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The diguanylate cyclase, Rrp1, regulates critical steps in the enzootic cycle of the Lyme disease spirochetes

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Abstract

Rrp1 is the sole c-di-GMP producing protein (diguanylate cyclase) of *Borrelia burgdorferi.* To test the hypothesis that Rrp1 regulates critical processes involved in the transmission of spirochetes between ticks and mammals, an *rrp1* deletion mutant (B31-Δ*rrp1*) and a strain that constitutively produces elevated levels of Rrp1 (B31-OV) were constructed. The strains were assessed for progression through the enzootic cycle using an *Ixodes* tick/C3H-HeJ mouse model and tick immersion feeding methods. B31-Δ*rrp1* infected mice as efficiently as wild type but had altered motility, decreased chemotactic responses to N-acetylglucosamine (NAG) and attenuated ability to disseminate or colonize distal organs. While this strain infected mice, it was not able to survive in ticks. In contrast, the B31-OV displayed normal motility patterns and chemotactic responses but was non-infectious in mice. Using immersion feeding techniques we demonstrate that B31-OV can establish a population in ticks and survive exposure to a natural bloodmeal. The results presented here indicate Rrp1, and by extension, c-di-GMP, are not required for murine infection, but are required for the successful establishment of a productive population of *B. burgdorferi* in ticks. These analyses provide significant new insight into the genetic regulatory mechanisms

Keywords

Borrelia; Lyme disease; Rrp1; cyclic-di-GMP; diguanylate cyclase

INTRODUCTION

Lyme disease is a tick borne infection caused by *B. burgdorferi*, *B. garinii*, and *B. afzelii* (Benach *et al.*, 1983; Burgdorfer *et al.*, 1982). The Lyme disease spirochetes must adapt to different environmental conditions as they cycle between mammals and *Ixodes* ticks (Barbour and Hayes, 1986). Recent studies suggest that c-di-GMP, a ubiquitous bacterial nucleotide secondary messenger molecule (Cotter and Stibitz, 2007; Galperin, 2006; Hengge, 2009; Romling and Amikam, 2006), is a key regulator of *B. burgdorferi* adaptive responses and may play an important role in the enzootic cycle (Freedman *et al.*, 2009;

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Pitzer *et al.*, 2011; Rogers *et al.*, 2009b; Ryjenkov *et al.*, 2005; Sultan *et al.*, 2010). In *B. burgdorferi*, c-di-GMP is produced by a single diguanylate cyclase, designated as Rrp1 (Galperin *et al.*, 2001; Ryjenkov *et al.*, 2005). The global regulatory ability of Rrp1, and by extension c-di-GMP, was revealed through microarray analysis of an *rrp1* deletion mutant (Rogers *et al.*, 2009b). The Rrp1 regulon, which consists of ~10% of the genome, includes genes that encode proteins spanning a wide range of functional categories with a concentration on motility, chemotaxis, and metabolism. Several of the Rrp1 regulated genes encode proteins that are likely to be in involved in the enzootic cycle.

As in other bacterial systems, c-di-GMP levels in *B. burgdorferi* are thought to be controlled by the opposing activities of diguanylate cyclases and phosphodiesterases. *Borrelia* strains encode a diguanylate cyclase (Rrp1), EAL domain phosphodiesterase (PdeA) and HD-GYP domain phosphodiesterase (PdeB) (Rogers *et al.*, 2009b; Ryjenkov *et al.*, 2005; Sultan *et al.*, 2010). Little is known regarding the effector mechanisms of c-di-GMP in the Lyme disease spirochetes and arthropod-borne pathogens in general. In other bacteria, the regulatory effects of c-di-GMP have been linked to interactions with GEMM riboswitches (Smith *et al.*, 2009; Sudarsan *et al.*, 2008), ribonucleoprotein complexes (Tuckerman *et al.*, 2011), transcriptional regulators (Hickman and Harwood, 2008), PilZ domain-containing proteins (Amikam and Galperin, 2006; Ryjenkov *et al.*, 2006), and proteins with cyclic nucleotide monophosphate domains (Tao *et al.*, 2011). Two PilZ domain containing proteins (PlzA and PlzB) have been identified in *Borrelia* strains and demonstrated to bind c-di-GMP in a highly specific manner (Freedman *et al.*, 2009). All strains produce the chromosomally encoded PlzA protein. Subsets of strains carry a second gene encoding a c-di-GMP binding protein designated as PlzB. PlzB is encoded by a variably size linear plasmid (Freedman *et al.*, 2009). The effector functions of these proteins have not been defined.

