Synthesis of RpoS Is Dependent on a Putative Enhancer Binding Protein Rrp2 in *Borrelia burgdorferi*

Zhiming Ouyang, Jianli Zhou, Michael V. Norgard*

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas, United States of America

**Abstract**

The RpoN-RpoS regulatory pathway plays a central role in governing adaptive changes by *B. burgdorferi* when the pathogen shuttles between its tick vector and mammalian hosts. In general, transcriptional activation of bacterial RpoN (σ^54^)-dependent genes requires an enhancer binding protein. *B. burgdorferi* encodes the putative enhancer binding protein Rrp2. Previous studies have revealed that the expression of σ^54^-dependent *rpoS* was abolished in an *rrp2* point mutant. However, direct evidence linking the production of Rrp2 in *B. burgdorferi* and the expression of *rpoS* has been lacking, primarily due to the inability to inactivate *rrp2* via deletion or insertion mutagenesis. Herein we introduced a regulatable (IPTG-inducible) *rrp2* expression shuttle plasmid into *B. burgdorferi*, and found that the controlled up-regulation of Rrp2 resulted in the induction of σ^54^-dependent *rpoS* expression. Moreover, we created an *rrp2* conditional lethal mutant in virulent *B. burgdorferi*. By exploiting this conditional mutant, we were able to experimentally manipulate the temporal level of Rrp2 expression in *B. burgdorferi*, and examine its direct impact on activation of the RpoN-RpoS regulatory pathway. Our data revealed that the synthesis of RpoS was coincident with the IPTG-induced Rrp2 levels in *B. burgdorferi*. In addition, the synthesis of OspC, a lipoprotein required by *B. burgdorferi* to establish mammalian infection, was rescued in the *rrp2* point mutant when RpoS production was restored, suggesting that Rrp2 influences *ospC* expression indirectly via its control over RpoS. These data demonstrate that Rrp2 is required for the synthesis of RpoS, presumably via its action as an enhancer binding protein for the activation of RpoN and subsequent transcription of *rpoS* in *B. burgdorferi*.

**Introduction**

*Borrelia burgdorferi*, the causative agent of Lyme disease, is sustained in nature via a complex life cycle involving an arthropod tick vector (*Ixodes scapularis*) and mammals [1,2]. During its transit between these two markedly different host and tick milieus, *B. burgdorferi* undergoes significant adaptive changes. In *B. burgdorferi*, host adaptation is achieved by dramatic changes in gene expression in response to various tick or host stimuli [3–9]. Among a number of potential regulators that have been postulated to be present in *B. burgdorferi* [10–45], a novel genetic regulatory pathway, the RpoN-RpoS pathway (or the σ^54^-σ^54^ cascade) [18], plays a central role in modulating *B. burgdorferi* host adaptive responses and virulence expression. In this pathway, one alternative sigma factor (σ^54^, RpoN) controls the expression of another alternative sigma factor (σ^54^, RpoS) through binding to a canonical −24/−12 promoter sequence [12,26,40]. Once RpoS is produced, it functions as a master regulator to modulate the expression of a number of virulence-associated outer membrane lipoproteins such as outer surface lipoproteins (Osp) C and A, and decorin binding proteins (Dbp) B and A [1,3,8,12–14,18,29,32,36,40,46–57].

Transcriptional activation of σ^54^-dependent genes in bacteria requires a bacterial enhancer binding protein (hEBP), which is an AAA+ activator ATPase [58–63]. Sequence analyses have indicated that Rrp2 (BB0763) is composed of three structural domains, including an N-terminal regulatory domain (R), a central AAA+ ATPase core domain (C), and a C-terminal DNA binding domain (D), suggesting that Rrp2 may function as a hEBP to activate σ^54^ dependent *rpoS* transcription in *B. burgdorferi* [15,45]. Previously, by exploiting a variant carrying a point mutation G239C in the C domain of RpoS, we [10,29,45] reported that the *rrp2* point mutant has been hitherto the only evidence to support the role of Rrp2 in the activation of the RpoN-RpoS regulatory pathway. Despite this important finding, there remain unanswered questions concerning the roles of Rrp2 in *B. burgdorferi* gene regulation. In particular, the finding that expression of *rpoS* was abolished in the *rrp2* point mutant has been hitherto the only evidence to support the role of Rrp2 in the activation of the RpoN-RpoS regulatory pathway. A direct link between Rrp2 protein levels produced in *B. burgdorferi* and the expression of *rpoS* has been lacking, primarily due to the inability to inactivate *rrp2* via deletion or insertion mutagenesis. The G239C point mutation in *rrp2* presumably abolishes the putative ATPase activity required for σ^54^-dependent *rpoS* transcriptional activation. However, it also remains possible that the G239C point mutation causes a change in Rrp2’s overall conformation, thereby preventing *rpoS* transcription in the *rrp2* point mutant. In addition, although the expression of *ospC* was found to be lost in the *rrp2* point mutant, how Rrp2 ultimately controls the expression of this key virulence-associated
lipoprotein remains unknown. Rrp2 may indirectly modulate ospC expression via its control over RpoS. Alternatively, given that ospC was found to be constitutively expressed in B. burgdorferi when ospH and rpoS were inactivated [64], Rrp2 may control ospC expression through another RpoS-independent factor(s). It is also possible that Rrp2 may directly modulate ospC expression by binding to its promoter. To address these questions, we employed an artificial gene expression system [65,66] to experimentally control the protein levels of Rrp2 synthesized in B. burgdorferi, and examined its impact on rpoS expression. Such a strategy affects only the levels of Rrp2 produced in B. burgdorferi, and does not alter the overall structure of the protein. Our data show that the expression level of Rrp2 correlates closely with the expression of rpoS, indicating that Rrp2 activates the expression of σ54-dependent rpoS which, in turn, modulates ospC expression in B. burgdorferi.

