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The molecular basis of protein toxin HicA–dependent binding of the protein antitoxin HicB to DNA

Ashley J. Winter¹, Christopher Williams¹, Michail N. Isupov², Hannah Crocker¹, Mariya Gromova¹, Philip Marsh¹, Oliver J. Wilkinson³, Mark S. Dillingham², Nicholas J. Harmer², Richard W. Titball²*, and Matthew P. Crump¹,*

From the ¹School of Chemistry, University of Bristol, Cantock's Close, Bristol, BS8 1TS, UK; ²Biosciences, College of Life and Environmental Sciences, University of Exeter, EX4 4QD, UK; ³School of Biochemistry, Biomedical Sciences Building, University Walk, Bristol, BS8 1TD.

Running title: Structure of HicAB

* To whom correspondence should be addressed: Matt.crump@bristol.ac.uk; R.W.Titball@exeter.ac.uk

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ABSTRACT

Toxin–antitoxin (TA) systems are present in many bacteria and play important roles in bacterial growth, physiology, and pathogenicity. Those that are best studied are the type II TA systems, in which both toxins and antitoxins are proteins. The HicAB system is one of the prototypic TA systems, found in many bacterial species. Complex interactions between the protein toxin (HicA), the protein antitoxin (HicB), and the DNA upstream of the encoding genes regulate the activity of this system, but few structural details are available about how HicA destabilizes the HicB–DNA complex. Here, we determined the X-ray structures of HicB and the HicAB complex to 1.8 and 2.5 Å resolution respectively and characterized their DNA interactions. This revealed that HicB forms a tetramer and HicA and HicB form a hetero-octameric complex that involves structural reorganization of the C-terminal (DNA-binding) region of HicB. Our observations indicated that HicA has a profound impact on binding of HicB to DNA sequences upstream of hicAB in a stoichiometric-dependent way. At low ratios of HicA:HicB, there was no effect on DNA binding, but at higher ratios, the affinity for DNA declined cooperatively, driving dissociation of the HicA:HicB:DNA complex. These results reveal the structural mechanisms by which HicA de-represses the HicB–DNA complex.

Toxin-antitoxin (TA) systems are found in a wide range of bacteria and the best studied are the type II TA systems, where both the toxins and the antitoxins are proteins. Type II TA systems are structurally and functionally diverse, with fourteen separate families classified by either three-dimensional structure or mode of action (see 1-3 for recent reviews). The operons for these systems are typically organised so that the antitoxin precedes the toxin encoding gene. Whilst the functions of TA systems are not fully clarified, they are known to play roles in post-segregational killing and abortive infection (cell-death prior to bacteriophage replication). They have also been proposed to play roles in the formation of metabolically quiescent persister cells, which can survive exposure to otherwise supra-lethal doses of antibiotic, and subsequently grow when the antibiotics are removed (1,4-6). However, the evidence for a role of TA systems in persister cell formation is controversial (7-9).

Different toxins have different molecular targets in the cell, and are able to modify functions such as transcription, translation or DNA replication (2,10,11), leading to growth arrest. The action of the toxin can be blocked after binding by the cognate antitoxin (1,12). Protein antitoxins characterised to date consist typically of two
recognition sites, one involved in toxin binding and the other in DNA binding. Monomeric and multimeric forms of both toxins and antitoxins can exist in solution and higher order complexes have been reported (2,12).

A range of factors determine the relative levels of toxin and antitoxin in the cell. Compared to toxins, antitoxins are generally sensitive to degradation by proteases, including Clp and Lon (13,14). In addition, there are interactions between the promoter region of the TA operon and the antitoxin or the toxin-antitoxin complex that regulates transcription. These interactions differ depending on the TA system (1,15).

A prototypic model, describing regulation of the operon, indicates that antitoxin alone binds weakly to the promoter region. However, introduction of the toxin can modify the DNA binding affinity of the antitoxin in a concentration dependent fashion (15,16). At low toxin:antitoxin ratios binding is enhanced (17-26). At high toxin:antitoxin ratios the toxin acts as a repressor and DNA binding affinity is reduced (15,20,22,27,28). This phenomenon of TA transcription being regulated by the toxin acting as either a co- or de-repressor is referred to as conditional cooperativity. (2,16). Aside from DinJ/YafQ (29) and MqsRA (30), the majority of TA systems are believed to display conditional cooperativity.

The HicAB system is widely distributed in a range of bacteria, and there is evidence that the toxins are RNases (31-33). We have previously studied the HicAB system in Burkholderia pseudomallei, a pathogen found in tropical regions of the world (34) which frequently causes chronic or latent infections of humans (35). The B. pseudomallei HicAB system plays a role in regulating the frequency of persister cells and may therefore play a role in disease (36). Recently, a truncated version of the HicAB complex from a different bacterium, Yersinia pestis, was solved by X-ray crystallography and biochemical studies provided direct evidence of HicA3 acting as an mRNase and HicB3 as a autoregulatory DNA binder (33). The HicAB system from Escherichia coli has also been studied and an excess of HicA shown to de-repress a HicB-DNA complex and restore transcription of HicB (37).

The X-ray structures of complexes representing many TA classes have been solved but to date there have been few examples of complexes of which HicA de-represses the fully de-repressed state (15,38). In particular an atomic level description of how HicA destabilizes the HicB-DNA repressor complex has not been reported. Here we report the X-ray structures of the B. pseudomallei HicB and the HicAB complex and the characterisation of their DNA interactions. Together these results reveal the structural mechanisms by which HicA de-represses the HicB-DNA complex.

Results

Structure determination of HicB.

Purified HicB (sequence in Fig. 1A, Table S1) formed a tetramer (Fig. S1) and yielded crystals that diffracted to 1.8 Å (Table S2). Crystals of a mutant of HicB with two methionine substitutions (HicB-I51M/I99M) (33) incorporating selenomethionine, diffracted to 3 Å which yielded a partial model. To assist in finding a molecular replacement (MR) solution, we also purified a HicB N-terminal domain (N-domain) construct (HicB-NT, residues 1-86 lacking the C-terminal domain (C-domain)) (Fig. S2) and crystals diffracted to 1.5 Å. Using a combination of these datasets the complete structure of HicB was solved by molecular replacement and exists as a tetramer (Fig. 1B) (Table S3). Each subunit consists of the N-domain (M1-L85), a linker region (S86-E93) and a C-domain (R94-K135) (Fig. 1C). The N-domain adopts an antiparallel β1β2β3α1α2α3β4 fold (Fig. S2) where the central helix α1 (I32-L53) lies within a cleft formed by β1β2β3 strands. The C-domain adopts a ribbon-helix-helix (RHH) (β5α5α6) motif that forms a strand swapped dimer with a partner C-domain. This occurs at two sites between subunits 1/3 and 2/4, forming two intact RHH domains (Fig. 1B). The tetramer has a single two-fold symmetrical axis with dimerization interfaces between adjacent N- and C-domains. The subunits of HicB can adopt one of two different configurations where the N-domain α1 helices are either sequestered and lie parallel to the C-domains (subunits 1 and 4) or are solvent exposed and orient perpendicularly to the C-domains (subunits 2 and 3) (Fig. 1D, Fig. S3A, B). This non-symmetric arrangement leads to two distinct sets of hydrophobic and electrostatic interactions between
the N- and C-domains (Fig. S3C). N-domains of subunits 1 and 4 interact with the C-domains of subunits 2 and 3 respectively via principally electrostatic interactions whilst the reverse set of interactions (e.g. N-domain of subunits 2 with C-domain of subunit 4) employ predominantly hydrophobic interactions. The interface between adjacent RHH domains is comprised of only alanine residues 109 and 112 and histidine 115 that also provide little interaction. This arrangement creates two dimeric C-domains that interact minimally with each other and the N-domains (Fig. 1D).

HicA binding drives conformational rearrangement from a closed to an open form of HicB.

A HicAB complex (Fig. S4) was prepared and crystals diffracted to 2.5 Å from which the structure was solved via MR (Table S3). The asymmetric unit of the HicAB complex contains four HicA and four HicB protomers arranged as a 222 symmetric hetero-octamer (Fig. 2A) with four identical HicA interaction sites at the N-domains of HicB. The positively charged face of the β-sheet of HicA binds to the α1 helix and β3 strand of HicB (Fig. 2B). In the complex the double-stranded RNA binding domain-like (dsRBD-like) fold of the HicA monomer is retained. However, the β1-β2 loop which includes the functionally important residues G22 and H24 in the wild-type HicA, is buried and projects towards a polar pocket on HicB formed by S27, N38 and E41 (Fig. 2C). A complete list of interactions between the two proteins is given in Table S4.

The conversion of the HicB tetramer to the higher symmetry HicAB hetero-octamer requires extensive conformational rearrangements that generate a more open conformation (Fig. 2A). These conformational rearrangements are most marked for subunits 1 (blue) and 4 (yellow) of HicB that undergo a domain rearrangement to flip the α1 helix from a sequestered conformation into an exposed conformation that can bind HicA. Conversely, helix α1 which is already partially solvent exposed in subunits 2 (pink) and 3 (green) of HicB undergoes a smaller 2.4 Å lateral displacement (Fig. 3A).

