GENES RESPONSIBLE FOR ANTIBIOTIC TOLERANCE IN ESCHERICHIA COLI

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ABSTRACT OF DISSERTATION

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ABSTRACT

Bacterial populations stochastically produce a small number of non-growing or dormant persister cells that are tolerant to antibiotics. Persisters are phenotypic variants that are genetically identical to the susceptible cells within a clonal population. Persisters contribute to the antibiotic recalcitrance of biofilm infections. A number of recent studies point to the involvement of toxin/antitoxin (TA) systems in persister formation. A strain with two point mutations in hipA, the toxin of the hipBA TA system, produced 10,000 times more persisters than the wild type. Similarly, overexpression of HipA caused a sharp increase in the persister fraction. However, deletion of hipA did not produce a phenotype suggesting that multiple genes/pathways cause persister formation. The antitoxin HipB represses the hipBA operon by cooperative binding to four operator sites and inactivates the toxin HipA. The crystal structure of the HipA-HipB-DNA complex revealed that one HipB dimer is sandwiched by one molecule of HipA on each side and that HipB induces a 70° bend in the operator DNA. HipA is a kinase with eukaryotic serine/threonine fold. EF-Tu was identified as a target of HipA suggesting that HipA causes protein synthesis inhibition and dormancy upon phosphorylation of EF-Tu. For HipA to be active, HipB must be removed or degraded. Antitoxins are typically degraded by one of the ATP-dependent proteases. HipB was stabilized in lon· background and also degraded by Lon in vitro demonstrating that Lon is the main protease responsible for HipB proteolysis. The unstructured C-terminus of HipB is critical for rapid proteolysis as a truncated HipB appears to be stabilized.

To identify additional genes responsible for persister formation, we performed a screen for mutants with altered antibiotic tolerance using an ordered library of 3,985 Escherichia coli knockout strains. We exposed stationary-state cultures in 96-well plates to ofloxacin at a
concentration well above MIC and determined the persister cell level of each culture. 10 mutants with decreased persistence but no difference in growth rate or ofloxacin MIC compared to wild type were identified. Two putative persister genes, yigB and ygfA, encoding a FMN phosphatase and a 5-formyl tetrahydrofolate (THF) cyclo-ligase, respectively, were further validated.
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Chapter 1: Introduction

Bacterial Persistence

Bacterial populations harbor a subpopulation of persister cells that survive lethal doses of antibiotics. The surviving persister fraction makes up $10^{-6}$ to $10^{-4}$ of the population in exponentially growing cultures and $\sim 10^{-2}$ in stationary phase (25). Upon reinoculation in fresh medium persisters produce a culture with a similar level of persister cells demonstrating that persisters are phenotypic variants, which are genetically identical to the susceptible cells within a population, rather than resistant mutants (Fig.1) (19).

Figure 1. Biphasic killing of a bacterial culture and persister heritability. An exponentially growing culture of \textit{E. coli} was exposed to a high dose of ampicillin. The bulk of the population collapses rapidly leaving onlypersisters intact. After lysis surviving persisters were reinoculated and reexposed to ampicillin. The procedure was repeated 3 times. The dashed line indicates how an ampicillin resistant strain would have behaved. Adapted from (23) and based on (19, 42).
Addition of a second bactericidal antibiotic to the persister fraction produced little or no additional killing showing that persisters are multi-drug tolerant (42). Persisters are tolerant to all currently available antibiotics and are found in all species examined (25).

Analysis of single cells of an *E. coli* high-persistence mutant, *hipA7*, treated with ampicillin revealed that the surviving cells were slow or non-growing already prior to treatment (3). In addition, persisters were isolated from exponentially growing *E. coli* wild type cells on the basis of the expression of a transcriptional reporter in non-persisters, indicating that persisters are in a dormant state (37). It appears that bactericidal antibiotics fail to be effective against persister cells because their targets are inactivated or non-functional in dormant cells. In contrast, resistant mutants survive because the antibacterial agent fails to reach or bind its target (Fig. 2) (23, 25). Moreover, recently it has been shown that fluoroquinolones generate some double strand breaks even in persister cells and that these are actively repaired suggesting that critical repair pathways are active in otherwise metabolically down regulated cells (12).

![Figure 2](image.png)

**Figure 2. Model of antibiotic tolerance in comparison to antibiotic resistance.** Bactericidal antibiotics kill cells by forcing the active target to produce corrupted products. Persister proteins act by blocking the target, so no corrupted product can be produced. By contrast, all resistance mechanisms prevent the antibiotic from binding to the target. Adapted from (23).
Persister cells and infectious disease

Biofilms are implicated in an estimated 60% of all bacterial infections. (9, 11, 14, 29, 33). Biofilms are bacterial communities, which have settled on a surface and are protected by an exopolymer matrix (14). The vast majority of bacterial species forms biofilms. Biofilms of medical relevance are produced e.g. by *Pseudomonas aeruginosa* which causes infections in the lung of cystic fibrosis patients and *Staphylococcus aureus* and *Staphylococcus epidermidis* which cause infections of indwelling devices (25, 30, 38). Though biofilm infections are notoriously hard to eradicate, biofilms are not more resistant to antibiotics than planktonic since cells in the biofilm do not grow in the presence of an antibiotic (6, 25). The recalcitrance of biofilms has been attributed to the exopolymer matrix, which serves as physical barrier for certain antibiotics and immunity factors (26), to slow growth of cells within the biofilm (26), and to the presence of persister cells in the biofilm (24, 25, 39). Fluoroquinolones and metal oxyanions are bactericidal agents, which pass the matrix and are effective against slow or non-growing cells (16, 17, 39) but even these agents cannot eliminate a subpopulation of tolerant cells in the biofilm. Therefore, persister cells largely contribute to the recalcitrance of biofilm infections. In an *in vitro* setting, the number of tolerant persister cells in the biofilm was similar to the number of persister cells in planktonic stationary phase cells. However, *in vivo* biofilms present a bigger challenge than planktonic cells, since immune factors eliminate planktonic cells more easily than biofilm persister cells (Fig. 3).
Figure 3. Model of relapsing biofilm infections. Regular cells and persister cells form in the biofilm and are shed off into surrounding tissue and the bloodstream. Antibiotics kill regular cells, and the immune system eliminates escaping persister cells. The matrix protects persister cells from the immune system, and when the concentration of the antibiotic drops, they repopulate the biofilm, causing a relapse. Adapted from (23).

Moreover, recently it has been shown that *P. aeruginosa* high persister (*hip*) mutants were selected for over the course of infection in cystic fibrosis patients, and also *Candida albicans hip* mutants were selected for in patients with oral thrush (22). These findings indicate that persisters are largely responsible for failure to eradicate chronic infections (23).

Understanding the mechanism of persister cell formation is likely to lead to new effective therapies for the treatment of biofilm infections. However, research in this area has been hindered by the difficulties in identifying persister genes.
Mechanism of Antibiotic Tolerance

The first gene linked to persistence was identified in a targeted search for *E. coli* mutants with high levels of persistence. A strain with two point mutations in *hipA* (hipA7 allele) was discovered which produced 1,000-10,000 times more persisters than the wild type (4, 5, 31, 32, 35). Wild type HipA also leads to growth arrest and high persistence when overproduced (8, 21). *hipA* is part of a two gene operon, *hipBA* which encodes a toxin/antitoxin (TA) pair. TA systems were originally discovered on plasmids where they serve as maintenance systems but are also common on the chromosome where their role is largely unknown (13). HipA is a kinase with a eukaryotic Ser/Thr kinase-like fold (8). In contrast, all other toxins of type II TA modules (protein antitoxin/protein toxin) group into either gyrase inhibitors (ParD, CcdB), mRNA interferases (RelE, MazF, YoeB, HicA, Doc) (13, 18, 41) or PIN domain fold proteins (VapC) (1, 28). Replacing the conserved amino acids in the phosphorylation site (S150A) or the Mg\(^{2+}\)- or catalytic binding sites (D332Q and D309Q, respectively) of HipA abolishes the ability to confer growth arrest and antibiotic tolerance. We identified Elongation factor Tu (EF-Tu) as a HipA target suggesting that HipA causes persistence via phosphorylation of EF-Tu (36). Indeed, Thr\(^{382}\)-phosphorylated EF-Tu leads to stasis since it can no longer bind aminoacyl-tRNA (36). It is likely that EF-Tu is not the only target of HipA since overexpression arrests both transcription and translation (21, 36).

The antitoxin HipB represses the *hipBA* operon by cooperative binding to four operator sites (4, 5, 36). HipB also binds to and thereby neutralizes the toxin HipA. The crystal structure of HipA-HipB-DNA revealed that a HipB dimer is sandwiched by one molecule of HipA on each side. Under the standard regime of batch culture growth HipA is inhibited by the tight interaction of HipB. To activate HipA and its persistence function, the antitoxin HipB has to be
removed or degraded. The C-terminus of HipB appears to be unstructured which suggests a mechanism of HipA activation by proteolysis of HipB (36). Proteolytic regulation of the antitoxin has been demonstrated for several TA modules. In *E. coli*, the chromosomally encoded TA modules MazEF, RelBE, YefM/YoeB, HicAB and DinJ/YafQ are regulated by the AAA+ ATP-dependent proteases Lon and/or ClpPX (7, 13, 34). ClpPA degrades PhD antitoxin of the plasmid-encoded PhD/Doc TA module (13). The other two ATP-dependent proteases HslVU and FtsH do not have any known antitoxin substrates. HipB does not share any homology with any of the known antitoxins. Neutralization of its cognate toxin also differs mechanistically from other TA modules (13). We show that despite structural and functional differences, Lon is also the main protease responsible for HipB degradation. Our data suggests that Lon recognizes the unstructured C-terminus of HipB.

While the gain-of-function allele *hipA7* and HipA overproduction caused high levels of persistence, a deletion of *hipA* gene produced no phenotype (20), arguing that multiple redundant pathways are likely involved in persister formation and additional components remain to be discovered. Concordantly, ectopic expression of two other toxins, RelE and MazF, also strongly increased tolerance to antibiotics, whereas a deletion of the toxin had no phenotype (25).

One exception of a toxin/antitoxin system where the deletion of the toxin showed a pronounced decrease in the level of persisters is the toxin TisB. The toxin is part of the type I TA module (small RNA antitoxin/protein toxin) *tisAB*. It is a membrane-acting peptide, producing dormancy due to decreases in proton motif force and ATP levels (12). TisB is induced by the SOS response, and becomes the main mechanism of persister formation during SOS response, therefore under specific conditions a deletion has a phenotype.
Not only TA systems play a role in the molecular mechanism underlying persistence. Expression cloning points to enzymes of phospholipid synthesis, in particular the glycerol 3-phosphate dehydrogenase GlpD and the glycerol 3-phosphate acyltransferase PlsB, being involved in persister maintenance (40). Initial efforts to find specific persister genes by analyzing the levels of persisters in clones from a transposon insertion library have been unsuccessful (27, 40). PhoU, the repressor of the pho regulon was considered as a persister gene. However, deletion of phoU alters susceptibility of the cell, and therefore points to a resistance mechanism rather than a persistence mechanism (27).

We decided to revisit this approach using an ordered knockout library of all ORF-coding non-essential E. coli gene disruptions (Keio collection) (2, 15). We introduced several changes that considerably improved the quality of screening: the growth medium was optimized to decrease variability among parallel samples; the screen was performed in stationary state, where persisters are most abundant, and all cultures reach a similar growth end-point; a method that allows for sensitive screening without multiple dilutions was introduced. This involved plating persister cells surviving antibiotic challenge on a medium containing a different antibiotic, mecillinam. In this way, only spontaneous mutants resistant to mecillinam survive, which reduced the number of surviving cells, obviating the need for multiple dilutions. We find that several global regulators (DnaK, DnaJ, DksA, HU, IHF) as well as phosphatase YigB, and 5-formyl tetrahydrofolate (5-formyl THF) cyclo-ligase YgfA are involved in persister formation. It is important to stress that none of the knockouts had a complete lack of persisters. In most cases the drop was around 10-fold. Redundancy of persister genes has also been observed in P. aeruginosa. A screen of a transposon insertion library produced also produced multiple hits with moderate phenotype (dinG coding for a helicase under SOS control, spuC coding for a putrescin
aminotransferase and two genes of unknown function) but failed to identify a strain that produces no persisters (10).

The preponderance of global regulators and chaperones among the genes affecting persistence strongly suggests that the function is encoded by redundant genes and/or mechanisms. Persister genes are probably among the many that are controlled by these regulators. This strongly points to redundancy in persister genes.

