Structural insights into the PrpTA toxin–antitoxin system in *Pseudoalteromonas rubra*

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Bacteria could survive stresses by a poorly understood mechanism that contributes to the emergence of bacterial persisters exhibiting multidrug tolerance (MDT). Recently, *Pseudoalteromonas rubra* prpAT module was found to encode a toxin PrpT and corresponding cognate antidote PrpA. In this study, we first reported multiple individual and complex structures of PrpA and PrpT, which uncovered the high-resolution three-dimensional structure of the PrpT:PrpA²:PrpT heterotetramer with the aid of size exclusion chromatography-multi-angle light scattering experiments (SEC-MALS). PrpT:PrpA²:PrpT is composed of a PrpA homodimer and two PrpT monomers which are relatively isolated from each other and from ParE family. The superposition of antitoxin monomer structures from these structures highlighted the flexible C-terminal domain (CTD). A striking conformational change in the CTDs of PrpA homodimer depolymerized from homotetramer was provoked upon PrpT binding, which accounts for the unique PrpT-PrpA[RHH] mutual interactions and further neutralizes the toxin PrpT. PrpA²−54 form I and II crystal structures both contain a doughnut-shaped hexadecamer formed by eight homodimers organized in a cogwheel-like form via inter-dimer interface dominated by salt bridges and hydrogen bonds. Moreover, PrpA tends to exist in solution as a homodimer other than a homotetramer (SEC-MALS) in the absence of flexible CTD. Multiple multi-dimers, tetramer and hexamer included, mediated by the symmetric homodimer interface and the complicated inter-dimer interface could be observed in the solution. SEC-MALS assays highlighted that phosphate buffer (PB) and the increase in the concentration appear to be favorable for the PrpA²−54 oligomerization in the solution. Taken together with previous research, a model of PrpA²−54 homotetramer in complex with prpAT promoter and the improved mechanism underlying how PrpTA controls the plasmid replication were proposed here.

**KEYWORDS**
PrpTA, toxin–antitoxin system, MDT, conformational changes, protein oligomerization, plasmid replication, RHH
Introduction

Drug-resistant bacterial pathogens, such as *Mycobacterium tuberculosis*, *Cryptococcus neoformans*, and *Cryptococcus gattii*, are of significant concern in recent years (Huemer et al., 2020; Tharapel et al., 2022). Multidrug tolerance (MDT) is directly caused by a small fraction of phenotypically variant subpopulation-persister cells, which are substantially dormant and characterized by slow growth rates, high expression of stationary phase markers, reduced protein synthesis, and low DNA replication (Schumacher et al., 2012, 2015; Page and Peti, 2016; Paul et al., 2019; Ma et al., 2021; Xia et al., 2021). MDT provides the basis for the subsequent rapid evolution of resistance and is thought to be the prerequisite for drug resistance and the primary reason for ineradicable bacterial and chronic infections (Fleming, 1929; Cohen et al., 2013; Maisonneuve and Gerdes, 2014; Lee and Lee, 2016; Levin-Reisman et al., 2017; Xie et al., 2018; Paul et al., 2019; Ma et al., 2021). Under endogenous or exogenous stress, the toxin–antitoxin system (TAS) could simultaneously mediate and accelerate the development of persister cells and MDT. Upon the removal of stress, persisters will resuscitate and restore normal growth leading to clinical recurrent infections, especially those caused by biofilms, and subsequent treatment failures (Cohen et al., 2013; Paul et al., 2019; Zhou et al., 2021).

Toxin–antitoxin modules were initially identified as a plasmid stability factor in the conjugative plasmids in the 1980s (Ogura and Hiraga, 1983; Jaffe et al., 1985; Gerdes et al., 1986; Cooper and Heinemann, 2000; Van Melderen, 2010; Ni et al., 2021). Later, they turned out to be widely distributed on the chromosomes and mobile genetic elements (MGEs) in bacteria, archaea, and bacteriophage, especially pathogenic bacteria (Ogura and Hiraga, 1983; Hayes and Van Melderen, 2011; Fraikin et al., 2020; Leroux et al., 2020; Kamruzzaman et al., 2021; Ni et al., 2021; Xue et al., 2022). It is worth noting that MGE is closely related to the genetic stability and formation of persisters through horizontal transfer and vertical transmission (Cooper and Heinemann, 2000; Costa et al., 2001; Schumacher et al., 2012, 2015; Blair et al., 2015; Levin-Reisman et al., 2017; Ni et al., 2021; Xia et al., 2021). However, there is an ongoing controversy concerning the direct link between TAS persistence, and it is believed that at least not all TASs are necessarily related to persistence (Kim and Wood, 2016; Edelmann et al., 2020; Song and Wood, 2020). TAS, directly or indirectly, participates in the regulation of intracellular physiological activities including bacteriophage resistance, antibiotic tolerance, biofilm formation, and response to oxidative stress (Paul et al., 2019, 2022; Bertelsen et al., 2021; Kamruzzaman et al., 2021; Li et al., 2021; Ma et al., 2021; Qi et al., 2021; Xia et al., 2021; Du et al., 2022). Followed by the degradation of the corresponding antitoxin partner, the active toxin component was, thus, released to interact with cellular pathways to activate potentially deleterious toxic activities (Muthuramalingam et al., 2016; Leroux et al., 2020; Sarpang and Murphy, 2021; Xia et al., 2021). Furthermore, the type II TAS, which is studied the most and best, is the most abundant in bacterial MGEs among eight TAS types investigated to date (Xie et al., 2018; Kamruzzaman et al., 2021; Sarpang and Murphy, 2021; Zhang et al., 2021; Xue et al., 2022). For instance, *E. coli* and *S. enterica* TAS from *Edwardsiella piscicida* are thought to be the model organism for the study of intracellular infections and refer to antibiotic resistance and host infection (Ma et al., 2021; Du et al., 2022).