The displacement of the α1 helix in subunits 1 and 4 requires a reshuffling of packing interactions within the N-domains and the interface to the C-domain (Fig. 2D). For example, the electrostatic interactions that tethered the N-domain of subunit 1 to the C-domain of subunit 2 (Fig. S3C) are lost. These and are replaced by a hydrophobic network between N-domain residues I51, V57, F59 and P92 and P100/F102 of an inter-subunit C-domain (Fig. 2D). Supplementary ionic interactions between adjacent subunits, e.g. E58-K106 form additional inter-subunit interactions to stabilise the symmetrical conformation of HicB. In the complex, L85 rotates 60°, breaking the tethering interaction to the C-domains of subunit 1 and 4 (P100 and F102) that were present in free HicB (Fig. S3C). L85 is instead drawn into an intra-subunit hydrophobic network (I22, I43, V47, V57 and V83) with the N-domain (Fig. 2D) which stabilises the open conformation. Similarly, L88 rotates into an empty pocket created by the 2.8 Å shift of E48 that now forms a salt-bridge with R19 of HicA (Fig. 2B). This movement breaks the L88-F102 interaction between adjacent subunits in free HicB.

The loss of these packing interactions allows the HicB C-domains to rotate outwards in HicAB (Fig. 3A) without any significant rearrangements within the C-terminal domains themselves (hydrophobic packing is 80% conserved within the C-domains between HicB and HicAB). Residues from the C-terminal helix (R124 and L127) form new intra-subunit interactions with residues F59, D84, S86 and Q87 from the N-domain.

Analysis of the surface charge distribution for HicB revealed a clustering of positive charge on each of the RHH C-domains (Fig. 3B). In the free form the C-domains are oriented in such a way as these clusters align to form an extended positively charged patch. When viewed from a 90° rotated angle this patch can be seen to encompass an obvious groove at its centre. In the HicAB structure however, these domains reorient to move these clusters of charges to opposite sides of the HicAB complex, breaking the positively charged patch apart (Fig. 3C).

Small angle X-ray scattering (SAXS) was employed to confirm the solution structure of HicB. The shape envelope of HicB fitted the crystal structure of the tetrameric form of HicB (Fig. 4A, Statistics given in Table S5). These results confirm that HicB is a tetramer in solution.
By contrast, the symmetrical HicB tetramer component from the HicAB complex gave a poor fit ($\chi^2 = 8.94$, Fig. S5A) to this envelope. SAXS data collected on the HicAB complex generated a shape envelope that that could accommodate the symmetrical HicB tetramer component of the HicAB crystal structure (Fig. 4B, $\chi^2 = 2.89$). Conversely, the reverse analysis showed that the HicB tetramer could not be fitted to the SAXS shape envelope of the HicAB complex (Fig. S5B, $\chi^2 = 8.06$). These results support our crystallographic data that the HicB to HicAB transition involves substantial conformational changes both in the crystal and solution forms.

**HicB binds a symmetry DNA motif in the hicAB operon promoter region.**

Electrophoretic mobility shift assays (EMSA) were applied to investigate whether multimers of HicB or HicAB bound to synthetic oligonucleotides upstream of hicAB (Fig. S6A and B). This revealed a binding region for HicB (S1-2) 17-36 bp upstream of the start codon (Fig. 5A). This region contained a single palindromic sequence containing two sites (S1 and S2) spanning -24 to -19 (ACACA) and -34 to -29 (TGTGT). HicB-NT lacking the C-terminal RHH domain did not bind (Fig. S6C). Mutating nucleotides within the palindromic sequence from ACACA (S1) to AGGGA (MS1) or TGTGT (S2) to TCCCT (MS2) also abolished binding of these oligonucleotides to HicB (Fig. S6D).

Binding of fluorescently labelled S1-2 (Hex-S1-2) to HicB was also measured using fluorescence anisotropy (FA) (Fig. 5B) and the $K_d$ determined to be 3.0 ± 0.4 nM. We did not detect binding of HicB-NT or HicA to DNA (Fig. S6E, F, G).

In the presence of sub-stoichiometric ratios of HicA (HicA:HicB 0.5:1) the $K_d$ was broadly similar (1.9 ± 0.2 nM) (Fig. 5C) to the $K_d$ of HicB alone. However, when the HicA:HicB ratio was increased to greater than one (1:2:1), the titration did not reach saturation and the $K_d$ was estimated to be in excess of ~0.4 µM (Fig. 5D). When HicA was titrated into a preformed complex of HicB-HEX-S1-2 (80% bound), we observed dissociation of HicB from the DNA at HicA:HicB > 1 ($IC_{50} = 63.5 ± 0.9$ nM, Hill coefficient = 2.6) and full dissociation of the HicB-DNA complex was observed after the addition of ~300 nM HicA (Fig. 5E). The dissociation curve showed a sigmoidal dependence on the concentration of HicA indicative of a cooperative binding mechanism. The presence of free DNA at excess HicA:HicB ratios was also observed by EMSA (Fig. S6E).

The concentration of positively charged and polar groups on the RHH C-terminal domains of HicB (Fig. 3B) suggested that these residues might be involved in DNA binding. Purified HicB variants with R94A, R94E, N96A, N96Q, S98A and S98T mutations were tetrameric and bound to HicA. Compared to wild-type HicB, gel shift assays for R94A, R94E, N96A and S98A all showed loss of binding to S1-2 DNA (Fig. S7 and Fig. S8). Mutants N96Q and S98T both showed a marked reduction in binding (Fig. S8D).

**Discussion**

In this study we have solved the high-resolution X-ray structures of *B. pseudomallei* HicB and the complete complex of HicAB. The PISA server (39) confirmed the HicB oligomeric state (Table S6) and the dimerization interfaces within the tetramer were specific to HicB family members, with minor similarity to a putative RNA binding protein (Table S7). DALI (40) also calculated structural similarity with other HicB family members, including putative anti-toxin structures, but not non-HicB type 2 antitoxins (Table S8). HicB resembled several RNA binding proteins and endoribonuclease/helicases, consistent with the observation that HicB contains a partially degraded RNase H fold and a DNA binding domain (32). The PISA server also confirmed the HicAB oligomeric state (Table S9) and that the HicAB interface was specific to HicAB family members (Table S10). Our model supports the previous findings that *Y. pestis* HicB3 (33) binds to HicA3 and buries functionally essential residues of the toxin (Fig. S9). This may indicate a general mechanism for HicA recognition by HicB. A recent study has reported the HicBA crystal structure from *Streptococcus pneumoniae* and this also supports this mechanism (41). Although this complex is also hetero-octameric, the authors did not observe the open conformation that we report here.

Our work also provides new insight into HicB recognition of DNA. The palindromic DNA binding sites (S1-2) we have identified overlap with the predicted -10 sequence and CRP sites (Fig. 5). The DNA binding sites we have identified are
distinct from those previously seen in studies with *E. coli* HicAB and show that HicB from different species can recognise different DNA motifs. A single promoter has been experimentally mapped to a similar upstream region of *hicAB3* in *Y. pestis*. However, we cannot exclude the possibility of a second promoter, as suggested upstream of *E. coli* hicAB (37). We have shown that residues in the RHH motif (β5) of the C-terminal domain (R94, N96 and S98), which form a contiguous positively charged patch in the unbound conformation of HicB (Fig. 3B), are important for DNA recognition. FA and EMSA experiments (Fig. 5 and S6) reported similar results, although it was not possible to quantify the unbound and bound DNA components in EMSA due to the observed streaking of some HicB-DNA complexes. The RHH motif is also present in DinJ, FitA and RelB antitoxins (29,38,42). A structural comparison revealed direct conservation of the proximal basic residue of the β strand (R94) and N96. R94, N96 and S98 have K95, N97 and T99 counterparts in HicB3 suggesting their more general importance (Fig. S10).

The broad mechanism by which HicB binding to DNA is modulated by HicA has previously been observed. EMSA data show that an excess of *E. coli* HicA resulted in the dissociation of *E. coli* HicB from DNA (37). In our distinct system, where HicB contains a different DNA binding motif, we observe a similar phenomenon. We have, however, now elucidated at a molecular level how sufficiently high concentrations of HicA attenuate HicB:DNA interactions by stabilising a rearranged conformation of HicB that binds DNA weakly. Whilst the free HicB tetramer contains two RHH domains that are oriented to contact S1 and S2 sites of the palindromic DNA sequence, the RHH domains are separated by 70 Å in the hetero-octamer and cannot make the necessary simultaneous interactions with both S1 and S2 to achieve strong DNA binding.

