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c-di-GMP regulates RNA chaperone activity of PlzA in the Lyme disease spirochete

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Abstract

PlzA is a c-di-GMP-binding protein crucial for adaptation of the Lyme disease spirochete *Borrelia* (*Borreliella*) *burgdorferi* during its enzootic life cycle. Unliganded *apo*-PlzA is important for vertebrate infection, while liganded *holo*-PlzA is important for tick acquisition; however, the biological function of PlzA has remained enigmatic. Here we report that PlzA has RNA chaperone activity that is inhibited by c-di-GMP binding. *Holo*- and *apo*-PlzA bind RNA and accelerate RNA annealing, while only *apo*-PlzA can strand displace and unwind double-stranded RNA. Guided by the crystal structure of PlzA, we identified several key aromatic amino acids protruding from the *N*- and *C*-terminal domains that are required for RNA binding and unwinding activity. Our findings illuminate c-di-GMP as a switch controlling the RNA chaperone activity of PlzA and we propose that complex RNA-mediated modulatory mechanisms allow PlzA to regulate gene expression during both the vector and host phases of the *B. burgdorferi* life cycle.

INTRODUCTION

The Lyme disease agent *Borrelia* (*Borreliella*)*burgdorferi* is maintained in an enzootic cycle traversing between*Ixodes* ticks and vertebrates (1,2). The spirochete regulates gene expression in a phase-specific fashion as it cycles between its tick vector and its vertebrate hosts (3,4). *Ixodes* larvae feed on an infected host and acquire the spirochetes, which persist in the midgut. Following the molt, nymphs feed on a vertebrate and the spirochetes migrate from the midgut through the hemocoel to the salivary glands and transmit to the naïve host (5,6). Gene expression is globally regulated by the alternative sigma factors RpoS (σ^S) and RpoN (σ^N) (7-9), the stringent response via guanosine tetraphosphate and pentaphosphate [(p)ppGpp] (10,11), and cyclic dimeric GMP (c-di-GMP) (12-15). While transcriptomics studies of these signaling systems have revealed gene products important for infectivity and persistence, our understanding of post-transcriptional regulation remains sparse.

Many mechanisms of post-transcriptional gene regulation rely on RNA chaperones, a heterogeneous group of proteins that modulate RNA structures by disrupting RNA secondary and tertiary structure (unwinding, unfolding, and strand-displacing) or accelerating base pairing of RNAs (annealing) (16-18). Bacterial RNA chaperones include Hfq, StpA, FinO/ProQ, CsrA, cold shock proteins (CSPs), and the ribosomal proteins S1 and S12; mutants have pleiotropic phenotypes including reduced virulence, impaired growth, and reduced

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ability to respond to and survive environmental stresses (19,20). RNA chaperones can display sequence-independent, transient annealing and strand displacement activity (17,18,21). Hfq and, likely, ProQ are considered "matchmaker" chaperones that facilitate base pairing of*trans* -acting small regulatory RNAs and their RNA targets (19,22-24). *B. burgdorferi* harbors several RNA-binding proteins that regulate post-transcriptional gene expression, but their RNA chaperone activity and mechanisms of regulation have not been extensively studied (4). In 2010, we identified BB0268 as a unique RNA chaperone (25). BB0268 partially complements an *Escherichia colihfq* mutant and the *hfq* gene of *E. coli* partially complements several defects of the pleiotropic *bb0268* mutant of*B. burgdorferi* ; however, BB0268 has negligible homology with Hfq orthologs from well-studied model bacteria (25).

Small RNAs (sRNAs) regulate gene expression, which depends on their three-dimensional structure as well as RNA chaperones (26,27). Many pathogenic bacteria utilize sRNAs to regulate virulence (20,28-30). Only three sRNAs to date have been functionally characterized in *B. burgdorferi* : $\mathrm{DsrA_{Bb}}$, a *trans* -acting sRNA, regulates translation of an *rpoS* mRNA species (31); Bb6S RNA, a protein-interacting sRNA, globally regulates gene expression by affecting RNA polymerase σ factor selectivity (32); and *ittA* , another sRNA, affects expression of several genes and is required for dissemination in the vertebrate host by an unknown molecular mechanism (33). However, the catalog of non-coding sRNAs in *B. burgdorferi*increased dramatically following high-throughput sequencing studies (4,34-38).

c-di-GMP is a global secondary messenger responsible for numerous physiological adaptations of bacteria, including biofilm formation, virulence, motility, phage resistance, and cell morphology (39-43). Diguanylate cyclases synthesize c-di-GMP from two GTP molecules and phosphodiesterases degrade c-di-GMP into linear 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) and/or GMP. In *B. burgdorferi* , c-di-GMP is produced by a sole diguanylate cyclase, Rrp1, and degraded by two phosphodiesterases, PdeA and PdeB (44-47). Rrp1 forms a prototypical two-component signal transduction system with histidine kinase 1 (Hk1), although the activating ligand remains unknown (12,48). *rrp1* and *hk1* mutants have wild-type transmission to and colonization of vertebrates via needle inoculation; however, both mutants are quickly destroyed in the tick midgut during larvae and nymph blood meal feeding (12,13,49,50). In other bacteria, c-di-GMP regulates gene expression at the transcriptional, post-transcriptional, and post-translational levels by binding to different effectors (riboswitches, transcriptional regulators, enzymes, and proteins) (51). In most *B. burgdorferi* , PlzA is the only c-di-GMP effector identified to date (52-55). Mouse infectivity is markedly impaired in a*plzA* mutant (52,56,57), unlike the *hk1* and *rrp1*mutants. Groshong et al. (14) recently demonstrated that a *plzA*point mutant unable to bind c-di-GMP (R145D) is fully infectious in mice confirming that the function of PlzA in mammals is c-di-GMP-independent. The c-di-GMP-dependent role of PlzA in the tick is controversial. Kostick-Dunn et al. (56) found that PlzA is not required to establish infection in ticks, while Pitzer et al. (55) report that *plzA*mutants do not survive during acquisition.

