Structure of FitAB from *Neisseria gonorrhoeae* Bound to DNA Reveals a Tetramer of Toxin-Antitoxin Heterodimers Containing Pin Domains and Ribbon-Helix-Helix Motifs*

Kirsten Mattison†, J. Scott Wilbur‡, Magdalene So§, and Richard G. Brennan∗,†

From the Departments of chemistry and Molecular Biology and Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, Oregon 97239

*This work was supported by National Institutes of Health Grant AI47260 (to M. S.) and funds from the Robert A. Welch Foundation (G-0040). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Neisseria gonorrhoeae** is a sexually transmitted pathogen that initiates infections in humans by adhering to the mucosal epithelium of the urogenital tract. The bacterium then enters the apical region of the cell and traffics across the cell to exit into the subepithelial matrix. Mutations in the fast intracellular trafficking (fitAB) locus cause the bacteria to transit a polarized epithelial monolayer more quickly than the wild-type parent and to replicate within cells at an accelerated rate. Here, we describe the crystal structure of the toxin-antitoxin heterodimer, FitAB, bound to a high affinity 36-bp DNA fragment from the fitAB promoter. FitA, the antitoxin, binds DNA through its ribbon-helix-helix motif and is tethered to FitB, the toxin, to form a heterodimer by the insertion of a four turn α-helix into an extensive FitB hydrophobic pocket. FitB is composed of a PIN (PilT N terminus) domain, with a central, twisted, 5-stranded parallel β-sheet that is open on one side and flanked by five α-helices. FitB in the context of the FitAB complex does not display nuclease activity against tested PIN substrates. The FitAB complex points to the mechanism by which antitoxins with RHH motifs can block the activity of toxins with PIN domains. Interactions between two FitB molecules result in the formation of a tetramer of FitAB heterodimers, which binds to the 36-bp DNA fragment and provides an explanation for how FitB enhances the DNA binding affinity of FitA.

**Neisseria gonorrhoeae** (GC)† is the agent of the sexually transmitted disease, gonorrhea. The mechanisms used by GC to initiate infection have been very well characterized. Gonococci adhere via a multistep cascade and subsequently enter cells forming the epithelial barrier of the urogenital tract, traffic across these cells and exit into the subepithelial matrix (1, 2). Although studies have identified many of the molecular mechanisms used by GC to adhere to and enter cells, our knowledge of the mechanisms that operate in the later stages of infection is limited.

GC are able to survive and grow within epithelial cells (3); they also traverse the epithelial monolayer to infect the stromal tissue of the subepithelium (2). The immune response to bacteria in the subepithelium produces the inflammation and purulent discharge characteristic of gonorrhea (4, 5). On occasion, GC establish a carrier state in which an asymptomatic individual harbors cultivable and transmissible bacteria. These carriers are key to the spread of gonococcal disease, as humans are the only known reservoir for GC (6). The mechanisms by which GC maintains this persistent state are unknown. One hypothesis is that the organism resides within the epithelial cells instead of crossing into the subepithelium, thus evading the host immune response. The gene product(s) that affect GC intracellular growth and transcytosis are therefore important for the maintenance of gonococci in the human population.

The fitAB operon was identified in a screen for GC mutants with a fast intracellular trafficking phenotype across polarized epithelial monolayers (3). A GC mutant that lacks fitAB grows normally extracellularly, but has an accelerated rate of intracellular replication with a concomitant increase in the rate at which this mutant traverses a monolayer of polarized epithelial cells. Thus, either FitA or FitB, or their complex, is hypothesized to slow intracellular replication and intracellular trafficking of GC.

FitA is an 8.4-kDa protein with a predicted N-terminal ribonucleoside-binding domain (RHH) DNA binding motif (7, 8). FitB is a 15.3-kDa protein with a predicted PIN (PilT-N terminus) domain according to the BLAST search tool (9). The function of the PIN domain is unknown; however many proteins that contain a PIN domain are thought to perform roles in nucleic acid metabolism including synthesis and remodeling. In genome studies on Archaea and thermophilic bacteria, sequences predicted to encode PIN domain-containing proteins are found in regions predicted to encode DNA polymerases, helicases, and nucleases (10). In addition, the Dis3p exonucleases from *Saccharomyces cerevisiae* and nonsense-mediated mRNA decay (NMD) proteins in *Caenorhabditis elegans* are predicted to

