Published in final edited form as:

Mol Microbiol. 2017 January ; 103(1): 86–98. doi:10.1111/mmi.13545.

The putative Walker A and Walker B motifs of Rrp2 are required for the growth of *Borrelia burgdorferi*

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Summary

Rrp2 encodes a putative bacterial enhancer binding protein (bEBP) in *Borrelia burgdorferi*. Point mutation (G239C) of Rrp2 abolishes the transcriptional activation of σ^{54} -dependent *rpoS*. In contrast to canonical bEBPs that are dispensable for bacterial growth, Rrp2 is essential for borrelial growth in BSK medium. It has been believed that Rrp2's ATPase activity is not required for cell growth, but experimental evidence supporting this notion has been lacking. In particular, it has remained unclear whether the residue G239 is involved in Rrp2's presumptive ATPase activity. To address these information gaps, we examined the roles of Rrp2's potential strategic signatures including the G239 residue and the putative Walker A and Walker B motifs. Herein we show that Rrp2 has ATP binding and hydrolysis activities engendered by the Walker A and B motifs, respectively. However, these activities were not significantly impaired by a G239C mutation. Further mutagenesis analyses indicated that Rrp2's Walker A and B motifs are required for borrelial growth; mutations of key residues in these two motifs were lethal to *B. burgdorferi*. The combined data suggest that the Walker A and Walker B motifs of Rrp2 are involved in the control of another unknown RpoS-independent gene product(s) associated with borrelial replication.

Graphical Abstract

Rrp2 acts in concert with BosR to activate σ^{54} -dependent *rpoS* transcription in *Borrelia burgdorferi*. In contrast to canonical bacterial enhancer binding proteins, Rrp2 is essential for the growth of *B. burgdorferi*. We demonstrate that the Walker A and B motifs constituting ATP binding and hydrolysis activities of Rrp2 are required for borrelial growth, suggesting that the

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Walker motifs are involved in Rrp2's control over another unknown RpoS-independent gene product(s) associated with cell replication.

Introduction

Borrelia burgdorferi, the etiological agent of Lyme disease, is maintained in nature via an enzootic life cycle involving a small arthropod tick vector (*Ixodes scapularis*) and small rodents (Burgdorfer *et al.*, 1982, Steere *et al.*, 1983). As *B. burgdorferi* encounters diverse tick and mammalian stimuli, significant adaptive changes occur (Anguita *et al.*, 2003, Brisson *et al.*, 2012, Caimano *et al.*, 2016, Hu & Klempner, 1997, Liang *et al.*, 2002, Liang *et al.*, 2004, Pal & Fikrig, 2003, Radolf *et al.*, 2012, Rosa *et al.*, 2005, Samuels, 2011, Samuels & Radolf, 2010, Singh & Girschick, 2004, Skotarczak, 2009). These changes usually are achieved through differential gene expression, which is controlled by global regulatory mechanisms such as the RpoN (σ^{54})-RpoS (σ^S) regulatory pathway (Hubner *et al.*, 2001, Radolf *et al.*, 2012, Samuels, 2011). Specifically, this pathway consists of two alternative sigma factors, in which σ^{54} directly controls the transcription of *rpoS* via a canonical -24/-12 promoter (Burtnick *et al.*, 2007, Hubner *et al.*, 2001, Smith *et al.*, 2007). Once RpoS is synthesized, it modulates the expression of major outer surface lipoproteins (Osp) that facilitate *B. burgdorferi*'s ability to colonize and infect ticks or mammals (Caimano *et al.*, 2005, Caimano *et al.*, 2007, Dunham-Ems *et al.*, 2012, Fisher *et al.*, 2005, Hubner *et al.*, 2001, Ouyang *et al.*, 2008, Ouyang *et al.*, 2012, Xu *et al.*, 2012).

Typically, activation of a bacterial σ^{54} system requires a bacterial enhancer binding protein (bEBP) (a.k.a., AAA+ activator ATPase) to provide energy for the formation of the σ^{54} open complex (Bush & Dixon, 2012, Rappas *et al.*, 2007, Studholme & Dixon, 2003, Wigneshweraraj *et al.*, 2008). In general, bEBPs consist of three structural domains, including a N-terminal regulatory (R) domain (a.k.a., the receiver domain) for sensing environmental cues, a central AAA+ core (C) domain for ATPase activity, and a C-terminal DNA binding (D) domain. Bioinformatic analyses have suggested that *B. burgdorferi* Rrp2 (BB0763) also is composed of these three domains (Fig. 1), implying that Rrp2 acts as a bEBP to activate the transcription of σ^{54} -dependent *rpoS*. To test this assumption, an *rrp2* point mutant (*rrp2*[G239C]) (carrying a point mutation in the C domain) was created (Yang *et al.*, 2003); the G239 residue is conserved among bEBPs and was presumed to be essential for Rrp2's ATPase activity and subsequently, the activation of σ^{54} -dependent *rpoS*. Accordingly, analyses showed that synthesis of RpoS and RpoS-dependent virulence factors (e.g., OspC and DbpA) was abolished in this mutant, and thus this mutant was incapable of infecting mammalian hosts (Boardman *et al.*, 2008, Ouyang *et al.*, 2008, Yang *et al.*, 2003). More recently, by introducing a regulatable (IPTG-inducible) *rrp2* expression shuttle plasmid into *B. burgdorferi*, we found that the controlled up-regulation of Rrp2 resulted in the induction of σ^{54} -dependent *rpoS* expression (Ouyang *et al.*, 2014). Additionally, synthesis of RpoS was dependent on and coincident with IPTG-induced Rrp2 levels in an *rrp2* conditional lethal mutant (Ouyang *et al.*, 2014), thereby providing a direct link between the production of Rrp2 in *B. burgdorferi* and the expression of *rpoS*. As such, the role of Rrp2 in the transcriptional activation of σ^{54} -dependent *rpoS* has been solidly established.

