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HicAB toxin–antitoxin complex from *Escherichia coli*: expression and crystallization

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Toxin–antitoxin (TA) systems are widespread in both bacteria and archaea, where they enable cells to adapt to environmental cues. TA systems play crucial roles in various cellular processes, such as programmed cell death, cell growth, persistence and virulence. Here, two distinct forms of the type II toxin–antitoxin complex HicAB were identified and characterized in *Escherichia coli* K-12, and both were successfully overexpressed and purified. The two proposed forms, HicAB^L and HicAB^S, differed in the presence or absence of a seven-amino-acid segment at the N-terminus in the antitoxin HicB. The short form HicAB^S readily crystallized under the conditions 0.1 M Tris–HCl pH 8.0, 20% (w/v) PEG 6000, 0.2 M ammonium sulfate. The HicAB^S crystal diffracted and data were collected to 2.5 Å resolution. The crystal belonged to space group *I*222 or *I*2₁2₁2₁, with unit-cell parameters *a* = 67.04, *b* = 66.31, *c* = 120.78 Å. Matthews coefficient calculation suggested the presence of two molecules each of HicA and HicB^S in the asymmetric unit, with a solvent content of 55.28% and a Matthews coefficient (*V*_M) of 2.75 Å³ Da^{−1}.

1. Introduction

Toxin–antitoxin (TA) systems are abundant amongst bacteria and archaea, where they function to enable cells to adapt to environmental cues (Gerdes *et al.*, 2005). TA systems have been proposed to play crucial roles in various cellular processes, such as programmed cell death, cell growth, persistence and virulence. To date, hundreds of plasmid-encoded or chromosome-encoded bacterial TA modules have been characterized, some by intensive investigation into their phylogeny and gene regulation (Buts *et al.*, 2005; Gerdes *et al.*, 2005; Hayes, 2003). TA systems are usually found as a bicistronic gene pair encoding a toxin protein and an antitoxin. The antitoxins are either proteins or RNAs. Depending on the nature of the antitoxin and its mechanism of action, TA systems are commonly grouped into six distinct types (types I–VI; Schuster & Bertram, 2013). In type I and type III TA systems the antitoxin is a small noncoding RNA that interferes with cognate toxin production or sequesters toxin activity, respectively, whereas in the other four TA types (types II, IV, V and VI) the antitoxin is a low-molecular-weight protein that counteracts the toxicity of its cognate toxin in distinct ways (Yamaguchi *et al.*, 2011).

Being abundant in bacteria, type II TA systems are the best-studied type of TA module to date (Chan *et al.*, 2016). In normal growing cells, binding of a type II antitoxin to the toxin blocks the activity of the toxin, and thereby simultaneously autoregulates the expression of its own operon (Chan *et al.*,

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Table 1
Macromolecule-production information.

Source organism	<i>E. coli</i> K-12
DNA source	Genomic DNA
Forward primer†	CGGGATCCATGAAACAAAGCGAGTTCAGAC
Reverse primer†	CCGCTCGAGTTAAACCATCACCAGCGATAAC
Cloning vector	pET-28a(+) (Novagen)
Expression vector	pET-28a(+) (Novagen)
Expression host	<i>E. coli</i> Rosetta 2 (Novagen)
Complete amino-acid sequences of the constructs produced	
HicA‡	MGSSHHHHHHSSGENLYFQGSMSKQSEFRRWLESQ GVDVANGSNHLKLRFRHGRSVMRPHPCDEIKE PLRKAILKQLGLS
HicB§	MR ETVEIM RYPTLTTPAPEGGYMSVFDIPEALT QGETVAEAMEAAKDALLTAFDFYFEDNELIPL PSP LN SHDHFIEVPLSVASKVLLNNAFLQSEI TQQELARRIGKPKQEITRLFNLHHATKIDAVQ LAAKALGKELSLVMV

† Restriction sites for BamHI and XhoI are shown in bold in the forward and reverse primers, respectively. ‡ The His-tag fusion appended at the N-terminal end of HicA is underlined. § The two alternative methionines in HicB that initiate translation of HicB^L and HicB^S are shown in bold.

