS and DNA repair mutants with ofloxacin following PQ treatment. If the concentration of ofloxacin is reduced in the cells, more cells would survive by repairing the damage and forming persisters through the SOS response. Since persisters bear relatively mild DNA damage and undergo relatively mild SOS induction, the absence of SOS should have more significant impact on the tolerance of PQ-treated cultures. As shown in Figure 2.11, PQ treatment increased the survival in all strains. The inability to induce SOS (\textit{lexA3}) results in >10-fold decrease in tolerance compared to control samples, and 1000-fold decrease in the PQ-treated samples. This difference is even more significant in \textit{ΔrecA} mutant, deficient in both the
SOS induction and DNA repair. SOS inducible helicase UvrD is involved in DNA repair, and the deletion of *uvrD* resulted in reduced tolerance, but the decrease was less pronounced than in the case of *lexA3* and *recA*. However, the reduction was more significant for in PQ-treated samples of all mutants except *tisB*. TisB promotes tolerance during exposure to high FQ doses and does not contribute significantly to tolerance upon challenge with lower concentrations of FQ (Figure 2.12). Compared to the wild type cells in control samples (no PQ pretreatment) the tolerance level of *tisB* mutant was ≥20-fold lower above 10-fold MIC and barely affected below that concentration of ofloxacin. In PQ-treated cultures the tolerance was dependent on TisB (≥20-fold reduction) above 20-fold MIC, consistent with PQ treatment lowering the intracellular concentration of ofloxacin.

**Figure 2.11.** The ofloxacin challenge (10x MIC) following 0.8 mM PQ treatment of DNA repair and SOS mutants. The ofloxacin concentrations used to strains are: BW25113, mutants *lexA3* and *tisB*, 1 µg/ml; *uvrD* 0.5 µg/ml; *recA* 0.3 µg/ml.
Figure 2.12. The 24-hour ofloxacin challenge following 0.8 mM PQ treatment of BW25113 and mutant tisB.

The level of protection conferred by PQ or salicylate treatment is consistent with the upregulation of the efflux pump and the production of superoxide. To further explore the correlation between the pump induction, superoxide generation, the SOS induction and the tolerance to FQs, we measured the promoter activity of several genes during treatment with PQ, salicylate, and different antibiotics (Figure 2.13). The reporter strains are from E. coli promoter collection which carry gene encoding GFP under control of different promoters (128). Genes of the efflux components acrA, acrB and tolC are under the regulation of soxRS and marRAB regulons (Figure 2.15). Redox-cycling agents such as PQ are traditionally used to induce soxRS. Salicylate binds to MarR to de-repress MarA. Gene sulA is upregulated during SOS induction. The reporter strains of acrA, acrB, and marA are not included in the collection. Therefore, the promoter activity of the genes soxS, tolC, sulA, and sodA, which is an indicator of superoxide production, was tested (Figure 2.13). The promoter activity during the treatment with aminoglycosides could not be measured in this way since aminoglycosides inhibit the synthesis of GFP.
The results in Figure 2.13 show that the efflux pump is significantly induced by PQ and to the lesser extent by salicylate. PQ upregulated \textit{soxS} dramatically so after 130-min PQ treatment, the GFP signal was above upper detection limit of the plate reader. \textit{tolC} was significantly induced which is expected since it has a \textit{sox} box in the promoter region (Figure 2.15). Ofloxacin alone did not affect the expression of \textit{tolC}. However, when it was added following PQ treatment, \textit{tolC} was upregulated 4-fold. Salicylate had no effect on \textit{soxS}. Surprisingly, it failed to induce \textit{tolC} since it induces MarA, a regulator of \textit{tolC} (130). However, together with ofloxacin, salicylate upregulated \textit{tolC} by 1.5-fold after 3-hour ofloxacin treatment, showing the synergy between salicylate and ofloxacin.

SOS was strongly induced by ofloxacin treatment alone. Pretreatment with salicylate slightly reduced the induction by ofloxacin whereas PQ pretreatment reduced it significantly (Figure 2.13 E). The decrease in SOS induction and increase in tolerance are proportional to the induction of \textit{tolC} by salicylate and PQ. This observation indicates that the induced pump AcrAB-ToIC effluxes ofloxacin and thereby reduces DNA damage, increasing the formation of persisters through the SOS response.

Ofloxacin alone upregulated \textit{sodA} 2-fold, indicating the formation of superoxide. PQ upregulated \textit{sodA} 3-fold, with further increase upon the addition of ofloxacin, indicating an even higher level of superoxide. The accumulation of superoxide in the cells promoted the formation of persisters. Salicylate had no effect on \textit{sodA}.

Compared to ofloxacin, ampicillin alone had no effect on the induction of the genes tested. Reduced signal from PQ or salicylate treatment in \textit{soxS}, \textit{tolC} and \textit{sulA} was due to the cell lysis by ampicillin. Ofloxacin treatment induces SOS, and PQ or salicylate can enhance persister formation by mediating the expression of efflux pumps. Ampicillin does not induce SOS, which
explains why the pump has no effect on the tolerance to β-lactams. High concentration of superoxide also enhances persister formation. Superoxide is formed during ofloxacin, but not during ampicillin treatment (Figure 2.13 G and H), which further explains why PQ influences the tolerance to ofloxacin only.
Figure 2.13. The promoter activity during 3-hour treatment with ofloxacin or ampicillin following the pretreatment with PQ or salicylate. (A) soxS, ofloxacin; (B) soxS, ampicillin; (C) tolC, ofloxacin; (D) tolC, ampicillin; (E) sodA, ofloxacin; (F) sodA, ampicillin; (G) sulA, ofloxacin; (H) sulA, ampicillin. Time in minutes after the addition of antibiotics is indicated on x-axis. PQ or salicylate was added 30 min before the antibiotics.
Discussion

One of the main lines of defense against pathogens is the production of reactive oxidizing species by immune system. As a part of the cytotoxic response activated phagocytes produce both ROS and RNS (60) which damage almost every part of the target bacterial cell. This is an efficient way of killing pathogens because it is not possible to escape this part of the immune response by mutation of a single molecular target.

The action of bactericidal antibiotics has been reported to generate ROS as well. Therefore, during infection and antibiotic treatment, bacteria are likely to be exposed to ROS in host environment and undergo oxidative damage (60). Damage caused by ROS elicits oxidative stress responses which promote survival through production of proteins able to inactivate ROS and repair the damage.

Bacteria are able to survive antibiotic treatment by acquiring a mutation(s) conferring the resistance or by entering a persistent state. Resistant cells keep growing in the presence of the antibiotic whereas persister cells do not grow but do not die, neither. Persistent state can be induced by certain types of damage and can be influenced by bacterial stress responses. Tolerance to FQ antibiotics which cause extensive fragmentation of DNA is dependent on the SOS gene network inducible by the DNA damage and aiming at DNA repair.

We hypothesized that the oxidative stress might also influence tolerance to antibiotics. Using PQ as a superoxide generating agent, we found that PQ treatment significantly increases the persister level to FQs, but not to β-lactams and aminoglycosides. This protection is a result of the superoxide formation and of the induction of AcrAB-TolC, the major efflux pump in gram negative bacteria (Figure 2.14).
The redox-cycling agent PQ shuttles electrons from NADPH to oxygen, generating superoxide. Its cytotoxicity has been ascribed to the hydroxyl radical generation through the Fenton reaction (Figure 2.1). PQ has been reported to be protective against norfloxacin (123) and bleomycin (20), both of which are DNA damaging agents. We found that superoxide increases bacterial tolerance to FQs but not to β-lactams and aminoglycosides (Figure 2.10). FQs kill bacteria by introducing DSBs to DNA by corrupting gyrase and topoisomerase function. Superoxide may elicit certain cellular responses to protect DNA. However, this protection is not expected to be concentration-dependent because it is not affected by the major efflux pump AcrAB (Figure 2.6 and 2.10 A). Similarly, superoxide protection against bleomycin, a DNA cleaving agent, was shown to be independent of SoxS and RecA (20). The mechanism of superoxide protection is not known.