Interestingly, recent studies suggest that Rrp2 has additional unique features that distinguish it from other canonical bEBPs. First, Rrp2 may employ unconventional mechanisms to activate σ^{54} -dependent *rpoS* transcription. Typically, bEBPs bind to DNA within about 1 kb upstream of the σ^{54} binding site (Bush & Dixon, 2012, Rappas *et al.*, 2007, Studholme & Dixon, 2003, Wigneshweraraj *et al.*, 2008). However, despite a predicted DNA-binding domain in Rrp2, all efforts to date have failed to substantiate the specific binding of Rrp2 to DNA upstream or downstream of *rpoS* (Blevins *et al.*, 2009, Burtnick *et al.*, 2007), suggesting that Rrp2 activates σ^{54} -dependent *rpoS* transcription independent of DNA binding. Second, in addition to functioning as a bEBP (to activate σ^{54} -dependent *rpoS* transcription), Rrp2 may be involved in other cellular physiology and metabolism processes in *B. burgdorferi*. To date, it has not been possible to eliminate Rrp2 from *B. burgdorferi*; all efforts to obtain a null *rrp2* mutant in *B. burgdorferi* have been unsuccessful, suggesting that Rrp2 is essential for borreliar growth. This hypothesis was confirmed recently; by exploiting *rrp2* conditional mutants, we (Ouyang *et al.*, 2014) and others (Groshong *et al.*, 2012, Yin *et al.*, 2016) found that abolishment of Rrp2 is lethal for *B. burgdorferi* growth in BSK medium. These more recent data imply that Rrp2 may activate or repress other as yet uncharacterized σ^{54} /RpoS-independent genes essential for borreliar replication *in vitro*, because mutations in *rpoN* and *rpoS* are not lethal for the growth of the spirochete (Caimano *et al.*, 2005, Dunham-Ems *et al.*, 2012, Fisher *et al.*, 2005, Hubner *et al.*, 2001, Ouyang *et al.*, 2008). The requirement of Rrp2 for *B. burgdorferi* viability differs dramatically from the traits of other conventional bEBPs, because (i) for canonical bEBPs, they are dispensable for bacterial growth, and thus they typically can be inactivated via insertion or deletion mutagenesis (Bush & Dixon, 2012, Rappas *et al.*, 2007, Studholme & Dixon, 2003, Wigneshweraraj *et al.*, 2008); and (ii) canonical bEBPs usually activate only σ^{54} -dependent genes. These caveats strongly implicate Rrp2 as an unconventional bEBP (i.e., different from prototypical NtrC-like bEBPs).

To date, very little is known regarding the molecular bases underlying Rrp2's surprising dual functions. While a recent study (Yin *et al.*, 2016) reported that Rrp2's R and D domains are associated with borreliar growth, the contributions of the central C domain and Rrp2's ATPase activity to cell replication remain unclear. Based on (i) the readiness to obtain a *rrp2*(G239C) point mutant in *B. burgdorferi* and (ii) the assumption that the G239C mutation abolishes Rrp2's presumptive ATPase activity, it has been postulated that Rrp2's ATPase activity is dispensable for the growth of *B. burgdorferi*. However, whether Rrp2 binds ATP or catalyzes ATP hydrolysis has not been decisively determined. More importantly, it remains unknown whether the G239 residue is involved in protein's ATPase activity. For all bEBPs, the ATP binding and hydrolysis activities are defined, respectively, by the Walker A motif (GXXXXGK[T/S]) and Walker B motif (hhhhDE, where h stands for any hydrophobic amino acid) in the C domain (Bush & Dixon, 2012, Rappas *et al.*, 2007, Studholme & Dixon, 2003, Wigneshweraraj *et al.*, 2008). These two motifs are universally required for the formation of the σ^{54} open complex. *In silico* analyses predict that Rrp2's C domain contains a putative Walker A motif (GESGVGKE at positions 173–180) and a putative Walker B motif (TIFLDE at positions 240–245) (Fig. 1), plausibly associated with ATP binding and hydrolysis. In particular, the predicted Walker motifs do not encompass the G239 residue. To directly address whether Rrp2's potential ATPase activity contributes to borreliar growth, we

herein investigate the roles of these prominent signatures (i.e., the putative Walker A and B motifs in Rrp2's C domain) by exploiting a new *rrp2* conditional mutant, combined with a systematic mutagenesis approach.

Results

Rrp2 binds ATP via the putative Walker A motif

Fluorescent nucleotide binding experiments using mantATP were employed to assay the ATP binding of Rrp2. After purification of recombinant Rrp2 (rRrp2) from *E. coli* via Ni-NTA column and FPLC size-exclusion chromatography, we obtained rRrp2 of >95% homogeneity (Fig. S1). Purified rRrp2 was then used in a binding reaction to assess its nucleotide binding. As shown in Fig. 2A, compared with the emission spectra of the reactions containing rRrp2 alone or mantATP alone, the mixture of rRrp2 with mantATP clearly exhibited an increase in fluorescence intensity, indicating that rRrp2 bound mantATP. By plotting the change in fluorescence against mantATP concentrations, a *K_d* of 2.58 μ M was obtained for mantATP binding to rRrp2 (Fig. 2B). Competition experiments were also performed to assess the specificity of ATP binding to rRrp2. As shown in Fig. 2C and Fig. S2A, although the fluorescence intensities were only slightly decreased when 50- or 100- μ M of unlabeled ATP was added into the reaction, the fluorescence intensity of the reaction was significantly reduced when 1000 μ M of unlabeled ATP was added, suggesting that rRrp2 binds ATP specifically. As a control, we also purified recombinant BosR (rBosR) as previously described (Ouyang *et al.*, 2011). When the ATP binding of rBosR was measured, rBosR did not show detectable ATP binding activity (Fig. S2B).

The potential contributions of Rrp2's putative Walker A motif to ATP binding were also investigated. To this end, point mutations (G173A, G178A, or E180A) (Fig. 1) were introduced into the rRrp2 expression construct pOY131 via site-directed mutagenesis. Mutant proteins were purified and incubated with mantATP. As shown in Fig. 2D, when compared with WT rRrp2, all of G173A, G178A, and E180A showed a significantly (~43–50%) reduced ATP binding, suggesting that these residues (and the Walker A motif) are required for the ATP binding activity of Rrp2. We also introduced point mutations (i.e., D244N or E245Q) (Fig. 1) into the Walker B motif of rRrp2. Our results showed that, relative to WT rRrp2, D244N or E245Q still showed ~70% or ~78% of ATP binding, respectively (Fig. 2D).