Materials and Methods

Bacterial Strains and Culture Conditions

All strains and plasmids used in this study are described in Table 1. Low-passage infectious wild-type B. burgdorferi strain 297 [67], and the rpoS point mutant OY01 [29], were routinely cultured at 37°C and 5% CO2 in either BSK-II medium [68] or BSK-H medium (Sigma) supplemented with 6% rabbit serum (Pel-Freeze). When appropriate, supplements were added to media at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 100 μg/ml; streptomycin, 100 μg/ml. Spirochetes were enumerated by dark-field microscopy. E. coli strains were cultured in Lysogeny Broth (LB) supplemented with appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 100 μg/ml.

| Table 1. Strains and plasmids used in this study. |
|--------------------------------------------------|
| **Strains or plasmids** | **Description** | **Source** |
| **B. burgdorferi** | | |
| 297 | Infectious B. burgdorferi, human spinal fluid isolate | [67] |
| OY01 | 297, rpoS(G239C) point mutant | [29] |
| AH206 | 297, rpoS:ermC | [18] |
| OY159 | 297 transformed with pRrp2 | This study |
| OY160 | OY01 transformed with pRrp2 | This study |
| OY173 | OY01 transformed with pRrp2-FLAG | This study |
| OY179 | rpoS conditional lethal mutant, OY173 transformed with pOY202 | This study |
| **E. coli** | | |
| Top10F | F’ lacIq Tn10(Tet') mcrA Δ(mnr-hsdRMS-mcrBC) Δ800lacZAM15 ΔlacX74 recA1 ara139 Δ (ara-leu)7697 galiU galiK rpsL (Str') endA1 nupG | Invitrogen |

| **Plasmids** | | |
| pGEM-Teasy | TA cloning vector; Amp' | Promega |
| pJSB275 | Shuttle vector, Spec/Strep' | [69] |
| pRpoS | IPTG-inducible rpoS expression construct | [30] |
| pOY100 | PCR product of 86F/87R cloned into pGEM-Teasy, amp' | This study |
| pJD55 | Shuttle vector, Spec/Strep' | [46] |
| pOY202 | Plg8-kan cloned into pOY100, Amp/Kan' | This study |
| pRrp2 | PCR product of 303F/303R cloned into pOY202, Spec/Strep' | This study |
| pRrp2-FLAG | PCR product of 303F/263R cloned into pJSB275, Spec/Strep' | This study |

Generation of the IPTG-inducible rrp2 Expression Construct

To experimentally control rrp2 expression in B. burgdorferi, one rrp2 expression construct pRrp2 was generated by using the lac-based gene inducible expression system [65,66]. Briefly, rrp2 was amplified from B. burgdorferi using primers ZM303F and 303R (Table S1), and then cloned into pJSB275 [69] at the NdeI site. In pRrp2, rrp2 transcription is directly controlled by the IPTG-inducible T5 promoter (a.k.a. the promoter PpQE30) from plasmid pQE30 (Qiagen).

Generation of the rrp2 Conditional Lethal Mutant OY179

To create an rrp2 conditional mutant, we first generated one shuttle plasmid pRrp2-FLAG in which rrp2 expression was placed under the control of the IPTG-inducible promoter PpQE30. For creating pRrp2-FLAG, a DNA fragment encoding Rrp2-FLAG was amplified by using ZM303F and 303R (Table S1), and cloned into pJSB275 [69] at the NdeI site. This strategy adds a FLAG-tag (DYKDDDDK) to the C-terminus of Rrp2, thereby facilitating the detection of Rrp2 in B. burgdorferi. The plasmid pRrp2-FLAG was then electroporated into the rrp2 point mutant OY01, yielding the streptomycin-resistant strain OY179. B. burgdorferi transformation was performed as previously described [29,70].