Our structural and functional data and related functional data for HicAB from *E. coli* and *Y. pestis* showed that HicA binding decreased the affinity of HicB for DNA (37,41). However, in the case of HicAB from *S. pneumoniae* (41) HicA appeared to induce a modest increase of HicB:DNA binding affinity ($K_d$ 8 µM for HicB:DNA, 4 µM HicAB:DNA) inferring that HicA acts as a co-repressor rather than a de-repressor. Since no free HicB structure has been reported for *S. pneumoniae* (41), and the *Y. pestis* HicAB structure lacked the C-terminal DNA binding domains (33) (Fig. S9), the conformational changes induced by HicA cannot be elucidated. The two high-resolution structures presented herein delineate the conformational changes in HicB that modulate DNA binding.

Conditional Cooperativity has been demonstrated in a number of systems where there are multiple antitoxin binding sites within the regulatory regions of DNA (15,16,20,27,28). Three distinct molecular mechanisms have been proposed to explain Conditional Cooperativity: steric exclusion between non-repressing TA complexes; low-high affinity switches of toxin-antitoxin interaction sites; and allosteric communication between antitoxin domains (16). The RelBE system operates via a steric exclusion mechanism where free toxin can compete with a high affinity toxin/antitoxin-DNA complex (Fig. 6A) (24,38). In contrast, in the Pho-Doc (15) (Fig. 6B) and CcdAB (27) systems, when the antitoxin is in excess it binds both high and low affinity sites on the toxin. However when the toxin is in excess, only high affinity binding sites are occupied by the antitoxin that results in conditional cooperativity as the DNA-TA complex is disrupted. Pho (antitoxin) binding to the target operon is also allosterically regulated by the toxin Doc by forming a more structured DNA binding domain upon association with sub-stoichiometric ratios of Doc (15,43). The HicAB system described here falls into a class of TA systems including DinJ/YafQ (29) (Fig. 6C) and MqsRA (30) (Fig. 6D) where antitoxin binds to one or two operator sites but these are not individually or cross stabilised by toxin. Less is known about the mechanism of de-repression or the fully de-repressed state in these cases. In the DinJ/YafQ system, the toxin YafQ has no influence on DinJ binding affinity for DNA and the mechanism for dissociation is currently unknown. MqsRA on the other hand appears to operate under what has been termed a simplified conditional cooperativity model. Here the toxin is not a co-repressor but does act as a de-repressor, therefore ensuring that excess toxin is still effectively countered at the transcriptional level (16). The
MqsRA operator contains two palindromic regions, each of which can independently bind an MqsA dimer. There is however no stabilising interaction with MqsR and instead MqsR acts as a de-repressor due to overlap of the MqsR and DNA binding sites on MqsA (30). In the case of HicAB, sub-stoichiometric levels of HicA have no discernible effect on DNA binding. In contrast, an excess of HicA causes dissociation of the HicB-DNA complex through a large-scale conformational reorganisation of HicB to form a hetero-octameric complex (Fig. 6E). The competitive mechanism for DNA binding is similar to other related systems (30) but we have shown de-repression is cooperative at high concentrations of HicA. This allosteric regulation of interdomain interactions has also been proposed as a mechanism for this single site Conditional Cooperativity, but had not previously been experimentally demonstrated (16).

Whilst the structures defining the two endpoints of this process (i.e. HicB in forms that are and are not competent to bind DNA) have been determined here, further intermediate states are likely to exist. We have for example observed HicA1-HicB and HicA2-HicB complexes by native mass spectrometry (SEC-MALS) (33). Under experimental conditions, the FA data indicates that HicB can bind two HicA molecules with little change in the affinity of HicB for DNA (Figure 6E). Therefore, in the presence of DNA, the population of free HicA2HicB in the open non-DNA binding form is low. However, in accordance with a previous study with E. coli HicAB, competition experiments monitored with EMSA were unable to confirm formation of a stable DNA-HicB-HicA1/2 complex (37). When HicB is in an open conformation four molecules of HicA can be accommodated. In this conformation the DNA binding sites are separated and cannot cooperatively bind the target. Consequently, the $K_d$ for HicB binding to DNA increases from 3 nM to 0.4 mM. Whether the HicB promoter site is occupied by HicB or not will be influenced by the balance of two effects: the affinity of the closed form of HicB to DNA versus the affinity/competition of HicA for HicB that stabilizes the open, non-DNA binding form. Within the cell the interplay between HicB, DNA and HicA interactions will be complex. In a typical bacterium, the DNA target is at an effective concentration of ~ 1-2 nM (44) and on the order of the HicB-DNA dissociation constant (3 nM). At the point at which the pool of free HicB$^\text{T}$ has been largely bound as hetero-octamers by HicA and approaches the concentration of DNA, a mixture of spontaneous dissociation and HicA-driven dissociation may operate. Further studies are required to determine the series of steps leading to the release of the final HicB molecule from DNA in the presence of increasing concentrations of HicA.

**Experimental procedures**

**Protein expression, purification and mutation.**

All primers are given in Table S11. hicB (B. pseudomallei) was cloned from K96243 genomic DNA with HicB FL primer 1 and 2 and subcloned into pOPINE vector (pOPINE-HicB FL) before transformation into E. coli T7 Express cells. Cultures were grown to OD = 0.6 in LB media (37 °C) and induced (0.3 mM Isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG)) at 16 °C (16 h). Harvested cells were sonicated (50 mM Tris-HCl, 0.5 M NaCl, 10% (v/v) glycerol, pH 8.0) and purified by immobilised metal affinity chromatography (IMAC) and SEC (HiLoad Superdex 75, 25 mM Tris-HCl, 150 mM NaCl, pH 7.5) (Fig. S1E and S1F). Secondary structure calculations predicted a stretch of disordered amino acids at the C-terminus and a construct lacking $^{15}$VRHA$^{138}$ was designed (HicB) as well as a construct containing only the N-terminal residues (1-85) (HicB NT) and both were amplified from K96243 genomic DNA using appropriate primers and sub-cloned into pOPINE (pOPINE-HicB and pOPINE-HicB-NT). The pOPINE-HicB plasmid was used to generate HicB DM using mutagenic primers I51M and I99M. HicB R94A, N96A, S98A and N96Q genes were synthesized (Thermo Scientific), cloned into pOPINE and also used to subsequently generate R94E and S98T. pOPINE-HicB DM was transformed into E. coli B834 and cultures grown in SelenoMethionine Nutrient base supplemented with SelenoMethionine Nutrient Mix and 40 mg/L selenomethionine (Molecular
Dimensions) at 37 °C to OD = 0.6, followed by addition of 20 mg/L selenomethionine and induction (IPTG 0.3 mM, 16 h, 25 °C). Purification buffers were supplemented with 2 mM TCEP.

The HicA (H24A) B. pseudomallei mutant bearing an N-terminal His6-tag and a TEV cleavage site (ENLYFQ) construct was generated by Thermo Fisher and sub-cloned into a pET151-D/TOPO plasmid and HicA expressed and purified (36).

Crystallisation and data collection

Crystallisation conditions for HicB, HicB_DM, HicB-NT and HicAB are given in Table S2. Diffraction data was recorded from cryo-cooled crystals (100 K) at the Diamond Light Source Synchrotron in Oxford (i03 and i04). Data were processed and integrated using XDS (45), Xia2 (46) and further analysed using Phenix (47).

Structure determination and refinement

HicB_FL crystals diffracted to 4 Å despite optimisation. Crystals of HicB diffracted to 1.8 Å but could not be solved using MR. HicB_DM diffracted to 3 Å and a partial structure of the $P2_12_12_1$ crystal of selenomethionine incorporated HicB-I51M/I99M was determined by single-wavelength anomalous diffraction (SAD). Identification of heavy atoms sites, the resulting phase calculations and building of an initial model was determined by Crank2 (48), REFMAC5(49) and Buccaneer (50). Iterative rounds of manual model building and refinement were carried out using Phenix and Coot (51). HicB-NT ($P2_12_12_1$) was solved via MR and the Autobuild Pipeline in Phenix (52) using the partial model derived from HicB_DM followed by manual refinement using Coot and REFMAC.

The structure of HicB ($P4_1$) was determined by MR (Phaser) using the HicB_NT structure and C-terminal domains were manually built. The HicB model comprises 1-131 residues of the native sequence.

The structure of HicAB was also solved via MR. MOLREP (53) identified high contrast solutions for the two C-domain dimers with rotation peaks 1 (5.74σ) and 5 (4.45σ). Fixing the C-domains allowed four N-domains of HicB to be located when 200 rotation peaks were used in the translation search (MOLREP option NP=200). The four correct rotation peaks were 5, 10, 162 and 4 in the list. Four copies of a single chain of HicA (PDB: 4C26) were positioned into the resulting electron density using the spherically averaged phased translation function (54) and phased translation function implemented in MOLREP. The four-fold NCS averaging in DM (55) was used for phase improvement of HicAB structure with masks and NCS operators calculated separately for HicA and each domain of HicB. Density modification phases were input for phase refinement (56) in REFMAC5 (49). Resulting electron density maps allowed building of the hinge regions of HicB monomers in Coot.