2016). As transcriptional repressors, both type II antitoxins and TA complexes are able to bind directly to the operator regions that overlap the −35 and −10 promoter sites, repressing transcription by restricting RNA polymerase access (Schumacher *et al.*, 2015). In stressed cells, however, type II TA expression is activated, while the labile antitoxin is selectively degraded by the cellular proteases Lon and Clp, resulting in the release and increased concentration of toxins (Muthuramalingam *et al.*, 2016). Toxins target important cellular processes such as DNA replication or protein translation, inhibiting cell growth or enabling cells to enter a metabolically dormant state until the stress is removed (Coussens & Daines, 2016). Generally, TA expression correlates with cell-growth conditions, and is beneficial to bacterial adaptation.

The *hicAB* module belongs to a large family of type II TA systems that are abundant in many bacteria and archaea. The *hicAB* module has been implicated in the stress response, virulence and persistence (Daimon *et al.*, 2015; Button *et al.*, 2007; Li *et al.*, 2016; Butt *et al.*, 2014). The *hicAB* operon was first identified as a bicistron inserted into a locus adjacent to the pilus gene *hif* cluster, and was thus termed the *hic* (*hif* contiguous') locus in *Haemophilus influenzae* (Mhlanga-Mutangadura *et al.*, 1998). The *hif* cluster, which represents a pathogenicity island, might be involved in the adherence and colonization of *H. influenzae*, and the chromosomal *hicAB* locus has also been implicated in *H. influenzae* virulence and the maintenance of genomic superintegrations (Syed & Gilsdorf, 2007). In *Burkholderia pseudomallei*, a *hicAB* homologue has been implicated in persister-cell formation (Butt *et al.*, 2013, 2014). Clinically, persister cells cause chronic and recurrent disease. Expression of the *B. pseudomallei* HicA toxin increases the number of persister cells that are tolerant to the antibiotics ciprofloxacin and ceftazidime, while its deletion significantly reduces persister-cell frequencies (Butt *et al.*, 2014). Depending on the species, bacterial genomes encode between one and several HicAB modules. For example, the *Yersinia pestis* genome harbours three members of the HicAB

family (Bibi-Triki *et al.*, 2014; Goulard *et al.*, 2010). Recently, the crystal structures of the *Y. pestis* HicAB family complex HicA3B3 and the sole antitoxin HicB3 have been resolved, revealing the architecture of the HicAB family (Bibi-Triki *et al.*, 2014). In the structure of the HicA3–HicB3–Nt complex, in which HicB3–Nt represents a version of HicB from which the C-terminal domain has been cleaved off, HicA3–HicB3–Nt forms a heterotetramer that contains two HicA3 toxins and two HicB3–Nt molecules. HicA3 adopts a double-stranded RNA-binding domain. The HicB3 N-terminal domain, which resembles the RNase H fold, contacts HicA3 and covers the HicA3 active site.

In *Escherichia coli* K-12 only one HicAB module was identified on the chromosome. The *hicAB* genes are arrayed on the genome in an unusual reverse order, with the toxin locus preceding the antitoxin gene. The *E. coli* toxin *hicA* and antitoxin *hicB* loci are separated by a 24- or 45-nucleotide intergenic region, potentially resulting in 145- or 138-amino-acid forms of HicB, which counteract a 58-amino-acid HicA (Makarova *et al.*, 2006; Jørgensen *et al.*, 2009; Turnbull & Gerdes, 2017). *E. coli* HicA is a small RNA interferase with a double-stranded RNA-binding fold. It has been demonstrated that HicA can cleave certain model mRNAs and tmRNA in a translation-independent manner, suggesting that HicA acts as a ribosome-independent mRNA interferase (Jørgensen *et al.*, 2009). The conserved residues His23 and His48 were critical for HicA activity. The antitoxin HicB has been predicted to comprise two structural domains: an N-terminal domain with an RNase H fold lacking the catalytic residues and a C-terminal DNA-recognition domain (Makarova *et al.*, 2006). The DNA-recognition domain of HicB in *E. coli* and *Pseudomonas aeruginosa* comprises a helix–turn–helix motif rather than the ribbon–helix–helix motif found in *Y. pestis* HicB (Makarova *et al.*, 2006).

