Structure of the *Proteus vulgaris* HigB-(HigA)₂-HigB Toxin-Antitoxin Complex

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Toxin-antitoxin (TA) systems are chromosomally or plasmid-encoded gene pairs found in free-living bacteria that aid in survival during environmental and chemical stresses (1). TA systems have been implicated in diverse functions such as programmed cell death, growth, and gene regulation, biofilm formation, and persistence during increased antibiotic exposure, but their precise physiological functions are controversial (2–8). Their roles in persistence, adaptation, and survival mechanisms underscore their great potential as novel antimicrobial targets (9).

Type II TA operons encode both a small antitoxin and toxin protein (8–12 kDa each) that under normal growth conditions form a tight, nontoxic complex. These complexes transcriptionally autorepress by binding at operator sequences in their promoter region (1). Upon stress, the antitoxin is degraded by proteases, allowing the toxin to target key cellular processes, including replication (DNA gyrase) and translation (free mRNA, ribosome-bound mRNA, or the ribosome itself) (10–17). Tightly regulating and/or reducing these energetically expensive processes leads to an overall decrease in metabolite consumption and halts cell growth. This bacteriostatic state continues until the stress passes (1).

ReEl is one of the best studied ribosome-dependent toxins and functions by degrading mRNAs preferentially at stop codons in the ribosomal A site (11). Recent evidence suggests ReEl may recognize additional codons, but the molecular details of this specificity remain unclear (18, 19). The additional ReEl family member YafQ cleaves at lysine codons, and member YoeB cleaves at both sense and stop codons (14, 20, 21). The host inhibition of growth B (HigB) protein from *Proteus* spp. is a ReEl family member with a relaxed codon specificity (13, 22). HigB preferentially degrades 5’-AAA-3’ codons (lysine), but codons containing only one adenosine are sufficient for degradation by HigB (13).

The *Proteus vulgaris* HigBA TA system was first discovered on an exogenous plasmid that conferred kanamycin resistance and post-segregational killing at elevated temperatures (23). This plasmid was isolated from a post-operative pyelonephritis, an ascending urinary tract infection (23, 24). The higBA gene pair is not found in *Escherichia coli* K12 but is found chromosomally in pathogens such as *Vibrio cholerae, Streptococcus pneumoniae*, *E. coli* CFT073, and *E. coli* O157:H7 (25).

The HigB toxin gene and protein are distinguished from those of other ReEl family toxins in three ways. First, the *higBA*
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operon has an inverted gene structure with the HigB toxin gene preceding its cognate antitoxin (Fig. 1A) (23). This gene arrangement is only seen in the MqsRA and hicAB TA systems (15, 25). Second, sequence alignments with other RelE family members indicate that HigB appears to lack conserved catalytic residues required for mRNA recognition and degradation (Fig. 2A). Third, a single adenosine in the context of a codon is sufficient for degradation by HigB (13). This contrasts with previously proposed strict mRNA sequence requirements for other toxins (11).

We report the structural and biochemical characterization of the novel TA pair HigBA. Remarkably, our structure shows that, unlike most antitoxins, HigA makes relatively few contacts with its toxin partner and does not cover the solvent-accessible HigB active site. This structural arrangement implies a possible novel model of inhibition. We also present biochemical data that demonstrate tetrameric HigBA (henceforth denoted as HigB-(HigA)$_2$-HigB to reflect its spatial organization) is required for productive binding to its own DNA operator sequences, validating the functional relevance of our structural data.

EXPERIMENTAL PROCEDURES

Plasmids pET21c-HigBA and pET28a-His$_6$HigBA were generous gifts from Dr. Nancy A. Woychik (Rutgers–Robert Wood Johnson Medical School). A C-terminal hexahistidine (His$_6$) tag encoded on the pET21c construct was added to HigA of the pET21c-HigBA construct by removal of the natural HigA stop codon using site-directed mutagenesis to create pET21c-HigBA-His$_6$. The pET28a-His$_6$HigBA (Δ84–104) plasmid was created by placing a premature stop codon in HigA after the codon 83. All sequences were verified by DNA sequencing (GeneWiz).