In this study, we test the hypothesis that Rrp1 and, by extension, c-di-GMP, regulate critical steps in the enzootic cycle of the Lyme disease spirochetes. *B. burgdorferi* strains that lack Rrp1 or that constitutively produced elevated levels of Rrp1 were generated and analyzed for their ability to infect and transit between mice and *Ixodes* ticks. Motility patterns, chemotactic responses to N-acetylglucosamine (NAG), and the expression of genes involved in NAG metabolism were also assessed. The data presented within demonstrate that Rrp1, presumably through its production of c-di-GMP, regulates critical processes required for completion of specific stages of the enzootic cycle. These analyses provide unique insight into a relatively unexplored area of the role of diguanylate cyclases in genetic regulation in *B. burgdorferi* and arthropod borne pathogens in general.

RESULTS

Generation and analysis of an *rrp1* deletion mutant, *rrp1* over-expressing strain and cis-complemented strain

To determine if Rrp1 regulates cellular processes required for completion of the enzootic cycle, *rrp1* of *B. burgdorferi* strain B31 clone 5A4 (an infectious clone) was replaced by a spectinomycin/streptomycin resistance cassette (*strep*^{R}) to yield strain B31-Δ*rrp1* (Figure 1A). A functional *cis*-complemented strain, B31-KI ("knock-in"), was generated by replacing the *strep*^{R} cassette of B31-Δ*rrp1* with wild-type *rrp1* and a downstream kanamycin resistance cassette (Figure 1B). To generate a strain that constitutively produces elevated levels of Rrp1, B31-wt was transformed with pBSV2-P$_{flaB}$*rrp1* (Figure 1C) to yield a strain carrying a plasmid and chromosomal copy of *rrp1*. Clonal populations of each strain were assessed for insertion of the cassette or for the presence of the pBSV2-P$_{flaB}$*rrp1* plasmid using PCR (Figure 1D & Table 1). The *Borrelia* genome is segmented and consists of a linear chromosome and 20 or more linear and circular plasmid (Barbour and Garon, 1987). Genetic manipulation can result in the loss of plasmids required for survival in

mammals (Labandeira-Rey and Skare, 2001; Purser *et al.*, 2003). To assess plasmid content, PCR was performed with plasmid specific primer sets (data not shown) (McDowell *et al.*, 2001). Only clones harboring the full set of plasmids were selected for further analyses. To evaluate Rrp1 protein and transcript levels, immunoblot and qRT-PCR analyses were conducted (Figure 1E &F, respectively). *rrp1* transcript and protein was detected in the B31-wt, B31-KI, and B31-OV strains, but not in B31-Δ*rrp1*. In vitro, B31-OV produced 2-fold more *rrp1* mRNA than B31-wt or B31-KI (Figure 1F).

In nature *B. burgdorferi* encounters radically different environment conditions as it cycles between ticks and mammals. One environmental variable that it must adapt as it cycles between ticks and mammals is temperature. Cultivation in the laboratory, at 25 to 27°C, is thought to mimic at least in part the tick environment. Hence, we determined growth rates at 27, 33, and 37°C (Figure 1G). B31-Δ*rrp1* displayed an extended lag phase at 27°C (7 days) and did not enter stationary phase for 13 days. All remaining strains entered log phase within 2 days and stationary phase by day 7. It can be concluded that Rrp1 is not required for growth in vitro, but its absence influences growth at lower temperatures. This suggests that Rrp1 may play an important role in the tick environment. Consistent with a potential role for Rrp1 and c-di-GMP in the tick, *rrp1* transcription has been demonstrated to be dramatically upregulated in ticks after ingestion of a bloodmeal (Rogers *et al.*, 2009b).