![Diagram of the model of the influence of PQ-induced oxidative stress on persistence to FQs](image)

**Figure 2.14.** The model of the influence of PQ-induced oxidative stress on persistence to FQs.
Superoxide is generated by bactericidal antibiotics through oxidation of the respiratory electron transport chain driven by \( \text{O}_2 \) and rapid consumption of NADH. It causes cell death by two means. It inactivates various enzymes through destabilization of their iron-sulfur clusters. Its conversion to \( \text{H}_2\text{O}_2 \) by superoxide dismutases fuels the FR and generation of hydroxyl radicals which damage many cellular components (60). If the bactericidal effect is due to hydroxyl radical, a degradation product of superoxide, then it should be reduced in anaerobic condition where superoxide is not generated. However, MICs and MBCs for ofloxacin and ampicillin did not change under anaerobic condition (Figure 2.7). The killing efficiency of aminoglycosides is lower under anaerobiosis which is likely due to the reduced PMF required for the uptake of aminoglycosides (19, 115). Although the supplementation with KNO\(_3\) as an alternative terminal electron acceptor can sustain the oxidative respiration, it is not as efficient as oxygen because of its lower electronegativity. Consistent with this observations, bactericidal antibiotics are efficient within the anaerobic host environment. Therefore, how and how much ROS contribute to the antibiotic killing remains an open question.

PQ treatment generates superoxide and upregulates SodA (Figure 2.13). However, PQ at 20 mM does not kill the wild type \( \text{E. coli} \) cells in exponential phase (data not shown). 0.8 mM PQ used for pretreatment does not even inhibit the growth. It follows that ROS are not as lethal as previously thought or they are lethal to the cells, but PQ induces a stress response which scavenges ROS and repairs the damage (Figure 2.1). The latter scenario may explain the observed spike of hydroxyl radicals after exposure to bactericidal antibiotics (57, 60). Cells under normal physiological conditions have mechanisms to keep the hydroxyl radical at low level. Bactericidal antibiotics interfere with those mechanisms so the ROS level builds up. However, the contribution of hydroxyl radical spike to the cell death remains to be established.
The oxidized PQ directly oxidizes the [2Fe-2S] cluster of SoxR, de-repressing SoxS, which induces the Sox regulon by binding to the sox-box in the promoter region of the genes it controls, including the genes encoding the major efflux pump AcrAB-TolC (15). The inner membrane transporters remove the toxic compounds from the cytoplasm, and AcrB captures them near the membrane/periplasm interface and passes them onto TolC which pumps them out of the cell (114). TolC is the key component of several proton-driven multidrug efflux pumps in *E. coli* (28).

In this study, bacteria were exposed to FQs, β-lactams and aminoglycosides following the PQ pretreatment. The pump protects against FQs but not against β-lactams and aminoglycosides. Aminoglycosides are substrates for AcrD, an AcrB paralog with different substrate specificity (2, 35, 98). *acrD* is not upregulated in the *E. coli* treated by PQ (15). FQs and β-lactams both can be effluxed by AcrB efficiently (72, 126), but only tolerance to FQs was increased by PQ pretreatment. The difference (Figure 2.11-13) is due to the mechanism of action of FQs and the mechanism of tolerance to FQs. FQ treatment introduces DSBs to DNA (75). During processing of DSBs, RecA protein is activated by binding to ssDNA and promotes the autocleavage of SOS repressor LexA leading to the SOS induction, which is required for the formation of the majority of persisters to FQs (31). Persister level to FQs depends on the extent of the SOS induction which in turn is a function of the FQ concentration (31). At high FQ concentration tolerance is largely dependent on SOS-regulated toxin TisB (18, Figure 2.12). PQ treatment decreases the intracellular concentration of FQ through the induction of the AcrAB-TolC pump which leads to an increase in persister level. The PQ treatment does not change the genetic requirements for persister formation but it affects the persister level dependency on FQ concentration.

Persistence still depends on SOS induction and repair functions carried on by RecA, LexA and UvrD (Figure 2.11). However, the FQ concentration at which the majority of persisters form through the action of TisB is significantly higher following the PQ treatment (Figure 2.12) as compared to non-treated cultures, which is expected if the effective antibiotic concentration is lowered.
The AcrAB-ToIC pump plays a major role in the PQ protection against FQs. Increased persistence by sodium salicylate (Figure 2.6) confirms that the upregulation of the AcrAB-ToIC pump is crucial for the increased persistence. The expression of *acrAB-toIC* is controlled by multiple regulators (Figure 2.15). Both *acrAB* and *toIC* are positively regulated by MarA, Rob and SoxS transcriptional regulators (124). These three regulators differently affect particular promoters (76) and also regulate each other (15). In addition, SdiA, a protein regulating cell division dependent on quorum sensing, positively regulates AcrAB in *E. coli* (34, 93, 126). This complex regulation of AcrAB-ToIC allows its upregulation by a variety of compounds including sodium salicylate, tetracycline, chloramphenicol, organic solvents, an uncoupling agent cyanide m-chlorophenylhydrazone, the redox-cycling agents, the host produced antimicrobials such as

![Figure 2.15](image-url)
polymyxin B, LL-37, and metabolites such as bile salts, fatty acids and dipyridyl (4, 6, 76, 91, 124) as well as the low Mg\(^{2+}\) concentration in the phagosome (130). The link between the increased tolerance to FQs and the upregulation of efflux pumps is probably universal among bacteria with MDR efflux pumps.

In the host the survival of bacteria to FQ treatment could be affected by NO and O\(_2^-\) produced by the macrophage and the low Mg\(^{2+}\) concentration in the phagosome. NO is a powerful inducer of SoxRS regulon, therefore it strongly induces the efflux pump. The low Mg\(^{2+}\) concentration in the phagosome can further upregulate the pump. High concentration of superoxide itself will increase the tolerance to FQs. It has been found that after incubation with murine macrophages for 8-hours, soxS expression in the phagocytosed bacteria was induced up to 30-fold (88). Bacterial MDR efflux pumps are activated during the incubation with macrophages (1). Besides macrophages, oral and gastrointestinal microbiome generates nitric oxide (NO) from nitrate/nitrite (33, 110, 111), which can also lead to the protection to the killing by FQs.

The role of multidrug efflux pump AcrAB-TolC in the increased tolerance to FQs demonstrates the synergy between drug resistance and drug tolerance. Combining FQs with the pump inhibitors may greatly improve the efficiency of the treatment of chronic infections.

**Conclusions**

Bacteria frequently encounter oxidizing agents in their natural environments. They counteract their damaging effects by inducing the oxidative stress response. Using PQ, a superoxide generating agent, we found that the induction of the AcrAB-TolC pump, which is a part of soxRS regulon, increases tolerance to FQs, but not to other classes of antibiotics, regardless of the presence of oxygen. Superoxide also contributes partially to the increase in tolerance through a
mechanism which remains to be elucidated. Other pump inducers, such as sodium salicylate, also increase persister level to FQs. The pump induction reduces the FQ-mediated DNA damage and the subsequent SOS induction by lowering the intracellular concentration of FQs. This finding demonstrates the synergy between bacterial tolerance and the resistance mechanisms, which is important for understanding the limitations of FQ treatment.
CHAPTER 3 Influence of Heat Shock on *Escherichia coli*

Drug Tolerance

Introduction

Bacteria have stress response systems that enable them to minimize deleterious effects, or repair damage so that they can adapt very rapidly to the chemical or physical change in their environment, including osmolarity, pH, temperature, and the concentration of reactive oxygen species (ROS). Heat-shock induces a large set of proteins, specifically heat shock proteins (HSPs), upon the shift to a higher temperature. This response is universal, and many of the HSPs are highly conserved among species (21). A lot of HSPs are molecular chaperones or proteases that facilitate refolding of damaged proteins or eliminate proteins that cannot be repaired.

Some genes are upregulated in different stress responses. Therefore, exposure of cells to one type of stress can enable them to fight against other stresses. For instance, in Chapter 2 we found that oxidative stress conferred cells the tolerance to FQs. Also, when bacteria are challenged with high osmolarity, they acquire increased resistance to a high temperatures and oxidative stresses (46). HSPs DnaK and GroEL were upregulated at the transcriptional and translational levels by different classes of antibiotics (21). DnaK is an ATP-dependent HSP70-type molecular chaperone. Consistent with its upregulation upon antibiotic treatment, the deletion mutant *dnaK* showed a lower level of drug tolerance after the challenge with different types of antibiotics (47). We wonder if induction of the heat shock response would confer higher persistence to bacterial cells. Using BW25113, the wild type strain of the KEIO collection we tested the effect of heat shock at different temperatures on the killing by ofloxacin. The results demonstrated that the 40-min heat shock at a temperature of 43°C or higher confers higher
tolerance to ofloxacin. The cells surviving after 40-min of heat shock at 50°C were largely
tolerant to the killing of ofloxacin. Under this condition, we screened over 100 genes of HSPs for
their roles in the increased tolerance to ofloxacin and found some interesting candidates for
further investigation.