**HigBA Expression and Purification**

*E. coli* BL21(DE3) cells harboring pET21c-HigBAHis$_6$ and pET28a-His$_6$HigBA were grown at 37 °C with shaking in Lysogeny Broth medium with either 100 μg/ml ampicillin or 10 μg/ml kanamycin, respectively. Protein expression was induced with 0.05 mM isopropyl 1-thio-D-galactopyranoside, and cultures were grown for an additional 3 h except for pET28a-His$_6$HigBA (Δ84–104), which was grown at 18 °C for 12 h after induction. All cells were pelleted at 4,000 × g for 15 min, washed with size exclusion buffer (40 mM Tris-HCl, pH 7.5, 250 mM KCl, 5 mM MgCl$_2$, and 5 mM β-mercaptoethanol), pelleted again at 7,000 × g for 10 min, and stored at −20 °C.

Cell pellets were thawed on ice, resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 10% (w/v) glycerol, 250 mM KCl, 5 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% (v/v) Triton X-100), and lysed by sonication. Each supernatant was collected by centrifugation at 39,000 × g for 45 min and filtered through a 0.45-μm filter (Millipore), prior to loading onto a 5-ml Ni$_2$-nitrilotriacetic acid column using an ÄKTApurifier10 (GE Healthcare) at 10 °C. The column was washed with buffer A (40 mM Tris-HCl, pH 7.5, 10% (w/v) glycerol, 250 mM KCl, 5 mM MgCl$_2$, 5 mM β-mercaptoethanol, and 50 mM imidazole) and eluted with a linear gradient of the same buffer supplemented with 500 mM imidazole. Elution fractions containing the target proteins were concentrated with a 3-kDa molecular mass cutoff concentrator (Millipore), filtered, and loaded onto a Superdex 200 16/60 column (GE Healthcare). Protein fractions determined to be >95% pure by SDS-PAGE were pooled and used for crystallization or biochemical analyses. Selenomethionine-incorporated HigBA-His$_6$ protein was expressed in *E. coli* BL21(DE3) cells as described (26) and purified as described above.

**Crystallization, X-ray Data Collection, and Structural Determination of HigBA Complexes**

HigBA-His$_6$ (Crystal Form 1)—Crystals of trypsinized selenomethionine-derivatized HigBA-His$_6$ were grown by sitting drop vapor diffusion in 3–10% PEG 3350, 0.2 M l-proline, and 0.1 M HEPES, pH 7.5, over approximately 2 days at 10 °C. Ethylen glycol was used as a cryoprotectant and added in two increments to a final concentration of 30%. Crystals were flash-frozen in liquid nitrogen, and a single anomalous dispersion dataset was collected at the Northeastern-Collaborative Access Team (NE-CAT) 24-IDC beamline at the Advanced Photon Source using 0.979 Å radiation (Table 1). A total of 113,311 reflections were collected, indexed, and reduced to 16,748 unique reflections (unmerged) to a resolution of 2.8 Å with the program HKL2000 (27). Phase determination was carried out using the intrinsic anomalous signals from selenium. A total of 11 heavy atom sites were identified and used for initial phases with the program Autosol of the PHENIX Suite (28). The starting model was initially built by PHENIX Autobuild (28), followed by manual building in Coot (29). During refinement, XYZ coordinates, real space, and B-factors (isotropic) were refined to a final R$_{work}$/R$_{free}$ of 19.7/23.8. The final model contained two HigB and two HigA molecules per asymmetric unit (Fig. 1 and supplemental Fig. S1A).

HigA-HigB (Crystal Form 2)—Crystals of His$_6$-HigBA were grown by sitting drop vapor diffusion in 90 mM sodium acetate, pH 4.6, 180 mM ammonium acetate, 25% PEG 4000, and 4% acetone at 20 °C in 1 week. For cryoprotection, dextrose was dissolved in the reservoir solution and added to the crystallization drop in 15% increments up to 30% (w/v) by exchanging the mother liquor. This was followed by 1–2 min of equilibration, flash frozen in liquid nitrogen, and a native dataset was collected at NE-CAT 24-IDE beamline. A total of 172,519 reflections were collected, indexed, and reduced to 31,287 unique reflections with the program XDS (30). The structure was solved to 2.2 Å by molecular replacement using the AutoMR PHENIX program (28) with one HigB and one HigA molecule from the previously solved HigBA complex as a search model (form 1). Three HigB and three HigA molecules were found in the asymmetric unit (supplemental Fig. S1B). A similar PHENIX refinement scheme was used as with form 1 but with the addition of TLS refinement. Manual model building in Coot was performed to a final R$_{work}$/R$_{free}$ of 17.3/21.1% (29).