To inactivate rrp2 in B. burgdorferi through homologous recombination, a suicide plasmid pOY202 was created. Briefly, the left arm for creating pOY202 was PCR-amplified using primers ZM86F and ZM86R, whereas the right arm was amplified using ZM87F and ZM87R (Table S1). After digestion with AscI, these two fragments were fused together. By using this ligated DNA as the template, PCR was employed to amplify a fragment comprising the upstream and downstream regions of rrp2 by using primers ZM86F and ZM87R. The obtained fragment was cloned into pJSB275 at the NdeI site.
into strain OY01 (rrp2) and cloned into pJSB275. The plasmid pRrp2 was then transformed into B. burgdorferi strain OY173. Transformants were isolated in the presence of kanamycin and streptomycin, along with 0.05 mM of IPTG to allow the production of Rrp2-FLAG during selection. IPTG-inducible expression shuttle plasmid. To create pRrp2, pRrp2 was amplified from B. burgdorferi strain OY01 (rrp2[G239C]), yielding OY160. SDS-PAGE (B) and immunoblot (C) were performed to analyze gene expression in OY160. Bacteria were grown at 37°C in BSK-II medium with various concentrations of IPTG. When bacterial growth reached ~10^8 cells per ml, spirochetes were harvested. Approximately 4×10^10 spirochetes were loaded onto each lane of a 12.5% SDS-PAGE gel. In (B), approximate molecular masses are indicated at the left in kDa; concentrations of IPTG are indicated above the image; and the arrow indicates OspC. Specific antibodies, denoted as α- used in the immunoblot (C), are indicated on the left.

RNA Isolation and qRT-PCR

RNA isolation and qRT-PCR were performed as previously described [29–31,71]. Briefly, when total RNA was isolated from B. burgdorferi by using Trizol (Invitrogen), RNase-free DNase I (GenHunter Corporation) was used to digest genomic DNA. After RNA was further purified using RNeasy Mini Kit (Qiagen), 1 μg of RNA was used to synthesize cDNA using the SuperScript III Platinum Two-step qRT-PCR kit according to the manufacturer’s protocol (Invitrogen). qRT-PCR was employed to examine gene expression, using the relative quantification method (ΔΔCT) as described [29–31,71]. Gene expression fold change was presented as mean ± SE values from three independent experiments. Statistical analyses of the data were performed using the Student’s t test.

Results and Discussion

Complementation of the rrp2 Point Mutation by using an IPTG-inducible rrp2 Expression Construct

Previously, we introduced a G239C mutation into rrp2 and created an rrp2 point mutant OY01 (rrp2[G239C]) in B. burgdorferi [29]. The expression of rpoS and ospC is abolished in OY01. To confirm that the loss of rpoS and ospC expression in the rrp2 point mutant was due solely to the mutation of rrp2, a trans-complementation approach was employed. To this end, an IPTG-inducible plasmid pRrp2 (Fig. 1A) was created by placing the expression of rrp2 under the control of the IPTG-inducible PpQE30 promoter. By adjusting the amount of the inducer (IPTG) added to the medium, the expression of rrp2 on pRrp2 could be controlled. pRrp2 was then introduced into the rrp2 point mutant OY01, yielding the streptomycin-resistant strain OY160.

To induce the expression of wild-type Rrp2, OY160 was grown in BSK-II medium containing varying concentrations of IPTG. In this experiment and subsequently, bacterial growth and morphology were not affected when spirochetes were grown in media with indicated levels of IPTG. Cells were collected when bacterial growth reached early stationary phase (~10^8 cells/ml). As shown in Fig. 1C, when 10-, 20-, 50-, or 100-μM of IPTG was added into the media, synthesis of Rrp2 was enhanced in a dose-dependent manner in B. burgdorferi. Of note, when IPTG was not added into the medium, a band was also detected in immunoblot by using the Rrp2 antibody; this band represents the existing mutated protein Rrp2[G239C]. The induction of Rrp2 also resulted in the synthesis of RpoS and OspC in OY160. As shown in Fig. 1B and Fig. 1C, when bacteria were grown in BSK-II containing 50- or 100-μM of IPTG, RpoS and OspC were readily detected in OY160, and the increased protein levels correlated well with the increased levels of Rrp2 in OY160. These data suggest that Rrp2 expressed from pRrp2 is capable of complementing the rrp2 point mutation, thereby activating the RpoN-RpoS pathway in B. burgdorferi.

Up-regulation of Rrp2 in B. Burgdorferi Induces the Expression of RpoS and OspC

The approach of gene overexpression has proven to be a highly valuable tool for examining gene functions, particularly for genes that cannot be inactivated [72]. This strategy was successfully employed previously to study the role of Rrp2 in B. burgdorferi, where it was reported that overexpression of Rrp2 in B. burgdorferi led to the induction of OspC [69]. However, it remained unknown whether RpoS synthesis was influenced by the overexpression of Rrp2 in B. burgdorferi. In this current study, the IPTG-inducible
A 297/pRrp2
IPTG, μM 0 10 20 30 50 100

B
α-Rrp2
α-RpoS
α-OspC
α-DbpA

Figure 2. Up-regulation of Rrp2 in B. burgdorferi induces the expression of rpoS. Gene expression in OY159 was analyzed by SDS-PAGE (A), immunoblot (B, C), and qRT-PCR analyses (D). In (A) and (B), spirochetes grown in BSK-II media containing varying concentrations of IPTG were harvested when bacterial growth reached early stationary phase (~10^6 cells per ml). In (C) and (D), spirochetes were grown in BSK-II medium. When bacterial growth reached mid-log phase (~10^7 cells per ml), various amounts of IPTG were added into culture. Cells were collected at 9 h post-induction. In (A) and (C), concentrations of IPTG are indicated above the image. The arrow indicates OspC in (A). Specific antibodies, denoted as α- in the immunoblot (B, C), are indicated on the left. In (D), data were collected from three independent experiments, and the bars represent the mean measurements ± standard deviation. The mean values between induced groups (100-, 200-, or 500-fold IPTG) and the uninduced group (0 μM IPTG) were compared using the Student’s t test and are significantly different (p<0.05). For data normalization, the B. burgdorferi flaB gene was used as an internal control.

