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Essential Role of the Response Regulator Rrp2 in the Infectious Cycle of *Borrelia burgdorferi*[▽]

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Alteration of surface lipoprotein profiles is a key strategy that the Lyme disease pathogen, *Borrelia burgdorferi*, has evolved to be maintained within its enzootic cycle between arthropods and mammals. Accumulated evidence indicates that the central regulatory pathway controlling differential gene expression by *B. burgdorferi* is the RpoN-RpoS pathway (the σ^{54}-σ^{S} sigma factor cascade). It was previously shown that activation of the RpoN-RpoS pathway is controlled by Rrp2, a two-component response regulator and σ^{54}-dependent transcriptional activator. The role of Rrp2 in the infectious cycle of *B. burgdorferi* has not been determined heretofore. In this report, we demonstrate that an *rrp2* mutant defective in activating σ^{54}-dependent transcription was unable to establish infection in mice, but the *rrp2* mutant was capable of surviving within ticks during and after tick feeding. Because the *rrp2* mutant was defective in the production of OspC, an outer surface lipoprotein essential for mammalian host infection, we further examined whether the loss of infectivity of the *rrp2* mutant was solely due to the inability to produce OspC. While transformation with a shuttle vector carrying *ospC* under the control of a constitutive *flaB* promoter restored infection to an *ospC* mutant in immunodeficient SCID mice, it could not rescue the avirulent phenotype of the *rrp2* mutant. These data indicate that, in addition to controlling OspC, Rrp2 controls another factor(s) essential for *B. burgdorferi* to establish infection in mammals. Furthermore, microarray analyses revealed that 125 and 19 genes were positively and negatively regulated, respectively, by Rrp2, which provides a foundation for future identification of additional Rrp2-dependent virulence determinants in *B. burgdorferi*.

Borrelia burgdorferi, the causative agent of Lyme disease, is maintained in nature in an enzootic cycle involving ticks (*Ixodes scapularis*) and mammals (*Peromyscus leucopus*) (4, 24, 47). *B. burgdorferi* adapts to diverse host environments by coordinately regulating the expression of numerous genes, many of which encode *Borrelia* surface lipoproteins (2, 33, 38, 42, 45, 51). In the past few years, efforts toward elucidating the underlying mechanisms of *Borrelia* differential gene expression have led to the identification of a novel regulatory pathway, the RpoN-RpoS pathway (also called the σ^{54}-σ^{S} sigma factor cascade), which is central to the infectious cycle of *B. burgdorferi* (5, 7, 8, 16, 20, 25, 28, 46, 56). In this pathway, the two-component response regulator Rrp2, along with the alternative sigma factor RpoN (σ^{54} or σ^{N}), directly activates transcription of *rpoS*, which encodes another alternative sigma factor, RpoS (σ^{S}). RpoS functions as a global regulator that controls the expression of more than 145 *Borrelia* genes (8, 16). Many RpoS-activated genes appeared to be differentially expressed during tick feeding, and some, including *ospC*, *dbpAB*, BBK32, *oppA5*, BBA64, and BBA66, have been shown to be required for or associated with mammalian host infection (10, 14, 15, 19, 21, 22, 29, 30, 43, 44). In addition, an increased level of RpoS leads to the repression of a group of genes that are associated with spirochetal colonization and survival in ticks, including *ospA* and BB0365 (6, 8, 34).

The finding that the RpoN-RpoS pathway activates the transcription of *ospC* and *ospC*-like genes while repressing *ospA* and *ospA*-like genes implies that this pathway is not operative in flat ticks and is activated when ticks take a blood meal. Indeed, a recent report by Caimano et al. showed that *rpoS* expression is upregulated during tick feeding (8). It has been postulated that RpoS functions as a gatekeeper that modulates differential gene expression during the process of tick feeding which ensures the successful establishment of infection within the mammalian host (8). Both RpoN and RpoS are essential for the infectious cycle of *B. burgdorferi*; neither an *rpoN* nor an *rpoS* mutant was able to establish infection in mammalian hosts (7, 16). The *rpoN* mutant also failed to enter the tick salivary glands (16). The avirulent phenotype of the *rpoN* and *rpoS* mutants in mammals is consistent with the fact that both mutants were unable to produce OspC, a virulence factor essential for *B. burgdorferi* to establish infection in the mammalian host (22, 50) and possibly for spirochetal transmission from the tick gut to the salivary glands (13, 35). However, it remains unclear whether the loss of infectivity of the *rpoN* and *rpoS* mutants is due solely to the abrogation of OspC or is also related to the loss of additional virulence determinants.

The upstream activator of the RpoN-RpoS pathway, Rrp2, is predicted to comprise three functional domains: an N-terminal receiver domain typical of a two-component response regulator, a central σ^{54}-dependent activation domain, and a

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TABLE 1. *B. burgdorferi* strains used in this study

Strain name	Description	Reference or source
5A4NP1[a]	BBE02::Kan^r, cp9$^-$	26
5A4NP1 *rrp2*(*G239C*)	Same as 5A4NP1, except *rrp2* was replaced with *rrp2*(*G239C*)-*ermC*	This study
5A4NP1 *rrp2*(*wt*)	Same as 5A4NP1 *rrp2*(*G239C*), except *rrp2*(*G239C*)-*ermC* was replaced with *rrp2*(*wt*)-*aadA*	This study
13A[a]	lp25$^-$, lp56$^-$	52
13A *ospC*::Gen^r	Same as 13A, except *ospC*::Gen^r	52
13A *ospC*::Gen^r/pBBE22-*flaB$_p$*-*ospC*	Same as 13A *ospC*::Gen^r, except carrying a shuttle vector with a copy of wild-type BBE22 and constitutive *flaB$_p$*-*ospC*	This study
13A *rrp2*(*G239C*)	Same as 13A, except *rrp2* was replaced with *rrp2*(*G239C*)-*ermC* and lp28-4 was lost	This study
13A *rrp2*(*G239C*)/pBBE22-*flaB$_p$*-*ospC*	Same as 13A *rrp2*(*G239C*), except carrying a shuttle vector with a copy of wild-type BBE22 and constitutive *flaB$_p$*-*ospC*	This study
13A *rrp2*(*wt*)	Same as 13A *rrp2*(*G239C*), except *rrp2*(*G239C*)-*ermC* was replaced with *rrp2*(*wt*)-*aadA*	This study
13A *rrp2*(*wt*)/pBBE22-*ospC*	Same as 13A *rrp2*(*wt*), except carrying a shuttle vector with a copy of wild-type BBE22 and constitutive *flaB$_p$*-*ospC*	This study

[a] A clonal isolate of strain B31.