Protein interfaces, surfaces, and assemblies (PISA) program was used to calculate molecular interfaces and oligomeric states (31), and ConSurf was used to map HigB sequence conservation onto the crystal structure (32). Sequence alignments were performed with ClustalW (33), and all figures were generated using PyMOL (34).
**Size Exclusion Chromatography (SEC) Assay**

One hundred microliters of 75/H9262 M protein in SEC buffer were loaded onto a Superdex 75 10/300 column (GE Healthcare). Estimated molecular weights were calculated by comparison with the molecular weight standards (Bio-Rad) (Fig. 5D). Peaks from the SEC chromatogram corresponding to different protein-protein complexes were run on a 15% SDS-polyacrylamide gel for analysis (Fig. 5E).

**Electrophoretic Mobility Shift Assay (EMSA)**

Assays were performed as described previously (35) but with slight modifications. Double-stranded DNA representing the Phig region was generated by mixing chemically synthesized DNA (IDT), heating to 90 °C for 2 min, and slowly cooling to room temperature (Fig. 5A). Protein at a final concentration of 0, 0.25, 0.5, 1, and 2/H9262 M was incubated with 10 ng of DNA for 20 min on ice along with 0.5 mg/ml BSA. Free and protein-bound DNA were resolved on a native 8% polyacrylamide gel prepared with Tris borate, pH 8, EDTA buffer (Fig. 5C). The gel was run at 10 °C for 1 h, and DNA was stained with SYBR Green dye (Invitrogen) and visualized using a Typhoon Trio (GE Healthcare).

**Molecular Modeling HigB on the 70 S Ribosome**

HigB was modeled on E. coli RelE bound to the Thermus thermophilus 70 S ribosome (PDB code 3KIQ) (36). The HigB coordinates were optimally superimposed onto RelE using secondary structure matching in Coot (37). Conserved secondary structural motifs of the RNase fold of RelE and HigB aligned with a root mean square deviation (r.m.s.d.) of 2.4 Å (for 63 equivalent α-carbon pairs) (supplemental Fig. S3).

**RESULTS**

**Structural Determination of the HigB-(HigA)₂-HigB Complex**

By placing the hexahistidine tag at either the N terminus of HigB or the C terminus of HigA, we were able to solve two different x-ray crystal structures of the HigBA complex (Fig. 1 and supplemental Fig. S1). Given how small each protein is (the antitoxin is 11.5 kDa and the toxin is 10.7 kDa), we were concerned that the affinity tag may influence potential crystal packing interactions and the overall oligomeric states. However, both crystal structures are entirely consistent, with an overall r.m.s.d. of 0.9 Å for 366 equivalent α-carbon pairs with only a single minor difference within loop 5 of HigB (supplemental Fig. S1C) (38).

The HigBA-His₆ complex (form 1) crystallized in the hexagonal space group P3₂1 with two HigBA heterodimers per asymmetric unit (supplemental Fig. S1A). The initial phases to 2.8 Å were obtained by single anomalous dispersion using selenomethionine-derivatized protein (Table 1). This model was used as an initial search model for the His₆-HigBA structure (form 2) and was solved using molecular replacement to 2.2 Å (Table 1). The form 2 complex grew in the hexagonal space group P6₃ with three HigB and three HigA molecules per asymmetric unit (Table 1 and supplemental Fig. S1B). Both forms 1 and 2 contain a HigB-(HigA)₂-HigB tetramer, although form 2 contains an additional HigBA dimer in the asymmetric unit. A full tetramer is formed by applying two-fold crystallographic symmetry (supplemental Fig. S1B). Thus, the overall subunit compositions of HigB and HigA are identical.

In form 1, residues 1–90 (92 total) were built for one HigB molecule, although unambiguous density allowed building of all HigB residues of the second molecule. The C terminus of the fully built HigB is involved in crystal contacts with a neighboring crystallographic symmetry-related molecule, which presumably stabilized this region. The side chain and backbone of HigB residues Lys-57, Asp-59, and Glu-61 have poor electron density in both crystal forms, and two out of the three HigB molecules from form 2 showed little to no Cα electron density for Lys-57 and Asp-59. Therefore, the backbone was built using...
This family shares a microbial RNase fold characterized by a tertiary fold consistent with the RelE/YoeB family (Fig. 2). HigB shows an overall similarity with these toxins (14–18%), HigB shares an overall tertiary fold consistent with the RelE/YafQ/YoeB (22, 25, 40). Despite low sequence identity with these toxins (14–18%), HigB shows an overall tertiary fold consistent with the RelE/YoeB family (Fig. 2, B and C). This family shares a microbial RNase fold characterized by a single α-helix that packs against an antiparallel β-sheet (Fig. 2B). HigB is a small globular protein consisting of two N-terminal α-helices (α1–2) flanked by three twisted, parallel β-strands (β1–3) and six loops (Fig. 2B). A Dali search reveals HigB is most similar to Mycobacterium tuberculosis RelK (PDB code 3OEL), E. coli YoeB (PDB code 2A6S), Pyrococcus horikoshii RelE (PDB code 1WMI), and M. tuberculosis RelE-2 (PDB code 3G5O), with Z-scores of 8.3, 8.4, 7.7, and 6.4, and r.m.s.d. of 2.7, 2.8, 2.7, and 2.7 Å, respectively (using 79, 80, 73, and 73 α-carbon positions. In form 2, residues 1–90 were built for all three HigB molecules. In both crystal forms, HigA (104 amino acids total) was modeled to either residue 92 or 93 as no interpretable electron density was seen beyond these positions.