The binding of c-di-GMP induces a large conformational change in PlzA (14,53). The crystal structure of *holo* -PlzA revealed a two-domain structure separated by a short linker (58). Both the *N* - and*C* -terminal domains contain seven-stranded β-barrels that are nearly superimposable. Differences between the two domains are found in the loops connecting the β-strands. The *N* -terminal domain has α helices joining the β-strands, while the *C* -terminal domain has disordered linkers. Notably, *apo* -PlzA did not yield crystals, suggesting more structural flexibility than the liganded form.

In this study, we demonstrate that PlzA has RNA chaperone activity that includes RNA annealing, strand displacement and unfolding. Our data show that c-di-GMP inhibits PlzA strand displacement and RNA unfolding activities *in vitro* and in a heterologous system *in vivo* . We hypothesize that unliganded *apo* -PlzA facilitates conformational changes in RNA that alter their expression, remodeling the RNA structure to stimulate or inhibit transcription or translation, thus enabling spirochete transmission and survival in the vertebrate. In contrast, c-di-GMP does not affect the RNA annealing activity of PlzA*in vitro* , as *apo* - and *holo* -PlzA have similar RNA annealing properties. We hypothesize that *holo* - and*apo* -PlzA are "matchmaker" RNA chaperones that facilitate sRNA and target RNA annealing resulting in the regulation of gene expression throughout the *B. burgdorferi* enzootic cycle. To our knowledge, this is the first protein

with RNA chaperone activity that is modulated by a second messenger.

MATERIALS AND METHODS

Bacterial strains and growth conditions

B. burgdorferi low-passage virulent B31-5A4 was cultivated in Barbour-Stoenner-Kelly II (BSK-II) complete medium at 34°C (59). Cultures were passaged to 1 × 10^5 cells/ml and grown to 1-3 × 10^7 cells/ml. Cell density was determined using a Cellometer counting chamber (Nexcelom). *E. coli* strains, HI-control 10G or BL21(DE3) cells, were grown overnight in lysogeny broth (LB) at 37°C to a density of ˜1 OD_{600}. Cells were then passaged 1:100 unless stated otherwise. Descriptions and genotypes of all strains used (*E. coli* and *B. burgdorferi*) can be found in Table S1.

Construction and expression of recombinant proteins

StpA and GrpE recombinant proteins were purified in *E. coli* using the pETite C-His vector utilizing the Expresso T7 system protocol (Lucigen) as described in Brandt et al. (60). Genes were amplified by PCR from *B. burgdorferi* strain B31-5A4 genomic DNA using primers listed in Table S2. pETite C-His plasmids were transformed into *E. coli* Top 10G Solo cells (Lucigen) and plated on LB plates supplemented with 50 μg/ml kanamycin. Positive transformants were screened by PCR and confirmed by sequencing. Correct plasmids were transformed into *E. coli* BL21(DE3) cells (Lucigen). Positive transformants were screened and grown in LB-kanamycin (50 μg/ml) overnight. The overnight culture was used as an inoculum at a 1:100 dilution in LB-kanamycin (50 μg/ml) in 100 ml for protein expression. Protein expression was induced by the addition of isopropyl-d-thiogalactopyranoside (IPTG; 1 mM) when cells were at mid-logarithmic phase (OD_{600} = 0.4-0.6). Cells were harvested at late-log-phase growth (3-4 h), and recombinant protein was purified under nondenaturing conditions using a nickel-nitrilotriacetic acid (Ni-NTA) Fast Start His tag affinity purification kit (Qiagen) per the manufacturer's protocol. Proteins were dialyzed into PBS (pH 7.4) and quantified by bicinchoninic acid (BCA) assay (Thermo-Fisher Scientific). Protein purification was examined by immunoblot and Coomassie brilliant blue staining.

plzA R145D and R145K mutant alleles were generated using splice overlap exchange techniques (Table S2) (53,61). The amplicons were digested with AgeI and XhoI, ligated into pET45b(+) using Instant Sticky-end Ligase Master Mix (New England Biolabs), and purified using a QIAquick PCR Purification Kit (Qiagen). *plzA* R145D F92A F95*A* mutant and *plzA* R145D F168A F218A mutant gene sequences were synthesized with flanking BamHI and EagI restriction sites and provided in pET45b(+) for expression with an *N* -terminal Hexa-His-tag (GenScript). To propagate the plasmids, *E. coli*NovaBlue(DE3) cells (Novagen) were transformed according to the manufacturer's protocol with the following modification: 100 ng of the plasmid was added to 20 μl of competent cells. Transformants were selected with 100 μg/ml ampicillin. Positive transformants were screened by PCR and confirmed by DNA sequencing. Isolated colonies were selected and transferred into 10 ml of prewarmed LB-ampicillin (100 μg/ml) and grown overnight (37°C; 250 rpm). The overnight culture was used as an inoculum at a 1:200 dilution in LB-ampicillin (100 μg/ml) in 500 ml for protein expression. Protein expression was induced by the addition of 1 mM IPTG when cells were at mid-logarithmic phase (OD_{600}= 0.4). Cells were harvested at late-log-phase growth (4 h) (5000 ×*g* ; 15 min; 4°C). The cell pellet was resuspended and vortexed in binding/washing (B/W) buffer (20 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4) containing protease inhibitor cocktail (1% v/v; Sigma) and Pierce Universal Nuclease for Cell Lysis (ThermoFisher; final concentration 0.5 μg/ml). The samples were passed through an EmulsiFlex-C3 high-pressure homogenizer (three times at 1000-1500 psi; 4°C), and the resulting slurry was centrifuged (15,500 × *g* ; 30 min; 4°C) to separate the soluble and insoluble fractions. To purify proteins from the soluble fraction, nickel affinity chromatography was performed using a Fast Protein Liquid Chromatography platform (ÄKTA Pure 25 M; Cytiva) with a 1-ml HisTrap FF column (Cytiva) at a flow rate of 1 ml/min (4°C). The column was equilibrated with five column volumes of B/W buffer. Samples were loaded into a 10 ml Superloop (Cytiva), followed by washing with 20 column volumes of B/W buffer. Proteins were eluted with five column volumes of elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole). One-ml fractions were collected from under the 280 nm peak, combined, and dialyzed into phosphate-buffered

