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Study of the Response Regulator Rrp1 Reveals Its Regulatory Role in Chitobiose Utilization and Virulence of *Borrelia burgdorferi*

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Life cycle alternation between arthropod and mammals forces the Lyme disease spirochete, *Borrelia burgdorferi*, to adapt to different host milieus by utilizing diverse carbohydrates. Glycerol and chitobiose are abundantly present in the *Ixodes* tick. *B. burgdorferi* can utilize glycerol as a carbohydrate source for glycolysis and chitobiose to produce *N*-acetylglucosamine (GlcNAc), a key component of the bacterial cell wall. A recent study reported that Rrp1, a response regulator that synthesizes cyclic diguanylate (c-di-GMP), governs glycerol utilization in *B. burgdorferi*. In this report, we found that the *rrp1* mutant had growth defects and formed membrane blebs that led to cell lysis when GlcNAc was replaced by chitobiose in the growth medium. The gene *chbC* encodes a key chitobiose transporter of *B. burgdorferi*. We found that the expression level of *chbC* was significantly repressed in the mutant and that constitutive expression of *chbC* in the mutant successfully rescued the growth defect, indicating a regulatory role of Rrp1 in chitobiose uptake. Immunoblotting and transcriptional studies revealed that Rrp1 is required for the activation of *bosR* and *rpoS* and that its impact on *chbC* is most likely mediated by the BosR-RpoS regulatory pathway. Tick-mouse infection studies showed that although the *rrp1* mutant failed to establish infection in mice via tick bite, exogenous supplementation of GlcNAc into unfed ticks partially rescued the infection. The finding reported here provides us with new insight into the regulatory role of Rrp1 in carbohydrate utilization and virulence of *B. burgdorferi*.

The spirochete *Borrelia burgdorferi* is the causative agent of Lyme borreliosis, the most frequently reported vector-borne disease in the United States and many parts of the globe (1). In its natural environment, *B. burgdorferi* cycles between the *Ixodes* tick vector and small mammals, such as the white-footed mouse (2, 3). During the transmission process, *B. burgdorferi* is challenged with rigorous changes in the surrounding milieus, such as shifts in temperature as well as in the availability of different carbon sources for growth. The necessity to utilize different carbohydrates is further supported by the presence of several carbon metabolism pathways and transporters in the genome of this spirochete (4). For instance, in addition to the glycolysis pathway utilizing glucose as the main carbon source, *B. burgdorferi* also encodes genes essential for metabolizing glycerol, chitobiose, and *N*-acetylglucosamine (GlcNAc) (4–8).

During the mammalian phase of the enzootic life cycle of spirochetes, glucose is abundantly present in the host blood which can be utilized by *B. burgdorferi* for metabolism and growth (9, 10). On the other hand, during the tick phase, alternative carbohydrates, such as glycerol and chitin, become available. Glycerol is produced by the *Ixodes* tick as one of the cryoprotective agents, and chitin is the major component of cuticle of arthropod, including ticks (11–13). *In vitro* growth analysis has shown that *B. burgdorferi* is able to utilize glycerol as its carbon source in the absence of glucose (5, 7, 14). Also, mutants defective in glycerol metabolism are attenuated in overall survival within the tick vector. These mutants exhibit delayed replication after a blood meal or are unable to persist effectively in feeding ticks, emphasizing the requirement of glycerol for maximal fitness of *B. burgdorferi* during the tick phase of the life cycle (5, 14).

The genome of *B. burgdorferi* encodes several genes that constitute the phosphotransferase system (PTS) required for chitobiose uptake and utilization (4, 6). Among the three putative chitobiose transporter genes, *chbA* (BBB05), *chbB* (BBB06), and *chbC* (BBB04), only *chbC* is essential for chitobiose utilization *in vitro* (6, 15). The expression of *chbC* was shown to be regulated by both RpoD and RpoS (16), and its expression is highly elevated in ticks (5). *In vitro* growth analysis showed that the *chbC* mutant is unable to utilize chitobiose as a carbon source when GlcNAc availability is limited. Membrane blebs formed on the surface of the *chbC* mutant cells when GlcNAc was replaced with chitobiose in the growth medium, indicating a defect in utilizing chitobiose as a source of GlcNAc for cell wall synthesis (6). However, despite its having the complete set of genes for chitobiose metabolism, *in vivo* studies indicated that the *chbC* mutant is able to survive in the tick and complete the enzootic life cycle, suggesting that the chitobiose metabolism system is not essential for the survival of *B. burgdorferi* (17).

During the enzootic cycle, *B. burgdorferi* differentially regulates its tick-phase and mammal-phase genes, primarily via the RpoN-RpoS regulon and two important two-component systems (TCS), the Hk2-Rrp2 and the Hk1-Rrp1 signaling pathways (for reviews, see references 18 and 19). The Rrp2-RpoN-RpoS regulatory system has been well investigated and is known for its essential role

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during the transition from tick to mammal as well as in the mammalian phase of the life cycle (20–24). Several important regulatory elements of this signaling pathway, such as $DsrA_{Bb}$, acetyl-phosphate, and BosR, have also been identified (for reviews, see references 18 and 19). However, little is known about the role of the second TCS. A recent breakthrough in investigating the function of Hk1-Rrp1 highlighted the significance of this TCS in the tick phase of the enzootic cycle (14, 25–27). Rrp1, a diguanylate cyclase, is the sole protein that produces cyclic diguanylate (c-di-GMP) in the genome of *B. burgdorferi* (28). Disruption of c-di-GMP production has been shown to result in global alteration of gene expression, as revealed by microarray analysis of an *rrp1* mutant (14, 27). *In vitro* transcriptional analysis indicated that *rrp1* is strongly induced upon tick feeding and that the induction is independent of changes in temperature (27). An *rrp1*-defective mutant is infectious but attenuated in overall virulence in mice via needle inoculation, and it failed to be transmitted to naïve ticks upon feeding (26). In addition, a mutant defective in *rrp1* has reduced fitness in unfed ticks and is quickly killed upon feeding (14). These observations imply that Rrp1 is required for the basic survival of *B. burgdorferi* in the arthropod vector as well as for protection from host factors/immunity, further emphasizing the importance of Rrp1 during the tick phase of the life cycle.

Based on transcriptional analysis, Rrp1 has been shown to regulate genes involved in the metabolism of GlcNAc (26). This is required for *in vitro* growth of *Borrelia* (10), presumably both for energy production through glycolysis and for synthesizing the precursor for cell wall construction (4). Live-imaging analysis from Dunham-Ems et al. showed that *B. burgdorferi* replicates extensively in the tick gut at the onset of feeding (29). In order to multiply, *B. burgdorferi* requires a large supply of GlcNAc for cell wall synthesis. The one possible source of GlcNAc available upon feeding comes from chitin/chitobiose, which is shed from the peritrophic matrix upon feeding (30). In this study, we sought to investigate the role of Rrp1 in the regulation of chitobiose metabolism by the Lyme disease spirochete. Using a mutant defective in *rrp1*, we showed that Rrp1 is essential for chitobiose utilization *in vitro* and that supplementation of GlcNAc in ticks successfully rescued the mutant transmission to mice via tick bite. Immunoblotting and quantitative reverse transcription-PCR (qRT-PCR) analyses showed that Rrp1 affects *chbC*, the chitobiose transporter gene, by regulating the level of RpoS, probably through BosR, an important modulator of RpoS (31, 32). A model for Rrp1 regulation on chitobiose utilization is also proposed in this report.

MATERIALS AND METHODS

Ethics statement. All animal experimentation was conducted following the NIH guidelines for housing and care of laboratory animals. The protocol of using mice and ticks was approved by the Committee on the Ethics of Animal Experiments and the Institutional Animal Care and Use Committee of the University of Maryland (permit number R-12-33).

Bacterial strains and growth conditions. Infectious clone A3-68 derived from the *B. burgdorferi sensu stricto* B31A3 strain was used in this study (33). Cells were maintained at 34°C in Barbour-Stoenner-Kelly (BSK-II) medium supplemented with 6% rabbit serum in the presence of 3.4% carbon dioxide. The strains were grown in the following appropriate antibiotic(s) for selection pressure as needed: kanamycin (300 μg/ml) and/or gentamicin (40 μg/ml). *Escherichia coli* TOP10 strain (Invitrogen, Carlsbad, CA) was used for DNA cloning and cultured in lysogeny broth (LB) supplemented with appropriate concentrations of antibiotics.

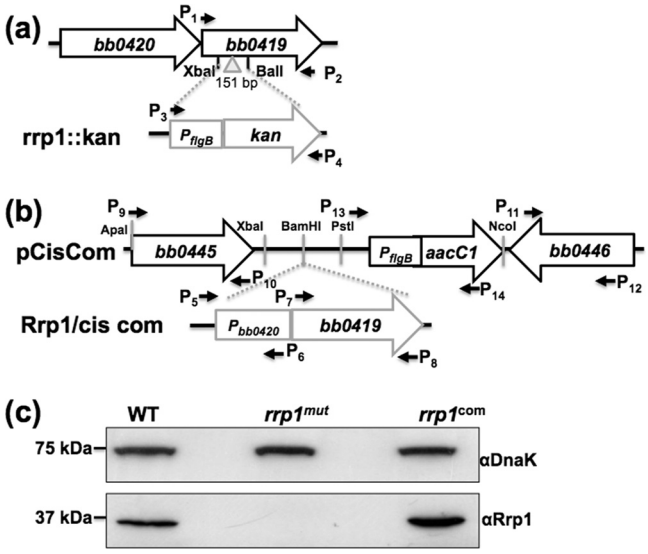

FIG 1 Construction of the *rrp1*^{mut} mutant and its complemented *rrp1*^{com} strain. (a) To construct the rrp1::kan plasmid, the entire ORF of *bb0419* (*rrp1*) was PCR amplified. A *kan* cassette was inserted into the XbaI and BalI cut sites that are present within the *rrp1* ORF, resulting in a 151-bp deletion, generating the rrp1::kan plasmid. (b) P_{bb0420}, 218 bp from the upstream region of *bb0420* that contains the native promoter of the *bb0419-bb0420* operon, was PCR amplified and fused to the 5′ end of *bb0419*. The obtained fragment was then inserted into the pCisCom construct at the BamHI cut site, yielding Rrp1/cis com. (c) Immunoblot analysis of the whole-cell lysate of the wild-type (WT), *rrp1*^{mut}, and *rrp1*^{com} strains probed with a specific antibody against Rrp1. DnaK was used as an internal control, as previously described (42). α, anti-.