doi:10.1371/journal.pone.0096917.g002

The rrp2 expression construct pRrp2 was introduced into B. burgdorferi wild-type strain 297, yielding the merodiploid strain OY159. Gene expression in these spirochetes was measured through SDS-PAGE, semi-quantitative immunoblot, and quantitative RT-PCR analyses. In this experiment, two strategies employing shorter or longer induction times were used to induce Rrp2 in B. burgdorferi. For the longer induction time, bacteria were continuously grown in BSK-II containing various amounts of IPTG for about 7 days and harvested when growth reached the early stationary phase (~10^6 cells/ml). As shown in Fig. 2B, synthesis of Rrp2 in OY159 was enhanced when bacteria were grown in media containing IPTG (compared with spirochetes cultivated in media without IPTG). Moreover, synthesis of RpoS and OspC was also found to be enhanced when IPTG was added into the medium (Fig. 2A, 2B). To exclude the possibility that gene induction might be an indirect effect resulting from prolonged exposure to IPTG, a shorter (9 h) induction period was also examined. When bacterial growth in BSK-II reached mid-log phase (~10^7 cells/ml), varying amounts of IPTG were added into the media. After 9 h of induction, spirochetes were collected and gene expression was examined. As shown in Fig. 2C, immunoblot analyses showed that the syntheses of both Rrp2 and RpoS were induced by IPTG in a dose-dependent manner. Gene expression was also measured by using real-time quantitative RT-PCR (qRT-PCR) analyses. When gene transcription in spirochetes grown in BSK-II with 20-, 100-, 200-, or 500-μM of IPTG was compared with gene expression in bacteria grown without IPTG, transcription of rrp2 was induced at 1.3-, 8.8-, 12.4-, or 18.9-fold, respectively; accordingly, rpoS transcription was induced at 0.9-, 5.6-, 9.4-, or 17.1-fold, respectively. These combined data strongly suggest that Rrp2 is responsible for the induction of RpoS in B. burgdorferi.

Generation of an rrp2 Conditional Mutant in B. burgdorferi

Given that many attempts to fully inactivate rrp2 in B. burgdorferi have failed, and that rrp2 thus seems to be essential for B. burgdorferi in vitro growth [69], we generated a rrp2 conditional mutant in B. burgdorferi using a similar approach as described previously [69, 73]. In this conditional lethal mutant, the wild-type chromosomal copy of rrp2 is disrupted; rrp2 is expressed from an IPTG-inducible shuttle plasmid. As a prelude to this approach, we first created another IPTG-inducible rrp2 expression construct pRrp2-FLAG (Fig. 3A). From this shuttle plasmid, the expression of rrp2 in B. burgdorferi is tightly controlled by IPTG added into the medium. To assist in the detection of Rrp2, a DNA fragment encoding the FLAG tag was fused to the 3’ of rrp2. Because adding a FLAG tag to Rrp2 could affect its general function, this plasmid pRrp2-FLAG was initially introduced into the rrp2 point mutant OY01 (rrp2[G239C]) to create the strain OY173. OY173 was then used to test whether Rrp2-FLAG was functional in activating σ54-dependent rpoS expression. OY173 bacteria were grown continuously in media with varying concentrations of IPTG, and collected when growth reached the early stationary phase. As shown in Fig. 3C, when IPTG was not added into the medium, Rrp2-FLAG was not detected. However, when 20-, 50-, 100-, or 200-μM of IPTG was added into the media, the synthesis of Rrp2-FLAG was enhanced in a dose-dependent manner. The produc-
**Figure 3. Gene expression in B. burgdorferi strain OY173.** (A) Construction of an IPTG-inducible *rrp2*-FLAG expression shuttle plasmid. The plasmid pRrp2-FLAG pRrp2 was introduced into strain OY01 (*rrp2*[G239C]), yielding OY173. SDS-PAGE (B), immunoblot (C, D), and qRT-PCR analyses (E) were performed to analyze gene expression. In (B) and (C), spirochetes grown in BSK-II medium containing varying concentrations of IPTG were harvested when bacterial growth reached early stationary phase (~10^8 cells per ml). In (D) and (E), spirochetes were grown in BSK-II medium. When bacterial growth reached mid-log phase (~10^7 cells per ml), various amounts of IPTG were added into culture. Cells were collected at 9 h post-induction. In (B) and (D), concentrations of IPTG are indicated above the image. The arrow indicates OspC in (B). Specific antibodies, denoted as a- used in the immunoblot (C, D), are indicated on the left. In (E), the bars represent the mean measurements ± standard deviation. The mean values between induced groups (100-, 200-, or 500 µM IPTG) and the uninduced group (0 µM IPTG) were compared using the Student’s *t* test and are significantly different (p < 0.05). For data normalization, the *B. burgdorferi flaB* gene was used as an internal control. doi:10.1371/journal.pone.0096917.g003

