Rrp2, a Prokaryotic Enhancer-Like Binding Protein, Is Essential for Viability of *Borrelia burgdorferi*

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The Lyme disease spirochete, *Borrelia burgdorferi*, exists in two diverse niches (i.e., an arthropod tick vector and mammalian host) during its enzootic life cycle. To effectively adapt to these unique environments, the bacterium alters the expression of numerous genes, including several major outer surface (lipo)proteins that are required for infection and transmission. An enhancer-binding protein (EBP), known as Rrp2, is one identified activator of the RpoN/RpoS alternative sigma factor cascade. Because initial efforts to generate an *rrp2* deletion strain were unsuccessful, the role of Rrp2 in the activation of the RpoN/RpoS pathway was first defined using a strain of *B. burgdorferi* carrying an *rrp2* point mutant that was defective in its ability to activate RpoN-dependent transcription. The fact that subsequent attempts to disrupt *rrp2* have also been unsuccessful has led investigators to hypothesize that Rrp2 has other undefined functions which are essential for *B. burgdorferi* survival and independent of its EBP function. We used a *lac*-based inducible expression system to generate a conditional *rrp2* mutant in virulent *B. burgdorferi*. In this strain, an isopropyl- β -D-thiogalactopyranoside-inducible copy of the *rrp2* gene is expressed in *trans* from a borrelial shuttle vector. We found that the chromosomal copy of *rrp2* could be inactivated only when *rrp2* was induced, and the maintenance of *rrp2* expression was required for the growth of the mutants. In addition, the overexpression of *rrp2* is detrimental to *B. burgdorferi* growth in a manner that is independent of the RpoN/RpoS pathway. These studies provide the first direct evidence that *rrp2* is an essential gene in *B. burgdorferi*.

During its natural enzootic life cycle, *Borrelia burgdorferi* transitions between an arthropod vector and a mammalian host (36). To adapt to the distinct environments represented within the tick and mammal, spirochetes must undergo significant changes in gene expression that are hallmarked by the inverse regulation of two major outer surface (lipo)proteins (Osps), OspA and OspC (1, 13, 14, 18, 27, 30, 33, 34, 38). The reciprocal regulation of *ospA* and *ospC* is mediated by an alternative sigma factor cascade in which one alternative sigma factor (RpoN, σ^N) controls the expression of a second alternative sigma factor (RpoS, σ^S) (5, 9–11, 16, 20, 28, 35, 42). Specifically, RpoN mediates *rpoS* expression at the level of transcription via a canonical $-24/-12$ RpoN-dependent promoter located upstream of *rpoS* (35). RpoS then serves to activate the transcription of *ospC* and other potential virulence- or transmission-associated proteins, such as decorin-binding protein A (DbpA), BBA52, and BBA07 (11, 15, 20, 28, 42). The Hk1-Rrp1 cyclic-di-GMP regulatory network has also been identified as an important regulator of numerous cellular processes in *B. burgdorferi* and has been shown to be critical for maximal dissemination in mammals and acquisition/survival within ticks (12, 19, 24, 32).

One of the key characteristics of RpoN-dependent gene transcription is the requirement for an additional activator known as an enhancer-binding protein (EBP) (8, 26, 37). The genome of *B. burgdorferi* encodes one such activator (17), *rrp2* (bb0763), which is homologous to the NtrC family of EBPs (41). NtrC EBPs contain an amino-terminal response regulator receiver domain, a central RpoN activation domain, and a carboxy-terminal DNA-binding domain (26). To activate RpoN-dependent transcription, the N-terminal domain of Rrp2 is presumed to be phosphorylated at the D52 residue in the amino-terminal domain (41). Phosphorylated Rrp2 activates RpoN-dependent transcription by binding to the RpoN-RNA polymerase holoenzyme and hydrolyzing ATP

to provide the energy necessary to form the open promoter complex (26, 31, 37). Because attempts to generate an *rrp2* deletion strain were unsuccessful, the role of Rrp2 in activating the RpoN/RpoS pathway in *B. burgdorferi* was initially demonstrated by targeting its ATPase activity (41). Specifically, a *B. burgdorferi* strain was generated that carried a point mutation in *rrp2* that replaced one of the key residues responsible for binding ATP [i.e., Rrp2(G239C)]. The Rrp2(G239C) mutant strain was viable; however, it was unable to activate the RpoN/RpoS pathway and subsequently was incapable of expressing known *rpoS*-regulated outer surface proteins (6, 28, 41). Therefore, Rrp2 has been identified as a key activator of the RpoN/RpoS pathway.

While prior studies have successfully defined the importance of Rrp2 in *B. burgdorferi* gene regulation (6, 9, 28, 41), they have also revealed several enigmatic features that distinguish it from prototypical EBPs found in other bacterial species. First, data suggest that Rrp2 activates RpoN-dependent transcription in the absence of apparent upstream enhancer elements that commonly are associated with other EBPs (5, 9). While this finding is not unique to *B. burgdorferi* (2, 7, 22), it distinguishes Rrp2 from the prototypical members of the NtrC family of EBPs (8). Second, despite numerous attempts to create an *rrp2* deletion mutant in *B. burgdorferi* (5, 9, 41), the only *rrp2* mutant strain that has been successfully generated is the Rrp2(G239C) mutant (41). This inability to produce an *rrp2* deletion mutant has led many investigators to

