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Cyclic Di-GMP Receptor PlzA Controls Virulence Gene Expression through RpoS in *Borrelia burgdorferi*

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As an obligate pathogen, the Lyme disease spirochete *Borrelia burgdorferi* has a streamlined genome that encodes only two two-component signal transduction systems, Hk1-Rrp1 and Hk2-Rrp2 (in addition to CheA-CheY systems). The output of Hk1-Rrp1 is the production of the second messenger cyclic di-GMP (c-di-GMP), which is indispensable for *B. burgdorferi* to survive in the tick vector. The output of Hk2-Rrp2 is the transcriptional activation of the global regulator RpoS, which is essential for the pathogen to accomplish its tick-mouse transmission and to establish mammalian infection. Although evidence indicates that these two systems communicate with each other, how they are connected is not fully understood. In this study, we showed that the c-di-GMP-binding protein PlzA, a downstream effector of Rrp1, positively modulates the production of RpoS, a global regulator and downstream target of Rrp2. Thus, PlzA functions as a connector that links Hk1-Rrp1 with Hk2-Rrp2. We further showed that PlzA regulates *rpoS* expression through modulation of another regulator, BosR, at both the transcriptional and the posttranscriptional levels. In addition, PlzA was also capable of regulating *rpoS* expression independently of Rrp1, suggesting that besides being a c-di-GMP-binding protein, PlzA has other functions. Along with the previous finding of PlzA controlling motility, these studies demonstrate that PlzA is a multifunctional protein. These findings further reinforce the notion that *B. burgdorferi* utilizes its limited signaling systems and regulators to govern multiple cellular processes during its complex enzootic cycle between ticks and mammals.

Borrelia burgdorferi, the Lyme disease spirochetal pathogen, is maintained in an enzootic cycle involving two markedly different hosts, an arthropod vector and a mammalian host (1–3). As an obligate pathogen, *B. burgdorferi* has a dramatically reduced genome. Remarkably, *B. burgdorferi* has evolved by utilizing its limited genomic capabilities to adapt to and survive in these two distinct host environments during its natural cycle (4, 5). In this regard, the *B. burgdorferi* genome only has two two-component signal transduction systems, Hk1-Rrp1 and Hk2-Rrp2 (in addition to the chemotactic CheA-CheY systems), and two alternative sigma factors, RpoS (σ^S) and RpoN (σ^{54}).

In the past 10 years, we and others have shown that response regulator Rrp2 activates transcription of *rpoS* from its RpoN (σ^{54})-type promoter. The stationary-phase sigma factor RpoS (σ^S) functions as a global regulator, controlling expression of many virulence genes, such as *ospC*, *dbpB/A*, *bbk32*, etc., prior to spirochetal transmission from ticks to mammals (6–10). This unique RpoN-RpoS (σ^{54}-σ^S) sigma cascade is essential for *B. burgdorferi* transmission from ticks to mammals and for the establishment of infection in the mammalian host. Recently, a Fur/PerR-like transcription factor, BosR, was also shown to be essential for *rpoS* expression (11–16). Although how BosR fits into Rrp2- and RpoN-dependent activation of *rpoS* remains unclear, *in vitro* data showed that BosR can directly bind to the promoter region of *rpoS* (13, 17). In addition, a small RNA-binding protein (DsrA), a ROC-type repressor (BadR), and a plasmid-coded protein (BBI16) have all been shown to be involved in regulation of RpoS level in *B. burgdorferi* (18, 19).

The second response regulator of *B. burgdorferi*, Rrp1, is the sole diguanylate cyclase that produces the secondary messenger cyclic di-GMP (c-di-GMP) (20). Degradation of c-di-GMP is carried out by two phosphodiesterases, the EAL domain protein BB0363/PdeA and the HD-GYP domain protein BB0374/PdeB, in *B. burgdorferi* (21, 22). Recently, three independent groups reported construction of infectious *hk1* or *rrp1* mutants and demonstrated that while Hk1-Rrp1 is not required for mammalian infection, it is essential for spirochetal survival in the tick vector (23–25). It appears that the defect of the *rrp1* mutant in ticks is, in part, due to a defect in motility and its inability to utilize glycerol, chitobiose, and *N*-acetylglucosamine (23–27). Glycerol is produced by some insects as well as arthropods as a cryoprotective molecule (28), whereas chitobiose (derived from chitin), a major component of the tick cuticle, is an important source of *N*-acetylglucosamine for cell wall synthesis of *B. burgdorferi* (29–31). It was found that expression of the glycerol uptake/metabolism operon *glpFKD*, a chitobiose transporter gene (*chbC*) (30, 31), and several glucosamine metabolism genes is governed by Rrp1 (23, 25, 32). Furthermore, constitutive expression of *glpFKD* in the *rrp1* mutant or supplementing *N*-acetylglucosamine in tick midguts partially rescued the *rrp1* mutant's survival in ticks or its transmission to the mammalian host (23, 32).