Construction and complementation of *B. burgdorferi rrp1*^{mut}. The rrp1::kan plasmid was constructed for the targeted mutagenesis of *rrp1* (*bb0419*) and the plasmid Rrp1/cis com for the complementation of the mutant. To construct the rrp1::kan plasmid, the entire open reading frames (ORF) of the *bb0419* gene and the kanamycin resistance gene (*kan*) were PCR amplified using primer pair P_1 and P_2 and primer pair P_3 and P_4, respectively (see Table S1 in the supplemental material). The resulting PCR products were cloned into pGEM-T-Easy vector (Promega, Madison, WI). A 151-bp DNA fragment was deleted from *bb0419* using the restriction cut sites XbaI and BalI. Then, the *kan* cassette was inserted into the obtained fragment at XbaI and BalI cut sites, generating the rrp1::kan plasmid (Fig. 1a).

Complementation of the *rrp1*^{mut} mutant was performed using a previously described approach (34). To construct the suicide vector pCisCom for *cis* complementation, the 5′ and 3′ arms for homologous recombination were PCR amplified using primer pair P_9 and P_{10} and primer pair P_{11} and P_{12}, and the *flgBp-aacC1* cassette (35) was amplified from pBSV2G vector (36) using primer pair P_{13} and P_{14}. The resulting fragments were cloned into pGEM-T vector (Promega). The *aacC1* cassette was inserted downstream of the 5′ arm at the engineered XbaI and NcoI restriction cut sites. The 5′ arm and the *aacC1* cassette were then placed upstream of the 3′ arm using ApaI and NcoI cut sites, yielding the pCisCom plasmid. To construct the Rrp1/cis com plasmid, the full length of *bb0419* and 218 bp from the upstream region of *bb0420* containing the native promoter of the *bb0419-bb0420* operon were amplified using primer pair P_5 and P_6 and primer pair P_7 and P_8 (see Table S1 in the supplemental material). The two resulting PCR fragments were then PCR ligated using primer pair P_5 and P_8. The resultant P_{bb0420}-*bb0419* fragment was ligated to the pGEM-T-vector (Promega). BamHI was then used to digest the resulting plasmid, and the obtained BamHI-cut P_{bb0420}-*bb0419* fragment was inserted into suicide vector pCisCom to generate Rrp1/cis com (Fig. 1b).

Measuring growth rates of *B. burgdorferi*. To measure the growth rates of the wild-type (WT) A3-68, *rrp1*^{mut}, *rrp1*^{com}, and *rrp1*^{mut} *chbC*-positive (*chbC*$^{+}$) strains, 5 μl of the stationary-phase cultures (1×10^8 cells/ml) was inoculated into 5 ml of normal BSK-II medium or BSK-II medium without added exogenous GlcNAc but supplemented with 20 μM chitobiose, in the form of *N*,*N*-diacetylchitobiose (designated BSK-II$_{(-\text{GlcNAc})}$+chitobiose), and incubated at 23°C and pH 7.6 (unfed tick condition) and at 34°C and pH 7.6 as described before (37). The bacterial concentrations of the cultures were measured every 24 to 48 h for up to 24 days using a Petroff-Hausser counting chamber (38). Counts were repeated in triplicate with at least two independent samples, and the results are expressed as the means ± standard errors of the means (SEM).

***chbC* rescues growth of the *rrp1*^{mut} mutant.** To rescue the growth of the *rrp1*^{mut} mutant in the BSK-II$_{(-\text{GlcNAc})}$+chitobiose medium, a plasmid that overexpresses *chbC* (*bbb04*) was constructed. The *flgBp* and the ORF of *chbC* were PCR amplified using primer pair P_{15} and P_{16} and primer pair P_{17} and P_{18}, respectively. The resulting fragments were cloned into pGEM-T vector (Promega). The *chbC* fragment was then digested with NdeI and SphI restriction enzymes and ligated to the pGEM-T-*flgBp* plasmid. The entire *flgBp-chbC* fragment was then digested with SphI and ligated to the shuttle vector, pBBE22G (39). The resultant vector was transformed into the *rrp1*^{mut} competent cells via electroporation.

SDS-PAGE and immunoblotting. *B. burgdorferi* cells were cultivated at 34°C and pH 7.6 until the stationary phase (~10^8 cells/ml) was reached. Cells were harvested for immunoblot analysis at the early stationary phase (day 1 [D1]) and at day 2 and day 3 (late stationary phase [D2 and D3, respectively]). Equal amounts (10 to 50 μg) of whole-cell lysates were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA). The immunoblots were probed with specific antibodies against various proteins, including BosR, DbpA, DbpB, OspA, OspC, Pta, Rrp1, Rrp2, RpoS, and DnaK (an internal control). These antibodies were kindly provided by F. T. Liang (Louisiana State University), X. F. Yang (Indiana University), M. J. Caimano (University of Connecticut Health Center), J. Skare (Texas A&M University), and J. Seshu (University of Texas at San Antonio). Immunoblots were developed using horseradish peroxidase secondary antibody with an enhanced chemiluminescence (ECL) luminol assay, as previously described (40).

RNA preparation and qRT-PCR. RNA isolation was performed as previously described (41, 42). Briefly, *B. burgdorferi* strains were cultivated at 34°C, and 50 ml (~ 10^8 cells/ml) of the stationary-phase cultures was harvested for RNA preparation. Total RNA was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO), following the manufacturer's instructions. The resultant samples were treated with Turbo DNase (Ambion, Austin, TX) at 37°C for 2 h to eliminate genomic DNA contamination. The resultant RNA samples were reextracted using acid phenol-chloroform (Ambion), precipitated in isopropanol, and washed with 70% ethanol. The RNA pellets were resuspended in RNase-free water. cDNA was generated from the purified RNA using an iScript cDNA synthesis kit (Bio-Rad). Quantitative reverse transcription-PCR (qRT-PCR) was performed using iQ SYBR green Supermix and a MyiQ thermal cycler (Bio-Rad). The *flaB* (*bb0147*) transcript was amplified and used as an internal control to normalize the qRT-PCR data as described before (43, 44). The enolase gene (*eno*; *bb0337*) was included as a positive-control housekeeping gene as described previously (41). The results were expressed as the expression ($2^{-\Delta\Delta CT}$) of the *rrp1*^{mut} transcripts relative to that of the wild type. The primers used for qRT-PCR are listed in Table S1 in the supplemental material.

Microinjection and analysis of ticks for the assessment of spirochete persistence and transmission from ticks to mice. C3H mice (Jackson Laboratory, Bar Harbor, ME) at 4 to 6 weeks of age were used in this study. *Ixodes scapularis* nymphal ticks (Oklahoma State University, Stillwater, OK) were microinjected with wild-type or genetically manipulated spirochetes as described earlier (37, 45, 46). Cultures of *B. burgdorferi* were grown in BSK-II medium at a density of 10^7 cells/ml and centrifuged at 4,000 rpm for 20 min. The resulting pellet was then washed 3 times with phosphate-buffered saline (PBS) and finally resuspended either in GlcNAc-free BSK-II medium or in the medium supplemented with 10 mM GlcNAc at a cell density of 10^9 cells/ml. Equal volumes of the resultant cell suspensions were injected into the rectal aperture of unfed nymph ticks using a femtojet microinjector system (Eppendorf AG, Hamburg, Germany) as previously described (45). After injection, ticks were allowed to remain in the incubator for 24 h for recovery and were subsequently placed on mice (3 animals/group) and allowed to engorge. Postfed ticks were collected and analyzed for the presence of bacteria using qRT-PCR as described previously (37). Parallel groups of microinjected unfed ticks were kept in the incubator for 5 days and used to assess the persistence of spirochetes using qRT-PCR. For assessment of pathogen transmission from ticks to the host, mice were sacrificed following 2 weeks of tick engorgement. The heart, bladder, skin, and joints were collected and subjected to qRT-PCR analysis for measuring *B. burgdorferi* burdens as previously detailed (37). Spleen tissues were collected from mice and assessed for the presence of viable spirochetes using culture analysis.

Sample preparation for quantification of intracellular *N*-acetylglucosamine. To measure the intracellular concentration of GlcNAc, *B. burgdorferi* cells were cultured in BSK-II or BSK-II$_{(-\text{GlcNAc})}$+chitobiose medium and harvested by centrifugation at 5,000 rpm. Cells were washed twice and resuspended in sterilized PBS. Cell densities were adjusted to 10^9cells/200 μl, and cells were sonicated for 20 s. Lysates were subjected to high-speed centrifugation at 15,000 rpm and 4°C for 20 min. The resulting supernatants were transferred to clean microcentrifuge tubes and analyzed by liquid chromatography tandem mass spectrometry (LC-MS).

