The response regulator Rrp2 is essential for the expression of major membrane lipoproteins in *Borrelia burgdorferi*

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***Borrelia burgdorferi* (*Bb*), the agent of Lyme disease, exists in nature through a complex enzootic life cycle that involves both ticks and mammals. As *Bb* transitions between its two diverse niches, profound adaptive changes occur that are reflected in differential patterns of gene expression, particularly involving lipoprotein genes. Using a mutagenesis approach, we show that Rrp2 (gene BB0763), one of the proteins predicted by the *Bb* genome (www.tigr.org) to be a response regulator of a two-component sensory transduction system, is a pivotal regulator governing the expression of major membrane lipoproteins such as OspC, DbpA, and Mlp8, as well as many other mammalian infection-associated immunogens of *Bb*. Sequence analysis additionally suggested that Rrp2 is a bacterial enhancer-binding protein, essential for σ^{54}-dependent gene activation. Mutagenesis of a key amino acid residue within a putative activation domain revealed that Rrp2 controlled lipoprotein expression by governing the expression of the alternative σ-factor σ^{s} in a σ^{54}-dependent manner. We therefore propose a signal transduction pathway involving Rrp2, σ^{54}, and σ^{s}, which in concert control the expression of key lipoproteins and other infection-associated immunogens in *Bb*.**

Borrelia burgdorferi (*Bb*), the spirochetal agent of Lyme disease, is maintained in nature through a complex enzootic life cycle that involves both tick and mammalian hosts (1). As *Bb* transitions between these two diverse environmental niches, profound adaptive responses occur, which are reflected in differential patterns of gene expression, the paradigm of which is the reciprocal regulation of outer surface (lipo)protein A (OspA) and outer surface (lipo)protein C (OspC) (2–6). These adaptive responses ostensibly are under the control of global regulatory mechanisms yet to be identified and characterized. In this regard, genome sequence information thus far has predicted relatively few potential global regulators in *Bb* (7). Among these are two alternative σ factors, sigmaN (RpoN; σ^{N}; σ^{54}) and sigmaS (RpoS; σ^{s}; σ^{38}) (8, 9). Studies on these two σ factors (10) have led to the discovery of the first regulatory (RpoN–RpoS) pathway in *Bb*, wherein σ^{54} controls the expression of σ^{s} which, in turn, regulates the expression of key membrane lipoproteins.

Among those membrane lipoproteins regulated by the RpoN–RpoS pathway are OspC and decorin-binding protein A (DbpA). Although the precise function of OspC is unknown, its upregulation during tick feeding suggests that it facilitates *Bb* migration to tick salivary glands and/or spirochete transmission into mammalian tissues (5, 6, 11). DbpA is purported to facilitate the adherence of *Bb* to extracellular matrix as the spirochete invades mammalian dermal tissues (12–14). Recent evidence (15–17) suggests that the expression of other lipoproteins (denoted as group I lipoproteins), such as those within the multicopy lipoprotein (Mlp) paralogous family, are also influenced by the RpoN–RpoS pathway. Thus, the RpoN–RpoS pathway appears to play a prominent role in regulating a number of membrane lipoproteins associated with borrelial host adaptation and/or virulence expression.

Despite the differential expression of *Bb* membrane lipoproteins under various environmental conditions (2–6, 16, 18–21), virtually nothing has been known about signal transduction processes that govern differential lipoprotein expression in *Bb*. In this regard, two-component systems are a mainstay of signal transduction pathways in bacteria (22). Such two-component systems are typically comprised of a histidine kinase sensor and a downstream response regulator (22). The *Bb* genome is predicted to encode two putative two-component response regulators, Rrp1 (gene BB0419) and Rrp2 (gene BB0763) (7). Herein, we provide experimental evidence that Rrp2 governs the expression of OspC, DbpA, Mlp8, and many other *Bb* immunogens, and that Rrp2 serves as the activator for the RpoN–RpoS regulatory pathway. The combined findings provide insights into the regulatory mechanism(s) by which *Bb* modulates key features of its differential expression of membrane lipoproteins involved in *Bb*'s complex life cycle and parasitic strategy.

Materials and Methods

Bacterial Strains and Growth Conditions. *Bb* strains used in this article are summarized in Table 1. An infectious clone of *Bb*, BbAH130, was recovered after plating 297 (*Bb* strain 297) (23) on Barbour–Stoenner–Kelly (BSK) agar medium. The clone was then needle-inoculated into mice and recovered from cultures of ear-punch biopsies. BbAH130 retains all 21 of the parental plasmids (determined by PCR; ref. 24), and is infectious for mice (unpublished data). *rpoN* or *rpoS* mutants, as well as a *rpoN*-complemented strain of *Bb* 297 were described (10). Borreliae were cultivated *in vitro* in BSK-H liquid medium (Sigma; ref. 25) under various environmental conditions. Conditions for adaptation of *Bb* to various culture temperatures were described (16). For all protein analyses, spirochetes were harvested at the late-logarithmic stage (*ca.* 3×10^7 cells per ml). *Escherichia coli* strain TOP 10 (Invitrogen) was used as a cloning host. *E. coli* strain BL21 was used as the host for recombinant protein expression and purification.

