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Molecular Mechanisms of HipA Mediated Multidrug Tolerance and its Neutralization by HipB

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Summary

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 Ser/Thr 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.

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} – 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 growth phase after antibiotic removal, allowing survival of the bacterial population. 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 co-transcribed 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 as wild type HipA cannot be expressed in the absence of HipB due to 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 an RNase/gyrase-like fold, RNase barnase-like structure or PIN domain (14). The corresponding antitoxins contain

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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 the only toxin that is a validated biofilm tolerance factor. 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. Due to wild type HipA mediated persistence we used the HipA mutant, 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; fig. S1) (16–18). The HipA structure has a globular fold with 15 β -strands and 15 α -helices and can be divided into an N-terminal α/β domain and an all α -helical C-terminal domain (Fig. 1A). Notably, density is missing for residues 185–195, which are near the active site and likely 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 α atoms, indicating that HipA represents a new class of protein kinase (20).

HipA contains all the catalytic residues found in protein kinases, including the putative catalytic base, Asp³⁰⁹ (20). The D309Q mutation abrogates persistence, strongly suggesting that kinase function is key to HipA-mediated MDT (15). Indeed, we found that HipA binds ATP with a K_d of 18.0 ± 2.0 μM , which is similar to the dissociation constants obtained for ATP binding to other Ser/Thr kinases (fig. S2) (20). To delineate the ATP binding mechanism of HipA, we determined the structure of the HipA-ATP-Mg²⁺ complex to 1.66 Å resolution and refined the structure to an $R_{\text{work}}/R_{\text{free}}$ of 18.4%/21.7%. Clear 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 Glu²³⁴, the amide nitrogen of Phe²³⁶ and the side chain N ϵ of Gln²⁵², respectively. The adenine ring stacks with Phe²³⁶ and Tyr³³¹ while Val⁹⁸, Val¹⁵¹, Ile¹⁷⁹ and the side chain methylene carbons of salt-bridged residues Asp²³⁷ and Arg²³⁵ provide hydrophobic interactions. The γ phosphate hydrogen bonds to both the side chain of His³¹¹ and the amide groups of Gly¹⁵³ and Ala¹⁵⁴, 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 apoHipA structure indicating that nucleotide binding is required for its stabilization. Two Mg²⁺ ions are also present in the HipA-ATP structure and likely 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 the N- and C-domains to undergo only a small rotation ($\sim 4^\circ$) 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 finding of a unique kinase fold and high affinity ATP binding supported strongly the hypothesis that HipA mediates persistence by phosphorylating one or more target proteins. To identify possible HipA targets, we carried out *in vitro* pull-down assays on candidate *E. coli* proteins. One protein, EF-Tu, was found to interact stringently with HipA in the presence of ATP-Mg²⁺ and 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-tRNA binding to the ribosome (22). Upon 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 Thr³⁸² by an unknown kinase(s) (23–24). The side chain of Thr³⁸² contacts Glu¹¹⁷ to stabilize the GTP-bound, closed state of EF-Tu. Phosphorylation of Thr³⁸² favors the GDP-bound, open form as it would lead to repulsion of Glu¹¹⁷ and prevent EF-Tu from adopting the GTP-bound closed conformation. Critically, Thr³⁸² phosphorylated EF-Tu cannot bind aminoacyl-tRNA and is therefore inactive in translation (23–24). To test if 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 anti-pThr/pSer/pTyr antibodies, 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, encompassing Thr³⁸² (bold) with a K_d of $15 \pm 5 \mu\text{M}$ (fig. S4). Subsequently, we solved a crystal structure of the HipA-(AMP-PNP)-IREGGRTVGA complex to 3.5 Å resolution. The structure revealed that the activation loop, residues 185–195, 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 strongly 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).

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 with 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 bp *hipB* operator (top strand ACTATCCCCTTAAGGGGATAG) was solved and refined to an $R_{\text{work}}/R_{\text{free}}$ of 22.5%/28.1% to 2.68 Å resolution (Table S1) (Fig. 3A–C).

HipB forms a compact dimer that specifically interacts with DNA through major groove contacts, while two HipA molecules sandwich the HipB-DNA complex by contacting the sides of the HipB dimer (Fig. 2A–C). Notably, 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 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 three 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). 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 Å, 1.60 Å and 1.51 Å for 59, 56 and 59 corresponding Ca atoms, respectively, thus placing it in the Xre-HTH family of transcriptional regulators (25). The homology between HipB and these proteins is confined to the four-helix bundle region as the β-lid is found only in HipB. Despite the similarities in DNA binding domains, these proteins bind their DNA sites differently as 434 Cro does not significantly distort its DNA site and biochemical data indicate that C.AhdI bends its DNA site by 47° (26–27). By 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.

HipB-induced DNA distortion aligns the recognition helices for specific binding to consecutive major grooves. Contacts from the HTH specify completely the nucleotides of the HipB signature motif, T₂A₃T₄C₅C₆ (Fig. 3A–B). Ser²⁹ from α2 makes hydrophobic contacts to the