Initial observation of the interplay between Hk1-Rrp1 and Hk2-Rrp2 was reported by Rogers et al., showing that deletion of *rrp1* resulted in reduced *rpoS* and *ospC* expression (26). More recently, Sze et al. further showed that Rrp1 influences *rpoS* by

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TABLE 1 Primers used in this study

Primer name	Sequence (5′–3′)	Purpose
bb0733-AF	CAGGTACCAATTCCAAGTATAGCTCCAAAACTTG	Construct pMH23
bb0733-AR	CAGGATCCGAATCCATAGAAATAGAATAAATTACTC C	Construct pMH23
bb0733-BF	CACTGCAGATTTTTAGTTTTGAATTTATAGATGGAG	Construct pMH23
bb0733-BR	CACTCGAGCTCTTAAGATGGCATCAATTAAATTTC	Construct pMH23
KPNflgBp-5	CAGGTACCATGTTTAAGGTTTATGATTTAG	Construct pMH105
flgBp-733-3	CCATAATCTCTTATTTTTCTAGATAAAAGCATATGGAAACCTCCCTCATTTAAAATTGC	Construct pMH105
flgBp-733-5	GCAATTTTAAATGAGGGAGGTTTCCATATGCTTTTATCTAGAAAAATAAGAGATTATGG	Construct pMH105
Pst-Flag-733-3	CACTGCAGTTACTTGTCGTCATCGTCTTTGTAGTCATTGAAATAATCATGGATCAAC	Construct pMH105
qBB0680F	TTGAGCAAATAGCCTCAGGT	qRT-PCR
qBB0680R	TCCACAATGTCTTGCATAGC	qRT-PCR
qBB0566F	GCCTGAAGGAGAGCTTGTAA	qRT-PCR
qBB0566R	ATCCCCTTCTTTCATTTTCTT	qRT-PCR
qBBA73F	AAACAACACAGAAGCGATAA	qRT-PCR
qBBA73R	AGATTTTGGGTGCTTACAAT	qRT-PCR
qBBA24F	GATACCCCACTACCCGTTTT	qRT-PCR
qBBA24R	CATGTGGACTAACAGGAGCA	qRT-PCR
qBBA07F	ACGAAGCAGATGCATCATAA	qRT-PCR
qBBA07R	AATGTTGCCAATGCTAAACA	qRT-PCR
qBB0771F	TAACCCTTTACCCGCATATC	qRT-PCR
qBB0771R	TACAAAGAGGCAATGCAAAA	qRT-PCR
qBB0733F	ATGCTTTTATCTAGAAAAATAAGAGATTATGG	qRT-PCR
qBB0733R	ATTGAAATAATCATGGATCAACATAG	qRT-PCR
qBB0844F	CATGCACCTCTGCTTGTATT	qRT-PCR and RT-PCR
qBB0844R	ATTAGCGATGGGAGTCTTGA	qRT-PCR and RT-PCR
qPCR-flaB-R	CAGCAATAGCTTCATCTTGGTTTG	qRT-PCR and RT-PCR
qPCR-flaB-F	ACCAGCATCACTTTCAGGGTCTCA	qRT-PCR and RT-PCR
qBB0733F	ATGCTTTTATCTAGAAAAATAAGAGATTATGG	qRT-PCR
qBB0733R	ATTGAAATAATCATGGATCAACATAG	qRT-PCR
qBBB19F	CGGATTCTAATGCGGTTTTACTTG	qRT-PCR
qBBB19R	CAATAGCTTTAGCAGCAATTTCATCT	qRT-PCR

modulating *bosR* transcription, which in turn modulates *chbC* expression (32). We also reported a nearly 3-fold reduction of *ospC* expression in the *rrp1* mutant by microarray analysis (23). However, how Rrp1 modulates the production of BosR and RpoS remains unclear.

PlzA belongs to a group of c-di-GMP receptors that contain a conserved PilZ domain (33, 34). Unlike most bacteria, which harbor multiple PilZ domain-containing proteins, *B. burgdorferi* strain B31 has only a single copy of one PilZ domain-containing protein, PlzA (35, 36). Some *B. burgdorferi* strains have a second copy on the plasmid (36). The *B. burgdorferi plzA* mutant has a defect in motility with reduced infectivity in mice (35). In ticks, expression of *plzA* is induced during feeding (35, 36). The *plzA* mutant showed decreased but not abolished survival, unlike the *hk1* and *rrp1* mutants (23–25, 35). In this study, we present the first account of a multifunctional PilZ domain c-di-GMP receptor. We provide evidence that Rrp1 influences BosR and RpoS through PlzA. In addition, we show that PlzA is also capable of regulating *rpoS* expression independently of Rrp1.