C-terminal DNA-binding domain. Multiple attempts to inactivate *rrp2* have not been successful (5, 56), suggesting that the abrogation of *rrp2* may be deleterious to cell survival. However, successful generation of an *rrp2* mutant encoding an Rrp2 variant with a point mutation of G239C in the central activation domain provided genetic evidence that Rrp2 is a σ^{54}-dependent activator and controls the activation of the RpoN-RpoS pathway (56). In addition, Burtnick et al. recently reported that unlike other σ^{54}-dependent activators that require an enhancer-binding site for activation, Rrp2 was capable of activating *rpoS* in an enhancer-independent manner (5).

In contrast to RpoN and RpoS, the role of Rrp2 in the infectious cycle of *B. burgdorferi* has not been examined due to the inability to generate any *rrp2* mutant and the isogenic complemented strain from an infectious strain of *B. burgdorferi*. Herein, we report the successful construction of an *rrp2* mutant from a virulent strain of *B. burgdorferi* and a corresponding complemented clone that retains full virulence. With these strains, we demonstrated that Rrp2 is required for mammalian infection but not for spirochetal survival in ticks. Furthermore, we show that constitutive expression of *ospC* could not rescue the avirulent phenotype of the *rrp2* mutant, indicating that Rrp2 controls additional virulence determinants essential for *B. burgdorferi* to establish infection in mammals. Lastly, as an initial approach to identify Rrp2-dependent virulence factors, we performed microarray analyses to determine the global influence of Rrp2 on gene expression in *B. burgdorferi*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. burgdorferi* strains used in this study are listed in Table 1. Strain 5A4NP1 (a gift of H. Kawabata and S. Norris at the University of Texas Health Science Center at Houston) is a B31 clone that contains all essential endogenous plasmids and is infectious in mice and ticks (26, 36). It has a kanamycin resistance marker inserted in the restriction modification gene BBE02 on plasmid lp25, resulting in increased transformation efficiency and allowing selection for clones that retain the essential plasmid lp25. Strain 13A is a B31 5A13 derivative that is missing plasmids lp25 and lp56 (36, 52). Borreliae were cultivated in vitro in modified Barbour-Stoenner-Kelly medium (BSK-H; Sigma, St. Louis, MO) supplemented with 6% normal rabbit serum (Pel Freez Biologicals, Rogers, AR) or BSK-H complete medium at 35°C unless indicated otherwise.

Generation of transformants. Electrocompetent *B. burgdorferi* cells were prepared and transformed as previously described (40, 58). Briefly, 20 to 50 μg of plasmid DNA was used in each transformation. After electroporation, the culture was incubated at 34°C overnight to allow recovery. Relevant antibiotics were then added to the cultures in the following final concentrations: 50 μg/ml for gentamicin, 50 ng/ml for erythromycin, 50 μg/ml for streptomycin, and 300 μg/ml for kanamycin. Cultures were then aliquoted into 96-well tissue culture plates (230 μl/well) for colony selection of transformants (58) instead of using semisolid medium as previously described (40). Two weeks after plating, wells containing positive cultures were identified by a color change in the medium, and the presence of viable spirochetes was verified by dark-field microscopy. To create the *rrp2*(*G239C*) mutant, 5A4NP1 and 13A were transformed with the suicide vector pXY201A containing the mutated *rrp2* gene linked to an *ermC* marker (41, 56). To confirm marker exchange, PCR was performed on whole-cell lysates of transformants and resulting PCR products were then subjected to DNA sequence analysis to verify the presence of the mutation corresponding to G239C. The resulting strains were designated 5A4NP1 *rrp2*(*G239C*) and 13A *rrp2*(*G239C*), respectively (Table 1). To generate the corresponding complemented strains, 5A4NP1 *rrp2*(*G239C*) and 13A *rrp2*(*G239C*) were transformed with the suicide vector pXY206A, which harbors a wild-type copy of *rrp2* linked to an *aadA* marker (17, 56). PCR and sequencing analyses were then performed to confirm the replacement of the mutant *rrp2* with a wild-type copy of *rrp2*. The resulting strains were designated 5A4NP1 *rrp2*(*wt*) and 13A *rrp2*(*wt*), respectively.

To generate strains with constitutive expression of *ospC*, strains 13A *ospC*::Gen^r (53), 13A *rrp2*(*G239C*), and 13A *rrp2*(*wt*) were transformed with the shuttle vector pBBE22-*flaB$_p$*-*ospC* (53), resulting in 13A *ospC*::Gen^r/pBBE22-*flaB$_p$*-*ospC*, 13A *rrp2*(*G239C*)/pBBE22-*flaB$_p$*-*ospC*, and 13A *rrp2*(*wt*)/pBBE22-*ospC*, respectively. Transformants were confirmed by the constitutive OspC expression via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The plasmid contents of transformants were surveyed by PCR (27). In addition to lp25 and lp56, which are not present in parental strain 13A (53), strains 13A *rrp2*(*G239C*)/pBBE22-*flaB$_p$*-*ospC* and 13A *rrp2*(*wt*)/pBBE22-*ospC* are missing lp28-4, which is not necessary for infectivity for the inoculum dose used in this study (10^5 spirochetes/mouse), despite the fact that it can partially contribute to infectivity; loss of lp28-4 resulted in an increase of the 50% infective dose of *B. burgdorferi* by approximately 1 log following intradermal inoculation (27, 36).

***B. burgdorferi* infection of mice via needle inoculation.** For mammalian infection studies, 3- to 4-week-old C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME) or C3H/SCID mice (Harlan, Indianapolis, IN) were inoculated intradermally with 1×10^5 spirochetes. At 10 to 14 days postinoculation, ear punch biopsy samples (and heart, spleen, and joint tissues for tick-infected mice) were collected and spirochetes were cultured in BSK-H medium supplemented with 1× *Borrelia* antibiotic mixture (Sigma). After 1 to 2 weeks, dark-field microscopy was used to examine cultures for the presence of motile spirochetes: a single growth-positive culture was used as the criterion for infection of each mouse. All animal and tick experimentation was approved by the University of Laboratory Animal Care Committee at Indiana University.