**Material and Methods**

**The effect of heat shock on the killing of ofloxacin.** *E. coli* strain BW25113 in -80°C glycerol
stock was inoculated in LB broth and incubated at 37°C with aeration and shaking at 220 rpm
overnight. The overnight culture was then diluted 1:100 and incubated under the same condition
for one hour at which time samples were taken to plate for colony-forming units (CFU) on LB
agar plates supplemented with pyruvate and magnesium sulfate. The culture was then
transferred into PCR tubes, with 100 µl/tube and 3 tubes for each temperature were tested. The
tubes were placed into a PCR machine with a 96-well capacity (Eppendorf, Mastercycler
gradient, USA). With the temperature gradient program of the PCR machine, the temperature
was the same for all of the wells in the each column, but a temperature gradient was distributed
across the wells in each row. In this work the temperature range was 37°C ~ 52°C. The tubes
were heated for 20 min, 40 min, or 1 hour, respectively. After the heat shock, a 10 µl sample per
tube was taken for CFU checking, and the rest culture in each tube was added ofloxacin of 1
µg/ml and mixed well. The tubes were then incubated at 37°C with aeration for 3 hours before
samples were serial diluted and plated for CFU.

**The screening for HSPs that confer high tolerance to ofloxacin.** *E. coli* strains BW25113
and its mutants with a single HSP gene deletion (from KEIO collection) were inoculated into LB
broth supplemented with 50 µg/ml kanamycin for the mutants and incubated overnight at 37°C
with aeration. The overnight cultures were diluted 1:100 into 1.2 ml same medium in a well of a
24-well plate, which was then incubated at 37°C with aeration for one hour. After plating for CFU, the culture in each well was transferred to 3 PCR tubes (100 µl/tube), which were then arranged in the 96-well PCR machine, where they were heated at 50°C for 40 min. After heat shock, samples were taken for CFU counting and the rest of the culture in the tubes was treated with ofloxacin at 1 µg/ml for 3 hours at 37°C with shaking at 220 rpm. After the ofloxacin treatment, samples were taken from the tubes for serial dilution and plating on LB agar plates supplemented with pyruvate.

Results and Discussion

The protection of heat shock on the killing by ofloxacin. As mentioned in the introduction, some HSPs are upregulated when bacterial cells are treated with antimicrobials. We wondered if the induction of heat shock response would prepare the cells for better survival against antimicrobials. *E. coli* strain BW25113 in exponential phase was incubated at various temperatures from 37°C to 52°C for 20 min, 40 min, or 1 hour before being challenged with ofloxacin at 1 µg/ml. With incubation longer than 20 min, the killing effect of heat shock alone emerged at around 46°C. For all heat shock durations, heat shock above 42°C increased the tolerance to ofloxacin, and the extent of the protection increased with the temperature. With heat shock for 40 min or 1 hour at 50°C, most cells that survived the heat shock could also survive the ofloxacin treatment (Figure 3.1).

The screening for HSPs that confer high tolerance to ofloxacin. To find the HSPs responsible for the increased tolerance against ofloxacin, we screened a collection of mutants where a single gene of HSPs was deleted. The collection of HSPs were gathered from the literature (22, 46, 48, 95-97) and are listed in Table 3.1. The genes listed before gene *adhE* and
marked in gray were not included in the KEIO collection, therefore they were not screened in this work.

(A) Heat shock 20 min

(B) Heat shock 40 min
Figure 3.1. The effect of heat shock on the ofloxacin killing to BW25113. (A) Heat shock for 20 min; (B) Heat shock for 40 min; (C) Heat shock for 1 hour.

Table 3.1. The genes of HSPs

<table>
<thead>
<tr>
<th>Gene</th>
<th>B name</th>
<th>Protein function</th>
</tr>
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<tbody>
<tr>
<td>\textit{b1903}</td>
<td>b1903</td>
<td>Orf, hypothetical protein</td>
</tr>
<tr>
<td>\textit{eno}</td>
<td>b2779</td>
<td>Enolase</td>
</tr>
<tr>
<td>\textit{fba}</td>
<td>b2925</td>
<td>Fructose-bisphosphate aldolase, class II</td>
</tr>
<tr>
<td>\textit{gapA}</td>
<td>b1779</td>
<td>Glyceroldehyde-3-phosphate dehydrogenase; Catalyzes 1st step in the 2nd phase of glycolysis; cytoplasmic.</td>
</tr>
<tr>
<td>\textit{groS} \ (\textit{mopB})</td>
<td>b4142</td>
<td>Chaperone for assembly of enzyme complexes; phage morphogenesis; GroESL small subunit</td>
</tr>
<tr>
<td>Gene</td>
<td>Accession</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>grpE</td>
<td>b2614</td>
<td>HSP24; with DnaJ, GrpE stimulates the ATPase activity of DnaK; heat shock protein; mutant survives induction of prophage lambda; nucleotide exchange function</td>
</tr>
<tr>
<td>hflB (ftsH)</td>
<td>b3178</td>
<td>Cell growth, septum formation, phage lambda development, essential inner membrane ATP-dependent protease, acting on SecY</td>
</tr>
<tr>
<td>htrA (degP)</td>
<td>b0161</td>
<td>Membrane-associated serine endoprotease; involved in protein degradation; essential for survival at 42°C; periplasmic</td>
</tr>
<tr>
<td>htrB(waaM)</td>
<td>b1054</td>
<td>Not under heat shock regulation; membrane protein affecting cell division, growth, and high-temperature survival</td>
</tr>
<tr>
<td>purB</td>
<td>b1131</td>
<td>Adenylosuccinate lyase</td>
</tr>
<tr>
<td>rpoE</td>
<td>b2573</td>
<td>RNA polymerase, sigma-E factor; heat-shock and oxidative stress</td>
</tr>
<tr>
<td>rpoH</td>
<td>b3461</td>
<td>RNA polymerase σ^{32} subunit; initiates transcription off heat shock promoters</td>
</tr>
<tr>
<td>slpA</td>
<td></td>
<td>FFKBP-type 16 kDa peptidyl-prolyl cis-trans isomerase [Escherichia coli CFT073]</td>
</tr>
<tr>
<td>topA</td>
<td>b1274</td>
<td>DNA topoisomerase type I, omega protein</td>
</tr>
<tr>
<td>ygiM</td>
<td>b3055</td>
<td>SH3 domain protein</td>
</tr>
<tr>
<td>yhgH</td>
<td>b3413</td>
<td>Orf, hypothetical protein</td>
</tr>
<tr>
<td>yi81</td>
<td></td>
<td>IS186 hypothetical protein</td>
</tr>
<tr>
<td>yi82</td>
<td></td>
<td>IS186 and IS421 hypothetical protein</td>
</tr>
<tr>
<td>yrfH</td>
<td>b3400</td>
<td>HSP15, an abundant heat shock protein that binds RNA and DNA.</td>
</tr>
<tr>
<td>yrfI</td>
<td>B3401</td>
<td>HSP3, molecular chaperone activated by disulfide bond formation; protects thermally unfolding proteins from aggregation; cytoplasmic.</td>
</tr>
<tr>
<td>adhE</td>
<td>b1241</td>
<td>Alcohol dehydrogenase, acetaldehyde dehydrogenase, CoA-linked allyl alcohol resistance; deactivase for PFL</td>
</tr>
<tr>
<td>Gene</td>
<td>Accession</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>adiA</td>
<td>b4117</td>
<td>Biodegradative arginine decarboxylase</td>
</tr>
<tr>
<td>ahpC</td>
<td>b0605</td>
<td>Alkyl hydroperoxide reductase small subunit</td>
</tr>
<tr>
<td>apaH</td>
<td>b0049</td>
<td>Diadenosine tetraphosphatase; stress response; complex operon</td>
</tr>
<tr>
<td>b1593</td>
<td>b1593</td>
<td>Orf, hypothetical protein</td>
</tr>
<tr>
<td>bfr</td>
<td>b3336</td>
<td>Bacterioferritin, iron storage and detoxification protein</td>
</tr>
<tr>
<td>cadA</td>
<td>b4131</td>
<td>Lysine decarboxylase 1</td>
</tr>
<tr>
<td>cadB</td>
<td>b4132</td>
<td>Transport of lysine</td>
</tr>
<tr>
<td>carA</td>
<td>b0032</td>
<td>Carbamoyl-phosphate synthetase, glutamine (small) subunit</td>
</tr>
<tr>
<td>carB</td>
<td>b0033</td>
<td>Carbamoyl-phosphate synthase large subunit</td>
</tr>
<tr>
<td>cheA</td>
<td>b1888</td>
<td>Sensory transducer kinase between chemo- signal receptors CheB and CheY</td>
</tr>
<tr>
<td>cheW</td>
<td>b1887</td>
<td>Positive regulator of CheA protein activity</td>
</tr>
<tr>
<td>clpA</td>
<td>b0882</td>
<td>ATP-binding component of serine protease; directs protease to specific substrates and complexed with ClpP; it appears to function in the degradation of unfolded/abnormal proteins; cytoplasmic</td>
</tr>
<tr>
<td>clpB</td>
<td>b2592</td>
<td>Thought to be an ATPase subunit of an intracellular ATP dependent protease</td>
</tr>
<tr>
<td>clpP</td>
<td>b0437</td>
<td>ATP-dependent proteolytic subunit of ClpA-ClpP serine protease, heat-shock protein F21.5</td>
</tr>
<tr>
<td>clpS (yljA)</td>
<td>b0881</td>
<td>Regulatory protein for ClpA substrate specificity; clpS is a specificity adapter for the ClpAP protease complex, targeting it to aggregated proteins, N-end rule substrates, and others. [More information is available at EcoCyc: G6463 and EcoGene: EG14241].</td>
</tr>
<tr>
<td>clpX</td>
<td>b0438</td>
<td>ATP-dependent specificity component of clpP serine protease, chaperone; directs partner protease to specific substrates.</td>
</tr>
</tbody>
</table>
**codA** b0337  Cytosine deaminase