Rrp2 has ATPase activity that is dependent on both Walker A and B motifs

A sensitive and direct colorimetric ATPase assay was employed to examine whether rRrp2 has ATP hydrolysis activities. If rRrp2 acts as an ATPase, it would catalyze the decomposition of the substrate (i.e., ATP) into ADP and, at the same time, release free phosphate ion (Pi). Free Pi then reacts with an orange dye, resulting in a stable product with a dark green color. As shown in Fig. 3A, incubation of rRrp2 with ATP and the orange dye yielded green products. By plotting against a standard curve produced with a series of Pi, the free Pi released from the reaction was quantitated. As shown in Fig. 3B, incubation of 1-, 2-, or 5- μ M of rRrp2 with the ATP substrate caused the release of increasing amounts of free Pi. These data substantiate that rRrp2 functions as an ATPase. As a recombinant protein

purification control, we tested the ability of rBosR to hydrolyze ATP and found that this protein preparation was incapable of catalyzing ATP hydrolysis (Fig. 3B).

In general, the Walker B motifs of bEBPs are responsible for ATP hydrolysis. To test whether this is true for Rrp2's putative Walker B motif, rRrp2 variants (D244N or E245Q) were purified and then tested in ATPase assays. When compared with that of WT rRrp2, the ATPase activities of both mutated proteins were significantly reduced (Fig. 3C). In particular, when compared with WT rRrp2, D244N or E245Q displayed only ~11% or ~21% of ATPase activity, respectively. These data suggest that the putative Walker B motif is required for the ATP hydrolysis activity of Rrp2.

For some bEBPs, such as NtrC (Rombel *et al.*, 1999), PspF (Schumacher *et al.*, 2004), and XylR (Perez-Martin & de Lorenzo, 1996), their ATP hydrolysis activities are also dependent on the Walker A motifs (a.k.a., their ATP binding potential). We therefore examined whether Rrp2's Walker A motif mutants were capable of catalyzing ATP hydrolysis. Relative to that of WT rRrp2, ~10–17% of free Pi was released from the reaction containing the Walker A mutants of G173A, G178A, or E180A (Fig. 3C). These data suggest that Rrp2's Walker A motif (and its ATP binding activity) also is involved in its ATPase activity.

A G239C point mutation does not abolish the ATPase activity of Rrp2

To test whether the G239C mutation abolished the potential ATPase activity of Rrp2, we also introduced a G239C mutation into the rRrp2 expression construct pOY131. The mutant protein G239C was then used to examine whether the mutant binds mantATP and hydrolyzes ATP. When measured via fluorescence spectrometry, relative to that of WT rRrp2, the ATP binding activity of the mutant G239C protein was not substantially reduced (still exhibited ~90% activity of WT rRrp2) (Fig. 2D). When used in the ATPase assay, the G239C mutant protein was still capable of hydrolyzing ATP. Specifically, when compared with that of WT rRrp2, the levels of free Pi released from the incubation of G239C with ATP was at ~70% (Fig. 3C).

Generation of an *rrp2* conditional mutant in *B. burgdorferi*

Previously, *rrp2* conditional lethal mutations were created by three different groups (Groshong *et al.*, 2012, Ouyang *et al.*, 2014, Yin *et al.*, 2016). All of those three conditional *rrp2* mutants were merodiploid strains, in which Rrp2 was expressed from a shuttle vector carrying PpQE30-*rrp2*. To investigate the potential contribution of Rrp2's C domain in influencing borrelian growth, we generated another *rrp2* conditional mutant OY180 (Fig. S3). Briefly, we first created a recombinant *B. burgdorferi* strain OY158 expressing LacI by introducing the pBBE02::lacI-Gent^R into strain B31. Following that, the suicide construct pOY275 was electroporated into OY158. In the resultant conditional mutant OY180, the chromosomal copy of *rrp2* in strain B31 was replaced with a PflgB-aadA-PpQE30-*rrp2* cassette, which was confirmed via PCR analyses (Fig. S3A, B). The constitutive expression of LacI (the repressor for the PpQE30 promoter) in this strain was confirmed via immunoblot (Fig. S3C). These strategies allowed *rrp2* expression in OY180 to be solely and tightly controlled by the IPTG-inducible promoter PpQE30 (Fig. 4A). The growth of the *rrp2* conditional mutant OY180 was assessed by cultivating spirochetes in BSK-II containing

various concentrations of IPTG. In these experiments, starter cultures of OY180 were grown in BSK-II medium with 50 μM IPTG. When growth reached the log phase ($\sim 2.5 \times 10^7$ spirochetes ml^{-1}), cells were harvested and washed thrice with PBS. After a final wash, spirochetes were inoculated into BSK-II medium containing varying concentrations of IPTG. As shown in Fig. 4B, OY180 failed to grow in BSK-II with 0-, 10-, or 20- μM IPTG. In contrast, OY180 grew in BSK-II with 30-, 50-, 100-, 200-, 500-, 1000-, or 2000- μM IPTG, reaching a similar final cell density as the WT strain B31 (Fig. 4B). Analyses of cell pellets via SDS-PAGE and immunoblot indicated that the levels of Rrp2, RpoS, and OspC produced in OY180 were closely dependent on the concentrations of IPTG used (Fig. 4C and D). These data substantiate the essentiality of Rrp2 in promoting the *in vitro* growth of *B. burgdorferi*.