A binary type II TAS consists of a stable metabolic inhibitor—toxin and corresponding labile antitoxin (Muthuramalingam et al., 2016; Leroux et al., 2020; Song and Wood, 2020; De Bruyn et al., 2021; Srivastava et al., 2021; Xia et al., 2021; Zhang et al., 2021; Jurenas et al., 2022). As for the antitoxin serving as a repressor, it is comprised of the N-terminal DNA-binding domain and the C-terminal toxin-neutralizing domain (Bobay et al., 2005; Coles et al., 2005; Xue et al., 2022). Taken together with the strand β1, the helix–turn–helix (HTH) motif is supposed to be expanded into a ribbon–helix–helix (RHH) motif, as found in *CopG/MetJ/Arc* repressor superfamily which includes the plasmid transcriptional repressor prototype SaCopG (PDB: 1EA4) from *Streptococcus agalactiae*, methionine repressor protein EcMetJ (PDB: 1MJK) from *Escherichia coli*, and Arc repressor from *Salmonella* virus P22 (PDB: 1PAR) (Raumann et al., 1994; Garvie and Phillips, 2000; Costa et al., 2001; Schreiter and Drennan, 2007). RHH superfamily transcription factors (TFs) are of physiological importance toward the recognition between human pathogens and hosts (Schreiter and Drennan, 2007). RHH superfamily TFs always dock into the major grooves of duplex nucleic acid as a multi-dimer and further transcriptionally autoregulate (Knight and Sauer, 1989; Raumann et al., 1994; Schreiter and Drennan, 2007; Schumacher et al., 2015; Garcia-Rodriguez et al., 2021). Even though some of them are dimeric in solution, they will instantly build up contacts in the corresponding DNA-bound complex with the exception of *TraY* whose polypeptide has two repeats of the RHH motif (Raumann et al., 1994; Costa et al., 2001; Solar et al., 2002; Schreiter and Drennan, 2007).

Recently, *PrpTA* was found to be a pMBL6842-encoded type II TAS with the unique function to regulate the plasmid replication in *Pseudalteromonas rubra*. It consists of stable *PrpT* toxin and labile *PrpA* antitoxin canonically positioned adjacently within the same operon (Li et al., 2016; Ni et al., 2021). So far, there are few reports referring to the structural details of type II TAS systems in marine bacteria. In this study, we reported for the first time the high-resolution structure of the *PrpT*:PrpA<sub>2</sub>:PrpA<sub>2</sub> heterotetramer and two forms of crystal structures of truncated antitoxin-PrpA<sub>2</sub>. Moreover, a series of size exclusion chromatography-multi-angle light scattering experiments (SEC-MLSA) assays were performed to investigate the oligomeric states of the *PrpTA* system. Our
results reflected that the α3 helices of PrpA will bend obviously toward the PrpA$^{\text{RHH}}$ domains of homodimer depolymerized from homotetramer upon PrpT binding, which consequently leads to the extra mutual interactions between toxin PrpT and N-terminal PrpA$^{\text{RHH}}$ domains. PrpT together with PrpA finally forms a stable PrpT:PrpA$_2$:PrpT heterotetramer, thus restricting the flexibility of PrpA$_{\text{CTD}}$ and neutralizing the toxin PrpT. Furthermore, PrpA$_2$-54-form I and II crystallize as a doughnut-shaped hexadecamer formed by eight vicinal homodimers via an inter-dimer interface dominated by salt bridges and hydrogen bonds. The incomplete hexadecameric rings, tetramer and hexamer included, could be observed in the solution presumably due to the unstructured C-terminal domain (CTD) that is absent in the crystallized entity like V. cholerae ParD2. It seems that the oligomerization of PrpA$_2$-54 is partially affected by protein concentration and solution conditions. Moreover, a knowledge-based model for PrpA$_2$-54 tetramer-PrpAT promoter was proposed and discussed here. Overall, the PrpTA system assembly mechanism, antitoxin PrpA oligomerization, and the structural details of the mechanism underlying how PrpTA TAS controls plasmid replication could be further elucidated or improved.

Materials and methods

Protein expression and purification

All vectors were transformed into Rosetta (DE3) competent cells using the heat shock method. A single colony was inoculated in Luria-Bertani (LB) medium supplemented with the corresponding antibiotic, such as ampicillin (100 μg/ml) and kanamycin (50 μg/ml) at 310.15 K (37°C) and 220 rpm. Protein expression was induced with 1 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) once the bacterial growth reached OD$_{600}$ of 0.8 and further incubated at 289.15 K (16°C) for 20 h. Bacterial cells were harvested by centrifugation at 8,000 rpm for 6 min and 277.15 K (4°C), followed by resuspension in 0.3 M NaCl and 0.05 M Tris pH 8.0, and further lysed with ultrasonicator (Qsonica; USA) in an ice bath. After centrifugation at 12,000 rpm for 30 min and 277.15 K (4°C), the protein was purified from the supernatant utilizing Ni-NTA resin (GE Healthcare: USA), followed by Superdex 75 pg column (HiLoad$^{\text{TM}}$ 16/600, GE Healthcare; USA). The purity and concentration of the proteins were assessed with SDS-PAGE and OneDrop$^{\text{TM}}$ OD-1000 + spectrophotometer (WINS; China), respectively. In addition, antitoxin PrpA$_{\text{FL}}$ (expressed by vector PrpA-N-his-pET28a) carries a 6 x his tag on the N-terminus after being translated, and PrpA$_{254}$ (expressed by vector PrpA$_2$-54-C-his-pET28a) carries a 6 x his tag on the C-terminus. Toxin PrpT [expressed by vector PrpT-pET22b(+)]] carries no tag. Their recombinant sequences could be accessed in Supplementary Table 1. The theoretical relative molecular weight of PrpT, PrpA$_{\text{FL}}$ with N-terhexahistidine, and PrpA$_2$-54 with C-terhexahistidine is ~11.4, ~11.66, and ~7.05 kDa, respectively. Moreover, all vectors and oligonucleotide fragments used in the current study were obtained from Sangon Biotech Co., Ltd (Shanghai, China).