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saline (PBS, pH 7.4) overnight at 4°C using Spectra/Por 1 (6–8 kDa cutoff) dialysis membranes (Spectrum Laboratories). The concentrations of the recombinant proteins were determined using a BCA assay (Pierce) as instructed by the supplier.

Circular Dichroism Spectroscopy

An Applied Photophysics Chirascan Circular Dichroism (CD) spectrophotometer was used to acquire CD spectra of wild-type and mutant forms of PlzA. All measurements were made in a temperature-regulated sample holder at 25°C in 10 mM sodium phosphate buffer, pH 7.0, using a 1-mm pathlength quartz cuvette. Prior to measuring CD spectra, protein samples were diluted to approximately 20-25 μM and their UV-Vis spectra measured using a Beckman DU 800 UV-Vis spectrometer. An extinction coefficient of 14,900 M^{-1} cm^{-1} at 280 nm, determined by the method of Pace and coworkers (62) using the Expasy ProteinParam tool (63) was used to evaluate concentration from the UV spectra. Samples were diluted fivefold further to concentrations ranging from 4.2-5.5 μM so that high-quality spectra could be obtained down to 180 nm without saturating the CD detector. Spectra were collected from 260 nm to 180 nm using a 1-nm bandwidth, a 1-nm step size and 5 s sampling time per point. Ellipticity was converted to Molar Residue Ellipticity (MRE) using equation 1, where ϑ is the ellipticity in degrees, l is the pathlength in cm, c is concentration in mol/L and n is the

$$MRE = \left(\frac{100\times\theta}{l\times c\times n}\right) \text{(Eq. 1)}$$

number of residues in the protein (261 for PlzA). The STRIDE webserver (64) was used to evaluate secondary structure in the PDB file for PlzA (7MIE). The DichroWeb server (65) was used to analyze the CD data using a truncated (190-240 nm) version of the SP175 reference set (66) and the CDSTTR method (67). The normalized root mean squared deviation NRMSD between the experimental and the spectra calculated by CDSTTR with the SP175 reference set ranged from 0.019-0.024.

Gel RNA annealing assay

The gel annealing assay protocol was adapted from Doestch et al. (68), except manganese was used instead of magnesium in the FRET buffer (69,70). The assay utilizes two short unstructured complementary 21-mer RNAs, termed J1 and M1, that are labeled with Cy5 and Cy3, respectively, and are found in Table S3. All RNAs were synthesized by Dharmacon. RNA annealing reactions were performed in 1× $MnCl_2$ FRET buffer (50 mM Tris-HCl, pH 7.5, 1 mM $MnCl_2$, 10 mM NaCl, 1 mM DTT) at 37°C. Two controls were included with each annealing assay. Control 1 (C1) is single stranded M1 RNA (10 nM M1, 1× $MnCl_2$ FRET Buffer) and control 2 (C2) is preformed J1M1 dsRNA (10 nM M1, 10 nM J1, 1×$MnCl_2$ FRET Buffer). C2 dsRNA was formed by heating the two RNAs (J1 and M1) at 95°C for 2 min and then slowly cooling to room temperature. A master mix for the annealing reaction was assembled (10 nM J1 RNA in 1× $MnCl_2$ FRET buffer) on ice and moved to 37°C to equilibrate. A time point zero aliquot (10 μl) was taken from the master mix, added to M1 RNA and 2.5 μl of 5× stop/load buffer (150 mM EDTA, 2% SDS, 15% (w/v) sucrose, 12.5% (w/v) Ficoll 400, 0.2 mg/ml yeast tRNA and 3 μM of unlabeled M1 (uM1) and immediately loaded into a running (100V) 20% native polyacrylamide gel (20% TBE, 12 well, Novex precast gel). M1 RNA was added to the RNA annealing master mix to start the annealing reaction. Time points were withdrawn from the annealing reaction, stopped with 5× stop/load buffer and loaded on the running gel. To track the progression of the running gel, a lane was loaded with 5× stop/load buffer with the addition of Gel loading buffer II (Invitrogen). Proteins were added to reactions for a final concentration of 1.2 μM, which is similar to protein concentrations used by others to test for non-specific RNA chaperone activity (18,21,68,71). Gels were run at 100 V at 4°C for 3 h. RNA was visualized using Cy3 and Cy5 channels on an Azure Sapphire using the SmartScan feature and overlays for each channel were used for gel images. Quantification of band intensity was measured using the rectangle and gel plot function in ImageJ. The rate of dsRNA production was quantified by plotting the amount of dsRNA (J1M1) divided by the total RNA (J1M1, J1uM1) over time, which was normalized to the zero time point and graphed using GraphPad Prism with linear regression analysis. The amount of RNA was quantified using the Cy5 channel. Differences between the assay endpoints (at 15 min) for each annealing reaction were determined using Welch's t -test (GraphPad Prism).

