Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells

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(Article begins on next page)
Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells

Graphical Abstract

Highlights

- Persisters accumulate fewer antibiotics as a direct result of increased efflux rate
- Persisters show higher expression of efflux-associated genes
- High expression of tolC is critical to promote persister formation
- Persisters combine active efflux and passive dormancy to survive antibiotic attack

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In Brief

By using in vivo fluorescent imaging and next-generation sequencing, Pu et al. demonstrate that bacterial persisters exhibit enhanced efflux activity in addition to physiological quiescence, a double insurance strategy to ensure its survival under antibiotic attack.
Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells

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SUMMARY

Natural variations in gene expression provide a mechanism for multiple phenotypes to arise in an isogenic bacterial population. In particular, a sub-group termed persisters show high tolerance to antibiotics. Previously, their formation has been attributed to cell dormancy. Here we demonstrate that bacterial persisters, under β-lactam antibiotic treatment, show less cytoplasmic drug accumulation as a result of enhanced efflux activity. Consistently, a number of multi-drug efflux genes, particularly the central component TolC, show higher expression in persisters. Time-lapse imaging and mutagenesis studies further establish a positive correlation between tolC expression and bacterial persistence. The key role of efflux systems, among multiple biological pathways involved in persister formation, indicates that persisters implement a positive defense against antibiotics prior to a passive defense via dormancy. Finally, efflux inhibitors and antibiotics together effectively attenuate persister formation, suggesting a combination strategy to target drug tolerance.

INTRODUCTION

Multi-level stochasticity arising from mRNA transcription and protein translation leads to remarkable phenotypic heterogeneity in a population of cells with an identical genome (Elowitz et al., 2002; Lidstrom and Konopka, 2010; Raj and van Oudenaarden, 2008). Such non-genetic individuality is often proposed as a “bet-hedging” mechanism (Beaumont et al., 2009; Losick and Desplan, 2008; Veening et al., 2008), whereby a clonal cell population maximizes its survival chance under rapidly changing environment by exploring diverse phenotypic solutions. One phenotype may gain a higher fitness under a given condition, and other phenotypes may be better adapted to certain other environmental situations. In the case of bacteria under drug treatment, while the majority of cells are quickly eradicated by bactericidal antibiotics, a very small group of phenotypic variants, termed persisters, show strong drug tolerance. This phenomenon was first reported in 1944 by Joseph Bigger when he studied the lethal effect of penicillin on Staphylococcus aureus (Bigger, 1944). Bacterial persisters have also been observed in other pathogenic species, and they may play a role in the recurrence of chronic infections. Their existence is believed to prolong and exacerbate the treatment of diseases, such as tuberculosis, cystic fibrosis associated lung infections, and candidiasis (Boucher, 2001; Chao and Rubin, 2010; Fauvart et al., 2011; LaFleur et al., 2006; Mulcahy et al., 2010).

To develop new drugs targeting chronic infections requires a deep understanding of the mechanisms underlying persister formation. However, the extremely low percentage of persister cells in a bacterial population and complex pathways involved in persister formation have delayed the study of this phenomenon. Previous studies associated persister formation mainly with cell dormancy (Balaban et al., 2004; Lewis, 2007; Shah et al., 2006), in which the important contribution from toxin-antitoxin (TA) loci has been highlighted (Balaban et al., 2004; Dörr et al., 2010; Keren et al., 2004; Vázquez-Laslop et al., 2006). TA locus encodes two components: a stable toxin that can interrupt essential cellular pathways and induce a dormancy-like state and a labile antitoxin that can conjugate the toxin to nullify such toxicity. TA modules are most likely activated by stress responses through the alarmone guanosine tetraphosphate (ppGpp) pathway (Maisonneuve et al., 2013). Furthermore, the SOS response induces persister formation through activating TisB overexpression (Dörr et al., 2010), a member of the toxin family as well. Screening a complete bacterial knockout library also identified a number of global regulators involved in persister formation (Hansen et al., 2008). For example, overexpression of ygtA downregulated overall transcription, and overexpression of relE led to a decreased protein synthesis rate (Maisonneuve et al., 2011), both of which assist bacterial drug tolerance through inducing a dormant state of the cell.