Crystallization

The finely purified PrpA$_2$-54 and PrpTA were concentrated to ~5.2 and ~3.6 mg/ml, respectively, in 0.1 M NaCl with 0.05 M Tris (pH 8.0). Initial crystal screening was performed by mixing an equal volume of protein and reservoir solution utilizing the sitting drop vapor diffusion method at 289.15 K (16°C). Two days post-crystallization, the crystal of PrpA$_2$-54-form I was observed in a condition containing 0.2 M sodium acetate trihydrate, 0.1 M sodium citrate (pH 5.5), and 5% (w/v) PEG 4000. Similarly, crystals for PrpA$_2$-54-form II were obtained in 0.2 M magnesium chloride hexahydrate, and crystals for PrpTA complex were obtained in 0.1 M HEPES sodium at pH 7.5, 30% (v/v) PEG 400, 0.1 M sodium cacodylate (pH 6.0), and 15% (w/v) PEG 4000.

Data collection and structure determination

Crystals were picked with nylon loops and cryoprotected in a reservoir solution supplemented with 20% glycerol. All the diffraction datasets were collected at the beamlines in Shanghai Synchrotron Radiation Facility (SSRF) utilizing the single-wavelength small-angle oscillation method. All datasets were initially indexed and integrated with XDS, followed by scaling with an aimless module integrated into CCP4i (v7.1). The initial structural model of PrpA$_2$-54 was determined by Phaser in PHENIX (v1.19.2) with VcParD2 antitoxin (PDB: 7B22) as a search template utilizing molecular replacement method, followed by model building with Autobuild module in PHENIX. Similarly, the structure of the PrpTA complex was searched against the refined structure of the PrpA$_2$-54 monomer and Caulobacter vibrioides ParE toxin monomer. The structures were iteratively refined using Refmac5, WinCoot (v0.9.6), and phenix.refine offered by PHENIX. The detailed statistical information about these datasets is summarized in Supplementary Table 2.

SEC-multi-angle light scattering experiments

SEC-multi-angle light scattering experiments were conducted using an AKTATM pure HPLC system (GE Healthcare, USA) connected in-line with a DAWN HELEOS
II (Wyatt Technology, Santa Barbara, CA, USA) eight-angle light-scattering detector, followed by a refractive-index detector (Wyatt Technology, Santa Barbara, CA, USA). SEC-MALS system was equilibrated with the corresponding running buffer at 0.5 mL/min for 12 h prior to the sample loading. A series of PrpA, PrpA$^{2−54}$, and PrpTA samples were prepared in several kinds of buffers (including 50 mmol/L tris–HCl and 100 mmol/L NaCl pH 8.0, 50 mmol/L tris–HCl and a300 mmol/L NaCl pH 8.0, 50 mmol/L tris–HCl and 500 mmol/L NaCl pH 8.0, 50 mmol/L MES and 500 mmol/L NaCl pH 5.5, and 100 Mm (NaH$_2$PO$_4$/Na$_2$HPO$_4$) pH 8.0), and 0.1 mL was injected into the loop for each dilution. Accordingly, the absolute molar mass of each protein sample could be determined based on the data processed by the ASTRA (v7.0.1) offered by Wyatt company.

Circular-dichroism spectroscopy

Circular-dichroism spectra were recorded at room temperature using a Chirascan™ qCD Spectrometer (Applied Photophysics Limited, UK) at a concentration of 0.4 mg/mL in 100 Mm (NaH$_2$PO$_4$/Na$_2$HPO$_4$) pH 8.0 based on 0.5 mm optical path. For each CD experiment, the background was measured one time, the buffer was measured two times, and samples were measured three times to reduce error and noise. All data were acquired with the subsequent parameters, bandwidth: 1 nm, wavelength scanning range: 180–260 nm, and time per point: 0.5. Pro-Data (v4.5.1825.0), and BeStSel were used to view and process CD spectra data to consequently access information on secondary structure composition.

Modeling for PrpA$^{2−54}$ tetramer complex with prpAT promoter

Sequence-specific interactions between antitoxin PrpA and duplex oligonucleotide are mostly mediated by N-terminal RHH domains of PrpA via recognizing the conserved 5′- (G/A)TTTG(T/A)AT(A/G)-3′ motif which could be pseudopalindromic or asymmetric (Ni et al., 2021). Based on the structure of transcriptional repressor CopG in complex with 22 bp dsDNA [PDB: 1EA4, which revealed a tetramer consisting of two dimers and associated by a crystallographic dyad, interacting in the same way as two dimers in the unliganded structure (Del Solar et al., 1989; Gomis-Ruth et al., 1998; Costa et al., 2001)], a possible dsDNA-RHH interaction mode was generated by NUCBIND. Combined with the structure of PrpA$^{2−54}$ antitoxin homotetramer, a knowledge-based model of PrpA$^{2−54}$ homotetramer in complex with prpAT promoter was proposed and later improved by energy minimization refinement and water refinement successively using refinement module offered by HADDOCK 2.4 with default parameters. The iteratively refined model was finally validated by the PISA server.