The HicAB family shares limited amino-acid identity among its homologues. The primary sequences of each HicAB component share less than 30% identity between *Y. pestis* and *E. coli* K-12. In this study, the *E. coli* HicAB complex was purified and the *hicAB* operon was found to encode two possible forms of the HicAB complex. Only the complex containing the short form HicAB^S was successfully crystallized. Preliminary X-ray crystallographic characterization of the *E. coli* HicAB complex was performed.

2. Materials and methods

2.1. Macromolecule production

Using gene-specific DNA primers (forward primer 5'-CGGGATCCATGAAACAAAGCGAGTTCAGAC-3' and reverse primer 5'-CCGCTCGAGTTAAACCATCACCAGCGATAAC-3'), the bicistronic *hicAB* coding region was amplified by polymerase chain reaction (PCR) from *E. coli* K-12 (DH5α) genomic DNA. The amplified DNA fragment was then subcloned into pET-28a(+) vector between the BamHI and XhoII restriction sites (underlined in the primer sequences), generating the pET-28a-*hicAB* construct. To

Table 2
Crystallization data.

Method	Sitting-drop vapour diffusion
Plate type	48-well plate (XtalQuest)
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	20
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5 mM EDTA
Composition of reservoir solution	0.1 M Tris-HCl pH 8.0, 20%(w/v) PEG 6000, 0.2 M ammonium sulfate
Volume and ratio of drop	2 µl (1:1 protein:reservoir solution)
Volume of reservoir (µl)	100

create pET-28a-*hicA* to produce HicA, a site-directed mutagenesis method was employed with pET-28a-*hicAB* as the template with the pair of primers hicAFP (5'-ACAACCTCGGTTTGAGTTAACTCGAGCACCACCACCAC-3') and hicARP (5'-TTAACTCAAACCGAGTTGTTTCAGGATTGCTTTACGCA-3'), resulting in deletion of the *hicB* coding region and the intergenic sequence upstream of *hicA* in pET-28a-*hicAB*. To generate pET-28a-*hicAB*^S and pET-28a-*hicAB*^L-(M8I) to produce the two complexes HicAB^S and HicAB^L-(M8I), the first and second in-frame ATG codons of HicB were separately mutated to ATC in pET-28a-*hicAB*. HicAB^S and HicAB^L-(M8I) represent two forms of the HicAB complex in which HicA either forms a complex with a 138-amino-acid HicB (HicB^S) or with a 145-amino-acid HicB with a methionine-to-isoleucine mutation at position 8 [HicB^L-(M8I)]. Each construct was transformed into the expression host *E. coli* Rosetta 2 (Novagen) to overexpress the two HicAB complex derivatives with an N-terminal 6×His-tag fusion (Table 1). Similarly, the *E. coli* HicA protein was successfully overexpressed and purified from the overexpression strain *E. coli* Rosetta 2 at 310 K, indicating that a limited amount of HicA did not harm cell growth. The expression strain was grown in shaker flasks to an OD₆₀₀ of approximately 0.8 at 310 K and was induced at 289 K for 18 h with 0.5 mM isopropyl β-D-1-thiogalactopyranoside. After induction, the cells were harvested and the pellets were flash-frozen. To perform protein purification, the cell pellets were suspended in 50 mM Tris-HCl pH 8.0, 20%(w/v) sucrose, 500 mM NaCl, 20 mM imidazole, 0.1% Tween 20, 1 mM PMSF, 1 mM β-mercaptoethanol, 10 µg ml⁻¹ DNase. The cell suspension was disrupted by a high-pressure homogenizer and the cell homogenate was then centrifuged at 22 900g for 60 min at 277 K to remove cellular debris. The supernatant was subjected to nickel-nitrilotriacetic acid bead column purification. The His-tagged protein or complex was then eluted in 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. The proteins were then fractionated with a Superdex 200 column (GE Healthcare) equilibrated with buffer consisting of 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.5 mM EDTA, 1 mM β-mercaptoethanol. The HicAB fractions were pooled and desalted in buffer A (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 1 mM β-mercaptoethanol) and finally purified using a HiTrap SP column (GE Healthcare) eluted with an NaCl gradient in buffer A. Both forms of N-terminally His-tagged recombinant HicAB toxin-antitoxin complex, HicAB^L-(M8I) and HicAB^S,

and the HicA toxin of *E. coli* K-12 were successfully over-expressed and purified.