The leading theory explaining antibiotic tolerance of persister cells lies in the fact that even though antibiotic molecules bind their targets, their lethal effects have been disabled due to the extremely slow metabolic and proliferation rates in those
dormant cells (Lewis, 2007). However, one fundamental question remains poorly addressed: do antibiotics effectively enter and accumulate in persister cells to exert their lethal effects? Antibiotic accumulation in Gram-negative bacteria is primarily influenced by two factors, membrane permeability and efflux activity. Hydrophobic antibiotics, such as aminoglycosides and macrolides, gain access into the cell through the membrane by metabolic activity-dependent diffusion, while hydrophilic antibiotics, such as β-lactam, enter the cell through porin channels to reach their targets (Delcour, 2009). Inversely, bacterial multi-drug efflux systems actively pump antibiotics out to reduce cellular drug accumulation, thus facilitating bacterial survival (Sun et al., 2014; Webber and Piddock, 2003). Therefore, stochastic and heterogeneous expression of porin or efflux proteins could lead to uneven antibiotic accumulation and consequently different drug tolerance across a bacterial population. Whether these two systems play roles in bacterial persistence thus deserves in-depth investigation.

In this study we combined in vivo fluorescent imaging and next-generation sequencing to explore the critical biological mechanisms that produce bacterial persistence. We found that enhanced efflux activity contributed strongly to persister formation and that bacterial persisters adopt a two-pronged strategy to ensure survival under antibiotic attack. Most biological processes were slowed down during dormancy, yet the efflux systems, on the contrary, displayed greater activity that further increased persister tolerance to antibiotics.

RESULTS

Antibiotic Accumulates at a Lower Level in Persister Cells

To investigate the antibiotic accumulation in bacterial persisters, we used a fluorescent β-lactam antibiotic BOCILLINTM FL Penicillin (BOCILLIN) to measure quantitatively single cell intracellular drug concentration. We isolated persisters after 150 μg/ml carbenicillin treatment (see Experimental Procedures). These drug tolerant cells had not genetically acquired resistance—after removing the antibiotic, they regrew a new bacterial population in which the majority of cells was still vulnerable to the same antibiotic (Figure S1A). Furthermore, according to previously established staining protocol (Orman and Brynildsen, 2013a), we combined fluorescence microscopy with colony counting assay to confirm that persister cells, not the viable but non-culturable cells (VBNCs), were the main cell type in these drug tolerant population (Figures S1B–S1E). After isolating persister cells, we incubated them for 30 min at 37°C (Figure 1A) with BOCILLIN. In parallel, the same staining process was carried out on a population of untreated cells that contained both persisters and susceptible cells. The fluorescent signal corresponding to antibiotic concentration in individual cells was then imaged and normalized by the cell size (the 2D projection area of the cell in bright field imaging). We followed previously described methods (Taniguchi et al., 2010) to remove background fluorescence and cell auto-fluorescence, and signal from dead cells (Figure S1B). After isolating persister cells, we incubated them for 30 min at 37°C (Figure 1A) with BOCILLIN. In parallel, the same staining process was carried out on a population of untreated cells that contained both persisters and susceptible cells. The fluorescent signal corresponding to antibiotic concentration in individual cells was then imaged and normalized by the cell size (the 2D projection area of the cell in bright field imaging). We followed previously described methods (Taniguchi et al., 2010) to remove background fluorescence and cell auto-fluorescence, and signal from dead cells (Figure S1B). After isolating persister cells, we incubated them for 30 min at 37°C (Figure 1A) with BOCILLIN. In parallel, the same staining process was carried out on a population of untreated cells that contained both persisters and susceptible cells. The fluorescent signal corresponding to antibiotic concentration in individual cells was then imaged and normalized by the cell size (the 2D projection area of the cell in bright field imaging). We followed previously described methods (Taniguchi et al., 2010) to remove background fluorescence and cell auto-fluorescence, and signal from dead cells (Figure S1B). After isolating persister cells, we incubated them for 30 min at 37°C (Figure 1A) with BOCILLIN. In parallel, the same staining process was carried out on a population of untreated cells that contained both persisters and susceptible cells. The fluorescent signal corresponding to antibiotic concentration in individual cells was then imaged and normalized by the cell size (the 2D projection area of the cell in bright field imaging). We followed previously described methods (Taniguchi et al., 2010) to remove background fluorescence and cell auto-fluorescence, and signal from dead cells (Figure S1B). After isolating persister cells, we incubated them for 30 min at 37°C (Figure 1A) with BOCILLIN. In parallel, the same staining process was carried out on a population of untreated cells that contained both persisters and susceptible cells. The fluorescent signal corresponding to antibiotic concentration in individual cells was then imaged and normalized by the cell size (the 2D projection area of the cell in bright field imaging). We followed previously described methods (Taniguchi et al., 2010) to remove background fluorescence and cell auto-fluorescence, and signal from dead cells (Figure S1B). After isolating persister cells, we incubated them for 30 min at 37°C (Figure 1A) with BOCILLIN. In parallel, the same staining process was carried out on a population of untreated cells that contained both persisters and susceptible cells. The fluorescent signal corresponding to antibiotic concentration in individual cells was then imaged and normalized by the cell size (the 2D projection area of the cell in bright field imaging).
Figure 1B) was only ~1/5 of that in total cells (720.5 AU/pixel, Figure 1C), indicating that the accumulated antibiotic concentration in persisters was substantially lower than that in total cells.