Analysis of c-di-GMP levels

C-di-GMP production by each strain was assessed through HPLC analysis of nucleotides extracted from in vitro cultivated bacteria. This approach has been successfully applied to measure c-di-GMP in *E. coli* (Antoniani *et al.*, 2010) and it has a sensitivity of 0.3 pmol nucleotide/mg cells (data not shown). *B. hermsii* DAH (tick-borne relapsing fever) and *Treponema denticola* (periodontal disease) were included as controls. *B. hermsii* harbors orthologs of each of the proteins that have been demonstrated to participate in the synthesis and breakdown of c-di-GMP in *B. burgdorferi* and hence we reasoned that it would behave similarly to *B. burgdorferi. T. denticola* was included because it actively produces several diguanylate cyclases during in vitro cultivation (R.T. Marconi, unpublished data), thereby making it a good positive control for c-di-GMP detection. In addition, because *B. hermsii* and *T. denticola* have a similar membrane composition to *B. burgdorferi* (no lipopolysaccharide with a lipoprotein rich outer membrane) we reasoned that they could serve as controls for the nucleotide extraction process. C-di-GMP was readily detected in *T. denticola* but not in *B. hermsii* (data not shown), *B. burgdorferi* B31-wt, B31-Δ*rrp1* B31-KI or B31-OV (Figure 1H) demonstrating that c-di-GMP concentrations are inherently low in cultivated *Borrelia*. This is consistent with the low level expression of *rrp1* during in vitro cultivation (Rogers *et al.*, 2009b).

Demonstration that Rrp1 directly or indirectly influences infectivity and dissemination and or secondary colonization of *B. burgdorferi* in mice

Mice were subcutaneously needle inoculated with each strain and infection assessed by seroconversion (ELISA and immunoblotting) 4 weeks post-inoculation (Figure 2A). All mice inoculated with B31-wt, B31-Δ*rrp1*, and B31-KI were seropositive with equivalent IgG titers. The B31-OV strain had a significantly lower IgG titer that all other strains ($p<0.01$). The low level IgG titer elicited by the B31-OV strain is similar to that observed for other non-infectious isolates and it presumably results an antibody response to the inoculum itself (Earnhart *et al.*, 2010). To compare antibody responses evoked by each strain, the overall pattern of immunoreactive proteins detected was determined by immunoblotting (Figure 2B). Consistent with the ELISA data, serum from mice inoculated with B31-OV did not recognize any proteins in the immunoblot analyses. No significant

differences in immunoreactive profiles were observed with the serum collected from mice infected with B31-wt, B31-Δ*rrp1*, and B31-KI.

To further assess infectivity, dissemination, and or secondary colonization, biopsies from each mouse were collected from organs and tissues distal to the inoculation site and placed in BSK-H media for spirochete cultivation. Cultures were obtained from all clinical specimens recovered from B31-wt and B31-KI inoculated mice (Table 2). None of the heart biopsies and only a subset of the bladder and skin samples harvested from B31-Δ*rrp1* inoculated mice yielded positive cultures. Positive cultures were not obtained from mice inoculated with B31-OV. This observation is consistent with the immunological data described above. It can be concluded that Rrp1 is not required for infectivity but is required for maximal dissemination and or secondary colonization.

Rrp1 is essential for spirochete acquisition by *Ixodes scapularis*

To assess the ability of each strain to transit from infected mammals to feeding ticks, larval stage *Ixodes scapularis* ticks were fed to repletion on mice (4 weeks post-inoculation), collected, DNA extracted, and qPCR was performed (Figure 3A & B). Ticks fed on B31-wt and B31-KI infected mice had an average of ~2.0 and ~2.4 *flaB* copies per 1000 tick genome equivalents indicating establishment of a productive population in ticks. Spirochetes were not detected in ticks fed on mice inoculated with B31-OV or B31-Δ*rrp1*. The results with the B31-OV strain are as expected since this strain did not establish a detectable infection in mice. To determine if the B31-OV and B31-Δ*rrp1* strains can survive in ticks, a mouse independent route of infecting ticks was employed. Larval ticks were submerged in cultures of each strain and then fed on mice to provide bloodmeal derived nutrients (Figure 3B). The efficiency of infecting ticks by this approach was 20 to 40% with each strain except B31-Δ*rrp1* (0%). These data suggest that Rrp1 directly or indirectly regulates processes that are required for survival in ticks.