Microinjection of *B. burgdorferi* into ticks. Pathogen-free *Ixodes scapularis* nymphs were obtained from the Tick Rearing Facility at Oklahoma State University. Microinjection was used to introduce spirochetes into the guts of *I. scapularis* nymphs as previously described (35, 58). Briefly, each *B. burgdorferi* variant was cultivated under normal conditions in BSK-H medium in the presence of selective antibiotics. Bacteria were harvested by centrifugation and concentrated in phosphate-buffered saline (PBS) to a density of 10^8 spirochetes per ml. Ten microliters of the cell suspension was then loaded into a 1-mm-diameter glass capillary needle (World Precision Instruments Inc., Sarasota, FL) by use of a microloader (Eppendorf AG, Westbury, NY). The bacterial suspension was then injected into the rectal apertures of unfed nymphal ticks by use of a FemtoJet microinjector system (Eppendorf AG). The parameters for injection were a pressure of 1,000 hPa, an injection time of 0.1 s, and a compensation pressure of 0 hPa, which delivered an average volume of 0.15 μl (1×10^4 to 2×10^4 spirochetes).

Transmission of *B. burgdorferi* to mice via tick bite. After microinjection, ticks were allowed to recover for 2 to 4 h and then placed on C3H/HeJ mice (~15 ticks/mouse). To confine the infected ticks to the mammalian host, nymphs were placed in containment capsules as previously described (55). To construct the capsules, the bottoms of screw-cap microcentrifuge tubes were cut 5 mm below the screw threads and attached to the shaved backs of mice with a melted mixture (weight/weight) of 4 parts rosin and 1 part beeswax. Mesh was placed over a hole made in the screw-cap lid and secured with Super Glue (Loctite). Ticks were then placed in the containment unit, which was quickly closed with the screw cap. Ticks were allowed to feed to repletion (4 to 5 days) and then collected.

Immunofluorescence assays. The entire contents of the fed nymphs were placed onto silylated microscope slides (CEL Associates, Pearland, TX). Slides were allowed to air dry before being placed on a 65°C heating block for 25 min, followed by submersion in acetone for 5 min to complete fixation. Slides were incubated at 37°C for 1 h with blocking solution (PBS-Tween 20 with 5% goat serum) in a humid chamber. The blocking solution was replaced with BacTrace fluorescein isothiocyanate-conjugated goat anti-*B. burgdorferi* antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a 1:100 dilution in blocking solution. The slides then were incubated for 1 h at 37°C in a dark, humid chamber. Slides then were washed twice in PBS-Tween 20 and counterstained with 20 μg/ml propidium iodide in PBS for 3 min. Slides were washed twice with PBS-Tween 20 and then mounted with antifade light mounting medium (Molecular Probes, Eugene, OR). Samples were observed for *B. burgdorferi* by using an Olympus BX50 fluorescence microscope with a 40× objective equipped with a charge-coupled-device camera (CCD-100S; DAGE-MTI, Michigan City, IN) and Olympus DP Controller software.

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting were carried out as previously described (55). Cells were loaded in gel lanes at 5×10^7 cells per lane. For immunoblotting, SDS-PAGE gels were transferred to nitrocellulose in a Bio-Rad Mini Trans-Blot transfer cell at room temperature. Protein bands were detected using a 1:50 dilution of mixed monoclonal antibodies against FlaB and OspC (54, 57) and a 1:1,000 anti-mouse immunoglobulin G peroxidase conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibody and developed with 4-chloro-1-naphthol as the substrate.

RNA extraction, microarray, and quantitative reverse transcription-PCR (qRT-PCR) analyses. RNA was extracted from three biological replicates of 5A4NP1 and 5A4NP1 *rrp2*(*G239C*) by use of Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Digestion of contaminating genomic DNA in the RNA samples was performed using RNase-free DNase I (GenHunter Technology, Nashville, TN), and removal of DNA was confirmed by PCR amplification using primers specific for the *B. burgdorferi flaB* gene. RNA quality was determined using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

Oligonucleotides representing 1,723 putative open reading frames of *B. burgdorferi* B31MI and 19 random-sequence 70-mer negative controls were synthesized by Qiagen-Operon (Alameda, CA) as described previously (49). The oligonucleotides were resuspended in 150 mM sodium phosphate (pH 8.5; Microarrays Inc., Nashville, TN) to a concentration of 40 μM and printed on CodeLink activated slides (Amersham Biosciences, Piscataway, NJ) by use of a custom arrayer (Microarrays Inc.). Each oligonucleotide was printed in quadruplicate on each array. Arrays were blocked postprinting per CodeLink instructions with 50 mM ethanolamine. The attachment of probe DNA was confirmed by Microarrays' proprietary Veriprobe assay (Microarrays Inc.).

cDNA was synthesized and labeled with Cy3 or Cy5 by use of the Amersham postlabeling kit according to the manufacturer's instructions, with minor modifications (Amersham Biosciences). Briefly, 10 μg of total RNA was converted to cDNA by use of CyScript RT in the presence of 1 μl of random nanomers (Amersham Biosciences) and 4.5 μg of random hexamers (Invitrogen). Each cDNA sample was labeled with Cy3 and Cy5 separately. Cy3- or Cy5-labeled cDNA from parental B31 RNA was then combined with the Cy5- or Cy3-labeled cDNA from the mutants. Labeled probes were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA) and then applied in the microarray experiment. With three pairs of samples plus dye switching, we made a total of six hybridized slides. Hybridized slides were then scanned on an Axon 4000B microarray scanner using GenePix Pro 6.1 (Molecular Devices, Sunnyvale, CA). The image was analyzed using the GenePix program, and data were then analyzed with Acuity 4.0 (Molecular Devices) by using the ratio-based normalization method and a cutoff value of a threefold change. Statistical analyses were performed using the one- and two-sample significance test ($P < 0.05$) in the Acuity program.

qRT-PCR was performed with RNA samples used for microarray analysis. cDNA was synthesized using the ThermoScript RT system (Invitrogen). qPCR was performed in triplicate on an ABI 7000 sequence detection system using Platinum SYBR green qPCR SuperMix (Invitrogen). Calculations of the relative expression of the gene of interest were normalized to *flaB* gene expression by using the threshold cycle ($\Delta\Delta C_T$) method. Comparisons of *B. burgdorferi* gene expression between the wild type and the *rrp2* mutant were performed using the Student *t* test.

Microarray data accession number. The array data have been deposited at http://www.ncbi.nlm.nih.gov/geo/ (accession number GSE11284).