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dsRNA formation and purification

dsRNA was formed and purified using the J1M1 RNAs described above. 40 μM M1J1 dsRNA was formed in 1× FRET buffer by heating at 95°C for 5 min and then slow cooling to room temperature. The reaction was stopped in 5× stop/load buffer and separated on a pre-poured native gel (Novex 20% TBE gel at 100 V for 1-3 h). The dsRNA was purified from the gel via electrophoresis. Gel slices containing the dsRNA were placed in preparative well of a 1% agarose gel. Four to six 1-inch pieces of Whatman paper were placed in the prep well (between the gel slices and the gel) the dsRNA was electrophoresed (100 V for 3 min) from the gel slices onto the Whatman paper. The Whatman paper was imaged on a BioRad ChemiDoc Gel Imager to verify dsRNA migration. Each Whatman paper was placed in a 1.7-ml light sensitive Eppendorf tube with 500 μl of 10× $MgCl_2$ FRET buffer (500 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 10 mM DTT). Tubes were placed in a microshaker (1400 rpm, 4°C) overnight to extract dsRNA. The 10× $MgCl_2$ FRET buffer (˜500 μl) was removed from the Eppendorf tubes and added to 3 volumes of ethanol (EtOH), 0.3 M sodium acetate, and 15 μg of GlycoBlue? (Invitrogen). RNA was incubated at -20°C overnight. RNA was precipitated at 19000 × g for 45 min at 4°C. Pellets were washed with 200 μl of 70% EtOH and spun again at 19000 × g for 20 min at 4°C. The supernatant was decanted and RNA was resuspended in water. The purified dsRNA was quantified by nanodrop and used for the strand displacement gel assay.

Gel strand displacement assay

Gel strand displacement assays were adapted from Doestch et al. (68), with Mn in the buffer instead of Mg (69,70). Strand displacement assays utilize M1, J1 and uM1 RNAs, as described above. RNA strand displacement reactions were performed in 1× $MnCl_2$ FRET buffer (50 mM Tris-HCl, pH 7.5, 1 mM $MnCl_2$, 10 mM NaCl, 1 mM DTT) at 37°C. Two controls were included with each strand displacement assay. Control 1 (C1) is purified J1M1 dsRNA (30 nM purified M1J1, 1× $MnCl_2$ FRET Buffer) and control 2 (C2) is J1uM1 dsRNA (30 nM purified M1J1, 0.3 μM uM1, 1× FRET $MnCl_2$ Buffer). C2 dsRNA J1uM1 was formed by heating purified dsRNA J1M1 and uM1 at 95°C for 2 min and slowly cooling to room temperature. Strand displacement master mix was prepared with 30 nM purified dsRNA in 1× $MnCl_2$ FRET buffer. A zero time point was removed from the master mix, added to 0.3 μM uM1 and 2.5 μl of 5× stop/load buffer, and immediately loaded into a running (100 V) 20% native polyacrylamide gel (20% TBE, 12 well, Novex precast gel). Strand displacement reactions were started by adding uM1 RNA to the strand displacement master mix at 37°C. Time points were withdrawn from the reaction, stopped with 2.5 μl of 5× stop/load buffer. Gels were run at 100 V at 4°C for 3 h. Gels were visualized using Cy3 and Cy5 channels on an Azure Sapphire using the SmartScan feature and overlayed for gel images. Quantification of band intensity was measured using the Cy5 channel with the rectangle and gel plot function in ImageJ. The amount of strand displacement in each lane was quantified by plotting the amount of J1M1 (Cy5) dsRNA divided by the total RNA (J1M1, J1uM1 and M1; Cy5), which was normalized to the amount of dsRNA (J1M1; Cy5) at time point zero and graphed using GraphPad Prism with second order polynomial regression analysis. Differences between the assay endpoints (at 15 min) for each strand displacement reaction were determined using Welch's t -test (GraphPad Prism).

RL211 antitermination assay

E. coli RL211 harbors the *cat* gene preceded by a Rho-independent *trpL* terminator and is sensitive to chloramphenicol (Cm). To test antitermination activity as described in Ammerman et al. (72), Bae et al. (73), and Landick et al. (74), the genes encoding StpA in the pET7C-HIS plasmid and PlzA, PlzA R145K and PlzA R145D in pET-45b(+) plasmid were electroporated into *E. coli*RL211 strain provided by Dr. Robert Landick (74). Transformed RL211 strains were grown in either with 100 μg/ml ampicillin or 50 μg/ml kanamycin overnight (OD_{600} 0.9-1.0). Cultures were spotted (5 μl) on LB plates with and without 34 μg/ml Cm. Cells were grown for 1-3 d at 37°C. Plates were imaged in an Azure Sapphire imager.

Filter binding assay

10 nM single-stranded Cy3 labeled M1, *trpL* RNA or purified 10 nM double-stranded J1uM1 RNA were titrated with PlzA proteins (50 nM, 100 nM, 200 nM, 300 nM, 500 nM, 800 nM, 1000 nM, and 2000 nM) in

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1× binding buffer (50 mM Tris-HCl pH 7, 2 mM DTT, 6% glycerol) and incubated at room temperature for 5 min. Nitrocellulose (Whatman PROTRAN BA 83 0.2 μm) and nylon membranes (GE Healthcare positively charged nylon transfer membrane 0.45 μm) were pre-equilibrated in 1× binding buffer and loaded into a slot blot apparatus (Hoefer Scientific Instruments PR 600 slot blot). Wells were washed with 1× binding buffer and pulled through the membranes with a vacuum. 90 μl of a 100-μl reaction was applied to the wells and vacuumed. PlzA-RNA complexes bound to the nitrocellulose membrane and the free RNA flowed through and bound to the nylon membrane. Wells were washed four times with 90 μl 1× binding buffer. RNA was detected using the Cy3 channel on both membranes with a Azure Sapphire imager. RNA bound to each membrane was quantified using ImageJ and the fraction of PlzA bound RNA was calculated as a function of PlzA concentration. At least three replicates were performed for each PlzA protein. Mean curves and standard error of the means were calculated and the data were fit to the Hill equation for multiple binding sites, which yielded dissociation constants.