**Figure 4. Generation of an *rrp2* conditional lethal mutant in B. burgdorferi.** (A) Schematic representation of the *bb0764–bb0761* genes in the *B. burgdorferi* chromosome and the insertion of *PflgB-kan* cassette into *rrp2* by homologous recombination. Arrows indicate the approximate positions of the oligonucleotide primers used for subsequent analyses. (B) Analyses of the wild-type 297 and the *rrp2* conditional lethal mutant OY179 by PCR. The specific primer pairs are indicated on the right. Lanes WT, 297; lanes M1, M2, and M3, three clones of OY179. doi:10.1371/journal.pone.0096917.g004

**Figure 5. Gene expression in the *rrp2* conditional lethal mutant OY179.** SDS-PAGE (A) and semi-quantitative immunoblot (B) analyses were performed to analyze gene expression. Bacteria were grown at 37°C in BSK-II medium with various concentrations of IPTG. When bacterial growth reached ~10^7 cells per ml, spirochetes were harvested. Approximately 4 x 10^7 spirochetes were loaded onto each lane of a 12.5% SDS-PAGE gel. Concentrations of IPTG are indicated above the image, and the arrow in (A) indicates OspC. Specific antibodies, denoted as a- used in the immunoblot (B), are indicated on the left. doi:10.1371/journal.pone.0096917.g005
Also, PCR amplification revealed that strain OY179 contained the rpoS copy from the conditional mutant OY179 (lanes M1, M2, and M3). The inactivation of chromosomal rrp2 was targeted (conferring streptomycin resistance) (Fig. 4B). The growth of the conditional mutant OY179 was assessed by cultivating the bacteria in BSK-II containing 30-, 50-, or 100-µM IPTG. OY179 grew in BSK-II medium containing various concentrations of IPTG and gene expression was analyzed by SDS-PAGE (A) and immunoblot (B). The arrow in (A) indicates OspC. Specific antibodies, denoted as α, used in the immunoblot (B) are indicated on the left.

doi:10.1371/journal.pone.0096917.g006

Expression of rrp2 Correlates with the Expression of RpoS and OspC in the rrp2 Conditional Mutant

To assess whether the protein level of Rrp2 correlated with the expression of rpoS, gene expression in OY179 was measured via SDS-PAGE and semi-quantitative immunoblot analyses. As this end, OY179 was grown in BSK-II with varying concentrations of IPTG and cultures were harvested when cell growth reached early stationary phase. For bacteria grown under these conditions, no obvious differences were observed when spirochete morphology and motility were examined using dark-field microscopy. As shown in Fig. 5B, immunoblot analyses revealed that OY179 produced Rrp2-FLAG but not native Rrp2. Moreover, the levels of Rrp2-FLAG were dependent on the concentrations of the inducer IPTG. Specifically, when 20-µM IPTG was added into the medium, the level of Rrp2-FLAG produced in OY179 was ∼3-fold lower than the level of Rrp2 observed in WT strain 297. When bacteria were cultivated in BSK-II containing 30-µM IPTG, Rrp2-FLAG was produced at a level commensurate with the level of Rrp2 in WT strain 297. When 50- or 100-µM IPTG was added into the medium, Rrp2-FLAG produced in OY179 was 3.5-fold or 10.3-fold higher, respectively, than the level of Rrp2 in strain 297. In addition, there was a tight correlation between the levels of Rrp2-FLAG and the levels of RpoS and OspC (Fig. 5A and 5B). Taken together, the increased synthesis of RpoS was coincident with the IPTG-inducible production of Rrp2-FLAG, supporting that Rrp2 activates the RpoN-RpoS pathway in B. burgdorferi.

Rrp2 Indirectly Controls the Expression of OspC and DbpA via RpoS

Although the expression of ospC and dbpA was found to be lost in the rrp2 point mutant [10,29,45], how Rrp2 ultimately controls the expression of these key lipoproteins has remained somewhat unclear. Given that (1) the expression of ospC and dbpA is directly regulated by RpoS through RpoS-specific promoters [74–76], and (2) rpoS transcription is abolished in the rrp2 point mutant [10,29,45], we have hypothesized that Rrp2 likely regulates the expression of rpoS which, in turn, influences ospC and dbpA expression. To further test this hypothesis, we generated an IPTG-inducible rpoS expression shuttle construct pRpoS (i.e., pOY110) [30], in which rpoS expression is controlled solely by the IPTG-inducible PpQF30 promoter. This construct was introduced into the rrp2 point mutant OY01, to investigate whether the IPTG-induced RpoS could restore ospC and dbpA expression in this mutant. As shown in Fig. 6A and 6B, when RpoS was induced from pRpoS by IPTG, production of OspC and DbpA was consequently rescued. As aforementioned, when the rrp2 point mutation was complemented by the IPTG-inducible rrp2 expression construct pRrp2, expression of ospC was restored in the complemented strain OY160 (Fig. 1B and 1C). Consistent with previous findings [69], however, when pRrp2 was introduced into a B. burgdorferi rpoS mutant AH206 (ΔrpoS), RpoS and OspC were not produced in this strain, despite the fact that Rrp2 synthesis was induced by IPTG (Fig. S2). These combined data suggest that controlled induction of RpoS can overcome the Rrp2 deficiency, which constitutes compelling evidence that Rrp2 indirectly controls ospC and dbpA expression via RpoS.
Conclusions