Mass spectrometry.

For native electrospray ionisation (ESI) mass spectrometry, 20 μM samples of HicB were dialysed in 100 mM ammonium acetate. Nano-ESI experiments were performed on a Synapt G2-Si (Waters) using acquisition parameters adapted from (57). Complexes of HicAB were formed by mixing appropriate ratios of HicA and HicB, then dialysed in 100 mM NH₄Cl and analysed as described for HicB.

Analytical Size Exclusion Chromatography.

HicAB was pre-formed at 50 μM and incubated at 25 °C (1 h) and analysed with a calibrated (58) Superdex 75 10/300 GL pre-packed column (Table S12).

Small angle X-ray scattering.

In line SEC-SAXS for HicB and HicAB, were collected at Beamline 21, Diamond Light Source. using an Agilent 1200 HPLC and 2.4 mL Superdex S200 column (Table S5). 50 μL of protein at an appropriate concentration was loaded (0.04 mL/min) onto an equilibrated S200 column (25 mM Tris-HCl, 150 mM NaCl, pH 7.5). Frames were collected at 3 seconds per frame, 25 °C. X-ray scattering was recorded (Pilatus 2M detector) with a fixed camera length of 4.014 m, at 12.4 keV. Angular range q data were collected between 0.006-0.042 Å⁻¹. ScAtter (www.bioisis.net) determined the radius of gyration ($R_g$), the maximum particle dimension ($D_{max}$) and the pair distribution function ($P(r)$) (59,60). Ab initio bead density shape envelope models for HicB and HicAB were generated by DAMMIF (61), averaging twenty three independent runs using the program DAMAVER, before a single DAMMIN run refinement (62). Ab initio bead density shape
envelope models were aligned to both ab initio models and crystal structures using SUPCOMB (63). FoXS (64) was used to compare the calculated X-ray scattering of crystal structures with the experimental scattering profile of each protein. Experimental SAXS data and derived models of both HicB4 and HicAB4 have been deposited in the Small Angle Scattering Biological Databank (SASBDB) (65) with the accession codes SASDD45 and SASDD55.

**DNA binding assays**

Gel shift assays were carried out with specific annealed pairs of oligonucleotides (Integrated DNA Technologies) and serial dilutions of protein. For determination of the DNA binding region, 12.8 μM HicB4m (3.2 μM tetramer concentration) was equilibrated at room temperature with 3 μM DNA for 30 minutes in 25 mM Tris, 100 mM NaCl, 10% glycerol and loaded onto a 10% Tris-MMEA gel. HicA depression of HicB.S1-2 complex, [HA] is the anisotropy of the fully bound HicB-S1-2 complex, [X] is the concentration of protein. For competition assays involving HicA, data were fitted to a four-component inhibition equation to determine the IC50 value via equation (3).

\[
Y = A_D + \frac{(A_{DP} - A_D)}{1 + \frac{[HA]}{IC_{50}}}
\]

(3)

Where \( A_D \) is the anisotropy of free S1-2, \( A_{DP} \) is the anisotropy of the saturated HicB-S1-2 complex, \([HA]\) is the concentration of HicA, \( H \) is the Hill slope.

The atomic coordinates of HicB-NT, HicB and the HicAB complex have been deposited to the Protein Data Bank under accession codes: PDB 6G1C, 6G1N and 6G26 respectively. Experimental SAXS intensity of HicB, HicAB are deposited in SASBDB database (Accession entry SASDD45 and SASDD55).
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**FOOTNOTES**

The abbreviations used are: dsRBD-like, double-stranded RNA binding domain-like; HicA, protein toxin; HicB, protein antitoxin; HicB-NT, N-terminal domain of HicB; HicB_FL, full length HicB; MR, molecular replacement; RHH, ribbon helix helix; SAXS, small angle X-ray scattering; TA, toxin-antitoxin.
**Figure 1.** Crystal structure of HicB. (A) Sequence of *B. pseudomallei* HicB and comparison to the closest structural homologue, HicB3 from *Y. pestis*. Symbols indicate a conserved residue (*), conservative mutation (:) and a semi-conservative mutation (.). Underlined C-terminal residues were deleted in the His6-tagged construct of HicB used in this study. (B) The tetrameric crystal structure of HicB with subunits highlighted and dimerization interfaces at both the N terminal and C-terminal across adjacent subunits. (C) Single subunit of HicB with secondary structure elements labelled. (D) HicB tetramer with the C-terminal domains rendered with a solid surface and residues at the interfaces between C-terminal pairs are annotated.
**Figure 2.** The HicAB hetero-octameric complex. (A) Cartoon representation of the HicAB hetero-octamer where HicA (red) binds to the N-terminal domain of each HicB subunit (blue, pink, green and yellow). (B) HicA (red) has a triple stranded β-sheet that interacts with the α1 helix of HicB (blue). (C) The catalytic residue H24 (H24A in the crystal structure) is predicted to project into a polar pocket formed by S27, E41 and N37 of HicB. (D) Cartoon representation of new interaction sites across the HicB tetramer. Box 1 highlights the new internal hydrophobic network formed due to the rotation of L85 and L88 to interact with I22, I43, V47 and V83 within subunit 1 of HicB (blue). Box 2 indicates new inter-subunit interactions between adjacent subunits (1 and 3) of HicB (blue and green). P100 and F102 form hydrophobic contacts to I22, I43, V47, V57 and V83, while E58 interacts with K106. Due to the symmetrical nature of HicB within the hetero-octamer, these interactions sites are conserved between subunits of HicB. This is highlighted for HicB subunit 2 (box 3 and 4).
Figure 3. Conformation rearrangements induced by HicA binding and forming the complex HicAB. (A) HicA (red) interacts with the $\alpha$1 helix of each subunit of HicB (highlighted blue, pink, green and yellow) in the unbound conformation to form the hetero-octameric HicAB complex (PDB: 6G26), with $\beta$5 strands of HicB also highlighted to illustrate their rotation upon complexation. (B) Surface representation of HicB showing clustering of positive charges on one face mapped to R94, N96 and S98 of the C-terminal domain of HicB. (C) Surface representation of HicAB highlighting perturbation of the positively charged patch of the C-terminal domain (R94, N96 and S98) due to rotation of the two RHH domains. HicA is represented as grey to emphasize the surface charge of HicB.
Figure 4. Small angle X-ray scattering of HicB and HicAB. (A) *Ab initio* modelling of the crystal structure of the dimer of dimers form of HicB into the shape envelope of HicB (white), with each subunit of HicB appropriately coloured: blue, pink, green and yellow. The FoXS profile of the proposed scattering profile for the crystal structure (red) against the experimental raw scattering data (black) is underneath. ($\chi^2 = 1.98$). (B) *Ab initio* modelling of the HicB component of the HicAB crystal structure into the shape envelope of HicAB (white), with the corresponding FoXS profile underneath ($\chi^2 = 2.89$).
Figure 5. Overview of HicB binding to S1-2. (A) Overview of the hicAB operon. The palindromic region within the upstream region of hicAB that binds HicB. S1 and S2 are highlighted in red and blue. (B) Quantification of HicB binding to HEX-S1-2. Samples contained 7.5 nM HEX-S1-2 in DNA binding buffer. The proportion of HEX-S1-2 bound by increasing concentration of HicB was followed (n=1). Data of three independent repeats were fit to equation (2). (C) Quantification of HicA/HicB binding to HEX-S1-S2 (n=1) at HicA:B 0.5:1. Again 3 repeats were fit to equation (2). (D) Quantification of HEX-S1-2 binding to HicAB at 1.2:1 for 3 independent repeats (n=1). Data were fit to equation (2). (E) Quantification of HicA binding to a preformed complex of HicB M (40 nM) with HEX-S1-2 (7.5 nM) corresponding to 80% bound, HicB M refers to the monomer concentration of HicB. The proportion of substrate displaced by increasing concentrations of HicA was calculated for three independent repeats via equation (3) with a Hill coefficient of 2.6. For each experiment the mean value is plotted with error bars representing the S.E.M. Standard errors of $K_d$ values were calculated in GraphPad Prism.
A

Formation of a hetero-hexamer (RelE, RelB)$_2$

ReIE:B >1:1

Formation of RelE-RelB$_2$-RelE

Steric clashes between RelE causes derepression

B

Negative cooperativity between Phd dimers

Doc bridges dimers

Further Doc binds Phd via high affinity site

Phd:Doc> 1:1

Phd only binds to high affinity site of Doc (Doc-Phd-Doc)

Derepression of operon

C

DinJ

YafQ

LexA binding site

DinJ:YafQ> 1:1

Derepression?