Immunoblotting

Recombinant purified protein (10 μl) or eluates (10 μl) and 2× Laemmli buffer (Sigma) (10 μl) were run on a precast Criterion Any kD TGX Stain-Free gel (BioRad) at 150 V for 1 h. Gels were blotted onto prepackaged Iblot2 stacks (PVDF membrane, Invitrogen) using a custom Iblot2 program (20 V 3 min, 23 V 1 min, 25 V 1 min). Membranes were blocked for 15 min in SuperBlock, TBS (Thermo-Fisher Scientific). Recombinant proteins were detected using mouse anti-6x-His antibody (Clonetech) [1:20,000 1×TBST (1.5 M NaCl, 10 mM Tris-HCl pH 7.5, 0.05% tween 20), 1 h at room temperature] and goat anti-mouse HRP (1:20,000 1×TBST, 1 h at room temperature) (Seracare). PlzA protein was detected using rat anti-PlzA antibody (1:5,000 1×TBST, 1 h at room temperature) and goat anti-rat antibody (BioRad) (1:40,000 1×TBST, 1 h at room temperature). Membranes were washed with 1×TBST three times for 5 min between antibodies and before detection. Membranes were developed using chemiluminescence (ECL select, Amersham) on an Azure Sapphire imager using the chemiluminescence setting with High Dynamic Range (HDR), auto exposure, and cumulative capture. Membranes were also stained with Coomassie brilliant blue to visualize protein purity.

PlzA RNA binding assay

The Pierce His protein interaction pulldown kit was used following the manufacturer's instructions with some modifications. Briefly, spin columns were prepared with 50 μl of slurry and equilibrated with a 1:1 TBS: Pierce Lysis Buffer with 10 mM imidazole. 150 μg of recombinant His-tagged PlzA proteins were immobilized on the slurry by incubating in the spin columns for 30 min at 4°C on a rotating platform. Unbound protein was removed and columns were washed. 50 ml of B31 5A4 *B. burgdorferi* were grown to a cell density of 1×10^8 cells/ml in BSK-II at 34°C and 5% CO_2. Cells were pelleted at 5,000 × *g* and frozen at -80°C. Pellets were thawed on ice and resuspended in wash solution (1:1 ratio of Pierce lysis buffer and BupH Tris Buffered Saline; 500 μl total volume). Cells were lysed in a 2-ml microfuge tube with 500 μl of 0.1 mm glass beads with a Retsch at 30 Hz for 5-10 min in a frozen cassette. Lysates were cleared at 12000 × *g* for 10 min, added to the spin column and rotated for 1 h at 4°C. Columns were centrifuged at 1250 × *g* for 30 sec and flow throughs were saved for SDS-PAGE analysis. The columns were washed five times with 400 μl of wash solution and centrifuged at 1250 × *g* for 30 s. Proteins were eluted with 250 μl of wash buffer containing 300 mM imidazole. The columns were rotated at 4°C for 5 min and then centrifuged at 1250 ×*g* for 30 s. Two eluates were acquired per sample. 10 μl of each eluate was saved for SDS-PAGE analysis. The remaining sample was prepared for hot phenol RNA isolation as previously described in Lybecker et al. (75). RNA concentration was measured using a Nanodrop spectrophotometer and analyzed on an Agilent 2100 Bioanalyzer picochip.

RESULTS AND DISCUSSION

PlzA has c-di-GMP-independent RNA annealing activity *in vitro*

The role of PlzA in the enzootic cycle of *B. burgdorferi* has been intensively studied (14,52,55-57), but the molecular basis for PlzA function remains unknown. A comparative genomic analysis revealed PlzA shares modest sequence similarity with ProQ (Fig. S1), an RNA chaperone (22,76-79). The recently determined

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crystal structure revealed that*holo* -PlzA has bilobed β-barrel domains connected by a short linker (58). Several RNA-binding proteins, including cold shock proteins A and E and eukaryotic Y-box proteins, utilize small β-barrels to interact with RNA (16). We hypothesized that PlzA has RNA chaperone activity, which could be modulated by the binding of c-di-GMP. To test this hypothesis, we assayed the RNA annealing activity of PlzA *in vitro* utilizing two artificial unstructured complementary 21-nucleotide RNAs, termed J1 and M1, that were 5' end-labeled with Cy5 or Cy3, respectively (68). *In vitro* assays that employ artificial RNA substrates are often used to test RNA chaperone activity. RNA chaperones typically possess non-specific RNA chaperone activities even if they have specific RNA targets. To assay "pure annealing" (without melting internal base pairs from structured RNA), the J1 and M1 RNAs were designed without internal secondary structure (68,71,80). The annealing reactions were initiated by adding the M1 RNA to the J1 RNA, samples were removed from the reaction over time, mixed with stop buffer and resolved on a native polyacrylamide gel (Fig. 1A). The stop buffer contains an excess of uM1 that binds to the remaining single-stranded J1, effectively stopping the annealing reaction (Fig. 1A and S2A). RNA annealing is visualized by a gel shift of the individual single-stranded RNAs (ssRNAs) green and red bands to a yellow/orange band double-stranded RNA (dsRNA) that migrates more slowly (Fig. S2B). Annealing of complementary RNAs occurs unaided *in vitro* ; however, addition of StpA, a protein with known RNA annealing activity (17,71), significantly accelerated the base pairing (Fig. S2B). To measure the rate of annealing, dsRNA formation was quantified using the ratio of J1M1 dsRNA (yellow band) to total RNA over time (Fig. 1B). We found that PlzA accelerated RNA annealing over time compared to the RNA only reaction and the GrpE (which lacks RNA chaperone activity) and c-di-GMP negative controls (Fig. 1B). Furthermore, the PlzA-mediated RNA annealing rate was independent of c-di-GMP (Fig. 1B, compare green and blue triangles). The amount of dsRNA formed by the endpoint of the assay with PlzA was significantly higher than the negative controls, albeit lower than the positive control (Figs. 1C and S3A).