Construction of Suicide Plasmids. All recombinant DNA experiments and the use of antibiotic resistance markers in strain 297 were reviewed and approved by the University of Texas Southwestern Medical Center Biological and Chemical Safety Advisory Committee. A 5-kb DNA fragment containing *rrp2* (gene BB0763) with flanking sequence was obtained by PCR amplification from 297 genomic DNA. PCR was performed by using the Expand High-Fidelity PCR system (Roche Diagnostics). The primer design was based on published gene sequences for *Bb* strain B31 (ref. 7 and primers A and E; see Table 2, which is

Abbreviations: *Bb*, *Borrelia burgdorferi*; 297, *B. burgdorferi* strain 297; Dbp, decorin-binding protein; EBP, enhancer-binding protein; Erm, erythromycin; Mlp, multicopy lipoprotein; Rrp, response regulator protein; Strep, streptomycin; Osp, outer surface protein; Hk, histidine kinase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY309266).

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Table 1. *Bb* strains used in this article

Strain name	Purpose	Ref(s).
BbAH130	Parental strain of an infectious clone of *Bb* strain 297	This article
RpoS^{mut}	*rpoS* mutant of *Bb* strain 297	10
RpoN^{mut}	*rpoN* mutant of *Bb* strain 297	10
Rrp2^{G239C}-Erm^{r}	Clone with the *rrp2* point mutation (G239C) linked to *ermC*	This article
Rrp2^{wt}-Erm^{r}	Clone harboring wild-type *rrp2* linked to an *ermC* gene	This article
Rrp2^{wt}-Strep^{r}	A wild-type *rrp2* allele was restored in the Rrp2^{G239C}-Erm^{r} mutant	This article
Rrp2^{G239C}-Strep^{r}	Clone with the *rrp2* point mutation (G239C) linked to *aadA*	This article
Rrp2-^{G239C}-Erm^{r} + P_{flgB}-rpoS	Clone Rrp2^{G239C}-Erm^{r} transformed with shuttle vector pALH227 that contains a constitutive *rpoS* driven by the *flgB* gene promoter of *Bb*	This article, 10

published as supporting information on the PNAS web site, www.pnas.org). The PCR fragment (GenBank accession no. AY309266) was then cloned into the pCR-XL-TOPO cloning vector (Invitrogen), resulting in plasmid pXY155. A *Bam*HI site was then created downstream of *rrp2* on pXY155 by using the QuikChange site-directed mutagenesis kit (Stratagene; primers F and G, Table 2). An *ermC* (from pJRS233) or *aadA* cassette [which confers streptomycin (Strep) resistance to *Bb*] from pKFSS1 (provided by S. Samuels, University of Montana, Missoula) was then amplified and the PCR fragment was inserted at the *Bam*HI site, resulting in plasmid pXY212 and pXY206A (Fig. 2*A*), respectively. Experiments revealed that the cassettes in either orientation relative to *rrp2* caused recombinants to behave similarly. To create the Rrp2^{G239C} mutation within the putative activation domain of Rrp2, a single-nucleotide change was created at the respective position in *rrp2* on plasmid pXY212 by using the QuikChange site-directed mutagenesis kit (primers H and I; Table 2); this change resulted in plasmid pXY201A (Fig. 2*A*). In pXY201A, the distance between the mutation site and the marker was ≈1 kb, which was flanked by ≈2 kb of additional *Bb* DNA on each side.

Generation of the Rrp2^{G239C} *Bb* Mutant. BbAH130 was made electrocompetent, and was transformed as described (10, 26). Colonies on pBSK agar medium (26) containing erythromycin (Erm) (50 ng/ml) appeared 2 weeks after plating. To confirm marker exchange, PCR was performed on whole-cell lysates of transformants by using the amplification strategy shown in Fig. 2*A* (arrows). PCR fragments amplified with primers A and B were then subjected to DNA sequence analysis to verify the presence of the mutation corresponding to G239C. The resulting mutant was designated Rrp2^{G239C}-Erm^{r} (Fig. 2*A*). To restore a wild-type *rrp2* allele in this mutant, Rrp2^{G239C}-Erm^{r} was transformed with pXY206A. Clones resistant to 50 μg/ml Strep (Strep^{r} clones) were verified (by PCR) to contain the correct *aadA* marker insertion, and the pertinent amplicon was DNA-sequenced to distinguish those clones that contained the wild-type copy of *rrp2*. A clone in which the wild-type *rrp2* was successfully restored was designated as Rrp2^{wt}-Strep^{r} (Fig. 2*B*), and a clone that incorporated *aadA*, but retained the mutant *rrp2* gene, was designated Rrp2^{wt}-Strep^{r} (Fig. 2*B*).

Antibodies, Antisera, and Protein Analyses. Rat polyclonal antisera, as well as monoclonal antibodies (14D2-27 and 8H3-33) directed against OspC, DbpA, Mlp8, σ^{s}, P6.6, OspA, or FlaB were reported (16, 27, 28). Sera collected from C3H/HeJ mice postinfestation with *Ixodes scapularis* nymphs harboring wild-type 297 were described (15); for the purposes of this article, sera collected 2–16 weeks postinfestation of these mice were pooled and used as a single reagent.