**Figure 4. Redundant pathways of persister formation in E. coli.** Candidate persister genes and their targets, when known, are indicated. Abbreviations: FMN, flavin mononucleotide; pmf, proton motive force; TA, toxin/antitoxin modules. Adapted from (23).
REFERENCES


Chapter 2: Molecular Mechanisms of HipA-Mediated Multidrug Tolerance and Its Neutralization by HipB


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ABSTRACT

Bacterial multidrug tolerance is largely responsible for the inability of antibiotics to eradicate infections and is caused by a small population of dormant bacteria called persisters. HipA is a critical Escherichia coli persistence factor that is normally neutralized by HipB, a transcription repressor, which also regulates hipBA expression. Here, we report multiple structures of HipA and a HipA-HipB-DNA complex. HipA has a eukaryotic serine/threonine kinase–like fold and can phosphorylate the translation factor EF-Tu, suggesting a persistence mechanism via cell stasis. The HipA-HipB-DNA structure reveals the HipB-operator binding mechanism, ~70° DNA bending, and unexpected HipA-DNA contacts. Dimeric HipB interacts with two HipA molecules to inhibit its kinase activity through sequestration and conformational inactivation. Combined, these studies suggest mechanisms for HipA-mediated persistence and its neutralization by HipB.
INTRODUCTION AND RESULTS

Bacteria that are resistant or tolerant to antibiotics are an increasing threat to human health. Indeed, ~60% of infections in the developed world are caused by biofilms, which exhibit multidrug tolerance (MDT) (1, 2). MDT is caused by the presence of dormant bacterial cells called persisters, which account for only $10^{-6}$ to $10^{-4}$ cells in a growing population, making MDT difficult to study (3–5).Persisters are not mutants but phenotypic variants of wild-type cells that evade killing by somehow adopting a transient dormant state (6, 7). Dormancy provides protection because bactericidal antibiotics kill by corrupting their active targets into producing toxic byproducts. These protected persisters can then switch back to the growth phase after the removal of antibiotics, allowing the bacterial population to survive. The first high-persistence allele, $hipA7$ (high-persistence A), was identified in *Escherichia coli* and increased the frequency of persistence by 10,000 fold (8–10). *E. coli hipA* encodes a 440-residue protein, HipA, which is cotranscribed with a smaller upstream gene, hipB. HipB is an 88-residue protein that represses the *hipBA* operon by binding cooperatively to four operators upstream of *hipBA* (11, 12). HipB forms a complex with HipA, and because wild-type HipA cannot be expressed in the absence of HipB because of its deleterious effects on cell growth, *hipBA* has been categorized as a toxin/antitoxin (TA) module in which HipA, the toxin, is neutralized by the antitoxin, HipB (13, 14). Toxin proteins from chromosomally encoded TA modules, of which more than 10 have been identified in *E. coli*, appear to promote cell dormancy and may play roles in the development of persistence under certain conditions (5, 7). Chromosomal TA modules can be grouped into three main superfamilies based on whether the toxin has a ribonuclease (RNase)/gyrase–like fold, RNase barnase–like structure, or a PilT N-terminus (PIN) domain (14). The corresponding antitoxins contain DNA binding domains and C termini that are largely unfolded until bound by
the toxin (14). HipA and HipB show no homology to any member of these TA superfamilies. Moreover, HipA is one of the few validated biofilm tolerance factors. Indeed, it has been demonstrated that overexpression of the HipA protein leads to MDT in *E. coli* (2). However, the mechanism of HipA-mediated MDT is unknown.

To delineate the functions of HipA and HipB in MDT, we carried out biochemical and structural studies on HipA and HipA-HipB-DNA complexes. Because of wild-type HipA–mediated persistence, we used the HipA mutant Asp$^{309} \rightarrow$Gln$^{309}$ (D309Q) (referred to as HipA), which can be produced in large quantities in the absence of HipB (15). The structure of HipA was solved to 1.54 Å resolution and refined to an $R_{\text{work}}/R_{\text{free}}$ of 19.5/23.2% (table S1 and fig. S1) (16–18). The HipA structure has a globular fold with 15 $\beta$ strands and 15 $\alpha$ helices and can be divided into an N-terminal $\alpha/\beta$ domain and an all–$\alpha$-helical C-terminal domain (Fig. 1A). Density is missing for residues 185 to 195, which are near the active site and probably correspond to the activation loop of other kinases. Structure based homology searches revealed that HipA is most similar to human CDK2/cyclin A kinase (19). The structural homology between HipA and CDK2 was highest in the C-terminal region that contains CDK2 catalytic residues, suggesting that HipA functions as a protein kinase, as reported (15). Although HipA is most similar to CDK2, the proteins superimposed with a large root mean square deviation (RMSD) of 3.9 Å for 150 corresponding C$\alpha$ atoms, indicating that HipA represents a previously unknown class of protein kinase (20).

HipA contains all the catalytic residues found in protein kinases, including the putative catalytic base Asp$^{309}$ (20). The D309Q mutation abrogates persistence, strongly suggesting that kinase function is key to HipA-mediated MDT tolerance. Indeed, we found that HipA binds adenosine triphosphate (ATP) with a dissociation constant $K_d$ of $18.0 \pm 2.0 \mu$M, which is similar
to the dissociation constant obtained for ATP binding to other serine/threonine kinases (fig. S2) (20). To delineate the ATP binding mechanism of HipA, we determined the structure of the HipA-ATP-Mg$^{2+}$ complex to 1.66 Å resolution and refined the structure to an $R_{\text{work}}/R_{\text{free}}$ of 18.4/21.7%. Density for ATP is observed in the cleft between the HipA N and C domains (fig. S1). HipA binds ATP with high selectivity (Fig. 1B). Specifying contacts are provided to the adenine N6, N1, and N3 atoms by the carbonyl oxygen of Glu$^{234}$, the amide nitrogen of Phe$^{236}$, and the side chain Ne of Gln$^{252}$, respectively. The adenine ring stacks with Phe$^{236}$ and Tyr$^{331}$, whereas Val$^{98}$, Val$^{151}$, Ile$^{179}$, and the side-chain methylene carbons of salt-bridged residues Asp$^{237}$ and Arg$^{235}$ provide hydrophobic interactions. The γ phosphate hydrogen-bonds to both the side chain of His$^{311}$ and the amide groups of Gly$^{153}$ and Ala$^{154}$, which form part of a loop analogous to the Gly loops of other protein kinases (Fig. 1B). Residues 152 to 156 of this loop are less ordered in the substrate-free HipA (apoHipA) structure, indicating that nucleotide binding is required for its stabilization. Two Mg$^{2+}$ ions are also present in the HipA-ATP structure and probably function analogously to other protein kinases in facilitating phosphotransfer by accelerating substrate association and product dissociation (20, 21).

Comparison of the HipA-ATP and apoHipA structures revealed that binding ATP causes then and C domains to undergo only a small rotation (~4°) relative to each other (Fig. 1C). However, by analogy to other kinases, a more pronounced closure of the HipA domains upon binding the protein substrate is expected (20). The findings of a specialized kinase fold and high-affinity ATP binding strongly supported the hypothesis that HipA mediates persistence by phosphorylating one or more target proteins. To identify possible HipA targets, we carried out in vitro pulldown assays on candidate *E. coli* proteins. One protein, EF-Tu, was found to interact strongly with HipA in the presence of ATP-Mg$^{2+}$ and guanosine diphosphate (GDP) (Fig. 1D).
EF-Tu, the most abundant protein in *E. coli*, belongs to the guanosine triphosphatase superfamily and plays an essential role in translation by catalyzing aminoacyl–transfer RNA (tRNA) binding to the ribosome (22). Upon guanosine triphosphate (GTP) hydrolysis to GDP, EF-Tu undergoes a conformational change to an open form, which cannot bind the ribosome. Previous studies showed that EF-Tu is phosphorylated on residue Thr$^{382}$ by an unknown kinase or kinases (23, 24). The side chain of Thr$^{382}$ contacts Glu117 to stabilize the GTP-bound closed state of EF-Tu. Phosphorylation of Thr$^{382}$ favors the GDP-bound open form because it would lead to repulsion of Glu117 and prevent EF-Tu from adopting the GTP-bound closed conformation. Thr$^{382}$-phosphorylated EF-Tu cannot bind aminoacyl-tRNA and is therefore inactive in translation (23, 24). To test whether EF-Tu is a HipA substrate, we used an in vitro transcription/translation system to produce the toxic wild-type HipA enzyme (fig.S3). Immunoblotting studies, using antibodies to pThr/pSer/pTyr, indicated that HipA could phosphorylate EF-Tu in a manner stimulated by GDP (Fig. 1D). Moreover, fluorescence polarization studies revealed that HipA bound the EF-Tu peptide, IREGGRTVGA (25), encompassing Thr$^{382}$ (shown in bold here) with a $K_d$ of 15 ± 5 µM (fig. S4). Subsequently, we solved a crystal structure of the HipA–(AMP-PNP)–IREGRGRTVGA complex to 3.5Å resolution. The structure revealed that the activation loop, residues 185 to 195, was now folded and density was observed for the peptide near the active site and close to the activation loop (fig. S4). These combined data suggest that HipA may phosphorylate Thr382 to block aminoacyl-tRNA binding by EF-Tu. However, given that HipA affects multiple *E. coli* processes, other cellular targets are likely (9, 10).
Figure 1. HipA is a protein kinase that phosphorylates EF-Tu. (A) The *E. coli* apoHipA structure. β sheets and α helices are colored magenta and cyan, respectively. Secondary structural elements and the N and C termini are labeled. Disordered loops, including the putative activation loop (labeled AL), are indicated by dashed lines. (A) to (C) and Figs. 2, A to C; 3, B to D; and 4, A to D were made using PyMOL (31). (B) The ATP-binding pocket of HipA. Shown are ATP molecules (sticks), Mg$^{2+}$ ions (magenta spheres), water molecules (red spheres), and hydrogen bonds (dashed black lines). (C) Superimposition of the C domains of apoHipA (yellow) and HipA-ATP-Mg$^{2+}$ (blue) reveals only a small rotation upon ATP binding. ATP in the HipA-ATP structure is shown as sticks. (D) (Top) HipA–(GST-EF-Tu) pulldown (SDS-PAGE, stained with Coomassie brilliant blue). Lanes are as follows: 1, molecular weight ladder; 2, glutathione agarose (bead) retentate after addition of GST-EF-Tu, HipA, ATP, and GDP; 3, bead retentate after addition of GST-EF-Tu, HipA, ATP, and GDP, and washing; 4, bead retentate after addition of GST-EF-Tu, HipA, and no GDP or ATP; 5, bead retentate after addition of GST-EF- Tu, HipA, and no GDP or ATP, and washing. (Bottom) Wild-type HipA kinase assay using EF Tu as a substrate (immunoblot). The positions of GST-EF-Tu and X$_a$-cleaved EF-Tu are indicated by red and blue arrows, respectively. Lanes are as follows: 1, EF-Tu (cleaved) + wild type HipA + ATP + GTP; 2, EF-Tu (cleaved) + wild-type HipA + ATP + GDP; 3, EF-Tu (cleaved) + inactive HipA (D309Q) + ATP + GDP; 4, EFTu (uncleaved) + wild-type HipA + ATP + GDP; 5, EF-Tu (uncleaved) + wild-type HipA + ATP + GTP; 6, wildtype HipA + ATP; 7, native EF-Tu (cleaved) + ATP + GDP.
Under normal cellular conditions, the persistence function of HipA is somehow masked by its tight interaction with HipB (11, 12). HipB also functions as a transcriptional autoregulator of the hipBA operon by cooperatively binding four operators with the consensus sequence TATCCN8GGATA (where N indicates any nucleotide), located in the hipBA promoter region (11, 12). HipB binds these operators with high affinity, which is enhanced by the addition of HipA to the complex (12). To delineate the mechanism of HipB-mediated inhibition of HipA, the structure of the HipA-HipB complex bound to a 21–base pair hipB operator (top strand ACTATCCCTAAGGGGATAG) was solved and refined to an R_work/R_free of 22.5/28.1% to 2.68 Å resolution (table S1) (Figs. 2 and 3). HipB forms a compact dimer that specifically interacts with DNA through major groove contacts, whereas two HipA molecules sandwich the HipB-DNA complex by contacting the sides of the HipB dimer (Fig. 2). HipB binds far from the HipA active sites and, unlike other TA inhibition mechanisms, does not occlude the active site. The HipB dimer interface is extensive and buries 2700 Å² of accessible surface area (ASA), which accounts for over 36% of the total dimer ASA. HipB contains one β strand and four α helices with topology α1−α2−α3−α4−β1. Helices 2 and 3 form a canonical helix-turn-helix (HTH) motif. The first 3 and last 16 residues of each HipB subunit are disordered and located near a small β sheet that is composed of β1 and β1′ (from the other subunit) and forms a “β lid” (Fig. 2A).
Figure 2. Crystal structure of the HipA-HipB-DNA complex. (A) Ribbon diagram of the HipA HipB-DNA operator complex. The two HipA monomers are blue, and one subunit of the HipB dimer is yellow and the other orange. The N and C termini and secondary structural elements of one HipB subunit (orange) are labeled. β1′ is labeled for the yellow subunit. Also labeled are the N and C domains of each HipA molecule. The DNA is shown as sticks with carbon, nitrogen, oxygen, and phosphorus atoms colored green, blue, red, and magenta,
respectively. (B) Superimposition of substrate-free HipA (red) onto HipB-bound HipA (blue), showing their essentially identical conformations. For clarity, only one HipA molecule in the HipA-HipB-DNA complex is shown. (C) Closeup of the HipA-HipB interaction interface. Each lateral side of the HipB dimer, labeled subunits 1 and 2, interacts with the N and C domains of one HipA monomer. The DNA is shown as a gray surface. For clarity, only one HipA molecule is shown because the interaction interface between the other HipA molecule and the HipB dimer is identical. The residues that contribute to the interface are labeled and shown as orange sticks for HipB and dark blue sticks for HipA.