2.2. Crystallization

The optimal crystallization conditions were determined by the sitting-drop vapour-diffusion method in 48-well crystallization plates (XtalQuest). The purified wild-type HicAB (WT-HicAB) complex and the two derivatives HicAB^L-(M8I) and HicAB^S were concentrated to 20 mg ml⁻¹ in 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.5 mM EDTA by centrifugal filtration. Drops consisting of 1.0 µl protein solution and 1.0 µl reservoir solution were equilibrated against 100 µl reservoir solution. All crystallization experiments were carried out at 291 K. Eight different test screens were used: The Classics Suite, The Classics II Suite, The JCSG Core I-IV Suites, The Protein Complex Suite and The Nucleix Suite (Qiagen). Clusters of small crystals of the WT-HicAB and HicAB^S complexes were obtained within approximately 3 d using reservoir solutions consisting of 0.1 M Tris-HCl pH 8.0, 15% PEG 4000, 0.15 M ammonium sulfate and of 0.1 M Tris-HCl pH 7.5, 20% PEG 5000 MME, 0.2 M ammonium sulfate, respectively.

Optimization of the crystallization conditions from the initial screening results was performed by the sitting-drop vapour-diffusion method. We tested various precipitants (by replacing the precipitant with other polyethylene glycol species), protein concentrations and pH values, as well as different temperatures and additives. Superior crystals of the short form HicAB^S were only produced from a 1:1 mixture of 20 mg ml⁻¹ protein solution and 0.1 M Tris-HCl pH 8.0, 20%(w/v) PEG 6000, 0.2 M ammonium sulfate at 291 K (Fig. 1). Crystallization information is summarized in Table 2. Before flash-cooling in liquid nitrogen, crystals were harvested into crystallization solution with 25%(v/v) glycerol as a cryo-protectant.

Figure 1
Crystals of HicAB^S obtained by the hanging-drop method at 291 K in 0.1 M Tris-HCl pH 8.0, 20%(w/v) PEG 6000, 0.2 M ammonium sulfate. The scale bar is 100 µm in length.

2.3. Data collection and processing

Diffraction data were collected on beamline 3W1A at Beijing Synchrotron Radiation Facility (BSRF). Crystals were soaked in a 25%(v/v) glycerol solution, which acted as a cryoprotectant, before diffraction and were then flash-vitrified in liquid nitrogen at 100 K. Data were processed using the *HKL-2000* program package (Otwinowski & Minor, 1997). The details of data collection and processing are listed in Table 3.

3. Results and discussion

Initially, the toxin protein HicA and WT-HicAB with a hexahistidine tag at the N-terminus of HicA (Table 1) were separately expressed and purified from *E. coli* Rosetta 2. After crystallization trials, WT-HicAB occasionally produced crystals under certain crystallization conditions, but the crystals were hard to reproduce. The main reason for this appeared to be that the WT-HicAB preparation contained two complex species, HicAB^S and HicAB^L (HicA in complex with HicB^S or HicB^L; Fig. 2), leading to batch-to-batch variation. Moreover, two forms of HicB with lengths of 138 and 145 amino acids were annotated in *E. coli*. They are potentially translated from the same *hicAB* mRNA with two independent but in-frame translational start sites for HicB, and each forms a complex with HicA (Makarova *et al.*, 2006; Jørgensen *et al.*, 2009; Fig. 2). This suggested that the two annotated complexes might correspond to the observed forms in the gel, HicAB^S and HicAB^L, which might exist in *E. coli* as isoforms because of the use of the two alternative start codons or *via* proteolytic

Table 3
Data collection and processing.

Values in parentheses are for the highest resolution shell.	
Diffraction source	3W1A, BSRF
Wavelength (Å)	1.0
Temperature (K)	100
Detector	MAR CCD 165
Crystal-to-detector distance (mm)	180
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	40
Space group	<i>I</i> 222 or <i>I</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 67.04, <i>b</i> = 66.31, <i>c</i> = 120.78, α = β = γ = 90
Mosaicity (°)	0.99
Resolution range (Å)	50.0–2.50
Total No. of reflections	69417
No. of unique reflections	9548
Completeness (%)	100 (100)
Multiplicity	7.3 (7.2)
CC _{1/2}	0.990 (0.962)
⟨ <i>I</i> /σ(<i>I</i>)⟩	27.5 (4.9)
<i>R</i> _{meas}	0.077 (0.382)
Overall <i>B</i> factor from Wilson plot (Å ²)	27.9