LC-MS. All samples were analyzed on the LC-MS/MS system consisting of a Shimadzu Prominence high-performance LC (HPLC) system (Kyoto, Japan) and an API 3000 Turbo Ionspray ionization triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) controlled by Analyst 1.4.2 software (Applied Biosystems). A simple and rapid protein precipitation method was used for sample preparation. Two hundred microliters of acetonitrile containing 50 μM *N*-(2,4,5-trihydroxy-6-hydroxymethyl-tetrahydropyran-3-yl)-formamide (internal standard [IS]) was added to 100 μl of sample to precipitate protein. After mixing and centrifugation at 14,000 rpm for 10 min, 5 μl of supernatant was injected for LC-MS/MS analysis. Separation of analytes was achieved using a 3-μm-pore-size Thermo Aquasil C_{18} column (4.6 mm by 50 mm) maintained at 25°C and an isocratic elution with 80% methanol and 20% distilled water at a flow rate of 0.2 ml/min in which both contained 0.1% formic acid. The total run time was 5 min for each injection. The mass spectrometer was operated in the turbo ion spray mode with negative ion detection. The detection and quantitation of GlcNAc, *N*-valeryl-glucosamine, and the IS were accomplished by multiple-reaction monitoring (MRM) with the transitions *m/z* 220.2→118.9 for GlcNAc, 262.2→118.9 for *N*-valerylglucosamine, and 206.1→118.9 for the IS. The instrumental parameters were tuned to maximize the MRM signals. An online motorized six-port divert valve was used to introduce the LC eluent to the mass spectrometer over a period of 1.0 to 5.0 min for data acquisition, whereas eluent from 0 to 1.0 min was diverted to the waste.

Blank media were spiked with the standard working solution and then serially diluted with blank media to generate the following concentrations for both analytes: 10, 1.0, 0.5, 0.1, 0.01, 0.005, and 0.002 μM. These matrix-based calibration standards were treated the same as the samples. The calibration plots were constructed using linear regressions of the peak area ratio of analyte over IS (*y* axis) against the corresponding nominal concentration of the analyte (*x* axis). The calibration linear range of GlcNAc was 0.002 to 10 μM (on column). Three independent assays were conducted, and GlcNAc concentrations were expressed as averages of μM/10^8 cells for intracellular GlcNAc concentrations and μM for culture media GlcNAc concentrations.

Scanning electron microscope. Wild-type, *rrp1*^{mut}, *rrp1*^{com}, and *rrp1*^{mut} *chbC*$^{+}$ cells were centrifuged and washed once with PBS. Cells were then resuspended in fresh PBS. SEM determinations were performed

as described before (47, 48). Briefly, 10 μl of cell suspensions was settled on poly-L-lysine-coated round coverslips (BD BioSciences, San Jose, CA). Samples were fixed statically in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde, 0.075% ruthenium red, and 0.075 M lysine acetate for 1 h at room temperature. Three 10-min washes were performed using 0.2 M sodium cacodylate buffer (pH 7.2) containing 0.075% ruthenium red at room temperature without agitation. Samples were then dehydrated with a graded ethanol series (30, 50, 75, 95, and 100%) at room temperature for 10 min for each incubation step. Samples were exchanged into 100% hexamethyldisilazane and allowed to air dry before analysis was performed using a Hitachi SU-70 scanning electron microscope at an acceleration voltage of 10.0 kV.

Statistical analysis. The significance of differences between different experimental groups was evaluated with two-way analysis of variance (ANOVA) (P values of <0.05 were considered significant).

RESULTS

Isolation of the *rrp1*^{mut} mutant and its *rrp1*^{com} complemented strain. Inactivation of the *rrp1* gene (*bb0419*) was performed using the rrp1::kan vector (Fig. 1a) by first linearizing it with SphI and then transformation into infectious A3-68 (33) competent cells by electroporation (49). After 14 days of incubation, a previously described PCR method (50) was used to screen for clones with the desired targeted mutagenesis. One clone that contained the full plasmid profile of its parental strain (see Fig. S1a in the supplemental material) was selected for further analysis and was named *rrp1*^{mut}. Immunoblotting analysis using antiserum against Rrp1 confirmed that the production of the cognate gene product was abolished in the mutant (Fig. 1c). For the complementation of the *rrp1*^{mut} mutant, a *cis* complementation approach was performed by inserting the *bb0419* gene together with its native promoter into the intergenic region between *bb0445* and *bb0446* (34) at the BamHI-cut site as shown in Fig. 1b. The Rrp1/cis com vector was linearized with ApaI and electrotransformed into *rrp1*^{mut} competent cells. Immunoblotting was used to screen for complementation. One clone containing the same plasmid profile as its parental strain (see Fig. S1b in the supplemental material) was selected for further analysis and named *rrp1*^{com}. As shown in Fig. 1c, immunoblotting analysis confirmed that the expression of Rrp1 was restored in the *rrp1*^{com} strain.

Rrp1 affects the ability of *B. burgdorferi* to utilize chitobiose for its growth. Previous studies by Kostick et al. and He et al. have established the importance of Rrp1 for the survival of *B. burgdorferi* in the tick after a blood meal (14, 26). During the feeding process, chitin or chitobiose is shed from the peritrophic membrane, which can be utilized as a carbon source for growth (15, 30, 51). *In vitro* growth analyses have shown that *B. burgdorferi* is still able to grow when GlcNAc is replaced with chitobiose in the BSK-II medium (6, 15, 16). To investigate if the failure of the *rrp1*^{mut} mutant to survive in feeding tick was due to its inability to utilize chitobiose, which becomes available after a blood meal, we compared the growth of the *rrp1*^{mut} strain to that of the wild-type and *rrp1*^{com} strains in BSK-II medium as well as in BSK-II$_{(-\text{GlcNAc})}$+chitobiose medium in which GlcNAc was replaced with 20 μM chitobiose. The reason of choosing this concentration was that a previous study showed that addition of 18 μM is sufficient to restore normal growth of *B. burgdorferi in vitro* when GlcNAc is omitted from the BSK-II medium (6). Two conditions were included in the comparison to mimic the life cycle of the spirochete, the unfed-tick condition (23°C and pH 7.6) and the routine laboratory culture condition (34°C and pH 7.6), as described previously (37). In normal BSK-II medium, the *rrp1*^{mut} mutant exhibited a growth pattern similar to that exhibited by the wild type under both culture conditions (Fig. 2a and c), consistent with the observations made by He et al. (14). In BSK-II$_{(-\text{GlcNAc})}$+chitobiose medium, while the mutant had a growth pattern similar to that of the wild type at 23°C and pH 7.6 (Fig. 2b), it exhibited a severe growth defect at 34°C and pH 7.6 (Fig. 2d). Under this culture condition, the number of mutant cells rapidly declined after the level of growth reached the mid-log phase ($\sim 10^7$ cell/ml). At the stationary phase, the mutant cell density was approximately 100-fold less than that of the wild type. The observed growth defect was fully restored in the *rrp1*^{com} strain (Fig. 2d). Of note, the same experiment with independent cultures was repeated at least three times, and a similar growth pattern was observed. These results indicate that inactivation of *rrp1* somehow impairs the ability of *B. burgdorferi* to utilize chitobiose for *in vitro* growth.

The *rrp1*^{mut} mutant forms membrane blebs. As illustrated in Fig. 2d, after day 4 (D4), the number of *rrp1*^{mut} cells remained steady. However, dark-field microscopic analysis revealed that the mutant appeared to enter a "lysis phase" (Fig. 3a). During this phase, membrane blebs were progressively appearing on the cell surface. At D4, membrane blebs were seen forming on the cell surfaces in roughly 60% of the *rrp1*^{mut} mutant population (Fig. 3a). At day 6 (D6), more than 80% of the mutant population had visible membrane blebs whereas both the wild-type and *rrp1*^{com} strains remained normal (Fig. 3a). A scanning electron microscope was used to further visualize the membrane bleb formation throughout growth (from D2 to D8) in BSK-II$_{(-\text{GlcNAc})}$+chitobiose medium. At the time of initial growth (D2), the *rrp1*^{mut} cells appeared normal (Fig. 3b). When cells entered the lysis phase (D4 to D8), blebs began to form and multiply in size and number, which eventually led to cell lysis (Fig. 3b). The formation of membrane blebs was abolished upon complementation in *rrp1*^{com} cells (Fig. 3a; see also Fig. S2 in the supplemental material). These results indicate that inactivation of *rrp1* induces membrane bleb formation, which further leads to cell lysis of the mutant. *B. burgdorferi* needs exogenous GlcNAc to grow. Failure to utilize chitobiose as a source of GlcNAc for cell wall synthesis may explain the growth and morphological defects observed in *rrp1*^{mut} when GlcNAc is replaced by chitobiose in the growth medium.