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
have PIN domains (11, 12). Bicistronic operons where an RHH DNA-binding protein is juxtaposed with a PIN domain have been proposed to form one family of toxin/antitoxin systems (13). The PIN domain containing protein is thus predicted to act as a toxin; this is in agreement with the role of FitB in slowing GC replication when the FitA (“the antitoxin”)-mediated inhibition of FitB could slow intracellular GC replication by acting as a “toxin” when the FitA–mediated inhibition of FitB nuclease activity is relieved upon complex dissociation.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation, Crystallization, and X-ray Intensity Data Collection**—FitA and FitB were overexpressed in *E. coli* using a pET28b vector (Invitrogen) that encodes the intact FitAB operon (8). This vector incorporates a T7 promoter at the 5′-end of fitA and sequences encoding six histidine residues at the 3′-end of fitB. The C-terminal amino acid sequence of FitB was also slightly altered by the addition of an XhoI restriction site, changing from . . . NPWHLEHHHHHH to . . . NPWHLEHHHHHHH. The overexpressed FitAB complex was purified using nickel affinity chromatography (Qiagen). Purified FitAB complex was concentrated to 5 mg/ml in 25 mM Tris, pH 7.5, 500 mM NaCl, 200 mM imidazole.

The intact FitAB complex did not crystallize despite numerous attempts. Therefore, limited proteolysis was done on the FitAB complex in order to generate a stable core that might be more amenable to crystallization. Using 0.1 mg/ml trypsin (Sigma) the complex was digested for 30 min at 22 °C before crystallization trials. Trypsin inhibitor cross-linked to agarose beads (Sigma) was used to remove trypsin from the FitAB solution after digestion. Polyacrylamide gel electrophoresis and mass spectrometry analysis revealed that this treatment removed the ribbon-helix-helix motif of FitA and the resulting complex, termed FitcAB, does not bind DNA (data not shown). Crystallization was carried out using hanging drop–vapor diffusion where FitcAB was mixed 1:1 (v:v) with a reservoir solution of 0.26 M sodium phosphate/citrate pH 4.7 and 2.0 M ammonium acetate.

**Structure of the FitAB Complex Bound to DNA**

| TABLE 1 Selected crystallographic statistics | SeMet-FitcAB3(LxM) | Native FitcAB wild type | Native FitAB - IR36 DNA |
|----------------------------------------------|--------------------|-------------------------|------------------------|
| Space group | P2 2 2 1 | P2 2 2 1 | P2 2 2 1 |
| Cell constants (Å) | a = 49.1 | a = 70.0 | a = 75.0 |
| | b = 68.5 | b = 50.7 | b = 82.4 |
| | c = 104.5 | c = 48.3 | c = 135.5 |
| Wavelength (Å) | 0.9796 | 0.9686 | 0.9794 |
| Resolution (Å) | 57.74–2.00 | 57.74–2.00 | 57.74–2.00 |
| Overall R work (%) | 9.0 (32.5) | 9.5 (33.2) | 9.8 (33.5) |
| Overall R free | 3.7 (2.3) | 3.4 (2.2) | 3.1 (2.2) |
| Total reflections (#) | 196443 | 170802 | 32493 |
| Unique reflections (#) | 24539 | 13340 | 11340 |
| Completeness (%) | 96.5 (95.3) | 99.9 (99.9) | |
| Phasing resolution (Å) | 20.00–3.00 | 7.0 |
| Selenium sites (#) | 7 | 0.720 |
| Overall Figure of Merit | 19.1/22.4 | 19.1/22.4 |
| Refinement | 21.2/26.9 |
| Rwork/Rfree (%) | Rwork/Rfree (%) | |
| Rmsd Bond angles (°) | 1.28 | 1.27 |
| Bond lengths (Å) | 0.007 | 0.008 |
| B values (Å2) | 1.90 | 1.60 |
| Average B-values (Å2) | Overall 25 | 41 |
| Protein 25 | 38 |
| DNA 38 | 51 |
| A-tract 38 | 42 |
| Ramachandran analysis | 94/2/129 | 88.5/655 |
| Most favored (%/#) | 5.8/8 | 11.2/83 |
| Additional allowed (%/#) | 0/0 | 0.3/2 |
| Generously allowed (%/#) | 0/0 | 0.0/0 |
| Disallowed (%/#) | n/a, not applicable |

* Values in parentheses are for the highest resolution shell, 1.80 Å–1.99 Å for FitcAB, 3.00–3.14 Å for FitAB-IR36.

* Figure of Merit = (Σ|P(α)−P(α)'|)/ΣP(α)' where α is the phase and P(α)' is the phase probability distribution.