The HipB subunit structure showed significant homology to 434 Repressor, 434 Cro, and the restriction-modification controller protein C. AhdI from *Aeromonas hydrophila*, with RMSDs of 1.56, 1.60, and 1.51 Å for 59, 56, and 59 corresponding Cα atoms, respectively, thus placing it in the Xre-HTH family of transcriptional regulators (26). The homology between HipB and these proteins is confined to the four-helix bundle region because the β lid is found only in HipB. Despite the similarities in DNA binding domains, these proteins bind their DNA sites differently because 434 Cro does not significantly distort its DNA site, and biochemical data indicate that C. AhdI bends its DNA site by 47° (27, 28). In contrast, HipB induces a large, 70° bend in its operator (Fig. 3D). This bending may play a role in the cooperative binding of HipB to its four operator sites, which is predicted to involve DNA wrapping (11, 12). Indeed, the *hipBA* promoter also contains a binding site for the architectural protein IHF, which could further aid in DNA condensation.
Figure 3. HipB and HipA interactions with the hipB operator DNA. (A) Schematic representation of HipB HipA-DNA interactions. Only one half site of the 21-oligomer duplex is shown because the identical contacts are made with each half site. The strands are labeled 1A to 10A and 1B to 10B. Bases are represented as rectangles and labeled according to sequence. The ribose groups are shown as pentagons. The operator signature motif sequence, TATCC, is red.
HipB-DNA contacts are yellow. Hydrophobic contacts are indicated by lines and hydrogen bonds are indicated by arrows. Blue arrows indicate HipA-phosphate contacts. (B) HipB-DNA interactions. Only one HipB subunit-DNA half site is shown. The DNA and residues making side-chain contacts are shown as sticks. The signature motif sequence, TATCC, is labeled in red. For clarity, only the four-helix bundle is shown and labeled. (C) HipA-DNA contacts. The HipB dimer (yellow) is shown for reference. The location of the two DNA-interacting residues from HipA, K379 and R382, are shown as blue sticks and highlighted by mesh surface representations. The DNA is shown as sticks and colored as in (B). (D) HipA-HipB bound DNA is bent. This is an omit $F_o-F_c$ map in which the DNA was omitted from refinement to 2.68 Å resolution. The map is contoured at 2.8 $\sigma$. The DNA is shown as sticks and the bend angle is indicated.

HipB-induced DNA distortion aligns the recognition helices for specific binding to consecutive major grooves. Contacts from the HTH motif completely specify the nucleotides of the HipB signature motif, $T_2A_3T_4C_5C_6$ (Fig. 3, A and B). Ser$^{29}$ from $\alpha2$ makes hydrophobic contacts with the Thy2 methyl group. Residues of the recognition helix provide the remaining base-specifying contacts whereby two hydrogen bonds from Gln$^{39}$ read Ade3, whereas two hydrophobic contacts from Ala$^{40}$ and Ser$^{43}$ specify Thy4. Finally, Lys$^{38}$ makes hydrogen bonds with the guanine O6 oxygens of base pairs 5 and 6. HipB also makes 11 phosphate contacts to each half site. Deoxyribonuclease I protection studies showed that HipA binding to the HipB-DNA complex leads to an increase in protection and binding affinity (12). This is explained by the finding that HipA provides four phosphate backbone contacts to each half site from Lys$^{379}$ and Arg$^{382}$ (Fig. 3, A and C).
In the HipA-HipB-DNA complex, the HipB dimer is sandwiched on each side by one HipA molecule, and the complex is formed from noncontiguous (2 and 3C). This type of interaction contrasts sharply with structures of other TA modules in which the toxin interacts with a C-terminal region of the antitoxin that typically is structured only in the presence of toxin. Specifically, for the HipA-HipB pair, the HipA N domains interact with one HipB subunit, whereas the HipA C domains interact primarily with the other HipB subunit (Fig. 2C). This interaction interface is extensive, burying ~5000 Å2 of ASA, and involves both nonpolar and polar interactions. In the HipA N-domain–HipB interface, HipB residues from the turn before \( \alpha_1 \) interact with residues on HipA \( \beta_{15} \), and residues on HipB \( \alpha_1 \) make extensive contacts with HipA residues located on a long 310-like loop between \( \beta_3 \) and \( \alpha_1 \). The formation of the HipA C-domain–HipB interface primarily involves HipB residues from \( \alpha_2 \) and the turn between \( \alpha_4 \) and \( \beta_1 \). These residues interact primarily with HipA residues in the loop between \( \alpha_8 \) and \( \alpha_9 \) and the N terminus of \( \alpha_9 \). In addition, “cross-subunit” contacts are made between HipB residues Gln\(^{12} \) and HipA C-domain residue Gly\(^{284} \), and between HipB residue Tyr\(^{8} \) and HipA C-domain residue Ser\(^{286} \). These cross contacts, combined with the numerous interactions of each subunit in the HipB dimer with the N and C domains of HipA, lock HipA in an open and probably inactive conformation (Fig. 2C).

To activate HipA for persistence and free it from its DNA tether, HipB must be removed or degraded. Unlike most antitoxins, HipB interacts with HipA using residues from noncontiguous, well-ordered domains and not loops. Proteases that degrade toxins typically bind and tug on disordered regions to unfold the substrate. HipB contains an exposed and flexible 16-residue C terminus attached to the small b lid that covers the hydrophobic core of the protein and would appear to be an excellent candidate for protease attack (Fig. 4A). The structure of the
HipA-HipB-DNA complex also provides insight into the mechanism of increased persistence of the HipA7 protein. HipA7, which contains two substitutions, G22S and D291A, confers a high persistence phenotype on *E. coli* cells independent of HipB. Subsequent data revealed that the D291A mutation alone was sufficient for this phenotype (29). The HipA-HipB-DNA structure indicates that this phenotype probably results from a weakened HipA-HipB interaction, which unleashes HipA kinase activity. Specifically, Asp^{291} makes key contacts to stabilize the HipA-HipB interface, including hydrogen bonds to the side chain of Ser^{285}, which positions the Ser^{285} carbonyl oxygen to interact with HipB residue Gln^{62}, and hydrogen bonds to the amide nitrogen of Leu^{327}, which buttresses the HipA C-terminal region that interacts with HipB (Fig. 4B).

HipA undergoes only a small conformational change upon binding ATP, suggesting that HipA could bind ATP when in complex with HipB-DNA. Indeed, HipA(ATP)-HipB-DNA crystals isomorphous to HipA-HipB DNA crystals could be grown de novo. Alternatively, ATP could be soaked into preformed HipA-HipB-DNA crystals. In both cases, difference \( F_o - F_c \) electron density maps revealed clear density for ATP in the HipA active site (Fig. 4, C and D). In addition, isothermal titration calorimetry studies revealed a Kd of 15.0 \( \pm 1.0 \) mM for ATP-Mg\(^{2+}\) binding to HipA in the HipA-HipB-DNA complex, which is essentially identical to that obtained for ATPMg\(^{2+}\) binding to HipA alone (fig. S2). If the HipA active site is not blocked for ATP binding, then how does HipB binding neutralize HipA? Data from other protein kinase structures indicate that although ATP binding causes only small domain movements such as those we observed in HipA, the binding of protein substrates causes substantial domain closure (20, 21). This large-scale movement brings the two substrates into proximity for catalysis and precludes bulk solvent from the active site. HipB binding would appear to prevent this conformational change in HipA by locking the enzyme into an inactive open conformation by its
extensive interactions with the HipA N and C domains. Finally, the recent finding that *E. coli* EF-Tu is localized primarily to the cytosolic and membrane fractions, which is far from the nucleoid where the HipA-HipB DNA complex would reside, suggests that HipB-DNA binding may also inactivate HipA by its sequest ratio (30). These studies have provided important insight into the mechanisms by which HipA mediates persistence and HipB neutralizes HipA. The high conservation of HipA among Gram-negative bacteria indicates its central role in the development of persistence. Thus, inhibitors that specifically target the substrate-binding sites of HipA, may prove effective against persistence and MDT.
Figure 4. HipB is a vulnerable antitoxin that neutralizes HipA. (A) The HipB dimer is shown as ribbons with one yellow and one orange subunit. The DNA is shown as pink sticks. Hydrophobic core residues are shown as sticks and transparent surfaces. The β lid is composed of a short two-stranded β sheet. Disordered C-terminal residues extend from the lid and are depicted as colored dashes. (B) Mutation sites (G22S and D291A) in the HipA7 protein mapped onto HipA proteins of the HipA-HipB-DNA complex. The HipA molecules are cyan, the HipB subunits are yellow and orange, and the DNA is a pink ribbon. Residues G22 and D291 are shown as blue Corey, Pauling, Koltan structures (CPKs). HipB residue Q62 and HipA residues S285 and L327 are also shown as CPKs. A closeup of the interactions involving HipA residue D291 is shown (inset). Hydrogen bonds are indicated by dashed lines. (C) Structure of the HipA(ATP)-HipB-DNA complex. HipA molecules are yellow, the HipB dimer is cyan, and the DNA and ATP are shown as sticks. The blue mesh represents a 2.98 Å resolution Fo-Fc map, contoured at 4.0 σ, in which the ATP was omitted. (D) Closeup view of the ATP-binding pocket and omit map. Carbon, nitrogen, oxygen, and phosphorus atoms are colored magenta, blue, red, and purple, respectively.

MATERIALS AND METHODS

Crystallization of apoHipA and the HipA-ATP complex. To obtain milligram quantities of pure HipA, a HipA D309Q substitution was made that can be expressed in E. coli in the absence of HipB without leading to cell stasis (15). The mutant was subcloned into pBAD33 and the construct transformed into DH5α cells. The cloning placed a hexa-histidine tag plus serine and arginine immediately after the initiator methionine at the HipA N terminus. For overexpression, E. coli cells were inoculated with an overnight culture using chloramphenicol selection. Cells
were grown at 37 °C to an OD600 of 0.5-0.6 and induced with arabinose for 2.5 hrs. After pelleting, the cells were lysed using a French Press and the supernatant loaded onto a Ni-NTA column. The protein was purified using a 10-500 mM imidazole gradient. Purified fractions were pooled and concentrated for crystallization. ApoHipA crystals were grown at room temperature via hanging drop, vapor diffusion methods using a reservoir of 16% PEG 8000, 20% glycerol and 0.04 M potassium phosphate, pH 7.5. The crystals grow over a period of a week and diffract to beyond 1.6 Å resolution. The crystals take the monoclinic space group P21 with \( a = 48.7 \text{ Å}, b = 85.8 \text{ Å}, c = 49.7 \text{ Å} \) and \( \beta = 90.1° \). For cryoprotection, apoHipA crystals were taken straight from the drop and frozen in the cryostream. Intensity data were processed with MOSFLM. Crystals of the HipA-ATP complex were obtained by mixing the purified protein containing 1 mM ATP and 5 mM MgCl₂ with a reservoir of 30% PEG 600, 0.1 M CHES pH 9.5. The HipA ATP complex crystallized nonisomorphously with respect to the apo form of HipA and takes the monoclinic crystal form, space group P21 with \( a = 68.5 \text{ Å}, b = 84.1 \text{ Å}, c = 69.3 \text{ Å} \) and \( \beta = 91.5° \). For cryoprotection, HipA-ATP crystals were dipped for several minutes in the crystallization solution plus 30% glycerol. Intensity data were processed with MOSFLM.

**Structure determinations of apoHipA and the HipA-ATP complex.** To determine the HipA structures, selenomethionine-substituted HipA was produced using the methionine inhibitory pathway. The protein was crystallized using conditions for obtaining apo and ATP-bound HipA. Three wavelength MAD data sets were collected for selenomethionine substituted apoHipA and HipA-ATP crystals. The data were processed with MOSFLM and the selenium sites were located using SOLVE (16). The MAD maps were of excellent quality and allowed complete tracing of the HipA structure using O (Fig. S1) (18). Model building was carried out using O and
refinement with CNS (17-18). The final apoHipA structure, which contains one molecule per asymmetric unit (ASU), includes residues 2-109, 114-134, 146-184, 196-437, 1 chloride ion, 1 phosphate ion and 377 water molecules and has $R_{work}/R_{free}$ values of 19.5%/23.2%. The final HipA-ATP structure, which contains two molecules of HipA-ATP in the ASU, includes residues 2-105, 114-184, 196-437 of one molecule, residues 3-183, 198-435 of the other molecule, 2 ATP molecules, 4 Mg$^{2+}$ ions, 2 CAPS sulphate groups and 868 water molecules and has $R_{work}/R_{free}$ values of 18.4%/21.7%. The two HipA-ATP molecules in the ASU are essentially identical as their $C\alpha$ atoms can be superimposed with an RMSD of 0.30 Å. Both the apoHipA and HipA-ATP structures have excellent geometry as ascertained by Ramachandran analyses (Table S1).

**Crystallization of the HipA-HipB-DNA complex.** An artificial hipB gene was designed and purchased from Genscript Corporation, Piscatway, NJ, USA; Web: www.genscript.com. This gene was codon optimized for protein expression in *E. coli* and subcloned into the pET15b vector such that the N-terminal hexa-histidine tag was expressed for purification via Ni-NTA column chromatography. The expression vector was transformed into competent BL21(DE3) cells. The protein was expressed by the addition of 1.0 mM IPTG for 3 hrs at 20 °C. To form the HipA-HipB complex the two purified proteins were mixed stoichiometrically, ~1:1 (1 HipB dimer:2 HipA molecules) in Buffer A (25 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT, 5% glycerol). The complex was further purified by S200 size exclusion chromatography and concentrated to 10 mg/mL for crystallization. To obtain crystals of the HipA-HipB-DNA complex, several oligodeoxynucleotides (Oligos Etc., Wilsonville, OR), each of which encompassed the 20 bp *hipB* operator, were mixed in a ratio of 1 DNA duplex to (1 HipB dimer:2 HipA molecules) and used in crystallization screens. Data quality crystals were obtained
with a 21 mer DNA site, which has 5′ adenosine overhangs. To grow the crystals, the HipA HipB-DNA solution was mixed 1:1 with a reservoir of 0.5% PEG 5000, 0.8 M potassium/sodium tartrate tetrahydrate and suspended over the same reservoir. Crystals took several weeks to grow and diffracted anisotropically to a limiting resolution of 2.5 Å in the best direction and 3.3 Å in the worst direction. Crystals were cryopreserved by dipping for several minutes in saturated lithium sulphate and annealling. All data were processed with MOSFLM.