Both HigBA structures adopt nearly identical tertiary and quaternary structures (supplemental Fig. S1C). Two HigA molecules form a dimer similar to that observed in previous HigA crystal structures without the toxin (supplemental Fig. S2A) (39). Each HigB interacts with one HigB molecule to form a heterodimer that with additional HigA–HigA interactions completes a dimer of heterodimers (Fig. 1, B and C). Consistent with our structural results, PISA predicts the HigBA complex to exist as a tetramer (31). The structure of the P. vulgaris HigA dimer in the context of the TA complex is very similar to that of HigA alone from E. coli CFT073 (PDB codes 2ICT and 2ICP) (39) and Coxiella burnetii (PDB code 3TRB) with r.m.s.d. of 2.5, 1.6, and 1.6 Å, respectively. This indicates HigA does not undergo large conformational changes upon toxin binding (supplemental Fig. S2A).

HigB Adopts a Microbial RNase Fold—HigB is a member of the RelE toxin family, which includes ribosome-dependent toxins RelE, YafQ, and YoeB (22, 25, 40). Despite low sequence identity with these toxins (14–18%), HigB shows an overall tertiary fold consistent with the RelE/YoeB family (Fig. 2, B and C). This family shares a microbial RNase fold characterized by a neighboring residues as guides for α-carbon positions. In form 2, residues 1–90 were built for all three HigB molecules. In both crystal forms, HigA (104 amino acids total) was modeled to either residue 92 or 93 as no interpretable electron density was seen beyond these positions.

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utilize any of its five α-helices to conceal or wrap around HigB, demonstrating a novel mode of antitoxin interaction with HigB (Fig. 1B). HigA primarily contacts HigB at two positions via largely hydrogen bonding and electrostatic interactions (Fig. 3). The N terminus, α1, α5, and loop 5 of HigA pack directly against the HigB N terminus, α1, α2, and loop 3 (Fig. 1B). The HigA N terminus interacts with HigB α1 via hydrogen bonding of the backbone carbonyls of Phe-14 and Asn-31 to the backbone of HigA Phe-4 and the side chain of Gln-3, respectively (Fig. 3A). Two water-mediated interactions occur between HigA residues Lys-5 and Ser-7 and HigB residues Leu-13, Asn-31, Gln-35, and Asp-32, forming a hydrogen-bonding network (Fig. 3A). Four HigA residues, Arg-15, Asp-16, Asp-19 (α1), and Arg-69 (α5), make ionic interactions with HigB residues Asp-32 (α2), Arg-48 (loop 3), Arg-29 (α2), and Glu-45 (loop 3), respectively (Fig. 3, A and B). These ionic interactions surround a hydrophobic core mediated by HigA residues Met-12, Phe-66, and Leu-70 and HigB residues Ala-36, Thr-39, Leu-46, and

FIGURE 2. Highly conserved amino acids of HigB cluster on a concave, solvent-accessible surface. A, sequence alignments using ClustalW (33) of P. vulgaris HigB with other ribosome-dependent toxins showing residues with 50, 75, or 100% sequence identity as light, medium, or deep purple, respectively. Residues located within the HigB concave surface (purple circles) and E. coli RelE amino acids that recognize and/or degrade mRNA (black circles and triangles, respectively) are indicated. B, HigB toxin structure colored by amino acid conservation among HigB homologs according to the scale shown (1 is least conserved and 9 is the most conserved). Residues located on the concave surface proposed to contain active site residues are shown as sticks. HigB residues that make ionic interactions with HigA are also shown as sticks and colored by conservation. C, E. coli RelE R81A toxin structure (PDB code 4FXI) with residues identified as important for mRNA recognition or cleavage shown as sticks.