* Rwork = Σ|Fobs|/Σ|Fcalc| and Rfree = Σ|Fobs|−|Fcalc|/Σ|Fobs|−|Fcalc|; where all reflections belong to a test set of 10% randomly selected data.
nium sulfate. Crystals appeared overnight and grew to final dimensions of 0.1 mm × 0.1 mm × 0.02 mm in 3 days.

To generate selenomethionine (SeMet)-substituted FitAB complex, the expression vector described above was used as a template for standard PCR mutagenesis (Stratagene) of FitB, which contains no methionines, to yield a construct encoding FitAB where residues Leu43, Leu63, and Leu116 of FitB were substituted with methionines (FitAB3(LxM)). The FitAB3(LxM) protein was purified as described above. DNA binding assays confirmed that the FitAB3(LxM) complex has the same affinity for DNA as wild-type FitAB (data not shown). For overexpression of SeMet-substituted FitAB3(LxM), E. coli harboring the expression vector were grown in minimal medium lacking methionine with added SeMet as described (14). Using nickel affinity column chromatography, SeMet-containing FitA and FitB3(LxM) copurify as do the wild-type proteins. The SeMet-containing heterodimer was concentrated and trypsinized as described for wild-type FitAB. Crystallization of the SeMet-FitAB3(LxM) complex employed 0.26 M sodium citrate pH 5.6 and 2.0 M ammonium sulfate. Crystals with dimensions 0.2 mm × 0.2 mm × 0.2 mm were obtained after 4 weeks.

To crystallize the FitAB-DNA complex, 5 mg/ml of purified native FitAB3(LxM) was mixed in a 4:1 molar ratio with IR36 DNA (8), where two of the thymine bases were replaced with 5-iodouracil (I) (top strand, 5'IAGATTGCTATCATTTTTTTATTTTTGATAGCATTTG; bottom strand, 5'CATAATGCATTCTGAAAGGAATAAGCTGACAGATATTT). The protein-DNA complex was then mixed 1:1 (v/v) with a reservoir solution of 0.1 M sodium acetate, pH 4.0, 0.27 M sodium acetate, pH 7.0, 7.2% PEG 20,000, 7.2% PEG monomethyl ether 550.

FIGURE 1. Structure of the FitAB heterodimer. In all panels, FitA is shown in magenta and FitB is in cyan. a, ribbon diagram of the FitAB heterodimer. The α-helices and β-sheets for both proteins are labeled as are the N and C termini. b, sequence alignment of FitA and FitB proteins with homologues. Secondary structural elements of the proteins are shown above the alignment. Y4JX from Rhizobium sp. and StbCB from Pseudomonas syringae are highly homologous to FitAB by sequence analysis but no structural data are available for either of these systems. Arc and Mnt from Enterobacteria phage P22 are ribbon-helix-helix proteins with structural homology to FitA. PilT from Azotobacter vinelandii and PAE2754 from Pyrobaculum aerophilum are PIN domain containing proteins with structural homology to FitB. Alignments were generated with ClustalW (49). Residues involved in FitA-FitB heterodimerization are highlighted in yellow, those forming the FitA-FitA dimer interface are highlighted in green, and those making up the FitB-FitB dimer interface are highlighted in orange. Conserved residues involved in specific protein-DNA contacts are highlighted in blue and the conserved acidic residues forming the putative FitB active site are highlighted in red. c, stereoview of the FitA-FitB heterodimer interface. Residues involved in ionic interactions contributing to the stability of the interface are colored according to gray (carbon), red (oxygen), and blue (nitrogen). This extensive interface buries up to 1900 Å² accessible surface area.
structure was used as a model to solve the structure of the FitAB-IR36 complex by molecular replacement using CNS (18). The final model contains 1–139 of FitB, 46–64 of FitA, 92 water molecules, 1 acetate ion, 2 sulfate ions, and 3 magnesium ions. The final model was verified by inspection of $2F_o - F_c$ simulated annealing-composite omit maps.

The high resolution FitcAB structure was used as a model to solve the structure of the FitAB-IR36 complex by molecular replacement using CNS (19). After simulated annealing (SA) and extensive positional and thermal parameter refinement using CNS followed by model rebuilding in O, the $R_{work}$ and $R_{free}$ converged to 21.2% and 26.9%, respectively, at 3.0 Å resolution. The model was verified by inspection of the $2F_o - F_c$ simulated annealing-composite omit maps. The final model contains four molecules of FitA (residues 2–69, 2–65, 2–68, 2–64), four molecules of FitB (residues 1–143, 1–140, 1–143, 1–140), the complete 36 base pair double-stranded IR36 DNA fragment and 54 water molecules. Figures were made using Swiss-PDB Viewer (20) and POV-Ray.