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>E. coli</i> TOP10F'	F' [<i>lacI</i> ^q Tn10(Tet ^r)] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 ara</i> Δ139 Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^r) <i>endA1 nupG</i>	Invitrogen
<i>B. burgdorferi</i>		
Bb297	Strain 297; infectious, human spinal fluid isolate	21
BbIRp2	Bb297 transformed with piRrp2; Strep ^r	This study
BbIRp2-Δ <i>rrp2</i>	BbIRp2 with <i>rrp2</i> interrupted by Kan marker; Strep ^r Kan ^r	This study
BbJSB18-B2	Bb297 <i>rpoN</i> ::Pflg <i>B-aadA</i> mutant; Strep ^r	35
BbJSB19-A7B	Bb297 <i>rpoS</i> ::Pflg <i>B-aadA</i> mutant; Strep ^r	28
BbΔ <i>rpoN</i> /piRrp2	BbJSB18-B2 transformed with piRrp2(Kan); Strep ^r Kan ^r	This study
BbΔ <i>rpoS</i> /piRrp2	BbJSB19-A7B transformed with piRrp2(Kan); Strep ^r Kan ^r	This study
Plasmids		
pGEM-T Easy	TA cloning vector; Amp ^r	Promega
pJSB104	Shuttle vector with IPTG-inducible luciferase; Spec/Strep ^r	4
pJSB275	pJSB104 lacking the NdeI in the resistance marker; Spec/Strep ^r	This study
piRrp2	pJSB275 with <i>Bbluc</i> ⁺ replaced with <i>rrp2</i> ORF; Spec/Strep ^r	This study
pJSB194	Shuttle vector with IPTG-inducible BptA; Kan ^r	4
piRrp2(Kan)	pJSB194 with BptA replaced with <i>rrp2</i> ORF; Kan ^r	This study
pJSB386A	pGEM-T Easy <i>rrp2</i> ::Kan insertional activation construct; Kan ^r	This study

hypothesize that Rrp2 has other undefined functions which are essential for *B. burgdorferi* growth but independent of its EBP function (5, 9, 41). To test this hypothesis, we generated a conditional *rrp2* mutant in virulent *B. burgdorferi* using a shuttle vector-based *lac* repressor/operator-inducible expression system to express *rrp2* in *trans* (4). Using this approach, we found that we were only able to inactivate *rrp2* in a strain of *B. burgdorferi* expressing the inducible copy of *rrp2* carried by the shuttle vector. In addition, the induction and maintenance of *rrp2* expression were required for the growth of the mutant strains in which the native copy of *rrp2* was deleted. During these studies, it was also found that the overexpression of *rrp2* was detrimental to *B. burgdorferi* growth by a mechanism that is independent of *rpoN* and *rpoS*. These data provide the first definitive evidence that *rrp2* is necessary for the viability of *B. burgdorferi*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains and plasmids used in this study are described in Table 1. Infectious, low-passage *B. burgdorferi* strain 297 (Bb297) was used for these studies (21). *Escherichia coli* strain TOP10F' (Invitrogen, Carlsbad, CA) was used as a cloning host. *E. coli* transformants were selected in Luria-Bertani medium supplemented with 100 μg/ml of ampicillin, 100 μg/ml of spectinomycin, or 50 μg/ml of kanamycin. Unless noted otherwise, *B. burgdorferi* was grown in Barbour-Stoenner-Kelley-II (BSK-II) medium at 37°C with 3 to 5% CO₂ and 150 μg/ml of streptomycin or 150 μg/ml of kanamycin when appropriate (29).

Generation of the inducible *rrp2* shuttle vectors. The *rrp2* open reading frame (ORF) was PCR amplified with PrimeStar HS DNA polymerase (TaKaRa, Madison, WI) using Bb297 genomic DNA as a template and primers 297-Rrp2 ORF/NdeI-5' and 297-Rrp2 ORF/HindIII-3' (Table 2), which introduced NdeI and HindIII restriction sites to the 5' and 3' ends of the ORF, respectively. The PCR fragment was TA cloned into pGEM-T Easy (Promega Corp., Madison, WI), verified by DNA sequencing, excised with NdeI and HindIII, and ligated into a modified derivative of pJSB104 (i.e., pJSB275) that had been digested with the same restriction enzymes (4). pJSB104 and pJSB275 are borrelial shuttle vectors that contain a codon-adapted *lacI* repressor transcribed from the *PflaB* promoter and an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T5 pro-

motor derived from pQE30. To facilitate the inducible expression of a gene, the ORF for the gene of interest is fused downstream of the T5 promoter. The resulting shuttle vector carrying the inducible *rrp2* was designated piRrp2 (Fig. 1A). Because the mutations in our previously characterized Bb297 *rpoS* and *rpoN* mutants were marked with a Strep^r marker (28, 35), the Pflg*B-aadA* Strep^r marker in piRrp2 had to be replaced with an *aph*[3']-IIIa kanamycin resistance (Kan^r) marker (4). This construct, designated piRrp2(Kan), was generated by exchanging the NdeI/HindIII-flanked *bptA* ORF in pJSB194 (4) for the NdeI/HindIII-flanked *rrp2* ORF described above.