D

MqsA binding rotates N-terminal domain

MqsR competes for the overlapping binding site

N-terminal binds DNA via amino acids that overlap with the toxin binding site

E

HicB alone fully represses

HicA:HicB <1:1

HicA:HicB>1:1

Fully derepressed by HicA

IC$_{50}$ 63.5 nM
Figure 6. Overview of de-repression models for RelBE, Phd-Doc, DinJ-YafQ, MqsRA and HicAB.

For each panel, the toxin is highlighted in red and the antitoxin in blue, while DNA binding sites are represented by half arrows. (A) The RelB$_2$ dimer or RelB$_2$E complex can bind to one of the two adjacent DNA operator sites, but it is proposed that the formation of a W-shaped hetero-hexameric complex (RelB$_2$E)$_2$ may occupy both adjacent DNA operator sites simultaneously to confer full transcriptional repression of the relBE operon. Excess toxin (RelE) binds a second site of a RelB dimer forming a rigid RelB$_2$E$_2$ hetero-tetramer, two hetero-tetramers that cannot simultaneously bind both operator sites due to steric hindrance resulting in transcriptional de-repression. Transcription of the relBE operon returns RelB and RelE to stoichiometric levels (≠1:1). (B) Doc forms a hetero-pentameric complex with Phd (Phd$_2$-Doc-Phd$_2$) by binding to Phd$_2$ via low (L) and high (H) affinity sites to confer full repression of the phd-doc operon by binding two operator sites. Doc allosterically regulates Phd to form a structured DNA binding domain to ensure full transcription repression only occurs upon formation of a Phd-Doc complex. An excess of Doc preferentially binds Phd solely through H sites resulting in the formation of a rigid heterotetramer (Doc-Phd$_2$-Doc) that cannot occupy both operator sites due to steric clashes between adjacent heterotetramers. In contrast both DinJ-YafQ (C) and MqsRA (D) do not follow the model of conditional co-operativity, as both toxins act as de-repressors rather than co-repressors. (C) DinJ$_2$ fully represses its operator via a single palindromic site. Addition of YafQ forms a YafQ-DinJ$_2$-YafQ hetero tetrameric complex, but an excess does not result in de-repression of the DinJ/YafQ-DNA complex and the de-repression mechanism is unknown. (D) Likewise, MqsA fully represses in the absence of MqsR. Excess MqsR competes with an overlapping DNA binding site of MqsA (highlighted in red) and formation of a proposed hetero-tetrameric MqsRA complex results in de-repression (the published MqsRA complex is a partial model). MqsA cannot simultaneously bind both MqsA and DNA. (E) Like MqsA and DinJ, HicB alone results in saturation of the palindromic sequences (S1-2). HicA binds the surface exposed α1 helices of subunit 2 and 3 to form an intermediate HicA$_2$HicB complex that does not result in an increase of affinity to S1-2. At concentrations of HicA > HicB, there is binding of a further two HicA molecules that results in the 90° rotation of the ribbon-helix-helix motifs prevents binding to the palindromic sequences and dissociation of HicB from DNA. The intermediate steps of this pathway are unknown and either route, or an equilibrium between the two cannot be discounted as of present.
Supplementary Information

The molecular basis of protein toxin HicA–dependent binding of the protein antitoxin HicB to DNA

Ashley J. Winter1, Christopher Williams1, Michail N. Isupov2, Hannah Crocker1, Mariya Gromova1, Philip Marsh1, Oliver J. Wilkinson3, Mark S. Dillingham3, Nicholas J. Harmer2, Richard W. Titball2,* and Matthew P. Crump1,*

From the 1 School of Chemistry, University of Bristol, Cantock's Close, Bristol, BS8 1TS, UK; 2 Biosciences, College of Life and Environmental Sciences, University of Exeter, EX4 4QD, UK; 3 School of Biochemistry, Biomedical Sciences Building, University Walk, Bristol, BS8 1TD.

* To whom correspondence should be addressed: Matt.crump@bristol.ac.uk;
R.W.Titball@exeter.ac.uk

This PDF file includes:

Figs. S1 to S10
Tables S1 to S12
Figure S1. Characterisation of HicB and HicB_FL.

(A) SDS-PAGE following the expression and purification of HicB (lacking C-terminal residues 135-138, Table S1) from post induction to isolation via IMAC and SEC. (B) SEC of HicB, eluting as a single oligomeric species. (C) Native mass spectrometry of HicB. Peaks were assigned based on their charge state: monomeric (black), dimeric (blue) or tetrameric (red). (D) De-convoluted spectrum of the m/z envelope shown in e yielding tetrameric HicB. Expected molecular weight: 62,952 Da. (E) SDS-PAGE following the expression and purification of full length HicB (HicB_FL, see Experimental procedures and Table S1) from post induction to isolation via IMAC and SEC. ESMS (not shown) (expected 16,201 Da, observed 16,200 Da). (F) SEC of HicB_FL eluting as a single oligomeric species calculated as 64,800 Da (tetramer). Each SEC profile contains a calibration curve (inset) using known standards: Aprotinin (AP), Ribonuclease A (RA), Carbonic Anhydrase (CA), Ovalbumin (OV) and Conalbumin (CO).
Figure S2. Characterisation of HicB-NT.

(A) SDS-PAGE following the expression and purification of HicB-NT from initial induction to isolation via IMAC and SEC. (B) SEC of HicB-NT, eluting as a single oligomeric species. (inset) calibration curves using known standards: Aprotinin (AP), Ribonuclease A (RA), Carbonic Anhydrase (CA), Ovalbumin (OV) and Conalbumin (CO). (C) Native mass spectrometry of HicB-NT. Peaks were assigned on their charge state: monomeric (black) or dimeric (blue). (D) De-convoluted spectrum (spanning 20-20.5 kDa) of the m/z envelope shown in c yielding dimeric HicB-NT: Expected molecular weight: 20,266 Da. (E) Structural representation of dimeric HicB-NT with subunits coloured blue and red respectively, highlighting the hydrophobic dimerization interface. Adjacent subunits associate via parallel packing of the β4 strands (V77-S82), flanked by the α2 helix (V64-L67) of each subunit. Adjacent subunits form a hydrogen bond and salt-bridge network between the side chains of β4-β4 (V77-L80) β1-α1 (E2-K37), α2-α1 (E65-I32, V64-D33, E65-D33), together burying 1,271 Å² of the total surface area (10,466 Å²). The final HicB-NT model comprises 1-85 residues.
Figure S3  Tethering interactions between adjacent N-terminal and C-terminal domains of HicB in the unbound conformation.

(A) Cartoon representation of the hydrophobic and ionic interactions between adjacent C-terminal domains and N-terminal/hinge region. (B) Cartoon representation of the location of hydrophobic and ionic interaction sites that are dependent on the placement of the α1 helix. (C) Interaction sites within the tetramer. Box 1 highlight electrostatic interaction sites (Residues E48, E52, D89, R101 and H105) and box 2 indicates the absence of hydrophobic interaction sites (Residues I51, V57, F59, L85, L88 with P100 and F102) between adjacent subunits electrostatic sites. Box 3 and 4 show the respective presence and absence of hydrophobic interaction sites (Residues I51, V57, F59, L85, L88 with P100 and F102) and electrostatic sites (Residues E48, E52, D89 with R101 and H105) between adjacent subunits.
Figure S4. Binding of HicA to HicB.

(A) Analytical SEC profiles of HicB, HicAB and HicA. (B) SDS-PAGE of isolated fractions for each analytical S75 profile. The vertical black line indicates where the gel was spliced to remove two unwanted lanes and improve clarity. (C) Native mass spectrometry of HicB within the region 2500-3800 m/z with oligomeric states: dimeric (black) and tetrameric (blue) highlighted based on their charge state value. (D) Native mass spectrometry of HicAB within the region 2450-3150 with oligomeric states of HicB₄ (blue) and HicA₄-HicB₄ (purple) highlighted. Sub-stoichiometric complexes of HicA₁-HicB₄ (green), HicA₂-HicB₄ (red) and HicB₂ were also generated in the gas phase.
Figure S5. Small angle X-ray scattering of HicB and HicAB.