RESULTS AND DISCUSSION

Structure of the FitAB Heterodimer—The structure of the FitB protein complexed with a C-terminal fragment of FitA (FitcAB) was determined to 1.8 Å resolution by multiple wavelength anomalous diffraction using selenomethionine substituted proteins (“Experimental Procedures” and Table 1). This structure was used as a model to solve the structure of the full-length FitcAB complex bound to a 36-bp DNA molecule to 3.0 Å resolution by molecular replacement (“Experimental Procedures” and Table 1).

The FitA monomer has an extended structure, with the topology β1 (residues 4–7), α1 (residues 11–23), α2 (residues 28–43), α3 (residues 48–59) (Fig. 1a). Electron density is visible for the intact N terminus of FitA, beginning at Ala$^2$. Met$^1$ is not present in our preparation, as determined by N-terminal sequencing of the protein (data not shown). At the C-terminal end, variable electron density is seen for the four molecules of the asymmetric unit, the final 9–14 residues are not visible (depending upon the monomer). The first 45 residues of this protein (β1-α1-α2) are highly homologous to the RHH class of DNA-binding proteins, which includes the bacteriophage P22 proteins Mnt and Arc (Fig. 1b) (7, 8, 22). An overlay of the FitA

![Ribbon diagram of the structure of the FitAB-IR36 complex](image-url)

**FIGURE 2.** Ribbon diagram of the structure of the FitAB-IR36 complex. a, the FitA and FitB proteins are colored magenta and cyan, respectively. DNA is colored according to gray (carbon), red (oxygen), blue (nitrogen), and yellow (phosphorus). The four FitAB heterodimers are numbered from I to IV. b, view of a rotated to demonstrate that the two FitA β sheets bind on the same face of the DNA helix. c, sequence of the 36-bp IR36 site used for crystallization. The 8-bp inverted repeat half-sites are highlighted in magenta. FREE energy is determined for the two FitA subunits shown in red and blue, the FitA and FitB proteins are colored gray and cyan, respectively.
Structure of the FitAB Complex Bound to DNA

FIGURE 3. Stereoviews of the multiple oligomerization interfaces of the FitAB complex. FitA is in magenta and FitB is in cyan. Selected residues are colored according to gray (carbon), red (oxygen), and blue (nitrogen). a, FitA dimerization forms the DNA binding β-sheet, with β1 from each of the FitA subunits (I and IV or II and III). b, the two FitA RHH motifs form one globular domain, with interactions between α1 of one subunit and α2 of the dimer partner (I with IV and II with III). Two identical interfaces are formed in this manner per FitA dimer, which together with the β-sheet stabilize the domain and bury 2850 Å² accessible surface area. c, two FitB monomers have an extensive dimerization interface (I and II or III and IV), where α3 from one subunit contacts α5 from the other subunit. Half of this interface is shown here, as there is 2-fold symmetry around residue Phe78 (F78) in the complete interface. FitB homodimerization buries 1870 Å² accessible surface area.

and Arc repressor structures results in a root mean-squared deviation (RMSD) of 1.1 Å over the first 45 residues (23). An arginine found in β1 of the RHH proteins is conserved in FitA (Fig. 1b, highlighted in blue).

FitB forms a compact domain with an α/β/α-fold (Fig. 1a). This protein consists of a central 5-stranded parallel β-sheet with four α-helices packed on one side of the sheet and three α-helices on its other side. The topology is β1 (residues 1–5), α1 (residues 7–12), α2 (residues 19–26), β2 (residues 32–36), α3 (residues 38–48), α4 (residues 55–65), β3 (residues 74–76), α5 (residues 80–94), α6 (residues 102–112), β4 (residues 117–120), α7 (residues 124–128), β5 (residues 132–134) (Fig. 1a). Electron density for the entire FitB protein is visible, with only engineered histidine residues at the C terminus not observed in the structure (missing 3/6 His in 2 monomers and 6/6 His in 2 monomers). Searches, using both the DALI server (23) and the protein structure comparison service SSM (24) at the European Bioinformatics Institute, found structural homologues of FitB in PIN-domain containing proteins. Using the BLAST server, none of these PIN domain-containing proteins were found to have significant similarity at the primary structure level to FitB (9). The archetypical PIN domain is found in the PilT N terminus, and the closest FitB structural homologue is PAE2754 from Pyrococcus aerophilim (Fig. 1b) (25, 26). The functional significance of this domain is unknown. However, the PIN domain is found in a wide variety of systems, from bacterial FitB-like genes that are thought to be involved in plasmid maintenance, to the yeast Dis3p exonucleases (11, 27, 28). Despite a lack of sequence similarity, the four acidic residues absolutely conserved among PIN domains are present in FitB (Fig. 1b, highlighted in red).