This is the first study, to our knowledge, to show that the level of Rrp2 produced in *B. burgdorferi* directly correlates with RpoS levels. Moreover, our study provides further validation for the application of an IPTG-inducible expression system [65,66] for assessing *B. burgdorferi* gene regulation. By using this system, an rrp2 conditional lethal mutant was generated. Given the inability to inactivate rrp2 by conventional deletion or insertion mutagenesis, and the fact that both σ^54^ and RpoS can be readily inactivated in *B. burgdorferi* [14,18,29,48,49], Rrp2 likely also controls the expression of genes independent of σ^54^ or RpoS control, which in turn suggests that Rrp2 may function as a unique bEBP (other characterized bEBPs activate only σ^54^-dependent promoters). Our new conditional mutant thus provides an innovative way not only to define the direct control of Rrp2 over the central RpoN-RpoS pathway, but also to interrogate the overall regulatory role of Rrp2 in *B. burgdorferi* gene regulation. For example, by profiling gene expression in this new conditional mutant, it may be possible to identify novel Rrp2-controlled, σ^54^- or RpoS-independent genes essential for *B. burgdorferi* growth or survival. Such studies will likely uncover new virulence-associated genes important for spirochetal pathogenesis, but also will provide the first definitive evidence that Rrp2 acts as an atypical bEBP to orchestrate virulence expression in *B. burgdorferi*. Although Rrp2 was presumed to be activated through phosphorylation [10,29,43], it still remains unanswered whether or how phosphorylated Rrp2 dynamically controls rpoS expression. Future work, such as pulse-chase analyses of the rrp2 point mutant OY01 trans-complemented with rrp2 variants, may help address these questions.

Supporting Information

**Figure S1** Growth of the rrp2 conditional mutant OY179 in vitro. *B. burgdorferi* was inoculated into BSK-II medium with various concentrations of IPTG at 100 spirochetes/ml. Spirochetes were enumerated using darkfield microscopy. Values are the means from three independent experiments. Error bars indicate standard deviations (n = 3). (TIF)

**Figure S2** Overexpression of Rrp2 does not restore expression of *OspC* and *DbpA* in the RpoS mutant. The rpoS point mutant AH206 harboring the IPTG-inducible rrp2 construct (pRrp2) was grown at 37°C with various concentrations of IPTG and gene expression was analyzed by SDS-PAGE (A) and immunoblot (B). RpoS and OspC were not detected in this strain, which is consistent with previous findings [69]. Specific antibodies, denoted as α, used in the immunoblot (B) are indicated on the left. (TIF)

**Table S1** Oligonucleotide primers used in this study. (DOCX)

**Author Contributions**

Conceived and designed the experiments: ZO MVN. Performed the experiments: ZO JZ. Analyzed the data: ZO MVN. Wrote the paper: ZO MVN.