Additionally, we used single-cell time-lapse fluorescence microscopy to track antibiotic induced cell death and persister re-growth. As shown in Figures 1D and S1F and Movie S1, the cells with low antibiotic accumulation tolerated antibiotic attack, whereas the cells with high antibiotic accumulation gradually died and lysed. These persister cells exhibited dormancy during our time-lapse recording, featured by non-growth and non-division, but later resumed growth after the antibiotic was removed from the medium. Our observations revealed a negative correlation between the amount of antibiotic accumulated inside a cell and its probability of persisting. This proved that a reduction in the accumulation of antibiotic contributed directly to bacterial persistence.

Figure 2. High Efflux Activity in Persister Cells
(A and B) Histogram of antibiotic accumulation in total cells (n = 103) and persister cells (n = 144) of ΔompF strain.
(C and D) Histogram of antibiotic accumulation in total cells (n = 116) and persister cells (n = 109) of ΔompC strain.
(E and F) Histogram of antibiotic accumulation in total cells (n = 138) and persister cells (n = 84) of ΔtolC strain.
(G) Experimental procedure for measuring efflux rate of fluorescent antibiotic in bacterial cells.
(H) Intracellular fluorescent intensity decay after removing antibiotic in the medium is well fit by a single exponential function. Fluorescent intensity in a persister cell (red) decayed more rapidly than in a susceptible cell (green), while a cell of ΔtolC mutant (purple) showed little change in fluorescence intensity with time.
(I) Statistical analysis showing that the efflux rate of antibiotic in persister cells is significantly higher than that in total cells of wild-type E. coli (TC, total cells; PER, persisters) (p < 0.0001).

See also Figure S2 and Movie S2.

Persister Cells Exhibit Increased Efflux Pump Activity
Antibiotic accumulation in persister cells can be lowered by decreasing the membrane permeability, increasing the efflux rate, or a combination of both. To examine exactly how antibiotic levels were lowered in persisters, we constructed two porin knockout mutants (ΔompF and ΔompC) and an efflux knockout mutant (ΔtolC). Using our earlier method, we compared the effect of these knockouts on each subpopulation of persisters as well as the total cell population. OmpF and OmpC are the major porin channel proteins that facilitate penetration of β-lactam antibiotics through the outer membrane (Jaffe et al., 1982; Zier-vogel and Roux, 2013) and TolC is the central component of a number of bacterial efflux pumps, responsible for exporting cellular β-lactam antibiotics. First, for porin genes, we observed that the average fluorescence intensity of the total cell population in ΔompF mutant was 172.4 AU/pixel (Figure 2A), and in the ΔompC mutant the intensity was 181.8 AU/pixel (Figure 2C).
was 3,553.1 AU/pixel (Figure 2F), almost equal to the value in average fluorescence intensity of persisters in comparison with the value of the wild-type mutant was 3,346.6 AU/pixel (Figure 2E), which was a 4-fold decrease in intensity in comparison with the wild-type persister. This indicated that efflux plays a critical role in reducing cellular antibiotic concentration in persister cells of wild-type. Combined, these results suggest that both decreased membrane permeability and increased efflux activity contribute to the reduction of antibiotic accumulation in wild-type persisters, but that the latter may play a more critical role.

To determine quantitatively the different efflux rates between wild-type persister and total cells, we measured the fluorescence decay of individual cells incubated in microfluidic channels (Figure S2A) by time-lapse imaging, after a sudden removal of BOCILLIN in the medium (Figure 2G; Movie S2). The fluorescence intensity in each cell decayed very quickly in the initial 20 s and then plateaued (Figure 2H). We fit the initial 20 s decay of each cell to a single exponential function \( f(t) = a \exp(-bt) \), where \( a \) represented the initial intensity and \( b \) denoted the decay rate. The decay rate had contributions from both the efflux rate and the photobleaching rate of BOCILLIN. As a negative control, we showed that the fluorescence decay in the \( \Delta tolC \) mutant, where efflux had been deactivated, was very slow (Figure 2H).