In addition to the RHH and PIN domain-containing proteins, there is a group of prokaryotic proteins with a high level of sequence homology to FitAB (Fig. 1b). These typically consist of both a FitA and a FitB homologue in a conserved operon organization and little is understood about their biochemical function, although they are known to play a general role in plasmid stability and/or partition (27–31). These have been proposed to act as toxin/antitoxin pairs, with the RHH protein acting as the antitoxin, repressing the toxic activity of the PIN domain-containing protein (32). The structure of the FitAB complex is likely to predict the structures of these toxin/antitoxin proteins. The biochemical function of this group of proteins within prokaryotic cells is likely to be similar as that performed by FitAB. Sequence alignments of FitA and FitB with two examples of such systems (Y4jJ/K and StbCB) are shown in Fig. 1b.

The FitA-FitB Interface—The FitA and FitB proteins form a tightly associated dimer and the FitAB structure reveals the heterodimerization interface, which is formed predominantly by contacts between α3 and the C-terminal extended coil region of FitA and helices α1, α2, and α4 of FitB (Fig. 1, a and c). The interface buries 1900 Å² accessible surface area in which the FitA helix fills a large exposed hydrophobic groove on FitB resulting in a globular heterodimeric domain (Fig. 1, a and c).
These residues of the helix exist between Ile20 and Pro47 of FitB alpha (Fig. 1c). At the C terminus of FitA, outside of the helical region, the side chains of residue Ile59 contacts the side chains of residues Val38 and Leu39 from the subunit 2d of FitB (Fig. 1c). Other residues of the alpha and alpha-beta turn of FitB that are important components of the dimer interface are Ile67, Leu70, and Phe71, which contact Leu48, Met51, and Ile55 of FitA (Fig. 1c).

FitA helix alpha-3 binding to FitB is also stabilized by four ionic interactions, which serve to orient and buttress the two molecules, thereby facilitating a tight association between the two helices of the human nuclear receptors (22). The two helices of the RHH DNA-binding proteins (22), and its formation involves nearly every amino acid residue of beta1, alpha1, and alpha2 of FitA (Fig. 3a and b). The beta1-strands from each FitA monomer combine to form a two stranded antiparallel beta-sheet with four intersubunit hydrogen bonds between the backbone carbonyl oxygen and amide nitrogen atoms and a van der Waals contact between the side chains of Val5 and its dyadic mate (Fig. 3a). The two alpha-helices of the RHH domain contribute to the extensive interface whereby residues Thr13, Ala16, and Ile17 of alpha1 from one subunit form hydrophobic contacts to residues Leu36, Ile39, and Ala30 of alpha2 of the other subunit (Fig. 3b). This interface occurs twice in each RHH domain. In addition to these van der Waals contacts, anionic interaction is formed between the Ne group of arg20 from alpha1 and the Ce atom of Glu43 from alpha2 (Fig. 3b).

The FitA homodimer also buries a large accessible surface area (1870 A^2). At this interface, alpha3 from one FitB monomer contacts alpha5 from the other FitB monomer (Fig. 3c). As both for FitA, there are two such dyad related surfaces per homodimer. Phe78 in the alpha4-alpha5 loop is a key residue for FitB-FitB dimerization. The backbone amide and carbonyl atoms of Phe78 form hydrogen bonds to their counterparts in the adjacent monomer. In addition, the Phe78 aromatic ring contributes to the hydrophobic interface by approaching the C-beta methyl group of residue Ala57 from alpha3 (Fig. 3c). Other hydrophobic contacts involve the side chains of residues Ala41, Leu55, and Ala48 from alpha3 of one FitB subunit and Tyr86, Ala87, and Ser91 from alpha5 of the other FitB subunit (Fig. 3c). Arg42 and Glu50 form a salt bridge between the two helices that also serves to stabilize this extensive interface.