**codB** b0336  Cytosine permease

**corA** b3816  Mg$^{2+}$ transport system; mutants resistant to Co$^{2+}$, Mn$^{2+}$, and Ni$^{2+}$, insensitive to Ca$^{2+}$

**creB** b4398  Catabolic regulation response regulator

**cspD** b0880  Cold shock protein

**cusC (ylcB)** b0572  Putative resistance protein

**cutC** b1874  Copper homeostasis protein

**cypA** b2313  Membrane protein required for colicin V production

**cycA** b4208  Transport of D-alanine, D-serine, and glycine permease

**cysK** b2414  Cysteine synthase A, O-acetylserine sulfhydrolase A subunit; homodimer; selenate resistance, azaserine resistance

**dinl** b1061  Multicopy suppresses phenotype of cold-sensitive dinD filamentous mutation; inhibits RecA-mediated UmuC self-cleavage

**dld** b2133  Vinylglycolate resistance, FAD enzyme

**dnaJ** b0015  HSP40, stimulates the ATPase activity of DnaK; cytoplasmic

**dnaK** b0014  Chaperone Hsp70; DNA biosynthesis; autoregulated heat-shock proteins

**dps** b0812  Stress response DNA-binding protein; starvation induced resistance to H$_2$O$_2$ phase

**endA** b2945  DNA-specific endonuclease I

**fkpA** b3347  Peptidyl-prolyl cis-trans isomerase ; folding catalyst, accelerates the folding of proteins; periplasmic

**flgE** b1076  Flagellar biosynthesis, hook protein
**fliD** b1924 Flagellar biosynthesis; filament capping protein; enables filament assembly

**ftsJ (rrmJ)** b3179 23S rRNA Um522 methyltransferase involved in cell division and growth; FtsJ, ribosomal RNA large subunit methyltransferase

**fxsA(hslW)** b4140 Overexpression alleviates the exclusion of phage T7 in F plasmid bearing cells; inner membrane associated.

**gadE (yhiE)** b3512 Orf, hypothetical protein

**glyA** b2551 Serine hydroxymethyltransferase

**gntX (yhgH/hslQ)** b3413 Function uncharacterized.

**guaA** b2507 GMP synthetase (glutamine-hydrolyzing)

**guaB** b2508 IMP dehydrogenase

**hchA (yedU)** b1967 Hsp31 molecular chaperone; important for the processing of 8-12mer peptides *in vivo*; Hsp31 (YedU) is a chaperone that is active as a homodimer.

**hflD (ycfC)** b1132 Orf, hypothetical protein

**hflX** b4173 GTP–binding subunit of protease specific for phage lambda cII repressor

**hfq** b4172 Host factor I for bacteriophage Q beta replication, a growth-related protein

**hscA** b2526 Stress response gene; Hsp70 family

**hslI (ldhA)** b1380 Fermentative D-lactate dehydrogenase, NAD-dependent

**hslJ** b1379 Heat-inducible; regulatory gene; heat-inducible lipoprotein involved in novobiocin resistance

**hslO (yrfl)** b3401 Orf, hypothetical protein
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>hslR</em></td>
<td>Ribosome-associated heat shock protein Hsp15 (hsp15)</td>
</tr>
<tr>
<td><em>hslU</em> (clpY, HtpI)</td>
<td>ATPase subunit that interacts with HslV, chaperone subunit of a proteasome like degradation complex; cytoplasmic</td>
</tr>
<tr>
<td><em>hslV</em> (clpQ, HtpO)</td>
<td>ATP-dependent protease; probable subunit of a proteasome-like degradation complex; cytoplasmic.</td>
</tr>
<tr>
<td><em>htpG</em></td>
<td>Chaperone Hsp90 with ATPase activity; cytoplasmic.</td>
</tr>
<tr>
<td><em>htpX</em></td>
<td>Probable protease; overexpression leads to an increased degradation of abnormal proteins; integral membrane protein, inner membrane.</td>
</tr>
<tr>
<td><em>htpY</em> (htgA)</td>
<td>Positive regulator for $\sigma^{32}$ heat-shock promoters</td>
</tr>
<tr>
<td><em>htrC</em></td>
<td>Essential for growth at high temperature, under $\sigma^{32}$ (heat shock) regulation</td>
</tr>
<tr>
<td><em>htrE</em></td>
<td>Sequence homology with pilin protein PapC</td>
</tr>
<tr>
<td><em>hyaB</em></td>
<td>Hydrogenase-1 large subunit</td>
</tr>
<tr>
<td><em>hydN</em></td>
<td>Iron-sulfur protein required for Hyd-3 activity</td>
</tr>
<tr>
<td><em>ibpA</em> (hslT)</td>
<td>Inclusion body protein A, small heat-shock protein</td>
</tr>
<tr>
<td><em>ibpB</em> (hslS)</td>
<td>Inclusion body protein B, heat shock protein</td>
</tr>
<tr>
<td><em>intF</em></td>
<td>Putative phage integrase</td>
</tr>
<tr>
<td><em>lon</em></td>
<td>DNA-binding, ATP-dependent protease La; heat-shock K-protein</td>
</tr>
<tr>
<td><em>lpdA</em> (lpd on KEIO)</td>
<td>Lipoamide dehydrogenase (NADH); component of 2-oxodehydrogenase and pyruvate complexes; L-protein of glycine cleavage complex</td>
</tr>
<tr>
<td><em>lysU</em></td>
<td>Lysyl-tRNA synthetase, inducible</td>
</tr>
<tr>
<td>Gene</td>
<td>Accession</td>
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<tr>
<td>--------</td>
<td>-----------</td>
</tr>
<tr>
<td>marA</td>
<td>b1531</td>
</tr>
<tr>
<td>marR</td>
<td>b1530</td>
</tr>
<tr>
<td>metA</td>
<td>b4013</td>
</tr>
<tr>
<td>miaA</td>
<td>b4171</td>
</tr>
<tr>
<td>mipA</td>
<td>b1782</td>
</tr>
<tr>
<td>mopA (yeaF)</td>
<td>b4143</td>
</tr>
<tr>
<td>mutM</td>
<td>b3635</td>
</tr>
<tr>
<td>pflB</td>
<td>b0903</td>
</tr>
<tr>
<td>phoB</td>
<td>b0399</td>
</tr>
<tr>
<td>phoR</td>
<td>b0400</td>
</tr>
<tr>
<td>pphA (prpA)</td>
<td>b0441</td>
</tr>
<tr>
<td>ppiD</td>
<td>b0441</td>
</tr>
<tr>
<td>prlC</td>
<td>b3498</td>
</tr>
<tr>
<td>pspA</td>
<td>b1304</td>
</tr>
</tbody>
</table>
associated.