Constitutive expression of *chbC* rescues the growth defects of the *rrp1*^{mut} strain. The *chbC* gene encodes a key chitobiose transporter of *B. burgdorferi*. Previous studies showed that *chbC* mutants are defective in growth when GlcNAc is replaced by chitobiose (6, 15, 16), which is similar to the phenotype of the *rrp1*^{mut} strain (Fig. 2d). Upon analysis via qRT-PCR, we found that the transcript level of *chbC* in the *rrp1*^{mut} strain was decreased by approximately 70% compared to that of the wild type and was restored in the *rrp1*^{com} strain (Fig. 4a). On the basis of this observation, we hypothesized that the repression of *chbC* expression in the *rrp1*^{mut} strain accounts for the growth defect and membrane bleb formation. To test this hypothesis, the *rrp1*^{mut} *chbC*$^{+}$ strain, a mutant that constitutively expresses *chbC*, was constructed and experiments similar to those described above were repeated. Our results showed that constitutive expression of *chbC* fully restored the ability of the *rrp1*^{mut} strain to grow in BSK-II$_{(-\text{GlcNAc})}$+chitobiose medium whereas the empty pBBE22G vector alone failed to do so (Fig. 4b). In addition, dark-field and scanning electron microscopic analyses did not show significant membrane blebbing in *rrp1*^{mut} *chbC*$^{+}$

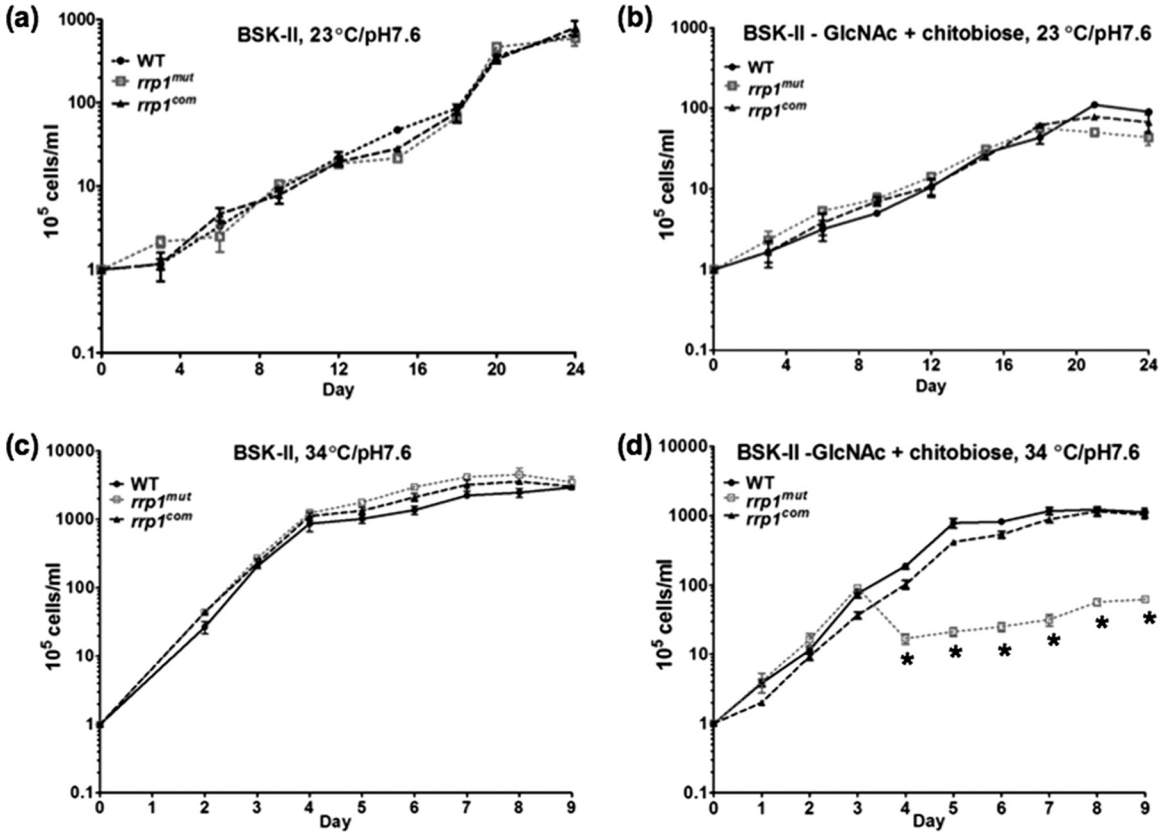

FIG 2 The growth curves of WT, *rrp1*^{mut}, and *rrp1*^{com} strains. Growth curves were measured under the following conditions: (a) cells were grown in BSK-II medium at 23°C and pH 7.6; (b) cells were grown in BSK-II$_{(-GlcNAc)}$+chitobiose medium at 23°C and pH 7.6; (c) cells were grown in BSK-II medium at 34°C and pH 7.6; and (d) cells were grown in BSK-II$_{(-GlcNAc)}$+chitobiose medium at 34°C and pH 7.6. Asterisks indicate that the difference in cell densities between the *rrp1*^{mut} mutant and the WT was statistically significant at a *P* value of <0.001. Cell counting was repeated in triplicate with at least two independent samples, and the results are expressed as means ± SEM.

cells during the growth in BSK-II$_{(-GlcNAc)}$+chitobiose medium (Fig. 3a; see also Fig. S2 in the supplemental material). Collectively, these results suggest that the observed growth defects of the *rrp1*^{mut} strain in BSK-II$_{(-GlcNAc)}$+chitobiose medium was most likely due to the repression of *chbC* expression in the mutant.

***B. burgdorferi* can utilize chitobiose to obtain GlcNAc.** We reasoned that the growth defects of the *rrp1*^{mut} strain in BSK-II$_{(-GlcNAc)}$+chitobiose medium may have been due to its inability of utilizing chitobiose. To test this hypothesis, we developed an LC-MS method (sensitivity reached the nanomole level) to directly measure the intracellular levels of GlcNAc in *B. burgdorferi* cells under different culture conditions. In normal BSK-II medium at 34°C and pH 7.6, the intracellular concentration of GlcNAc in the *rrp1*^{mut} cells (1.35 ± 0.23 μM, $n = 3$ independent samples) in the late stationary phase was similar to that in the wild-type (1.52 ± 0.027 μM, $n = 3$) and *rrp1*^{com} (1.34 ± 0.32 μM, $n = 3$) cells (Fig. 5a), suggesting that inactivation of *rrp1* has no impact on GlcNAc uptake and/or metabolism. Under the same culture conditions in BSK-II$_{(-GlcNAc)}$+chitobiose medium, the levels of GlcNAc detected in the wild-type (1.42 ± 0.57 μM, $n = 3$) and *rrp1*^{com} (0.96 ± 0.42 μM, $n = 3$) cells were similar to that in normal BSK-II medium. Of note, the level of GlcNAc in the *rrp1*^{com} cells was slightly less than that in normal BSK-II medium, but the results were not statistically significant ($P > 0.05$). We also found that the intracellular levels of GlcNAc in the wild-type and *rrp1*^{com} cells were augmented during the transition from the early log phase to the stationary phase (Fig. 5a). In contrast to the wild-type and *rrp1*^{com} strains, no evident GlcNAc peak was detected by LC-MS in the *rrp1*^{mut} cells after the early log phase in BSK-II$_{(-GlcNAc)}$+chitobiose medium (Fig. 5a). These results indicate that the intracellular level of GlcNAc in the *rrp1*^{mut} cells is too low to be detected when GlcNAc is replaced by chitobiose in the growth medium.

We also measured the basal level of GlcNAc in BSK-II medium without supplementing exogenous GlcNAc (BSK-II$_{(-GlcNAc)}$. The detected GlcNAc concentration was approximately 1.47 ± 0.06 μM (Fig. 5b). Addition of 20 μM chitobiose did not contribute significantly to the measurable amount of GlcNAc in BSK-II$_{(-GlcNAc)}$+chitobiose medium (1.73 ± 0.11 μM). The detected GlcNAc levels were much lower than that in normal BSK-II medium (1.92 ± 0.037 mM) or the minimal concentration of GlcNAc (180 μM) required to support high-cell-density growth (6). *B. burgdorferi* lacks a *de novo* biosynthesis pathway of GlcNAc, and there is no other significant source of GlcNAc in BSK-II$_{(-GlcNAc)}$+chitobiose medium. Conceivably, the GlcNAc detected in the wild-type and *rrp1*^{com} cells is converted from chitobiose in the medium and this conversion is severely impaired in the *rrp1*^{mut} mutant.