RESULTS

Rrp2 is essential for mammalian host infection via needle or tick inoculation. We previously showed that a point mutation (G239C) in the activation domain of Rrp2 abolishes temperature- and pH-induced activation of *rpoS* and *ospC*, providing genetic evidence that Rrp2 is the σ^{54}-dependent activator that governs expression of *rpoS* and, consequently, the production of OspC and other RpoS-dependent proteins. Despite the fact that the previously constructed *rrp2* mutant was generated from an infectious clone of *B. burgdorferi* strain 297, the corresponding *cis*-complemented strain (created upon replacing the mutant *rrp2* with a wild-type copy of *rrp2*) was avirulent and therefore rendered the *rrp2* mutant unsuitable for studying Rrp2 function in vivo (data not shown). To overcome this obstacle and to elucidate the role of Rrp2 in the infectious cycle of *B. burgdorferi*, a similar *rrp2* mutant was generated from infectious clone 5A4NP1 of *B. burgdorferi* B31 by use of the previously reported strategy (56). One *rrp2* mutant clone that has an endogenous plasmid profile identical to that of 5A4NP1 was subsequently selected for the generation of a complemented (replacement) strain by transforming a suicide vector carrying a wild-type *rrp2* linked to an *aadA* marker, which confers streptomycin resistance in *B. burgdorferi* (17, 56). Consistent with previous findings for *B. burgdorferi* 297, the G239C mutation in Rrp2 abolished the production of OspC in B31 (Fig. 1).

To examine the role of Rrp2 in spirochetal infection in mammals, groups of C3H/HeJ mice were inoculated intradermally with 10^5 spirochetes of either wild-type strain 5A4NP1, *rrp2* mutant 5A4NP1 *rrp2* (*G239C*), or *rrp2*-complemented strain 5A4NP1 *rrp2*(*wt*). Two weeks after inoculation, ear punch biopsy samples were harvested and cultured in BSK-H medium. As shown in Table 2, none of the mice infected with the *rrp2* mutant were culture positive for *B. burgdorferi* infection, suggesting that Rrp2 is required for mammalian infection. However, the *rrp2*-complemented strain was readily observed in all cultures of ear punch biopsy samples, indicating that the loss of virulence in the *rrp2* mutant was solely due to the mutation in the *rrp2* gene.

It has been reported that needle inoculation and tick chal-

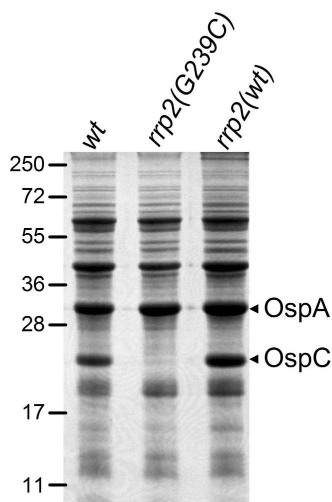

FIG. 1. Protein expression profiles of *B. burgdorferi* strains used for the infection and microarray analyses. Infectious clone 5A4NP1 [wild type (*wt*)], the isogenic *rrp2* mutant [*rrp2*(*G239C*)], and the complemented *rrp2* strain [*rrp2*(*wt*)] were cultivated in BSK-H medium and harvested at the late logarithmic phase of growth (5×10^7 spirochetes/ml), and whole-cell lysates (5×10^7 spirochetes/gel lane) were subjected to SDS-PAGE (Coomassie blue-stained gel). Numbers at the left denote protein molecular mass markers in kDa. The bands corresponding to OspC and OspA are indicated on the right.

lenge of mice may have profoundly different infection outcomes (23). Therefore, we further examined the infectivity of the *rrp2* mutant via tick bite. To do so, we employed a previously developed microinjection technique to artificially inject spirochetes into sterile nymph guts (35, 58). This technique allows the delivery of equal numbers of mutant and wild-type spirochetes directly into the tick gut via the tick rectum with virtually 100% efficiency. Approximately 1.5×10^4 spirochetes of 5A4NP1, the *rrp2* mutant, or the corresponding complemented strain were microinjected into flat *I. scapularis* nymphs. The injected ticks then fed on C3H/HeJ mice. Consistent with the results from needle inoculation, bacteria could not be recovered from any tissue (ear punch biopsy samples or heart, spleen, or joint tissue) of the mice challenged with the *rrp2* mutant strain, whereas all mice challenged by ticks injected with wild-type or *rrp2*-complemented strains yielded spirochetes from cultivated tissues (Table 2). These data indicate

TABLE 2. Rrp2 is required for mammalian infection

Strain	No. of mice infected by indicated route/total no. of mice	
	Needle inoculation	Tick bite
5A4NP1	5/5	4/4
5A4NP1 *rrp2*(*G239C*)	0/5	0/7
5A4NP1 *rrp2*(*wt*)	5/5	5/5
13A *ospC*::Gen^r/pBBE22-*flaB*$_p$-*ospC*	5/5	NA[a]
13A *rrp2(G239C)*/pBBE22-*ospC*	0/6	NA
13A *rrp2(wt)*/pBBE22-*ospC*	6/6	NA

[a] NA, not available.

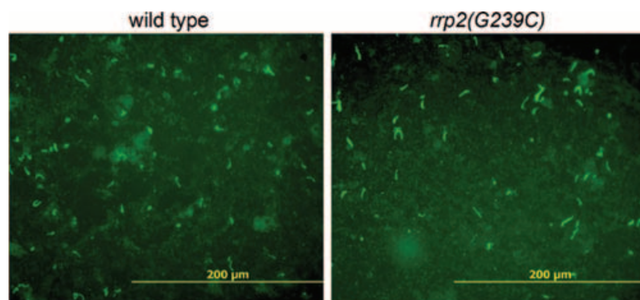

FIG. 2. Detection of wild-type or *rrp2* mutant spirochetes in ticks by immunofluorescence assays. Unfed *I. scapularis* nymphs were microinjected with either wild-type 5A4NP1 or isogenic *rrp2* mutant [*rrp2*(*G239C*)] spirochetes and allowed to feed to repletion on mice. After detachment, fed nymphs were dissected, and tick smear preparations were subjected to immunofluorescence assays with fluorescein isothiocyanate-labeled anti-*B. burgdorferi* antibody. Panels shown are representative images from three separate experiments.

that a functional Rrp2 is required for mammalian infection regardless of the infection route.

Rrp2 is not required for spirochetal survival during tick feeding. Because the *rrp2* mutant was incapable of infecting mice, the entire infectious cycle of the *rrp2* mutant between ticks and mammals could not be examined. However, the ability to generate artificially infected ticks allowed an examination of the mutant's behavior during tick feeding, the period when Rrp2 and the RpoN-RpoS pathway become activated (8). To do so, microinjected ticks carrying various *B. burgdorferi* strains fed on naïve mice and naturally detached ticks were collected. Fed nymphs were dissected and the tick smears were subjected to immunofluorescence assays. As shown in Fig. 2, both 5A4NP1 and the isogenic *rrp2* mutant were readily detectable in ticks and no obvious difference in spirochetal numbers was observed. These data indicate that the *rrp2* mutant is able to survive in the tick vector upon the intake of a blood meal, implying that Rrp2 does not control the production of molecules essential for spirochetal survival in the tick gut during host feeding.