To generate polyclonal antiserum directed against the *Bb* Rrp2 protein, a DNA fragment encoding full-length Rrp2 was amplified by PCR from 297 genomic DNA (primers J and K; Table 2). The PCR product was then restriction enzyme-digested and ligated into the corresponding polylinker sites of pPROEX-Htb (BRL). After cloning and expression in *E. coli*, the N-terminal His_6-tagged fusion protein was purified by affinity chromatography on a nickel-nitrilotriacetic acid matrix according to the manufacturer's instructions (Qiagen, Valencia, CA). Rat polyclonal antisera directed against Rrp2 was obtained by immunizing rats with the recombinant Rrp2 fusion protein according to published protocols (27). SDS/PAGE and immunoblotting were carried out as described (15); total protein from ≈5×10^{7} spirochetes was loaded per gel lane.

RT-PCR. Total RNA from wild-type BbAH130 or mutants was isolated by using the NucleoSpin RNA II purification kit (BD Biosciences Clontech, Palo Alto, CA), according to instructions provided by the manufacturer. RT-PCRs for amplification of *rpoS* and *ospC* transcripts were performed by using ≈50 ng of total RNA, pertinent oligonucleotide primers, and the Titan One tube RT-PCR system (Roche Diagnostics; ref. 10).

Results

Strategy for Mutating *rrp2* in *Bb*. Rrp2 has been predicted to be a response regulator in *Bb* (7), but the putative input signal and the potential output function(s) of Rrp2 have remained undefined. As a first approach toward elucidating the function of Rrp2, we attempted to disrupt the *rrp2* gene. However, multiple attempts were unsuccessful, suggesting that Rrp2 might be essential for cell survival.

Further BLAST searches revealed that Rrp2 could be divided into three putative functional domains: an N-terminal receiver domain typical of a two-component response regulator, a central activation domain reminiscent of a σ^{54}-dependent activator, and a C-terminal helix–turn–helix (HTH) motif, which is characteristic of a DNA-binding domain (Fig. 1). That the central domain of Rrp2 contains all seven motifs conserved among other σ^{54}-dependent activators (ref. 29, Fig. 1, and Fig. 9, which is published as supporting information on the PNAS web site), suggests that in response to receiving sensory information, Rrp2 regulates the transcription of σ^{54}-dependent genes.

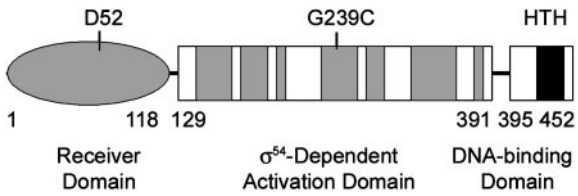

Fig. 1. Predicted domain structure for *Bb* Rrp2. The numbers denote amino acid residues. D52 is a putative phosphorylation site. The seven shaded boxes in the central domain represent motifs conserved among σ^{54}-dependent activators (EBPs). G239C denotes the altered amino acid residue in the *rrp2* mutant. HTH, helix–turn–helix motif.