MATERIALS AND METHODS

Bacterial strains. The low-passage, virulent *B. burgdorferi* strain 5A4NP1 (a gift from H. Kawabata and S. Norris, University of Texas Health Science Center at Houston) was derived from wild-type strain B31 by inserting a kanamycin resistance marker into the restriction modification gene *bbe02* on plasmid lp25 (37). *B. burgdorferi* was cultivated in Barbour-Stoenner-Kelly (BSK-II) medium supplemented with 6% normal rabbit serum (Pel Freez Biologicals, Rogers, AR) at 35°C with 5% CO_2. Relevant antibiotics were added to the cultures at the following final concentrations: 300 μg/ml for kanamycin, 100 μg/ml for streptomycin, and 50 μg/ml for gentamicin. The constructed suicide vectors were maintained in *Escherichia coli* strain TOP10.

Inactivation of *plzA*. To construct a suicide vector for generating the *plzA* mutant by homologous recombination, the 1.2-kb upstream region and the 1.0-kb downstream region of *plzA* were PCR amplified from strain B31 genomic DNA with primer pairs bb0733-AF/bb0733-AR and bb0733-BF/bb0733-BR (Table 1), respectively. The resulting DNA fragments were cloned upstream and downstream of a streptomycin-resistant cassette (*aadA*), respectively, in the pCR-XL-TOPO cloning vector (Invitrogen). The resulting suicide vector was confirmed by sequencing and was designated pMH23. pMH23 plasmid DNA was then transformed into *B. burgdorferi* strain 5A4NP1 using a previously described protocol (38, 39). Kanamycin- and streptomycin-resistant transformants were analyzed by reverse transcription-PCR (RT-PCR) and quantitative RT-PCR to confirm the loss of *plzA*. Plasmid profiles of the *plzA* mutant clones were determined by performing PCR with 21 pairs of primers specific for the endogenous plasmids (40, 41).

Construction of a shuttle vector for constitutive expression of *plzA*. The shuttle vector pMH105 was constructed by inserting a *flgB* promoter driving a *plzA* gene fused with a FLAG tag at the C terminus of PlzA into plasmid pTM61 (kindly provided by George Chaconas, University of Calgary, Canada) (42). This shuttle vector, pMH105, was then transformed into the *plzA* mutant, wild-type strain 5A4NP1, or the *rrp1* mutant (15). The transformants with proper antibiotic resistance were selected and subjected to reverse transcription-PCR (RT-PCR) or quantitative RT-PCR (qRT-PCR) analyses to confirm the *plzA* expression in these clones.

qRT-PCR. RNA samples were extracted from either *B. burgdorferi* cultures or ticks using an RNeasy minikit (Qiagen, Valencia, CA) accord-

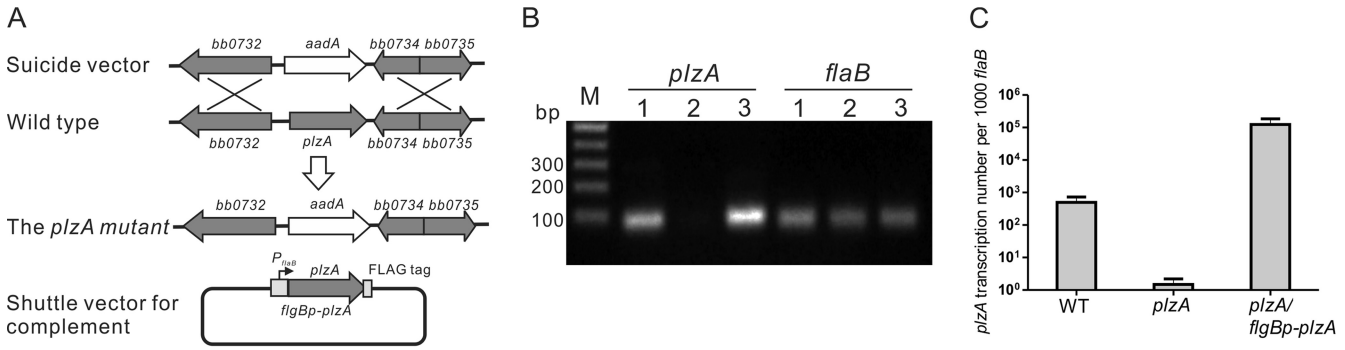

FIG 1 Construction of the *plzA* mutant and complemented strains. (A) Schematic representation of the genes *bb0732* to *bb0735* in the *Borrelia* chromosome and replacement of *plzA* (*bb0733*) with an *aadA* gene cassette (conferring streptomycin resistance) by homologous recombination. A shuttle vector carrying a copy of a constitutive *flgB* promoter-driven *plzA* gene encoding a FLAG-tagged PlzA was used for complementation of the *plzA* mutant (*plzA/flgBp-plzA*). (B) Confirmation of the *plzA* mutation and complementation by RT-PCR. *flaB* serves as a positive control. Lane 1, wild-type *B. burgdorferi* strain B31-5A4NP1; lane 2, the *plzA* mutant; lane 3, the *plzA* mutant carrying *flgBp-plzA*. (C) qRT-PCR analysis of *plzA* levels in the *plzA* mutant and the *plzA* mutant carrying *flgBp-plzA*.