processing. Based on the possibility of the two enzyme forms differing in the presence or absence of the N-terminal peptide, HicAB^S and HicAB^L-(M8I) were generated, which allowed us to investigate whether they crystallized differently. HicAB^S and HicAB^L-(M8I) represent two forms of the WT-HicAB complex (HicAB^S and HicAB^L) in which HicA either forms a complex with a 138-amino-acid HicB or a 145-amino-acid HicB with a methionine-to-isoleucine mutation at position 8. All of the recombinant HicAB complexes were successfully purified using the same procedure, *i.e.* sequential purification

Figure 2
(a) Molecular-weight estimation of HicAB complexes and HicA based on size-exclusion chromatography (Superdex 75 10/300, GE Healthcare). WT-HicAB (grey line), HicAB^S (dotted line), HicAB^L-(M8I) (dashed/dotted line) and HicA (grey dashed line) are shown. Molecular-weight standards are indicated to estimate the apparent molecular mass. (b) Analysis of purified WT-HicAB, HicAB^S and HicAB^L-(M8I) on a 4–20% gradient SDS-PAGE gel. HicA, HicB^S and HicB^L are labelled on the left. Lane *M* contains molecular-mass markers (labelled in kDa).

via an Ni-NTA resin column, a Superdex 200 column and a HiTrap SP column, demonstrating that HicA can interact with both forms of HicB (Fig. 2).

Both purified HicA and the HicAB complexes (20 mg ml⁻¹) were subjected to crystallization screening. Only crystals of HicAB^S were readily obtained under several crystallization conditions. After crystallization optimization, crystals suitable for diffraction data collection were grown from an optimized reservoir solution composed of 0.1 M Tris-HCl pH 8.0, 20% (w/v) PEG 6000, 0.2 M ammonium sulfate (Fig. 1). The HicAB crystals were flash-cooled and diffracted to 2.5 Å resolution (Fig. 3). A native data set was collected with 100% completeness. The data were processed with the *HKL-2000* program package, which suggested that the crystals belonged to space group *I*222 or *I*₂12₁2₁, with unit-cell parameters *a* = 67.04, *b* = 66.31, *c* = 120.78 Å, $\alpha = \beta = \gamma = 90^\circ$. Matthews coefficient calculation suggested the presence of two molecules of both HicA and HicB in the asymmetric unit, with a solvent content of 55.28% and a *V*_M of 2.75 Å³ Da⁻¹. The crystallographic parameters and data-collection statistics are shown in Table 3. To evaluate the HicAB complexes in solution, the purified proteins were subjected to analytical gel-filtration chromatography (Superdex 75 10/300, GE Healthcare). The representative elution volumes of 11.4 ml for the HicAB complexes [WT-HicAB, HicAB^S and HicAB^L-(M8I)] and 15.2 ml for HicA correspond to estimated molecular masses of 31.6 and 6 kDa for the HicAB complexes and HicA, respectively (Fig. 2). Based on theoretical molecular masses for HicA and HicB of 6.8 and 15.2 kDa, respectively, these results indicate that HicAB is heterotrimeric or heterotetrameric rather than heterodimeric in solution, while the isolated HicA toxin is monomeric.

Figure 3
X-ray diffraction image of an HicAB^S crystal.

Structure-solution trials by molecular replacement using distant homology models [*Y. pestis* HicA3-HicB3 (PDB entry 4p78; Bibi-Triki *et al.*, 2014) and HicB3 (PDB entry 4p7d; Bibi-Triki *et al.*, 2014) and *Thermus thermophilus* TTHA1913 protein (PDB entry 1whz; RIKEN Structural Genomics/Proteomics Initiative, unpublished work)] were unsuccessful. The structure of TTHA1913 (a hypothetical protein from *T. thermophilus*) is most closely related to that of HicA. Experimental phasing and other molecular-replacement attempts are currently under way. Structure determination of HicAB will help to reveal the overall architecture of the HicAB complex, providing insight into how the toxin HicA is neutralized by its cognate antitoxin HicB and how the antitoxin HicB interacts with the toxin HicA, potentially positioning its DNA-binding domain for effective functioning.

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