**Structure determinations of HipA-HipB-DNA and HipA(ATP)-HipB-DNA complexes.** The structure of the HipA-HipB-DNA complex was solved by Molecular Replacement using MolRep and the HipA (either apo or ATP bound) structure as a search model. The crystals contain one HipA monomer, one HipB subunit and one DNA half site in the ASU with crystallographic symmetry generating the dimeric complex. After initial refinement, difference Fourier electron density maps revealed clear density for HipB and the DNA, which were built using O (18). The final structure includes HipA residues 2-184, 196-437; HipB residues 4-74, all nucleotides of the DNA, 8 sulphate ions and 34 water molecules. HipB has topology $\alpha_1$-$\alpha_2$-$\alpha_3$-$\alpha_4$-$\beta_1$ ($\alpha_1$; residues 10-24, $\alpha_2$; 28-35, $\alpha_3$; 39-48, $\alpha_4$; 54-65, $\beta_1$; 66-72). To obtain the structure of the HipA(ATP)-HipB-DNA complex, ATP and MgCl$_2$ were added to final concentrations of 5 mM each to purified HipA-HipB solutions, which were then mixed with DNA in the same stoichiometry used to obtain HipA-HipB-DNA crystals. This *de novo* crystallization was carried out as per the HipA-HipB-DNA complex and isomorphous tetragonal crystals were obtained. Data collected on such crystals revealed clear density for ATP. Data were also collected for HipA-HipB-DNA crystals soaked with 5 mM ATP and 5 mM MgCl$_2$ for several days to 1 week. The crystals were cryoprotected as for the HipA-HipB-DNA complex using lithium sulphate,
however 5 mM ATP and 5 mM MgCl$_2$ were added to the cryoprotectant. Intensity data were collected at ALS Beamline 8.2.1 and processed with MOSFLM. Structures were solved by Molecular Replacement using the HipA-HipB-DNA complex as a search model. Clear density was observed for the ATP molecule in both ATP co-crystallized and soaked crystals after initial rounds of refinement. The best data was obtained for a soaked crystal (to 2.98 Å resolution compared to 3.2 Å resolution for the de novo crystallized complex) and the final R$_{factor}$/R$_{free}$ for this structure is 26.2%/28.2 % to 2.98 Å resolution. The final structure includes HipA residues 2-184, 196-437; HipB residues 4-74, all nucleotides of the DNA, 1 ATP molecule, 9 sulphate ions and 4 water molecules.

**Binding affinity of ATP for HipA and the HipA-HipB complex.** The binding affinities of HipA and HipA-HipB for ATP were determined by isothermal titration calorimetry (ITC). All protein samples were dialyzed extensively into Buffer B (25 mM Tris pH 7.5, 300 mM NaCl, 5% glycerol and 5 mM MgCl$_2$) while ATP was directly dissolved into this dialysis buffer. Prior to dialysis, the HipA-HipB complex was purified further via S200 size exclusion chromatography in order to ensure isolation of stoichiometrically bound HipA-HipB complex. Protein concentrations were measured by Bradford Assay (Biorad). Immediately prior to the ITC experiment, samples were degassed. ATP (3 mM) was titrated into 110 μM protein and the change in heat was monitored using a VP-ITC instrument (MicroCal, Inc.). Additional control experiments, in which 3 mM ATP was titrated into dialysis buffer alone and dialysis buffer was titrated into 110 μM HipA, yielded negligible heats of dilution. Thermogram analysis was performed using the Origin 5.0 package.
**Candidate HipA substrate pulldowns.** Candidate *E. coli* proteins included tryptophanase and EF-Tu. Tryptophanase has been implicated in biofilm formation and EF-Tu was known to be phosphorylated on Thr382 by an unknown kinase(s). Moreover, eukaryotic Ser/Thr kinases have been shown to be capable of phosphorylating *E. coli* EF-Tu residue Thr382 (23). Thus, both were deemed possible candidate substrates for HipA. Candidate proteins, which contained either GST affinity tags (EF-Tu) or no tags (tryptophanase) were incubated with purified His-tagged HipA D309Q for 30 min at room temperature and then either pulled down with glutathione agarose or Ni-NTA resin and washed with 25 mM Tris, pH 7.5, 100 mM NaCl, 5% glycerol before elution with either glutathione or imidazole. The pulldowned proteins were resolved by SDS PAGE and visualized by Coomassie brilliant blue. HipA alone was not retained on glutathione agarose beads (in the presence or absence of ATP and/or GDP) and was found in the eluate even without the wash step indicating that it has negligible affinity for glutathione agarose. To assess tryptophanase binding to HipA, HipA was bound to Ni-NTA and a lysate from cells containing induced, non-tagged tryptophanase was added, mixed for several hrs and eluted. From these studies, no binding between HipA and tryptophanase was observed. To examine EF-Tu binding to HipA, GST pulldowns were performed. GST-fused EF-Tu was adsorbed to glutathione agarose and HipA added, mixed and eluted. HipA was retained in samples containing ATP and GDP (Fig. 1D).

**In vitro translation of wild type HipA.** Wild type (wt) HipA cannot be produced in *E. coli* due to its inhibition of cell growth. Thus, to produce wt HipA protein, a wheat germ *in vitro* transcription/translation system was used (Roche Diagnostics, Switzerland). The *hipA* gene, including sequences encoding the N-terminal hexahistidine tag, was inserted into the pIVEX 1.3
WG vector between the NdeI and XmaI sites. Subsequently, 2 μg of recombinant DNA were added to 50 μL of the translation reaction, which was reconstituted as specified by the Roche manual (Germ CECF Kit (Roche Diagnostics)). Reactions were run at 24 °C with continuous shaking for 24 hrs. To purify wt HipA protein, a Ni-NTA batch purification was carried out. Reaction lysate was equilibrated with Ni-NTA resin (8:1 lysate volume to resin bed volume ratio) in a 1.5 mL Eppendorf microtube for 30 min on an orbital shaker, and the supernatant was then separated from the resin by centrifugation. Resin was washed with a total volume of 4 mL of Buffer B in increments of 0.75 mL. wt HipA was eluted with Buffer B plus 500 mM imidazole. Samples were run on an SDS gel to analyze purity, which ranged between 60-70%. Western blot analysis was done using an anti-His antibody to confirm that the protein, which migrated at a molecular weight of ~50 kDa, was Histagged wt HipA (fig. S3).

**EF-Tu phosphorylation by HipA.** The partially purified, in vitro translated wt HipA protein was used in EF-Tu phosphorylation assays. 30 μL reactions were run in a solution of 20 mM Tris, pH 7.5, 100 mM NaCl, and 10 mM MgCl2. Either wt HipA or HipA D309Q was added to an aliquot of EF-Tu (either GST-EFTu or factor Xa cleaved EF-Tu, which no longer contains the GST tag) with and without relevant nucleotide substrates. To test the effect of the wheat germ cell extract on the phosphorylation assay, a control vector was subjected to the same conditions as the hipA recombinant DNA and the product was then combined with EF-Tu. Each reaction contained 20 mM ATP. The reactions were incubated at room temperature for 1 hr and stopped by the addition of 15 μL 2X SDS-PAGE loading buffer and heated at 95 °C for 5 min. To assay for phosphorylation, an ImmunoBlot analysis was done. Samples were run on Tris-HCl SDS-PAGE 10-20% gradient gels (Biorad), transferred to PVDF membranes, and blocked with 2%
BSA in 1X phosphate buffered saline (PBS), 0.2% Tween-20 (PBST) for two hrs. The membranes were immunoblotted with primary antibodies specific for phosphoserine/phosphothreonine/ phosphotyrosine residues (AnaSpec, San Jose, CA) followed by incubation for two hrs. with horseradish peroxidase-conjugated anti-mouse secondary antibodies, both in 2% BSA, 1X PBST. The blot was visualized by chemiluminescence using an Alpha Imager (Alpha Innotech, San Leandro, CA).

**Fluorescence polarization (FP) binding of EF-Tu peptide by HipA.** FP binding studies were carried out with an N-terminal-fluorescein labelled peptide, IREGGRTVGA, where the position of EF-Tu Thr382 is bolded. The measurements were made using a PanVera Beacon 2000 Fluorescence Polarization System equipped with 490 nM excitation filters and 530 nM emission filters. Binding studies are carried out by titrating the D309Q protein (using typical protein concentrations of 10 μM - 250 μM) into 0.500 mL binding buffer (25 mM Tris pH 7.5, 300 mM NaCl, 5% glycerol, 1 mM ATP, 1 mM MgCl$_2$) containing 1 nM fluoresceinated peptide. The resulting binding isotherm was plotted by fitting to a simple bimolecular binding model using nonlinear regression. The Kd was $15 \pm 5 \text{ μM}$ for HipA binding to the EF-Tu peptide. As a control, another 10-mer peptide with the sequence ESTEQQNLEW was used for FP measurements under the identical conditions and revealed no saturable binding up to final HipA concentrations of 300 μM at which point the protein started to precipitate.

**Crystallization and structure determination of HipA-(AMP-PNP)-EF-Tu peptide complex.** To obtain crystals of the HipA-(AMP-PNP)-EF-Tu peptide complex, HipA at 5 mg/mL was mixed with 1 mM AMP-PNP, 1 mM MgCl$_2$ and the peptide, IREGGRTVGA, was added in
saturating amounts to the solution. The complex was mixed 1:1 with a reservoir of 1 M sodium acetate trihydrate and sealed over a dry reservoir. Crystals grew in 2-3 days and could be cryopreserved directly from the drop. Data were collected on an R-Axis-HTC and processed with CrystalClear. The crystals are tetragonal, I422, with $a = b = 128.4$ Å and $c = 203.9$ Å. Crystals typically diffract to 5.0 Å resolution. However, one crystal diffracted to 3.5 Å resolution and was collected for structure determination. The intensity data have an overall $I/\sigma I$ of 7.3 and 2.3 for the highest resolution shell. The $R_{\text{symm}}$ for all the data is 12.4% and 39.0% for the highest resolution shell. The structure was solved by Molecular Replacement using MolRep and the HipA-ATP structure as a search model. The crystals contain one HipA monomer in the ASU. After initial refinement, difference Fourier electron density maps revealed clear density for the AMP-PNP and activation loop residues 185-195, which were missing in all previous structures. After several rounds of positional refinement, contiguous density was observed near the active site, which could be fit with the peptide. However, because of the limited resolution the peptide was as built as a polyalanine chain. The final structure includes HipA residues 2-437, the AMPPNP molecule and 9 peptide residue built as a polyalanine chain. The structure has an $R_{\text{work}}/R_{\text{free}}$ of 29.1%/36.0% with excellent geometry.
**Figure S1. HipA binds ATP-Mg\(^{2+}\).** (A) Omit Fo-Fc map contoured at 4.5 \(\sigma\) and calculated to 1.66 Å resolution in which the ATP molecules were omitted from the HipA-ATP structure. HipA side chains are colored cyan and shown as sticks. The ATP molecule is shown as sticks with carbon, nitrogen, oxygen and phosphorus atoms colored yellow, blue, red and dark yellow, respectively. The electron density map is shown as a grey mesh. The Mg\(^{2+}\) ions, which were not omitted, and the ATP are labeled. (B) Experimental MAD map of the HipA-ATP structure contoured at 1.7 \(\sigma\) and calculated to 1.70 Å resolution. The electron density map is shown as a blue mesh and the side chains are shown as sticks with carbon, nitrogen, oxygen and phosphorus atoms colored yellow, blue, red and dark yellow, respectively. This figure shows a view centered on the ATP binding site. (C) A different section of the experimental MAD map shown in B. The contour level is 1.7 \(\sigma\) and the map was calculated to 1.70 Å resolution. These Figures were made using O (18).
Figure S2. Isothermal titration calorimetry fitted thermograms of ATP binding to HipA. (A) and to HipA in the HipA-HipB complex (B). The integrated binding isotherms are corrected for ligand and protein dilution effects. The integrated binding isotherms show essentially identical curves indicating that the HipA interaction with HipB does not obstruct or change the ability of HipA to bind ATP.
Figure S3. Immunoblot of *in vitro* transcription/translation experiments used to obtain wild type HipA. HipA contains an N-terminal hexa-histidine tag and was purified by Ni-NTA chromatography. The presence of the protein was probed with an anti-his-tag antibody and visualized by chemiluminescence exposure with an Alpha Inotech. The presence of the wild type protein was confirmed by this blot. The lanes correspond to the following:

1. 2 μL of purified wt HipA
2. 4 μL of purified wt HipA
3. 6 μL of purified wt HipA
4. 8 μL of purified wt HipA
5. MW Ladder (no his-tagged proteins)
6. 1 μL of hexa-histidine tagged D309Q produced from *E. coli*
7. 2 μL of Ni-NTA wash, before elution of wt HipA from Ni-NTA column
8. GUS Control Reaction (positive control, β-glucuronidase with His-tag)
Figure S4. (A) Representative fluorescence polarization binding isotherm of the HipA EF-Tu peptide, IREGGRTVGA, interaction. A $K_d$ of 15 ± 5 $\mu$M was obtained. (B) Ribbon diagram of the HipA-(AMP-PNP)-EF-Tu peptide structure solved to 3.5 Å resolution. The activation loop, which is only observed in the structure with the peptide, is colored yellow and the peptide is colored red. The AMP-PNP molecule is shown as CPK. (C) Views of the 2Fo-Fc map of the HipA-(AMP-PNP)-peptide structure contoured at 0.9 $\sigma$. (Left) Close up view of the AMP-PNP
molecule. (Middle) View of the excellent electron density for activation loop (residues 185-195). (Right) Views of the electron density of the peptide (red Cα trace). Density for the peptide was strongest near the active site pocket, but side chains were not clearly discernable and therefore the peptide was built as a polyalanine chain.

| Table S1: Selected crystallographic data for HipA and HipA-HipB-DNA complexes |
|-----------------|-----------------|-----------------|-----------------|
| Crystal         | apoHipA         | HipA-(ATP)      | HipA-HipB-DNA   |
| Space group     | P21             | P21             | P422            |
| Cell dimensions | a=48.7 Å        | a=68.5 Å        | a=166.3 Å       |
|                 | b=85.8 Å        | b=84.1 Å        | b=166.3 Å       |
|                 | c=49.7 Å        | c=69.3 Å        | c=62.1 Å        |
| β = 90.1°       | β = 91.5°       |                  |                 |
| Molecules/ASU (#) | 1              | 2               | 1               |
| Resolution (Å)  | 43.03-1.54      | 42.64-1.66      | 74.33-2.68      |
| Overall Rsym (%)a | 5.4 (38.7)     | 5.4 (29.5)      | 7.1 (38.3)      |
| Overall I/σ(I)  | 13.7 (2.0)      | 10.5 (3.2)      | 9.0 (2.0)       |
| Total Reflections (#) | 164732    | 361604         | 149024          |
| Unique Reflections (#) | 59003      | 89054          | 25017           |
| Completeness (%) | 98.1 (97.3)    | 96.3 (94.2)     | 99.7 (99.3)     |
| Refinement Statistics |
| Resolution (Å)  | 43.03-1.54      | 42.64-1.66      | 74.33-2.68      |
| Rwork/Rfree(%)c | 19.5/23.2       | 18.4/21.7       | 22.5/28.1       |
| Ramachandran analysis |
| Most favored (%/#) | 93.8/334       | 92.9/677       | 88.0/375        |
| Add. allowed (%/#) | 6.2/22          | 6.7/49         | 11.0/47         |
| Gen. allowed (%/#) | 0/0             | 0.3/2          | 0.9/4           |
| Disallowed (%/#) | 0/0             | 0.1/1          | 0/0             |

aRsyn = ΣΣ|Ihkkl-Ihkkl(j)|/ ΣIhkkl, where Ihkkl(j) is observed intensity and Ihkkl is the final average value of intensity.
b values in parentheses are for the highest resolution shell.
cRwork = Σ|Fobs| - |Fcalt|/Σ|Fobs| and Rfree = Σ|Fobs| - |Fcalt|/Σ|Fobs|; where all reflections belong to a test set of 5% randomly selected data.
REFERENCES AND NOTES


25. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.