Results

PrpTA complex exists as a heterotetramer in solution and contains a PrpA homodimer depolymerized from homotetramer

To investigate the assembly mechanism of the PrpTA system in solution, a series of SEC-MALS experiments were performed on purified PrpTA complex, PrpA$^{FL}$, and PrpA$^{2−54}$ samples in this study (Figure 1A and Supplementary Figures 1, 2). Moreover, we succeeded in crystallizing PrpTA but failed to crystallize PrpA$^{FL}$ due to the flexibility in the CTD reported before (Ni et al., 2021). However, we were lucky to crystallize truncated PrpA$^{FL}$ (PrpA$^{2−54}$) and finally solved two forms of PrpA$^{2−54}$ crystal structures and the high-resolution PrpTA crystal structure (Figure 1B and Supplementary Figure 3). Considering the theoretical relative molecular weight of PrpT ($\sim$11.4 kDa) and PrpA ($\sim$11.66 kDa), our SEC-MALS results reflected that PrpTA complex, PrpA$^{FL}$ and PrpA$^{2−54}$ present an estimated absolute mass matching tetramer, tetramer and dimer, respectively. These results highlighted that deletion of PrpA$^{CTD}$ (55–86 residues) could mediate the change in the oligomerization states of PrpA$^{FL}$ from tetramer to dimer, suggesting the importance of CTD for the PrpA$^{FL}$ homotetramer. Furthermore, it seems that the homodimer is actually the minimal assembly unit of PrpA$^{2−54}$ in the solution. Overall with the crystal structure of the PrpTA complex, it was certain that the PrpTA biological assembly unit indicated with PrpT:PrpA$_2$:PrpT (Figure 1Ba) contains two PrpT monomers (Figure 1Bb), which were isolated from each other, and a PrpA homodimer (Figure 1Bd). In other words, PrpT:PrpA$_2$:PrpT heterotetramer is maintained by one single PrpA$^{FL}$ homodimer interface formed by N-terminal PrpA$^{RHH}$ domains and two PrpT-PrpA$^{FL}$ heterodimer interfaces, which is similar to its structurally relevant complex, such as CvParDE, and consistent with the conclusion that PrpA dimer interface and PrpT-PrpA heterodimer interface with an estimated CSS (Complex Formation Significance Score, calculated by PISA server) value of 1 contribute to PrpTA heterotetramer complexation. To sum up, the PrpTA complex exists as the PrpT:PrpA$_2$:PrpT heterotetramer in solution and consists of two toxin monomers together with an antitoxin PrpA homodimer which appears to be depolymerized from homotetramer and the minimal functional unit of PrpA$^{FL}$.

Toxin PrpT belongs to the ParE family

PrpT toxin starts from a RHH fragment in the N-terminus, helices of which are connected by a single glycine residue, and the $\beta$ tends to be packed with the EF-P OB-like fold that is mainly composed of C-terminal 3-stranded antiparallel $\beta$ sheet
FIGURE 1
PrpT-PrpA$_2$-PrpT heterotetramer consists of a PrpA homodimer and two PrpT monomers isolated from each other. (A) PrpTA complex (red), PrpA (blue), and PrpA$_2$-54 (black) present an estimated absolute molecular weight matching tetramer, tetramer, and dimer, respectively. (B) Two views of PrpTA heterotetramer cartoon presentation (a). Cartoon representations of PrpT toxin monomer (b), PrpA$_{FL}$ antitoxin monomer (c), and PrpA$_{FL}$ homodimer (d) (The black solid ellipse represents the local twofold axis, and all secondary structure elements are defined by DSSP plugin in PyMol).

$\beta_3$–$\beta_5$), as shown in Figure 1Bb. To determine accurately the protein family of PrpT, the sequence alignment, phylogenetic analysis, and structural superposition analysis with its homologs were performed. The sequence alignment and phylogenetic tree reflected that PrpT and its sequence homologs could be divided into three subgroups (Supplementary Figures 4A,B). In addition to the residue Leu that is highly conserved in the $\alpha_1$ helix of all known structures, a conservative (Gln/Gly)-Gly diad is specific to members in group 1, that is, ParE family. Accordingly, the superposition of all solved structures of toxin proteins from the sequence alignment above could also be divided into three subgroups based on their structure deviations with toxin PrpT (Figure 2Aa). The crystal structure of PrpT is similar to structures of ParE family members with an average estimated r.m.s.d. of 0.855 Å and the main difference lies in the conformation of $\alpha_2$–$\beta_3$ and $\beta_4$–$\beta_5$ loops, which might play a key role in adjusting and maintaining the conservative three-dimensional structure (Figure 2Ab). However, structures from group 2 or group 3 could be distinguished apparently from PrpT due to large structure deviations mainly resulting from the flexibility of loops and the length differences of $\alpha_1$ and $\alpha_2$ with corresponding helices in PrpT (Figure 2Ac). Therefore, the toxin PrpT could be assigned to the ParE family.
PrpAFL monomer structure has a novel three-dimensional folding