Aberrant motility and chemotaxis patterns of the B31-Δ*rrp1* and B31-OV strains

Several approaches were employed to to determine if *rrp1* deletion or elevated production of Rrp1 regulates *B. burgdorferi* motility and or chemotaxis. General motility patterns were assessed for spirochetes in BSK-H media with and without 1% methylcellulose using dark-field and differential interference contrast (DIC) microscopy (with video tracking). Methylcellulose increases media viscosity in a matrix dependent manner (Ruby and Charon, 1998). All strains displayed wave propagation but their translational motion (directional forward movement) patterns and flex frequency differed (Figure 4). Note that all figures and supplemental videos of motility depict cells that are representative of the population as a whole. Consistent with earlier analyses of *B. burgdorferi* motility, B31-wt moves short distances (run speed of 4.14 μm sec^{-1}), flexes (0.1738 flexes sec^{-1}), and reverses direction (Figure S1 & Table 3) (Li *et al.*, 2002; Sultan *et al.*, 2010). Motion-tracking revealed that B31-Δ*rrp1* had a faster run speed (5.58 μm sec^{-1}) and a significantly decreased flex frequency (0.015 flexes sec^{-1}). Hence it was locked in a constant straight run (Figure S2 & Table 3). In contrast, elevated production of Rrp1 as in the B31-OV strain had no effect on motility patterns (Figure S4). As expected, the B31-KI strain (Figure S3) displayed motility patterns consistent with B31-wt. Motility was also assessed in semi-solid media using swarm assays. The B31-Δ*rrp1* swarm diameter was found to be reduced by 47% relative to B31-wt ($p<0.01$). The swarm diameters of B31-KI and B31-OV were similar to B31-wt (Figure 5A and 5B). The results demonstrate that Rrp1, presumably through its production of c-di-GMP, plays an important role in regulating flagellar motor function.

C-di-GMP has been demonstrated play a role in regulating chemotaxis in several gram negative enteric bacteria (Paul *et al.*, 2010). To determine if the presence or absence of Rrp1

directly or indirectly influences chemotaxis, capillary assays were performed using NAG (Figure 5C). NAG is a known chemoattractant of *B. burgdorferi* (Bakker *et al.*, 2007; Shi *et al.*, 1998). The B31-Δ*rrp1* strain was highly attenuated in its NAG driven chemotactic response ($p<0.05$) while the response of B31-OV was not statistically different from that of the B31-wt strain. Complementation of *rrp1* (B31-KI) restored wild type chemotactic responses to B31-Δ*rrp1*. Collectively, the data indicate Rrp1 is required for regulation of flagella motor activity and NAG driven chemotactic responses.

Transcriptional analysis of genes involved in NAG metabolism

NAG, which is abundant in ticks and in glycosaminoglycans of mammalian tissue, is required for in vitro growth of *Borrelia* (Barbour, 1984). To determine if Rrp1 regulates the transcription of genes involved in NAG metabolism, qRT-PCR analyses were performed. The expression levels of *nagA* (NAG-6-phosphate deaminase), *nagB* (glucosamine-6-phsophate-isomerase), *ptsG* (PTS system, glucose-specific IIBC component), and ORF BB0002 (β-N-acetylhexosaminidase; a putative chitobiase) were examined (Table 4). Deletion of *rrp1* resulted in a significant decrease in the amount of *nagA* and *nagB* mRNA but had no affect on BB0002 and *ptsG* (BB0645) transcription. B31-OV also had decreased *nagB* expression but transcription of the other 3 genes were not significantly affected. These results indicate that Rrp1 directly or indirectly regulates the transcription of important genes involved in the utilization of NAG. The decreased transcription of these genes could influence the ability of *B. burgdorferi* to survive in tissues rich in NAG and in the tick.