32. We thank C. R. Knudsen for her generous gift of the GST-EFTu expression construct. Coordinates and structure factor amplitudes for the apoHipA, HipA-ATP, HipA-HipB-DNA, and HipA(ATP)-HipB-DNA complexes have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (www.rcsb.org/) under accession codes 3DNU, 3DNT, 3DNV, and 3DNW, respectively. We acknowledge support from Burroughs Wellcome Career Development Award 992863 and NIH grant GM074815 (to M.A.S.), NIH grant GM061162 (to K.L.), and the Robert A. Welch Foundation (grant G0040) and NIH Grant AI048593 (to R.G.B.).
Chapter 3: Regulation of the *Escherichia coli* HipBA Toxin-Antitoxin System by Proteolysis

*In preparation for publication*

*Sonja Hansen, Marin Vulić, Maria A. Schumacher, Richard G. Brennan, Kim Lewis*

**ABSTRACT**

Bacterial populations produce antibiotic-tolerant persister cells. A number of recent studies point to the involvement of toxin/antitoxin modules in persister formation. The *hipBA* toxin/antitoxin (TA) module is a type II toxin/antitoxin module composed of the HipB antitoxin and the HipA toxin. HipA is an EF-Tu kinase which causes protein synthesis inhibition and dormancy upon phosphorylation of its substrate. Antitoxins are labile proteins that are degraded by one of the cytosolic ATP-dependent proteases. We followed the rate of HipB degradation in different protease deficient strains and found that HipB was stabilized in *lon* background. These findings were confirmed in an *in vitro* degradation assay, showing that Lon is the main protease responsible for HipB proteolysis. Moreover, we demonstrated that degradation of HipB is dependent on the presence of a carboxy-terminal stretch of 16 unstructured amino acid residues of HipB (residues 73-88).
INTRODUCTION

Bacterial populations stochastically produce a small number of non-growing or dormant persister cells that are tolerant to antibiotics. Persisters are phenotypic variants that are genetically identical to the susceptible cells within a clonal population. Thus, persistence is a non-inheritable, transient trait (18, 37). Previously, we have shown that the recalcitrance of biofilms is largely due to the presence of persisters (6, 34). Recent studies by our group link persistence to chronic infectious disease. In the case of cystic fibrosis patients infected with *Pseudomonas aeruginosa*, high persister (*hip*) mutants were selected for over the course of infection (24). Similarly, *Candida albicans hip* mutants were selected for in patients with oral thrush (23). These findings indicate that persisters are largely responsible for failure to eradicate chronic infections (24).

Non-growing persisters make up a small part of the population: $10^{-6}$ to $10^{-4}$ in exponentially growing cultures and $\sim 10^{-2}$ in stationary phase (25, 35). *In vitro* selection of *Escherichia coli* mutants with a 10,000 fold increase in the persister fraction produced a strain with two point mutations in *hipA*, G22S and D291A, (*hipA*7 allele) (3, 4, 26, 27, 31). *hipA* is the toxin of the *hipBA* toxin/antitoxin (TA) pair. The antitoxin HipB represses the *hipBA* operon by cooperative binding to four operator sites (3, 4, 32) and inactivates the toxin HipA. Ectopic expression of HipA causes multidrug tolerance (10). Despite the strong phenotype of the gain-of-function allele *hipA*7 and HipA overexpression, a deletion of *hipA* did not produce a phenotype (10, 16, 21, 22, 26). Toxin/Antitoxin systems are highly redundant and therefore only overproduction and not deletion of the toxin is expected to show a difference in persistence (12, 16, 24, 25, 33). Concordantly, ectopic expression of two other toxins, RelE and MazF, also strongly increased tolerance to antibiotics, whereas a deletion of the toxin had no phenotype (19).
One exception is the toxin TisB, a membrane-acting peptide, producing dormancy due to decreases in pmf and ATP levels. Deletion of the type I TA module (small RNA antitoxin/protein toxin) \textit{tisAB} led to a pronounced decrease in the level of persisters. TisB is induced by the SOS response, and becomes the main mechanism of persister formation during SOS response, so a deletion has a phenotype (7, 12). Unlike any other toxins of type II TA modules (protein antitoxin/protein toxin) which so far group mainly into either gyrase inhibitors (ParD, CcdB), mRNA interferases (RelE, MazF, YoeB, HicA, Doc) (13, 17, 35) or PIN domain fold proteins (VapC) (1, 7), HipA is a kinase with a eukaryotic Ser/Thr kinase-like fold (10). Replacing the conserved amino acids in the phosphorylation site (S150A) or the Mg\textsuperscript{2+}- or catalytic binding sites (D332Q and D309Q respectively) abolishes the ability to confer growth arrest and antibiotic tolerance (10). Elongation factor Tu (EF-Tu) was identified as a HipA target which points to a likely mechanism of HipA-mediated persister formation (13). HipA phosphorylates EF-Tu, and Thr\textsuperscript{382}-phosphorylated EF-Tu leads to stasis since it can no longer bind aminoacyl-tRNA (32). Under the standard regime of batch culture growth the persistence function of HipA is masked by its tight interaction with HipB. To activate HipA, the antitoxin HipB has to be removed or degraded. Proteolytic regulation of the antitoxin has been demonstrated for several TA modules. In \textit{E. coli}, the chromosomally encoded TA modules MazEF, RelBE, YefM/YoeB, HicAB and DinJ/YafQ are regulated by the AAA+ ATP-dependent proteases Lon and/or ClpPX (9, 13, 30). ClpPA degrades PhD antitoxin of the plasmid-encoded PhD/Doc TA module (7). The other two ATP-dependent proteases HslVU and FtsH do not have any known antitoxin substrates (7, 13). In this study we provide evidence that Lon is the main protease responsible for HipB degradation. Our data suggests that Lon recognizes the unstructured C-terminus of HipB.
**MATERIAL AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study and their relevant characteristics are listed in Table 1. Strains were grown in LB medium unless otherwise noted. When required, LB broth or LB agar were supplemented with ampicillin (100 µg/ml, Amp) and chloramphenicol (30 µg/ml, Cam).

**Plasmid constructions.** Primers P1hishipBXbaI (CGGTCTAGATAAGGAGATATATGGAATAATGACACCACCACCACCACCACATGAGCTTTCAGAAGATCTA) and P2hipBEcoRI (CCGGAATTCTTACCACCAGATTATTTGCTG) or P2hipBsEcoRI (CCGGAATTCGTGCATAGCGTCATTGAGAG) were used to amplify hipB from *E. coli* MG1655; P1GFPXbaI (CGGTCTAGATAAGGAGATATATGGAATAATGAGTAAAGGAGAAGAACT) and P2GFPEcoRI (CCGGAATTCTTACCACCAGATTATTTGCTGTTCTGTTGATTCTGGCAGGCATTTTTCGCTTTGTATAGTTCATCCATGC) or P2GHEcoRI (CCGGAATTCTTATTGTATAGTTCATCCATGC) were used to amplify GFP from pUA66. The PCR products were digested with *Xba*I and *Eco*RI and ligated into *Xba*I and *Eco*RI sites of pBRlac_tac creating pBRhipB or pBRhipB72 and pBRGFP or pBRGFP-H, respectively.

**Strain construction.** Precise deletion-replacement of *hslVU* was created by the method of Datsenko and Wanner (11).

**Protein expression and purification.** N-terminally hexa-histidine (his$_6$)-tagged HipB, HipB72, HipA and Lon were purified using strains and plasmids indicated in Table 1. Strains were induced by addition of 0.2 % arabinose or 1 mM IPTG for 4h. Cells were lysed by sonication in
the presence of 1 mg/ml lysozyme. Protein extracts were applied to a Ni-NTA resin (QIAGEN). The columns were washed with buffer containing 20 mM imidazole, and eluted with buffer containing 250 mM imidazole. The eluted protein was concentrated and dialyzed with protein storage buffer (50 mM Tris-HCl, pH 8, 250 mM NaCl, 5 mM dithiothreitol [DTT], 5% glycerol)(29).

**In vivo degradation and Western Blot analysis.** The rate of degradation of HipB, HipB72, GFP and GFP-H was determined using samples from exponentially growing cells. Expression of proteins from pBRlac_\_tac was induced by addition of 1 mM IPTG at OD$_{600}$ of 0.3. After 1h induction, protein synthesis was inhibited by the addition of 100 µg/ml Cam, and samples were removed at indicated time points. Protein levels were detected by Western blotting using either a monoclonal His-tag antibody (EMD Biosciences) or polyclonal antibody to GFP (Abcam) and a polyclonal goat-anti mouse IgG AP conjugate.

**In vitro degradation assay.** To monitor degradation of HipB, His$_6$-Lon (0.6 µM) and His$_6$-HipB (0.48 µM) or His$_6$-HipB72 (0.48 µM) were added to a degradation buffer [50 mM Tris-HCl (pH 8.0), 4 mM ATP, 7.5 mM MgCl$_2$] and incubated at 37°C. When indicated, His$_6$-HipB was mixed with either His$_6$-HipA (0.48 µM) lysozyme (0.48 µM) or duplex DNA containing a 21 bp hipB operator site (0.24 µM) (sequence of the top strand ACTATCCCCTTAAGGGGATAG) or a control oligo (sequence of the top strand ATGATGAGCTTTCAGAAGATC). Samples were removed at indicated times, and analyzed by SDS-PAGE.
Table 1 Strains and plasmids used in this study

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RESULTS

Rapid degradation of HipB is dependent on the presence of Lon. HipB does not share any homology with any of the known antitoxins. Neutralization of its cognate toxin also differs mechanistically from other TA modules. Typically, antitoxins block the active site of the toxin thereby inhibiting its activity. The interaction with the toxin involves the C-terminus of the antitoxin, which is structured only when bound to the toxin (7). HipB, however, does not make any contacts with HipA near the active site (32). One HipB dimer binds two HipA molecules involving interactions with both the N and the C domain of HipA (32). The C terminus of HipB remains unstructured in the presence of HipA (32). To test whether proteolytic regulation is a shared characteristic of HipB with the typical antitoxins of the mRNA interferase and gyrase inhibitor TA modules, despite functional and structural differences, we measured the rate of in vivo degradation of HipB in E. coli wild type (KLE901). Since endogenous HipB could not be detected by Western Blotting using a polyclonal antibody to HipB (data not shown), N-terminally six-his tagged HipB (His\textsubscript{6}-HipB) was expressed from a plasmid containing an IPTG inducible promoter (pBRhipB). After 60 min induction, protein synthesis was stopped by the addition of Cam and the rate of HipB proteolysis was determined by Western blotting (Fig. 1). His\textsubscript{6}-HipB was degraded with a t\textsubscript{1/2} of approximately 11 min (average of 3 experiments) in wild type cells confirming rapid degradation.
Figure 1. HipB is rapidly degraded in *E. coli* wild type. *E. coli* BW25113 pBR*hipB* (KLE901) was cultured in LB to OD\textsubscript{600nm} 0.3, 1mM IPTG was added for 1 h and then protein synthesis was stopped by the addition of 100 µg/ml Cam. Samples were collected over the course of 90 min. The presence of HipB in whole cell lysates was detected with an anti-his antibody and a polyclonal goat-anti mouse IgG AP conjugate by Western blotting. The graph is a representative of three independent experiments.

Next, we transformed with pBR*hipB* into protease deficient strains lacking *lon* (KLE902), *clpP* (KLE903) or *hslVU* (KLE904) to identify a protease responsible for HipB degradation. We compared the rate of *in vivo* degradation of HipB in wild type to the rate of degradation in the protease deficient strains. Turnover of HipB in Δ*hslVU* and in Δ*clpP* was comparable to wild type demonstrating that HslVU and ClpPA/ClpPX are not involved in HipB degradation. Deletion of *lon* stabilized HipB (Fig. 2), indicating that Lon is likely the main protease involved in HipB degradation *in vivo.*
Figure 2. HipB proteolysis in protease deficient strains. HipB was expressed from pBRhipB in BW25113 (KLE901) and its lon::kan (KLE902), clpP::kan (KLE903) or hslVU::FRT (KLE904) derivate. The strains were grown in LB medium, and at an OD$_{600}$ of 0.3 1mM IPTG was added. After 1h induction, protein synthesis was inhibited by the addition of 100 µg/ml Cam, and samples for Western blots were removed over the course of 30 min.