FIGURE 3. Minimal interface between HigB and HigA. A, zoomed in view of HigB-HigA salt bridge and hydrogen bonding interactions (dashed lines). Water molecules are shown as red spheres and color scheme is the same as in Fig. 1. B, ~45° rotated view of A highlighting additional salt bridge and hydrogen bonding interactions. C, hydrophobic interactions formed between HigA and HigB.
Tyr-51 (Fig. 3C). Both HigB and HigA residues involved in the HigA-HigA and HigA-HigB interfaces are not highly conserved among HigB and HigA homologues (Figs. 2A and 4A, circles and triangles).

**HigA Monomer Contains an Intact DNA Binding Domain**—The *P. vulgaris* HigA protein contains a compact five α-helical bundle and a disordered C terminus (residues 93–104) (Fig. 1B). All α-helices were juxtaposed, and their relative orientation is very similar to members of the xenobiotic response element-helix-turn-helix family (XRE-HTH) of DNA-binding proteins (49). Family members include the P22 C2 and phage 434 proteins, which transcriptionally repress specific genes by binding to their operator regions in the major groove in a sequence-specific manner (50, 51).

HigA has a number of unique structural characteristics in addition to the presence of the HTH motif. For example, each HigA monomer contains a defined hydrophobic core unlike other antitoxins that recognize RelE family members. Normally antitoxins only form a hydrophobic core upon self-dimerization and have typically been classified as partially unstructured (41, 43, 44, 52, 53). Additionally, most antitoxins that recognize RelE family members form one DNA-binding motif upon dimerization (41, 43, 44, 52, 53). In sharp contrast, each HigA monomer contains a complete DNA-binding motif. Therefore, the HigA dimer contains two DNA-binding motifs that fully extend over the two 9-nucleotide inverted repeats of the hig operator shown to interact with HigA through DNase protection assays (Figs. 1A and 5A and supplemental Fig. S2B) (35). These results imply that a single HigB-(HigA)2-HigB tetramer can repress an operator site consisting of two inverted repeat sequences (Figs. 1A and 5A).

**HigA Mediates the Formation of the HigB-(HigA)2-HigB Complex**—HigA dimerizes to form a dimer of heterodimers (Fig. 1, B and C). These HigA dimers interact in a two-fold symmetrical manner mainly stabilized by hydrophobic interactions (Fig. 4C). Loop 6 packs against a5 of its partner HigA and caps the junction formed by a1, a2, and a4 of the adjacent HigA molecule (Fig. 4, C and D). This 1,240 Å2 interface is mediated primarily via hydrophobic amino acids (Ile-54, Leu-68, Leu-76, Leu-79, Ile-83, Ile-88, and Tyr-91) from both molecules (Fig. 4C). For comparison, the HigB-HigB interface is 280 Å2 (Fig. 1B). Thus, the HigA-HigA interaction plays a major role in driving the formation of the tetrameric HigB-(HigA)2-HigB complex.
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**HigA Does Not Mask the HigB Active Site**—The HigB active site is likely located at a concave surface where a high density of conserved residues reside, including the proposed catalytic HigB amino acid His-92 (Fig. 2, A and B) (13). RelE amino acids that contact and cleave mRNA cluster in a similar concave surface (Fig. 2C) (36). This surface is located ~20 Å distal opposite to the HigA-HigB interface (Fig. 1B). Additionally, the active site is solvent-accessible, and this suggests that simple active site steric occlusion by HigA is not the mechanism of HigB inhibition.

**HigB-(HigA)₂-HigB Tetramer Is Required to Interact with Its DNA Operator**—Antitoxins generally require dimerization to form one DNA binding domain. These include members of the ribbon-helix-helix (RelB, VapB-3, and FitA), AbrB (MazE), and PhD/YefM superfamilies (22, 39, 41, 43, 44, 47, 53–56). There are two DNA-binding motifs in the context of the HigB-(HigA)₂-HigB structure. This implies that one tetramer could bind two inverted repeats of a single operator site (Fig. 5A and supplemental Fig. S2B). This is in contrast to canonical TA systems that appear to require two TA oligomeric complexes, or four antitoxins in total, to interact with a single operator site (55–57). To test whether a single HigA HTH can interact with DNA, we attempted to disrupt the HigB-(HigA)₂-HigB tetrameric state and tested whether this complex could productively interact with the higB operator. We truncated HigA at loop 6 (HigA Δ84–104) to disrupt the HigA-HigA interface (Fig. 5B).