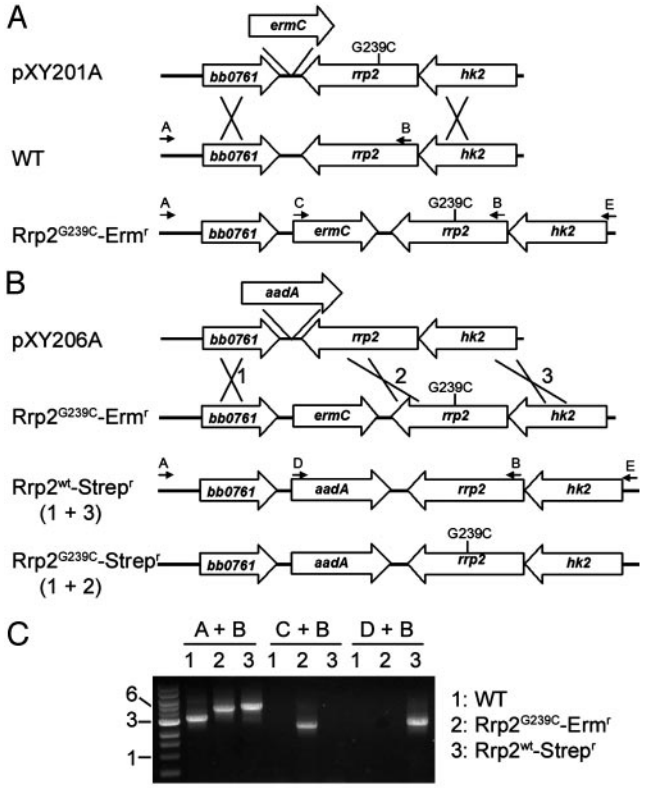

Fig. 2. Strategy for mutagenesis of *rrp2*. (*A*) Generation of a point mutation within *rrp2*. Suicide plasmid pXY201A, encoding a point mutation corresponding to G239C in *rrp2* linked to *ermC*, was transformed into *Bb*. On homologous recombination, strain Rrp2^{G239C}-Erm^{r} was generated. Crosses denote approximate positions of recombination (double crossover) events. Small arrows with letters denote positions and oligonucleotide primers used for PCR analyses. (*B*) Restoration of wild-type *rrp2* in Rrp2^{G239C}-Erm^{r}. Suicide plasmid pXY206A, encoding wild-type *rrp2* linked to *aadA*, was transformed into Rrp2^{G239C}-Erm^{r}. On homologous recombination, 80% of clones (denoted as Rrp2^{wt}-Strep^{r}) obtained the wild-type *rrp2* allele after crossovers at positions 1 and 3. The other 20% of clones (denoted as Rrp2^{G239C}-Strep^{r}) occurred as a result of crossovers at positions 1 and 2, and thus these clones retained the mutant version of *rrp2* linked to *aadA*. (*C*) PCR analysis of *rrp2* transformants. Letter combinations denote primer pairs used for PCR. The lane at the left is a kb ladder.

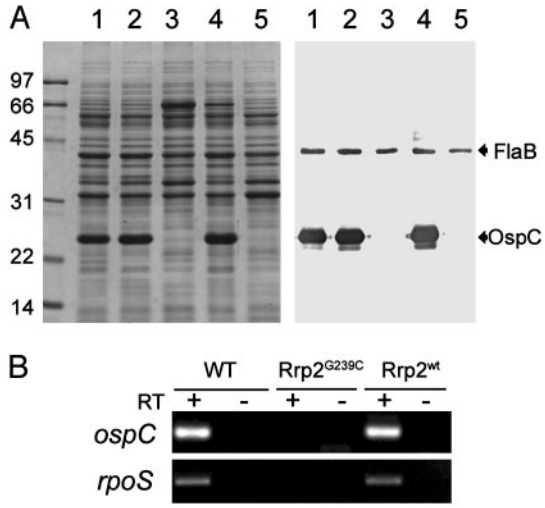

Fig. 3. (*A*) SDS/PAGE (Coomassie blue stain) (*Left*) and immunoblot (*Right*) of whole-cell lysates of *Bb* strains. Numbers at left denote protein molecular mass markers in kDa. For immunoblotting, antibodies directed against FlaB and OspC were pooled. Strain designations are as in Table 1. Lanes 1, wild-type *Bb*; lanes 2, Rrp2^{wt}-Erm^{r}; lanes 3, Rrp2^{G239C}-Erm^{r}; lanes 4, Rrp2^{wt}-Strep^{r}; lanes 5, Rrp2^{G239C}-Strep^{r}. (*B*) RT-PCR analysis for *ospC* and *rpoS* in various strains (designated at the top): WT, wild-type 297; Rrp2^{G239C}, Rrp2^{G239C}-Erm^{r}; and Rrp2^{wt}, Rrp2^{wt}-Strep^{r}. RT, presence (+) or absence (−) of reverse transcriptase in RT-PCRs.

It is well documented that to induce σ^{54}-mediated transcription in bacteria, an essential activator, also known as an enhancer-binding protein (EBP), first must bind to an enhancer region upstream of where the Eσ^{54} holoenzyme is bound (30, 31). The inactive form of the EBP then must be activated (e.g., through phosphorylation), whereupon it catalyzes ATP hydrolysis and interacts with Eσ^{54} to result in transcription. Point mutations within the ATP-binding motifs (C1 or C4; ref. 29) of the central activation domain of EBPs typically abolishes their activation ability (loss of ATP-binding/hydrolysis), whereas leaving functions engendered by their other domains (e.g., phosphorylation, DNA binding) intact (32). This feature was exploited as a potential means of circumventing our prior inability to obtain *rrp2* mutants, and to test the prediction that Rrp2 serves as a EBP in *Bb*. It was envisioned that obtaining such a mutant would enable us to elucidate the putative activation function of Rrp2 while, at the same time, avoiding lethality. To this end, we first performed *in vitro* site-directed mutagenesis to create a point mutation corresponding to G239C within the C4 ATP-binding motif of Rrp2 (Fig. 1). An *ermC* marker then was inserted downstream of the *rrp2* gene (pXY201A; Fig. 2*A*). After transformation of *Bb* with this suicide plasmid, >30 Erm^{r} recombinants were recovered containing the correct *ermC* insertion (verified by PCR; Fig. 2*C*). PCR fragments amplified by using primers A and B (Table 2 and Fig. 2*A*) from 10 random clones were then chosen for DNA sequencing. All 10 clones (denoted as Rrp2^{G239C}-Erm^{r}; Fig. 2*A*) harbored the desired mutation and the *ermC* marker. One clone was then selected for further sequence analysis of an amplicon obtained by using primers A and E (Fig. 2*A* and Table 2); data revealed that the genes flanking *rrp2* remained intact.