We performed filter-binding assays to determine the binding affinity of PlzA for the M1 ssRNA substrate (Fig. 1D). Cy3-labeled M1 RNA and serial dilutions of PlzA proteins were incubated together, applied to a slot blot apparatus, and filtered through two membranes (nitrocellulose and positively charged nylon). The protein and protein-bound RNA bind to nitrocellulose membrane, while unbound RNA flows through the nitrocellulose and binds to the nylon membrane. The fraction of bound RNA was calculated as a function of protein concentration (Fig. 1E). The disassociation constant (K_D) of StpA, the non-specific positive control, was 838.5 ± 171.9 nM (Fig. S4), which agrees with the K_D determined previously (81). Generally, non-specific interactions of RNA chaperones with RNA result in K_D in the low μM range, while specific interactions are in the low nM range (18,21,68,80). TheK_D of PlzA with and without c-di-GMP were 68.17 ± 7.77 nM and 93.37 ± 37.94 nM, respectively, which was not statistically different ($p = 0.4338$), demonstrating that c-di-GMP does not affect PlzA binding to single-stranded M1 (Fig. 1E).

c-di-GMP inhibits PlzA strand displacement activity *in vitro*

Proteins other than RNA chaperones can accelerate RNA annealing *in vitro* via indiscriminate mechanisms including molecular crowding and shielding repulsive charge interactions between RNA molecules (82). However, a hallmark of RNA chaperones is their ability to destabilize dsRNA helices and displace one of the strands in the helix with another RNA, termed strand displacement. Unlike RNA annealing, the melting and strand displacement of stable, completely complementary dsRNA does not occur without an RNA chaperone *in vitro* . We performed strand displacement gel assays (68) to determine if PlzA has strand displacement activity *in vitro* . Stable dsRNA (yellow band) was formed and purified using the two unstructured fluorescent-labeled complementary 21-nucleotide RNAs, J1 and M1, described above in Fig. 1. The strand displacement assay was initiated by adding an excess of M1 unlabeled competitor RNA (uM1) with or without an RNA chaperone to the J1M1 dsRNA; strand displacement activity is detected as a decrease in dsRNA J1M1 (yellow band) and increases in J1uM1 dsRNA (red band) and M1 ssRNA (green band), as the unlabeled competitor RNA, uM1, replaces the M1 RNA in the dsRNA (Fig. 2A). As noted, strand displacement did not occur without an RNA chaperone (Fig. S2C, RNA only), while the known RNA chaperone StpA had strong strand displacement activity (Fig. S2C, StpA). Strand displacement was quantified using the ratio of J1M1 dsRNA (yellow band) to total RNA and values graphed over time (Fig. 2B). We found

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that PlzA had increased strand displacement activity and the amount of the initial dsRNA at the endpoint of the assay was significantly lower compared to the three negative controls, RNA only, c-di-GMP alone and GrpE (Figs. 2B, 2C and S3B). However, the ability of PlzA to strand displace was not as efficient as the positive control StpA in the*in vitro* assay. Furthermore, PlzA strand displacement activity was modulated by c-di-GMP: the addition of c-di-GMP abrogated PlzA strand displacement activity (Fig. 2B and C, compare green triangles and blue inverted triangles). Inhibition of PlzA strand displacement activity was specific to c-di-GMP as c-di-AMP had no effect (Fig. 2B, compare green and orange triangles).

RNA-binding proteins affinity for RNA can be modulated by the binding of metabolites, sRNAs, and proteins as well as by phosphorylation. There are several RNA-binding proteins in *B. subtilis* , HutB, PyrR, and TRAP, that are activated by the binding of histidine and Mg^{2+}, UMP and UTP, and tryptophan, respectively (83), but there are no examples of RNA chaperone activity being regulated by a second messenger. We hypothesized that the *holo* -PlzA may have a lower affinity for dsRNA compared to *apo* -PlzA, thus bound c-di-GMP would decrease strand displacement activity. We tested PlzA affinity for dsRNA in the presence and absence of c-di-GMP by filter binding assays, similar to Fig. 1D, using M1uJ1 dsRNA. The data demonstrate c-di-GMP does not affect PlzA binding to double-stranded RNA and the disassociation constants are nearly identical:K_D of 61.7 ± 15.18 nM and 60.87 ± 8.45 nM with and without c-di-GMP, respectively (Fig. 2D). In agreement with previous reports, the K_D of StpA was in the low μM range (Fig. S4). Taken together, these data indicate that the binding of c-di-GMP affects PlzA strand displacement activity but does not affect the affinity of PlzA for ssRNA or dsRNA *in vitro* . In contrast to other known metabolite-sensing RNA-binding proteins (83), RNA binding by PlzA is not affected by c-di-GMP, but the RNA chaperone activity (strand displacement) of PlzA is inhibited by c-di-GMP. To our knowledge, we have identified the first RNA chaperone whose activity is regulated by a second messenger.