The higBA operator sequence used in the EMSAs includes two endogenous operator sites, both of which in turn contain two inverted repeats (Fig. 5A). Wild-type HigB-(HigA)₂-HigB binds to its own operator DNA with increasingly higher oligomeric states (Fig. 5C, lanes 2–5). This indicates more than one HigB-(HigA)₂-HigB complex interacts with its promoter. However, we found that HigBA(Δ84–104) is unable to bind to this same DNA promoter (Fig. 5C, lanes 7–10). Considering that each HTH motif of HigA is left intact in this mutant, it is surprising that HigBA(Δ84–104) is unable to bind DNA. Two possibilities for this result exist. The first is that the HigA mutation caused destabilization of the protein, and little to no soluble HigA is produced. The second possibility is that removal of the C terminus of HigA disrupts the HigA-HigA dimerization interface resulting in a HigBA heterodimer.

TheSEC results show wild-type HigB-(HigA)₂-HigB elutes as the expected tetrameric complex of 56 kDa, whereas purified HigBA(Δ84–104) elutes with an apparent molecular mass of 23 kDa (Fig. 5D). This is approximately the molecular mass of a dimer of HigA or HigB or HigBA heterodimer. To differentiate between these options, we analyzed the fractions of each peak with SDS-PAGE (Fig. 5E). The 56-kDa peak of the wild-type...
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HigB-(HigA)$_2$-HigB complex shows a large band at ~10 kDa, which is most likely both His$_6$-HigB (13.0 kDa) and HigA (11.5 kDa) (Fig. 5E, lane 1). These bands could be separated by treatment with thrombin to release the N-terminal His$_6$ tag and the linker region of HigB; this allows the identification of both HigB and HigA (Fig. 5E, lane 2). The HigBA(Δ84–104) complex that runs at ~23 kDa shows two distinct bands on the SDS-polyacrylamide gel (Fig. 5E, lane 3). Upon thrombin treatment to release the N-terminal His$_6$ tag and the linker region of HigB, we again see the appearance of tag-free HigB (Fig. 5E, lane 4). Therefore, HigBA(Δ84–104) is indeed a heterodimer of HigB and truncated HigA(Δ84–104). Taken together, these results demonstrate that the oligomeric state of the HigBA complex, specifically a HigB-(HigA)$_2$-HigB tetrameric state, is required for productive DNA interaction. This is despite each HigA monomer containing a full HTH motif.

**DISCUSSION**

TA systems commonly contain at least two operator regions with two imperfect inverted repeats comprising a single operator site (Fig. 5A). Antitoxin proteins belonging to the ribbon-helix-helix, AbrB, and PhD/YefM superfamilies require dimerization to form a single DNA binding domain that recognizes an inverted repeat (Figs. 1A and 5A). Direct binding of either an antitoxin dimer or a toxin-antitoxin complex confers transcriptional autoinhibition. The strength of the repression correlates to differences in binding affinities of either antitoxin dimers or TA complexes.

The crystal structures of the HigB-(HigA)$_2$-HigB complex presented here reveal the TA complex is a tetramer containing two, rather than one, DNA-binding motifs (Fig. 1, B and C). Our biochemical results indicate that a HigB-(HigA)$_2$-HigB tetrameric complex is essential for DNA binding (Fig. 5C). The loss of DNA binding upon disruption of HigA dimerization (thus forming a HigBA heterodimer) may result from a diminished interaction surface, culminating in reduced binding. Both the HigA dimer and HigB-(HigA)$_2$-HigB tetramer provide the same surface area for the inverted repeats to interact with, which is halved in the context of the HigBA dimer. Moreover, another possible reason for the HigBA dimer ablating DNA binding is that the HTH motifs, in the context of the HigB-(HigA)$_2$-HigB tetramer, are tethered or rigidly held in place because they are part of the HigA globular domain formed upon HigA dimerization (Fig. 1, B and C). In this manner, the precise structural arrangement may be functioning as a molecular ruler for specific recognition of both inverted repeats as seen in other HTH-containing proteins, such as Fis (58). In summary, both the DNA interaction area formed by the HigA dimer and the spatial organization of the HTH motifs coordinate to recognize DNA and render the area impenetrable to RNA polymerase.