**pta** b2297 Phosphotransacetylase

**purC** b2476 Phosphoribosylaminomimidazole-succinocarboxamide synthetase

**purE** b0523 Phosphoribosylaminomimidazole carboxylase; catalytic subunit

**purK** b0522 Phosphoribosylaminomimidazole carboxylase; CO(2)-fixing subunit

**purN** b2500 Phosphoribosylglycinamide formyltransferase 1

**pykF** b1676 Pyruvate kinase I (formerly F), fructose stimulated

**pyrB** b4245 Aspartate carbamoyltransferase, catalytic subunit

**pyrC** b1062 Dihydro-orotase

**pyrD** b0945 Dihydro-orotate dehydrogenase

**pyrI** b4244 Aspartate carbamoyltransferase, regulatory subunit

**pyrL** b4246 **pyrBI** operon leader peptide

**relB** b1564 Stringent/relaxed response; regulation of RNA synthesis

**rfaD** b3619 Heat inducible lipopolysaccharide, allows high temperature growth; RfaD, ADP-L-glycero-D-manno-heptose-6-epimerase

**rpmE** b3936 50S ribosomal subunit protein L31

**rpoD** b3067 RNA polymerase, $\sigma^{70}$ factor; regulation of proteins induced at high temperatures; initiates the vast majority of exponential phase transcription

**rseA** b2572 Membrane protein, negative regulator of $\sigma^E$

**sdaC** b2796 Probable serine transporter

**secG** b3175 p12 cytoplasmic membrane protein involved with protein export

**sgc** b4300 Putative DEOR-type transcriptional regulator
<table>
<thead>
<tr>
<th>Gene</th>
<th>_UCB</th>
<th>Function</th>
</tr>
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<td><strong>tdcE</strong></td>
<td>b3114</td>
<td>Probable formate acetyltransferase 3</td>
</tr>
<tr>
<td><strong>treC</strong></td>
<td>b4239</td>
<td>Trehalase 6-P hydrolase</td>
</tr>
<tr>
<td><strong>tsx</strong></td>
<td>b0411</td>
<td>Nucleoside channel; receptor of phage T6 and colicin K</td>
</tr>
<tr>
<td><strong>uhpA</strong></td>
<td>b3669</td>
<td>Response regulator, positive activator of <em>uhpT</em> transcription (sensor, <em>uhpB</em>)</td>
</tr>
<tr>
<td><strong>upp</strong></td>
<td>b2498</td>
<td>Uracil phosphoribosyltransferase</td>
</tr>
<tr>
<td><strong>uraA</strong></td>
<td>b2497</td>
<td>Uracil transport</td>
</tr>
<tr>
<td><strong>yafD</strong></td>
<td>b0209</td>
<td>Orf, hypothetical protein</td>
</tr>
<tr>
<td><strong>yafE</strong></td>
<td>b0210</td>
<td>Putative biotin synthesis protein</td>
</tr>
<tr>
<td><strong>yahA</strong></td>
<td>b0315</td>
<td>Orf, hypothetical protein</td>
</tr>
<tr>
<td><strong>yahB</strong></td>
<td>b0316</td>
<td>Putative transcriptional regulator LYSR-type</td>
</tr>
<tr>
<td><strong>yaiT</strong></td>
<td>b0371</td>
<td>Function unknown; interrupted by IS3B</td>
</tr>
<tr>
<td><strong>yaiU</strong></td>
<td>b0374</td>
<td>Putative flagellin structural protein</td>
</tr>
<tr>
<td><strong>ybaP</strong></td>
<td>b0482</td>
<td>GumN family protein</td>
</tr>
<tr>
<td><strong>ybbM</strong></td>
<td>b0491</td>
<td>Putative metal resistance protein</td>
</tr>
<tr>
<td><strong>ybbN</strong></td>
<td>b0492</td>
<td>Putative thioredoxin-like protein</td>
</tr>
<tr>
<td><strong>ybeY</strong></td>
<td>b0659</td>
<td>Metal-binding heat shock protein required for rRNA maturation</td>
</tr>
<tr>
<td><strong>ybeZ</strong></td>
<td>b0660</td>
<td>Putative ATP-binding protein in <em>pho</em> regulon</td>
</tr>
<tr>
<td><strong>ybjZ</strong></td>
<td>b0879</td>
<td>Putative ATP-binding component of a transport system; macrolide-specific ABC-type efflux carrier</td>
</tr>
<tr>
<td><strong>yccV</strong></td>
<td>b0966</td>
<td>A protein having an affinity for a hemimethylated oriC DNA and negatively controlling <em>dnaA</em> gene expression <em>in vivo</em></td>
</tr>
<tr>
<td><strong>yceP</strong></td>
<td>b1060</td>
<td>Orf, hypothetical protein; biofilm regulator <em>bssS</em> (not from <em>E. coli</em>)</td>
</tr>
<tr>
<td>Gene</td>
<td>Accession</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>--------------------------------------------------------------</td>
</tr>
<tr>
<td>ycfR</td>
<td>b1112</td>
<td>Biofilm, cell surface and signaling protein</td>
</tr>
<tr>
<td>(bhsA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ycgZ</td>
<td>b1164</td>
<td>Connector protein for RcsB regulation of biofilm and acid-resistance</td>
</tr>
<tr>
<td>ycjF</td>
<td>b1322</td>
<td>Uncharacterized protein in the <em>pspE-tyrR</em> intergenic region.</td>
</tr>
<tr>
<td>(hslG)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ycjX</td>
<td>b1321</td>
<td>Conserved protein with nucleoside triphosphate hydrolase domain</td>
</tr>
<tr>
<td>yeeD</td>
<td>b2012</td>
<td>Unknown conserved protein</td>
</tr>
<tr>
<td>yfiD</td>
<td>b2579</td>
<td>Autonomous glycyl radical cofactor</td>
</tr>
<tr>
<td>yfjD</td>
<td>b2613</td>
<td>Putative transport protein</td>
</tr>
<tr>
<td>ygiU</td>
<td>b3022</td>
<td>Orf, hypothetical protein</td>
</tr>
<tr>
<td>yhdN</td>
<td>b3293</td>
<td>Orf, hypothetical protein</td>
</tr>
<tr>
<td>yheL</td>
<td>b3343</td>
<td>Orf, hypothetical protein</td>
</tr>
<tr>
<td>yhgE</td>
<td>b3402</td>
<td>Putative transport</td>
</tr>
<tr>
<td>yidE</td>
<td>b3685</td>
<td>Putative transport protein</td>
</tr>
<tr>
<td>yjdE</td>
<td>b4115</td>
<td>Putative amino acid</td>
</tr>
<tr>
<td>(adiC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>yjeH</td>
<td>b4141</td>
<td>Putative transport</td>
</tr>
<tr>
<td>ymgB</td>
<td>b1166</td>
<td>Unknown</td>
</tr>
<tr>
<td>yrdB</td>
<td>b3280</td>
<td>Orf, hypothetical protein</td>
</tr>
<tr>
<td>yrfG</td>
<td>b3399</td>
<td>Putative phosphatase</td>
</tr>
<tr>
<td>ytfE</td>
<td>b4209</td>
<td>Orf, hypothetical protein</td>
</tr>
</tbody>
</table>
Figure 3.2. The screening of HSPs for the protection of heat shock from ofloxacin killing.
For every experiment, BW25113 was included as a control strain. The cultures of the strains in exponential phase were subjected to a 40 min heat shock at 50 °C and then challenged with ofloxacin at 1 µg/ml for 3 hours. The results are shown in Figure 3.2 A-F.