Constitutive expression of *ospC* did not restore virulence in an *rrp2* mutant. OspC production is controlled by the Rrp2-RpoN-RpoS regulatory cascade and is required for *B. burgdorferi* to infect mammals (5, 12, 20, 25, 46, 56, 57). A recent report showed that complementation of an *ospC* mutant with a copy of constitutively expressed *ospC* (under the *flaB* promoter of *B. burgdorferi*) restored infectivity in immunodeficient SCID mice, albeit the complemented strain did not sustain infection in immunocompetent mice (53). To determine whether the avirulence of the *rrp2* mutant was solely due to the loss of OspC, we generated an *rrp2*(*G239C*) mutant from 13A, the strain that was used for the *ospC* deletion and complementation studies with *flaB*$_p$-*ospC* (52, 53). The newly generated strain, namely, the 13A *rrp2*(*G239C*) mutant, along with the 13A *ospC*::Gen^r mutant, was then transformed with a shuttle vector, pBBE22-*flaB*$_p$-*ospC*. A wild-type copy of the BBE22 gene was also included on the shuttle vector, because 13A has lost lp25, which contains the BBE22 gene essential for spirochete growth in mice (37). As shown in Fig. 3, transformation

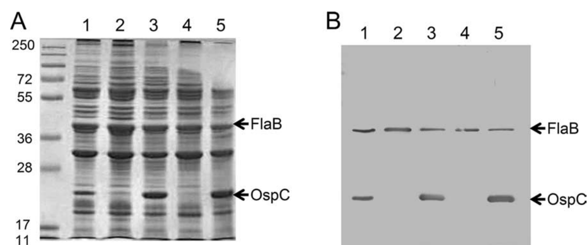

FIG. 3. Restoration of OspC expression with the pBBE22-*flaB*$_p$-*ospC* plasmid. SDS-PAGE (Coomassie blue-stained gel) (A) and immunoblot (B) analysis of whole-cell lysates of various strains of spirochetes harvested at the late logarithmic phase of growth (5×10^7 spirochetes/ml). Lanes for both panels: 1, 13A (wild type); 2, 13A *ospC*::Gen^r; 3, 13A *ospC*::Gen^r/pBBE22-*flaB*$_p$-*ospC*; 4, 13A *rrp2*(*G239C*); 5, 13A *rrp2*(*G239C*)/pBBE22-*flaB*$_p$-*ospC*. For immunoblotting, a 1:10 dilution of the samples used for SDS-PAGE analysis was loaded and monoclonal antibodies directed against FlaB (loading control) and OspC were pooled. The bands corresponding to FlaB and OspC are indicated by arrows.

with pBBE22-*flaB*$_p$-*ospC* restored expression of *ospC* to both the *ospC* and *rrp2* mutants during in vitro growth.

Groups of C3H/SCID mice were challenged with strains 13A *ospC*::Gen^r/pBBE22-*flaB*$_p$-*ospC* and 13A *rrp2*(*G239C*)/pBBE22-*flaB*$_p$-*ospC* or complemented strain 13A *rrp2*(*wt*)/pBBE22-*ospC* (Table 2). Whereas spirochetes were readily detected in tissues from mice infected with the *ospC* mutant carrying pBBE22-*flaB*$_p$-*ospC* (five mice infected of five mice total), no spirochetes were detected in tissues from mice inoculated with 10^5 *rrp2* mutant spirochetes harboring pBBE22-*flaB*$_p$-*ospC* (zero mice infected of six mice total) (Table 2). Replacement of *rrp2*(*G239C*) with wild-type *rrp2* restored infectivity (six mice infected of six mice total). These results showed that the avirulent phenotype of the *rrp2* mutant was not solely due to the loss of OspC production, indicating that Rrp2 controls the expression of an additional virulence determinant(s) that is indispensable for *B. burgdorferi* to establish infection in mammalian hosts.

Genome-wide analyses of Rrp2-controlled genes. As an initial approach to identify virulence determinants controlled by Rrp2, we performed microarray analyses of 5A4NP1 and the isogenic *rrp2* mutant spirochetes harvested from late-logarithmic-phase cultures grown at 35°C in BSK-H medium. When a cutoff value of a threefold change was used as the gene selection criterion, 125 genes showed higher expression in wild-type spirochetes than in the *rrp2* mutant (Table 3), suggesting that they are positively regulated by Rrp2. As expected, the expression of *rpoS* was 40-fold higher in the wild type than in the *rrp2* mutant. Consistent with this finding, the expression of *ospC*, *dbpBA*, BBK32, *oppA5*, BBA64, and BBA66, which are well-described RpoS-dependent genes (10, 19, 21, 22, 29, 30, 43, 44, 50), showed greater dependence on Rrp2 (9- to 90-fold difference in expression). Notably, more than half of the Rrp2-activated genes (60 genes, or 52%) have previously been identified by microarray analyses as differentially regulated genes under different conditions varying in terms of temperature, pH, the addition of blood, or host adaptation (namely, a dialysis membrane chamber [DMC] implanted in the rat peritoneal cavity) (2, 8, 33, 38, 51). These findings further reinforce the notion that Rrp2 is the major transcriptional regulator controlling differential gene expression in *B. burgdorferi*.

The genome distribution of the Rrp2-activated genes revealed that 94% of these genes are located on plasmids, mainly on linear plasmids lp54, lp28-2, and lp38, as well as on the cp32 circular plasmids (Fig. 4). This is consistent with the notion that the plasmids harbor the majority of differentially regulated genes (2, 32, 39, 51). Because most of the plasmid-located genes are annotated either as hypothetical or as conserved hypothetical genes, they provided limited clues about the physiological processes regulated by Rrp2. However, one prominent feature was that 26% of the Rrp2-activated genes encode known or putative surface lipoproteins, suggesting their potential roles in pathogen-host interactions, as has been demonstrated for *dbpBA* and BBK32 (14, 15, 43, 44).

In contrast to the large number of plasmid-contained genes controlled by Rrp2, there are only eight chromosomal genes shown to be positively regulated by Rrp2. In addition to *rpoS*, the chromosomal gene that displayed the greatest dependence on Rrp2 was BB0844 (38-fold difference in expression), which encodes a putative lipoprotein that shares no homology with any other protein in the database. Interestingly, BB0844 is one of the most highly regulated genes under every condition parameter tested, including temperature, pH, blood addition, and DMC, as revealed by previous microarray analyses (2, 33, 38, 51). The finding of its dependence on Rrp2 is also consistent with recent microarray and qRT-PCR analyses showing that BB0844 expression is RpoS dependent (8). Aside from BB0844, three out of eight total Rrp2-dependent chromosomal genes are related to chemotaxis (*mcp4*, *mcp5*, and *cheR1*). Although they present moderate differences in expression (four- to sixfold), further qRT-PCR analysis confirmed that the influence on expression of these genes by Rrp2 was significant (Fig. 5). This result is also consistent with the recent microarray findings that expression of these genes was influenced by temperature, blood, or DMC conditions (2, 32, 39, 51) and affected by the inactivation of *rpoS* (8).