Together, these interactions (Figs. 1 and 3) create a stable tetramer of FitAB heterodimers, which is in accord with the oligomerization state that was observed in previous solution studies (8). The biochemical significance of this unusual quaternary structure is underscored by the finding that FitA dimers bind the IR36 site with an affinity of 180 nM, while the FitAB tetramer of heterodimers binds this site with a much improved affinity (Kd, 4.5 nM). The increase in stability provided by FitB to the tetrameric complex might explain part of this 40-fold increase in DNA binding affinity, even though FitB does not interact directly with the DNA molecule (Fig. 2b). However, an equal or more important contributor to the higher affinity displayed by the FitAB tetramer of heterodimers is the increase in the local concentration of FitA dimer that is brought about by the dimerization of the FitB proteins.

The IR36 FitAB binding site is found upstream of the fitAB operon in N. gonorrhoeae. The inverted repeat half-sites (Fig. 2c) were defined in biochemical studies as the specific bases required for FitAB binding to this region (8). In the FitAB-IR36 complex structure, the two FitB-beta-sheets bind on the same face of the IR36 DNA (Fig. 2b). However, this positioning is unnecessary for high affinity DNA binding, as inverted repeats with various spacer lengths between the half-sites, ranging from 4 to as little as 2 base pairs, bind FitAB with equally high affinity (8). A 4-residue flexible loop that connects FitA helices alpha2 and alpha3 would allow facile rotation of the tetramer complex and

**TABLE 2**

| Selected characteristics of the IR36 DNA fragment | Average bend angle per base step | Average twist | Major groove width | Minor groove width |
|--------------------------------------------------|---------------------------------|---------------|---------------------|-------------------|
| All data were calculated using CURVES 5.1 (47).  |
| **Global bend** | deg | deg | A | A |
| IR36' | 44 | 1.32 | 34 |
| Inverted repeat | 13 | 1.83 | 32 | 13.4 | 7.1 |
| A tract | 14 | 1.08 | 35 | 13.7 | 3.7 |
| B form DNA' | 19 | 1.73 | 37 | 11.6 | 6.0 |

* a The overall bend angle is the total angle of curvature between the first and last helical axis segments. This value was divided by the number of base steps in the sequence (length minus one) for comparative purposes.

* b The average value calculated for the global interface pair twist (omega).

* c The terminal base pairs were deleted from IR36 prior to analysis, creating IR34.

* d The terminal bases are not tightly base paired, greatly changing the average values for the entire fragment when included.

* e The 7 base pairs of the inverted repeat, TGCTATC. Values are averaged per base pair over both inverted repeat segments, the global bend is reported for 1-7 bp segment.

* f The 14-base pair A tract in the center of the IR36 fragment.

* g The Dickerson dodecamer is used as an example of B form DNA (48).
FitA-DNA contacts. a, schematic diagram of the FitA-DNA contacts. The deoxyriboses of each nucleotide are numbered, labeled, and shown as pentagons. Side chain DNA hydrogen bonds are indicated by blue arrows, backbone amide-DNA hydrogen bonds are green arrows, and van der Waals contacts are shown as yellow arrows. Each FitA residue is from subunit I, II, III, or IV, as defined in the legend to Fig. 2. b, stereoview of the composite omit electron density map of one FitA-DNA interface contoured at 1.0σ to 3.0Å resolution (green mesh). The FitA protein is shown as magenta balls and sticks and the DNA is shown as balls and sticks where grey (carbon), red (oxygen), blue (nitrogen), yellow (phosphorus). Note the water (Wat1)-mediated contact between Asn8 and Thy32.
thereby provides an explanation for the observed high affinity binding of FitAB to IR sites with different relative orientations.

The IR36 fragment is interesting and unusual because the two half-sites are separated by a long (14-base pair) spacer region of AT-rich DNA. Sequences like the central region of IR36, containing four or more consecutive A-T base pairs are known as A-tracts (37). These sequences adopt a structure different from that of typical B-form DNA in which A-tracts are essentially straight and rigid (38, 39). In addition, these sequences deviate from B-form DNA by having a compressed minor groove and a shorter helical repeat of only 10 bp (37, 40). The rigidity of A-tracts is predicted to allow for sharp bends at their edges (37), however there is no pronounced local bending in the structure of IR36 (Fig. 2, a and b and Table 2). Rather the DNA is smoothly curved such that the end-to-end bend angle is 44°. As expected, the central 14 base pairs are straighter, i.e. more rigid, than the rest of the DNA; there is a greater bend in the inverted repeat sequence (1.83° per base step) than in the A-tract region (1.08° per base step) (Table 2). The rigidity of the A-tract region is also demonstrated by the thermal parameters of the structure; the average B factor for the entire DNA molecule is 51 Å², while it is only 42 Å² for the isolated A tract (Table 1). The average twist between base pairs is comparable between the A-tract and inverted repeat segments of the sequence with the inverted repeats having slightly less twist than the A-tracts (Table 2). The central sequence shares another characteristic of canonical A-tract DNA, a significantly compressed minor groove, which is underscored when compared with the minor groove widths of the flanking sequences. Minor groove widths in the A-tract are 3.7 Å on average, while in the flanking sequences the width of the minor groove averages 7.1 Å (Table 2). The functional significance of the IR36 A-tract is unknown. However, its complete solvent exposure would allow access to the replication and transcription machinery.