The first round of screening provided a list of interesting HSPs which might account for the increased tolerance to ofloxacin after the induction of heat shock, as marked by the red arrows in Figure 3.2. These genes and some findings about their protein products are listed in Table 3.2. The majority of these proteins are involved in resistance to different stresses. A few of them are of particular interest here because their functions show possible correlation with bacterial persistence when heat shock is not involved. YmgB decreases cellular motility and regulates the acid resistance through the influence of indole, which turns on drug efflux pumps and oxidative stress protection mechanisms (66). Persisters are cells with less motility, and as reported in the previous chapter, the induction of the efflux pump AcrAB-TolC and the oxidative stress response increases the tolerance to FQ killing. These findings together indicate that YmgB may correlate with the formation of persisters under some circumstances. Under anaerobic conditions PflB is induced and plays a role in the metabolism of glycerol (131). Some proteins in the metabolism of glycerol, aerobic sn-glycerol-3-phosphate dehydrogenase (GlpD), the anaerobic sn-glycerol-3-phosphate dehydrogenase (GlpABC), and the sn-glycerol-3-phosphate acyltransferase (PlsB) have been found related to the multidrug tolerance of E. coli (113). Protease ClpX activates ClpP. The protease complex ClpXP, and protease Lon cleaves the antitoxin MqsA (YgiT) in the TA module MqsRA, so the ribonuclease toxin MqsR (YgiU) is set free (58). The overexpression of MqsR increases the multidrug tolerance of bacterial cells (107) and induces another toxin gene, cspD (58). In addition, MqsR is involved in the regulation of motility by the quorum sensing signal autoinducer-2 (Al-2) (58). Interestingly, some quorum sensing signal molecules phenazine pyocyanin and the acyl-homoserine lactone 3-OC12-HSL
significantly increased the persister numbers in logarithmic P. aeruginosa PAO1 or PA14 cultures (79).

**HSPs ApaH, GlyA and ClpB which are probably related to bacterial drug tolerance play major roles in heat shock rescue.** Our original goal was to find the HSPs that confer high tolerance to antibiotics after the induction of the heat shock response. However, there are mutants, *apaH, glyA* and *clpB*, that demonstrate dramatic reduction in survival to heat shock, as marked by the green arrows in Figure 3.2. The literature review of these genes reveals that they are quite interesting for the investigation on persister formation. Gene *apaH* codes for Diadenosine tetraphosphatase, which hydrolyzes diadenosine tetraphosphate AppppA (Ap4A) producing two copies of ADP. The cellular concentration of adenylylated nucleotides, including Ap4A, rises rapidly upon heat shock or oxidative stress. Also, Ap4A binds to HSPs such as DnaK, GroEL and ClpB (37, 40), the mutant of which exhibited strong heat sensitivity in this study. A high level of Ap4A also causes early cell division (85). The deletion of *apaH* in *Salmonella enterica* serovar Typhimurium caused the accumulation of Ap4A, reducing the capacity of the *apaH* mutant to invade mammalian cells (53). Therefore, ApaH plays an important role in maintaining a healthy level of Ap4A. Interestingly, the single deletion mutant *apaH* showed a reduced level of persistence compared to the wild type during screening for candidate persister genes (47), indicating a role of Ap4A in persister formation. GlyA is a serine hydroxymethyltransferase, which plays a key role in the metabolism of folate (108). The deletion of a gene in the folate metabolism pathway, *ygfA* (5-formyl-tetrahydrofolate cyclo-ligase), resulted in approximately a 10-fold reduction in bacterial tolerance, and the overexpression of this gene produced increased tolerance to ofloxacin (47). ClpB is an ATP-dependent protease and a HSP100 chaperone. It is involved in stress tolerance of *Francisella tularensis in vivo* and is required for its multiplication in infected mice (77). Interestingly, this gene is upregulated in
the persister transcriptome (56) and interacts with Ap4A (40). From the observations in this work, apaH, glyA, and clpB apparently play major roles in rescuing cells from heat shock. Probably heat shock and stresses causing persister formation make similar damages which can be reduced or repaired by the products of apaH, glyA, and clpB.

Table 3.2. The genes of HSPs that may be involved in the increased tolerance to ofloxacin through heat shock response

<table>
<thead>
<tr>
<th>Gene</th>
<th>review</th>
</tr>
</thead>
</table>
| ymgB | • YmgB represses biofilm formation as a result of quorum sensing signal AI-2 or indole signaling, decreases cellular motility, and protects the cell from acid in E. coli.  
  • YmgB has been renamed as AriR for the regulation of acid-resistance influenced by indole.  
  • YmgB is a gene regulatory protein. ---see reference (7, 67, 117, 127) |
| pflB | • Inactivation of pyruvate formate-lyase (PflB) in Escherichia coli ATCC 8739 eliminated the production of formate and ethanol and reduced the production of acetate but increased the yield of succinate when glycerol was used as the carbon source in anaerobic condition (131).  
  • DNA repair was inhibited in mutant BW25113 ΔldhA ΔpflB (109). |
| yjeH | • The resistance to ceftriaxone of Salmonella enterica serovar Typhimurium is mediated by the putative transporter YjeH by its effect on the expression of the porin OmpD, putative outer membrane proteins STM1530 and STM3031, a subunit of the proton-pumping oxidoreductase NuoB and the heat shock protein MopA (51). |
| rfaD | • Gene htrM (rfaD) is essential for Escherichia coli viability only at elevated temperatures (94).  
  • A htrM (rfaD) null mutation mutant produces a defective
lipopolysaccharide that is unable to protect outer membrane proteins from degradation during folding (78).

*htpG*  
- HtpG is chaperone Hsp90 with ATPase activity and is induced under phenol treatment, heat shock, acid shock or nutrient starvation (100).

*ftsJ*  
- FtsJ regulates the accuracy of translation (125).

*sdaC*  
- Gene *sdaC* is also called *dcrA*. It is involved in serine uptake and is required for C1 phage adsorption. SdaC/DcrA protein also serves as a specific inner membrane receptor for colicin V, a peptide antibiotic that kills sensitive cells by disrupting their membrane potential once it gains access to the inner membrane from the periplasmic face (42).
- An active SdaC is essential for colonization of the avian gut by *Campylobacter jejuni* and the catabolism of L-serine is crucially important for the growth of this bacterium *in vivo* (120).

*rpmE*  
- RpmE contains one zinc ion per molecule of protein. In addition, mutagenesis of the *rpmE* gene encoding RpmE revealed that Cys-36 and Cys-39, located within a CxxC motif, are required not only for binding zinc but also for the accumulation of RpmE in the cell (83).

*clpX*  
- ClpX is a member of Hsp100 (heat-shock protein) family which is conserved among organisms. Hsp100/Clp implicates in stress resistance, intracellular protein turn-over, DNA replication and regulation of gene expression (122).
- ClpX is required for stress and desiccation tolerance in *Staphylococcus aureus* (39, 122).
- Proteases Lon and ClpXP are necessary for MqsR toxicity. MqsR (also named YgiU) is the toxin in TA module MqsR/MqsA (58). YgiU has been found to influence bacterial tolerance (18, 73, 107).

*ybbM*  
- A putative bacterial metal resistance protein (65).
- YbbM is also named as RsiW, a specific anti-sigma factor modulating the activity of the alternative, extracytoplasmic function sigma factor SigW. The *Bacillus subtilis* *sigW* regulon is induced by different stresses
such as alkaline shock, salt shock, phage infection and certain antibiotics that affect cell wall biosynthesis (103).

**purC**
- PurC is involved in purine synthesis and upregulated in biofilm population compared to the planktonic cultures of the soil saprophyte *Bacillus cereus* (121).

**hflD**
- HflD, an *E. coli* protein involved in the lambda lysis-lysogeny switch, impairs transcription activation by lambda CII (89).

**htpX**
- HtpX is a zinc-dependent endoprotease of the membrane-localized proteolytic system in *E. coli* (99).
- This protease contributes to the aminoglycoside resistance in *Pseudomonas aeruginosa* (50).

**rpoD**
- Survival and stress induced expression of *groEL* and *rpoD* of *Campylobacter jejuni* from different growth phases (59).

**ybaP**
- N/A

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**Future Work**

The killing by heat and ofloxacin of the wild type cells varies with experiment (Figure 3.2), suggesting that candidates from the first round of screening should be subjected to a second round to confirm the phenotype. In addition, during the first round of screening, the MIC of ofloxacin for each strain was not tested. The mutants with lower MICs would have fewer survivors. Therefore, candidates after the second round of screening should be further tested for MIC. Only the candidates with unaltered MICs will be further investigated.