ing to the manufacturer's protocols. For RNA analysis of *in vitro*-cultivated spirochetes, three independent culture samples were used for each strain. Contaminating DNA in RNA samples was digested using RNase-free DNase I (Promega, Madison, WI), and the absence of DNA was confirmed by PCR amplification using primers specific for the *B. burgdorferi flaB* gene. For cDNA synthesis, SuperScript III reverse transcriptase and random primers (Invitrogen, Carlsbad, CA) were used. Absolute quantitative PCR was performed in triplicate using the ABI 7000 sequence detection system and SYBR green PCR master mixture (ABI, Pleasanton, CA). A cloning vector containing a related DNA sequence was used as the standard template. A series of 10-fold dilutions of the standard template (concentrations, 100 to 10^7 copies per μl) was prepared and subjected to qPCR analysis. A standard curve was then generated by plotting the number of copies of the standard template against the cycle threshold (C_T) values. The numbers of copies of a specific gene were then extrapolated from the standard curve based on the C_T values obtained for each reaction.

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting were performed as previously described (43), with the exception that the images were developed using the chemiluminescent method (Pierce ECL Western blotting substrate; Thermo Scientific, IL). Monoclonal antibodies directed against OspC, RpoS, Rrp1, FlaB, or BosR have been described previously (23, 43–45).

Tick-mouse cycle of *B. burgdorferi*. All animal experiments and tick protocols described below were approved by the Institutional Animal Care and Use Committee at Indiana University. Pathogen-free *Ixodes scapularis* larvae were purchased from the Tick-Rearing Center at Oklahoma State University, Stillwater, OK. The tick-mouse experiments were conducted in the Vector-borne Diseases Laboratory at Indiana University School of Medicine, Indianapolis, IN. Pairs of C3H/HeN SCID mice were first needle infected with *B. burgdorferi* (10^5 spirochetes per mouse). Two weeks postinoculation, mouse infection was confirmed by cultivation of ear punch biopsy specimens for spirochete growth. Unfed larvae were then placed on the infected mice, and fully engorged larvae were subjected to qPCR analysis to determine the *B. burgdorferi* loads.

Statistical analyses. To determine the statistical significance of differences observed in qRT-PCR and qPCR, values were compared using an unpaired *t* test. The *P* values are given in each figure.

RESULTS

Construction of the *plzA* mutant and the complemented strains. To study the role of *plzA* in *B. burgdorferi*, we generated a *plzA* mutant of the infectious *B. burgdorferi* strain B31-5A4NP1 (37) by allele exchange as described previously (Fig. 1A) (39). A mutant containing all the same endogenous plasmids as the parental wild-type strain was chosen for further study. To complement the *plzA* mutant, a pBSV2G-based shuttle vector carrying a constitutively expressed FLAG-tagged PlzA was then transformed into the *plzA* mutant (Fig. 1A). The *plzA* mutation and complementation were confirmed by RT-PCR and qRT-PCR (Fig. 1B and C).

The *plzA* mutant is defective in RpoS and OspC production. To determine the impact of the *plzA* deletion on the protein profile of *B. burgdorferi*, whole-cell lysates harvested from stationary growth phase of the wild-type strain, the *plzA* mutant, and the complemented clone were subjected to SDS-PAGE analyses (Fig. 2, top). The result showed that production of OspC, the major RpoS-dependent surface lipoprotein and virulence factor (46–51), was dramatically reduced in the *plzA* mutant in comparison to the wild-type and complemented strains (Fig. 2, top). The defect in OspC and RpoS production was further confirmed by

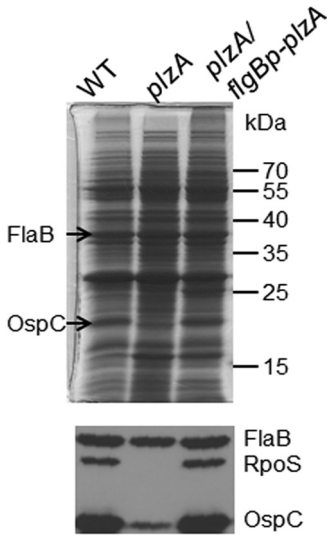

FIG 2 PlzA controls production of RpoS and OspC. Wild-type *B. burgdorferi* B31-5A4NP1 (WT), the *plzA* mutant (*plzA*), and the *plzA* mutant carrying a constitutive copy of *plzA* (*plzA/flgBp-plzA*) were harvested at late logarithmic phase, and the whole-cell lysates were subjected to SDS-PAGE (Coomassie blue stain) (top) and immunoblotting (bottom, using the chemiluminescent method). For immunoblotting, a mix of monoclonal antibodies against FlaB, RpoS, and OspC were used as primary antibodies, and the image was developed by chemiluminescence. FlaB served as a loading control.