In addition to Rrp2-activated genes, 19 genes showed a level of expression in the wild-type strain that was decreased by more than threefold compared to that in the *rrp2* mutant, suggesting that the expression of these genes is negatively regulated by Rrp2 (i.e., Rrp2-repressed genes) (Table 4). However, the overall range of the change in expression for this group of genes was much less (3- to 6.3-fold) than what was observed for Rrp2-activated genes (3- to 3,629-fold). Among them, five repressed genes were located on lp21, a plasmid not essential for mammalian infection. Interestingly, eight of the Rrp2-repressed genes belong to paralogous gene family 57, whose members are located on various plasmids (9). In addition, there are nine chromosomal genes that showed mild but statistically significant repression by Rrp2 (3.2- to 4.3-fold), two of which were confirmed by qRT-PCR analysis (Table 4 and Fig. 5C). Some of the Rrp2-repressed genes encode proteins with putative functions, including DNA helicase (BB010), putative flagellum protein (BB0180), asparaginyl-tRNA synthetase (BB101), alanine racemase (BB0160), and holo-acyl-carrier protein synthase (BB010) (18).

TABLE 3. Genes activated by Rrp2 (>3-fold)

Gene	Function(s)	Fold change
BBA06	Hypothetical protein	3,629.0
BBD07	Hypothetical protein	2,330.0
BBA34	OppAV, oligopeptide ABC transporter	542.8
BBG27	Conserved hypothetical protein	428.0
BBA72	Hypothetical protein	109.7
BBB19	OspC	90.1
BBA71	Hypothetical protein	78.0
BBA37	Hypothetical protein	71.9
BBG24	Hypothetical protein	62.7
BBA66	P35	56.9
BBA25	DbpB, decorin-binding protein	51.0
BBG26	Hypothetical protein	43.7
BB0771	RNA polymerase sigma factor RpoS	40.8
BB0844	Hypothetical protein	38.3
BBA73	Antigen, P35, putative	35.3
BBH41	Conserved hypothetical protein	34.9
BBA36	Lipoprotein	30.6
BBA05	S1 antigen	25.6
BBD24	Hypothetical protein	24.4
BBK32	P35, fibronectin-binding protein	23.5
BBA07	ChpAI protein, putative (homolog to Mlp)	23.3
BBA24	DbpA, decorin-binding protein	19.7
BBM27	Rev protein	16.5
BBP27	Rev protein	16.4
BBM28	MlpF, lipoprotein	15.3
BBJ001	Conserved hypothetical protein, pseudogene	14
BBG25	Conserved hypothetical protein	13.0
BBR29	Conserved hypothetical protein	12.9
BBA65	Hypothetical protein	10.3
BBJ01	Hypothetical protein	10.0
BBD19	Hypothetical protein	9.9
BBJ23	Hypothetical protein	9.3
BBA64	P35, antigen (Gilmore)	9.2
BBA26	Hypothetical protein	8.7
BBJ02	Hypothetical protein	8.4
BBM38	ErpK protein	7.2
BBG22	Hypothetical protein	7.1
BBA35	Hypothetical protein	7.0
BBS41	ErpG (OspG)	6.8
BBS42	Associated protein A (BapA)	6.7
BBE31	P35, putative	6.5
BB0680	Methyl-accepting chemotaxis protein Mcp-4	6.2
BBO39	ErpL protein	6.2
BBK07	Hypothetical protein	6.0
BBS28	Hypothetical protein	6.0
BB0519	GrpE protein	5.6
BBG10	Hypothetical protein	5.4
BBA33	Hypothetical protein	5.4
BBR44	Hypothetical protein	5.2
BBJ26	ABC transporter, ATP-binding protein	4.8
BBF01	ErpD-like protein, putative	4.8
BB0681	Methyl-accepting chemotaxis protein Mcp-5	4.7
BBP28	MlpA, lipoprotein	4.5
BBJ27	Hypothetical protein	4.5
BBQ64	Hypothetical protein	4.5
BBH09.1	Conserved hypothetical protein, pseudogene	4.4
BBA17	Hypothetical protein	4.4
BB0418	Hypothetical protein	4.3
BBQ65	Conserved hypothetical protein, pseudogene	4.3
BBJ25	Hypothetical protein	4.3
BB0040	Chemotaxis protein methyltransferase CheR-1	4.3
BBH10	Hypothetical protein	4.2
BB0509	Hypothetical protein	4.2
BBD001	Conserved hypothetical protein	4.2
BBN29	Hypothetical protein, authentic point mutation	4.2
BBL29	Conserved hypothetical protein	4.2
BBJ29	Hypothetical protein	4.2
BBR40	ErpH protein	4.0
BBG20	Hypothetical protein	4.0
BBG16	Hypothetical protein	4.0
BBJ28	Hypothetical protein	4.0
BBJ48	Hypothetical protein	4.0
BBH01	Conserved hypothetical protein	4.0
BBK48	P37, putative	3.9
BBG29	Conserved hypothetical protein	3.9
BBD20	Transposase-like protein, authentic frameshift	3.9
BBK52.1	Conserved hypothetical protein, pseudogene	3.9
BBF11.1	Conserved hypothetical protein, pseudogene	3.8
BBG19	Hypothetical protein	3.8
BBJ24	Hypothetical protein	3.8
BBL39	ErpA protein	3.8
BBP26	Conserved hypothetical protein	3.8
BBK53	Outer membrane protein	3.7
BBQ63	Hypothetical protein	3.7
BBR41	Conserved hypothetical protein	3.7
BBO29	Hypothetical protein	3.7
BBJ47	Hypothetical protein	3.6
BBG17	Hypothetical protein	3.6
BBL40	ErpB2 protein	3.6
BBO40	ErpM protein	3.5
BBM39	Hypothetical protein	3.5
BBR15	Hypothetical protein	3.5
BBI42	Outer membrane protein, putative	3.5
BBG03	Conserved hypothetical protein, authentic frameshift	3.5
BBR42	ErpY (OspF)	3.4
BBA32	Hypothetical protein	3.4
BBS27	Hypothetical protein	3.4
BBH09	Hypothetical protein	3.3
BBM26	Conserved hypothetical protein	3.3
BBR36	Conserved hypothetical protein	3.3
BBG14	Hypothetical protein	3.3
BBG21	Hypothetical protein	3.3
BBA57	Hypothetical protein	3.3
BBL36	Conserved hypothetical protein	3.3
BBH06	Hypothetical protein, CspZ	3.2
BBG18	Hypothetical protein	3.2
BBA01	Conserved hypothetical protein	3.2
BBP40	Hypothetical protein	3.2
BBK17	Adenine deaminase, AdeC	3.2
BBB23	Conserved hypothetical protein	3.2
BBM35	Conserved hypothetical protein	3.2
BBK24.1	Hypothetical protein	3.1
BBS38	Conserved hypothetical protein	3.1
BBN35	BdrD10	3.1
BBO36	Conserved hypothetical protein	3.1
BBB15	Hypothetical protein	3.1
BBR43	Hypothetical protein	3.1
BBN28	MlpI lipoprotein	3.1
BBB20	Hypothetical protein	3.1
BBK39	Hypothetical protein	3.1
BBJ43	Hypothetical protein	3.0
BBQ43	Conserved hypothetical protein	3.0
BBE11	Hypothetical protein	3.0
BBJ46	Hypothetical protein	3.0
BBH18	Hypothetical protein	3.0