Construction of the *rrp2*::Kan mutation vector. Regions of DNA flanking the *rrp2* ORF were amplified using primers that introduced an AscI restriction site on the 3' end of a 1.2-kb upstream flanking region, as well as a 5' AscI site and a 3' BssHII site on a 1.1-kb downstream flanking region (Table 2). These regions were amplified using PrimeStar HS DNA polymerase and TA cloned into pGEM-T Easy. To ligate the two flanking regions, the downstream flanking fragment was excised using BssHII and ligated into the vector containing the upstream region that was linearized with AscI. A Kan^r marker, designated *flgBp-aphI*-T7t, was provided by

TABLE 2 Oligonucleotide primers used in this study

Primer designation	Sequence ^a
297-Rrp2 ORF/NdeI-5'	CACATATGAGCAAAATACCTGTAGCTG
297-Rrp2 ORF/HindIII-3'	CAAGCTTACTATTGATCAATATTATTCG
Rrp2-F1-5'	GGATGGCAGTCCGGCATTGCTACT
Rrp2-F1/AscI-3'	GGCGCGCCTTGAAAGCGCATTATATTAT CAAAAGG
Rrp2-F2/AscI-5'	GGCGCGCCTTCATCCTCAAGATAAGTAGC
Rrp2-F2/BssHII-3'	GCGCGCTGAAGGAATCATTGTACTTGAC
5'-KanT7term/AscI	GGCGCGCCTAATACCGAGCTTCAAGGAAG
3'-KanT7term/AscI	GGCGCGCCAGATCCGGATATAGTTCCTCC TTTC
Rrp2 diag 5'	GTAAGGCCAGGCTCTGTAGCCAGC
Rrp2 diag 3'	GTGGAAGCAAGAAAGGCAAGGCAC
Rrp2 seq 3F	GCTTTTCAGAAAGTGTGCGCA
<i>aphI</i> -seq-out-3'	TCGTCTCGCTCAGGCGCAATC
priAH27	CGGGTCATATTTTTCAGCAGCTC
priAH131	GCAGGACAAATACAAAGAGGCAATGC
priAH59	GCTCCACCAACAGAGCTAAAAAGC
priAH61	GCAAGGTGGTTAATCGAATCCCTACG

^a Relevant restriction sites are indicated in bold.

FIG 1 piRrp2 inducible shuttle vector and immunoblot confirmation of BbiRrp2. (A) To create piRrp2, the *rrp2* ORF was amplified from Bb297 and cloned into a derivative of pJSB104. (B) Immunoblot analysis of Rrp2, OspC, and FlaB in wild-type Bb297 and BbiRrp2. Cultures were grown to the mid-exponential phase and either left untreated or supplemented with 1 mM IPTG. Antibodies used to detect the respective proteins are indicated at the right. Values at the left denote relevant molecular masses (kDa) of the standard (MW).

Scott Samuels at the University of Montana. The *flgBp-aphI-T7t* cassette was PCR amplified using Primestart HS DNA polymerase and primers 5'-KanT7term/Ascl and 3'-KanT7term/Ascl (Table 2), which introduced Ascl sites at the ends of the resistance marker. The Kan^r marker was then ligated into the unique Ascl restriction site at the junction between the two flanking regions. The final mutagenesis construct, designated pJSB386A, contained a Kan^r marker replacing a large internal region of the *rrp2* ORF between bp 75 and 1104.

Transformation of piRrp2 and piRrp2(Kan) into *B. burgdorferi*. The transformation of *B. burgdorferi* with piRrp2 was carried out as described by Yang et al. (43). Strep^r clones recovered after the electroporation of Bb297 with piRrp2 were designated BbiRrp2 (Table 1). piRrp2(Kan) was electroporated into the Bb297 *rpoS* and *rpoN* mutants to generate BbΔ*rpoS*/piRrp2 and BbΔ*rpoN*/piRrp2, respectively (Table 1). The PCR confirmation of the *rpoS* (priAH27 and priAH131) and *rpoN* (priAH59 and priAH60) mutations was performed as described by Hubner et al. (20). The PCR-based profiling of endogenous borrelial plasmids in transformants was performed as described by Blevins et al. (3).

Transformation of the *rrp2::Kan* mutagenesis construct into BbiRrp2. The pJSB386A construct was electroporated into BbiRrp2 as described above, and transformants were recovered in streptomycin, kanamycin, and 0.05 mM IPTG. Interruption of the native copy of *rrp2* by the Kan^r marker was confirmed by PCR with the following primer pairs (Table 2): Rrp2 diag 5' and Rrp2 diag 3' (P1 and P3), Rrp2 seq 3F and Rrp2 diag 3' (P2 and P3), and Rrp2-F2/BssHII-3' and *aphI*-seq-out-3' (P5 and P4). Confirmed *rrp2* conditional mutants were designated BbiRrp2-Δ*rrp2*. The PCR-based profiling of endogenous borrelial plasmids in transformants was performed as described by Blevins et al. (3).

IPTG induction of *rrp2* expression. To confirm *rrp2* expression, cultures were grown to the mid-log phase of growth (~1 × 10⁷ spirochetes/ml), at which time they were divided; 1 mM IPTG was added to one portion, while the remainder was left untreated. Bacteria were collected at

24 h postinduction for immunoblot analysis. To confirm the pH-dependent activation of induced Rrp2, cultures were inoculated at an initial density of 1 × 10³ bacteria/ml in BSK-II medium containing 0.05 mM IPTG that was adjusted to either pH 7.5 or 6.8. Bacteria were then collected at 7 days postinoculation (approximately 5 × 10⁸ bacteria/ml) for immunoblot analysis.