Mutation in the Activation Domain of Rrp2 Abolishes the Expression of OspC. A prominent phenotype of the Rrp2^{G239C}-Erm^{r} mutant was the absence of OspC expression, as revealed by both SDS/PAGE and immunoblot analyses (Fig. 3*A*, lanes 3). The abrogation of OspC expression in the Rrp2^{G239C}-Erm^{r} mutant was verified at the mRNA level by RT-PCR (Fig. 3*B*). The lack of OspC expression was not due to the insertion of the *ermC* gene within the *Bb* chromosome, inasmuch as a transformant with *ermC* linked to wild-type *rrp2* retained normal OspC expression (Fig. 3*A*, lanes 2).

To verify that the lack of OspC expression in the Rrp2^{G239C}-Erm^{r} mutant was due solely to the mutation, the mutant was transformed with a suicide vector (pXY206A; Fig. 2*B*) containing a wild-type *rrp2* gene linked to a *aadA* marker (conferring Strep resistance to *Bb*). After homologous recombination, ≈25 resulting Strep^{r}/Erm^{s} clones were obtained, and all clones yielded PCR amplification patterns that were consistent with the desired *aadA* insertion (Fig. 2 *B* and *C*). Further DNA sequencing revealed that 80% of the transformants (designated Rrp2^{wt}-Strep^{r}; Fig. 2*B*) incorporated a wild-type *rrp2* gene in place of the mutant allele. In these transformants, OspC expression was fully restored (Fig. 3*A*, lanes 4). Such restoration was not due to the presence of *aadA* marker alone, inasmuch as those 20% of Strep^{r}/Erm^{s} clones that recombined the *aadA* marker, but not the wild-type *rrp2* gene (designated Rrp2^{G239C}-Strep^{r}; Fig. 2*B*), still did not express OspC (Fig. 3*A*, lanes 5).

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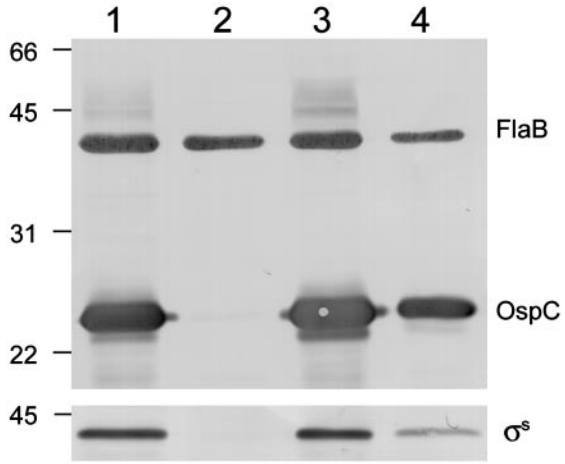

Fig. 4. Immunoblot of *Bb* strains. (*Upper*) The panel was probed with a mixture of antibodies against FlaB and OspC. (*Lower*) The panel was probed with antibodies against σ^s. Strains used in lanes 1–3 are denoted as in Table 1. Lane 1, wild-type *Bb*; lane 2, Rrp2^{G239C}-Erm^r mutant; lane 3, a wild-type *rrp2* allele was restored in the Rrp2^{G239C}-Erm^r mutant (Rrp2^{wt}-Strep^r); lane 4, the Rrp2^{G239C}-Erm^r mutant containing a shuttle plasmid encoding the constitutive expression of *rpoS*.

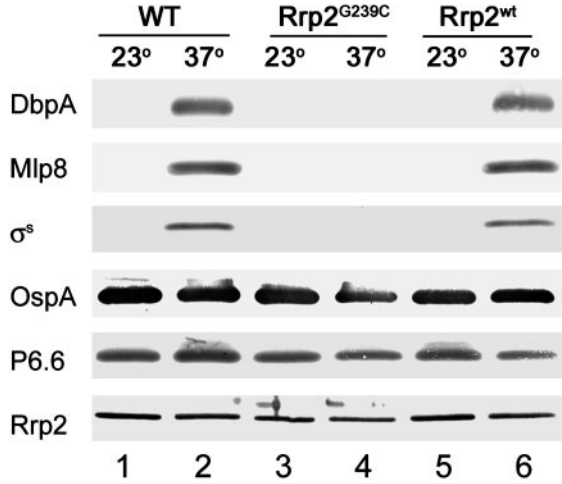

Fig. 5. Immunoblots of *Bb* strains grown at various temperatures (°C). Antibodies used to detect the respective proteins are indicated on the left. Strains used are labeled at the top as in Table 1. WT, wild-type; Rrp2^{G239C}, the Rrp2^{G239C}-Erm^r mutant; Rrp2^{wt}; the Rrp2^{wt}-Strep^r strain.