Furthermore, when treating with efflux inhibitor, the fluorescence stabilized in the persisters (Figure S2B). These results demonstrated that photobleaching and diffusion made only a minor contribution to the intensity decay and that the fast decay observed in wild-type cells arose primarily from the efflux. Also, if we recorded for a longer time, the fluorescence of all cell types reached a plateau close to cell auto-fluorescence, excluding the possibility that BOCILLIN was covalently incorporated into the peptidoglycan of cells. We evaluated ~50 cells for each cell type, and the mean time constant (\( b \)) for decay was 0.32/s in persisters but 0.095/s in normal cells (Figure 2I), indicating that the efflux rate in persisters was approximately 3 times (p value < 0.0001) larger than that in total cells. This result strongly suggests that the lower intracellular antibiotic accumulation observed in bacterial persisters is dependent on their increased efflux activities.

**Multi-Drug Efflux Genes Exhibit Significantly Higher Expression Levels in Persister Cells**

Based on the above results, we hypothesized that less antibiotic accumulated in persister cells due to lower expression of porin genes and/or higher expression of efflux genes. Therefore, we performed genome-wide gene expression profiling to compare persisters and total cells using RNA-seq. Two biological replicates of persisters and total cells were prepared to assure reproducibility, and these RNA-seq results are summarized in Table S1. A large number of multi-drug efflux-associated genes showed significantly higher expression (>3-fold, p value < 0.0001) in persisters, including tolC, acrA, acrB, acrD, acrF, emrA, emrB, macA, and macB (Figure 3A). TolC is a common channel protein of both major and minor efflux systems, enabling interaction with many translocase complexes (Figure 3B). Deletion of the tolC gene alone in E. coli largely abolishes efflux activity (Figures 2E and 2F). Further analysis of sequencing data showed that the expression level of marA, one important transcriptional activator of the tolC operon, exhibited a far higher expression level in persisters (>10-fold, p value < 0.0001), whereas other genes ygiA, ygiB, and ygiC, from the tolC operon (Figure 3C), showed similar expression levels to tolC. In contrast,
the porin genes ompF and ompC showed only slightly higher
transcriptional levels in persisters, indicating more porin chan-
nels on the membrane of those cells. Together these results
demonstrated that higher efflux rate, rather than lower mem-
brane permeability, was responsible for reduced antibiotic accu-
mulation in persister cells. qRT-PCR (Figure 3D) agreed with
sequencing results and confirmed that these efflux genes were
upregulated in persister cells, whereas β-lactam influx porin
genes ompF and ompC had a similar expression level or were
only slightly upregulated in persister cells. Both transcriptome
sequencing and qRT-PCR results revealed that tolC expression
in persisters was at least 8-fold greater than that in total cells,
making it a potential biomarker to differentiate persisters from
normal cells. Whole-genome sequencing (Table S2) and qRT-
PCR (Figure 3D) of persister and regrown populations confirmed
that enhanced expression of efflux genes is transient and revers-
able, featuring a non-genetic factor that assists bacterial
persistence.

High Expression Level of tolC Directly Assists Bacterial
Persistence

We next used single-cell fluorescence imaging to measure the
dependence of bacterial persistence on expression level of the
central efflux component TolC. First, we monitored the natural
fluctuation in concentration of TolC among the cell population.
Since trimers of TolC form barrel-like channels in functioning
efflux pumps on the outer membrane (Du et al., 2014), conven-
tional labeling of TolC by fluorescent proteins has been diffi-
cult. To solve this, we inserted a six amino acid tetracysteine tag into
the linker structure of chromosomally encoded tolC (TC-tag-TolC
strain, 76RP). This had little impact on either TolC expression
level or function (Figures S3A–S3C). In this way, the tagged
TolC protein was fluorescently labeled by tetracysteine-based
protein detection (FIAsH labeling) (Griffin et al., 1998). As shown
in Figure 4A (upper panel), the fluorescence intensity of TolC-
FIAsH across a population of total cells was distributed in a
broad gamma distribution with a long tail at high fluorescence,
indicating a noticeable heterogeneous expression of tolC among
different cells.

We monitored the antibiotic killing process under a microscope
using time-lapse imaging at controlled temperature. After 4 hr
treatment with high concentration (150 μg/ml) carbenicillin, the
majority of the cells had died and lysed, leaving only a few surviv-
ing persisters. By tracking the spatial coordinates of these persist-
er cells, we found their original positions in the cell popu-
lation and calculated their TolC-FIAsH fluorescent intensity prior
to the antibiotic attack. We found persisters emerged mostly from
the long high-intensity tail of the TolC-FIAsH fluorescence distri-
bution (81.0% of persister cells arose from 8.5% of total cell pop-
ulation with highest tolC expression) (Figure 4A, lower panel).