From another point of view, heat shock at 50°C presents a lethal stress to bacterial cells. The survivors of heat shock may be considered as heat shock persisters. The killing curve with time by heat shock at 50°C should be conducted to determine if and when the number of survivors
reaches a plateau. If the survivors of heat shock in Figure 3.2 are located in the plateau, then *apaH, glyA* and *clpB* will be very interesting candidate genes for the formation of heat shock persisters.
CHAPTER 4 *Escherichia coli* Native Persister Isolation

Introduction

Persisters are nongrowing, multidrug tolerant cells that form naturally in bacterial populations. In most bacterial populations such as *E. coli*, they account for $10^{-6}$ to $10^{-4}$ of a growing population and around $10^{-2}$ of a stationary population (55). Persistence is a transient state, since persisters resuscitate and form a population that is just as susceptible as the parental one when they are reinoculated to fresh medium. These characteristics make it challenging to isolate persisters. The first isolation method used ampicillin to lyse the growing population of *hipA7*, an *E. coli* mutant which produces a high level of persisters (56). However, ampicillin application introduces lethal stress to the population prior to obtaining persisters. It would then be very hard to exclude the impact of ampicillin on the obtained persister transcriptome. A more delicate method was developed for the sake of isolating native persisters (107). An unstable GFP was put under a growth-rate dependent ribosomal promoter such that fast growing cells are bright and persisters are dim. By fluorescence-activated cell sorting (FACS), the dim population was collected as persisters. The ofloxacin challenge following sorting showed that the dim population was much more tolerant than the bright one. The gene profile of sorted persisters significantly differs from that of active growing cells and non-growing stationary cells as well. The transcriptomes obtained by cell lysis and cell sorting methods are shown in Figure 4.1. Both transcriptomes point to phage shock genes and TA genes. However, many genes related to DNA damage such as *recA*, *recN*, *umuCD* and *uvrAB* were upregulated in persisters collected by cell lysis but not in sorted persisters, indicating the impact from antibiotic treatment. Metabolic genes involving in glycerol metabolism are upregulated in the sorted persisters but not in ampicillin treated persisters, indicating that the sorting method is better for preserving native information on persisters. Nonetheless, the sorting method is limited by its efficiency. Proteins and metabolites...
directly govern bacterial physiology, thus compared to transcriptome, the persister proteome and metabolome are more informative for studying persister formation and maintenance. However, they require larger samples of persisters. In addition, with cell sorting, cells are diluted in buffer, which sometimes resuscitates persisters. Therefore, this chapter focuses on the development of an efficient method to isolate native persisters.

Figure 4.1. Persister transcriptomes. (A) Transcriptome of persisters isolated by ampicillin lysis, from reference (56). (B) Transcriptome of persisters isolated by cell sorting, from reference (107).

The Plan for Native Persister Isolation

An *E. coli* strain lacking the *lamB* gene, which encodes a surface protein, will be engineered to contain a plasmid that carries the same gene under an inducible promoter. Based on the
assumption that persisters exist in an inactive state, upon induction \( lamB \) will only be expressed on the surface of non-persisters. This makes it possible to separate non-persisters from persisters using a ligand with high affinity to LamB. The details are illustrated in Figure 4.2. The ligand-LamB complex consists of amylose-coated magnetic beads (AMB), fusion protein MBPgpJ and \( lamB^+ \) cells. Amylose is a polymer of maltose, strongly interacting with the Maltose Binding Protein (MBP) part of the fusion protein. The gpJ domain of the fusion protein is the functional part of \( \lambda \) phage tail fiber J protein, strongly binding to LamB which is the receptor of phage \( \lambda \) (11). LamB is also a maltose receptor, and is highly expressed when the cell is incubated in medium supplemented with maltose (38).

![Figure 4.2. The principle of native persister isolation](image)

To isolate persisters, the strain will be cultured to the designated stage and then LamB will be induced for one hour. As shown in Figure 4.3, the cell samples will be washed and suspended in MBP column buffer, then incubated with MBPgpJ and AMB to form the complex shown in Figure 4.2. The mixture then will be placed against a magnet that pulls the complex into a pellet, leaving cells with a very low level of LamB in the supernatant (S/N). If the cell capture efficiency is not satisfactory after the first round of capture, the supernatant will be subjected to additional rounds of capture.
Material and methods

Strains and growth conditions. *E. coli* K-12 wild type MG1655 and its derivatives ∆*lamB* and ∆*lamB* ∆*lamB* were used as the objects of cell capture. *E. coli* Strain BL21DE3 (Novagen) was used for the host of plasmid pET28a-MBPgpJ overexpressing fusion protein MBPgpJ. The medium used for culture was Luria-Bertani Broth (10 g Bacto-tryptone, 5 g yeast extract and 10 g NaCl /liter, Fisher Scientific), sometimes supplemented with antibiotic marker, glucose or isopropyl β-D-1-thiogalactopyranoside (IPTG) as needed. The incubation was generally conducted at 37°C with shaking at 220 rpm.

The construction of strains. The strain, *E. coli* JM501pMAL-J, expressing fusion protein MBPgpJ was generously provided by Berkane et al. (11). The MBP was then replaced by a mutated version that has a higher affinity to amylose (pMAL system, New England Biolab). A
six-histidine tag was added to the N-terminal of the mutated MBP for downstream purification by a Ni-NTA column (Novagen). Subsequently the gene encoding the his-tagged MBPgpJ was cloned into the plasmid pET28a (Novagen) under a T7 promoter for a higher level of expression. The construct was used to transform the host cell BL21(DE3), which carries the gene for T7 RNA polymerase under the IPTG inducible promoter $Ptac$. The obtained strain BL21(DE3) pET28a-MBPgpJ overexpresses the fusion protein MBPgpJ when induced by IPTG. MG1655 $\Delta lamB$ was constructed by transducing MG1655 with the P1 stock of mutant $lamB$ from KEIO E. coli Knockout Collection. It was then transformed by various constructs where the gene $lamB$ was cloned under an inducible promoter to control the expression of protein LamB.

Results and Discussion

The overexpression of MBPgpJ. Around $10^8$ persister cells are needed to extract mRNA for DNA microarray analysis. More persister cells are required to analyze the proteome and metabolome of persisters. Considering that persisters are a tiny fraction of cells in a bacterial population and that, with this plan, non-persisters will be captured by the fusion protein, we need a large amount of MBPgpJ to coat AMB. Therefore, we constructed a strain, BL21(DE3) pET28a-MBPgpJ, to overexpress 6x his-tagged fusion protein MBPgpJ. The strain was cultured in LB at $37^\circ C$ until OD$_{600}$ 0.6 and then induced with IPTG of varying concentrations for a designated period of time. The subsequent protein analysis revealed that MBPgpJ was ectopically expressed from this construct when 1 mM IPTG was used (Figure 4.4).
Figure 4.4. The expression of 6xHis-MBPgpJ from 1 ml culture of BL21DE3 pET28a-MBPgpJ. The strain was cultured in LB to OD_{600} ~ 0.5 and then induced with IPTG at various concentrations for 3 hours. The cell pellet from 1 ml culture was then suspended and dissolved in 50 µl of SDS sample buffer at 95°C for 5 min. The obtained samples were diluted 5-fold by SDS sample buffer and loaded onto the gel at a volume of 20 µl per well. Lane 1 is the Marker. Lane 2 is the uninduced sample. Lane 3 is the sample induced with 0.5 mM IPTG and Lane 4 is the sample induced with 1.0 mM IPTG.

**The solubility of MBPgpJ.** The majority of the expressed MBPgpJ shown in Figure 4.4 was insoluble and went to inclusion bodies. The insoluble form of MBPgpJ cannot be used to test its specificity to LamB. The effort to concentrate and purify MBPgpJ from the soluble fraction of cell lysate was not successful. However, using an electro-elutor (BioRad, Model 422) we successfully obtained the functional fusion protein from its band on the NuPAGE 10% Bis-Tris gel (Invitrogen). The purified protein captured MG1655 cells cultured in LB supplemented with
0.2% maltose, but not MG1655 ΔlamB (data not shown). However, the reproducibility of both eluting the fusion protein and capturing the LamB⁺ cells was rather poor. The concentration of the eluted fusion protein was around 100 µg/ml (measured by Bradford Protein Assay), which

<table>
<thead>
<tr>
<th>1ml sample</th>
<th>Crude Extract</th>
<th>Crude extract</th>
<th>Insoluble</th>
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</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IPTG(mM)</td>
<td>0</td>
<td>0.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

![Image](image_url)