c-di-GMP inhibits the RNA unwinding activity of PlzA in *E. coli*

RNA chaperone activity has been assayed *in vivo* utilizing a transcription antitermination assay (74,84-86). *E. coli* strain RL211 harbors the chloramphenicol (Cm) acyltransferase gene (*cat*) preceded by a Rho-independent *trpL* transcriptional terminator. The terminator is a stem-loop followed by a poly(U) stretch that efficiently terminates transcription of the *cat* gene and results in susceptibility of the RL211 strain to Cm. However, proteins with RNA chaperone unwinding activity melt or unfold the *trpL* terminator leading to antitermination, transcription of the *cat* gene and resistance to Cm (Fig. 3A). We sought to determine if PlzA had antitermination activity *in vivo* in *E. coli* using the RL211 strain. Plasmids carrying *stpA* and *plzA* under the control of the *lac* promoter were transformed separately into the RL211 strain. Cultures were grown overnight in the absence of Cm and then spotted onto plates without (LB) and with Cm (Fig. 3B). As expected, all strains grew similarly on the LB plates (Fig. 3B). RL211 was unable to grow in the presence of Cm and growth was restored in the presence of Cm in the strain producing StpA, which is known to have RNA unwinding activity (71,87,88). The strain producing PlzA had minimal growth in the presence of Cm (Fig. 3B). We postulated that c-di-GMP may be inhibiting RNA unwinding in *E. coli,* an effect similar to what we observed in the strand displacement assay *in vitro* (Fig. 2).*E. coli* harbors 12 genes encoding diguanylate cyclases that generate c-di-GMP (89). Therefore, we tested the ability of a PlzA mutant that is unable to bind c-di-GMP (PlzA R145D) and a mutant with increased affinity for c-di-GMP (PlzA R145K) to rescue growth in Cm (53). Expression of the PlzA R145D mutant, which cannot bind c-di-GMP, rescued growth in Cm, while expression of PlzA R145K, which binds c-di-GMP with a high affinity, did not rescue growth in Cm (Fig. 3B). Taken together, these data suggest that PlzA has antitermination activity and this activity is inhibited by c-di-GMP *in vivo* , at least in a heterologous system. Therefore, both the RNA displacement and unwinding activities of PlzA, but not its RNA annealing activity, are modulated by c-di-GMP.

Potential RNA-binding sites of PlzA

The recently solved crystal structure of *holo* -PlzA reveals a bilobed protein of small *N* - and *C* -terminal β-barrels connected by a short linker region (58). The antiparallel seven-stranded β-barrels in the *N* - and *C* -terminal domains are under 100 amino acids each and have multiple aromatic and basic side chains (Fig. S5).

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Small β-barrel domains (SBB) are characterized by their β-strand-rich secondary structure and their small size, less than approximately 100 amino acids. Proteins containing SBB domains are functionally diverse and can bind to DNA, RNA and proteins (90). The*E. coli* major cold shock protein CspA is an SBB protein that binds RNA along one surface and is proposed to destabilize mRNA secondary structures at low temperatures (91). The RNA chaperone activity of CspA is dependent on three aromatic side chains protruding from the surface of the β-barrel, which disrupt the base pairs in an RNA hairpin via stacking interactions (92). The surrounding basic amino acids are also thought to contribute to the RNA chaperone activity of CspA by compensating for the negative charges on the RNA backbone (92). Other RNA-binding proteins also utilize the combination of aromatic and basic amino acid side chains to bind RNA (93). PlzA has numerous aromatic and basic amino acid side chains (Fig. 4 and Fig. S5). We propose that the protruding aromatic amino acids are necessary for RNA-binding and chaperone activity. The *N* -terminal F92 and F95 and *C* -terminal F168 and F218 amino acids are positioned to interact with RNA (Fig. 4A and Fig. S5) and were targeted for mutagenesis to investigate RNA-binding and unwinding activity (Fig. 5). Wild-type PlzA has modest RNA antitermination activity in *E. coli* , while the PlzA R145D mutant has strong RNA antitermination activity (Fig. 3), consequently we mutated the aromatic amino acids in an R145D background. F92 and F95 in the *N* -terminal domain and F168 and F218 in the *C* -terminal β-barrel were mutated to alanine, resulting in two triple-mutant *plzA* alleles (F92A/F95A/R145D and F168A/F218A/R145D). We measured the circular dichroism (CD) spectra of wild-type PlzA and the mutants to determine if the mutations affected the structure of the protein. The CD spectra of wild type and all the mutants are characteristic of well-folded proteins (Fig. 4B). There are small deviations between the wild-type and mutant CD spectra, but these differences have small effects on the secondary structure content of the variants relative to wild-type PlzA determined from the CD spectra (Table S4). The secondary structure content differs somewhat from that observed for the X-ray structure of PlzA, which was obtained in the presence of c-di-GMP. The increase in coil and the decrease in β-sheet observed with CD data for wild type and all mutants of PlzA in the absence of c-di-GMP are consistent with the structure of *apo* -PlzA being more flexible than *holo* -PlzA.