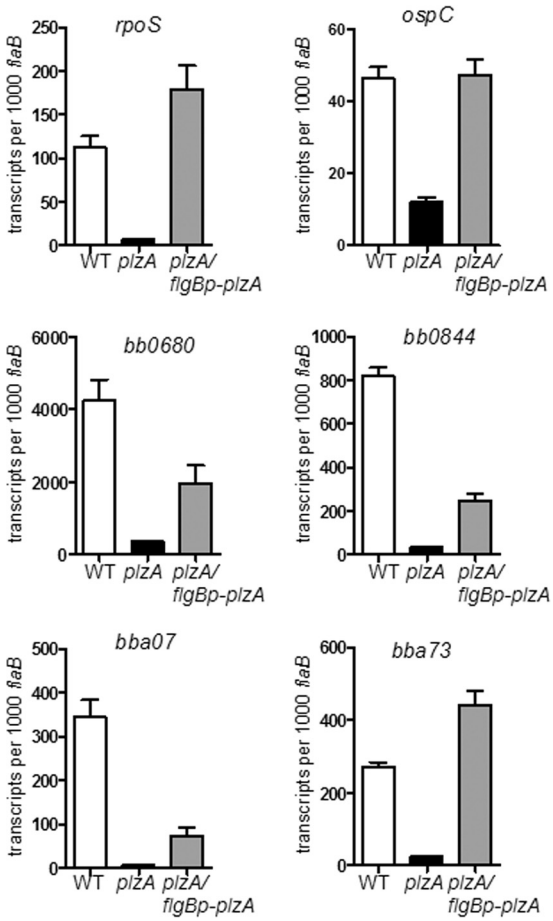

FIG 3 PlzA controls expression of *rpoS* and RpoS-dependent genes. *B. burgdorferi* strains were harvested at late logarithmic phase and subjected to RNA extraction and qRT-PCR analyses for expression of the RpoS-dependent genes indicated at the tops of the graphs. qRT-PCR data were from three independent samples.

immunoblotting analysis (Fig. 2, bottom). These results were also confirmed using another *plzA* mutant previously reported by Pitzer et al. (35) (data not shown). Thus, PlzA appears to modulate production of the major virulence factor OspC through RpoS.

RpoS is known to be regulated at various levels in other bacteria, including transcription, translation, proteolysis, and protein activity (52). First, we tested mRNA levels of *rpoS*. qRT-PCR analyses demonstrated that PlzA influences *rpoS* expression at the mRNA level (Fig. 3). As expected, the transcript level of *ospC*, as well as the transcript levels of several other RpoS-dependent genes, including *bb0680*, *bb0844*, *bba07*, and *bba73*, were greatly reduced in the *plzA* mutant in comparison to the wild-type and the complemented strains (7, 45, 53, 54) (Fig. 3). Taken together, these results indicate that PlzA positively regulates the activation of the RpoS regulon in *B. burgdorferi*.

Inactivation of *rrp1* reduces the production of RpoS and OspC, which can be overcome by overexpression of *plzA*. Previous reports indicated that Rrp1 influences *rpoS* expression (26, 32). Given that PlzA is a c-di-GMP-binding protein, we further investigated whether the effect of Rrp1 on *rpoS* is mediated by PlzA. We first confirmed the effect of Rrp1 on *rpoS* and *ospC* expression using our previously reported *rrp1* mutant (23). Deletion of *rrp1* reduced OspC and RpoS protein levels (Fig. 4A). Interestingly, lowering the culture pH (pH 7.0) could partially overcome the defect of *rrp1* (Fig. 4B). Deletion of *plzA* also resulted in impaired RpoS and OspC levels (Fig. 4A). *plzA* appeared to have a greater impact on OspC levels than *rrp1* at both pH 7.5 and pH 7.0 (Fig. 4B).

The above observation suggests that the defects in the *plzA* and *rrp1* mutants are similar but not identical. To further examine the relationship of PlzA and Rrp1 on influencing *rpoS* expression, we overexpressed PlzA in the *rrp1* mutant. The rationale is that if the function of PlzA is solely dependent on c-di-GMP, then overproduction of PlzA in the *rrp1* mutant should not rescue *rpoS* and *ospC* expression, since Rrp1 is the sole diguanylate cyclase in *B. burgdorferi*. Surprisingly, the result showed that overexpression of *plzA* could fully restore expression of *rpoS* and *rpoS*-controlled genes in the *rrp1* mutant (Fig. 4). This result suggests that PlzA can influence *rpoS* expression independently of Rrp1.

PlzA controls RpoS through BosR. Sze et al. reported that Rrp1 influences *rpoS* expression through BosR, a Fur/PerR homolog transcription regulator that positively regulates *rpoS* expression (32). Therefore, we examined whether PlzA also affects *rpoS* expression through BosR. As shown in Fig. 5A, deletion of *rrp1* greatly reduced the BosR level. However, PlzA had a more dramatic effect than Rrp1: BosR was virtually undetectable in the *plzA* mutant. Furthermore, overexpression of *plzA* was able to restore BosR production in the *rrp1* mutant (Fig. 5A).