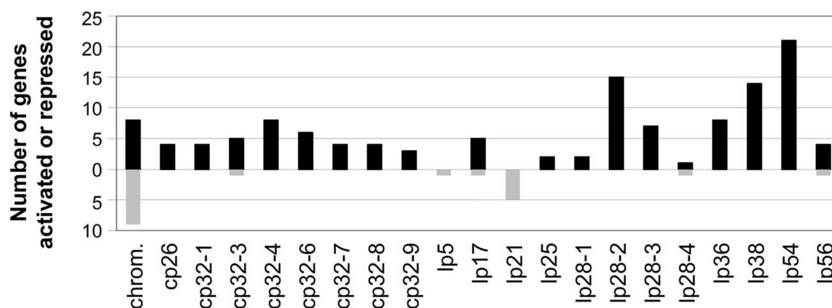

FIG. 4. Summary of genes activated or repressed by Rrp2. Numbers represent the total numbers of genes activated (black bars) or repressed (gray bars) for each plasmid. Plasmid cp9 is absent from the parental strains and is not presented in the diagram. No genes on lp5 or lp21 were found to be activated by Rrp2. Only genes on the chromosome (chrom.), lp17, lp28-4, lp56, cp32-3, lp5, and lp21 were found to be repressed by Rrp2.

DISCUSSION

Two-component systems are a mainstay of signal transduction pathways in bacteria (48). In contrast to what is the case for free-living bacteria such as *Escherichia coli*, which has more than 30 two-component systems, the *B. burgdorferi* genome encodes only two sets of two-component systems, Hk1-Rrp1 and Hk2-Rrp2, in addition to the CheY-CheA systems involved in chemotaxis (18, 31). We and others have previously shown that Rrp2 controls the activation of the RpoN-RpoS regulatory pathway, which in turn governs the expression of numerous *B. burgdorferi* genes (5, 7, 8, 16, 25, 56). In this report, we further show that Rrp2 is indispensable for spirochetes to establish infection in the mammalian host and that the avirulent phenotype of the *rrp2* mutant is not solely due to the abrogation of OspC. Thus, Rrp2 likely controls additional factors essential for mammalian host infection of *B. burgdorferi*.

The avirulent phenotype of the *rrp2* mutant, which is consistent with the phenotypes of the *rpoN* and *rpoS* mutants, was anticipated, since the mutant could no longer produce OspC, a virulence factor essential for mammalian host infection. However, it was not clear heretofore whether the loss of virulence in an *rrp2*, *rpoN*, or *rpoS* mutant was solely due to the loss of OspC. In addition to OspC, three Rrp2-dependent lipoproteins, DbpA, DbpB, and the BBK32 protein, have been shown to contribute to mammalian infection to various degrees (1, 11, 43, 44). Disruption of the *dbpBA* locus or the BBK32 gene exhibited a 4-log or 1-log decrease in infectivity with needle inoculation, respectively. However, both the *dbpBA* and the BBK32 mutants were capable of infecting mice through the natural route, i.e., tick bite (1, 44). Thus, the lack of DbpA, DbpB, and the BBK32 protein could not fully account for the avirulent phenotype of the 13A *rrp2*(*G239C*)/pBBE22-*ospC* strain (Table 2). Future work is needed to determine the infectivity of an *rrp2* mutant with simultaneous constitutive expression of *ospC*, *dbpBA*, and BBK32. Nevertheless, the result that constitutive expression of *ospC* in the *rrp2* mutant did not restore the infectivity in SCID mice indicates that the Rrp2-RpoN-RpoS pathway controls an additional yet-to-be-identified virulence determinant(s) important to the mammalian infection.

In addition to the role of Rrp2 in mammals, its potential function in ticks was also examined in this study. Upon artificially delivering spirochetes into tick guts via microinjection, we were able to examine the mutant's phenotype during tick feeding, the time when Rrp2 is predicted to become activated and when spirochetes encounter the most dramatic environmental changes in ticks. Our data showed that the *rrp2* mutant

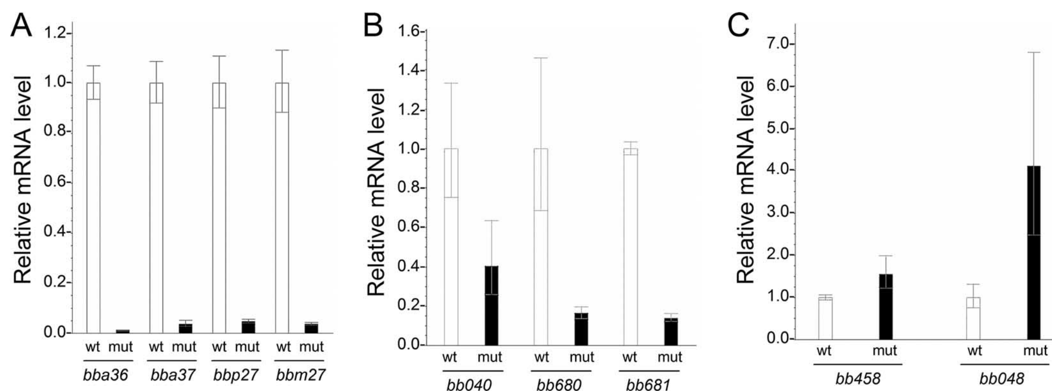

FIG. 5. qRT-PCR analysis of representative genes regulated by Rrp2. mRNA levels of four plasmid-contained, Rrp2-activated genes (A), three chromosome-contained, Rrp2-activated, chemotaxis-related genes (B), and two Rrp2-repressed genes (C) in the 5A4NP1 *rrp2*(*G239C*) mutant (mut) relative to the levels in the 5A4NP1 wild type (wt) (value = 1). The level of *flaB* mRNA was used for normalization of the relative mRNA level of each gene. Data were calculated from three independent cultures, and the differences in mRNA levels between the wild type and the *rrp2* mutant are statistically significant ($P < 0.05$).