**HipB is a substrate of the ATP-dependent protease Lon.** To determine whether the HipB is directly recognized and degraded by Lon we purified His$_6$-Lon and His$_6$-HipB for *in vitro* degradation studies. Lon was efficiently degrading HipB *in vitro* confirming our *in vivo* results (Fig. 3A). Substrate degradation by Lon requires ATP hydrolysis and Mg$^{2+}$ (8). HipB degradation was indeed dependent on the presence of ATP or MgCl$_2$ in the reaction buffer demonstrating that degradation of HipB is specific to the addition of active enzyme to the buffer (Fig. 3B). HipB functions as an inhibitor of HipA and as an autoregulator of the *hipBA* operon by cooperatively binding to the consensus sequence TATCCN$_8$GGTA. We tested whether Lon degrades HipB when it is bound to DNA or HipA. Addition of a 21 bp oligo ACTATCCCTTAAGGGGATAG (sequence of top strand) spanning the *hipBA* operator
sequence or addition of purified His$_6$-HipA reduced the rate of degradation (Fig. 3C). Addition of a control oligo (sequence of top strand ATGATGAGCTTTCAGAAGATC) or a control protein (lysozyme) had no effect on the rate of HipB proteolysis (data not shown).

![Figure 3](image_url)

**Figure 3. Lon degradation of HipB in vitro.** 0.6 μM His$_6$-Lon and 0.48 μM His$_6$-HipB were incubated in reaction buffer at 37°C (50 mM Tris-HCl (pH 8.0), 4 mM ATP, 7.5 mM MgCl$_2$) for indicated times with or without the component specified and subjected to SDS-PAGE and silver staining. (A) In vitro degradation of His$_6$-HipB by His$_6$-Lon. (B) ATP or MgCl$_2$ were omitted. (C) Addition of an oligodeoxynucleotide encompassing the 21 bp hip operator or His$_6$-HipA partially inhibited degradation of His$_6$-HipB.
The unstructured C-terminus of HipB is critical for degradation. HipB is composed of a hydrophobic core, a β-lid and an unstructured 16 amino acid C-terminus (AKNASPESTEQQNLEW) (32). Proteases typically bind disordered regions of their substrate, thus the unstructured C terminus appears to be an excellent recognition site for protease attack (32). To test the hypothesis that the 16 residue C-terminal stretch is critical for degradation, we cloned a truncated HipB (HipB72) lacking the last 16 residues of HipB into pBR creating pBRhipB72. We measured the rate of in vivo degradation of HipB and HipB72 in wild type (KLE901 and KLE905, respectively) and Δlon (KLE902 and KLE906, respectively) (Fig. 4). Interestingly, HipB72 is indeed substantially more stable than full length HipB when expressed in wild type and also in Δlon indicating that the unstructured C terminus of HipB is essential for degradation by Lon protease (Fig.4A). We purified the truncated HipB (His6-HipB72) and tested it in the Lon in vitro degradation assay. After 120 min the majority of HipB72 (68.1%) was still present whereas was 87.3% of HipB degraded (data represent the average of 5 independent experiments) (Fig. 4B).
Figure 4. The 16 C-terminal amino acid residues of HipB are required for degradation. (A) Degradation of HipB and HipB72 in vivo. HipB and HipB72 were expressed from a pBRlacitac promoter in BW25113 (KLE901 and KLE905, respectively) and its lon::kan derivate (KLE902 and KLE906, respectively). The strains were grown in LB medium, and at an OD$_{600}$ of 0.3 1mM IPTG was added. After 1h of induction, protein synthesis was inhibited by the addition of 100 μg/ml Cam, and samples for Western blots were removed over the course of 30 min. HipB wild type data (first lane) same as Fig. 2 (first lane). (B) Degradation of HipB and HipB 72 in vitro. His$_6$-HipB72 was purified and added to the Lon degradation assay.
To confirm that the unstructured C terminus of HipB is a degradation signal for Lon protease, we fused the C terminus of GFP with the unstructured C-terminal tail of HipB (creating pBRGFP-H, KLE908), and tested whether addition of the carboxy-terminal stretch of HipB (residues 73-88) causes degradation of GFP, which by itself is stable over the time period of the experiment (90% remaining after 60 min) (Fig. 5). The GFP-HipB tail hybrid was less stable, only 50% was detected after 60 min confirming that the C-terminus of HipB is critical for rapid proteolysis of HipB.

Figure 5. *In vivo* degradation of GFP and a GFP-HipB hybrid. GFP and GFP with C-terminal fusion to the C terminus of HipB were expressed from a pBRlacitac promoter in BW25113 (KLE907 and KLE908, respectively). The strains were grown in LB medium, and at an OD$_{600}$ of 0.3 1mM IPTG was added. After 1h of induction, protein synthesis was inhibited by the addition of 100 μg/ml Cam, and samples for Western blots were removed over the course of
60 min. Closed squares, GFP; open squares GFP-HipB(73-88). The graph represents the average of five independent experiments.

The hipBA locus has been associated with high persistence. Persistence is the ability to survive exposure to lethal doses of stresses, e.g. antibiotics, without expressing a resistance mechanism. A strain with two point mutations in hipA, (hipA7 allele) as well as overexpression of HipA, lead to high persistence when exposed to a number of different antibiotics. However, deletion of the hipBA operon had no phenotype, possibly due to redundant mechanisms of persister cell formation. Deletion of hipB alone is not possible due to toxicity of HipA. Our results suggest that deletion of the unstructured C terminus of HipB creates a more stable form of HipB that potentially neutralizes HipA more efficiently. We created a scar-less chromosomal deletion of the C terminus of HipB and tested whether a stable HipB has an effect on the surviving persister fraction after ofloxacin. Since the deletion of the entire operon had no appreciable effect on the surviving persister fraction, we expected only a moderate change in persister cells for the partial deletion. We observed a 3.5 fold decrease in the persister fraction of the mutant compared to the wild type in the exponential phase after 5 hours of ofloxacin treatment, and a 2 fold difference in stationary phase after 6 h of treatment (data not shown).

DISCUSSION

The hipBA toxin/antitoxin locus shares several characteristics with other TA modules, such as the genetic organisation in an operon with the antitoxin overlapping the toxin by one base pair, tight regulation of the operon by the antitoxin and inhibition of the toxin by its antidote. In addition, ectopic expression of the toxin confers growth arrest, which can be overcome by antitoxin expression. However, HipBA does not group into the three common toxin families of
RelBE-, the MazEF- and VapBC-like members. Toxin and antitoxin are structurally and mechanistically distinct from all other characterized TA pairs. HipA is a kinase, and HipB belongs to the Xre-helix-turn-helix family of transcriptional regulators. Binding of HipA-HipB2-HipA to DNA introduces a $70^\circ$ bend in the operator (32). In contrast to other antitoxins, HipB interacts with HipA via the N and C domain and the C terminus of HipB remains unstructured in the presence of the toxin (32). Despite functional differences, regulation by proteolysis is a shared characteristic with all other protein-coding antitoxins. HipB is a substrate of Lon protease since HipB is stabilized in the absence of Lon and degraded by Lon in vitro. Under standard growth conditions HipB neutralizes HipA and represses transcription of the *hipBA* operon. However, when Lon activity reaches elevated levels, e.g. during exposure to various stresses, HipB might be getting degraded faster than new HipB is produced resulting in free HipA. HipA can then in turn phosphorylate EF-Tu and potentially act on additional targets leading to the shutdown of essential cellular functions and thus dormancy.

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Chapter 4: The Role of Global Regulators and Nucleotide Metabolism in Antibiotic Tolerance in *Escherichia coli*
ABSTRACT

Bacterial populations produce a small number of dormant persister cells that exhibit multidrug tolerance. Persisters are largely responsible for the antibiotic recalcitrance of biofilm infections. The mechanism of persister formation remains largely unknown due to challenges in identifying persister genes.

We screened an ordered comprehensive library of 3985 Escherichia coli knockout strains to identify mutants with altered antibiotic tolerance. Stationary state cultures in 96-well plates were exposed to ofloxacin at a concentration which only allows tolerant persister cells to survive. The persister level of each culture was determined. 150 mutants with decreased persistence were identified in the initial screen, and subsequent validation confirmed 10 of them not affected in growth rate nor in MIC to ofloxacin. These were 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 (FMN phosphatase). The prominent presence of global regulators among these strains pointed to the likely redundancy of persister formation mechanisms – elimination of a regulator controlling several redundant persister genes would be expected to produce a phenotype. This observation is consistent with previous findings for a possible role of redundant genes such as toxin/antitoxin (TA) modules in persister formation. ygfA and yigB were of special interest. The mammalian homolog of YgfA, methenyltetrahydrofolate synthetase (MTHFS) catalyzes the conversion of 5-formyl THF into
the rapidly degraded 5,10-methenyl THF, depleting the folate pool. The YigB protein is a phosphatase of FMN which would deplete the pool of this cofactor. Stochastic overexpression of these genes could lead to dormancy and hence tolerance by depleting folate and FMN pools, respectively. Consistent with this scenario overexpression of both genes produced increased tolerance to ofloxacin.

INTRODUCTION

Persisters are multidrug tolerant cells that contribute to the antibiotic recalcitrance of biofilm infections (12, 27, 53). Biofilms are implicated in many bacterial infections, including those of indwelling devices, dental disease, endocarditis and cystic fibrosis (16, 18, 23, 38, 46). Understanding the mechanism of persister formation/maintenance is likely to lead to new effective therapies to treat biofilm infections. However, research in this area has been hindered by the difficulties in identifying persister genes.

Persisters are phenotypic variants of the wild type that are tolerant to killing by antibiotics (8, 27). They are survivor cells that make up a small part of the population, $10^{-6}$-$10^{-4}$ in exponentially growing cultures, and $\sim 10^{-2}$ in stationary phase (33). A targeted search for high-persistence mutants of Escherichia coli lead to the finding of a hipA7 allele, but a knockout of the gene appeared to have no phenotype (9, 10, 39, 40, 49), and work of the early pioneers of this field remained largely unknown. Persisters are tolerant to all currently available antibiotics, and are found in all species examined (33). Recent progress in this area can be summarized as follows: persisters are non-growing (6), dormant cells (50); persistence is not heritable (27); persisters probably form a single subpopulation tolerant to unrelated antibiotics (65); a transcriptome of isolated persisters points to toxin/antitoxin modules as possible persister genes.
expression cloning points to enzymes of phospholipid synthesis being involved in persister maintenance (54).

Analysis of a transposon (Tn) mutagenized library seemed a straightforward way to identify persister genes. However, initial efforts to find specific persister genes by analyzing levels of persisters in clones from a Tn insertion library have been unsuccessful (25, 54)

We decided to revisit this approach using an ordered knockout library of all ORF-coding non-essential E. coli gene disruptions (Keio collection) (4), and a sensitive method for detecting tolerance. We find that several global regulators (DnaK, DnaJ, DksA, HU, IHF) as well as phosphatase YigB, and 5-formyl tetrahydrofolate (5-formyl THF) cyclo-ligase YgfA are involved in persister formation.

MATERIAL AND METHODS

Bacterial strains and culture conditions. The Keio collection of E. coli knockout strains (4) was generously provided by Hirotada Mori. E. coli K-12 BW25113 is the parental strain of the ordered knockout library. Precise deletion-replacement of ssrS was created by the method of Datsenko and Wanner (17). To create double deletions the kanamycin resistance cassette replacing one gene was removed by FLP recombinase leaving a FLP recognition target site (FRT). A second deletion allele was then moved into that strain by P1 transduction selecting for kanamycin resistance. Bacteria were cultured with aeration at 37°C in Luria-Bertani (LB) medium or morpholinepropanesulfonic acid (MOPS) minimal medium (42) supplemented with 0.1% succinate and 0.05% casamino acids. When required the medium was supplemented with antibiotics at the following final concentration: mecillinam, 4 μg/ml; ofloxacin 5 or 10 μg/ml; chloramphenicol; 30 μg/ml, kanamycin, 50 μg/ml; streptomycin 10 μg/ml; benzalkonium
chloride 25 µg/ml; ampicillin 100 µg/ml, ciprofloxacin 1 µg/ml; and MgSO₄ at the final concentration of 20 mM. Mecillinam was gift from Leo Pharmaceutical Products.

**Frequency of mecillinam resistant mutants.** The fraction of spontaneous mecillinam resistant mutants was determined by plating serially diluted stationary state culture on LB agar supplemented with 4 µg/ml mecillinam. A sample of the culture was diluted and spotted on LB agar to determine the initial cell count.

**Persistence assay.** Persistence was measured by determining survival upon exposure to antibiotics. To determine the number of persisters in stationary phase, cells were cultured in 1 ml or 200 µl volumes for one overnight (ON) in LB medium, diluted 1000- or 200-fold, respectively into MOPS minimal medium and cultured for two additional ONs. Cultures were exposed to 5 µg/ml or 10 µg/ml ofloxacin for indicated times. 10 µl samples were removed before and after ofloxacin challenge, diluted, and spot plated on LB agar. To determine the number of persisters in a concentration-dependent assay, the concentration of ofloxacin varied as indicated, and the time of challenge was 6 h. Strains harboring expression vector pALS21 and derivatives thereof were assayed in the presence of 0.1 mM IPTG. To determine the number of persisters in exponential phase, cells were cultured in 1 ml volumes for one ON in LB medium, and diluted 1:100 into MOPS minimal medium for 5 hours (final concentration ~2×10⁸ CFU/ml). Cultures were exposed to various antibiotics as indicated for 3 hours. 10 µl samples were removed before and after ofloxacin challenge, diluted, and spot plated on LB agar.
**Screen for persistence mutants.** The mutant library was grown in 200 µl LB medium in 96 well plates overnight at 37°C, diluted into to 200 µl MOPS minimal medium, and incubated for two additional overnights. The library was challenged with 5 µg/ml ofloxacin for 6 h. The contents of the wells were then plated by transferring 10 µl volumes with a 96-pin replicator on LB agar containing 20 mM MgSO$_4$ and 4 µg/ml mecillinam and incubated overnight. The number of mecillinam resistant mutants was established by colony count. The growth rate of mutant strains was determined by measuring OD$_{600}$ in MOPS minimal medium. The minimal inhibitory concentration (MIC) of ofloxacin was determined by the standard NCCLS broth microdilution method (1). Strains which produced no mecillinam resistant mutants or fewer than the wild type were retested in persistence assays as described above.