Immunoblot analyses. Immunoblotting was performed as described by Blevins et al. (5). For the colorimetric detection of Rrp2, FlaB, and OspC, 4-chloro-1-naphthol was used as the substrate. Antibodies and antisera used to detect Rrp2, FlaB, and OspC were described in prior studies (3, 4, 35). In all experiments, cell lysate from 1 × 10⁷ bacteria was loaded in each gel lane. FlaB detection was included in all comparisons to confirm that equivalent concentrations of lysates were loaded. The All Blue Precision Plus molecular weight standard (Bio-Rad, Hercules, CA) was used for all immunoblots.

Growth curve analyses. For growth curve analyses, strains were inoculated to an initial density of 1 × 10³ bacteria/ml, and IPTG was added when necessary. Beginning at 3 days postinoculation, culture densities were determined daily by enumerating spirochetes using dark-field microscopy; 10 fields were counted per sample. To assess the effect of Rrp2 depletion on BbiRrp2-Δ*rrp2*, cultures were inoculated as described above. When the culture reached the early exponential phase of growth (1 × 10⁶ bacteria/ml), the spirochetes were collected by centrifugation and washed twice with sterile saline. The washed cell pellet was resuspended in BSK-II to a final density of 1.7 × 10⁶ bacteria/ml. The culture was then divided into three portions and supplemented with either 0.05 or 1 mM IPTG or left untreated. Cell growth was monitored as described above. To assess Rrp2 production, bacteria were collected at 24, 48, and 72 h postdepletion for immunoblotting.

RESULTS AND DISCUSSION

Generation and characterization of the IPTG-inducible *rrp2* strain. Prior attempts by numerous investigators to generate a mutant of *B. burgdorferi* in which *rrp2* is deleted have been unsuccessful (5, 9, 41). While these data suggest that *rrp2* is essential for growth, it is also possible that recombination into this region is inefficient. However, Yang et al. (41) initially defined the RpoN-activating function of Rrp2 by replacing the native copy of *rrp2* in the *B. burgdorferi* chromosome with an Rrp2(G239C) point mutant allele. Therefore, this inability to disrupt *rrp2* is likely not due to reduced recombination frequency at this locus. To test whether *rrp2* was indispensable, we generated a conditional *rrp2* mutant in *B. burgdorferi*. To achieve this, the *rrp2* ORF was cloned into a derivative of the previously described pJSB104 borrelial inducible expression vector (4). The resulting vector, designated piRrp2 (Fig. 1A), carries the *rrp2* ORF fused to an IPTG-inducible promoter and a constitutively expressed *lacI* repressor that has been codon optimized to enhance expression in *B. burgdorferi*. piRrp2 was then transformed into wild-type Bb297 (21) to generate strain BbiRrp2 (Table 1). PCR-based plasmid profiling revealed that the endogenous plasmid profile of BbiRrp2 matched that of Bb297 (data not shown). To confirm that piRrp2 was functional in *B. burgdorferi*, a culture of BbiRrp2 was grown to the mid-exponential phase of growth, and IPTG was added to induce Rrp2 production. At 24 h postinduction, cells were collected and protein expression evaluated by immunoblotting (Fig. 1B). The production of Rrp2 was elevated in IPTG-treated cultures of BbiRrp2, whereas the treatment of wild-type Bb297 with IPTG did not affect Rrp2 levels (Fig. 1B). The induction of *rrp2* in BbiRrp2 also resulted in increased levels of OspC (Fig. 1B). This is somewhat surprising, since BbiRrp2 was not cultivated under pH conditions to specifically trigger the Rrp2-mediated activation of RpoN (40). It is pos-

FIG 2 Construction and characterization of the BbiRrp2- Δ *rrp2* mutant. (A) Diagram illustrating the strategy for inactivation of *rrp2*. pJSB386A, pertinent region of the suicide vector used to disrupt *rrp2*; WT, *rrp2* and the flanking regions in the *B. burgdorferi* chromosome; Δ *rrp2*, predicted genomic structure of the *rrp2*::Kan mutant. The solid black arrows denote relative positions of primers used for the PCR characterization of *rrp2* mutants. (B) PCR confirmation of disruption of *rrp2*. Two individual clones of BbiRrp2 and BbiRrp2- Δ *rrp2* were screened. Oligonucleotide pairs used are designated to the right of each panel. P1 and P3 amplify across the inserted marker to generate different-sized amplicons, 1.6 kb (wild type) and 2.1 kb (Δ *rrp2*). The P5 and P4 pair is specific for *rrp2*::Kan, and P2 and P3 only amplify a chromosomal copy of intact *rrp2*. Bb297, wild-type *B. burgdorferi*; H₂O, negative control for amplification; pJSB386A, *rrp2*::Kan suicide vector used to interrupt *rrp2*. DNA size standards (MW), indicated to the left of the panel, are shown in kilobases. (C) Immunoblot analysis of Rrp2, OspC, and FlaB in Bb297 and BbiRrp2- Δ *rrp2* cultivated *in vitro* at pH 7.5 or 6.8. Antibodies used to detect the respective proteins are indicated at the right. Values at the left denote relevant molecular masses (kDa) of the standard (MW).

sible that a small proportion of Rrp2 is spontaneously phosphorylated during *in vitro* cultivation by small phosphodonors (e.g., Xu et al. demonstrated that acetyl phosphate can activate the Rrp2/RpoN/RpoS pathway [39]). Therefore, increasing the overall intracellular concentration of Rrp2 may serve to increase the level of this nonspecifically activated Rrp2 present in the spirochete. Nevertheless, these results suggest that Rrp2 expressed from piRrp2 is functional.