This figure uses the same scattering and shape data as shown in Figure 4, but is demonstrating the poor FoXS fit when the HicB crystal structures used to fit the data are swapped. (A) Ab initio modelling of HicB component of the HicAB crystal structure into the shape envelope of HicB (white). The FoXS profile of the proposed scattering for the crystal structure (red) against the experimental raw scattering data (black) is underneath ($\chi^2 = 8.94$). (B) Ab initio modelling of the crystal structure of the dimer of dimers form of HicB into the shape envelope of HicAB (white), with the corresponding FoXS profile underneath ($\chi^2 = 8.06$).
A

| DNA Fragment | 0-48 | 48-96 | 96-144 | 144-196 | 196-240 | 240-279 |
|--------------|------|-------|--------|---------|---------|---------|
| HicB (μM)    | 3.2  | 3.2   | 3.2    | 3.2     | 0       | 3.2     |

Bound DNA →

Free DNA →

B

| DNA Fragment | 0-20 | 0-25 | 0-30 | 0-35 | 0-40 |
|--------------|------|------|------|------|------|
| HicB (μM)    | 3.2  | 3.2  | 3.2  | 3.2  | 0    |

Bound DNA →

Free DNA →

C

| Protein | HicB (μM) | HicB NT (μM) |
|---------|-----------|--------------|
| Protein (μM) | 0 | 0.8 | 1.6 | 2.4 | 3.2 | 0 | 0.8 | 1.6 | 2.4 | 3.2 |

Bound DNA →

Free DNA →

D

| 17-36 DNA | WT | MS1 | MS2 | MS1+MS2 |
|-----------|----|-----|-----|---------|
| HicB (μM) | 0  | 3.2 | 3.2 | 0       |

Bound DNA →

Free DNA →

E

**HicA-S1-S2**

Anisotropy

[HicA] [nM]

F

**HicB-NT-S1-2**

Anisotropy

[HicB-NT] [nM]

G

| HicB (μM) | 0 | 3.2 | 0 | 3.2 | 3.2 | 3.2 | 3.2 | 3.2 | 3.2 |
|-----------|---|-----|---|-----|-----|-----|-----|-----|-----|
| HicA (μM) | 0 | 0   | 3.2 | 1.6 | 3.2 | 6.4 | 12.8 | 25.6 | 65.8 |

Bound DNA →

Free DNA →
Figure S6. Overview of DNA binding with HicB

(A) Gel-shift analysis investigating HicB binding of individual fragments of the intergenic region of DNA upstream of the *hicAB* operon. Concentrations quoted at (µM)$_T$ correspond to the concentration of the HicB tetramer. (B) Gel-shift assay probing the HicB DNA target site within the 0-48 bp region. (C) Gel shift assay of HicB and HicB-NT (Concentrations quoted at [µM]$_T$ or [µM]$_D$ correspond to the concentration of the HicB tetramer or HicB-NT dimer respectively) binding to 2 µM S1-2. (D) Mutations within S1-2 (2 µM) at either S1 or S2 abolishes binding to HicB (3.2 µM). (E) Quantification of HicA binding to HEX-S1-2 (n=1). Three independent repeats were fit to equation (2). (F) Quantification of HicB-NT binding to HEX-S1-2. The proportion of HEX-S1-2 bound by varying concentrations of HicB-NT was followed (n=1). Again 3 independent repeats were fit to equation (2). For each experiment the mean value is plotted with error bars representing the S.E.M. Standard errors of K$_d$ values were calculated in GraphPad Prism. (G) HicA acts as a de-repressor. EMSA gel of varying HicA ratios (0.8 µM – 50.8 µM) titrated into HicB (3.2 µM) resulting in de-repression of HicB-S1-2 binding upon an excess of HicA.
Figure S7. Gel shift assays of DNA binding with HicB alanine mutants and gel filtration.

(A) Gel-shift analysis of HicB-R94A against S1-2 (17-36 bp). (B) Gel-shift analysis of HicB-N96A against S1-2. (C) Gel-shift analysis of HicB-S98A against S1-2. (D) Structure of the RHH domain, with key DNA binding residues highlighted. (E) Analytical SEC traces of HicB variants R94A, N96A and S98A and Blue Dextran. (F) Analytical SEC of HicB variants R94A, N96A and S98A complexed with HicA.
Figure S8. Gel shift assays of DNA binding of HicB semi-conservative mutants and gel filtration.

(A) Gel-shift assay of HicB-R94E binding S1-2. (B) Gel-shift assay of HicB-N96Q binding S1-2. (C) Gel-shift assay of HicB-S98T binding S1-2. (D) Gel shift assay of HicB variants, R94E, N96Q and S98T at 1000 µM binding to 2 µM S1-2. (E) Analytical SEC traces of all HicB semi conservative mutants with Blue Dextran standard. (F) Analytical SEC of HicB semi-conservative mutants complexed with HicA.
Figure S9. Comparison of HicB and HicAB crystal structures to HicB3 and HicA3B3.

(A) Superimposition of HicB (Blue) to HicB3 (Light grey), RMSD backbone atoms: 3.58 Å. (B) Comparison of the tetrameric organisation of HicB (Left) and HicB3, PDB:4P7D (Right). Individual subunits are highlighted in blue, red, green and yellow. (C) Superimposition of HicA (red) to HicA3 (Dark grey) (PDB:4P78), RMSD backbone atoms: 2.20 Å (D) The superimposition of HicA3(red)HicB1 (blue) to HicA31 (Dark grey) HicB3-NT (Light grey) (PDB:4P78), RMSD backbone atoms: 3.60 Å. (E) Sequestration of G26 and H28 in HicA3HicB3-NT model. (F) The HicA32HicB32 dimer (PDB: 4P78) that was reported (top) and extension to the hypothesized HicA34HicB34 structure with C-domains modelled. This revealed steric clashes between HicA3 monomers and the C-domains of subunit 1 and 4 of HicB3 as the N-terminal domains of complexed HicA3B3 are not correctly rearranged as seen in HicAB.
Figure S10. Comparisons of the RHH of HicB to other RHH containing proteins.

(A) Superimposition of HicB (blue) to HicB3 (PDB: 4P7D, red: RMSD: 1.43 Å), DinJ (PDB: 4Q2U, orange: RMSD: 2.66 Å), FitA (PDB: 2H10, green: RMS: 2.05 Å) and RelB (PDB: 4FXE, purple: RMSD: 2.71 Å). Direct conservation of the proximal basic residue is seen. (B) Overlay of Arc (PDB: 1BAZ, red), CopG (PDB: 2CPG, green), ORF Omega (PDB: 2BNZ, light orange) and PutA (PDB: 2AY0, orange) with HicB highlighting conservation of the proximal basic residue and the polar amino acid that flanks this in canonical DNA binding proteins. Individual superimpositions of HicB (Blue) with these protein domains are as follows: Arc (RMSD: 1.3 Å), CopG (RMSD: 1.3 Å), ORF Omega (RMSD: 1.6 Å) and PutA (RMSD: 1.8 Å).
Table S1. Sequences of the HicB constructs used in this study. Residues in bold indicate the additional His6-tag or cloning artefacts.

| Construct | Sequence |
|-----------|----------|
| HicB      | MEFPIAVHKDDGSVYGTVPDPFGVHSWGETIDDAIKNTREAIVGHVETLIEGEDEFTSTEELVAKPEYAGAV
            |          |
|           |          |
| HicB_FL   | MEFPIAVHKDDGSVYGTVPDPFGVHSWGETIDDAIKNTREAIVGHVETLIEGEDEFTSTEELVAKPEYAGAV
            |          |
|           |          |
| HicB_NT   | MEFPIAVHKDDGSVYGTVPDPFGVHSWGETIDDAIKNTREAIVGHVETLIEGEDEFTSTEELVAKPEYAGAV
            |          |
|           |          |
| HicB_DM   | MEFPIAVHKDDGSVYGTVPDPFGVHSWGETIDDAIKNTREAIVGHVETLIEGEDEFTSTEELVAKPEYAGAV
            |          |
### Table S2. Crystallisation conditions for each protein in this study.

Proteins at appropriate concentrations (25 mM Tris-HCl, pH 7.5, 150 mM NaCl) were obtained from the hanging drop vapour diffusion method at 16 °C. Crystals were flash frozen in liquid nitrogen prior to diffraction analysis.

| Protein   | Conditions                                                                 | Cryoprotectant |       |
|-----------|-----------------------------------------------------------------------------|----------------|-------|
| HicB-NT   | 0.1 M NaOAc, pH 4.6, 8% (w/v) PEG 4000                                      | 10% (v/v) glycerol |       |
| HicB_DM   | 0.1 M NaOAc, pH 4.6, 2.0 M HCOONa, 11% (v/v) glycerol                       | N/A            |       |
| HicB      | 0.1 M NaOAc, pH 4.2, 0.02 M CaCl₂•dH₂O, 15% (v/v) MPD                      | 30% (v/v) glycerol |       |
| HicAB     | 0.1 M MES pH 6.5, 0.2 M NH₄SO₄, 16% (w/v) PEG 5000 MME                     | 25% (v/v) glycerol |       |
Table S3. Crystallography table with statistics