DISCUSSION

In the Lyme disease spirochete, *B. burgdorferi*, c-di-GMP has been postulated to regulate cellular processes required for completion of the enzootic cycle (Freedman *et al.*, 2009; Pitzer *et al.*, 2011; Rogers *et al.*, 2009b; Sultan *et al.*, 2010). Rrp1 is the sole diguanylate cyclase encoded by the *Borrelia* genome and deletion of *rrp1* results in pronounced changes in its transcriptome (Rogers *et al.*, 2009b). Since Rrp1 lacks a DNA binding domain or other known functional domains, its regulatory capability is thought to be linked specifically to its diguanylate cyclase activity. Until this study, the potential requirement for c-di-GMP over the course of the enzootic cycle of a tick-borne bacterial pathogen has not been assessed. Recent analyses of *B. burgdorferi pdeA* (EAL domain phosphodiesterase) and *plzA* (PilZ domain c-di-GMP binding protein) mutants, which display attenuated virulence, have provided indirect evidence that c-di-GMP is an important regulatory molecule in vivo (Pitzer *et al.*, 2011; Sultan *et al.*, 2010). The Lyme disease spirochetes offer an excellent model system for studying diguanylate cyclase mediated regulation over the course of the enzootic cycle because *B. burgdorferi* can be genetically manipulated and there is a well characterized and biologically relevant mouse-tick enzootic model.

In this study, we test the hypothesis that high c-di-GMP levels interfere with passage of spirochetes from ticks to mammals (transmission stage) while the absence of c-di-GMP attenuates the ability of *B. burgdorferi* to transit from mammals to ticks (acquisition phase). To assess these possibilities, strains that lack *rrp1* (B31-Δ*rrp1*) or produce it at elevated levels (B31-OV) were generated. While B31-Δ*rrp1* retained its ability to infect mice the B31-OV strain was not infectious. B31-Δ*rrp1* was also found be less efficient at dissemination and/or secondary colonization as it was not cultivated from heart biopsies and was inconsistently cultured from biopsies collected from sites distal to the inoculation site. In addition, while B31-Δ*rrp1* readily infected mice, natural tick feeding experiments revealed that this strain is not able to transit into ticks and establish a productive population. Because B31-OV cannot infect mice we were not able to assess its potential to transit from mammals to ticks via natural tick feeding. To allow us to assess the ability of this strain to survive in ticks an immersion feeding approach was employed (Policastro and Schwan,

2003). B31-OV established a productive population in ticks while B31-Δ*rrp1* did not. The data presented here and in earlier studies suggest that c-di-GMP levels regulate processes involved in transmission, acquisition and survival of *B. burgdorferi* in ticks (Rogers *et al.*, 2009b; Sultan *et al.*, 2010).

The inability of the B31-Δ*rrp1* to transit from ticks to mammals and or survive in ticks could be due to impaired chemotactic responses or the inability to efficiently utilize tick derived nutrients. Earlier transcriptional analyses demonstrated that genes that encode proteins involved in chemotaxis (*che* and *mcp*) and NAG utilization (*nagA* and *nagB*) are positively regulated by Rrp1 (Rogers *et al.*, 2009b). Consistent with the transcriptional data, a 75% reduction in the chemotactic response of B31-Δ*rrp1* to NAG was observed. Complementation restored wild type chemotactic responses to NAG. Wild type levels of *nagA* and *nagB* transcription were also restored by complementation. NAG, which is a major constituent of chitin and chitobiose, is highly abundant in the peritrophic membrane of the tick midgut (Rudzinska *et al.*, 1982; Zhu *et al.*, 1991). The inability of B31-Δ*rrp1* to pass from infected animals into ticks could be due to the reduced ability of this strain to sense tick derived NAG and migrate to the tick bite site. It stands to reason that efficient acquisition would be dependent on such responses and that the ability of *B. burgdorferi* to survive in ticks would require efficient utilization of NAG.