Generation and confirmation of the *B. burgdorferi* conditional *rrp2* mutant. To generate a conditional *rrp2* mutant, the pJSB386A knockout construct was electroporated into BbiRrp2, and allelic exchange between pJSB386A and the chromosome disrupted the native copy of *rrp2* with an *aphI* Kan^r resistance marker (Fig. 2A). If *rrp2* is essential, the Rrp2 expressed from piRrp2 should compensate for the loss of *rrp2* in the chromosome. In agreement with prior studies (5, 9, 41), no Kan^r clones were recovered following the electroporation of Bb297 with pJSB386A

(data not shown). In contrast, Kan^r transformants were readily obtained after the electroporation of BbiRrp2 with pJSB386A when cultures were supplemented with IPTG. PCR analyses of individual Kan^r clones confirmed that the chromosomal copy of *rrp2* was interrupted by *aphI* in these transformants (Fig. 2B). Thus, the resulting strain, BbiRrp2- Δ *rrp2*, contains a disrupted chromosomal *rrp2* and an intact copy of *rrp2* under the control of an IPTG-inducible promoter. Further PCR-based plasmid profiling identified two clones with an endogenous plasmid profile matching that of Bb297 and BbiRrp2 (data not shown).

To confirm that Rrp2 expressed from piRrp2 was functional in BbiRrp2- Δ *rrp2*, we assessed the activation of the RpoN/RpoS regulatory network in the mutant. To test this, BbiRrp2- Δ *rrp2* was grown at 37°C with IPTG to a high cell density in BSK-II medium, which had been adjusted to pH 6.8; these conditions are known to activate the Rrp2/RpoN/RpoS pathway of *B. burgdorferi* *in vitro* (40). An additional set of cultures was similarly grown in BSK-II at the standard pH of 7.5. Bb297 was cultivated under similar conditions as a control. When the cultures reached the stationary phase of growth, bacteria were collected for immunoblot analysis (Fig. 2C). In contrast to the bacteria cultivated at pH 7.5, which showed limited OspC production, increased OspC expression was observed in spirochetes from the pH 6.8 cultures. Slightly higher levels of OspC were also observed in pH 7.5 cultures of BbiRrp2- Δ *rrp2* than in cultures of Bb297. The elevated OspC levels in BbiRrp2- Δ *rrp2* may be due to a modest increase in Rrp2 concentration in the mutant strain (Fig. 2C). Regardless, these results showed the pH-dependent activation of the RpoN-RpoS pathway in BbiRrp2- Δ *rrp2*, similarly to that observed in wild-type *B. burgdorferi*.

***In vitro* growth characteristics of the conditional *rrp2* mutant.** To assess whether *rrp2* is essential for *B. burgdorferi* growth, cultures of BbiRrp2- Δ *rrp2* were grown in the presence or absence of IPTG. A control culture of BbiRrp2 was similarly inoculated and treated. Starting at 3 days postinoculation, cell densities in each culture were enumerated daily using dark-field microscopy. While both BbiRrp2 and BbiRrp2- Δ *rrp2* grew similarly in the presence of IPTG, no measurable growth was detected at any of the time points measured in the culture of BbiRrp2- Δ *rrp2* from which IPTG was omitted (Fig. 3). These data provide the first definitive evidence that *rrp2* is essential in *B. burgdorferi*.

In the experiments described above, the essentiality of *rrp2* was demonstrated by the inability of BbiRrp2- Δ *rrp2* to grow when inoculated into media lacking IPTG. If *rrp2* is truly indispensable, the removal of inducer from an established culture of BbiRrp2- Δ *rrp2* should have a similar effect. To test this, a culture of BbiRrp2- Δ *rrp2* was grown to the early exponential growth phase in the presence of IPTG. Spirochetes were collected from the culture by centrifugation, washed with saline to remove inducer, and resuspended in fresh BSK-II medium to a final density of 1.7×10^6 bacteria/ml. One portion of the postwash culture remained untreated, while IPTG was added to a final concentration of 0.05 or 1 mM in the remaining two aliquots. Cell densities of each culture were monitored daily, and bacteria were collected for immunoblot analyses to assess Rrp2 levels. While there was a brief lag in cell growth immediately after the cells were washed, the BbiRrp2- Δ *rrp2* culture supplemented again with 0.05 mM IPTG reached the postexponential stage of growth at approximately 5 days after the depletion of the IPTG-treated culture (see Fig. 4A). This was similar to the growth rate observed with BbiRrp2- Δ *rrp2* (Fig. 3).

FIG 3 *In vitro* growth characteristics of the conditional *rrp2* mutant. Cultures of BbiRrp2 and BbiRrp2-Δ*rrp2* were grown for 7 days in the presence (+ IPTG) or absence (– IPTG) of 0.05 mM IPTG. Values in the growth curves represent the mean cell counts ± standard deviations (SD) from two trials.

Therefore, the process of washing and diluting the initial IPTG-treated BbiRrp2-Δ*rrp2* culture did not adversely affect cell growth and viability. During the first 3 days, there was a modest increase (approximately 1 log) in the bacterial density of the BbiRrp2-Δ*rrp2* culture from which IPTG was omitted (Fig. 4A). However, between days 2 and 3 the growth rate in the untreated culture was substantially lower than that observed in the 0.05 mM IPTG-treated culture. Moreover, the cell density began to decrease in the untreated culture on the fourth day posttreatment.