Construction of a shuttle vector to complement the conditional *rrp2* mutant

To verify that the observation of failed growth of OY180 in medium without IPTG was due solely to the abolishment of Rrp2 synthesis, we introduced another shuttle plasmid pOY405 into this strain. In pOY405 (Fig. 5A), constitutive expression of Rrp2-3 $\times$ FLAG was driven by the promoter PflgK. Like OY180, the resultant strain OY266 was capable of growing in BSK medium with varying levels (20-, 50-, 100-, or 200- μM) of IPTG (Fig. 5B); analyses of cell pellets from these cultures via immunoblot revealed that spirochetes produced both native Rrp2 (driven from PpQE30 on chromosome and induced by IPTG) and Rrp2-3 $\times$ FLAG (from the shuttle vector pOY405 and promoted by PflgK) (Fig. 5D). More importantly, OY266 also grew in BSK without IPTG, under which spirochetes synthesized Rrp2-3 $\times$ FLAG only, but not native Rrp2 (Fig. 5D). These combined data suggest that the production of Rrp2-3 $\times$ FLAG from pOY405 was able to replace the requirement for native Rrp2 expression for borreliac growth *in vitro*. Also, in spirochetes grown in BSK with varying IPTG, as well as in cells cultivated in medium without IPTG, synthesis of RpoS and RpoS-dependent OspC was readily detected (Fig. 5C and D). More specifically, the levels of RpoS and OspC closely correlated with the total amounts of Rrp2 (including native Rrp2 and Rrp2-3 $\times$ FLAG), implying that Rrp2-3 $\times$ FLAG, like native Rrp2, is functional in activating σ^{54} -dependent *rpoS* and RpoS-dependent *ospC* expression in *B. burgdorferi*.

The Walker A and Walker B motifs of Rrp2 are essential for the *in vitro* growth of *B. burgdorferi*

To determine whether Rrp2's Walker A and B motifs were required for borreliac growth *in vitro*, we introduced point mutations (Fig. 1) into the Rrp2-3 $\times$ FLAG expression vector pOY405 through site-directed mutagenesis. The resulting constructs were transformed into the *rrp2* conditional mutant OY180 via electroporation. As shown in Fig. 6A–D, strain harboring a mutated *rrp2*-3 $\times$ FLAG on pOY405 (i.e., *rrp2*[G173A], *rrp2*[G178A], *rrp2*[E180A], or *rrp2*[D244N]) was capable of growing in BSK-II containing 100 μM IPTG. However, in contrast to OY266 (Fig. 5B), all of these strains were incapable of growing in medium lacking IPTG (Fig. 6A–D). Analyzing spirochetes cultivated in medium containing 100 μM IPTG via immunoblot revealed that Rrp2 (including native Rrp2 and FLAG-tagged Rrp2 mutant), RpoS, and OspC were produced in these cells (Fig. 6E). These data strongly support that the residues (i.e., G173, G178, E180, and D244) and the Walker A and B motifs

of Rrp2 are required for borreliac growth, which in turn implies that the ATP binding and ATPase activities of Rrp2 are needed for spirochetal viability.

We also introduced a G239C mutation into the *rrp2*-3×FLAG on pOY405. The shuttle vector was then introduced into the *rrp2* conditional mutant OY180. The resultant strain was able to grow in BSK-II containing 100 μM IPTG, as well as in medium lacking IPTG (Fig. 7A). These data substantiate previous observations that Rrp2's G239 residue is not required for spirochetal growth *in vitro* (Boardman *et al.*, 2008, Ouyang *et al.*, 2008, Yang *et al.*, 2003). Immunoblot analyses revealed that only FLAG-tagged Rrp2(G239C), but not native Rrp2, was produced in *B. burgdorferi* cultivated in BSK-II without IPTG (Fig. 7B); spirochetes also failed to synthesize RpoS or OspC under this condition (Fig. 7B). When native WT Rrp2 was induced by IPTG (in cells grown in medium with 100 μM IPTG), expression of RpoS and OspC were also detected (Fig. 7B). These data are in agreement with previous observations (Blevins *et al.*, 2009, Boardman *et al.*, 2008, Ouyang *et al.*, 2014, Yang *et al.*, 2003) that the mutant Rrp2(G239C) was incapable of activating the expression of σ^{54} -dependent *rpoS* in *B. burgdorferi*.

Discussion

B. burgdorferi alters its gene expression patterns when cycling between its tick vector and mammalian host(s). This modulation first requires spirochetes to detect and respond to a complex series of environmental cues. As a bEBP, Rrp2 plays a key role in sensing signals as ticks taking blood meal, consequently activating the RpoN-RpoS regulatory pathway and promoting spirochetal tick-mammal transmission. Usually, a bEBP catalyzes ATP hydrolysis, releases free energy, and then stimulates the conversion of a σ^{54} -closed complex to an open complex. The ATPase activity generally can be examined via an ATPase assay that measures free phosphate (Pi) released from a reaction between recombinant bEBP (usually ~1 to 10 μM) and the substrate ATP (Bush & Dixon, 2012, Perez-Martin & de Lorenzo, 1996, Rombel *et al.*, 1999, Schumacher *et al.*, 2004). Despite the long standing belief that Rrp2 is a bEBP in *B. burgdorferi*, definitive evidence supporting that Rrp2 functions as an ATPase has been lacking. Previously, Blevins *et al.* (Blevins *et al.*, 2009) reported that when recombinant Rrp2 was mixed with BeF3 and the substrate ATP, a fivefold increase (relative to reactions without BeF3) in Pi release was detected. In current study, we also measured the ability of rRrp2 to catalyze ATP hydrolysis by using purified recombinant Rrp2. Our data showed that when as little as 1 μM of rRrp2 was incubated with ATP, significant free Pi was detected. Analyzing other Rrp2 variants also indicated that Rrp2's ATPase activity depends on the signature Walker A and B motifs. Our data also revealed, for the first time, that Rrp2 binds ATP, which requires the Walker A motif. Taken together, these new data strongly support that Rrp2 functions as a bEBP ATPase.