As illustrated in Figure 1Bc, PrpAFL begins with an extended conformation (β1) spanning from 5 to 9 aa, followed by the α1 helix (11–23 aa) and the α2 helix (30–45 aa). The C-terminal PrpT neutralizing domain (49–85 aa), which is also the tetramerization domain, is connected with the C-terminus of the α2 helix by a hinge region composed of AGS triad [Figure 2B (upper)]. Due to the presence of glycine residue in the N-terminus of α1 helix, the centroidal axis is rotated ~70° in relation to helix α2. For a similar reason, the direction...
of the helix α1 is actually rotated ∼80° with respect to the strand β1 (Supplementary Figure 5c). Compared to the ∼47.9% helix content of PrpAFL in solution [Figure 2B (bottom)], the total helix content of PrpAFL in the PrpTA complex is ∼55.7%. Higher helix content seems to hint at a conformational change occurring in the PrpA CTD upon PrpT binding. The DALI topological analysis revealed not only the C-terminal diversity of CopG/MetJ/Arc family with the conservative RHH motif but also the structure uniqueness of PrpAFL. Despite the higher sequence similarity, PrpAFL exhibits a difference in the secondary structural elements (α2–α3 region) with its homologs and obvious structural deviation in the backbone track of CTD (Figure 2C). Even though VcParD2 (PDB: 7B22) and EcParD (PDB: 5CZE) exhibit structural similarity to a different degree with the N-terminal PrpA RHH domain (r.m.s.d. = 1.56 Å) and the PrpA CTD (r.m.s.d. = 4.41 Å), respectively, the structure of PrpAFL is still unique compared to structures already deposited in PDB together with the structure predicted by AlphaFold (r.m.s.d. = 5.41 Å). In summary, the folding of PrpAFL is definitely novel and distinct from any other structures deposited in PDB.

N-terminal 2-stranded antiparallel β-sheet located in ribbon–helix–helix domain is the oligomerization basis of PrpT:PrpA2:PrpT and PrpA

To investigate the structural details of PrpT:PrpA2:PrpT, crystal structures of the PrpTA complex and PrpA2−54 were all carefully analyzed. Like other members from the RHH family, PrpA monomer polymerizes in solutions and crystals in a highly symmetrical manner via a local twofold axis to form a two-stranded antiparallel β-sheet (Figure 3A). The symmetric dimerization interface could be found in PrpT:PrpA2:PrpT (excluding residue Ser3B) or either form of PrpA2−54 crystal structures (Figures 3A,B). Moreover, the comparative structural analysis highlighted that PrpA2−54 dimers are similar to each other with an r.m.s.d. value of ∼1.38 Å over 96 residues (Supplementary Figure 3). Thus, only the interface that existed in PrpA2−54-form II will be described in detail here. PISA analysis reflected that the hydrogen bond/salt bridge interaction network of dimerization
interface is primarily offered by Met6, Val8, Asp9, Ser31, and Arg35 from each monomer together with Arg15A/B, Thr10A/B, Arg54A, Arg4B, and Asp26B for the PrpA\(^2\)\(^{−54}\) (or symmetric Thr7A/B, Glu13A/B and asymmetric Ser3B for PrpA\(^{FL}\) in PrpTA complex). The dimerization interface in PrpTA or PrpA\(^2\)\(^{−54}\)-form II buries roughly 1700 Å\(^2\) with the nearly identical negative ΔG value (\(\sim\)24 kcal/mol) corresponding to hydrophobic interfaces. It is presumably because bonds are broken by even small shifts in distance or orientation caused by the tag that interface symmetry of terminus that carries the tag of PrpA\(^{FL}\) or PrpA\(^2\)\(^{−54}\) is always worse than that of the other one. In addition, a number of hydrophobic interactions between α helices could also partly contribute to the formation of a stable PrpA\(^{FL}\) homodimer. Crystal structures of PrpT:PrpA\(^2\)\(^{−54}\):PrpT heterotetramer complex and PrpA\(^2\)\(^{−54}\) suggested the significance of RHH dimer for the PrpT-PrpA recognition and the formation of PrpA\(^{FL}\) multi-dimer. Overall with LigPlus analysis (Supplementary Figure 6), it is rational to assert that the 2-stranded antiparallel β-sheet formed by two adjacent ribbons spanning from 5 to 9 aa is the basis of oligomerization of the PrpTA system.

**Flexibility-to-stability transition of PrpA\(^{CTD}\) upon the PrpT binding**

To investigate whether PrpA\(^{CTD}\) undergoes a conformational transition upon toxin PrpT binding, structural superposition of PrpA\(^{2\sim54}\) monomers with PrpA\(^{FL}\) was conducted. As demonstrated in Figure 4A, the results uncovered the obvious orientation change of α3 upon PrpT binding, which is consistent with the flexibility feature of the PrpA\(^{CTD}\). Except for the asymmetric Arg54A-ASP26B contact in PrpA\(^2\)\(^{−54}\), the protein backbones of PrpA\(^2\)\(^{−54}\) or PrpA\(^{FL}\) project away from the dimer interface without further intra- or inter-contacts, suggesting the flexibility of PrpA\(^{CTD}\) (Figure 3B). That is, the PrpT binding could result in the dramatic flexibility-to-stability transition of PrpA\(^{CTD}\), which provokes the de-tetramerization of PrpA\(^{FL}\) antitoxin and the rotation of PrpA\(^{CTD}\) by \(\sim\)80° around the AGS residue triad, further leading to the extra interaction between toxin PrpT and RHH domains of PrpA homodimer (Figure 4B). PrpT:PrpA\(^2\)\(^{−54}\):PrpT heterotetramer is formed via a local twofold axis and highlights two extremely similar PrpT-PrpA\(^{FL}\) contact interfaces. Therefore, only