During the first 3 days following depletion, immunoblot analysis was used to evaluate Rrp2 production (Fig. 4B). In the culture that did not receive IPTG, we were able to detect a very small amount of Rrp2 at the earliest time point, but Rrp2 was not evident at 48 and 72 h postdepletion. The treatment of the depleted BbiRrp2-Δ*rrp2* culture with 0.05 or 1 mM IPTG resulted in readily detectable levels of Rrp2. In addition, Rrp2 levels were higher in cultures that received 1 mM IPTG. These data confirm the hypothesis that the inability to inactivate *rrp2* using classical allelic exchange was due to *rrp2* being required for *B. burgdorferi* growth (5, 9, 41). Although this is not the first reported use of a conditional mutation system to identify essential genes in *B. burgdorferi* (25), this study confirms that our shuttle vector-based *lac* repressor/operator system is suitable for such an application.

Inhibition of *B. burgdorferi* growth in response to *rrp2* overexpression. An additional finding that came out of these studies was that the overexpression of *rrp2* was detrimental to cell growth. The inhibition of bacterial growth in cultures treated with excess IPTG (e.g., 1 mM) was observed in both BbiRrp2 (data not shown) and BbiRrp2-Δ*rrp2* (Fig. 4A). It was noted that the induction of *rrp2* in BbiRrp2 also results in the upregulation of OspC (Fig. 1B). Given that the overproduction of RpoS also negatively affects *B. burgdorferi* growth (A. M. Groshong and J. S. Blevins, unpublished results), it is possible that the growth inhibition following *rrp2* overexpression is due to the activation of the RpoN/

FIG 4 Effect of removing inducer on growth of the conditional *rrp2* mutant. A culture of BbiRrp2-Δ*rrp2* was grown with 0.05 mM IPTG to the early log phase. Cells were then washed to remove inducer and resuspended in media supplemented with various concentrations of IPTG. (A) Bacterial growth in each culture was assessed, and the values in the graphs represent the mean cell counts ± SD. Duplicate growth curves were performed with equivalent results; data from a representative study are shown. Values shown in the graph legend refer to the IPTG concentration added to the respective cultures. (B) Immunoblot analysis of Rrp2 production in IPTG-depleted BbiRrp2-Δ*rrp2* cultures cultivated in the presence of different concentrations of inducer. Antibodies used to detect the respective proteins are indicated at the right. Values at the left denote relevant molecular masses (kDa) of the standard (MW).

RpoS pathway. To test this, Bb297 *rpoN* and *rpoS* mutants were transformed with the piRrp2(Kan)-inducible *rrp2* shuttle vector; confirmed transformants were designated BbΔ*rpoN*/piRrp2 and BbΔ*rpoS*/piRrp2. BbΔ*rpoN*/piRrp2 and BbΔ*rpoS*/piRrp2 were cultivated in the presence or absence of IPTG, and bacterial growth was monitored daily. BbΔ*rpoN*/piRrp2 and BbΔ*rpoS*/piRrp2 cultures grown without IPTG showed normal growth, whereas no growth was detected in the cultures supplemented with excess IPTG (Fig. 5A). The results with BbΔ*rpoN*/piRrp2 and BbΔ*rpoS*/piRrp2 were identical to those obtained with BbiRrp2 and BbiRrp2-Δ*rrp2* (data not shown and Fig. 4A). To confirm that the RpoN/RpoS regulatory network was not activated in BbΔ*rpoN*/piRrp2 and BbΔ*rpoS*/piRrp2, mutants were grown to the mid-exponential phase of growth and IPTG was added to induce *rrp2* expression. At 24 h postinduction, protein expression was assessed by immunoblotting (Fig. 5B). As expected, IPTG-treated BbΔ*rpoN*/piRrp2 and BbΔ*rpoS*/piRrp2 did not express OspC. While the mechanism by which the overexpression of *rrp2* inhibits *B. burgdorferi* growth is unknown, these data clearly demonstrate that growth inhibition arising from *rrp2* overexpression is not a consequence of RpoN/RpoS activation.

Summary and conclusions. While we do not know the identity of the specific essential genes that are regulated by *rrp2*, the application of the inducible system will make it possible to identify genes which are differentially regulated in BbiRrp2-Δ*rrp2*. We have shown that the BbiRrp2-Δ*rrp2* mutant can only grow in the presence of IPTG. Therefore, it is possible to wash the cells to remove IPTG so that we can assess gene expression changes when *rrp2* is not expressed. The identity of the gene(s) whose expression is affected by *rrp2* during overexpression also remains to be determined. Although the biological relevance of *rrp2* overproduction is unknown, it would be interesting to determine whether genes that are responsible for growth inhibition when *rrp2* is overex-

FIG 5 Effect of overexpression of *rrp2* in *rpoN* and *rpoS* mutant backgrounds. Cultures of *BbΔrpoN/piRrp2* and *BbΔrpoS/piRrp2* were grown in the presence (+ IPTG) or absence (– IPTG) of 1 mM IPTG. (A) Bacterial growth in each culture was assessed, and the values in the graphs represent the mean cell counts $\pm$ SD from two trials. (B) To assess *OspC* levels following the induction of *rrp2* with IPTG, cultures of *BbiRrp2*, *BbΔrpoN/piRrp2*, and *BbΔrpoS/piRrp2* were grown to the mid-exponential phase and then either left untreated or supplemented with 1 mM IPTG. Antibodies used to detect the respective proteins are indicated at the right. Values at the left denote relevant molecular masses (kDa) of the standard (MW).