References

1. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, et al. (1982) Lyme disease—a tick-borne spirochetal illness? Science 216: 1317–1319.
2. Steere AC (1993) Current understanding of Lyme disease. Hosp Pract (Off Ed) 28: 37–44.
3. Croither TR, Champion CI, Whitlegg JP, Aguila R, Wu XY, et al. (2004) Temporal analysis of the antigenic composition of *Borrelia burgdorferi* during infection in rabbit skin. Infect Immun 72: 5063–5072.
4. de Silva AM, Telford SR 3rd, Brunet LR, Barthold SW, Fikrig E; (1996) *Borrelia burgdorferi* OspA is an arthropod-specific transmembrane protein of the Lyme disease vaccine. J Exp Med 183: 271–275.
5. Liang FT, Nelson FK, Fikrig E (2002) Molecular adaptation of *Borrelia burgdorferi* in the murine host. J Exp Med 196: 273–280.
6. Montgomery RR, Malaisse SE, Feen KJ, Bockerstedt LK (1996) Direct demonstration of antigenic substitution of *Borrelia burgdorferi* ex vivo: exploration of the paradox of the early immune response to outer surface proteins A and C in Lyme disease. J Exp Med 183: 261–269.
7. Onishi J, Piezman 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.
8. Schwan TG, Piezman 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: 382–384.
9. Schwan TG, Piezman 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.
10. Boardman BK, He M, Ouyang Z, Xu H, Pang X, et al. (2008) Essential role of Fur in virulence factor RpoS for dissemination within the vector *Borrelia burgdorferi*. Mol Microbiol 65: 277–289.
11. Boylan JA, Posey JE, Gherardini FC (2003) *Borrelia* oxidative stress response regulator, OsrR: a distinctive Zu-dependent transcriptional activator. Proc Natl Acad Sci U S A 100: 11064–11069.
12. Burtnick MN, Downey JS, Beett PJ, Boylan JA, Frye JG, et al. (2007) Insights into the complex regulation of *OspA* in *Borrelia burgdorferi*. Mol Microbiol 65: 277–297.
13. Dunham-Emo SM, Caimano MJ, Eggers CH, Rudolf JJ (2012) *Borrelia burgdorferi* requires the alternative sigma factor RpoS for dissemination in the vector during tick-to-mammal transmission. PLoS Pathog 8: e1002532.
14. Fisher MA, Grimm D, Henson AK, Elias AF, Stewart PE, et al. (2005) *Borrelia burgdorferi* σ^54^-dependent *fur* expression is required for mammalian infection and vector transmission but not for tick colonization. Proc Natl Acad Sci U S A 102: 5162–5167.
15. Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, et al. (1997) Genomic sequence of a Lyme disease spirochete, *Borrelia burgdorferi*. Nature 390: 580–586.
16. Freedman JC, Rogers EA, Kostick JL, Zhang H, Iyer R, et al. (2010) Identification and molecular characterization of a cyclic-di-GMP effector protein, PlzA (BB0733): additional evidence for the existence of a functional cyclic-di-GMP regulatory network in the Lyme disease spirochete, *Borrelia burgdorferi*. FEMS Immunol Med Microbiol 58: 295–299.
17. He M, Ouyang Z, Trexell B, Xu H, Moh A, et al. (2011) Cyclic-di-GMP is essential for the survival of the Lyme disease spirochete in ticks. PLoS Pathog 7: e1002133.
18. Hubner A, Yang X, Norden DM, Popova TG, Cabell BC, 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.
19. Hyde JA, Shaw DK, Smith Iii R, Trzeciakowski JP, Skare JT (2009) The BosR regulatory protein of *Borrelia burgdorferi* interfaces with the RpoS regulatory pathway and modulates both the oxidative stress response and pathogenic properties of the Lyme disease spirochete. Mol Microbiol 74: 1344–1355.
20. Jutras BL, Cenail AM, Carroll DW, Miller MC, Zhu H, et al. (2013) Bpnr, the Lyme disease spirochete’s PUR domain protein: identification as a transcriptional modulator and characterization of nucleic acid interactions. J Biol Chem 288: 26220–26234.
21. Jutras BL, Verma A, Adams CA, Brissette CA, Burns LH, et al. (2012) BpaB and EfbC, DNA-binding proteins regulate production of the Lyme disease spirochete’s infection-associated lipoprotein protein. J Bacteriol 194: 775–786.
22. Karna SL, Sanjuan E, Esteve-Gassent MD, Miller CL, Mamakova M, et al. (2011) CorA modulates levels of lipoproteins and key regulators of gene expression critical for pathogenic mechanisms of *Borrelia burgdorferi*. Infect Immun 79: 732–744.
23. Katona LJ, Tskarz R, Kuhlbov C J, Benach J, Benach JL (2004) The fur homologue in *Borrelia burgdorferi*. J Bacteriol 186: 6443–6456.
24. Kostick JL, Szkotnicki LT, Rogers EA, Bocci P, Raffaelli N, et al. (2011) The dimethylated cyclic-di-GMP effector Rrp1 regulates critical steps in the enzootic cycle of the Lyme disease spirochete. Mol Microbiol 81: 219–231.
25. Lybecke MC, Abel CA, Feig AL, Samuels DS (2010) Identification and function of the RNA chaperone Hfq in the Lyme disease spirochete *Borrelia burgdorferi*. Mol Microbiol 78: 622–633.
26. Lybecke MC, Samuels DS (2007) Temperature-induced regulation of RpoS by a small RNA in *Borrelia burgdorferi*. Mol Microbiol 64: 1075–1089.
27. Medrano MS, Policastro PF, Schwan TG, Coburn J (2010) Interaction of *Borrelia burgdorferi* Hbb with the p66 promoter. Nucleic Acids Res 38: 414–427.
44. Xu Q, Shi Y, Dadhwal P, Liang FT (2012) RpoS regulates essential virulence determinants. Infect Immun 72: 6433–6445.

45. Tong L, Breitling R, Telford SR 3rd, Spielman A (2003) Standardization of medium for culturing Lyme disease spirochetes. J Clin Microbiol 31: 1251–1255.

46. Hughes CA, Kodner CB, Johnson RC (1992) DNA analysis of Borrelia burgdorferi. J Clin Microbiol 30: 690–703.

47. Pollack RJ, Telford SR 3rd, Spielman A (1993) Standardization of medium for culturing Lyme disease spirochetes. J Clin Microbiol 31: 1251–1255.

48. Groshong AM, Gibbons NE, Yang XF, Blevins JS (2012) Rrp2, a prokaryotic enhancer-like binding protein, is essential for viability of Borrelia burgdorferi. J Bacteriol 194: 3336–3347.

49. Samuels DS (1995) Electromorphism of the spirochete Borrelia burgdorferi. Methods Mol Biol 47: 253–259.

50. Ouyang Z, He M, Oman T, Yang XF, Norgard MV (2007) Allosteric regulation of a bacterial DNA polymerase. FEMS Microbiol Rev 31: 611–627.