Rrp2 Controls the Expression of OspC Through σ^s. Experiments above indicated that the activation function of Rrp2 was essential for OspC expression. We reported earlier (10) that OspC expression also is controlled by the RpoN–RpoS regulatory pathway; in this pathway, σ^{54} is responsible for the production of σ^s which, in turn, initiates *ospC* transcription. Given that Rrp2 was predicted to be an EBP that activates σ^{54}-dependent genes, it thus was reasonable to examine whether Rrp2 also controlled the expression of σ^s. As reported (10) for *rpoN* and *rpoS* mutants, expression of σ^s was abrogated in the Rrp2^{G239C}-Erm^r mutant strain (Fig. 4, lane 2). Restoring the *rrp2* mutant with a wild-type copy of *rrp2* (Rrp2^{wt}-Strep^r) rescued the expression of σ^s (Fig. 4, lane 3). In both instances, protein expression data (Fig. 4) were consistent with the absence or presence, respectively, of mRNAs detectable by RT-PCR (Fig. 3*B*). To further corroborate the idea that Rrp2 influenced OspC expression through σ^s, the Rrp2^{G239C}-Erm^r mutant was then transformed with a shuttle vector harboring a wild-type copy of *rpoS* driven by the constitutive *Bb flgB* promoter (P_{flgB}) (10). In this strain, σ^s and OspC both were readily detectable by immunoblot (Fig. 4, lane 4), indicating that Rrp2 regulates the expression of OspC through its control of σ^s.

Other Lipoprotein Genes Controlled by Rrp2. OspC, σ^s, DbpA, and Mlp8 have been denoted as group I proteins, which are up-regulated in spirochetes growing at elevated temperature (37°C), reduced pH (6.8), or increased cell density (16). Given Rrp2's influence on OspC and σ^s expression, we examined whether Rrp2 similarly influenced the expression of other group I proteins. As shown (16), DbpA, Mlp8, and σ^s all were up-regulated in wild-type spirochetes cultivated at 37°C (Fig. 5, lane 2). In contrast, neither DbpA nor Mlp8 was detectable in the Rrp2^{G239C}-Erm^r mutant, regardless of culture temperature (Fig. 5, lanes 3 and 4). The clone in which the *rrp2* mutation was restored with wild-type *rrp2* (Rrp2^{wt}-Strep^r) regained temperature-induced DbpA and Mlp8 expression (Fig. 5, lane 6). Moreover, as in the case for OspC (Fig. 4, lane 4), *rpoS* driven by the *pflgB* promoter in the *rrp2* mutant also restored the expression of DbpA and Mlp8 (data not shown). As predicted, levels of OspA and Lp6.6, lipoproteins that are down-regulated during mammalian infection (group II proteins) (2, 3, 6, 16, 27), were not affected in the Rrp2^{G239C}-Erm^r mutant (Fig. 5).

Infection-Associated Immunogen Profile of the *rrp2* Mutant Differs Markedly from That of Wild-Type *Bb*. As an initial assessment of a role for Rrp2 in the global regulation of other mammalian infection-associated immunogens, whole-cell lysates of wild-type 297 or the Rrp2^{G296C}-Erm^r mutant were subjected to immunoblotting by using pooled sera collected from mice postinfestation with 297-infected *I. scapularis* nymphs. As shown in Fig. 6, markedly fewer infection-associated immunogens were detected within cell lysates of the Rrp2^{G296C}-Erm^r mutant (lane 2), as opposed to the wild-type (lane 1). Replacement of the mutant *rrp2* allele with a wild-copy of *rrp2* (Rrp2^{wt}-Strep^r) essentially restored the immunogen profile of the mutant to that of parental 297 (Fig. 6, lanes 5).

To gain further insight into additional potential overlapping control of protein expression in *Bb* by all three global regulators

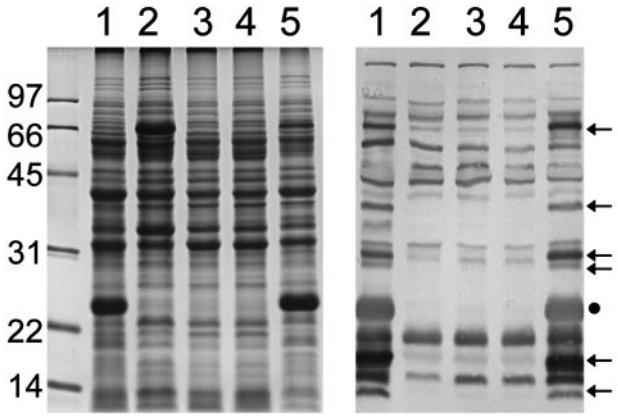

Fig. 6. SDS/PAGE (Coomassie blue stain) (*Left*) and immunoblot (*Right*) of *Bb* strains. Numbers at the left denote protein molecular mass markers in kDa. For immunoblotting, pooled antisera were used from mice infected with *Bb* 297 by means of a tick bite. Lanes 1, wild-type *Bb*; lanes 2, Rrp2^{G239C}-Erm^r mutant; lanes 3, *rpoN* mutant; lanes 4, *rpoS* mutant; lanes 5, a wild-type *rrp2* allele was restored in the Rrp2^{G239C}-Erm^r mutant (Rrp2^{wt}-Strep^r). Arrows denote antigenic proteins not expressed in the *rrp2*, *rpoN*, or *rpoS* mutants. The dot at the right indicates OspC.