| Project     | HicB-NT  | HicB     | HicB SeMet | HicAB   |
|-------------|----------|----------|------------|---------|
| Wavelength (Å) | 0.9763   | 0.9763   | 0.9790     | 0.9795  |
| Resolution range (Å) | 54.30 - 1.56 (1.62-1.56) | 39.42 - 1.85 (1.92 - 1.85) | 59.85-2.73 (2.86-2.73) | 34.02 – 2.49 (2.58-2.49) |
| Space group | P2₁2₁2₁  | P 4₁     | P2₁2₁2₁    | P2₁     |
| Unit cell (Å) | 63.5 76.7 76.9Å 90 90 90° | 62.6 62.6 173.5Å 90 90 90° | 58.7 59.9 172.8Å 90 90 90° | 85.1 72.2 85.3Å 90 90 1.90° |
| Multiplicity | 13.2 (13.0) | 4.7 (4.7) | 12.7 (13.1) | 6.8 (6.8) |
| Completeness (%) | 100.0 (100.0) | 99.0 (99.0) | 99.3 (99.3) | 99.9 (99.8) |
| Mean I/σ(I) | 29.9 (2.1) | 9.84 (0.40) | 16.0 (1.5) | 14.1 (1.4) |
| Wilson B-factor (Å²) | 36.69 | 54.74 | 71.3 | 66.6 |
| R-meas | 0.04 (1.26) | 0.07 (3.96) | 0.11 (2.09) | 0.12 (1.23) |
| CC1/2 | 1.000 (0.805) | 0.998 (0.129) | 0.998 (0.621) | 0.998 (0.692) |
| DelAnom correlation between half-sets | - | - | 0.493 (-0.03) | - |
| R/Rfree for partial model | - | - | 0.327/0.390 | - |
| Reflections used in refinement | 53900 | 55828 | - | 35696 |
| R-work | 0.2001 (0.3972) | 0.2016 (0.4480) | - | 0.1814 (0.330) |
| R-free | 0.2337 (0.4530) | 0.2355 (0.4517) | - | 0.2204 (0.375) |
| Number of protein atoms | 2502 | 4327 | - | 6272 |
| RMS (bonds) | 0.013 | 0.008 | - | 0.034 |
| RMS (angles) | 1.14 | 0.86 | - | 1.71 |
| Average B-factors | - | - | - | - |
| Protein | 41.97 | 67.95 | - | 56.73 |
| Solvent | 47.56 | 53.65 | - | 52.17 |
Table S4. List of interactions between HicA and HicB.

Residues that have direct equivalents within the HicA3B3 interaction site are underlined.

| Interaction | HicA-HicB |
|-------------|-----------|
| Hydrophobic |           |
| V18-L53     |           |
| A24-A42     |           |
| L35-L50     |           |
| L35-L53     |           |
| P39-W28     |           |
| P41-W28     |           |
| L45-V14     |           |
| L45-W28     |           |
| P46-V14     |           |
| Hydrogen bonds |     |
| S23-W28     |           |
| T37-H46     |           |
| Electrostatic |        |
| R19-E48     |           |
| R19-E52     |           |
| K28-E55     |           |
| K43-E30     |           |
### Table S5. SAXS analysis for HicB and HicAB

#### Data-collection parameters

| Instrument                          | SEC-SAXS at B21 Diamond Light Source |
|-------------------------------------|---------------------------------------|
| SEC column                          | Supdex 200 (GE Healthcare)            |
| Temperature                         | 25 °C                                 |
| q range (Å⁻¹)                       | 0.006-0.40                            |

#### Structural parameters

|                      | HicB                     | HicAB                     |
|----------------------|--------------------------|---------------------------|
| I(0) (cm⁻¹) [from P(r)] | 0.00378 ± 0.0000089       | 0.00349 ± 0.0000375       |
| Rg (Å) [from P(r)]    | 30.12 ± 0.24             | 31.31 ± 0.21              |
| I(0) (cm⁻¹) (from Guinier) | 0.00382 ± 0.0000089       | 0.00363 ± 0.0000092       |
| Dmax (Å)              | 97                       | 100                       |
| Rg (Å) (from Guinier) | 30.67 ± 0.37             | 31.90 ± 0.47              |
| Porod volume estimate (Å⁻³) | 100,000                  | 117,000                   |
| χ²                    | 0.07                     | 0.75                      |

#### Molecular-mass determination

|                      |                          |                          |
|----------------------|--------------------------|--------------------------|
| Partial specific volume (cm³ g⁻¹) | 0.738384                 | 0.738384                 |
| Contrast (Δρ x 10³⁰ cm⁻²) | 3.047                    | 3.047                    |
| Molecular mass M_r [from I(0)] | 67,000                   | 74,000                   |
| Calculated M_r from sequence (kDa) | 62,952                   | 91,164                   |
| From Porod Volume [Vporod/1.7] (Da) | 58,823                   | 73,000                   |

#### Software employed

|                      |                          |
|----------------------|--------------------------|
| Primary data reduction | GDA (Diamond Light Source) |
| Data processing      | Scatter                  |
| Ab initio analysis   | DAMMIN                   |
| Validation and averaging | DAMAVER                  |
| Rigid-body modelling | N/A                      |
| Computation of model intensities | FoXS                    |
| Three-dimensional graphics representations | Pymol                   |
Table S6 PISA analysis of HicB tetramer observed. Monomers were investigated for their total number of atoms (Nat), residues (Nres), total number of surface atoms (Sat) and residues (Sres). The total surface area (Area) and solvation energy of folding (∆G) were calculated. Assemblies (Crystal splits) were investigated with the Accessible Solvent Area (ASA), Buried Surface Area (BSA), Standard Free Energy of dissociation into nearest stable assemblies (∆G_diss), Standard Free Energy of dissociation into monomeric units (∆G0) and Composition of assembly reported. It was reported that the tetramer was stable in solution. Chains A,B, C and D refer to subunits 2, 3, 1 and 4 in figure 1.

| Monomer | Nat  | Nres | Sat  | Sres | Area (Å²) | ∆G (kcal/mol) |
|---------|------|------|------|------|-----------|---------------|
| A       | 1019 | 132  | 724  | 128  | 9294.5    | -105.8        |
| B       | 1019 | 132  | 719  | 128  | 9332.4    | -106.9        |
| C       | 1041 | 135  | 729  | 130  | 9737.4    | -108.8        |
| D       | 1041 | 135  | 725  | 129  | 9675.3    | -107.1        |

Table S7: Structural conservation of the HicB interfaces within the PDB. The PISA server reports the PDB entry, interface number, mmSize (number of macromolecular monomers within the assembly), Q score (relates from 0 to 1 for unrelated-identical proteins), Sequence identity (Seq ID), interface area, ∆G and Complexation significance score (CSS, relates from 0 to 1 as the interface relevance increases).

| Entry | Interface Number | mmSize | Q score | Seq Id | Interface area, Å² | ∆G kcal/mol | CSS |
|-------|------------------|--------|---------|--------|--------------------|--------------|-----|
| 4P7D  | 1                | 4      | 0.395   | 0.273  | 2089.2             | -30          | 0.844 |
| 4P78  | 3                | 4      | 0.369   | 0.268  | 951.5              | -21.7        | 0.931 |
| 5YRZ  | 3                | 4      | 0.284   | 0.194  | 604.6              | -7.7         | 0.268 |
| 3KWR  | 2                | 4      | 0.269   | 0.096  | 523.7              | -9.4         | 0.815 |
**Table S8: DALI.** The DALI server search result against a single subunit of HicB. The PDB chain, Z score, RMSD, sequence identity and description are highlighted. The Z score relates to the structural similarity between proteins. Proteins with a Z score below 3 were not included in this table.

| Chain   | Z score | Rmsd | Identity | Description                                      |
|---------|---------|------|----------|--------------------------------------------------|
| 4P7D-A  | 11.8    | 10.9 | 25       | Antitoxin HicB3 from *Yersinia pestis*           |
| 5YRZ-A  | 9.9     | 2.6  | 23       | Antitoxin HicB from *Streptococcus pneumoniae*  |
| 3KWR-B  | 10      | 3.7  | 15       | Putative RNA binding protein                     |
| 2DSY-C  | 6.1     | 2.2  | 21       | Hypothetical protein TTHA0281                    |
| 3K6Q-A  | 5.0     | 2.3  | 14       | Putative anti-toxin from *Syntrophomonas Wolfei*|
| 1ZBT-A  | 4.5     | 10   | 13       | Peptide chain release factor                     |
| 6CI7-F  | 3.9     | 14.3 | 8        | YCAO                                             |
| 5CFF-F  | 3.7     | 2.2  | 10       | Miranda/Staufen dsRBD5 complex                   |
| 3VYY-A  | 3.6     | 3.5  | 10       | ATP-Dependent RNA helicase A                     |
| 4WYQ-B  | 3.5     | 2.6  | 8        | Endoribonuclease DICER                          |
| 2MDR-A  | 3.5     | 2.2  | 4        | Double-stranded RNA-specific adenosine deaminase|
| 2LTR-A  | 3.4     | 2.6  | 7        | Protein RDE-4                                   |
| 3P1X-B  | 3.4     | 2.0  | 8        | Interleukin enhancer binding factor 3            |
| 3ADL-A  | 3.3     | 2.4  | 14       | RISC-loading complex subunit TARBP2              |
| 1WHQ-A  | 3.1     | 2.9  | 11       | RNA helicase A                                  |
Table S9 PISA analysis of HicAB hetero-octamer. Monomers were investigated for their total number of atoms (Nat), residues (Nres), total number of surface atoms (Sat) and residues (Sres). The total surface area (Area) and solvation energy of folding (ΔG) were calculated. Assemblies (Crystal splits) were investigated with the Accessible Solvent Area (ASA), Buried Surface Area (BSA), Standard Free Energy of dissociation into nearest stable assemblies (ΔG_diss), Standard Free Energy of dissociation into monomeric units (ΔG0) and Composition of assembly reported. It was reported that the tetramer was stable in solution. Chains A,B,C,D refer to Subunit 4, 1, 3 and 2 of HicB, while chains F, E, G and H refer to HicA moieties bound to each respective subunit.