Studies of other bacteria indicate an inverse correlation between c-di-GMP levels and bacterial motility (Jonas *et al.*, 2009; Kim and McCarter, 2007; Martinez-Wilson *et al.*, 2008; Paul *et al.*, 2010; Simm *et al.*, 2004; Wolfe and Visick, 2008). The influence of Rrp1 on motility of *B. burgdorferi* was first assessed by evaluating basic flagella motor function with wave propagation as a read out. All strains displayed wild type wave propagation patterns. To assess translational motion (i.e., directional movement away from the point of origin) methylcellulose was added to the media (Ruby and Charon, 1998). In standard BSK-H media, which lacks methylcellulose, spirochetes display limited translational motion due to low viscosity and the absence of an inherent matrix. For spirochetes to undergo translational motion, the flagella motors located at each end of the cell must rotate in opposite directions (i.e., clockwise and counterclockwise).

When the flagella motors rotate in the same direction, the cells flex and when both flagella motors reverse direction, a change in direction occurs (Charon *et al.*, 2009). All strains had translation motion but B31-Δ*rrp1* was drastically reduced in flex frequency and was locked in constant one-directional motion (see supplementary movie files and Table 3 for rates). In *E. coli*, c-di-GMP has been demonstrated to influence protein-protein interactions that are control flagella motor rotation (Fang and Gomelsky, 2010; Paul *et al.*, 2010). The motility phenotype of B31-Δ*rrp1* suggests that c-di-GMP may contribute to flagella motor activity in *B. burgdorferi* as well. However, the wild type motility phenotype of the B31-OV strain, which would be predicted to have elevated levels of c-di-GMP, seems at odds with the paradigm that high levels of c-di-GMP inhibit motility and promote a sessile life style (Hengge, 2009). Because the diguanylate cyclase activity of Rrp1 is strictly dependent on its phosphorylation state (Freedman *et al.*, 2009; Ryjenkov *et al.*, 2005) elevated production of Rrp1 may not necessarily lead to an increase in c-di-GMP. Rrp1 is cotranscribed with the histidine kinase, Hpk1, which is thought to serve as its phosphate donor (Rogers *et al.*, 2009b). Increased production of c-di-GMP may require elevated production and autophosphorylation of Hpk1. The series of events required for this to occur may be dependent on environmental stimuli that are unique to the tick-mammal interface (Rogers *et al.*, 2009b). These stimuli may not be encountered during in vitro cultivation.

To determine if Rrp1 production levels correlate with c-di-GMP concentration, we sought to measure the relative intracellular c-di-GMP concentration for each strain. Nucleotide

extracts were assayed using reverse phase-high performance liquid chromatography. Precedent for the application of this highly sensitive approach comes from studies of c-di-GMP levels in *E. coli* (Antoniani *et al.*, 2010). However, c-di-GMP levels in in vitro cultivated *B. burgdorferi* proved to be below the detection threshold. This raises the question: if the concentration of c-di-GMP is so low that it can't be detected during in vitro cultivation, is it even produced in vitro and is it plausible that the unique properties of the B31-Δ*rrp1* strain are in fact due to the loss of diguanylate cyclase activity? The recent demonstration that c-di-GMP pools are asymmetrical and localized within a cell offers a possible explanation (Christen *et al.*, 2010). It is possible that *B. burgdorferi* flagellar motor function is controlled by a concentrated local pool of c-di-GMP that represents a small fraction of the total cellular nucleotide pool. The deletion of *rrp1* would eliminate this pool and thus result in aberrant motility and chemotactic responses. Additional evidence for c-di-GMP production by the *Borrelia* during in vitro growth has come from the study of a *B. hermsii pdeA* deletion mutant. Deletion of *pdeA*, the primary phosphodiesterase involved in c-di-GMP breakdown in the *Borrelia*, leads to the accumulation of c-di-GMP thus allowing it to be detected by HPLC (R.T. Marconi, unpublished results). While it would be informative to measure c-di-GMP levels in spirochetes in vivo, such analyses are technically challenging due to the low numbers of spirochetes in mammals and ticks. It may be possible in future analyses to employ FRET based c-di-GMP biosensor approaches to assess changes in c-di-GMP levels in response to environmental stimuli (Christen *et al.*, 2010).