The *rrp1*^{mut} mutant has decreased RpoS expression. A previous study by Rhodes et al. showed that RpoS regulates *chbC* ex-

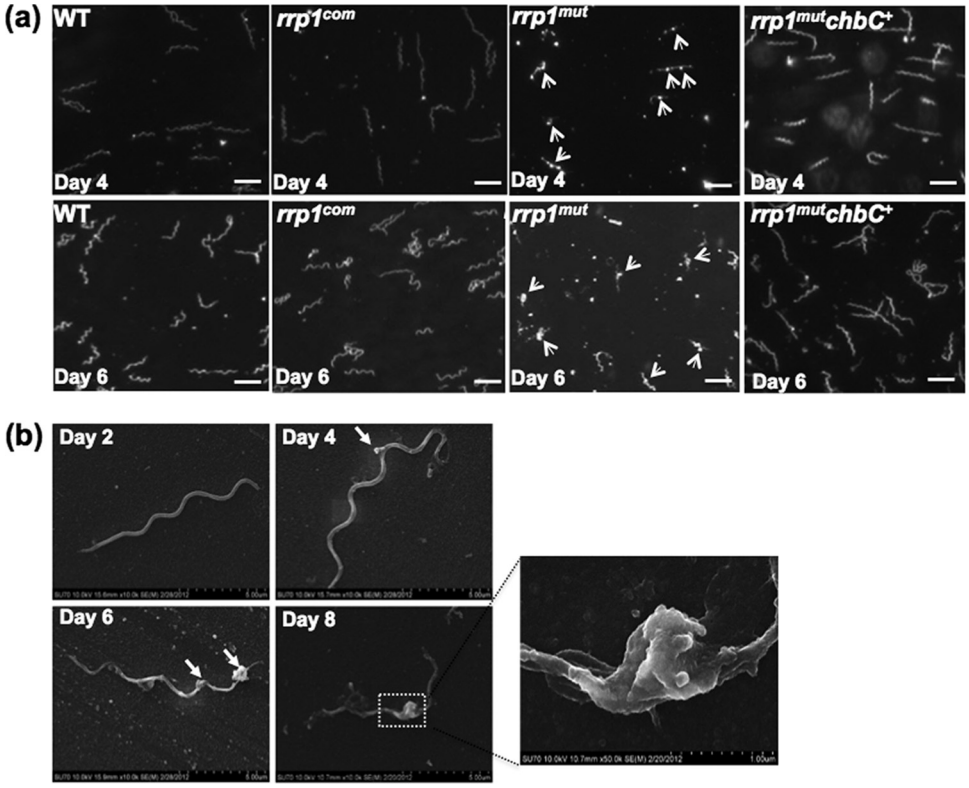

FIG 3 The *rrp1*^{mut} mutant forms membrane blebs in chitobiose-supplemented medium. (a) Dark-field microscopic analysis of *B. burgdorferi* strains grown in BSK-II$_{(-GlcNAc)}$+chitobiose medium at 34°C and pH 7.6. The cultures were collected at day 4 and day 6 and *B. burgdorferi* cells observed under dark-field illumination at ×200 magnification using a Zeiss Axiostar Plus microscope. Scale bars represent 10 μm. (b) Scanning electron microscope analysis of the *rrp1*^{mut} membrane bleb formation. A total of 10^5 cells/ml of the *rrp1*^{mut} mutant were inoculated into 5 ml BSK-II$_{(-GlcNAc)}$+chitobiose medium, and cells were collected for scanning electron microscope analysis at the different time points indicated to observe the formation of membrane blebs. Arrowheads point to membrane blebs observed.

pression (16). Mutation of *rpoS* led to a delay in chitobiose utilization, and the mutant formed membrane blebs in a chitobiose-supplemented growth medium, similar to the phenotype of the *rrp1*^{mut} mutant (Fig. 2d and 3; see also Fig. S2 in the supplemental material). Based on these facts, we hypothesized that inactivation of *rrp1* affects the level of RpoS, which in turn leads to the repression of *chbC* expression in the *rrp1*^{mut} mutant. To test this hypothesis, the levels of RpoS in the wild-type, *rrp1*^{mut}, and *rrp1*^{com} strains were evaluated by immunoblot analyses. We found that the levels of RpoS were substantially repressed in the *rrp1*^{mut} mutant (Fig. 6b).

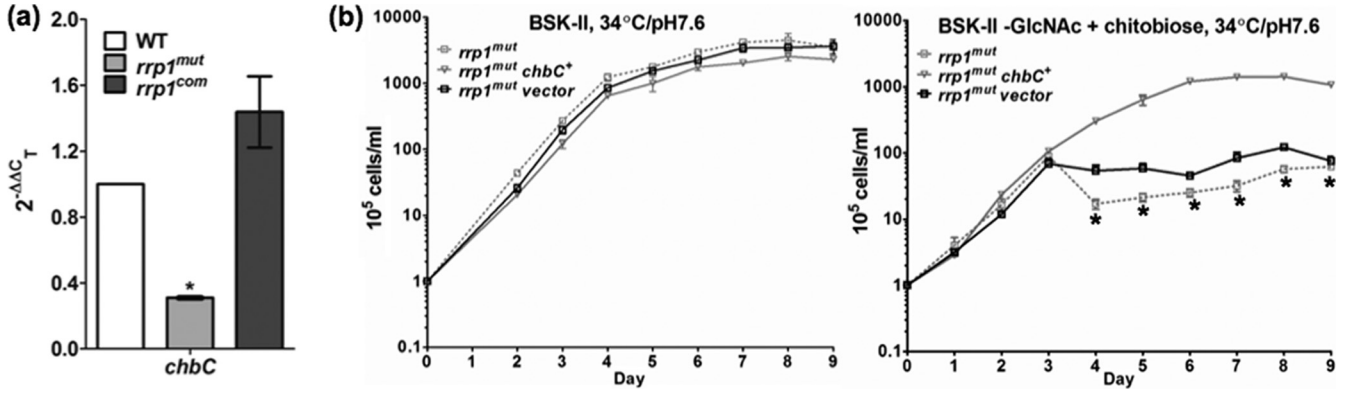

FIG 4 Constitutive expression of *chbC* restored the growth defects of the *rrp1*^{mut} mutant. (a) The *rrp1* mutant has decreased *chbC* transcript. The WT *rrp1*^{mut}, and *rrp1*^{com} strains were cultured at 34°C and pH 7.6 and harvested at the early stationary phase (~10^8 cells/ml). Total RNA was extracted for qRT-PCR. Data are expressed as expression of the *rrp1*^{mut} or *rrp1*^{com} transcripts relative to the wild-type level. Asterisks indicate that the difference between WT and *rrp1*^{mut} transcript levels was statistically significant at a *P* value of <0.01 (two-way ANOVA). (b) Constitutive expression of *chbC* rescues the growth defects of the *rrp1*^{mut} mutant. Growth curve experiments similar to those described for Fig. 2 were repeated using BSK-II and BSK-II$_{(-GlcNAc)}$+chitobiose media at 34°C and pH 7.6. Cell counting was repeated in triplicate with at least two independent samples, and the results are expressed as means ± SEM. Asterisks indicate that the difference between the *rrp1*^{mut} and *rrp1*^{mut} *chbC*$^{+}$ strains in cell densities was statistically significant at a *P* value of <0.001.

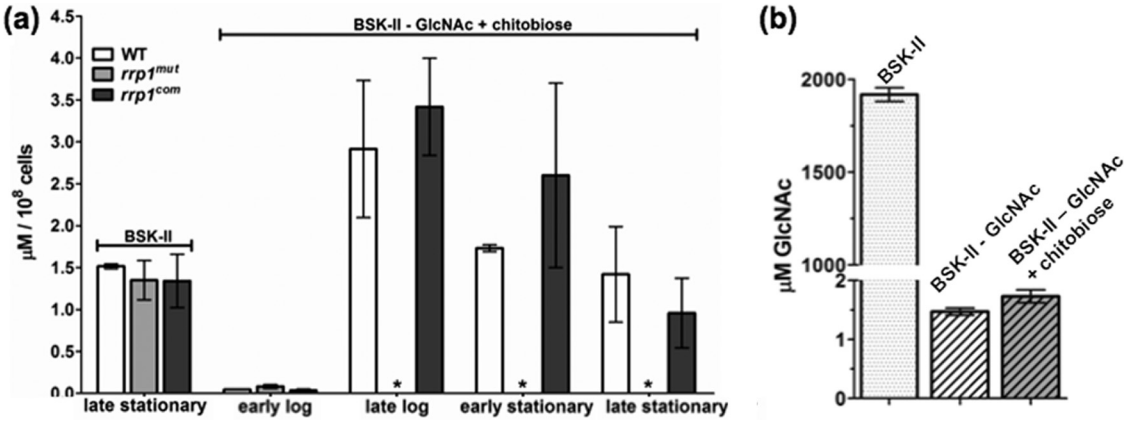

FIG 5 Detecting *N*-acetylglucosamine (GlcNAc) in *B. burgdorferi* culture media and cells. The intracellular level of GlcNAc and the level of GlcNAc in culture media were measured using LC-MS/MS. (a) The intracellular concentration of GlcNAc was determined at various stages of the growth curve for BSK-II$_{(-\text{GlcNAc})}$+chitobiose medium as indicated and at the late stationary phase for cultures in BSK-II medium. Samples were measured in triplicate, and data are represented as means ± SEM in μM/10^8 cells. (b) Concentration of GlcNAc in normal BSK-II, GlcNAc-depleted BSK-II BSK-II$_{(-\text{GlcNAc})}$ and BSK-II$_{(-\text{GlcNAc})}$+chitobiose. Asterisks indicate no GlcNAc peak detected.

At the early (D1) and middle (D2) stationary phase, RpoS was almost undetectable in the mutant; only a trace amount of RpoS was detected when the mutant entered the late stationary phase (D3) (Fig. 6a and b). To confirm this result, we examined the levels of expression of other known RpoS-regulated genes (e.g., *ospC*, *dbpA*, and *dbpB*) (20, 24, 52), as well as that of *ospA*, a gene that is not activated by RpoS (53, 54). Immunoblotting analyses revealed that the expression levels of OspC, DbpA, and DbpB were substantially decreased in the *rrp1*^{mut} mutant and were restored to the wild-type levels in the *rrp1*^{com} strain. In contrast, the level of OspA was unaffected in the *rrp1*^{mut} strain (Fig. 6c). Consistent with the immunoblot results, qRT-PCR analysis showed that the transcript level of *rpoS* in the *rrp1*^{mut} mutant was less than 10% of the wild-type level (Fig. 7b). Collectively, these data support the hypothesis that the impact of Rrp1 on *chbC* expression is mediated via RpoS.