**FitA-DNA Contacts**—FitA makes few specific contacts to the DNA and all contacts to base pairs are mediated by its residues from the conserved \( \beta \) sheet. As predicted, the highly conserved RHH residue Arg\(^7\) is crucial for DNA recognition (Fig. 4). The guanidinium side chain of Arg\(^7\) from each FitA subunit hydrogen bonds with the O6 and N7 atoms of the most 5'-guanine base of the inverted repeat sequence (Fig. 4b) as well as the thymine base on the 5'-side of this guanine. In subunits I and IV, this thymine base forms a water-mediated hydrogen bond to Asn\(^8\) as well (Fig. 4). The only other specific contact is a van der Waals interaction between the side chain of Val\(^5\) and the thymine from the T/A sequence central to each inverted repeat (Fig. 4a). In addition to these base contacts, a number of residues from the N-terminal end of FitA helix \( \alpha 2 \) contact the phosphate-sugar backbone of the DNA molecule. The side chains of Arg\(^{33}\) and Ser\(^{27}\) from all FitA subunits are involved in structures. The two proteins share only 13% amino acid identity over these aligned regions, the conserved acidic residues that define the PIN domain cluster in a surface pocket at the C-terminal end of the central, 5-stranded \( \beta \) sheet. FitB is in cyan and PAE2754 is in blue. c. In FitB, the putative active site for nuclease activity is blocked by the presence of Arg\(^{68}\) from FitA. FitA is in magenta and FitB is in cyan. Residues are colored gray (carbon), red (oxygen), blue (nitrogen).
such interactions, as are the amide nitrogens of Thr²⁸ and Glu²⁹ (Fig. 4a).

FitB Contains a PIN Domain—FitB has a high degree of structural homology to the PIN domain containing protein PAE2754. 93 corresponding Ca atoms of the two proteins overlay with an RMSD of 1.9 Å (Fig. 5a). PIN domains contain four highly conserved acidic residues that cluster at the C-terminal end of the β-sheet and form a negatively charged pocket near the center of the molecule (25, 41). This acidic pocket of the PIN domain containing flap endonuclease-1 is the active site for its exonuclease activity (41) and that of PAE2754 has been proposed to carry out an exonuclease function as well (25). In FitB these conserved residues are Asp⁵, Glu⁴², Asp¹⁰⁴, and Asp¹²² and they cluster to form an acidic pocket just as those from PAE2754 (Fig. 5, b and c).

In an initial attempt to identify a nuclease activity for FitB, several in vitro assays were carried out using a variety of nucleic acid substrates, including single and double-stranded RNA and DNA and Flap structures. However, no nucleic acid cleavage was detected in the context of the FitAB complex. Unlike some homologous PIN domain containing exonucleases, FitB has a dimerization partner, FitA. Intriguingly, an arginine residue, Arg⁶⁸, at the C terminus of FitA is located in the FitB acidic pocket, potentially blocking access of potential substrates and thus, inhibiting any enzymatic function (Fig. 5c). The guanidinium group of Arg⁶⁸ interacts with the carboxyl groups of residues Asp⁵, Glu⁴², and Asp¹⁰⁴ from FitB, forming strong electrostatic interactions that would not be easily displaced by a competing nucleic acid substrate. These negatively charged residues form the Mg²⁺ binding pocket in PAE2754 (29) and may similarly bind Mg²⁺ in FitB when Arg⁶⁸ from FitA is not present. Indeed, the trypsinized FitcAB molecule, the structure of which was solved as a part of this work, bound two solvent molecules in this acidic pocket that are very likely magnesium ions (“Experimental Procedures” and data not shown). A FitB(Mg²⁺)−dependent nuclelease might be activated in the cell when the FitA-FitB complex dissociates in response to some unknown signal, allowing Mg²⁺ to bind in the place of Arg⁶⁸ from FitA. This dissociation event is proposed to release the FitA antitoxin and allow the induction of a bacteriostatic action by the FitB toxin, by analogy to other proposed toxin-antitoxin systems (42–44). A common feature of toxin-antitoxin structures is that an otherwise unstructured peptide or RNA substrate that meets certain structural requirements. While the YoeB and RelE toxins have been shown to act as nucleases, their structure and active site residues are very different from those seen in FitB and thus they are not suitable as models for the putative FitB nuclease activity (35, 45). The identity of the putative FitB substrate(s) is the subject of ongoing biochemical and biological studies.