Transcriptional autoinhibition by TA complexes has been proposed to occur by regulation of the overall molar ratio of toxins and antitoxins as shown in vivo for RelEB (57). By varying the molar ratio, different stoichiometric complexes form, which may function to repress transcription to different magnitudes or cause complete derepression. In vitro biochemical experiments for the RelEB, PhD-Doc, and CcdAB TA systems demonstrate that once a saturated TA-DNA complex is formed, increasing the amount of free toxin protein destabilizes the DNA-TA interaction and probably allows for derepression of transcription (56, 57, 59, 60). These studies led to a model referred to as conditional cooperativity (56, 57). This model can help explain why toxins function as either anti- or corepressors depending upon environmental changes that require bacteria to respond and regulate metabolic processes quickly.

Despite these studies, the mechanism by which different TA complexes repress transcription is still not entirely clear. Structural and modeling studies of the RelEB and the PhD-Doc TA complexes suggest two tetrameric TA complexes sterically clash at single operator sites, although in the case of RelEB, modeling studies indicated that trimeric complexes can coexist (44, 56). However, the proposed trimeric complexes of RelEB and PhD-Doc have not been observed structurally. Therefore, it is not obvious how the diverse oligomeric states from structural studies fit with these models (43, 44, 56).

In contrast, structures of TA complexes such as N. gonorrhoeae FitAB and Rickettsia felis VapBC bound to two inverted repeats suggest that higher oligomeric states can simultaneously bind to two inverted repeats without steric clashes (55, 61). Because the spacing between inverted repeats may play a role in which oligomeric complexes fit, it is interesting that the fitAB promoter contains 12 bp between inverted repeats, although the vapBC promoter has only two. So, in this context, both short and long spacings between inverted repeats give rise to a higher oligomeric state binding to DNA. Modeling of our HigB-(HigA)$_2$-HigB structure on the structure of phage 434 bound to a double-stranded 20-nucleotide DNA (PDB code 1RPE) indicates that it is possible for both HTH motifs of the HigB-(HigA)$_2$-HigB tetramer to interact with one complete operator site (two inverted repeats) without steric clashes (supplemental Fig. S2B).

HigA is not the only antitoxin that contains an HTH motif. MqsA and HipB antitoxins also possess HTH motifs, but there are key structural and functional differences among the three (62, 63). First is the location of the HTH motif relative to the toxin (supplemental Fig. S2C). The HipA toxin binds to HipB at a location orthogonal to its HTH motif, although MqsA has a separate N-terminal toxin neutralization domain and a C-terminal DNA binding domain (supplemental Fig. S2C). The HipA toxin is also a much larger protein and is not homologous to either MqsR or HigB (63). Second, HipA contacts two HipB antitoxins, whereas HigB and MqsR only contact one. Finally, an important difference is that the MqsA antitoxin alone, and not the MqsRA TA complex, binds DNA (64). Thus, in this example, the toxin does not appear to act as a corepressor. In summary, although all three have an HTH motif in common, they show substantial functional and structural differences underscoring antitoxin plasticity.

HigB is a ribosome-dependent RNase that cleaves codons containing at least a single adenosine located, most likely, in the A site of the ribosome (13). Our HigB structure reveals a solvent-exposed concave surface containing highly conserved residues (Fig. 2B). Several lines of evidence suggest this HigB concave surface is its active site. Microbial RNases such as RNase T1 or RNase Sg contain similar concave surfaces, and ribosome-dependent toxins have been proposed to degrade mRNA...
in an analogous manner (36, 65, 66). An x-ray crystal structure of RelE bound to the 70 S ribosome shows the same concave surface interacts with mRNA (36). Additionally, mutagenesis experiments of other toxins also implicate the same concave surface residues as important for function (36, 41, 62). In summary, HigB appears to use the same tertiary fold and surface to recognize ribosome-bound mRNA.

A hallmark of toxin inactivation is a direct interaction in which the antitoxin wraps around the toxin much like a pincer (Fig. 4E) (43, 44, 52, 56, 61, 62, 67–69). This toxin-antitoxin interaction ablates activity of the toxin, and although the precise mechanism is unknown, it has been proposed to occur via antitoxin masking of the toxin active site. Our structure reveals that the antitoxin HigA does not wrap around and mask the active site of HigB. Instead, only two regions of contact are made, both of which are distant from the active site (Figs. 1 and 3). The MqsRA and the HipBA TA pairs also do not wrap around their cognate toxins but interact in a manner and location distinct from HigB-(HigA)$_2$-HigB (Fig. 4F and supplemental Fig. S2C) (62, 63).