TABLE 4. Genes repressed by Rrp2 (>3-fold)

Gene	Function(s)	Fold change
BB0762	Hypothetical protein	6.3
BBI02.2	Brute force ORF[a]	5.3
BBU04	Conserved hypothetical protein	5.0
BBU07	Conserved hypothetical protein	4.8
BBU10	Hypothetical protein	4.8
BB0773	Hypothetical protein	4.3
BBD14	Conserved hypothetical protein	3.8
BBU06	Conserved hypothetical protein	3.7
BB0111	Replicative DNA helicase	3.7
BBQ38	Conserved hypothetical protein	3.7
BB0180	Flagellar protein, putative	3.4
BB0128	Cytidylate kinase	3.4
BB0101	Asparaginyl-tRNA synthetase	3.4
BBU05	Plasmid partition protein, putative	3.3
BB0010	Holo-acyl-carrier protein synthase, putative	3.3
BB0048	Hypothetical protein	3.3
BBT04	Conserved hypothetical protein	3.3
BB0160	Alanine racemase	3.2
BBS33	Conserved hypothetical protein	3.0

[a] ORF, open reading frame.

was able to survive in ticks upon feeding. This result is consistent with previous findings for the *rpoN* mutant (16). Interestingly, the *rpoN* mutant was shown to be incapable of entering tick salivary glands (16). Further studies are needed to determine whether the *rrp2* mutant shares a similar defect. In addition, the ability of the *rrp2* mutant to survive through the tick molting process also warrants further study.

Global analyses of gene expression revealed numerous genes controlled by Rrp2, which provides a foundation for the further identification of new virulence determinants. In this study, we focused only on the genes that showed greater-than-threefold transcriptional changes, which should have reduced the number of genes that are false positives. Our analyses revealed that 125 and 19 genes appeared to be activated and repressed by Rrp2, respectively. Because Rrp2 and RpoN act in concert to activate transcription, we anticipate that the *rrp2* and *rpoN* mutants affect similar groups of genes. In this regard, Fisher et al. showed that inactivation of *rpoN* (*ntrA*) affected a much higher number of genes (305 genes) (16). This is most likely due to the fact that the previous study included all genes that had statistically significant changes in expression (i.e., without a minimal cutoff of change level). It is noteworthy that both studies showed that a large number of Rrp2- and RpoN-influenced genes are located on lp54 and cp32s (16).

Since *rpoS* is the only gene that has been experimentally demonstrated to have a σ^{54} promoter and to be directly activated by Rrp2 and RpoN (5, 25, 28, 46, 56), one would expect that genes dependent on RpoS should also be dependent on Rrp2. The influence of RpoS on global gene expression in both strains B31 and 297 has been analyzed under the in vitro standard cultivation condition as well as under the host adaptation DMC condition (8, 16). A comparison of the Rrp2-dependent and the RpoS-dependent gene profiles revealed that 46 of the top 50 RpoS-activated genes were shown to be Rrp2 dependent, further reinforcing the Rrp2-RpoS linear relationship. On the other hand, nearly half of the top 50 Rrp2-activated genes (22 genes, or 44%) were not present in the RpoS-activated gene profile under either in vitro or DMC conditions. One possibility is that the expression of these genes may be dependent on Rrp2 but not on RpoS. It is also possible that they are RpoS dependent but were missed in the analysis when the B31-based microarray was used for analyzing transcription in strain 297, which is the case for at least one of the genes, *ospC* (8).

In contrast to the Rrp2-activated genes, which largely overlap with the RpoS-activated genes, none of the 19 Rrp2-repressed genes were shown to be repressed by RpoS (8). Similarly, no RpoS-repressed genes identified in the DMC condition were repressed by Rrp2 in vitro. This is not surprising, since the RpoS-repressed genes, such as *ospA* and *lp6.6*, are known to be downregulated under the DMC condition but not under the standard in vitro culture condition that was used in the current study (35°C, pH 7.5). Therefore, they were not expected to be identified as Rrp2-repressed genes by this study. Future work is needed to identify Rrp2-repressed genes under the DMC growth condition.

One caveat of the current study is that a mutant strain carrying the Rrp2 variant with only a G239C point mutation was used rather than an Rrp2-deficient strain. Consequently, this study was limited to elucidating the roles of the central activation domain of Rrp2, but not the entire Rrp2 protein, in the infectious cycle of *B. burgdorferi*. As pointed out earlier, generating an Rrp2-deficient mutant was not achievable despite multiple attempts (5, 56). It has been postulated that full-length Rrp2 is required for cell survival during in vitro cultivation. For instance, in addition to being a σ^{54}-dependent transcriptional activator, Rrp2 may function as a repressor via its C-terminal putative DNA-binding domain, which suppresses genes that are otherwise lethal for spirochetal growth in vitro. This and other potential functions of Rrp2 remain to be elucidated.

The major temporal environmental change *B. burgdorferi* encounters during its infectious cycle is the process of tick feeding. During this process, *B. burgdorferi* in the tick midgut faces a sudden influx of mammalian blood as well as increased tick components (e.g., secreted proteases). In order to adapt to such dramatic changes and prepare for successful transmission and establishment of mammalian infection, spirochetes must evolve strategies that provide quick and global responses to environmental stimuli. In this regard, *B. burgdorferi* deploys a two-component system, Hk2-Rrp2, to modulate both of the alternative sigma factors present in the genome, σ^{54} and σ^{S}, to fulfill this crucial task. The unique feature of such a regulatory system is that while the activation of Rrp2 allows a quick and tight response to environmental signals (3), having Rrp2 and RpoN (σ^{54}) control the production of the global regulator RpoS (σ^{S}), primarily through transcriptional activation (5, 8, 28, 56), provides a global influence on gene expression. Rrp2, as the key regulator of this pathway, plays a central role in the infectious cycle of *B. burgdorferi*, and the current study further supports this notion. This study also provides a foundation for further identification of the unknown virulence determinants controlled by Rrp2. In addition, the importance of Rrp2 warrants further studies to elucidate the upstream signaling events that lead to the activation of Rrp2 and the RpoN-RpoS pathway (5).

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