We generated another *rrp2* conditional lethal mutant (OY180) to investigate the contributions of Rrp2's Walker A and B motifs to borreliac growth. It is noteworthy that the chromosomal *rrp2* locus was replaced with a PpQE30-*rrp2* cassette in this new mutant, allowing regulatable, IPTG-inducible expression of *rrp2* from the chromosome. Growth curve studies indicated that the conditional mutant OY180 only replicated in BSK containing IPTG, but failed to grow in medium lacking IPTG. When we introduced a shuttle

vector expressing WT Rrp2-3×FLAG into the conditional mutant OY180, the new merodiploid strain OY266 was capable of growing in BSK lacking IPTG, wherein spirochetes produced only Rrp2-3×FLAG from the shuttle vector (but not native Rrp2 from the chromosome). However, in those borreliae containing shuttle vectors encoding Rrp2 variants with point mutations in the Walker A or B motif, spirochetes only grew in medium containing IPTG (to induce native Rrp2 expression), but failed to grow in medium lacking IPTG. It is noteworthy that mutant Rrp2 variants were produced in these spirochetes when cultured in medium containing IPTG, suggesting that the growth phenotype is not resulted from protein stability. Collectively, these data clearly indicate that Rrp2's Walker A and B motifs, and also the ATP binding and hydrolysis activities, are required for the viability of *B. burgdorferi*. To the best of our knowledge, this is the first demonstration that the ATPase activity of a bEBP is required for bacterial growth.

Our findings that Rrp2's Walker A and B motifs are essential for spirochetal viability are quite profound. Compared with the other two structural domains (i.e., R and D), the central C domain is highly conserved among bEBPs (Bush & Dixon, 2012, Rappas *et al.*, 2007, Studholme & Dixon, 2003, Wigneshweraraj *et al.*, 2008). Moreover, previous studies have indicated that the G239 residue in Rrp2's C domain could readily be mutated in *B. burgdorferi* (Blevins *et al.*, 2009, Boardman *et al.*, 2008, Groshong *et al.*, 2012, Ouyang *et al.*, 2008, Yang *et al.*, 2003, Yin *et al.*, 2016). Of note, the residue G239 is located just upstream of the Walker B motif (positions 240–245) in Rrp2's C domain (Fig. 1). More importantly, G239 has been proposed to be essential for Rrp2's ATP hydrolysis, and thus mutation of this residue (i.e., G239C) abolishes Rrp2's ATPase activity (Boardman *et al.*, 2008, Ouyang *et al.*, 2008, Yang *et al.*, 2003). As such, it has been presumed that Rrp2's ATPase activity, as well as its highly conserved Walker A and B motifs, are not required for the growth of *B. burgdorferi*, which runs counter to our results. To resolve this discrepancy, we introduced the G239C mutation into the Rrp2 overexpression construct and obtained recombinant protein rRrp2(G239C). Our data revealed that, like WT rRrp2, rRrp2(G239C) still allowed significant ATP binding and hydrolysis capabilities. More significantly, we found that Rrp2(G239C)-3×FLAG is able to overcome the requirement of native Rrp2 for borrelial growth, further substantiating that the G239 residue is dispensable for the growth of *B. burgdorferi*. Consistent with previous observations (Blevins *et al.*, 2009, Boardman *et al.*, 2008, Ouyang *et al.*, 2014, Yang *et al.*, 2003), expression of *rpoS* and RpoS-dependent *ospC* were undetectable in the strains producing only Rrp2(G239C) (i.e., when bacteria were grown in medium without IPTG). These data collectively suggest that the loss of σ^{54} -dependent *rpoS* transcription in the *B. burgdorferi* *rrp2*(G239C) point mutant may result from the effect (of the G239C mutation) on other unknown activities of Rrp2, rather than from its effect on Rrp2's ATPase activity.

How Rrp2 promotes borrelial growth remains a mystery. Inasmuch as both *rpoN* and *rpoS* have been readily disrupted in virulent *B. burgdorferi*, and both mutants exhibit growth comparable to the WT strain (Caimano *et al.*, 2005, Fisher *et al.*, 2005, Hubner *et al.*, 2001, Ouyang *et al.*, 2008), RpoN and RpoS are not essential for the growth of *B. burgdorferi* *in vitro*. Thus, we propose that Rrp2 may activate or repress the expression of other *B. burgdorferi* genes associated with cell replication, synthesis of essential metabolic intermediates or products, and/or other borrelial essential physiology. Abnormal expression

of those σ^{54} - or RpoS-independent genes would be deleterious for borrelial growth *in vitro*. In bEBPs, the R domain typically functions as a phosphorylation-mediated switch to promote the oligomerization of the bEBP's C domain, whereas the D domain directs bEBPs binding to a specific DNA sequence (i.e., enhancer), and facilitates and stabilizes the oligomerization process of bEBPs (Bush & Dixon, 2012, Morett & Segovia, 1993, Rappas *et al.*, 2007). Furthermore, many structural and biochemical studies (Batchelor *et al.*, 2008, Bush & Dixon, 2012, Morett & Segovia, 1993, Osuna *et al.*, 1997, Pelton *et al.*, 1999, Rappas *et al.*, 2007, Rappas *et al.*, 2006, Ray *et al.*, 2005, Sallai & Tucker, 2005, Wigneshweraraj *et al.*, 2008) have indicated that nucleotide binding and hydrolysis cause significant conformational changes of bEBPs, which is directly linked to the activation of σ^{54} -dependent genes. Despite that Rrp2's D domain has been suggested to be dispensable for the activation of σ^{54} -dependent *rpoS* transcription (Blevins *et al.*, 2009, Burtnick *et al.*, 2007), one recent study reported that Rrp2's D domain, as well as its R domain, is required for spirochetal growth (Yin *et al.*, 2016); Rrp2 variants with the D domain or R domain truncated were incapable of supporting borrelial growth in BSK medium. Our study here adds that Rrp2's ATPase activity also is required for borrelial viability and the regulation over σ^{54} /RpoS-independent essential genes. It is possible that Rrp2 may employ different conformations to activate σ^{54} -dependent *rpoS* transcription or modulate σ^{54} /RpoS-independent genes associated with borrelial viability. Rrp2 possibly directly modulates the expression of those σ^{54} - or RpoS-independent essential genes when it is in one specific conformation (or state associated with ATP binding and hydrolysis). While it remains to be more fully addressed, mutation of the Walker A or B motifs (but not G239) may alter the conformation of Rrp2 which, in turn, may cause the loss of control by Rrp2 over those other essential genes. Alternatively, introducing mutations to the Walker motifs (but not G239) may change the orientations of the helices predicted in Rrp2's D domain and thus Rrp2's DNA binding activity (which is required by Rrp2 to modulate expression of its essential gene targets). In contrast, mutation of the G239 residue may only alter the unique interactions between Rrp2 and factors (such as σ^{54} and BosR) involved in the formation of a σ^{54} open complex and the subsequent activation of *rpoS* transcription. Future structural analyses of WT Rrp2 and Rrp2 variants may provide further insights into the molecular bases underlying Rrp2's unconventional dual function(s).