Typical examples capturing the dynamic process of persister
formation are shown in Figures 4B and S3D and Movie S3. In a
single field of view, many cells were susceptible to carbenicillin
and lysed gradually. They were featured by low TolC-FIAsH in-
tensity. In contrast, persisters occurred rarely and showed high
intensity in TolC-FIAsH fluorescence. We confirmed again that
the persister cells neither grew nor divided during our time-lapse
recording, consistent with cell dormancy. We then tested
whether these cells could resurrect when antibiotics were
removed from the growth medium. As shown in Figure 4B and
Movie S3, a short delay after antibiotic removal, the persisters,
indicated by high fluorescence intensity of TolC-FIAsH, began
to elongate, divide, and produce new microcolonies.

Having observed that all persisters exhibit higher tolC expres-
sion, we sought to examine whether all cells displaying high tolC
expression were more likely to form persisters. We utilized FACS
to group cells according to their TolC level, quantified by FIAsH
staining in the 76RP strain (Figure 4C). Both staining and cell
sorting did not affect persister formation frequency (Figure S3E).
The antibiotic sensitivity assay was subsequently performed on
each group of cells to evaluate the frequency of persister forma-
tion. As presented in Figures 4D and 4E, the highly fluorescent
subpopulation (1%, group C) expressing TC tag-TolC showed
18-fold enrichment in the number of persister cells in comparison
with that of the total population (100%, group A), and 24-fold
enrichment in comparison with the remaining dimmer population
(97.8%, group B). Altogether, this positive correlation between
tolC expression level and probability of persister formation sug-
gested that high expression level of tolC is critical to promote
persister formation.

Bacterial Persistence Negatively Correlates with
Intracellular Antibiotic Accumulation and Positively
Correlates to Efflux Activity

We quantified the correlation between efflux gene expression;
intracellular antibiotic accumulation; and persister formation fre-
quency using wild-type E. coli, ΔtolC, tolC overexpression, and
tolC rescue strains. First, we measured the tolC gene expression
by qRT-PCR. In the overexpression strain, tolC concentration
was about 3-fold of that in the wild-type strain; no tolC expres-
sion was detected in the ΔtolC strain; and in the rescue strain,
tolC expression returned to a similar level as that in the wild-
type strain (Figure 5A). We measured the antibiotic accumulation
in these strains with BOCILLIN using the above method. Fluores-
cent antibiotic accumulation was negatively correlated with tolC
expression, with significantly higher intensities (~2,600 AU/pixel)
in the ΔtolC strain and lower intensities (~10 AU/pixel) in the
overexpression strain. In the rescued strain, antibiotic accumula-
tion was at a similar level as that in the wild-type strain (Fig-
ure 5B). We then used an antibiotic sensitivity assay to measure
the frequency of persister formation. The initial concentration of
these strains were all set to be ~5 × 10^5 cells/ml before antibiotic
treatment. While wild-type E. coli produced ~3 × 10^4 cells/ml
antibiotic-tolerant persisters, the ΔtolC mutant produced only
~50 cells/ml (Figures 5C and 5D). The overexpression strain pro-
duced ~1.6 × 10^5 cells/ml persister cells, roughly 5-fold the
number produced by the wild-type strain (Figures 5C and 5D).
The rescued strain recovered the low-persister frequency in the
ΔtolC strain (Figures 5C and 5D). Thus, the tolC expression
level was negatively correlated to the intracellular antibiotic
accumulation, and positively correlated to persister formation.

Efflux Pump Inhibitors Combined with Antibiotics to
Eradicate Persisters

Since persister formation frequency was dramatically reduced in
efflux knockout strains (Figures S4A and S4B), we tested a
therapeutic strategy that combined antibiotics with efflux pump inhibitors (EPIs). Phenylalanine arginyl β-naphthylamide (PAβN) and 1-(1-Naphthylmethyl) piperazine (NMP) are well studied inhibitors that can effectively block TolC-composed efflux pumps through competitive substrate export. When treating wild-type E. coli with carbenicillin, the addition of PAβN had little effect, but the addition of NMP greatly improved lethality. The persister number was reduced to less than 20% of the number surviving when carbenicillin was applied alone (Figure 5E). We further tested if such potentiating effect was observable with other antibiotics. Starting again from an initial concentration of 5 × 10^7 cells/ml, the persister fraction in cells treated with cloxacillin (β-lactam) was ~5 × 10^4 cells/ml. The addition of PAβN and NMP reduced this number to ~8 × 10^3 cells/ml and ~3 × 10^4 cells/ml (Figure 5F), about 17% and 12% of the number that survived in the absence of inhibitors, respectively. The same effect was also observed in bacteria treated with nalidixic acid (quinolone), which produced ~2 × 10^6 cells/ml persisters. The addition of PAβN and NMP reduced this number to ~2.5 × 10^6 cells/ml and ~5 × 10^4 cells/ml (Figure 5G), about 0.5% and 3% of the number surviving without inhibitors, respectively. Those EPIs alone did not influence cell growth (Figure S4C). These results show that the combining antibiotics with EPIs offers therapeutic promise in the fight against bacterial persisters.
Active Efflux Plays a Primary Role in the Drug Tolerance of Persister Cells