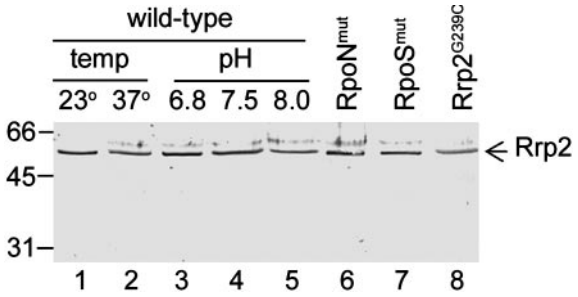

Fig. 7. Rrp2 is constitutively expressed by *Bb* under various culture temperatures, at various pH levels, and in various genetic backgrounds. Immunoblotting was used to detect Rrp2. Lanes 1–5, wild-type *Bb*; lanes 6 and 7, the *rpoN* mutant and the *rpoS* mutant (Table 1), respectively; lane 8, $Rrp2^{G239C}$ represents the $Rrp2^{G239C}$-Erm^r mutant.

Rrp2, σ^{54}, and σ^s, the immunogen profiles of the three mutants were also compared. Although the immunogen profiles of the *rpoN* and *rpoS* mutants also differed dramatically from that of wild-type 297 (Fig. 6, lanes 3 and 4), they were strikingly similar to that of the $Rrp2^{G239C}$-Erm^r mutant (Fig. 6, lanes 2, 3, and 4).

Rrp2 Is Constitutively Expressed in *Bb*. The combined data above support the contention that Rrp2 is a σ^{54}-dependent activator that controls the expression of σ^s, which, in turn, controls the expression of other lipoproteins. Because it is common that σ^{54}-dependent activators autoregulate their own expression by amplification in response to environmental stimuli (33), the expression of *rrp2* in various *Bb* mutants was examined. As shown in Figs. 5 and 7 (lanes 6–8), Rrp2 levels did not differ among wild-type or any mutants deficient in either Rrp2, σ^{54}, or σ^s. Given that genes controlled by Rrp2 are responsive to cultivation temperature and pH (10, 16), Rrp2 levels also were examined in wild-type 297 cultivated under these differing parameters. Rrp2 levels were not affected by any of the differing culture conditions tested (Figs. 5 and 7), suggesting that *rrp2* is constitutively expressed in *Bb*.

Discussion

A significant challenge facing the postgenomic era is to ascribe actual functions to proteins of predicted annotated genes (34). In our initial efforts to elucidate signal transduction pathways that allow *Bb* to accommodate its complex life cycle, genetic studies described herein show an important regulatory role for Rrp2; one of two two-component response regulators predicted to be present in *Bb* (7). It was found that Rrp2 controlled the expression of a number of differentially regulated borrelial lipoproteins, including OspC, DbpA, and Mlp8, as well as many other mammalian infection-associated immunogens. Moreover, Rrp2 exhibits its control over OspC and other lipoproteins via the described RpoN–RpoS regulatory pathway (10).

An initial technical obstacle to studying Rrp2 function was the inability to generate Rrp2-deficient mutants after >10 attempts by using various transformation conditions, markers, and insertion sites. As such, we hypothesized that a gross defect in Rrp2 may be lethal for *Bb*. Bioinformatics performed by we and others (8, 35, 36) revealed that Rrp2 had a predicted central activation domain with homology to other σ^{54}-dependent activators known as EBPs. To induce σ^{54}-mediated transcription, an essential EBP must first bind to an enhancer region ≈100–1,000 base pairs upstream of where the Eσ^{54} holoenzyme is bound (at a distinctive −24/−12 promoter) (30, 31). The inactive form of the EBP must then be activated (e.g., through phosphorylation), whereupon it subsequently catalyzes ATP hydrolysis (the EBP is an ATPase), and interacts with the Eσ^{54} holoenzyme to result in open-complex formation (culminating in transcription) (30, 31). Given that *rpoN* mutations are not lethal in *Bb* (10), we postulated that a mutation in Rrp2 targeting only its σ^{54} activation function also should not be lethal. To accomplish this objective, we first created a point mutation *in vitro* in the *rrp2* DNA sequence putatively encoding ATP binding (C4 motif) (Fig. 9 and ref. 29). Although a C1 ATP binding motif also is predicted in Rrp2 (Fig. 9 and ref. 29), the C4 motif is closer in proximity to the 3′ end of *rrp2*. The mutation was created at G239, a conserved residue shown in other systems to be essential for ATP binding (32). The defective *rrp2* gene was then linked to an antibiotic resistance marker (*ermC*). On homologous recombination by means of a double crossover event, we predicted that some of the Erm^r clones should acquire the *rrp2* mutation from the suicide vector. In fact, all 10 random Erm^r clones tested contained the desired mutation. Similarly, when we attempted to restore a wild-type copy of *rrp2* into one of these $Rrp2^{G239C}$-Erm^r mutants, 80% of $Strep^r$ recombinants acquired wild-type *rrp2*. This mutagenesis strategy therefore appears to be relatively efficient, and thus should be broadly applicable to studying the functions of other *Bb* genes.