CONCLUSION

In conclusion, FitA and FitB form a heterodimer in which FitA is the DNA binding subunit and FitB contains a nuclease activity that is blocked by the presence of FitA and activated by an as yet unidentified intracellular signal, which dissociates the FitAB complex. Four such FitAB heterodimers associate into a novel tetrameric structure that binds to the IR36 sequence from the fitAB promoter region with high affinity. Many PIN domain-containing proteins are involved in nucleic acid metabolism and/or remodeling, with the prokaryotic FitAB and its homologues responsible for controlling rates of DNA replication and/or plasmid maintenance (3, 27, 28). This structure illustrates the mechanism by which antitoxins with RHH motifs are able to block the activity of PIN domain toxins in prokaryotes (46). Future studies on the activity of FitB will help us understand both generally how PIN domains control such diverse processes as replication and nonsense-mediated mRNA decay and specifically the role of FitAB in GC virulence (3, 11).

Acknowledgments—We thank Drs. Hans Peter Bächinger, Kerry Maddox, and Cory Bystrom for N-terminal sequencing and mass spectroscopy analysis of proteins and Dr. Corie Ralston for help with data collection. The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, Materials Sciences Division, of the United States Department of Energy under Contract No. DE-AC03-76SF00098 at Lawrence Berkeley National Laboratory.

REFERENCES

1. Apicella, M. A., Ketterer, M., Lee, F. K., Zhou, D., Rice, P. A., and Blake, M. S. (1996) J. Infect. Dis. 173, 636–646
2. Merz, A. J., and So, M. (2000) Annu. Rev. Cell Dev. Biol. 16, 423–457
3. Hopper, S., Wilbur, J. S., Vasquez, B. L., Larson, J., Clary, S., Mehr, I. J., Seifert, H. S., and So, M. (2000) Infect. Immun. 68, 896–905
4. Edwards, J. L., and Apicella, M. A. (2004) Clin. Microbiol. Rev. 17, 965–981
5. Holmes, K. K., Counts, G. W., and Beaty, H. N. (1971) Ann. Intern. Med. 74, 979–993
6. Turner, C. F., Rogers, S. M., Miller, H. G., Miller, W. C., Gribble, J. N., Chromy, I. R., Leonard, P. A., Cooley, P. C., Quinn, T. C., and Zenilman, J. M. (2002) J. Am. Med. Assoc. 287, 726–733
7. Raumann, B. E., Rouldl, M. A., Pabo, C. O., and Sauer, R. T. (1994) Nature 367, 754–757
8. Wilbur, J. S., Chivers, P. T., Mattison, K., Potter, L., Brennan, R. G., and So, M. (2005) Biochemistry 44, 12515–12524
9. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
10. Makarova, K. S., Aravind, L., Grishin, N. V., Rogozin, I. B., and Koonin, E. V. (2002) Nucleic Acids Res. 30, 482–496
11. Clussold, P. M., and Ponting, C. P. (2000) Curr. Biol. 10, R888–R90
12. Makarova, K. S., Aravind, L., Galperin, M. Y., Grishin, N. V., Tatusov, R. L., Wolf, Y. I., and Koonin, E. V. (1999) Genome. Res. 9, 608–628
13. Pandey, D. P., and Berendzen, J. (2005) Nucleic Acids Res. 33, 966–976
14. Doublet, S. (1997) Methods Enzymol. 276, 523–530
15. Powell, H. R. (1999) Acta Crystallogr. D. Biol. Crystallogr. 55, 1690–1695
16. Potterton, E., Briggs, P., Turkenburg, M., and Dodson, E. (2003) Acta Crystallogr. D. Biol. Crystallogr. 59, 1131–1137
17. Teverwilliger, T. C., and Berendzen, J. (1999) Acta Crystallogr. D. Biol. Crystallogr. 55, 849–861
18. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gos, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. D. Biol. Crystallogr. 54, 905–921
Structure of the FitAB Complex Bound to DNA