It was previously reported that *bosR* expression is often regulated at the protein level, rather than the transcript level, by environmental factors such as CO_2 and Mn^{2+} concentrations (43, 55). Thus, levels of *bosR* mRNA were determined in the *plzA* mutant as well as the *rrp1* mutant. As shown in Fig. 5B, despite the obvious difference in BosR protein levels between wild-type and the *rrp1* mutant, there was no significant difference in *bosR* mRNA levels between the two strains. This result indicates that Rrp1 influences *bosR* at a posttranscriptional level. On the other hand, the effect of PlzA on *bosR* expression appeared to differ from that of Rrp1. Although there was about a 2- to 3-fold reduction of *bosR* mRNA level in the *plzA* mutant compared to wild-type *B. burgdorferi*, this moderate reduction of *bosR* mRNA could not fully account for the lack of detectable BosR in the *plzA* mutant (Fig. 5B). These data suggest that PlzA influenced *bosR* both transcriptionally and posttranscriptionally. Overexpression of *plzA* in the *rrp1* mutant resulted in 3-fold-higher levels of *bosR* mRNA than in the wild-type strain, leading to restoration of the RpoS level in the *rrp1* mutant (Fig. 5A and B).

Overexpression of PlzA partially rescues the *rrp1* mutant's defect in ticks. The *rrp1* mutant failed to survive in ticks, at least in part due to the inability to utilize glycerol and chitobiose (23–25, 32). Since Rrp1 influences the chitobiose transporter ChbC via RpoS (32), we sought to determine whether overexpression of *plzA* would affect the *rrp1* phenotype in ticks. The rationale was that although overexpression of *plzA* in the *rrp1* mutant background could not restore the function of PlzA that is dependent on c-di-GMP, it was able to rescue RpoS production in the *rrp1* mutant. Pathogen-free larval ticks fed on mice that were infected with either wild-type *B. burgdorferi*, the *rrp1* mutant, the wild-type strain overexpressing *plzA*, or the *rrp1* mutant overexpressing *plzA*. After ticks were replete (4 to 5 days of feeding), engorged larvae were collected for qPCR analyses to determine *B. burg-*