**Construction of YigB and YgfA overexpression vectors.** pALS21 was constructed by inserting the $\text{lacIq}$ fragment from pCA24N (29) into $\text{ClaI}$ and $\text{BspHI}$ sites of pACYC184 using primers 5’-ATGCATCGATTTCGCGGTATGGCATGATAG-3’ and 5’-ATCGTCATGATACGAGCCGGAAGCATAAAG-3’ and the multiple cloning site from pAH153 (22) into $\text{ScaI}$ and $\text{EagI}$ sites using primers 5’-ACGTAGTACTAAGCAGAAGGCCATCCTGAC-3’ and 5’-TGACCGGCCGACCCAGCCTCGCTTTGTAAC-3’. Primers pORFup 5’GAGCTCGCCCTTTCGTCTTCAC-3’ and pORFdown 5’-CCCGGGTCAGTCACGATGAATTCC-3’ were used to insert the $\text{yigB}$ and $\text{ygfA}$ ORFs including $\text{P}_{\text{T5-lac}}$ promoter region from pCA24N (29) into $\text{SmaI}$ and $\text{SacI}$ sites of pALS21 creating pALS22 and pALS23, respectively. Vectors were transformed into BW25113 by standard procedures.

**RESULTS**
Screening an ordered library for persister genes. A screen of an ordered library (Keio collection) was developed to identify all single knockout mutants exhibiting low persistence. The Keio collection contains 3985 strains created by replacing single genes with a kanamycin cassette (17) in *E. coli* K-12 BW25113 (4, 5). The library was designed to create in-frame single gene deletion mutants after excision of the kanamycin resistance cassette with FLP recombinase to avoid polar effects. Persister levels can vary strongly in parallel samples within a single experiment (65), which made our initial screening attempts unmanageable due to a large numbers of false positives. Apparently, each culture creates slightly different conditions in the process of growth, which can not be controlled for. We therefore reasoned that simplifying the growth medium may decrease culture-to-culture variation. Several conditions were tested, and we found that variation in the level of persisters was decreased in a defined MOPS minimal medium (42) with 0.1% succinate and 0.05% casamino acids compared to LB medium (data not shown). The level of persisters strongly increases with cell density, reaching a maximum in stationary state (27). Variations are therefore likely to be less prominent once all cultures reach the same density at stationary state, and this was chosen for testing. Another problem with screening is that multiple serial dilutions of a culture have to be made and plated to count for surviving colonies. Given the large number of strains and the need for replicating the results, this becomes a very laborious task. We therefore developed a screening approach which allowed for direct plating without dilutions. The rationale is to plate cells on a medium containing an antibiotic, and count surviving colonies of spontaneous antibiotic-resistant mutants. Since the number of resistant mutants is a fraction of the parent strain, this sharply reduces the number of colonies and obviates the need for dilution. We chose mecillinam, a beta-lactam antibiotic that targets penicillin-binding protein 2 (PBP2), as a selecting agent. Spontaneous resistance to
mecillinam occurred with a frequency of $5 \times 10^{-5}$ in the wild type, determined by serially diluting the wild type in two-fold steps and plating 10 µl drops on LB agar supplemented with 4 µg/ml mecillinam. A good correlation between cell density and occurrence of mecillinam resistant mutants was observed (Fig. 1a), which allowed us to use this indirect enumeration method in the screen.

To screen the Keio collection for strains with low persistence, the mutant library was incubated in MOPS minimal medium. After growth for two overnights 5 µg/ml ofloxacin (50 x MIC) was added for 6 h to the stationary phase cultures killing the bulk of the population. At this concentration and within this time period only persisters survive (Fig. 1b) (27). The contents of the wells were plated by transferring 10 µl drops on LB agar containing 20 mM MgSO$_4$ and 4 µg/ml mecillinam and incubated at 37°C. Mg$^{2+}$ inhibits the penetration of ofloxacin and minimizes carry-over effects (32). The number of spontaneous mecillinam resistant mutants was then counted in the wild type and deletion strains. In a pilot experiment, the wild type control produced $5 \times 10^8$ CFU/ml after growth for 48 h in MOPS minimal medium. After ofloxacin challenge, the persister level of the wild type was $3 \times 10^7$ CFU/ml, allowing 15 spontaneous mecillinam resistant colonies to grow on LB supplemented with mecillinam and MgSO$_4$ from the 10 µl aliquot. Thus, under these conditions a low persistence mutant will either not produce any or at least fewer mecillinam resistant colonies than the wild type (Fig. 1c). Since slight day-to-day variations in the numbers of mecillinam resistant colonies of the wild type were observed, relative rather than absolute numbers of resistant mutants in wild type and knockout strains were compared.
Figure 1. Screen for low persistence mutants. (A) Frequency of mecillinam resistant mutants in *E. coli* K-12 BW25113. Stationary phase culture in LB medium was serially diluted in two-fold steps and plated on LB agar supplemented with 4 μg/ml mecillinam. Samples from each
dilution were also removed for colony count. The frequency was $5 \times 10^{-5}$ based on initial cell counts and mecillinam resistant mutants arising on LB agar supplemented with mecillinam. (B) Survival of *E. coli* BW25113 stationary cells challenged with ofloxacin. Stationary phase cultures grown in MOPS minimal medium were treated with ofloxacin at indicated concentrations for 6 h and plated for colony counts. The experiment was performed in triplicate and error bars represent standard deviation. (C) Survival of strains from the Keio collection. Mutant strains cultured in MOPS minimal medium were treated with ofloxacin for 6 h and samples were plated on LB agar supplemented with 4 $\mu$g/ml mecillinam and 20 mM MgSO$_4$. Two hits are encircled.

**Low persistence mutants.** 150 mutants with reduced or no colony count were identified in the screen (3.7% hit rate). To distinguish between mutants that were impaired in persistence and those that produced fewer mecillinam resistant colonies due to lower starting cell numbers or increased sensitivity to ofloxacin or mecillinam, the growth rate and the MIC of these hits were examined. Strains that grew poorly in the minimal medium were identified (*atpE, atpF, carB, cyaA, purA, purD, purF, purK, purL, pyrD, pyrF, pyrI, sdhA, sdhB, sdhC, sdhD*). The expected increased sensitivity to ofloxacin was found in strains deleted in genes involved in recombination and repair (*recA, recB, recG, recJ, recN, recQ, ruvA, ruvB, xseA, xseB*) (47, 14, 26, 35, 36, 62) and in a strain deleted in *tolC*, an outer membrane component of several MDR pumps that extrude fluoroquinolones (43). These mutants were not considered further. The expected increased sensitivity to mecillinam was found in a strain deleted in *ponB*, coding for penicillin-binding protein 1B (20) which was re-tested in subsequent persistence assays. The wild type phenotype of three mutants impaired in cysteine metabolism, $\Delta$*cysHIJ*, was restored when 0.8
mM cysteine was added to the growth medium, showing that impaired survival was due to cysteine limitation (data not shown). Identification of these mutants, which were expected to fail to produce colonies on mecillinam supplemented medium helped validate the screen. The glpD deletion strain previously linked to persistence by our group (54), was among the remaining low persistence mutants and served as a further internal control.

All remaining hits were re-tested by performing persistence assays in stationary phase with 10 µg/ml ofloxacin in 96-well plates. A considerable number of hits did not show significant changes in persister level upon retesting.

The strains that were validated and had the greatest reduction in persister levels compared to the wild type were, 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 (FMN phosphatase) (Fig. 2).
Figure 2. **Survival of mutants affected in putative persister genes.** Cells were grown to stationary phase in MOPS minimal medium in 96-well plates (final concentration $\sim 1 \times 10^9$ CFU/ml) and treated with 5 $\mu$g/ml ofloxacin for 6 h. Samples were diluted and spot plated on LB agar. The experiment was performed in triplicate and error bars represent standard deviation.

**Time-dependent killing studies of low persistence mutants.** The low persistence phenotype of the mutant strains was further characterized in time-dependent killing studies which were performed in MOPS minimal medium in 1 ml cultures in 24-well plates. Stationary phase cultures were challenged with ofloxacin and sampled for CFU counts after 1, 3, and 6 hours of incubation. The absolute survival rate observed in the time-dependent killing studies is different from that in the original selection. In our experience the level of persistence is highly dependent on culture conditions, such as volume, aeration, carbon source, and pH. Increasing the culture volume from 200 $\mu$l in 96-well plates to 1 ml in 24-well plates mostly affects aeration. In 1 ml cultures, the low persistence phenotype was lost in strains with deletions of dnaJ, fis, hns, and hnr (data not shown). Strains with deletions of apaH and surA were moderately affected in persistence after 6 hours of exposure to ofloxacin with 1.3% and 1.7% survival, respectively, compared to 3.3% survival of the wild type. Four strains exhibited a prominent phenotype of an at least 4-fold decrease in persistence compared to the wild type: yigB (30-fold), dnaK (22-fold), dksA (6-fold), and ygfA (4-fold) (Fig.3).
Figure 3. Time-dependent killing studies of mutants affected in persister genes. 1 ml cultures were grown to stationary phase in MOPS minimal medium (final concentration ~1×10^9 CFU/ml) and treated with 10 µg/ml ofloxacin for indicated times. Samples were diluted and spot plated on LB agar. The experiment was performed in triplicate and error bars represent standard deviation.

The frequency of persisters in the dnaK deletion strain was highly variable in parallel replicates. Replicates either had a reduced growth rate at 37°C, and were lower in persistence than the wild type or picked up suppressor mutations which restored normal growth at 37°C and wild type persistence. dnaK null strains have been reported to frequently acquire suppressor mutations, for example in rpoH and rpoD (13, 51).

ygfA, is co-transcribed with ssrS coding for a small RNA, 6S RNA, which acts a repressor of σ70-dependent transcription in stationary phase (63). The location of ygfA directly
downstream of ssrS is conserved in α- and γ-proteobacteria as well as certain β-proteobacteria, which suggests functional relevance of the operon structure (7). Indeed, YgfA has been shown to stabilize 6S RNA in an overexpression system (63). Thus, deleting ygfA apparently diminishes the level of the ssrS transcript. In order to test the phenotype of an ssrS mutant (absent in the Keio library which only contains strains with deletions in protein-coding ORFs) an ssrS deletion (KLE910) as well as an ssrS ygfA double deletion (KLE911) was created. Persistence was tested in 200 µl MOPS minimal medium where ΔygfA had a stronger effect on persistence. We did not detect a phenotype for ΔssrS. The ΔssrS ΔygfA strain appeared to be affected in growth as well as persistence (Fig. 4).

**Figure 4. Effects of ΔygfA and ΔssrS mutations on persister formation.** *E. coli* BW25113 wild type, ΔygfA, KLE910 (ssrS), and KLE911 (ssrS ygfA), were grown to stationary phase in MOPS minimal medium and treated with 10 µg/ml ofloxacin for 6 h. Samples were diluted and
spot plated on LB agar. The experiment was performed in triplicate and error bars represent standard deviation.

To test for the robustness of the low persistence phenotype of the identified mutants, we investigated whether the number of persisters produced in the mutant strains is also lower in growing cultures. We diluted the deletion strains 1:100 in MOPS minimal medium and exposed them to 5 µg/ml ofloxacin in exponential phase after 5 hours of growth. We found that all mutants were also impaired in persistence during exponential phase (at least 4-fold decrease) (Fig. 5a). Similarly to what we observed in stationary phase, after 3 hours of exposure to ofloxacin strains with deletions of dnaK (27-fold), ygfA (17-fold), dksA (11-fold), and yigB (11-fold), showed the most prominent reduction in persistence compared the wild type. Additionally, a strain with a deletion of dnaJ was also strongly affected in persistence (41-fold decrease). We validated the reduction in persistence in exponential phase in these strains in a time-dependent-killing assay and observed the typical persister plateau for all strains (Fig. 5b).
Figure 5. Survival of mutants affected in putative persister genes in exponential phase. (A) 1 ml cultures were grown to exponential phase in MOPS minimal medium and treated with 5 μg/ml ofloxacin 3h. (B) Time-dependent killing studies of mutants affected in persister genes. Mutants with a prominent low persistence phenotype in log phase were exposed to 5 μg/ml
ofloxacin for indicated times. Samples were diluted and spot plated on LB agar. The experiment was performed in triplicate and error bars represent standard deviation.