The *rrp1*^{mut} mutant has decreased BosR expression. Several regulators, including Rrp2, RpoN, BosR, and Pta, modulate the transcription of the *rpoS* gene (for reviews, see references 18 and 19). We hypothesized that Rrp1 may regulate the expression of *rpoS* via one of these known regulators. To test this hypothesis, immunoblotting analyses were carried out to measure the levels of these regulators in the *rrp1*^{mut} mutant. Our results showed that the levels of both Rrp2 and Pta were not significantly affected in the *rrp1*^{mut} mutant (Fig. 7a). However, the level of BosR was significantly repressed in the mutant compared to the wild type. Complementation of Rrp1 restored the expression of BosR in the

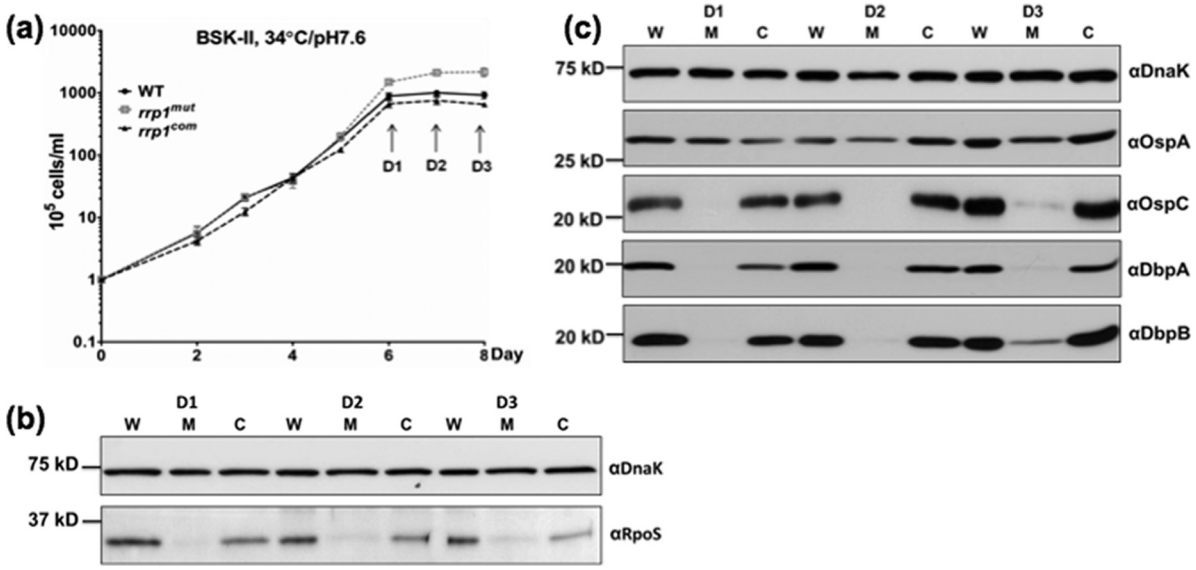

FIG 6 The *rrp1*^{mut} mutant has decreased RpoS expression. A total of 10^5 cells/ml of the WT, *rrp1*^{mut}, and *rrp1*^{com} strains were inoculated into BSK-II and cultivated at 34°C and pH 7.6. (a) Cells were harvested after the stationary phase (10^8 cells/ml) at the indicated time points (D1 to D3). Similar amounts of whole-cell lysates were analyzed by SDS-PAGE. (b) Detection of RpoS. (c) Detection of OspA, OspC, DbpA, and DbpB. DnaK was used as an internal control as previously described (42). W = WT strain, M = *rrp1*^{mut} mutant, C = *rrp1*^{com} strain.

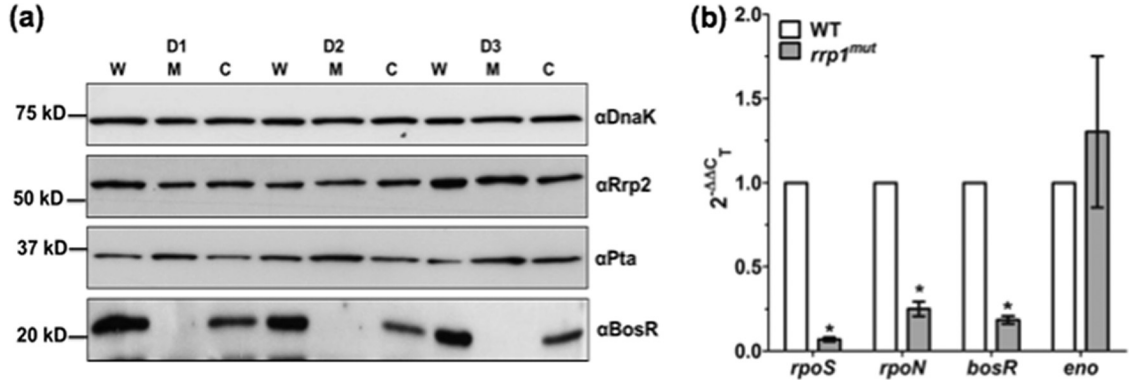

FIG 7 Rrp1 regulates RpoS expression through BosR. (a) Detection of BosR, Pta, and Rrp2 via immunoblot analyses was performed as described for Fig. 6. (b) qRT-PCR analysis of *bosR*, *rpoN*, *rpoS*, and *eno* transcripts was performed as described for Fig. 4a. Data are expressed as expression of the *rrp1*^{mut} transcripts relative to that of the corresponding wild-type transcripts. Asterisks indicate that the differences between WT and *rrp1*^{mut} transcript levels were statistically significant at a *P* value of <0.01 (two-way ANOVA).

rrp1^{com} strain (Fig. 7a), which indicates that Rrp1 most likely regulates RpoS via BosR, an activator of *rpoS* expression (31, 32). Consistent with the immunoblot results, the qRT-PCR analysis showed an approximately 80% reduction at the transcript level of *bosR*, suggesting that the impact of Rrp1 on *bosR* occurs at the transcriptional level (Fig. 7b). We also examined the transcript level of *rpoN* and found that it was also significantly downregulated in the *rrp1*^{mut} mutant (Fig. 7b). In contrast to *rpoS*, *rpoN*, and *bosR*, the expression level of the enolase (*eno*) gene remained unchanged, indicating that the observed reduction of transcript levels in the mutant is gene specific and not due to global repression of transcriptome following the mutagenesis of *rrp1*. Collectively, these results suggest that Rrp1 can regulate the expression of RpoS, probably through both BosR and RpoN.

Supplementation of GlcNAc enables the *rrp1*^{mut} mutant to be transmitted to mice via tick bite. During a blood meal, chitobiose shed from the tick peritrophic membrane (30) can be potentially metabolized and provide a sufficient amount of GlcNAc for *B. burgdorferi* to rapidly replicate, which may facilitate the transition of the spirochetes from the tick to a mammalian host (55). Previous studies showed that a mutant defective in *rrp1* expression fails to be transmitted to mice upon tick bite (14, 26). We reasoned that this failure is probably due to the defect of the *rrp1* mutant in utilizing chitobiose during the transmission. If this hypothesis is correct, we would expect that external supplementation of GlcNAc should rescue, at least in part, the observed transmission defect. To test this hypothesis, naïve *Ixodes scapularis* nymphs were artificially infected with equal numbers of the wild type, the *rrp1*^{mut} mutant, the *rrp1*^{com} strain, and the *rrp1*^{mut} mutant supplemented with 10 mM GlcNAc via microinjection. Infected nymphs were allowed to feed on naïve C3H mice until repletion. The ticks were then subjected to qRT-PCR analyses to determine the spirochete burden. Using *flaB* as a surrogate marker, we were able to detect the *rrp1*^{mut} mutant in replete ticks, albeit at a much lower burden ($P < 0.05$) than in both the wild-type- and *rrp1*^{com} strain-infected ticks. Supplementation of 10 mM GlcNAc did not increase the bacterial load of the *rrp1*^{mut} mutant in replete ticks (Fig. 8a). To investigate if supplementation of GlcNAc can assist in transmission of the *rrp1*^{mut} mutant from tick to mammal, C3H mice were sacrificed 14 days after tick feeding and transmission was assessed via tissue culture and qRT-PCR. Spleen tissue cultures from mice fed upon by both the wild-type- and *rrp1*^{com}-infected ticks were positive 1 week after isolation but were negative for spirochetes from mice fed upon by *rrp1*^{mut} mutant-infected nymphs even after 1 month of culturing (Table 1), which is consistent with previous observations (14, 26). However, this phenotype was partially rescued by providing GlcNAc to the unfed ticks prior to feeding. Two out of 3 mice fed upon by the nymphs infected with the *rrp1*^{mut} mutant with supplementation of 10 mM GlcNAc were positive for spirochetes in spleen tissue culture, indicating a successful transmission from ticks to mice (Table 1). Follow-up qRT-PCR analysis on tissues from mice fed upon by the nymphs infected with the *rrp1*^{mut} mutant with supplementation of 10 mM GlcNAc showed positive results for the *flaB* transcripts (Fig. 8b), further validating the tissue culture results. This finding supports our hypothesis that the defect in utilizing chitobiose as a source of GlcNAc contributes to the failure of the *rrp1* mutant in the transmission from ticks to mice.