Thy2 methyl group. Residues of the recognition helix provide the remaining base specifying contacts whereby two hydrogen bonds from Gln³⁹ read Ade3, while two hydrophobic contacts from Ala⁴⁰ and Ser⁴³ specify Thy4. Finally, Lys³⁸ makes hydrogen bonds with the guanine O6 oxygens of base pairs 5 and 6. HipB also makes 11 phosphate contacts to each half site. DNase 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 Lys³⁷⁹ and Arg³⁸² (Fig. 3A,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 regions of both HipA and HipB (Fig. 2; Fig. 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 while the HipA C-domains interact primarily with the other HipB subunit (Fig. 2C). This interaction interface is extensive, burying ~5000 Å² ASA, and involves both nonpolar and polar interactions. In the HipA N-domain-HipB interface, HipB residues from the turn before α1 interact with residues on HipA β15 and residues on HipB α1 make extensive contacts to HipA residues located on a long 3₁₀-like loop between β3 and α1. The formation of the HipA C-domain-HipB interface primarily involves HipB residues from α2 and the turn between α4 and β1. These residues interact primarily with HipA residues in the loop between α8 and α9 and the N-terminus of α9. In addition, “cross subunit” contacts are made between HipB residues Gln¹² and HipA C-domain residue Gly²⁸⁴ and HipB residue Tyr⁸ and HipA C-domain residue Ser²⁸⁶. Notably, these cross contacts, combined with the numerous interactions made by each subunit in the HipB dimer to the N- and C-domains of HipA, lock HipA in an open and likely, 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 and degrade the substrate. HipB contains an exposed and flexible 16 residue C-terminus attached to the small β-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 by the HipA7 protein. HipA7, which contains two substitutions, G22S and D291A, confers a high persistence phenotype to *E. coli* cells independent of HipB. Subsequent data revealed that the D291A mutation alone was sufficient for this phenotype (28). The HipA-HipB-DNA structure indicates that this phenotype likely results from a weakened HipA-HipB interaction, which unleashes HipA kinase activity. Specifically, Asp²⁹¹ makes key contacts to stabilize the HipA-HipB interface, including hydrogen bonds to the side chain of Ser²⁸⁵, which positions the Ser²⁸⁵ carbonyl oxygen to interact with HipB residue Gln⁶², and to the amide nitrogen of Leu³²⁷, 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. 4C–D). In addition, ITC studies revealed a K_d of 15.0 ± 1.0 μM for ATP-Mg²⁺ binding to HipA in the HipA-HipB-DNA complex, which is essentially identical to that obtained for ATP-Mg²⁺ 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 indicates that while ATP

binding causes only small domain movements such as those we observe in HipA, binding of protein substrates causes significant 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 sequestration (29).

In conclusion, these studies have provided important insight into the novel mechanisms by which HipA mediates persistence and HipB neutralizes HipA. The high conservation of HipA amongst Gram-negative bacteria indicates its central role in the development of persistence. Thus, inhibitors that target specifically the substrate binding sites of HipA, may prove effective against persistence and multidrug tolerance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 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. This Figure and Figures 1B–C, 2A–C, 3B–D and 4A–D were made using PyMOL (30).

(B) The ATP binding pocket of HipA. Shown are ATP molecule (sticks), Mg²⁺ ions (magenta spheres), waters (red spheres) and hydrogen bonds (dashed black lines).

(C) Superimposition of the C-domains of apoHipA (yellow) and HipA-ATP-Mg²⁺ (blue) reveals only a small rotation upon ATP binding. The location of ATP in the HipA-ATP structure is shown as sticks.

(D) *Top panel* shows HipA-(GST-EF-Tu) pulldown (SDS-PAGE, stained with Coomassie brilliant blue). Lanes are (1) MW ladder (2) Glutathione agarose (bead) retentate after adding GST-EF-Tu, HipA, ATP, GDP (3) Bead retentate after adding GST-EF-Tu, HipA, ATP, GDP and washing (4) Bead retentate after adding GST-EF-Tu, HipA, no GDP or ATP (5) Bead retentate after adding GST-EF-Tu, HipA, no GDP or ATP, and washing.

Bottom panel wt 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 (1) EF-Tu (cleaved)+wt HipA+ATP+GTP, (2) EF-Tu (cleaved)+wt HipA+ATP+GDP, (3) EF-Tu (cleaved)+inactive HipA (D309Q)+ATP+GDP, (4) EF-Tu (uncleaved)+wt HipA+ATP+GDP, (5) EF-Tu (uncleaved)+wt HipA+ATP+GTP, (6) wt HipA+ATP (7) native EF-Tu (cleaved)+ATP+GDP.

Fig. 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, one subunit of the HipB dimer is colored yellow and the other orange. The N- and C-termini and secondary structural elements of one HipB subunit (orange) are labeled. β_1' 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 apoHipA (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) Close up of the HipA-HipB interaction interface. Each lateral side of the HipB dimer, labeled subunit 1 and 2, interacts with the N- and C-domains of one HipA monomer. The DNA is shown as a grey surface. For clarity, only one HipA molecule is shown as 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.

Fig. 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-mer duplex is shown as the identical contacts are made to each half site. The strands are labeled 1A–10A and 1B–10B. Bases are represented as rectangles and labeled according to sequence. The ribose groups are shown as pentagons. The operator consensus sequence, TATCC, is colored red. HipB-DNA contacts are colored yellow. Hydrophobic contacts are indicated by lines and hydrogen bonds, 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 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 Figure 3B.

(D) HipA-HipB bound DNA is bent. 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 σ . The DNA is shown as sticks and the bend angle indicated.

Fig. 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 CPK. HipB residue Q62 and HipA residues S285 and L327 are also shown as CPK. A close up 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 colored yellow, the HipB dimer is cyan, the DNA and ATP are shown as sticks. The blue mesh represents an $2.98 \text{ \AA}$ resolution F_0-F_c map, contoured at 4.0σ in which the ATP was omitted.

(D) Close up view of the ATP binding pocket and omit map. Carbon, nitrogen, oxygen and phosphorus atoms are colored magenta, blue, red and purple, respectively.