Experimental Procedures

Bacterial strains and culture conditions

Infectious *B. burgdorferi* strain B31, an isolate recovered from mice infected with B31 MI (Johnson *et al.*, 1984), was used as the wild type (WT) strain throughout this study. Spirochetes were routinely cultivated at 37°C in a 5% CO₂ incubator in Barbour-Stoenner-Kelly II (BSK-II) medium (Pollack *et al.*, 1993) supplemented with 6% rabbit serum (Pel-Freez Biologicals, Rogers, AR). When appropriate, kanamycin (Kan), streptomycin (Strep), or gentamycin (Gent) was added to BSK at a final concentration of 160, 150, or 50 $\mu\text{g ml}^{-1}$, respectively. Growth of *B. burgdorferi* was monitored by enumerating spirochetes via dark-field microscopy and growth curve analysis was performed as previously described (Ouyang *et al.*, 2009, Ouyang *et al.*, 2014). *Escherichia coli* strain NEB 10-beta (New England BioLabs, Ipswich, MA) was used as the cloning host for plasmid construction, and strain

BL21(DE3) (ThermoFisher Scientific, Grand Island, NY) was used for protein production. *E. coli* strains were routinely grown at 37°C in Luria-Bertani broth (LB) supplemented with ampicillin (100 µg ml⁻¹), kanamycin (30 µg ml⁻¹), or chloramphenicol (20 µg ml⁻¹), as indicated.

Construction of an *rrp2* conditional mutant in *B. burgdorferi* strain B31

A conditional *rrp2* mutant in strain B31 was generated by replacing the chromosomal copy of *rrp2* with a PflgB-aadA-PpQE30-*rrp2* cassette. To this end, we first generated a recombinant *B. burgdorferi* strain OY158 constitutively expressing LacI, by introducing a suicide vector pBBE02::lacI-Gent^R (Gilbert *et al.*, 2007) (kindly provided by Scott Samuels) into strain B31. Via allelic exchange, lacI-Gent^R was integrated into *B. burgdorferi* *bbe02*. Transformants were selected by using gentamycin, and confirmed by PCR using primers 311F/311R and immunoblot by using anti-LacI antibody (Abcam, Cambridge, MA).

Next, a PpQE30-*rrp2* was amplified from the plasmid pRrp2 (Ouyang *et al.*, 2014) via PCR by using primers 302F and 303R (Supplementary Table S1). Another DNA fragment spanning *bb0764-rrp2* was amplified from B31 genome by PCR using primers 87.2R and 87F. After digested with AscI, these two fragments were ligated and cloned into pGEM-Teasy (Promega, Madison, WI), which results in pOY272. The PflgB-aadA fragment was cut out from pJD54 with AscI, and cloned into pOY272 linearized with AscI. In the resultant plasmid pOY275, PflgB-aadA is oriented in an opposite direction of PpQE30-*rrp2*. This suicide plasmid was then introduced into *B. burgdorferi* strain OY158 through electroporation, and the recombinants were selected by using streptomycin and gentamycin and confirmed by PCR, SDS-PAGE, and immunoblot.

Generation of an *rrp2*-3×FLAG constitutive expression construct

The shuttle vector pOY405 was created through a series of overlapping extension PCR cloning. First, the 66-bp 3×FLAG was synthesized by IDT DNA Technologies, Inc. (Coralville, IA). *rrp2* was PCR amplified from *B. burgdorferi* by using primers 232F and 299R. Based on these two fragments, *rrp2*-3×FLAG was amplified through overlap PCR using primers 232F and 290.5R. Next, the *B. burgdorferi* *flgK* promoter (PflgK) (Ge *et al.*, 1997) was amplified by PCR using primers 430F and 430R. After digested with NdeI, these two DNA fragments (PflgK and *rrp2*-3×FLAG) were ligated together. Using the ligated DNA as template, PCR was performed by using primers 430F and 290.5R. The resultant PflgK-*rrp2*-3×FLAG was then cloned into pJD44 at XbaI and BamHI sites, resulting in pOY405.

Cloning, overexpression, and purification of recombinant Rrp2 protein

To express recombinant Rrp2 (rRrp2) from *E. coli*, codon-optimized *rrp2* was PCR amplified from the plasmid pBAD-*rrp2*OP+ (Burtnick *et al.*, 2007) (kindly provided by Julie Boylan) using primers 151F and 151R. Purified DNA was cloned into the expression vector pET SUMO (ThermoFisher Scientific) via TA cloning. The resultant construct pOY131 was then transformed into the expression host strain BL21(DE3). To induce protein expression, *E. coli* was grown in LB medium at 37°C. When growth reached an OD₆₀₀ value of ~0.6, the expression of rRrp2 was induced by 1 mM IPTG for 15h at 16°C. After induction, cells

were collected through centrifugation. Pellets were lysed by sonication, and protein was purified by using Ni-NTA agarose (Qiagen, Valencia, CA) under native conditions according to the manufacturer's instruction. Eluted protein was then buffer exchanged into Buffer A (20 mM HEPES, 100 mM NaCl, 100 mM L-Arginine, pH 6.5) using an Amicon ultra centrifugal filter unit (EMD Millipore). Concentrated protein solution was loaded onto a Superdex 200 10/300 GL column connected with an ÄKTA FPLC system (GE Healthcare Life Sciences, Marlborough, MA) previously equilibrated in Buffer A. Protein fractions were collected and analyzed by SDS-PAGE. Concentration of protein was determined by using a Pierce BCA protein assay kit (ThermoFisher).