Further analysis of our transcriptome sequencing data identified several other groups of genes that showed differential expression between persister and total cells (Figure 6A; Table S1), implying multiple pathways that can be involved in persister formation. Global regulators that mediated downstream pathways leading to cell dormancy, including dnaJ, dnaK, hupA, hupB, ygfA, and yigB (Hansen et al., 2008), presented higher expression level in persister cells than in total cells. Toxin genes and their regulators from the TA modules hipA, crp, dksA, spoT, lon, and relA displayed upregulation in persister cells, whereas antitoxin gene hipB showed no changes between the two cell types (Balaban et al., 2004; Amato et al., 2013; Hansen et al., 2008; Maisonneuve et al., 2011, 2013). Oxidative stress response related genes, including soxS (Wu et al., 2012), pspA (Vega et al., 2012), and ansA, were also upregulated in persisters, as well SOS response genes dinG, uvrD, ruvA, and ruvB (Theodore et al., 2013). Finally, slow metabolism-related genes plsB (Spoering et al., 2006) and phoU (Li and Zhang 2007) were also highly expressed in persisters. The higher expression of these genes promotes a cell’s entry into the dormant state. However, unexpectedly, efflux pumps were actively working in those dormant cells, conferring upon them an increased capacity to tolerate antibiotic attack.

In order to rank the individual genes contributing to persistence, we constructed two libraries: a fluorescent protein EGFP fusion library and an ordinary knockout library covering reported genes associated with persistence. For the EGFP fusion library, we again used FACS to isolate cells with high fluorescence intensity (1%) and then performed the antibiotic sensitivity assay on these cells and compared that with the assay on the total population (100%). The results revealed that cells with higher expression levels of tolC showed the largest increase in bacterial persister enrichment (18.6-fold, p value = 0.00008) (Figure 6B). On the other hand, higher expression of other persistence related genes had less impact on persister enrichment (Figure 6B), of which pspA was the only gene that caused a significant increase in persister formation (3.2-fold, p value = 0.007). For the knockout library, we performed the antibiotic sensitivity assay to determine the effect of each knockout on bacterial survival rate. The strain carrying a deletion of tolC suffered the greatest reduction (29-fold) in persister formation following treatment with carbenicillin. Deletion of other individual persistence-related genes had no impact on persister enrichment.
genes caused a lower reduction in persister formation (Figure 6C), including ΔansA (14-fold), Δcrp (14-fold), ΔdnaK (6-fold), ΔhupA (3-fold), ΔhipA (9-fold), and ΔuvrD (11-fold). According to previous research (Maisonneuve et al., 2011), single deletion of lon led to a significant reduction in persister formation (similar to our results here), and the effect was comparable to that caused by deletion of 10 TA genes together. Of note, our results show that deletion of tolC results in a 29-fold reduction in persister formation, a three-times further decrease when compared to that caused by deletion of lon.

The above results suggest that multiple mechanisms can lead to persistence, and while the contribution of each mechanism varies, the efflux system holds the most significant role in persister formation, at least in our experimental conditions. Our observations highlight the precise control behind persister formation. Bacterial persisters manage to combine two seemingly contradictory mechanisms to survive antibiotic attack: they slow down most physiological processes during dormancy and at the same time activate their efflux systems to remove intracellular antibiotics.

**DISCUSSION**

Gene expression is intrinsically stochastic. The variation in synthesis and breakdown of molecules in biochemical reactions that initiate and regulate transcription and translation gives rise to the heterogeneity in amount of protein expressed in a population of cells (Huang, 2009; Elowitz et al., 2002; Raj and van Oudenaarden, 2008). This was recently demonstrated by a high-throughput system-wide analysis (Taniguchi et al., 2010). In our experiments, we have observed that populations of bacteria have a heterogeneous distribution of efflux pumps due to stochastic gene expression. The subpopulation with the higher expression of efflux genes is able to pump more antibiotics outside the cell to reduce intracellular antibiotic accumulation and is thus more likely to persist. Our observation is consistent with a previous study (Allison et al., 2011a) in which the authors introduced metabolites to induce antibiotic uptake, eradicating bacterial persisters and implying that a lower antibiotic concentration inside the cell is key to being persistent.