**Survival of mutants exposed to different classes of antibiotics.** Persister cells are characterized by multidrug tolerance. The screen for low persistence mutants was performed in stationary phase where the choice of antibiotic limited us to fluoroquinolones which are able to kill non-growing cells. In growing cultures, however, we were able to test whether the identified mutants are affected in tolerance when subjected to different antibiotics. We cultured the wild type and the 5 mutants with the strongest decrease in persistence in exponential phase as described above and exposed them to different classes of antibiotics for 3 hours (Fig. 6): another fluoroquinolone, ciprofloxacin; the aminoglycoside streptomycin; the beta-lactam antibiotic ampicillin; and the quaternary ammonium compound benzalkonium chloride. When exposed to ciprofloxacin all mutants showed a prominent decrease in persistence compared to the wild type, ranging from 4-fold ($\Delta ygfA$) to a 111-fold ($\Delta dnaK$) (Fig. 6a). After exposure to streptomycin $\Delta ygfA$ again showed the weakest decrease (3-fold), whereas $\Delta dksA$ (6-fold), $\Delta yigB$ (10-fold), $\Delta dnaK$ (66-fold), and $\Delta dnaJ$ (87-fold) were strongly affected in survival (Fig. 6b). $\Delta ygfA$ however showed a prominent effect after ampicillin challenge (82-fold). Persistence of $\Delta yigB$ was highly variable in this set of experiments and averaged to a 2-fold decrease. $\Delta dksA$ (40-fold), $\Delta dnaJ$ (13-fold), and $\Delta dnaK$ (46-fold) were strongly affected in persister levels (Fig. 6c). The difference in the levels of persisters between wild type and mutants was less pronounced when exposed to benzalkonium chloride. $\Delta dnaK$ had the strongest effect with an 11-fold decrease (Fig. 6d). In summary, these results show that all identified mutant are affected not only in ofloxacin tolerance but in multidrug tolerance.
Figure 6. Survival of low persistence mutants exposed to different classes of antibiotics. 1 ml cultures were grown to exponential phase in MOPS minimal medium and challenged for 3 hours with (A) 1 μg/ml ciprofloxacin, (B) 10 μg/ml streptomycin, (C) 100 μg/ml ampicillin, and (D) 25 μg/ml benzalkonium chloride. Samples were diluted and spot plated on LB agar. The experiment was performed in triplicate and error bars represent standard deviation.
Effect of YgfA and YigB overexpression on persistence. *ygfA* and *yigB* were overexpressed in order to further characterize their effect on persistence. Overexpressing persister genes has been shown to be challenging since overproduction of some proteins causes non-specific toxicity, shutting down cell growth and leading to tolerance (60). Thus, a low copy vector (pACYC184) was used to clone these genes under their native promoters in order to achieve moderate overexpression. Both constructs conferred high persistence in stationary phase after exposure to ofloxacin (Fig. 7). Strains overexpressing global regulators involved in persistence were not tested due to the multitude of phenotypes associated with proteins of global regulatory function and the genes that these regulators are controlling.

![Figure 7](image.png)

**Figure 7. Effects of *ygfA* and *yigB* overexpression on persister formation.** *E. coli* BW25113 cells transformed with pALS21 control (empty vector), pALS22 (*yigB*) and pALS23 (*ygfA*) were grown in MOPS minimal medium in the presence of 0.1 mM IPTG to stationary phase. Cultures
were treated with ofloxacin for indicated times. Samples were diluted and spot plated on LB agar. Wild type: closed squares, KLE921 (pALS21): open squares, KLE922 (pALS22): open diamonds, and KLE923 (pALS23): closed diamonds. The experiment was performed in triplicate and error bars represent standard deviation.

**Role of HU and IHF in persistence.** Two of the low persistence mutants had deletions of \textit{hns} and \textit{fis} which encode nucleoid binding proteins with global regulatory functions. In stationary phase, the low persistence phenotype was only apparent when tested in 200 µl medium in 96-well plates. When tested in 1 ml cultures, persistence of the \textit{hns} and \textit{fis} deletion strains was almost indistinguishable from the wild type. In exponentially growing cultures, \textit{Δhns} and \textit{Δfis} both had a 4-fold reduction in the level of persisters. Interestingly, deletions of the HupA subunit of HU, and the IhfB subunit of IHF, two other nucleoid binding proteins which are global regulators as well, also showed up as hits in the screen. However, upon retesting, the \textit{hupA} deletion was not significantly impaired in persister formation, and the \textit{ihfB} deletion even showed an increase in persistence. We decided to revisit persistence of \textit{hupA} and \textit{ihfB} deletions, and test persistence of deletions of their respective second subunits, \textit{hupB} and \textit{ihfA} and of HU (KLE912) and IHF (KLE913) null strains. It is known that HupB and HupA, and IhfA and IhfB have partial functionality when expressed individually (15, 64, 67). We observed a decrease in persistence in the \textit{ΔhupB} strain which was even more pronounced in the double mutant (Fig. 8a). We confirmed the increase in persistence in the \textit{ΔihfB} strain and the IHF null strain whereas the \textit{ihfA} mutant showed no phenotype (Fig. 8b). We then tested the IHF and HU null strains in a time-killing study in 1 ml volumes, and found that both null strains exhibited robust phenotypes (Fig. 8c). In exponentially growing cultures, the HU null strain had strongly reduced persistence.
levels, 40-fold compared to the wild type when exposed to ofloxacin for 3 hours, whereas persistence of the IHF null strain fluctuated highly in parallel replicates of the same experiment and from day-to-day in exponential phase (data not shown).

Figure 8. Effects of mutations in global regulators IHF and HU on persister formation. (A) and (B) Cultures of *E. coli* BW25113 wild type and mutant strains were grown to stationary phase and treated with ofloxacin for 6 h. Samples were diluted and spot plated on LB agar. (A) HU mutants. Δ*hupA, ΔhupB*, and KLE912 (*hupA hupB*) (B) IHF mutants. Δ*ihfA, ΔihfB*, and
KLE913 (ihfA ihfB). (C) Time-dependent killing studies of HU and IHF mutants. Cultures were grown to stationary phase in MOPS minimal medium and treated with 10 μg/ml for indicated times. Samples were diluted and spot plated on LB agar. Wild type: closed squares, KLE912 (hupA hupB): closed triangles, and KLE913 (ihfA ihfB): open squares. All experiments were performed in triplicate and error bars represent standard deviation.

**DISCUSSION**

**Screening a knockout library for persister genes.** Persisters are dormant, multidrug-tolerant cells and play a major role in biofilm recalcitrance to antimicrobials (12, 27, 53, 50, 33). In this study, we examined a comprehensive knockout library of *E. coli* ORFs with the aim of identifying persister genes. Finding genes coding for a complex biological function is usually straightforward, and screening transposon insertion libraries has been very successful in identifying components of sporulation, the SOS response, biofilm formation, and many others. Indeed, most *spo* genes, for example, were discovered by examining mutants defective in sporulation (37). However, screening knockout libraries for changes in persister levels has been largely unsuccessful (25, 34). Finding mutants with diminished persister levels proved to be highly challenging. The possible reason for this is the high level of redundancy in the mechanism coding for persistence. Previous research suggests that this may indeed be the case. Thus transcription profiling of isolated persisters pointed to overexpression of TA modules, and ectopic expression of several different toxins such as RelA, MazF, HipA, and YgiU causes reversible stasis and multidrug tolerance, emulating persistence (28, 50, 60). Another problem with screening for persister genes is the high level of variability in persister levels among parallel
samples, making the background false positives and negatives unmanageable for a large experiment (65).

We decided to revisit the screening approach, taking an advantage of the recently created complete, ordered knockout library of *E. coli* ORFs. We introduced several changes that considerably improved the quality of screening: (1) simplified the growth medium, which decreased variability among parallel samples; (2) screened in stationary state, where persisters are most abundant, and all cultures reach a similar growth end-point; (3) introduced a method that allows for sensitive screening without multiple dilutions. This involved plating persister cells surviving antibiotic challenge on a medium containing a different antibiotic, mecillinam. In this way, only spontaneous mutants resistant to mecillinam survive, which reduced the number of surviving cells, obviating the need for multiple dilutions.

It is important to stress that none of the knockouts had a complete lack of persisters, in most cases the drop was around 10-fold. The preponderance of global regulators and chaperones among the genes affecting persistence strongly suggests that the function is encoded by redundant genes/mechanisms. Persister genes are probably among the many that are controlled by these regulators. This strongly points to redundancy in persister genes. Note that there have been reports of strong phenotypes of single-gene deletions. We reported previously that *hipA* had a significantly decreased persister level in stationary state; however, that deletion extended into the non-coding *dif* region, important for chromosome partitioning. A precise deletion of *hipA* does not show a persister phenotype. A recent report described *phoU* as a gene whose disruption caused complete loss ofpersisters in the presence of an antibiotic (34). However, the *phoU* strain grows slowly and had decreased MIC towards a number of antibiotics tested. Strains that showed a considerably slower growth or changes in MIC may be pleiotropic mutants with changes in
resistance, rather than being affected in persister genes controlling dormancy and antibiotic
tolerance.

The most prominent finding from the screening was the identification of genes that act as
global regulators. The regulators identified were DksA, SsrS-YgfA, DnaKJ, HupAB, and IhfAB.

DksA is a ppGpp-dependent modulator of RNA polymerase. ppGpp is formed in
response to carbon and amino acid starvation which leads to inhibition of transcription from
rRNA and tRNA promoters and directs transcription towards genes needed for synthesis and
transport of amino acids (44, 45). Considering that persisters are dormant cells, this makes sense,
since stochastic increase in ppGpp or DksA, or both, would contribute to dormancy and then
knocking out dksA would decrease the level of persisters. The strain deleted in the ppGpp
synthetase RelA was identified as a hit in the primary screen. However, a ΔrelA strain grows
very poorly in minimal medium (unless suppressor mutations in RNA polymerase subunits are
acquired) (24); moreover, resistance to mecillinam is conferred by an increase in ppGpp
concentration (61). Thus, relA was removed from our set of low persistence hits. Note that a relA
and relA spoT deletion has previously been linked to persistence in a hipA7 mutant and in the
wild type (30).

ygfA knockout produced a robust phenotype of decreased persister formation. YgfA
apparently codes for a 5-formyl THF cyclo-ligase involved in folate biosynthesis. The
mammalian homolog of YgfA, methenyltetrahydrofolate synthetase (MTHFS) catalyzes the
conversion of the stable folate storage form 5-formyl THF into the rapidly degraded 5,10-
methenyl THF thereby depleting cellular folate pools (2). If the E. coli enzyme has the same
function, then in a simple scenario, stochastic overexpression of YgfA will lead to folate
deficiency impairing biosynthesis of purines, thymidilate, and methionine (7) and will contribute
to persister formation. Artificial overexpression of YgfA indeed produced a phenotype of increased tolerance to ofloxacin. Knocking out ygfA would be expected to decrease the level of persisters, which we have observed. Interestingly, ygfA is a member of a two-gene ssrS ygfA operon. ssrS codes for the small regulatory 6S RNA which interacts with σ70-RNA polymerase (RNAP) and thereby inhibits transcription of most σ70 dependent promoters in stationary phase. Common among most inhibited promoters is an extended -10 element present in more than 100 σ70-dependent promoters (59, 63). 6S RNA is present throughout all growth phases but is most abundant during stationary phase when >75% of σ70-RNAP complexes are associated with 6S RNA (63). In addition, 6S RNA also indirectly activates transcription of σS-dependent promoters (59). It appears that these two elements potentially control two different aspects of RNA synthesis – 6S RNA acts as an inhibitor of RNA polymerase, while YgfA can inhibit synthesis of nucleotides. This two-gene module seems like an attractive candidate for a persister operon. Knocking out ssrS produced no phenotype. However, it is known that ygfA stabilizes ssrS (63) and an ygfA knockout is therefore functionally closer to an ssrS ygfA double knockout. It may be that in vivo, changes in both of these functionally similar redundant components are necessary to observe a change in persister levels.

Another interesting gene that can potentially affect dormancy is yigB. The YigB protein was shown to act as a phosphatase of FMN (31) which would deplete the pool of this important cofactor in the cell upon overproduction, potentially contributing to dormancy. Overproduction of YigB produced high persistence, consistent with a possible role in persister production. Three other phosphatases of the same group, YnjI, Cof, and YrfG, were also shown to have specificity for FMN. To investigate whether involvement in persister formation is specific to YigB or
shared among FMN phosphatases, we retested strains with deletions of \textit{ynjI}, \textit{cof}, and \textit{yrfG}. None of the strains had a phenotype in regards to low persistence (data not shown).

DnaK chaperone assists in protein folding of nascent polypeptides and requires ATP and its co-chaperones DnaJ and GrpE (55, 56). DnaK also acts as a regulator of a large set of genes induced by heat shock and general stress response by regulating the activity and stability of the alternative sigma factors $\sigma^{32}$ and $\sigma^{5}$, respectively (41, 57, 58, 66). A decreased level of persisters in a \textit{dnaK} strain suggests that it may be required for maintaining persisters, and is thus a persister maintenance gene (similarly to previously described persister maintenance gene \textit{plsB}). It is also possible that DnaK controls several redundant persister genes, and the phenotype is only observed when their regulator is absent. A decrease in persistence was also found in a \textit{dnaK} deletion strain in \textit{S. aureus} when challenged with oxacillin (52).

Other global regulators which showed a phenotype when deleted were HU and IHF. A $\Delta hupB$ strain showed a decrease in persistence which was even more pronounced in the \textit{hupAB} double mutant. The opposite effect was observed in an IHF null strain and an \textit{ihfB} single deletion which are both high persistence mutants. IHF binds to specific DNA sequences of 30-35 bp (21) and directly or indirectly regulates transcription of more than 100 genes (3). Thus, fluctuation in the level of persistence in an exponentially growing culture of an IHF null strain might be explained by the deregulation of numerous genes. In contrast, HU binding to DNA is non-specific (11). HU acts as a regulator by promoting and inhibiting DNA binding of transcriptional regulators such as CRP protein, Lac repressor, Gal repressor, Trp repressor, and LexA (19, 48).

The genes identified by this screen provide good clues to the mechanism of persister formation and maintenance and will serve as a starting point for a more detailed investigation of the molecular mechanism of multidrug tolerance. What this screen missed are possible highly
redundant persister genes such as the TA modules, the essential genes, and most regulatory RNAs. It appears that a combination of several independent approaches will be required to obtain a comprehensive set of genes participating in persistence.

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