Site-directed mutagenesis

Point mutations of *rrp2* in pOY131 or pOY405 were created through site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. The primers used for site-directed mutagenesis are listed in Table S2. The introduced mutations were confirmed by DNA sequencing analysis.

SDS-PAGE and immunoblot

When bacterial growth reached late-log phases (i.e., $\sim 10^8$ cells ml⁻¹), spirochetes were collected via centrifugation. Cells were then washed three times with PBS, and resuspended in appropriate SDS protein sample buffer. Samples were loaded onto a gel for SDS-PAGE analyses as previously described (Ouyang *et al.*, 2008, Ouyang *et al.*, 2011, Ouyang *et al.*, 2014). For immunoblot, proteins in the gel were electro-transferred onto a nitrocellulose membrane and probed against specific antibodies including the monoclonal anti-FLAG M2 antibody (Sigma, St. Louis, MO), anti-RpoS monoclonal antibody 6A7-101 (Ouyang *et al.*, 2008), anti-BosR rat polyclonal antibody α -BosR (Ouyang *et al.*, 2009), anti-OspC monoclonal antibody 1B2-105A (Smith *et al.*, 2007), or anti-Rrp2 monoclonal antibody 5B8-100-A1 (Yang *et al.*, 2003). Immunoblots were developed colorimetrically using 4-chloro-1-naphthol as the substrate or by chemiluminescence using an ECL Plus Western blotting detection system (GE Healthcare Life Sciences).

Fluorescent ATP binding assays

To assess whether rRrp2 binds ATP, ATP binding assays were carried out by using N-Methylanthraniloyl ATP (mantATP) (ThermoFisher Scientific), a fluorescent analog of ATP, as previously described (Ni *et al.*, 2000, Jain *et al.*, 2013, Jeong *et al.*, 2004, Srinivasan *et al.*, 2016). Briefly, binding reaction was carried out by incubating different concentrations of mantATP with rRrp2 in Buffer A. Fluorescence was measured using the FluoroEssence program on a Horiba Fluorolog spectrophotometer (Horiba Scientific, Edison, NJ). The excitation wavelength was set at 350 nm, and emission spectra were recorded in the wavelength range of 380–600 nm. Binding of rRrp2 or its variants was calculated by measuring the average fluorescence emitted at 438–450 nm. As controls, reactions including the protein alone or the mantATP alone were also performed. The change of fluorescence intensity (fluorescence unit) was obtained by subtracting the background fluorescence (from mantATP alone and protein alone control reactions) from the total fluorescence observed in the reaction including both protein and mantATP. The *K_d* value was determined

in SigmaPlot by using the ligand binding module. For competition experiments, varying amounts of unlabeled ATP (Sigma) were added to the rRrp2-mantATP incubation reaction and the fluorescence was monitored.

ATPase assays

ATPase activity was assayed by measuring the production of inorganic phosphate (Pi) via a sensitive colorimetric assay using the high sensitivity ATPase assay kit (Innova Biosciences, Cambridge, UK) following the manufacturer's instructions. Briefly, rRrp2 was incubated with 0.5 mM ATP in Buffer A at 37°C. After 30 min, reactions were stopped by addition of the 'PiColorLock Gold' reagent (a superior malachite green reagent) (Innova Biosciences) and further incubated at room temperature for 30 min. Absorbance at 620 nm was determined by using a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA). The amount of free phosphate (Pi) released from reaction was calculated by plotting the absorbance against a standard curve created by a series of Pi standards (Innova Biosciences).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Scott Samuels for generously providing the plasmid pBBE02::lacI-Gent^R, and Julie Boylan for sharing the plasmid pBAD-rp2OP+. We thank Martin Goldberg, Ranjit Deka, and Wei Liu for assistance with FPLC. We also gratefully acknowledge Saipraveen Srinivasan for technical help in fluorescence assays. This work was supported by Grant R21AI119437 (to Z.O.) from the National Institute of Allergy and Infectious Diseases.

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Fig. 1. Architecture of Rrp2's domain structure

B. burgdorferi Rrp2 was predicted to act as a bacterial enhancer binding protein (bEBP). Bioinformatics analyses indicate that Rrp2 consists of three domains including an N-terminal regulatory domain (R), a central AAA+ core domain (C), and a C-terminal DNA binding domain (D). In bEBPs, the Walker A and B motifs are involved in ATP binding and hydrolysis, respectively. When aligned with the consensus sequences of Walker A and B motifs of other bEBPs, putative Walker A and B motifs were predicted in Rrp2 (conserved residues are underlined). The numbers represent relative positions of the Walker motifs in Rrp2. The relative position of the G239 residue is also shown; this residue is important for the transcriptional activation of σ^{54} -dependent *rpoS* in *B. burgdorferi*. Rrp2 variants created in this study are also shown, and the residues subjected to site-directed mutagenesis are indicated in red.

Fig. 2. *B. burgdorferi* Rrp2 is an ATP binding protein

(A) Fluorescent ATP binding experiments were carried out by incubating 1 μM rRrp2 with 1 μM or 5 μM mantATP. Binding was monitored by measuring fluorescence emission at 380–600 nm. The spectra are labeled and color-coded as indicated in the inset. One representative experiment from three biological replicates is shown. (B) Determination of the dissociation constant (K_d) between rRrp2 and mantATP. In each of the three independent experiments, 1 μM rRrp2 and various concentrations (0–20 μM) of mantATP were used. Change in fluorescence intensity (fluorescence unit) was calculated by subtracting fluorescence of mantATP alone and protein alone from that of mantATP with rRrp2. When plotting the change in fluorescence against mantATP concentrations, a $K_d=2.58$ μM was obtained. (C) Rrp2 binds ATP specifically. Varying concentrations (0–1000 μM) of unlabeled ATP were added to the reaction containing 1 μM rRrp2 and 1 μM mantATP. Average fluorescence at 438–450 nm was calculated and change in fluorescence (fluorescence unit) is shown. (D) ATP binding of Rrp2 variants. Binding reaction was performed by incubating 1 μM rRrp2 or each variant with 1 μM mantATP. Change in fluorescence (fluorescence unit) of each protein was calculated. The ratio of the fluorescence units between the mutant and WT rRrp2 was determined and shown. In (C) and (D), bars represent the mean values $\pm$ standard deviations from three biological replicates. The asterisk denotes statistical significance using Student's *t* test ($P < 0.05$) when compared with reactions containing 0 μM unlabeled ATP (C) or WT rRrp2 (D).