**Pre-existing versus Induced Expression of tolC in Persister Cells**

Whether this overexpression of efflux genes preempts drug treatment or is induced by antibiotic attack as a protective response in persisters remains inconclusive. Figure 4A shows that surviving persister cells belong to cells with higher fluorescent intensity of TolC-FIAsh, indicating such upregulation of efflux genes in persister cells may preexist. However, we cannot rule out the possibility that some of this higher expression was induced by drug treatment. Recent reports have found that indole signaling contributes to persister formation, possibly via upregulation of the efflux pumps through activation of the two-component systems BaeS-BaeR or CpxA-CpxR and the PspAB and OxyR pathways as a result of stress response (Vega et al., 2012; Hirakawa et al., 2005). Additionally, at a population level,
indole molecules produced by highly resistant cells have been shown to provide protection to other more susceptible cells by activating their drug efflux pumps and oxidative-stress protective mechanisms (Lee et al., 2010). This facilitated the survival of the whole population. We believe this population effect may play a role in persister formation; however, the exact ratio between pre-existing and drug induced persisters deserves further investigation.

**Lack of Cooperation between Porin Genes and Efflux Genes**

There are two ways to reduce intracellular antibiotic concentration: to lessen the membrane permeability by expressing fewer porin channels and to enhance efflux pump expression and activity. In our study, we proved that the expression of efflux genes was upregulated in persister cells. However, unexpectedly, we found that the porin gene *ompF*, which is critical for β-lactam antibiotic transportation, was also slightly upregulated in persister cells. Such increased expression of porin genes would amplify antibiotic entry and therefore lead to cell death, attenuating the chance of persisting. We do not understand the mechanism underlying this upregulation of *ompF* in persister cells, yet this further highlights the significant role of efflux pumps in bacterial persistence. It is possible that TolC protein might exert its effect on *ompF* promoter area or the terminal end of *ompF* gene to regulate gene expression (Misra and Reeves, 1987). Therefore, the higher expression of *ompF* in persister cells might be a side-effect caused by upregulation of tolC, as previous work has shown that downregulation of tolC led to a reduction in *ompF* expression (Morona and Reeves, 1982).

**Use of Efflux Inhibitors in Eradicating Bacterial Persisters**

To effectively treat chronic infectious diseases, specific anti-persistence therapies are required to eradicate persisters or to prevent their formation. Several genes that contribute to persister formation are current targets for drug design, including *hipBA*, *tisAB*, *phoU*, *edpA*, and *plsB* etc. (Fauvart et al., 2011). EPIs can be combined with conventional antibiotics to reduce efflux pump-associated intrinsic resistance or acquired multidrug resistance (Zechini and Versace, 2009). For example, broad-spectrum EPI PAIPiN enhanced the lethality of levofloxacin against drug-resistant *Pseudomonas aeruginosa* (Lomovskaya et al., 2001), and another pump inhibitor-verapamil reduced macrophage-induced drug tolerance in *Mycobacterium marinum* (Adams et al., 2011). In our current study, we have demonstrated that EPIs work synergistically with different antibiotics to reduce persister formation. This further emphasized the need to match specific EPIs with antibiotics to treat persistent bacterial infections.

**Latent but Active: Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells**

Previously, cell dormancy was considered as the leading mechanism resulting in bacterial persistence (Balaban et al., 2004; Rotem et al., 2010). In these cells, antibiotics failed to eradicate bacterial cells due to dormant downstream pathways, though antibiotics still bound to their molecular targets. It has long been proposed that multiple mechanisms contribute to bacterial persistence (Lewis, 2010; Allison et al., 2011b), and previous studies have identified many dormant pathways and genes related to persistence. These include global regulators (Hansen et al., 2008) that lead to slower cell growth, toxin genes in TA modules (Balaban et al., 2004; Maisonneuve et al., 2011) that induce a dormant state, oxidative stress response pathways (Vega et al., 2012; Wu et al., 2012), SOS response pathways (Theodore et al., 2013), and metabolism pathways (Spoering et al., 2006; Li and Zhang 2007). However, dormancy alone cannot explain persistence (Orman and Brynildsen, 2013b). We used transcriptome sequencing to identify upregulation of an additional group of efflux genes that contribute to persister formation and further established that cells with upregulated efflux genes showed elevated persistence while efflux knockout mutants showed attenuated persistence. Together, this suggests efflux pumps play a critical role in bacterial drug tolerance.