In summary, this study demonstrates that Rrp1 regulates stage specific steps in the enzootic cycle of the Lyme disease spirochetes. Deletion of *rrp1* prevented the establishment of a productive population in ticks, while elevated production blocked infectivity in mice. The presence or absence of Rrp1 was found to be intricately linked to flagellar motor regulation and chemotactic responses, processes which are likely to be critical for completion of the enzootic cycle. Since diguanylate cyclase activity is the only known function of Rrp1, it is highly probable that c-di-GMP levels are responsible for the phenotypes observed for the strains generated in this report. In constructing future models for *Borrelia* pathogenesis, genetic regulation and motility, it will be important to consider the far reaching regulatory potential of c-di-GMP.

EXPERIMENTAL PROCEDURES

Bacterial strains and cultivation conditions

Borrelia strains were cultivated in BSK-H media with 5% CO_2 at 25, 33 or 37° C. BSK-H media was prepared using bovine serum albumin from Gemini Bio-Products, Inc (lot #C54) as previously described (Samuels *et al.*, 1994). Spirochetes were harvested by centrifugation and washed with phosphate buffered saline (PBS).

Allelic exchange mutagenesis

Allelic exchange replacement of *rrp1* with an antibiotic resistance cassette (Frank *et al.*, 2003) was performed as previously described using plasmid pΔBB0419 (Rogers *et al.*, 2009b). Prior to introduction of DNA into *B. burgdorferi* by electroporation, the suicide plasmids were linearized with *Nco*I and *Sca*I. This was done to increase transformation efficiency (Samuels *et al.*, 1994) and to inactivate the kanamycin and ampicillin resistance cassettes. Electroporation was done using previously described conditions (Earnhart *et al.*, 2010). After allowing the cells to recover, the strains were grown in BSK-H media containing 75 μg ml^{-1} streptomycin and clonal populations obtained by sub-surface plating.

To complement the *rrp1* deletion, *rrp1* was reintroduced back into the chromosome of B31-Δ*rrp1* using allelic exchange. To generate the construct for complementation, a PCR product

extending from 1 kb upstream of *rrp1* to the 3′ end of *rrp1* (with 3′ flanking *Aat*II and *Asc*I sites) was cloned into pCR2.1 using TOPO-TA approaches (Invitrogen) to generate pBB0419-UP. A kanamycin resistance cassette, derived from pBSV2 (Stewart *et al.*, 2001), with a 5′ *Aat*II site, was fused by overlap extension to the downstream 1 kb sequence flanking *rrp1* (3′ *Asc*I site). The kanamycin-downstream fusion was cloned into pCR2.1, then digested and inserted into the pBB0419-UP to create pBB0419-kan. The resulting plasmid was purified, linearized with *Sca*I and *Nco*I, and electroporated into B31-Δ*rrp1* to produce the complemented strain, B31-KI. Selection was achieved with 75 μg ml^{-1} kanamycin and clonal populations were obtained by sub-surface plating.

Plasmid content analysis

The plasmid content of all clones selected for analysis was confirmed by PCR using plasmid specific primer sets exactly as previously described (Rogers *et al.*, 2009b).

Generation of a strain that overproduces *rrp1*

Constitutive overexpression of *rrp1* was accomplished by using a pBSV2 based plasmid (Stewart *et al.*, 2001) with *rrp1* expression under the control of the *flaB* promoter. The *flaB* promoter and complete *rrp1* gene were PCR amplified from B31-5A4 genomic DNA in separate PCR reactions and cloned into pCR2.1-TOPO to yield pCR2.1-P_{flaB} and pCR2.1-*rrp1*. The plasmids were digested with *Cla*I and *Sal*I. The *rrp1* containing fragment was excised from a gel and ligated into the pCR2.1-P_{flaB} to yield pCR2.1-P_{flaB}-*rrp1*. pBSV2 and pCR2.1-P_{flaB}-*rrp1* were linearized with *Bam*HI and *Sal*I. The P_{flaB}-*rrp1* fragment was ligated into the pBSV2 vector to generate pBSV2-P_{flaB}-*rrp1*. The final plasmid was propagated and electroporated into B31-5A4 cells. Selection of the overexpression strain (B31-OV) was achieved using kanamycin (200 μg ml^{-1}). Clones were selected by sub-surface plating and plasmid content determined by PCR.