Fig. 3. Rrp2 has ATPase activity

(A) ATP hydrolysis was carried out by using a sensitive ATPase assay. After incubating rRrp2 with ATP, the released free phosphate (Pi) reacted with the 'PiColorLock Gold' reagent and yielded stable green products. (B) The absorbance of reactions containing ATP and rRrp2 or rBosR at 620 nm was recorded. By plotting against a standard curve generated from a series of Pi standards, the amount of free Pi released from reactions were calculated. (C) Mutation in Walker A or B motif significantly attenuated the ATPase activity of Rrp2. Free Pi released from reactions between ATP and 1 μM rRrp2 and variants were calculated. In (B) and (C), bars stand for mean values $\pm$ standard deviations from three biological replicates. The asterisk denotes statistical significance using Student's *t* test ($P < 0.05$) when compared with reactions containing WT rRrp2.

Fig. 4. Analyses of the *rrp2* conditional lethal mutant OY180

(A) Schematic representation of the *rrp2* conditional mutant OY180. (B) Growth curve of OY180 in medium containing IPTG. BSK-II medium with various concentrations of IPTG was inoculated with 10^4 spirochetes. Bacterial growth was monitored daily via dark field microscopy. Data are presented as the mean values $\pm$ standard deviations drawn from three independent tests. Of note, it is impossible to enumerate spirochetes in some samples (e.g., growth in BSK with 0-, 10-, or 20- μM IPTG) because the cell density is too low (<1 per field under microscope). SDS-PAGE (C) and immunoblot (D) analyses were performed to analyze gene expression in OY180. Bacteria were grown at 37 $^{\circ}\text{C}$ in BSK-II medium with various concentrations of IPTG. When bacterial growth reached $\sim 10^8$ cells ml^{-1} , spirochetes were harvested. In (A), approximately 4×10^7 spirochetes were loaded onto each lane of a 12.5 % SDS-PAGE gel. Concentrations of IPTG are indicated above the image. The arrow indicates OspC, and the molecular masses indicated in kDa are shown on the left. In (B), $\sim 4 \times 10^7$ (for detecting Rrp2 or RpoS) or $\sim 2 \times 10^7$ (for detecting OspC) spirochetes were loaded onto each lane. Specific antibodies, denoted as α -, are indicated on the left.

Fig. 5. Trans-complementation of the *rrp2* conditional mutant OY180 by using a shuttle vector pOY405

(A) Schematic representations of *rrp2* in *B. burgdorferi* chromosome and the shuttle vector pOY405. (B) Growth curve of OY266 in medium containing IPTG. BSK-II medium with various concentrations of IPTG was inoculated with 10^4 spirochetes. Bacterial growth was monitored daily via dark field microscopy. Data are presented as the mean values $\pm$ standard deviations drawn from three independent tests. Of note, it is impossible to enumerate spirochetes in some samples (e.g., growth on day 3) because the cell density is too low (<1 per field under microscope). (C, D) Gene expression in OY266 was assessed by SDS-PAGE (C) or immunoblot (D). Spirochetes grown in BSK-II medium containing varying concentrations of IPTG were harvested at early stationary phase ($\sim 10^8$ cells ml^{-1}). In (C), approximately 4×10^7 spirochetes were loaded onto each lane of a 12.5 % SDS-PAGE gel. Concentrations of IPTG are indicated above the image. The arrow indicates OspC, and the molecular masses indicated in kDa are shown on the left. In (D), $\sim 4 \times 10^7$ (for detecting FLAG, Rrp2, or RpoS) or $\sim 2 \times 10^7$ (for detecting OspC) spirochetes were loaded onto each lane. Specific antibodies, denoted as α -, are indicated on the left.

Fig. 6. The Walker A and B motifs of Rrp2 are required for the growth of *B. burgdorferi* Specific point mutation (as indicated) was introduced into the Rrp2-3×FLAG in pOY405, and the resultant shuttle vector was transformed into the *rrp2* conditional mutant OY180. (A–D) Growth analyses of *B. burgdorferi*. BSK-II medium containing 0- or 100-μM of IPTG was inoculated with 10^4 spirochetes. Bacterial growth was monitored daily via dark field microscopy. Data are presented as the mean values $\pm$ standard deviations drawn from three independent tests. (E) Gene expression in *B. burgdorferi* was assessed by immunoblot. Spirochetes grown in BSK-II medium containing 100-μM IPTG were harvested at early

stationary phase ($\sim 10^8$ cells ml^{-1}). Each lane contains approximately 4×10^7 spirochetes. Specific antibodies, denoted as α -, are indicated on the left. mRrp2-3 $\times$ FLAG, mutated Rrp2-3 $\times$ FLAG (containing mutated Walker A or B motif).

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Fig. 7. The G239 residue of Rrp2 is dispensable for the growth of *B. burgdorferi*, but is essential for the expression of *rpoS*

A G239C mutation was introduced into the Rrp2-3×FLAG in pOY405, and the resultant shuttle vector was transformed into the *rrp2* conditional mutant OY180. (A) Growth analyses of *B. burgdorferi*. BSK-II medium containing 0- or 100-μM of IPTG was inoculated with 10^4 spirochetes. Bacterial growth was monitored daily via dark field microscopy. Data are presented as the mean values $\pm$ standard deviations drawn from three independent tests. (B) Gene expression in *B. burgdorferi* was assessed by immunoblot. Spirochetes grown in BSK-II medium containing 0- or 100-μM IPTG were harvested at early stationary phase ($\sim 10^8$ cells ml^{-1}). Each lane contains approximately 4×10^7 spirochetes. Concentrations of IPTG are indicated above the image. Specific antibodies, denoted as α -, are indicated on the left. mRrp2-3×FLAG, Rrp2(G239C)- 3×FLAG.