51. Pal U, de Silva AM, Montgomery RR, Fish D, Anguita J, et al. (2000) Attachment of Borrelia burgdorferi within Ixodes scapularis mediated by outer surface protein A. J Clin Invest 106: 561–569.

52. Pal U, Yang X, Chen M, Bockenstedt LK, Anderson JF, et al. (2004) OspC facilitates Borrelia burgdorferi invasion of Ixodes scapularis salivary glands. J Clin Invest 113: 220–230.

53. Shi Y, Xu Q, McShan K, Liang FT (2008) Both decorin-binding proteins A and B are critical for overall virulence of Borrelia burgdorferi. Infect Immun 76: 1239–1246.

54. Tilly K, Krum JG, Bestor A, Jewett MW, Grimm D, et al. (2006) Borrelia burgdorferi OspC protein required exclusively in a crucial early stage of mammalian infection. Infect Immun 74: 3545–3564.

55. Weening EH, Parveen N, Trzezciakowski JP, Leong JM, Hook M, et al. (2008) Borrelia burgdorferi lacking DlpBa exhibits an early survival defect during experimental infection. Infect Immun 76: 5064–5070.

56. 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: 641–649.

57. Bush M, Dixon R (2012) The role of bacterial enhancer-binding proteins as specialized activators of σ54-dependent transcription. Microbiol Mol Biol Rev 76: 497–529.

58. Morett E, Segovia I (1993) The σ54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. J Bacteriol 175: 6067–6074.

59. Rappas M, Bose D, Zhang X (2007) Bacterial enhancer-binding proteins: unlocking σ54-dependent gene transcription. Curr Opin Struct Biol 17: 110–116.

60. Wiggsworthwarz S, Bose D, Burrows PC, Joly N, Schumacher J, et al. (2006) Mola operons of the bacterial RNA polymerase containing the σ54 promoter-specificity factor. Mol Microbiol 68: 538–546.

61. Ghosh T, Bose D, Zhang X (2010) Mechanisms for activating bacterial RNA polymerase. FEMS Microbiol Rev 34: 611–627.

62. Studholme DJ, Dixon R (2003) Domain architectures of σ54-dependent transcriptional activators. J Bacteriol 185: 1757–1767.

63. He M, Oman T, Xu H, Blevins J, Norgard MV, et al. (2008) Allosteric activation of σ54-dependent RpoS pathway (σ54-σ54 cascade) in Borrelia burgdorferi. Mol Microbiol 70: 1435–1446.

64. Blevins JS, Revel AT, Smith AH, Bachlan GI, Norgard MV (2007) Adaptation of a luciferase gene reporter and lac expression system to Borrelia burgdorferi. Appl Environ Microbiol 73: 1501–1513.

65. Gilbert MA, Morton EA, Bundle SF, Samuels DS (2007) Artificial regulation of σ54 expression in Borrelia burgdorferi. Mol Microbiol 63: 1259–1273.

66. Hughes CA, Kodner CB, Johnson RC (1992) DNA analysis of Borrelia burgdorferi. J Clin Microbiol 30: 690–703.

67. Pollack RJ, Telford SR 3rd, Spielman A (1993) Standardization of medium for culturing Lyme disease spirochetes. J Clin Microbiol 31: 1251–1255.

68. Groshong AM, Gibbons NE, Yang XF, Blevins JS (2012) Rrp2, a prokaryotic enhancer-like binding protein, is essential for viability of Borrelia burgdorferi. J Bacteriol 194: 3336–3347.

69. Samuels DS (1995) Electromorphism of the spirochete Borrelia burgdorferi. Methods Mol Biol 47: 253–259.

70. Ouyang Z, He M, Oman T, Yang XF, Norgard MV (2009) A manganese transporter, BB0184, is required for viability of the Lyme disease spirochete, Borrelia burgdorferi. Proc Natl Acad Sci U S A 106: 3449–3454.

71. Dill B, Dixon R (2003) Domain architectures of σ54-dependent transcriptional activators. J Bacteriol 185: 1757–1767.

72. He M, Oman T, Xu H, Blevins J, Norgard MV, et al. (2008) Allosteric activation of σ54-dependent RpoS pathway (σ54-σ54 cascade) in Borrelia burgdorferi. Mol Microbiol 70: 1435–1446.

73. Blevins JS, Revel AT, Smith AH, Bachlan GI, Norgard MV (2007) Adaptation of a luciferase gene reporter and lac expression system to Borrelia burgdorferi. Appl Environ Microbiol 73: 1501–1513.

74. Gilbert MA, Morton EA, Bundle SF, Samuels DS (2007) Artificial regulation of σ54 expression in Borrelia burgdorferi. Mol Microbiol 63: 1259–1273.

75. Hughes CA, Kodner CB, Johnson RC (1992) DNA analysis of Borrelia burgdorferi. J Clin Microbiol 30: 690–703.

76. Pollack RJ, Telford SR 3rd, Spielman A (1993) Standardization of medium for culturing Lyme disease spirochetes. J Clin Microbiol 31: 1251–1255.