The *in vivo* antitermination assay was performed in the RL211 strain with plasmids expressing the PlzA triple mutant proteins. We found that *E. coli* expressing PlzA containing the mutated aromatic residues were unable to grow in Cm, suggesting that these amino acids are important for unwinding RNA and the antitermination activity of PlzA (Fig. 5). We hypothesized that the triple mutants were unable to antiterminate *in vivo* because of a lower affinity for the RNA. To examine the role of the aromatic residues in RNA binding, we performed filter binding assays with ssRNA, dsRNA and the *trpL* terminator RNA (Fig. 5B). The data demonstrate that the triple mutants have diminished (PlzA F92A/F95A/R145D) or abolished (PlzA F168A/F218A/R145D) RNA binding to all the RNA substrates. Apparent disassociation constants could not be calculated for all RNAs tested for PlzA F168A/F218A/R145D and ssRNA and dsRNA for PlzA F92A/F95A/R145D. TheK_D for PlzA F92A/F95A/R145D could be calculated but was over sevenfold higher than wild-type PlzA for the *trpL*RNA (Fig. S5). Again, the K_D for StpA was in the low μM range, as expected (Fig. S5). The R145D mutant PlzA protein also had a lower affinity for all RNAs tested compared to the wild type, but still efficiently bound RNA (Fig. 5B and Table S5). Regardless, PlzA R145D efficiently antiterminates transcription *in vivo* in the RL211 strain. The data suggest that the reduced affinity for the*trpL* RNA *in vitro* does not affect the antitermination activity of the PlzA R145D mutant *in vivo* . Surprisingly, the data show that PlzA binding to RNA does not necessarily result in RNA unwinding and antitermination. Wild-type PlzA can bind RNA, but in the presence of c-di-GMP cannot antiterminate. PlzA R145D binds RNA, albeit with a reduced affinity, but cannot bind c-di-GMP and has antitermination activity. However, the mutation of several key aromatic residues in the R145D background results in abolished or diminished RNA binding and loss of antitermination activity. Taken together, the data suggest that PlzA must be able to bind RNA and be in its apo-form to unwind RNA and antiterminate transcription *in vivo* . To our knowledge this is the first example of a small molecule regulating the activity of an RNA binding protein without affecting its ability to bind RNA.

RNA chaperones use multiple RNA-binding domains or multiple copies of the same protein for their various activities, including refolding RNA and annealing two RNAs as a matchmaker (16). The multiple RNA-

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binding sites of PlzA may facilitate binding to multiple RNAs, thus allowing them to interact. In addition, multiple interactions with the same RNA may allow PlzA to rearrange contacts with the RNA without releasing it.*Apo* -PlzA could not be crystalized and has more coil structure when analyzed, which indicates the protein structure may be dynamic in the absence of c-di-GMP and suggests that PlzA becomes less flexible upon c-di-GMP binding, possibly serving as a mechanism to inhibit the RNA unfolding activity.

RNA-binding proteins and DNA-binding proteins both rely on positively charged and polar amino acids for interacting with RNA and DNA. However, the chemical and structural differences of RNA and DNA result in distinct types of interactions with these residues (94-99). Despite the dissimilar mechanisms of RNA binding and DNA binding, numerous proteins bind both DNA and RNA, including Hfq, StpA and H-NS (100-102). The domains, or specific residues, required to bind RNA and DNA may be different in a protein. For example, human ADAR1 binds both RNA and Z-form DNA via two separate domains. The electrostatic surface of PlzA (Fig. 4A) reveals several electropositive surfaces including a deep, electropositive groove where c-di-GMP binds. The groove is larger than the c-di-GMP-binding site. Perhaps this surface interacts with DNA and/or RNA. Further experimentation is required to examine the role of these surfaces in nucleic acid binding. Notably, our data demonstrate that the electropositive surfaces of PlzA are not sufficient to bind RNA as mutating two aromatic amino acids diminished or abolished RNA binding.

PlzA binds *B. burgdorferi* RNAs *in vitro*

To determine if PlzA can bind *B. burgdorferi* RNA, we performed an*in vitro* RNA-binding assay using PlzA as bait. His-tagged wild-type and mutant PlzA proteins were immobilized on a cobalt column and *B. burgdorferi* cleared lysates were passed over the column (Fig. 6A). Columns were washed and PlzA was eluted with imidazole. Eluates were immunoblotted to assay for PlzA protein (Fig. 6B), and RNA bound to PlzA was isolated and run on a bioanalyzer (Fig. 6C). Immunoblot analyses demonstrate that PlzA proteins were found in both eluates but were more concentrated in the first fraction (Fig. 6B, E1). We found that wild-type PlzA and PlzA R145D, which does not bind c-di-GMP, both bind *B. burgdorferi* RNA, while neither of the PlzA triple mutant proteins nor the control without PlzA protein bind RNA (Fig. 6C). These results support the hypothesis that the aromatic amino acids in the *N* - and *C* -terminal domains are important for binding RNA and corroborate the *in vitro* filter binding data. These results also demonstrate that the positive surface charges of PlzA are not sufficient to bind *B. burgdorferi* RNA. Notably, the RNA detected on the bioanalyzer was relatively small (25-150 nucleotides), which suggest PlzA binds predominantly sRNAs, but the specific RNAs bound by PlzA awaits further investigation.

Concluding remarks

Apo - and *holo* -PlzA function differently in the vertebrate and tick phases of the enzootic cycle of *B. burgdorferi*(14,52,55-57). We and others have suggested that the effector functions of *apo* -PlzA and *holo* -PlzA reflect distinct activities and interaction partners (14,47,53,54,57). Here, we demonstrate that c-di-GMP modulates the RNA strand displacement and unwinding activity of PlzA, suggesting these activities may be important for the vertebrate phase of the life cycle, but not the tick phase. Conversely, PlzA has c-di-GMP-independent RNA annealing activity, and we postulate that PlzA may act as a matchmaker RNA chaperone aiding in sRNA and target RNA interactions throughout the enzootic cycle of the spirochete (Fig. 7).

DATA AVAILABILITY

All data are available in the main article and Supplementary Data.

SUPPLEMENTARY DATA

Supplementary Data are available online.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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