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**FIGURE 4**

Conformational change in the PrpA\(^{CTD}\) triggered by toxin PrpT binding. (A) Cartoon representation of structural superposition of PrpA\(^{FL}\) monomer with PrpA\(^2\)\(^{−54}\) monomers (left). The magnified version of α3 helices is highlighted in the right panel (The centroid axis of each helix fragment is shown as cylinder utilizing UCSF Chimera software suite). (B) The proposed model of conformational change in the PrpA\(^{CTD}\) upon toxin PrpT binding. (C) The cartoon representation of the PrpT-PrpA heterodimer contact interface. Subdomains of PrpT are enclosed with black-dotted lines and labeled in red.
one heterodimer interface will be discussed here. PrpA and PrpT interact via two hydrophobic subdomains surrounded by electrostatic interactions (Supplementary Figures 5, 7). The α3 and α4 make up the core PrpT binding region, the surface polarities of which are exactly opposite to the two subdomains of PrpT, which are mainly comprised of N-terminal ribbon–helix-helix and the C-terminal 4-stranded anti-parallel β-sheet (∝2–∝5), respectively (Figure 4C). The heterodimer interface dominated by a series of salt bridges/hydrogen bonds covers a surface of ~2000 Å² with an estimated ΔG value of −17 kcal/mol. Even though the number of extra contacts between PrpT and PrpA_RHH accounts for just ~20% of all PrpT-PrpA_IF interactions according to PISA, they are deemed to limit the swinging ability of PrpA_CTD in the local space, thus further enhancing the stability of PrpT:PrpA2:PrpT heterotetramer. Together, we infer that the conformational changes in the PrpA_CTD are significant for neutralizing the toxin.

Furthermore, compared with relevant toxin–antitoxin complexes reported before, as demonstrated in Figure 5, PrpT-PrpA2:PrpT heterotetramer is the only toxin–antitoxin complex whose toxin monomer could simultaneously interact with the CTD and the NTDs of the antitoxin homodimer, thus neutralizing the toxin. A similar strategy is also found in MtRelBE2 (Figure 5F), of which adjacent α2–α3 loop could interact with each other limiting the flexibility of CTD, meanwhile, blocking the contacts between antitoxin

![Comparison of the inter-molecular contact patterns in the toxin–antitoxin complexes with relevant structures. The PrpT toxin monomer in PrpT-PrpA2:PrpT heterotetramer could simultaneously interact with one CTD and both NTDs of the PrpA2 homodimer after the CTDs undergo an obvious spatial change. This interaction mode is thought to enhance the stability of CTD of each PrpA2 monomer by restricting its swinging ability in the local space and, therefore, further makes the heterotetramer much more stable neutralizing PrpT toxin.](image-url)
NTD and toxin monomer. In brief, the PrpA dimerization interface, PrpA^{CTD}-PrpT contacts, and PrpA^{RHH}-PrpT contacts all contribute to PrpTA oligomerization. In actual fact, except for EcParDE (Figure 5E) and PhRelBE (Figure 5G) whose oligomerization is maintained by toxin–toxin contacts that are actually absent in PrpT-PrpA_{2-PrpT} heterotetramer, the oligomerization of CvParDE (Figure 5B), PaParDE (Figure 5C), MoParDE (Figure 5D), MtRelBE (Figure 5F), and MjRelBE (Figure 5H) all only depend on their NTDs, especially the N-terminal antiparallel β sheet. To sum up, the PrpT-PrpA_{2-PrpT} heterotetramer displays a novel interaction profile between toxin and antitoxin, which contributes to the stability of the PrpTA complex.

![Image](https://example.com/image.png)

**Figure 6**

All monomers in PrpA_{2-Prp}^5 homotetramer participate in tetramerization and have much more extensive contacts. (A) A view of the cartoon representation of the PrpA_{2-Prp}^5 homotetramer, and the relevant residues in the dimer–dimer interaction interface are shown as cyan stick (chain C and C#) or blue stick (chain A and D) and labeled in black. The dimer–dimer interaction interface is magnified in the right panel (Hydrogen bonds or salt bridges are indicated by the short black-dotted lines and their distances labeled in black). (B) Cartoon representation of the VcParD2 tetramer (upper, PDB ID:7B22) and SaCopG tetramer (bottom, PDB ID:1B01) (Residues involved in the inter-dimer interface are highlighted by the same color as the corresponding chain). (C) The superposition of inter-dimer interface of VcParD2 tetramer (gray sticks) and PrpA_{2-Prp}^5 inter-dimer (red sticks) (Residues presented with lines in corresponding color actually do not participate in the tetramerization and are only used for comparison).
Phosphate buffer (PB) and the increase in protein concentration contribute to PrpA$_{2-54}$ oligomerization. (A) Size exclusion chromatography-multi-angle light scattering experiments (SEC-MALS) assays are conducted in Tris–HCl buffers with different pH and ion strength. (B) Two forms of PrpA$_{2-54}$ could assemble into a higher-order hexadecamer in crystal. The maximum diameters of a PrpA$_{2-54}$ hexadecamer and the hole located in the center are $\sim 80.9$ and $\sim 22.4\AA$, respectively. The 2-stranded antiparallel $\beta$-sheets of adjacent PrpA$_{2-54}$ homodimers produce an arc of $-\pi/4$. The ability of RHH motif dimerization and the highly symmetric inter-dimer interface is the basis of the formation of multi-dimer unit.

PrpA is stable

Structural insights into the molecular mechanism underlying how PrpTA TAS controls plasmid replication. The schematic diagram presented above is drawn based on the model proposed by Ni et al. (2021).
Dimer–dimer interface in PrpA<sup>2−54</sup> multi-dimer possesses more extensive inter-monomer contacts

Similar to the arrangement of CopG (PDB: 2CPG) (Gomis-Ruth et al., 1998; Costa et al., 2001) monomers in the asymmetric unit (AU), PrpA<sup>2−54</sup>-form I monomers are arranged in a cogwheel-like form in the AU [Supplementary Figure 3 (right)]. Apart from a type A-D dimer, either B or C could further dimerize with its symmetric mate generating type B-B# and type C-C# dimers (# represents symmetry mate). Since the dimer–dimer interfaces in AD-BB# and AD-CC# are similar to each other with an overall r.m.s.d. of 1.05 over 144 residues, only the AD-CC# interface will be, thus, discussed at length here.