Comparison of toxin active sites in toxin alone, toxin-anti-
toxin, and toxin bound to the ribosome structures reveals there are only minor structural rearrangements of the toxin (36, 41, 53). To further explore the inhibitory mechanism of HigB by HigA, we superimposed the HigB-(HigA)$_2$-HigB complex on the structure of RelE bound to mRNA on 70 S (36). A steric clash exists between HigB-(HigA)$_2$-HigB and ribosomal protein S12 and 16 S rRNA helix 18 (h18). The position of a large portion of the N terminus of HigA (α1–4) overlaps with the entire h18 (Fig. 6, clash 1). The second clash site is with the C terminus of HigA$'$ that overlaps with S12 residues 108–113 and the tetraloop of h18 (Fig. 6, clash 2). Thus, we propose binding of HigA sterically inhibits HigB from interacting with mRNA in the A site of the ribosome.

Taken together, our results expand the molecular understanding of how diverse antitoxins counteract the activity of toxin proteins. As Blower et al. (70) described, the tertiary structures of toxins are a static scaffold that may contain myriad possible active site residues that dictate substrate specificity. This appears to be consistent with what is known about ribosome-dependent toxins. However, the antitoxin structure and interaction with its cognate toxin varies and can be structurally divergent depending upon its DNA binding domain and the structural features of the antitoxin and toxin interface. This antitoxin structural plasticity underscores the expansive nature of TA-mediated bacterial survival mechanisms.

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FIGURE 6. HigB-(HigA)$_2$-HigB complex clashes with S12 and 16 S rRNA in the A site of the ribosome. HigB was modeled on the RelE-70 S complex (36) (PDB code 3KIO). HigA (gray) bound to HigB (green) directly clashes with 16 S rRNA h18 (tan) (Clash 1), and HigA$'$ (blue) of the other HigBA$'$ dimer clashes with h18 and S12 (red) (Clash 2). The active site of HigB (green) is located at the light blue circle. P-site tRNA and mRNA are shown in magenta and purple, respectively.
Toxin-Antitoxin HigB-(HigA)₂-HigB Structure


Toxin-Antitoxin HigB-(HigA)₂-HigB Structure


Structure of the *P. vulgaris* HigB-(HigA)_{2}-HigB toxin-antitoxin complex*

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**FIGURE S1.** HigBA forms a heterotetramer in both crystal forms. A. View of the asymmetric hexagonal unit of Form 1 with the color scheme the same as in Fig. 1. B. Two views of the asymmetric unit of Form 2 (left) with HigBA tetramer 1 colored as in panel A (boxed). The heterodimer HigBA of tetramer 2 is shown behind in green (HigB) and red (HigA). A ~110° rotation about the vertical axis emphasizes the second tetramer 2 (boxed) with HigA (red) and green HigB (green) from the same unit cell as the tetramer 1 on the left. HigA’ (gray) and HigB’ (black) comprise tetramer 2 but are from an adjacent asymmetric unit. C. Overlay of the three HigBA tetrameric complexes from Form 1 and Form 2. Loop 5 of HigB is boxed to emphasize the slight differences between the complexes.
**FIGURE S2.** Structural comparisons of HigA antitoxin. A. Comparison of the *P. vulgaris* HigA dimer (our structure) to *E. coli* CFT073 (PDB IDs 2ICT and 2ICP) and *C. burnetti* HigA dimers (PDB ID 3TRB). For *E. coli* CFT073 HigA dimers, one HigA molecule is present in each asymmetric unit and the dimer is formed between two asymmetric units related by 2-fold symmetry. B. Model of the tetrameric HigBA complex bound to DNA using the X-ray crystal structure of phage 434 bound to a 20mer DNA (PDB ID 1RPE) as an alignment guide. Alignments were performed in Coot using secondary-structure matching (SSM) (1). A HigBA tetramer can easily span an entire palindromic site. C. Alignment of the HigBA, MqsRA (gray), and HipAB (red; PDB ID 3DNV) TA complexes displays the different binding surfaces of antitoxins relative to their cognate toxin domain despite all three containing a HTH motif (circled). Alignments were based on the position of the HTH motif (orange for the helices and yellow for the loop). Only one toxin and antitoxin pair is shown for each complex. The MqsRA full length model is derived from the full-length antitoxin structure (gray; PDB ID 3GN5) and the MqsA N-terminus bound to MqsR structure (gray; PDB ID 3HI2).
**FIGURE S3.** HigB (green) alignment with RelE (white; PDB ID 3K1Q). A. Alignment of HigB and RelE was performed using secondary-structure matching in Coot in the context of the 70S-RelE complex (1). B. Rotation of panel A to emphasize the active sites. RelE has a C terminal $\alpha$ helix that is not present in HigB.

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