DISCUSSION

Owing to its complex enzootic life cycle alternating between arthropod vector and mammalian hosts, highly coordinated expression of the host-specific genes is indispensable for the survival and persistence of *B. burgdorferi* in nature. These extensive transcriptional adjustments are primarily governed by the Rrp2-RpoN-RpoS regulon and the Hk1-Rrp1 signaling pathway (for recent reviews, see references 18 and 19). Exhaustive investigations on the Rrp2-RpoN-RpoS regulatory pathway have provided compelling evidence that it is responsible for the differential expression of various genes required for infection and persistence in the mammalian host, including several outer surface proteins essential for the pathogenicity of *B. burgdorferi* such as OspC and DbpA (19–21, 23, 24, 56, 57). The second signaling pathway is comprised of a diguanylate cyclase (Rrp1) and a histidine kinase (Hk1) (25, 28). Recent studies by three independent laboratories have established the significance of both Rrp1 and Hk1 in the survival of *B. burgdorferi* during the blood meal (14, 25, 26). Rrp1 mutants and Hk1 mutant exhibited similar defects in feeding ticks, where the spirochetes were killed during the blood meal, indicating that the Hk1-Rrp1 TCS is essential during the transmission process (25, 26).

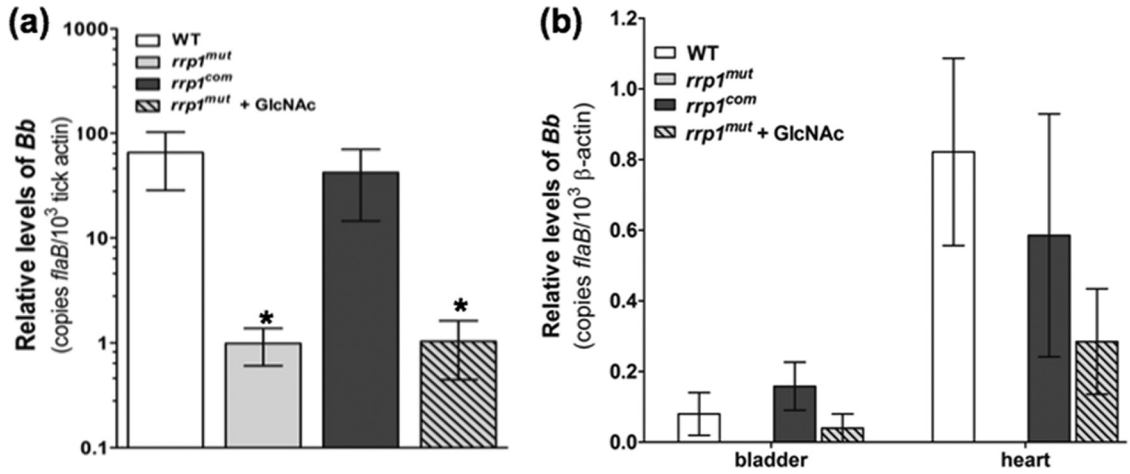

FIG 8 Supplementation of GlcNAc fails to rescue the *rrp1*^{mut} bacterial load in fed ticks but enables transmission via tick bite. (a) RNA samples were extracted from whole fed ticks (after repletion; 5 to 7 days) and subjected to qRT-PCR analysis. The bacterial burdens in ticks were measured by the number of copies of *flaB* mRNA compared to the number of copies of tick-actin transcript as previously described (37). The data are presented as the means of relative levels of *flaB* transcript ± SEM for three groups (each group contained 4 replete ticks) for each strain (WT, *rrp1*^{mut} mutant, and *rrp1*^{com} strain). Asterisks indicate that the differences in bacterial load between the WT strain, the *rrp1*^{mut} mutant, and the *rrp1*^{mut} mutant supplemented with GlcNAc were statistically significant at a *P* value of <0.05. (b) Detection of spirochete burdens in C3H mice infected via tick bite using qRT-PCR. At day 14 after tick feeding, mice were sacrificed. Tissues (skin, heart, joint, and bladder) were collected and total RNA from heart and bladder tissues subjected to qRT-PCR analysis as described in Materials and Methods. Statistical analysis showed no significant difference between the WT, the *rrp1*^{com} strain, and the *rrp1*^{mut} mutant supplemented with GlcNAc at $P = 0.05$.

The role of c-di-GMP in virulence in many pathogenic bacteria has been well established (58–60). Rrp1 is the sole c-di-GMP-synthesizing protein encoded in the genome of *B. burgdorferi* (28), and mutagenesis of *rrp1* has been shown to result in alteration of the *B. burgdorferi* transcriptome (14, 27). Among the genes affected by Rrp1 are carbohydrate metabolic pathways and the GlcNAc transporter systems (27). In this study, we provided evidence showing that Rrp1 is involved in the regulation of chitobiose metabolism in *B. burgdorferi*. *In vitro* growth analysis indicated that the *rrp1*^{mut} mutant is unable to grow in medium containing chitobiose in place of GlcNAc as the carbon source, and restoration of Rrp1 in the mutant successfully rescued the growth defect (Fig. 2d). In addition, overexpression of a single chitobiose transporter gene, *chbC*, is able to salvage the growth of the *rrp1*^{mut} mutant in chitobiose-supplemented medium (Fig. 4b), further supporting our hypothesis that Rrp1 can regulate the chitobiose metabolism pathway and, more specifically, the *chbC* gene. Furthermore, transcriptional analysis showed that the transcript level of *chbC* was significantly repressed in the *rrp1*^{mut} mutant relative to wild-type expression, suggesting that Rrp1 is an activator of the *chbC* gene (Fig. 4a).

A previous report established that RpoS is involved in the transcription of the *chbC* gene (16), which prompted us to investigate the linkage between Rrp1 and RpoS. Using immunoblotting analyses, we showed that the *rrp1*^{mut} mutant has reduced levels of RpoS protein and of several proteins encoded by RpoS-dependent genes, including OspC, DbpA, and DbpB (Fig. 6b and c). Upon analysis of several known regulatory elements of *rpoS*, we found that the protein level of BosR was also downregulated in the *rrp1*^{mut} mutant (Fig. 7a), which indicates that Rrp1 regulates RpoS, probably by affecting the expression of BosR. The oxidative stress response regulator BosR is an important activator of the RpoS pathway (31, 32, 61). The expression of *bosR* is induced upon tick feeding and reaches the maximal level in the murine host (62). As *rrp1* is also highly expressed in the feeding tick (27), it may assist the induction of *bosR* following a blood meal. Thus, a decrease in *bosR* expression in the *rrp1*^{mut} strain leads to a reduction in *rpoS* expression, which subsequently results in reduced transcription of the RpoS-dependent *chbC* gene.

Cultivation of the *chbC* mutant in chitobiose-supplemented medium has been shown to lead to the formation of membrane-attached masses (6). In addition, when the *rpoS* mutant is cultured in a chitobiose-supplemented medium, membrane blebs similar to those in the *chbC* mutant were observed (16). Our scanning electron microscope analysis showed that similar round membrane blebs were seen forming on the *rrp1*^{mut} cells when cultured in BSK-II$_{(-\text{GlcNAc})}$+chitobiose medium and that the formation of the blebs progressed throughout growth and, eventually, cell lysis (Fig. 3). The formation of membrane blebs was reversible upon overexpression of the *chbC* gene in the *rrp1*^{mut} background (Fig. 3a and 4b; see also Fig. S2 in the supplemental material), suggesting that the mutant has a defect in chitobiose utilization and that the membrane blebs were most likely formed due to failure to convert chitobiose to GlcNAc for cell wall synthesis. Dunham-Ems et al. recently showed that the *rpoS* mutant, when present in fed ticks, formed an unusual morphotype, which they termed "round bodies" (63). The observation of formation of such "round bodies" may be related to the blebbings observed in the

TABLE 1 Tick-to-mouse-infection study

Strain	No. of mice infected/ total no. of mice[a]
Wild type	3/3
rrp1^{mut}	0/3
rrp1^{com}	3/3
rrp1^{mut} + GlcNAc	2/3[b]

[a] Mouse spleen tissues were used for the reisolation.
[b] Spirochete cells were observed 2 weeks after tissue isolation.

chbC and *rpoS* mutants as well as in our *rrp1*^{mut} mutant (6, 16). Under unfavorable environmental conditions, initial membrane blebbing occurs, which then progresses to cell lysis. Part of the cell may convert to a cystic form or "round bodies," which, upon encountering a suitable environment, can be quickly reverted to a live spirochete (64–66). The initial failure to utilize chitobiose after a blood meal could cause an unfavorable environment for growth, which subsequently may induce the formation of "round bodies" as a mechanism of protection against hostile conditions.

How does regulation of chitobiose metabolism integrate into the life cycle of *B. burgdorferi*? Previous reports by Tilly et al. indicated that a *chbC* mutant can complete the enzootic cycle and that chitobiose metabolism is likely unimportant for *B. burgdorferi* growth *in vivo* (17). In their study, the *chbC* mutant was able to infect mice via needle inoculation, be acquired by naïve ticks, survive the molting process, and complete the transmission to naïve mice (17), implying that chitobiose metabolism is not essential for the life cycle of this spirochete. The genome of *B. burgdorferi* includes several copies of chitobiose transporter genes that are predicted to form the chitobiose PTS, which includes *chbA* (*bbb05*), *chbB* (*bbb06*), and *chbC* (*bbb04*) (4, 6). Although *in vitro* growth analysis indicated that both *chbA* and *chbB* do not play a role in chitobiose metabolism, they may be able to compensate for the loss of *chbC* during the *in vivo* growth in the arthropod vector, which could potentially rescue the *chbC* mutant during the enzootic cycle and subsequently enable transmission. An *in vivo* study using various *chb* mutants (i.e., double or triple *chb* mutants) would help to clarify the exact role of these chitobiose transporter genes in the infectious cycle of *B. burgdorferi*. In addition to the chitobiose transporter system, the genome of *B. burgdorferi* also encodes several other phospotransferase components for carbohydrate uptake (4). The import of GlcNAc into the *B. burgdorferi* cell is believed to be performed through the glucose PTS transporters (7), similar to the case in other bacteria such as *E. coli* (8). Hence, it is possible that an alternative uncharacterized PTS with a loose specificity for carbohydrate may be able to transport chitobiose, albeit at a lower capacity than the characterized chitobiose transporters. Identifying an additional PTS(s) for chitobiose uptake will definitely help increase understanding of the significance of chitobiose in the life cycle of this spirochete.