As demonstrated in Figure 6A (left), a functional PrpA<sup>2−54</sup> tetramer is defined by inter-dimer crystallographic contacts between RHH domains with positively charged DNA-binding surfaces exposed to the solvent environment. The AD and CC# interact via a surface that is electrostatically complementary and somewhat slightly hydrophobic, especially the interface formed by α2 helices of molecule AD together with almost equivalent parts of a molecule CC# [Figure 6A (right)]. In addition, the interface generated by AD and CC# dimers covers a total surface area of ~1100 Å<sup>2</sup>, which is a little smaller than that of the dimerization interface. The inter-dimer interface of PrpA<sup>2−54</sup> is thought to be strong enough since it can still exist as a multi-dimer in certain cases in the absence of PrpA<sup>CTD</sup> (Figure 7A). A similar dimer–dimer interface dominated by hydrogen bonds and salt bridges was also reported in VcParD2 tetramer [Figure 6B (upper)], especially the symmetric residues E32, R35, and R39 (matching E31, R34, and R38 in SaCopG, respectively) are extremely conserved in primary and tertiary structures. However, they are all totally different with inter-dimer interface dominated by hydrophobic van de Waals interaction that existed in SaCopG tetramer bound to DNA [Figure 6B (bottom)]. However, the inter-dimer interface of PrpA<sup>2−54</sup> displays a novel feature that all monomers could participate in the formation of tetramer suggesting a much more extensive and strong contact. In contrast, few residues from one single monomer from each dimer, that is, only two chains mediate the corresponding inter-dimer interface for SaCopG and VcParD2. The difference in the inter-dimer interface presumably lies in the different conformations caused by residue substitutions, such as Asp9, Glu43, and Lys28 (corresponding to Thr8, Gly27, and Asn42 in VcParD, respectively), which allows much more complex contacts between PrpA<sup>2−54</sup> monomers (Figure 6C). Moreover, an unfavorable positive-positive interaction is abolished by Gly47 substitution matching the Lys46 in VcParD. In general,
the dimer–dimer interface in PrpA2−54 multi-dimer possesses much more extensive inter-monomer contacts.

An increase in the protein concentration and phosphate buffer contributes to the PrpA2−54 oligomerization

A circular hexadecamer (≈112.58 kDa) consisting of 8 PrpA2−54 dimers could be found in both forms of PrpA2−54 crystals (Figure 7B). Higher-order form of PrpA2−54 is maintained by a dimer interface and inter-dimer interface. The maximum diameter of the annular doughnut-like hexadecamer is ≈80 Å and that of the hole in the center is approximately 22 Å. The C-terminal α2 helix of each PrpA2−54 monomer stretches outward from the inner side of the hexadecamer; overall with the flexibility of CTD, it is reasonable to infer that the replacement of PrpAFL would provide an entropic penalty for oligomerization, which is similar to the effect made by the IDR of VcParD2. To further investigate which factors could mediate the oligomerization of PrpA2−54, a series of SEC-MALS experiments were performed (Figure 7A). PrpA2−54 tends to present an absolute molecular weight matching PrpA2−54 dimer in most cases, and no clear dependency on the ion strength or pH of solutions could be observed. However, the increase in protein concentration within a limited range seems to result in a higher absolute molecular weight (41.38–42.41 kDa, probably hexamer). In addition, the hexadecamer could be occasionally observed in solution with an extremely smaller proportion (≈4%). The PrpA2−54 has a tendency to assemble into a relatively stable homotetramer in phosphate buffer (PB), which is distinct from its performance in Tris–HCl buffers. Taken together, the increase in the protein concentration and PB are favorable for the oligomerization of PrpA2−54 in the solution.

Conclusion and discussion

Toxins from the ParE/RelE family, PrpT included, share higher sequence and structural similarities with one another (Garcia-Rodriguez et al., 2021a; Klemencic et al., 2021; Ni et al., 2021; Zhou et al., 2021a). In contrast, antitoxins from the RHH superfamily could be different from one another in sequence with diverse CTD conformations (Raumann et al., 1994; Costa et al., 2001; Weihofen et al., 2006; Schreiter and Drennan, 2007). The antitoxin PrpAFL with an extremely conservative N-terminal RHH motif exists as a homotetramer in solution; however, PrpAFL tends to exist as the minimal functional homodimer other than homotetramer in the absence of PrpA2CTD. The symmetric homodimer interface and the inter-dimer interface with much more complicated inter-monomer contacts mediate PrpA2−54 homotetramer and homohexamer in solution and appear to be a potential prerequisite for assembling into a circular hexadecamer. PB and the increase in concentration will have a small impact on the oligomerization of PrpA2−54. In addition, comparative analysis of PrpAFL with PrpA2−54 monomers in different oligomeric states reflected that the PrpA2CTD is relatively flexible, which is presumably concerned mainly with the inconsecutive α2–α3 helix and little contacts between CTDs of PrpAFL homodimer. However, the relatively flexible PrpA2CTD could become stable upon PrpT binding, which might be involved in the extra contacts between toxin PrpT and PrpA2CTD domains caused by the conformational change in PrpA2CTD.