At least two lines of evidence from this article support the idea that Rrp2 is an EBP for σ^{54}-dependent gene activation in *Bb*. First, a single amino acid change within the putative activation domain of Rrp2 abolished the expression of *rpoS*, a known σ^{54}-dependent gene (10). Second, both the *rrp2* and *rpoN* mutants displayed virtually identical immunogen profiles when they were immunoblotted with sera from *Bb*-infected mice, indicating that Rrp2 and σ^{54} control similar genes. We therefore conclude that Rrp2 is a σ^{54}-dependent activator that works in concert with σ^{54} to control the expression of σ^s and other genes in *Bb*. Furthermore, inasmuch as the immunogen profile of the *rpoS* mutant mirrored those of the *rrp2* and *rpoN* mutants, it appears that *rpoS* is a major downstream target of control by σ^{54}. In addition, the control of σ^s by σ^{54} and Rrp2 likely is direct, inasmuch as computer analysis predicts a perfect −24/−12 σ^{54} promoter upstream of *rpoS* (8, 37).

Because we were unable to obtain *rrp2*-disrupted mutants, we postulated that in addition to serving as a σ^{54}-dependent activator (EBP), Rrp2 may have other as yet undefined functions associated with *Bb*'s survival. Rrp2 likely is a DNA-binding protein, and thus one such putative function would be to repress other borrelial genes whose expression otherwise is deleterious, at least under the *in vitro* cultivation conditions tested herein. Further studies will be necessary, however, to investigate other putative functions for Rrp2 in *Bb*.

Although Rrp2 appears to control σ^s and other lipoproteins differentially regulated by temperature in *Bb*, which is consistent with previous microarray data (38), Rrp2 was constitutively produced in spirochetes cultivated at 23°C or 37°C (Fig. 7). This finding would appear to be counterintuitive, inasmuch as σ^s and other proteins (e.g., OspC) are not expressed when *Bb* is cultivated at 23°C. One possibility in explaining this paradox is that although Rrp2 is expressed at 23°C, it is not activated. This explanation is logical, given that Rrp2 is a putative response regulator that ostensibly is activated *only* after phosphorylation. That is, Rrp2 may not be phosphorylated at 23°C, and temperature shift to 37°C may lead to phosphorylation and subsequent activation. An alternative explanation is that at either temperature, Rrp2 is both expressed and activated (phosphorylated; i.e., is capable of activating σ^s at 23°C), but at the 23°C condition there is an additional layer of regulation to counteract Rrp2-mediated activation of σ^s. The implication of this scenario is that Rrp2 may not regulate gene expression in a temperature-dependent manner, but rather in response to some other as yet unknown environmental signal(s). Critical to understanding how Rrp2 is activated is predicated ultimately on further identification and characterization of Rrp2's cognate histidine kinase (Hk; which is responsible for sensing the pertinent environmental

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stimulus). In this regard, the *Bb* gene immediately upstream of *rrp2* is predicted to encode a Hk (BB0764) (7). Computer analysis further predicts that BB0764 and *rrp2* are operonic (www.tigr.org). Therefore, the *bb0764* gene product is the primary candidate Hk for Rrp2 and, as such, we herein designate BB0764 as Hk2. Interestingly, Galperin *et al.* (39) reported that the BB0764 (Hk2) also has a PAS domain; PAS domains typically bind organic molecules that are sensitive to oxygen tension, light, redox potentials, small ligands, and overall energy states of the cell (40). This result raises the provocative possibility that activation of Rrp2 ultimately occurs not in response to temperature *per se*, but rather in response to Hk2 receiving other sensory information such as oxygen tension, redox potential, etc. All of these intriguing possibilities remain to be more fully explored.

Data from earlier work (10) and this article, as well as the computer predictions for Hk2, allow us to propose a model in which Rrp2 ultimately controls the expression of key lipoproteins and other protein antigens in *Bb* (Fig. 8). Sensory information likely channels into the sensor domain of Hk2 and triggers Hk2 autophosphorylation at its relevant His residue (His-162 is the reasonable candidate site). Phosphorylated Hk2 then ostensibly phosphorylates Rrp2 at its conserved residue (Asp-52). With phosphorylation, Rrp2 becomes activated. The active form of

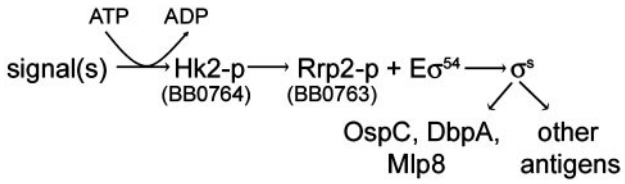

Fig. 8. Proposed mechanism of Rrp2 control over the RpoN–RpoS regulatory pathway in *Bb* (see text).

Rrp2 then hydrolyzes ATP, interacts with Eσ^{54} holoenzyme, and activates transcription of σ^{54}-dependent genes. *rpoS* is a principal target activated by this pathway, culminating in the expression of OspC, DbpA, Mlp8, and other infection-associated protein antigens of *Bb*. Although data provided in this article firmly demonstrate the control by Rrp2 over the expression of key *Bb* membrane lipoproteins, further experiments are warranted to investigate the molecular features of Hk2 and its putative relationship to Rrp2.

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