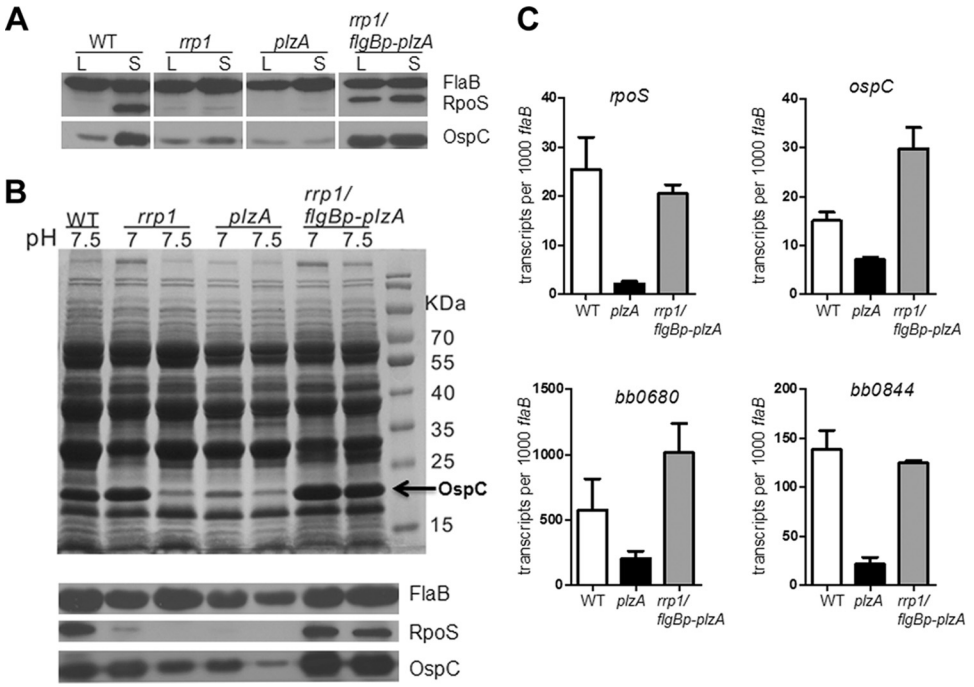

FIG 4 The defect of the *rrp1* mutant in *rpoS* expression can be overcome by overexpression of *plzA* or by low pH. (A) Wild-type *B. burgdorferi* B31-5A4NP1 (WT), the *rrp1* mutant (*rrp1*), the *plzA* mutant (*plzA*), and the *rrp1* mutant carrying a constitutive copy of *plzA* (*rrp1/flgBp-plzA*) were harvested at late logarithmic (L) or stationary (S) phase, and the whole-cell lysates were subjected to immunoblot analyses by chemiluminescence using a mix of monoclonal antibodies against RpoS and FlaB or antibody against OspC. FlaB serves as a loading control. (B) Cells were grown in BSK-II at pH 7.5 or 7.0 and harvested at stationary phase. Whole-cell lysates were subjected to SDS-PAGE (top) or immunoblotting (bottom) by chemiluminescence using monoclonal antibodies against RpoS, OspC, or FlaB. (C) qRT-PCR analysis of *rpoS* and RpoS-regulated genes in *B. burgdorferi* strains grown in standard BSK-II medium and harvested at late logarithmic phase.

dorferi burdens. As shown in Fig. 6, both the *rrp1* mutant and the *rrp1* mutant overexpressing *plzA* had dramatically lower spirochete loads than the wild-type strain. However, overexpression of *plzA* in the *rrp1* mutant resulted in 5-fold-higher spirochetal loads than in the *rrp1* mutant, suggesting that overexpression of *plzA* can partially rescue the survival defect of the *rrp1* mutant in ticks.

DISCUSSION

Accumulated evidence indicates that the two two-component systems in *B. burgdorferi*, Hk1-Rrp1 and Hk2-Rrp2, control spirochetal adaptation to each of its two hosts. While Hk2-Rrp2 is essential for mammalian infection (6, 10, 47), Hk1-Rrp1 is required for tick colonization (23–25). In the feeding gut, when spirochetes migrate from ticks to mammals, both Hk1-Rrp1 and Hk2-Rrp2 are activated (6, 24, 56). It is therefore not surprising that there is cross talk between these two systems. In this study, we provide evidence that the sole known c-di-GMP receptor protein PlzA links the two systems together and helps *B. burgdorferi* coordinate responses to multiple signals to modulate virulence gene expression (Fig. 7).

We first confirmed the previous observation that Rrp1 affects the RpoS regulon, the downstream targets of Rrp2 (23, 26, 32). It is noteworthy that although Rrp1 influences expression of *rpoS* and RpoS-regulated genes, the degree of its impact on RpoS is much less than that reported previously for Rrp2, RpoN, or BosR: mutation of *rrp2* or deletion of *rpoN* or *bosR* virtually abolishes the expression of *rpoS* and *ospC*, whereas deletion of *rrp1* only partially reduces *ospC* expression (Fig. 4). In addition, certain growth conditions, such as lowered pH (pH 7.0), significantly restored OspC production in the *rrp1* mutant (Fig. 4B).

Our observation that both the *rrp1* and *plzA* mutants showed defects in *rpoS* expression suggests that Rrp1 may regulate *rpoS* through PlzA (Fig. 2 and 4). In addition, our data reveal that PlzA can influence *rpoS* expression even in the absence of c-di-GMP. Several lines of evidence support this notion. First, the *plzA* mutant had a more severe defect in OspC production than the *rrp1* mutant (Fig. 4). Second, deletion of *rrp1* affected only the level of BosR protein, not the level of *bosR* mRNA, suggesting that Rrp1 influences BosR at the posttranscriptional level, such as with protein stability. In contrast to Rrp1, PlzA appears to regulate BosR levels at both mRNA and protein levels, because a 2-fold reduction in the *bosR* mRNA levels could not fully account for the lack of detectable BosR protein in the *plzA* mutant (Fig. 5). Lastly, overexpression of *plzA* overcomes the defect of *rrp1* on RpoS production (Fig. 4). It is worth noting that Sze et al. previously showed that their *rrp1* mutant has a defect in *bosR* transcription (32). The reason for the discrepancy remains unclear. However, it has been reported that regulation of *bosR* often occurs at the posttranscriptional level (43, 55). Based on our findings, we postulate that PlzA influences *rpoS* expression via BosR through two mechanisms: (i) upon PlzA binding to c-di-GMP, PlzA–c-di-GMP positively regulates the BosR level by affecting translation efficiency or protein stability; (ii) in the absence of c-di-GMP, PlzA functions as a positive regulator for *bosR* transcription (Fig. 7). The c-di-GMP-independent functions of PlzA may explain why the *plzA* mutant