pressed represent the same group of *rrp2*-regulated genes that are required for *B. burgdorferi* growth. The ideal approach to identify genes whose expression is altered during *rrp2* overexpression would be to utilize a strain in which the RpoN/RpoS pathway is inoperative, or to clone Rrp2(G239C) in an inducible expression construct and then assess changes in gene expression upon the induction of the point mutant in a wild-type *B. burgdorferi* background (41). Since EBPs such as *rrp2* control the transcription of their target genes through *rpoN*, it is difficult to predict the mechanism by which the expression of the genes in this branch of *rrp2*-dependent regulation would be controlled. To date, no one has been able to confirm that Rrp2 interacts with a specific DNA sequence upstream of the RpoN-dependent promoter of *rpoS* (5, 9). This presumed lack of specificity could provide a potential for promiscuity in activating genes at the transcriptional level, but how *rrp2*-dependent activation would function in the absence of *rpoN* is unclear.

In conclusion, we have provided the first definitive evidence that *rrp2* is essential in *B. burgdorferi*. The fact that the *B. burgdorferi* Rrp2(G239C) mutant described by Yang et al. (41) is unable to activate RpoN-dependent transcription but exhibits no growth

defect suggests that the essential function that *rrp2* serves is independent of its EBP activity. While there is evidence for at least one bacterial species in which RpoN is essential (e.g., *Myxococcus xanthus*) (23), this would mark the first demonstration of an NtrC family EBP serving a role, independent of its cognate EBP function, that is indispensable for bacterial viability, likely by controlling genes involved in cellular metabolism. Studies are under way to identify the *rrp2*-regulated gene products that contribute to borrelial growth and the manner by which their expression is regulated. Once these genes have been identified, we can begin to elucidate the mechanism by which Rrp2 controls genes independently of RpoN-mediated transcription.

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REFERENCES

- Anguita J, et al. 2000. *Borrelia burgdorferi* gene expression in vivo and spirochete pathogenicity. Infect. Immun. 68:1222–1230.
- Beck LL, Smith TG, Hoover TR. 2007. Look, no hands! Unconventional transcriptional activators in bacteria. Trends Microbiol. 15:530–537.
- Blevins JS, Hagman KE, Norgard MV. 2008. Assessment of decorin-binding protein A to the infectivity of *Borrelia burgdorferi* in the murine models of needle and tick infection. BMC Microbiol. 8:82.
- Blevins JS, Revel AT, Smith AH, Bachlani GN, Norgard MV. 2007. Adaptation of a luciferase gene reporter and *lac* expression system to *Borrelia burgdorferi*. Appl. Environ. Microbiol. 73:1501–1513.
- Blevins JS, et al. 2009. Rrp2, a σ^{54} -dependent transcriptional activator of *Borrelia burgdorferi*, activates *rpoS* in an enhancer-independent manner. J. Bacteriol. 191:2902–2905.
- Boardman BK, et al. 2008. Essential role of the response regulator Rrp2 in the infectious cycle of *Borrelia burgdorferi*. Infect. Immun. 76:3844–3853.
- Brahmachary P, Dashti MG, Olson JW, Hoover TR. 2004. *Helicobacter pylori* FlgR is an enhancer-independent activator of σ^{54} -RNA polymerase holoenzyme. J. Bacteriol. 186:4535–4542.
- Buck M, Gallegos MT, Studholme DJ, Guo Y, Gralla JD. 2000. The bacterial enhancer-dependent σ^{54} (σ^N) transcription factor. J. Bacteriol. 182:4129–4136.
- Burnnick MN, et al. 2007. Insights into the complex regulation of *rpoS* in *Borrelia burgdorferi*. Mol. Microbiol. 65:277–293.
- Caimano MJ, Eggers CH, Gonzalez CA, Radolf JD. 2005. Alternate sigma factor RpoS is required for the *in vivo*-specific repression of *Borrelia burgdorferi* plasmid lp54-borne *ospA* and *lp6.6* genes. J. Bacteriol. 187:7845–7852.
- Caimano MJ, et al. 2007. Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals provides insight into RpoS function during the enzootic cycle. Mol. Microbiol. 65:1193–1217.
- Caimano MJ, et al. 2011. The hybrid histidine kinase Hk1 is part of a two-component system that is essential for survival of *Borrelia burgdorferi* in feeding *Ixodes scapularis* ticks. Infect. Immun. 79:3117–3130.
- de Silva AM, Fikrig E. 1997. Arthropod- and host-specific gene expression by *Borrelia burgdorferi*. J. Clin. Invest. 99:377–379.
- de Silva AM, Telford SR, III, Brunet LR, Barthold SW, Fikrig E. 1996. *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. J. Exp. Med. 183:271–275.
- Eggers CH, Caimano MJ, Radolf JD. 2004. Analysis of promoter elements involved in the transcriptional initiation of RpoS-dependent *Borrelia burgdorferi* genes. J. Bacteriol. 186:7390–7402.
- Fisher MA, et al. 2005. *Borrelia burgdorferi* σ^{54} is required for mammalian infection and vector transmission but not for tick colonization. Proc. Natl. Acad. Sci. U. S. A. 102:5162–5167.