During the tick phase of the enzootic cycle, *B. burgdorferi* encounters different carbohydrates for metabolism, one of which is glycerol, the natural antifreeze agent produced by the *Ixodes* tick (12, 13). Studies have shown that the *rrp1* mutant is defective in glycerol metabolism and fails to survive after tick feeding (14). A different group also reported that mutagenesis of a glycerol utilization gene, glycerol 3-phosophate dehydrogenase (*glpD*), led to a reduced spirochete burden in unfed ticks and impaired replication in feeding ticks, emphasizing the importance of glycerol metabolism during the tick phase of the life cycle (5). Microarray analysis indicated that the expression of several glycerol metabolism genes is regulated by Rrp1 (14). Constitutive expression of the glycerol metabolic operon in the *rrp1* mutant was shown to partially rescue the mutant in ticks after a blood meal. However, the mutant remained incapable of transmission to mice, implying that another Rrp1-regulated factor(s) may be at play (14). It is reasonable to suggest that the chitobiose metabolic pathway (in addition to the glycerol metabolism) may be one of the factors that is required to fully rescue the *rrp1* mutant in ticks. In the unfed tick, glycerol is the major carbon source available to the spirochete. Glycerol can be channeled into the glycolysis pathway for energy production, and thus a mutant defective in glycerol metabolism would have reduced metabolic fitness. Upon feeding, chitobiose becomes available and can be converted into GlcNAc for cell wall synthesis. The *rrp1* mutant, which has a preexisting reduced fitness, encounters a second defect in chitobiose utilization, further compromising the condition of the spirochetes. Together, these defects eventually result in clearance of the spirochetes from the vector. This may explain why overexpressing the glycerol utilization pathway alone is unable to salvage the *rrp1* mutant in the feeding tick. In addition, our *in vitro* growth analysis showed that the *rrp1*^{mut} strain can grow in the chitobiose-supplemented medium under unfed tick conditions but not at elevated temperature (Fig. 2). As *chbC* is dually regulated by both RpoD and RpoS (16), under unfed tick conditions where RpoS is not expressed, *chbC* is solely regulated by RpoD, which can explain the lack of growth defect in the *rrp1*^{mut} mutant (Fig. 2b). Upon transition to an elevated temperature where RpoS is induced (56), a low level of RpoS in the *rrp1*^{mut} mutant leads to decreased transcription of *chbC*, which subsequently results in failure to utilize chitobiose for growth. Both *in vitro* growth analysis (Fig. 2) and transcriptional (Fig. 4a and 7b) and translational (Fig. 6) analyses seemed to support this proposition. Along with this proposition, a study from Rhodes et al. showed that at elevated temperature (33°C), the *rpoS* mutant showed reduced transcription of *chbC* as well as a defect in utilizing chitobiose for growth (16), emphasizing the importance of RpoS in the regulation of chitobiose metabolism.

In our tick infection study, when 10 mM GlcNAc was supplemented, we were able to partially rescue the *rrp1*^{mut} mutant in fed ticks to allow transmission to mice (Table 1). Although the bacterial burden (as measured by qRT-PCR) in fed ticks remained low in the GlcNAc-supplemented *rrp1*^{mut} mutant group, this could be explained by the additional defect in the glycerol metabolic pathway in the *rrp1* mutant as mentioned earlier. Addition of GlcNAc may provide only enough of a utilizable carbon source to rescue a small percentage of the spirochetes to multiply and complete the transmission process following a blood meal (Fig. 8). In order to fully rescue the mutant phenotype, both glycerol and chitobiose metabolism pathways may be necessary, as the former is necessary for glycolysis while the latter is needed for GlcNAc production for cell wall synthesis (4). In our tick study, we did not include the *rrp1*^{mut} *chbC*$^{+}$ strain as a rescue strategy due to several concerns. First, we observed a significantly higher expression of RpoS in this strain when we cultivated it under routine laboratory culture conditions (data not shown). Second, the *rrp1*^{mut} *chbC*$^{+}$ strain exhibited poor growth in normal BSK-II medium under conditions mimicking the unfed-tick conditions (data not shown). We reasoned that the untimely and high induction of RpoS may have been responsible for the poor growth pattern observed in this strain. We currently do not have an explanation as to how overexpression of the *chbC* gene can affect RpoS expression. Perhaps constitutive expression of the chitobiose transporter forces a switch in the carbohydrate metabolism preferences and stimulates the spirochete to induce fed-tick or mammal-phase genes, such as RpoS. The expression of RpoS as well as other important surface lipoproteins such as OspC is necessary to establish an infection in mammals (see reviews in references 18 and 19). *In vitro* immunoblot results indicated that the *rrp1*^{mut} mutant has minimal expression of RpoS and RpoS-regulated proteins (Fig. 6b and c). Reduced expression of these important virulence factors may explain

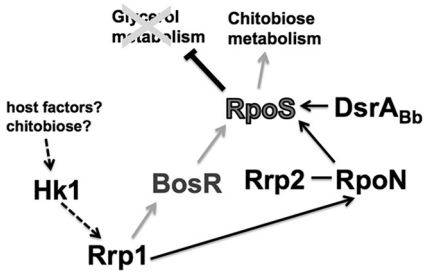

FIG 9 Model for Rrp1 regulation of chitobiose metabolism in *B. burgdorferi*. During the feeding process, certain host factors or chitobiose or both serve as a ligand(s) to activate the histidine kinase, Hk1, leading to phosphorylation of the response regulator, Rrp1. Phosphorylated Rrp1 activates BosR (the oxidative stress regulator) and RpoN, which then activate the transcription of *rpoS*. The expression of RpoS is further enhanced via the signaling by Rrp2-RpoN as well as other regulators such as DsrA$_{Bb}$. An increase in the level of RpoS leads to repression of glycerol metabolism genes and activation of the components of the chitobiose metabolic pathway, especially the chitobiose transporter gene, *chbC*. Expression of the chitobiose utilization pathway allows *B. burgdorferi* to utilize chitobiose shed from the peritrophic membrane which is formed after a blood meal as a source for GlcNAc, a precursor for cell wall synthesis. A switch from glycerol to chitobiose for utilization as the main carbon source allows the spirochete to multiply in numbers and completes the transmission cycle.

the partial restoration of transmission to mice (Table 1) as well as the late emergence of spirochetes from the tissue infected by the *rrp1*^{mut} mutant plus GlcNAc even though qRT-PCR analysis showed no significant difference between the WT and the *rrp1*^{mut} mutant plus GlcNAc in the spirochete burden of infected mice tissues (Fig. 8b).

A study by Caimano et al. showed that RpoS acts as a repressor of the glycerol metabolic pathway (20). In addition, RpoS has been shown to be involved in the transcription of the *chbC* gene (16). Based on this information together with the data in this study, we propose a regulatory model for Rrp1 during the feeding-tick stage of the *B. burgdorferi* enzootic cycle (Fig. 9). During a blood meal, host factors or chitobiose shed from the peritrophic membrane or both serve as a signal(s) to activate the Hk1 histidine kinase, leading to the phosphorylation of the response regulator, Rrp1. Activated Rrp1 induces BosR and RpoN expression, which, together with other factors (such as the Rrp2-RpoN signaling and DsrA$_{Bb}$), leads to the expression of RpoS. Induction of RpoS expression results in repression of the glycerol metabolism genes and activation of the chitobiose transporter *chbC* gene. Activation of the chitobiose utilization pathway allows a switch in carbohydrate metabolism, changing from glycerol to chitobiose as the main carbon source for replication and subsequent transmission to a mammalian host.

The regulation by c-di-GMP can occur at various stages, including both transcriptional and posttranscriptional stages (58, 67–69). C-di-GMP affects a cellular function by binding to its receptor proteins, such as PilZ domain-containing proteins (69). Our transcriptional analysis indicated a reduction in transcripts of both the activator of the *rpoS* gene, *bosR*, and *rpoN* (Fig. 7b), suggesting that transcriptional regulation is involved. Currently, little is known about the regulation by c-di-GMP in *B. burgdorferi*. A c-di-GMP effector protein, PlzA, which contains a PilZ domain, has been identified in *B. burgdorferi* (70). Whether PlzA or an additional unidentified effector protein(s) is involved in the regulation by Rrp1 on *bosR*/*rpoN* requires further investigation. In summary, our findings on Rrp1 in this study show an interaction between the two important signaling pathways in *B. burgdorferi*, Hk1-Rrp1 and Rrp2-RpoN-RpoS. By acting as an activator of RpoS, Rrp1 serves as a carbohydrate switch in *B. burgdorferi* during the tick phase, allows glycerol metabolism in the unfed tick stage, and switches the carbon metabolism to chitobiose upon a blood meal by activating RpoS expression via BosR. Studies to elucidate the underlying molecular mechanism as well as the effector protein(s) involved are necessary to fully understand the function of Rrp1 in the enzootic cycle.

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