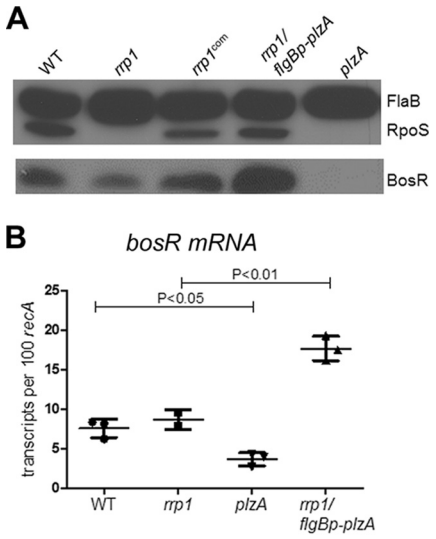

FIG 5 Transcriptional and posttranscriptional control of *bosR* expression by PlzA. (A) Wild-type *B. burgdorferi* B31-5A4NP1 (WT), the *rrp1* mutant (*rrp1*), the *rrp1* mutant with a wild-type copy of *rrp1* (*rrp1*^{com}), the *rrp1* mutant carrying a constitutive copy of *plzA* (*rrp1/flgBp-plzA*), and the *plzA* mutant (*plzA*) were harvested at stationary phase, and the whole-cell lysates were subjected to immunoblot analyses using the chemiluminescent method. (B) RNAs were extracted from the cells described above and subjected to qRT-PCR analysis for *bosR* transcript levels.

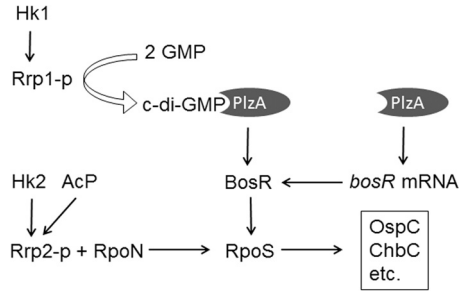

FIG 7 Working model of PlzA connecting the two signaling systems in *B. burgdorferi*, Hk1-Rrp1 and Hk2-Rrp2. The product of the Hk1-Rrp1 signaling system, c-di-GMP, binds to the effector PlzA and positively regulates the BosR protein level. PlzA also regulates the *bosR* mRNA level independently of c-di-GMP. An elevated BosR level increases the transcription of *rpoS* from an RpoN (σ^{54})-type promoter whose activation is governed by RpoN and phosphorylated Rrp2 by Hk2 or acetyl phosphate (AcP). RpoS, in turn, controls expression of many genes, such as *ospC* (essential for mammalian infection) and *chbC* (important to tick colonization). For simplicity, other functions of Rrp1 and PlzA, such as motility and glycerol utilization, as well as other factors involved in RpoS regulation, are not included in the model.

had a more severe defect than the *rrp1* mutant in mammalian infection (23, 25, 35).

The inability of the *rrp1* mutant to survive in ticks is likely due to defects in several cellular functions. First, additional c-di-GMP-binding effectors, besides PlzA, may exist in *B. burgdorferi*. Second, as shown in this study and by Pitzer et al. (35), PlzA modulates multiple cellular functions, including cellular motility and transcriptional and posttranscriptional regulation of virulence determinants. Some of the functions of PlzA depend on c-di-GMP, while other functions may be c-di-GMP independent. Since overexpression of *plzA* in the *rrp1* mutant could restore only c-di-GMP-independent functions of PlzA, it is not surprising that overexpression of *plzA* could not fully restore the *rrp1* mutant's survival in ticks (Fig. 6). The result of partial restoration of the *rrp1* mutant's survival in ticks further emphasized the important role of c-di-GMP in this process. Nevertheless, our finding that PlzA is a multifunctional c-di-GMP receptor appears to be a unique feature of *B. burgdorferi* signal transduction. Bacteria with numerous c-di-GMP signaling pathways often dedicate specific c-di-GMP receptors to control individual targets, in contrast to *B. burgdorferi*, which has a very limited number of signaling pathways (34).

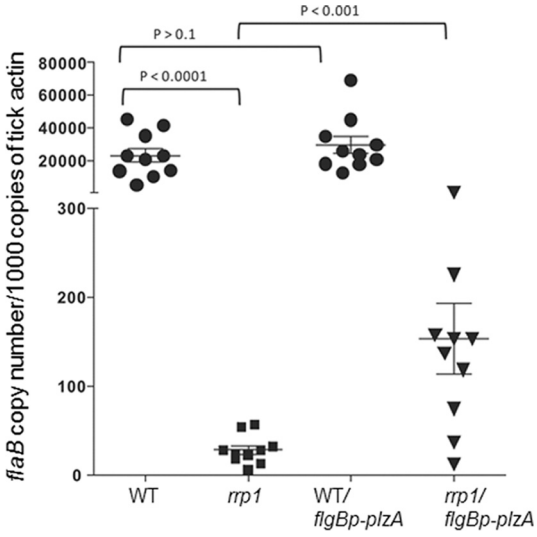

FIG 6 Overexpression of PlzA partially rescues the *rrp1* mutant's defect in ticks. Pathogen-free unfed *I. scapularis* larvae fed on mice infected with various *B. burgdorferi* strains, and fed larvae were subjected to DNA extraction and qPCR analyses for spirochete burden. Each dot represents the *B. burgdorferi* *flaB* copy number in one fed larval tick (a total of 10 ticks examined for each group). The horizontal bar represents the mean value for each group.

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