Overall with the structural evidence in our study, the model proposed before for the molecular mechanism [proposed by Ni et al. (2021)] underlying how PrpT/PrpA system controls plasmid replication could be further improved and the updated schematic diagram is shown in Figure 8. When PrpA is intact, antitoxin PrpA tends to exist as a functional homotetramer defined by the PrpA2RHH, PrpA2RHH contacts and unknown PrpA2CTD-PrpA2CTD contacts between PrpAFL homodimer. The flexible CTDs of PrpA2CTD are supposed to swing in a small local space, especially those in PrpAFL homodimer. After the PrpA2CTD comes across the cognate toxin PrpT, the PrpAFL homotetramer depolymerized into two isolated homodimers establishing strong interactions with toxin PrpT monomers, which results in the formation of PrpT:PrpA2:PrpT heterotetramer and the neutralization of PrpT toxicity. In other words, the PrpAFL homodimer tends to neutralize toxin PrpT in preference to binding to another PrpAFL homodimer, and the homodimer seems to be the most unstable form due to free CTDs. The binding of PrpT to the PrpA2CTD actually results in an obvious conformational change, rotating the PrpA2CTD nearly 80° in relation to AGS triad to establish extra PrpT:PrpA2RHH mutual interactions, further stabilizing PrpT:PrpA2:PrpT heterotetramer. Meanwhile, PrpA homotetramer alone and PrpT:PrpA2:PrpT heterotetramer could bind to the prpAT operon, resulting in the transcriptional inhibition of the PrpTA module. In addition, PrpA could also competitively bind to the tetron sequences in the ori, interfering with the binding of replication initiator RepB to the ori site, thus preventing the overrepllication of the plasmid. In contrast, PrpA is degraded during the stress condition, thus abolishing the inhibition of the RepB binding to ori (Ni et al., 2021).

Due to the high symmetry of the PrpA homodimer interface and inter-dimer interface, and structural features of members from the RHH family, multiple multi-dimeric (in complex with dsDNA) structures of PrpA2−54 could be predicted. In this study, a model of PrpA2−54 in complex with promoter dsDNA was modeled to investigate possible interaction profiles between PrpA2−54 and dsDNA. The model highlighted that antiparallel β-sheets of PrpA2−54 tetramer could establish interactions with the duplex prpAT promoter [Figure 9A (upper)]. Sequence-specific and non-specific contacts could be mediated mainly by β-sheets...
and helices, respectively, with N-terminal, the 2-stranded antiparallel β-sheet of PrpA<sup>2−54</sup> docking favorably into the major grooves of prpAT promoter, thus inducing dsDNA to bend obviously to interact better with positively charged DNA-binding surfaces of PrpA<sup>2−54</sup> [Figures 9A (bottom), B]. Compared to an ideal type B dsDNA, the negatively charged phosphodiester backbone of the promoter bent more than 30° due to compression of both major and minor grooves facing the tetramer. Bent dsDNA accounts for the base pairs adjacent to the center of the promoter being somewhat inclined; however, the phosphodiester backbone of the promoter excluding the central part still stretches along a flat track. In contrast, TFs such as CopG, Arc, and MetJ could bend the minimal cognate operator with two repressor binding sites by 40° to 60°, which is a bit higher than that of PrpA<sup>2−54</sup> and the discrepancy seems to result mainly from the varying spacer between DNA-binding sites (Raumann et al., 1994; Gomis-Ruth et al., 1998; Garvie and Phillips, 2000; Welshofen et al., 2006). In addition, CopG and MetJ could recognize the pseudo- or palindromic sites, however, Arc does not. Another thing is that most of the sidechains situated in the corresponding positions of the β-sheets in Arc, MetJ, and CopG make contacts with different operator base positions (Raumann et al., 1994), which indicates that the N-terminal β-sheet is the DNA-binding recognition element, such as the second α helix of HTH motif. Although PrpA and Arc all undergo conformational changes, it is the PrpA<sup>CTD</sup> that would suffer dramatic conformational changes upon the corepressor binding, which is not the same case with Arc whose N-terminal β-sheets do undergo conformational changes. These results are consistent with the conclusion that the RHH superfamily could recognize and bind nucleotides in the major grooves of duplex DNA by changing the conformation of β-sheet, sugar-phosphodiester backbone track, and sequence identity together with fine-tuned sidechains to build specific or unspecific contacts (Raumann et al., 1994; Gomis-Ruth et al., 1998; Costa et al., 2001; Schreiter and Drennan, 2007). However, due to the flexibility in PrpA<sup>CTD</sup>, it is difficult to accurately predict the model of PrpA<sup>FL</sup> homotetramer in complex with duplex DNA. Based on our structural insights into the PrpTA system, we speculated that CTDs in PrpA<sup>FL</sup> homotetramer appear to feature with (a) solvent-exposed PrpT binding surface; (b) weaker contact interface than PrpA<sup>RHH</sup>-PrpA<sup>RHH</sup> interface and PrpT-PrpA interface; and (c) some flexibility to arrest toxin PrpT. The interaction mode found in DNA-bound TraM (PDB: 3ON0) from <i>Escherichia coli</i> appears to satisfy all hypotheses mentioned above. In summary, our results are supposed to contribute to elucidating the PrpTA complex assembly mechanism, protein oligomerization, and the mechanism underlying how PrpTA TAS controls plasmid replication, which may help to understand the emergence of drug-resistant bacteria and MDT via a similar mechanism.

**Data availability statement**

The data presented in this study are deposited in the Protein Data Bank (PDB) repository, and the accession numbers are 7YCU, 7YCV, and 7YCW (https://www.rcsb.org/).

**Author contributions**

LN, ZZ, and CW conceived the idea and designed the frame of this study. CW and CN performed the experiments and analyzed the data. CW wrote the manuscript. KH and LX revised the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Supplementary material**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1053255/full#supplementary-material
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