| Monomer | Nat  | Nres | Sat  | Sres | Area (Å²) | ΔG (kcal/mol) |
|---------|------|------|------|------|-----------|--------------|
| A       | 1019 | 132  | 724  | 128  | 9294.5    | -105.8       |
| B       | 1019 | 132  | 719  | 128  | 9332.4    | -106.9       |
| C       | 1041 | 135  | 729  | 130  | 9737.4    | -108.8       |
| D       | 1041 | 135  | 725  | 129  | 9675.3    | -107.1       |
| E       | 479  | 61   | 297  | 56   | 4061.0    | -55.1        |
| F       | 466  | 60   | 296  | 56   | 3944.6    | -53.1        |
| G       | 472  | 60   | 294  | 56   | 4002.5    | -54.7        |
| H       | 461  | 59   | 305  | 57   | 4185.2    | -49.4        |

Stable crystal assemblies

| Split No | Size | Type | ASA (Å²) | BSA (Å²) | ΔG_diss (kcal/mol) | ΔG0 (kcal/mol) | Composition |
|----------|------|------|----------|----------|--------------------|---------------|-------------|
| 1        | 8    | 1    | 36213.4  | 17619.7  | 8.8                | 74.1          | ABCDEFGH    |
| 2        | 4    | 2    | 19521.3  | 7473.8   | 9.2                | 32.6          | ADFH        |
| 3        | 2    | 3    | 11537.3  | 1876.5   | 5.2                | 5.2           | CG          |
| 4        | 2    | 3    | 11710.0  | 1845.3   | 4.9                | 4.9           | DH          |
| 4        | 2    | 3    | 11611.9  | 1827.9   | 4.9                | 4.9           | AF          |
| 4        | 2    | 3    | 11567.2  | 1856.8   | 4.2                | 4.2           | BE          |

Metastable crystal assemblies

| Split No | Size | Type | ASA (Å²) | BSA (Å²) | ΔG_diss (kcal/mol) | ΔG0 (kcal/mol) | Composition |
|----------|------|------|----------|----------|--------------------|---------------|-------------|
| 5        | 4    | 5    | 22004.7  | 4974.7   | -0.9               | -0.9          | BDEH        |
| 6        | 4    | 5    | 21905.1  | 4948.5   | -1.3               | -1.3          | ACFG        |

Marginally stable assemblies

| Split No | Size | Type | ASA (Å²) | BSA (Å²) | ΔG_diss (kcal/mol) | ΔG0 (kcal/mol) | Composition |
|----------|------|------|----------|----------|--------------------|---------------|-------------|
| 6        | 4    | 4    | 27426.7  | 10213.1  | 9.6                | 56.9          | ABCD        |
| 7        | 2    | 6    | 17460.6  | 1272.6   | -0.2               | -0.2          | BD          |
| 2        | 6    | 2    | 17662.5  | 1244.1   | -0.6               | -0.6          | AC          |
**Table S10: Structural conservation of the HicAB interface within the PDB** The PISA server reports the PDB entry, interface number, mmSize (number of macromolecular monomers within the assembly), Q score (relates from 0 to 1 for unrelated-identical proteins), Sequence identity (Seq ID), interface area, ΔG and Complexation significance score (CSS, relates from 0 to 1 as the interface relevance increases).

| Entry | Interface Number | mmSize | Q score | Seq Id | Interface area, Å² | ΔG kcal/mol | CSS  |
|-------|------------------|--------|---------|--------|--------------------|-------------|------|
| 5YRZ  | 6                | 4      | 0.782   | 0.246  | 1114.5             | -7.9        | 0.468|
| 4P78  | 2                | 4      | 0.710   | 0.328  | 1179.3             | -7.5        | 0.608|
| 3KWR  | 1                | 4      | 0.241   | 0.091  | 674.4              | -12.5       | 0.436|
| Oligonucleotides | IDT | Sequence                                      |
|------------------|-----|-----------------------------------------------|
| 0-48 Primer 1    | IDT | gatcgtgattggatgtgtataattacacacaagaacatccgcgggagct |
| 0-48 Primer 2    | IDT | agctcccccgaatgtcttgtatcttatactacatccacaaggaagt |
| 48-96 Primer 1   | IDT | atagggggcaacacataattacacacatcgaagctttgctacgacgaggaagt |
| 48-96 Primer 2   | IDT | cctcggctgtgctgtgaatcttacacatccacacatcgaaggaagt |
| 96-144 Primer 1  | IDT | tgcgaggccgcggcgcagatcttcagctctgtgaatcttcagctct |
| 96-144 Primer 2  | IDT | ttctttctcgcgtacgctcaatcagctcttcgctcagctct |
| 144-196 Primer 1 | IDT | ggcctcgcgcggcgcagatcttcagctcttcgctcagctct |
| 144-196 Primer 2 | IDT | cattccttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| 192-240 Primer 1 | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| 192-240 Primer 2 | IDT | cattccttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| 240-279 Primer 1 | IDT | ggcctcgcgcggcgcagatcttcagctcttcgctcagctct |
| 240-279 Primer 2 | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| 0-20 Primer 1    | IDT | acaagacatctggggagct |
| 0-20 Primer 2    | IDT | agctcccccgaatgtcttgt |
| 0-25 Primer 1    | IDT | tgcgaggccgcggcgcagatcttcagctcttcgctcagctct |
| 0-25 Primer 2    | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| 0-30 Primer 1    | IDT | atagggggcaacacataattacacacatcgaagctttgctacgacgaggaagt |
| 0-30 Primer 2    | IDT | cctcggctgtgctgtgaatcttacacatccacacatcgaaggaagt |
| 0-35 Primer 1    | IDT | ttctttctcgcgtacgctcaatcagctcttcgctcagctct |
| 0-35 Primer 2    | IDT | cattccttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| 0-40 Primer 1    | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| 0-40 Primer 2    | IDT | cattccttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| 17-36 Primer 1   | IDT | atagggggcaacacataattacacacatcgaagctttgctacgacgaggaagt |
| 17-36 Primer 2   | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| MS1 Primer 1     | IDT | atagggggcaacacataattacacacatcgaagctttgctacgacgaggaagt |
| MS1 Primer 2     | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| MS2 Primer 1     | IDT | atagggggcaacacataattacacacatcgaagctttgctacgacgaggaagt |
| MS2 Primer 2     | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| MS1MS2 Primer 1  | IDT | atagggggcaacacataattacacacatcgaagctttgctacgacgaggaagt |
| MS1MS2 Primer 2  | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| HicB_FL Primer 1 | IDT | cttccggatttaatgctgggtgatgtttaacgcagctgggtgatgtttaacgcagctg |
| HicB_FL Primer 2 | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| HicB Primer 1    | IDT | cttccggatttaatgctgggtgatgtttaacgcagctgggtgatgtttaacgcagctg |
| HicB Primer 2    | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| I51 M Primer 1   | IDT | atagggggcaacacataattacacacatcgaagctttgctacgacgaggaagt |
| I51 M Primer 2   | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| I99 M Primer 1   | IDT | cttccggatttaatgctgggtgatgtttaacgcagctgggtgatgtttaacgcagctg |
| I99 M Primer 2   | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| Mutant Primer 1  | IDT | cttccggatttaatgctgggtgatgtttaacgcagctgggtgatgtttaacgcagctg |
| Mutant Primer 2  | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| R94E Primer 1    | IDT | cttccggatttaatgctgggtgatgtttaacgcagctgggtgatgtttaacgcagctg |
| R94E Primer 2    | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
| S98T Primer 1    | IDT | cttccggatttaatgctgggtgatgtttaacgcagctgggtgatgtttaacgcagctg |
| S98T Primer 2    | IDT | gttcttcgctgctgtgaatcttacacatccacacatcgaaggaagt |
Table S12. Samples analysed by SEC.

Elution volumes were used to calculate partition coefficient to indirectly determine the molecular weight based off the calibration curve.

| Sample  | Mw (Da) | Calculated Mw (Da) | Log₁₀Mw | Vₑ (ml) | Kₑv |
|---------|---------|--------------------|---------|--------|------|
| HicA    | 7,053   | 8,240              | 0.92    | 14.5   | 0.44 |
| HicB    | 62,952  | 65,610             | 1.82    | 9.03   | 0.11 |
| HicAB   | 91,164  | 71,870             | 1.86    | 8.79   | 0.10 |