**Figure 4.5.** Solubility of the fusion protein MBPgpJ. Strain BL21DE3 pET28a-MBPgpJ was cultured (A) in 100 ml LB with or without 1% glucose at 37°C to OD₆₀₀ 0.6 before one aliquot was induced with 0.3 mM IPTG for 3 hours; (B) in 100 ml LB supplemented with 1% glucose at 37°C in a shaker until OD₆₀₀ reached 0.6 and then 4 aliquots of the culture were induced with IPTG at 0 mM, 0.1 mM, 0.3 mM, and 0.5 mM respectively before being shifted to a shaker at 16°C where the cultures were incubated overnight. The cell lysate of each aliquot was centrifuged to obtain the soluble and insoluble parts. The samples were heated at 95°C for 5 min before loading onto a NuPAGE 10% Bis-Tris gel. The bands of MBPgpJ are marked with a red box.
may be too low to keep the protein stable. This problem was finally solved by the optimization of
the incubation conditions for BL21DE3 pET28a-MBPgpJ. When incubated in LB supplemented
with 1% glucose at 37°C to OD$_{600}$ ~ 0.6 and then induced by 0.1 mM IPTG and immediately
shifted to 16°C for overnight incubation, BL21DE3 pET28a-MBPgpJ expressed most of the
MBPgpJ in the soluble form. As shown in Figure 4.5 A, even with 1% glucose in the medium,
the fusion protein was still insoluble if, after induction, the cells are incubated at 37°C. While at
16°C, the fusion protein could be expressed in the soluble form, provided that the concentration
of IPTG did not exceed 0.5 mM. As shown in Figure 4.5 B, when IPTG concentration increased
from 0.1 mM to 0.5 mM, MBPgpJ shifts from the soluble to the insoluble form.

**The purification and storage of MBPgpJ.** BL21DE3 pET28a-MBPgpJ in exponential phase at
37°C was induced with 0.1 mM IPTG and then incubated at 16°C overnight. The cell lysate was
centrifuged to obtain the crude extract, which was passed through the Ni-NTA columns
according to the procedures described in the Ni-NTA Protein Purification Protocol 13 (The
QIAexpressionist™, QIAGEN, 2003). The buffer in the eluted fusion protein fractions was then
changed to MBP column buffer and concentrated using Amicon Ultra Centrifugal Filter Devices
(Millipore). The obtained fusion protein, with a concentration of around 2~4 mg/ml, remained
active at 4°C for up to 4 months. The protein in 50% glycerol stock remained active for an even
longer time. Glycerol can serve as a carbon source, and a persister candidate gene glpD, a
G3P dehydrogenase, is involved in the metabolism of glycerol (113). Therefore, caution shall
be used if the glycerol stock of MBPgpJ is used to isolate persisters.

**Cell capture capability of MBPgpJ.** The concentrated MBPgpJ stock was tested for its
capability of capturing lamB$^+$ cells. MG1655 and MG1655ΔlamB were cultured in LB to OD$_{600}$ ~
0.6 before they were induced with 0.2% maltose for 1 hour. Subsequently 1 ml aliquots were
centrifuged, washed, and resuspended in 1 ml MBP column buffer (200 mM NaCl, 20 mM Tris-HCl, recommended for AMB by New England Biolabs (NEB)). The suspension was diluted 10 times in MBP column buffer before 50 µl was incubated with 1 µl MBPgpJ stock of 2 mg/ml and 450 µl of AMB suspension (50 µl AMB in 400 µl MBP column buffer) on a rotator at 4°C for 1 hour. The mixture was then applied to a magnet (DYNAL MPC-L, Invitrogen) for 10 min. The supernatant went through another round of 1-hour incubation with 50 µl AMB stock and 1 µl fusion protein stock at 4°C before another magnet application. In the control samples, 1 µl MBP column buffer was used instead of the fusion protein. The previous persister isolation methods developed in our lab focused on fast-growing cells (56, 106), however persister levels are higher in stationary phase. It will be useful to use MBPgpJ to isolate persisters in stationary phase. The results of cell capture from both log and stationary phase are shown in Figure 4.6. The fusion

![Graph showing cell capture results](image)

**Figure 4.6.** Cell capture by MBPgpJ of *E. coli* cells. M stands for *lamB*+ strain *MG1655*, Δ for *MG1655 ΔlamB*, L for exponential phase, S for stationary phase, P for fusion protein usage, and φ for no fusion protein usage.
protein captured about 95% of MG1655 cells after the first round of cell capture and about 99% after the second round for exponential phase and around 97% after the second round for stationary phase. No significant cell capture was observed when cells are LamB− or when the fusion protein was not used. The specificity of MBPgpJ to lamB+ cells has been repeatedly confirmed during the optimization of cell capture procedures.

**LamB cloning for differentiating persisters from non-persisters.** As described in the plan, to efficiently isolate persisters from non-persisters, the non-persister cells must efficiently express LamB upon induction so that they would tightly attach to AMB, whereas the opposite is expected from persisters. Therefore, the gene of LamB was put under a promoter inducible by IPTG, which is preferred here since it neither imposes stress to a bacterial population nor resuscitates persister cells by serving as a carbon source like arabinose.

The first plasmid for LamB cloning was pZS*24, which is a low-copy number plasmid and has the IPTG and arabinose inducible promoter P\text{lac/ara}-1, with IPTG as the primary inducer and arabinose as the secondary inducer for higher level of expression (74). The results from λ infection test indicated the presence of LamB on the cell surface upon IPTG induction, as shown in Figure 4.7. However, the subsequent cell capture gave poor results (data not shown). Since pZS*24 is a low-copy number plasmid, the amount of LamB expressed after induction might be insufficient for the efficient cell capture. Therefore, \textit{lamB} was cloned into a high-copy number plasmid pZE34 which also has the promoter P\text{lac/ara}-1, and also into another high-copy number plasmid pQE60 (Qiagen) which combines a phage T5 promoter (recognized by \textit{E. coli} RNA polymerase) with a double \textit{lac} operator repression module for tightly regulated, high-level expression of target proteins in \textit{E. coli}. Subsequent λ test and cell capture experiments showed background \textit{lamB} expression and poor cell capture results, which were not improved by induction (data not shown). This could be due to a very low amount of LamB protein, or the poor assembly of LamB on the cell outer membrane. To be a functional porin for maltose absorption
and a receptor for λ phage, LamB must span the outer membrane as a trimer (10). At least a dozen proteins located in the cytoplasm, periplasm, inner membrane, and outer membrane are required to catalyze this complex assembly process (118). It is possible that upon the addition of inducer the export machinery could not keep up with LamB synthesis, causing mistakes in LamB assembly.

Figure 4.7. λ infection test for strain MG1655ΔlamB pZS*24 lamB. (A) MG1655; (B) MG1655ΔlamB pZS*24lamB, uninduced; (C) MG1655ΔlamB pZS*24 lamB induced with 0.2 mM IPTG. All plates were made of LB agar supplemented with 2 mM MgSO₄, and additional 0.2% maltose for (A), 50 µg/ml kanamycin for (B) and (C), and IPTG for (C). The same supplements were added in the top agar accordingly.
To quantify LamB expression from the plasmids described above, we developed an anti-LamB antibody for Western blot analysis. Based on the structure of LamB (102) and the software analysis (Primm Biotech, Inc., Cambridge, MA), peptide GNADNNANFGKAVPADFNGGSFGR (on LamB exterior loop 9) and peptide LRDNYRLVDGAS (on exterior loop 5) were synthesized and conjugated to the carrier protein Keyhole Limpet Hemocyanin (KLH). Anti-LamB monoclonal antibody was obtained by injecting the mixture of the conjugates into two rabbits. Western Blot analysis of cell lysates from MG1655 and MG1655ΔlamB confirmed that the antibody can specifically bind to LamB, which is in the solid fraction of the lysate, as shown in Figure 4.8. The antibody was then used to examine the level of the induced LamB expression from the aforementioned constructs pZS^+24-lamB, pZE34-lamB and pQE60-lamB. Only the band from MG1655 lysate (the positive control) was observed (data not shown), indicating that the levels of
expressed LamB from the constructs were far below the chromosomal expression level of the wild type cells, which is most likely the leading cause of poor cell capture.

Conclusion and Future Work

The cell capture results prove that the fusion protein MBPgpJ has great potential to enrich persisters if we can control the surface expression of LamB. To date, the cell capture technique has been conducted in buffer. Dilution in buffer might resuscitate persisters. Centrifuging cells and resuspending them in buffer would induce changes in the transcriptome of the collected persisters. Therefore, in later steps, the in situ capture (capture that occurs directly in culture) should be tested and optimized to obtain native persisters.
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