17. Fraser CM, et al. 1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature* 390:580–586.
18. Gilmore RD, Jr, Mbow ML, Stevenson B. 2001. Analysis of *Borrelia burgdorferi* gene expression during life cycle phases of the tick vector *Ixodes scapularis*. *Microbes Infect.* 3:799–808.
19. He M, et al. 2011. Cyclic di-GMP is essential for the survival of the Lyme disease spirochete in ticks. *PLoS Pathog.* 7:e1002133. doi:10.1371/journal.ppat.1002133.
20. Hubner A, et al. 2001. Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN-RpoS regulatory pathway. *Proc. Natl. Acad. Sci. U. S. A.* 98:12724–12729.
21. Hughes CA, Kodner CB, Johnson RC. 1992. DNA analysis of *Borrelia burgdorferi* NCH-1, the first northcentral U.S. human Lyme disease isolate. *J. Clin. Microbiol.* 30:698–703.
22. Joslin SN, Hendrixson DR. 2008. Analysis of the *Campylobacter jejuni* FlgR response regulator suggests integration of diverse mechanisms to activate an NtrC-like protein. *J. Bacteriol.* 190:2422–2433.
23. Keseler IM, Kaiser D. 1997. σ^{54} , a vital protein for *Myxococcus xanthus*. *Proc. Natl. Acad. Sci. U. S. A.* 94:1979–1984.
24. Kostick JL, et al. 2011. The diguanylate cyclase, Rrp1, regulates critical steps in the enzootic cycle of the Lyme disease spirochetes. *Mol. Microbiol.* 81:219–231.
25. Liang FT, et al. 2010. BB0250 of *Borrelia burgdorferi* is a conserved and essential inner membrane protein required for cell division. *J. Bacteriol.* 192:6105–6115.
26. Morett E, Segovia L. 1993. The σ^{54} bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. *J. Bacteriol.* 175:6067–6074.
27. Ohnishi J, Piesman J, de Silva AM. 2001. Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks. *Proc. Natl. Acad. Sci. U. S. A.* 98:670–675.
28. Ouyang Z, Blevins JS, Norgard MV. 2008. Transcriptional interplay among the regulators Rrp2, RpoN and RpoS in *Borrelia burgdorferi*. *Microbiology* 154:2641–2658.
29. Pollack RJ, Telford SR, III, Spielman A. 1993. Standardization of medium for culturing Lyme disease spirochetes. *J. Clin. Microbiol.* 31:1251–1255.
30. Ramamoorthy R, Philipp MT. 1998. Differential expression of *Borrelia burgdorferi* proteins during growth *in vitro*. *Infect. Immun.* 66:5119–5124.
31. Rappas M, Bose D, Zhang X. 2007. Bacterial enhancer-binding proteins: unlocking σ^{54} -dependent gene transcription. *Curr. Opin. Struct. Biol.* 17:110–116.
32. Rogers EA, et al. 2009. Rrp1, a cyclic-di-GMP-producing response regulator, is an important regulator of *Borrelia burgdorferi* core cellular functions. *Mol. Microbiol.* 71:1551–1573.
33. Schwan TG, Piesman J. 2000. Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. *J. Clin. Microbiol.* 38:382–388.
34. Schwan TG, Piesman J, Golde WT, Dolan MC, Rosa PA. 1995. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. *Proc. Natl. Acad. Sci. U. S. A.* 92:2909–2913.
35. Smith AH, Blevins JS, Bachlani GN, Yang XF, Norgard MV. 2007. Evidence that RpoS (σ^S) in *Borrelia burgdorferi* is controlled directly by RpoN (σ^{54}/σ^N). *J. Bacteriol.* 189:2139–2144.
36. Steere AC. 2001. Lyme disease. *N. Engl. J. Med.* 345:115–125.
37. Studholme DJ, Buck M. 2000. The biology of enhancer-dependent transcriptional regulation in bacteria: insights from genome sequences. *FEMS Microbiol. Lett.* 186:1–9.
38. Suk K, et al. 1995. *Borrelia burgdorferi* genes selectively expressed in the infected host. *Proc. Natl. Acad. Sci. U. S. A.* 92:4269–4273.
39. Xu H, et al. 2010. Role of acetyl-phosphate in activation of the Rrp2-RpoN-RpoS pathway in *Borrelia burgdorferi*. *PLoS Pathog.* 6:e1001104. doi:10.1371/journal.ppat.1001104.
40. Yang X, et al. 2000. Interdependence of environmental factors influencing reciprocal patterns of gene expression in virulent *Borrelia burgdorferi*. *Mol. Microbiol.* 37:1470–1479.
41. Yang XF, Alani SM, Norgard MV. 2003. The response regulator Rrp2 is essential for the expression of major membrane lipoproteins in *Borrelia burgdorferi*. *Proc. Natl. Acad. Sci. U. S. A.* 100:11001–11006.
42. Yang XF, et al. 2005. Analysis of the *ospC* regulatory element controlled by the RpoN-RpoS regulatory pathway in *Borrelia burgdorferi*. *J. Bacteriol.* 187:4822–4829.
43. Yang XF, Pal U, Alani SM, Fikrig E, Norgard MV. 2004. Essential role for OspA/B in the life cycle of the Lyme disease spirochete. *J. Exp. Med.* 199:641–648.