As demonstrated by our results, in addition to a “passive defense” via dormancy, bacterial persisters employ an “active defense” to pump antibiotics out and reduce intracellular drug concentration through enhanced efflux activity. Our findings indicate that while most biological processes in persisters are slowed down, the efflux systems, on the contrary, become more active and effectively promote antibiotic persistence. It is surprising to see that active efflux and dormancy coexist in many persister cells since dormancy seems to exclude active efflux. How bacterial cells control precisely which pathways to shutdown and which to activate, and whether dormancy and active efflux are intrinsically co-regulated, remains unknown. Our observation that persisters combine two contradictory mechanisms to survive antibiotic attack highlights the advanced network regulation endowed by natural selection, and also the challenges we face in eradicating these drug tolerant cells.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmid Construction**

Wild-type strain BW25113 and its tolC knockout strain (JW56503) were gifts from Yale Genetic Stock Center. The detailed information for the construction of tolC overexpression strain (Figure S5A), tolC rescued strain, 76RP strain (Figure S5B), and other strains are provided in Supplemental Experimental Procedures. Primers used are listed in Table S3.

**Antibiotic Killing and Persister Isolation**

Frozen stocks of *E. coli* strains were diluted by 1:1,000 into LB medium and cultured overnight. The overnight culture was diluted in fresh LB to appropriate concentration (OD600 = 0.2), which was then split into two flasks. To one flask carbenicillin (150 μg/ml, Sigma) was added at a desired concentration to yield persisters. To the other flask the same volume of sterile Milli-Q (which was the solvent of carbenicillin solution) was added to yield total cells. The cultures were returned to the 37°C shaker for 4 hr. Then the cells were collected by centrifuging at 4000 g for 5 min and washed with M9 minimal medium with 150 μg/ml carbenicillin three times to isolate unlysed persister cells and total cells. To confirm that the persisters we analyzed were from the second killing phase, the biphasic killing curves of each strain were shown in Figure S6.

**Fluorescent Microscopy**

All the imaging work was performed on an inverted microscope (Nikon Eclipse Ti). The illumination was provided by different solid state lasers (Coherent), at 488nm for BOCILLIN and FlAsH, and 532nm for propidium iodide, respectively. The fluorescent signal of cells was collected by an EMCCD camera (Photometrics Evolve 512). The appropriate filter sets were selected for each
Supplemental Information includes six figures, three tables, three movies, and six pages of text. The full information can be accessed at the provided DOI: 10.1016/j.molcel.2016.03.035.

Supplemental Information

Sequencing data have been deposited at the NCBI sequence read archive under accession number SRP072433.

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Flow Cytometry Analysis and Cell Sorting

All samples were measured on a BD FACSAria III flow cytometer. FISh labeling 76RP strain or chromosomal genex-egfp translational fusion strain in the stationary phase was resuspended in sterile 1XPBS, which was also used as sheath fluid in flow cytometer. Microorganisms were identified by FSC (forward scatter) and SSC (side scatter) parameters. Cells were sorted into three groups based on their fluorescence intensity (488-nm excitation with 530/30-nm-band-pass filter) using a 70 μm nozzle. Approximately 100,000 cells were collected in each group. The results were analyzed by FlowJo V10 software (Treestar, Inc.).

Antibiotic Sensitivity Assay

The overnight cultures of E. coli strains were diluted by 1:20 into fresh LB with antibiotic carbenicillin (final concentration of 0 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml, and 160 μg/ml, respectively). Then the culture was returned to the 37°C shaker for another 4 hr. Samples were withdrawn and appropriately diluted in LB medium and spotted on an LB agar plate for overnight culture at 37°C. Colony counting was performed the next day. For the inhibitor assay, PAIn (sigma) or NMP (sigma) at a final concentration of 100 μM was added into medium at the same time points with antibiotics.

ACCESSION NUMBERS

Sequencing data have been deposited at the NCBI sequence read archive under accession number SRP072433 (http://www.ncbi.nlm.nih.gov/sra/SRP072433).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, three tables, three movies, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.03.035.

AUTHOR CONTRIBUTION

Y.P., Z.Z., and Y.L. designed the study; carried out experiments; and analyzed the data; J.Z., Y. Zhao, and Y. Zhu performed mutagenesis experiments; Q.M., H.G., Y.S., Y.K., and M.A.B.B. analyzed the sequencing data and performed related bioinformatics analyses; F.B. and X.S.X. designed and supervised the study; Y.P., Z.Z., Y.L., and F.B. wrote the manuscript with contributions from H.C. and M.A.B.B.
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