Growth curve analysis

To determine growth rates for the B31-wt, B31-Δ*rrp1*, B31-KI, and B31-OV, an equal number of actively growing spirochetes (33°C) were transferred into fresh BSK-H complete media and incubated at 25, 33, and 37°. Cells were counted every 24h for 20 days using dark field microscopy. The average number of spirochetes per field was determined by averaging the counts from 10 fields for each time point.

SDS PAGE and immunoblotting

Lysates were fractionated using 12.5% Criterion Precast Gels (BioRad) and transferred to PVDF membrane as previously described (Rogers *et al.*, 2009a). The membranes were blocked (1xPBS, 0.2% tween, 5% non-fat dry milk) and screened with a variety of antisera including mouse anti-Rrp1 (1:1000), mouse anti-*Borrelia* infection sera (1:1,000), and mouse anti-FlaB (1:400,000). Antibody binding was detected using horseradish peroxidase-conjugated secondary antibody (Pierce −1:40,000) and SuperSignal West Pico chemiluminescence substrate (Pierce).

Determination of intracellular c-di-GMP concentration

C-di-GMP determination in *B. burgdorferi* acid soluble extracts was performed as previously described (Antoniani *et al.*, 2010) with minor modifications. Briefly, bacterial cells were collected, resuspended in 0.4 M ice-cold $HClO_4$, and lysed by sonication. After centrifugation (16,000 × g; 3 min; 4°C) to remove cell debris, supernatants were neutralized with 0.16 M K_2CO_3, kept on ice for 10 min, and centrifuged (16,000 × g; 3 min; 4°C). Neutralized samples were injected into an HPLC system equipped with a diode-array detector. Nucleotides separation was performed on a Sinergi 4.0 μ Fusion-RP 250 × 4.6 mm

column (18°C; Phenomenex, Inc). Elution conditions were 12 min at 100% buffer A (100 mM potassium phosphate, pH 6.0), 8 min up to 12% buffer B (buffer A containing 20% methanol), 3 min up to 45% buffer B, and 4 min up to 100% buffer B, holding at 100% buffer B for 7 min and returning to 100% buffer A in 6 min. Flow rate was maintained at 1 ml min^{-1}.

RNA isolation and real time reverse transcriptase polymerase chain reaction (qRT-PCR)

RNA was isolated from *Borrelia* cultures using the RNeasy Midi kit as described by the manufacturer (Qiagen) and treated with DNase I (Invitrogen). cDNA was generated using the Superscript III First Strand cDNA Synthesis kit (Invitrogen), 50 ng random hexamer primers, and 1 μg total RNA. RT-PCR was performed with SYBR green PCR Master Mix (Applied Biosystems) and the DNA Engine Opticon System (MJ Research) with primers listed in Table 1. The following cycle parameters were used: 1 cycle of 10 min at 95° followed by 40 cycles of 10 sec at 94°, 30 sec at 50°, and 30 sec at 72°. Melting curves were generated over the temperature range 45–95° to assess amplification specificity. All reactions were run in triplicate with three biological replicates and the data were normalized against enolase (*eno*; BB0377). Statistical analyses were performed using a paired, two-tailed t-test. Alterations of gene expression were considered significant if $p<0.05$.

Infectivity in mice: seroconversion and cultivation analyses

The potential ability of each strain to infect C3H-HeJ mice was assessed by subcutaneous needle inoculation of 10^4 spirochetes between the shoulder blades (in 100 μl BSK-H complete media). Four weeks post-inoculation, the mice were sacrificed and blood, organs, and tissues were harvested. Seroconversion was assessed by ELISA. The ELISA plates were coated with 0.1 OD B31-5A4 spirochetes ml^{-1} in 100 μl of carbonate buffer (pH 9.6). The plates were incubated for 2h at RT with shaking, blocked with 1% bovine serum albumin in PBS-T, and antiserum from each mouse was applied in serial 3-fold dilutions (1:50–1:109, 1:350). Then, the plates were washed three times and bound IgG detected with peroxidase conjugated goat-anti-mouse IgG antiserum (1:20,000) and ABTS chromagen. The plates were read at OD_{405}. Statistical analysis was performed using a one-way ANOVA. Seroconversion was also assessed through immunoblotting (1:1000). To further confirm infectivity, tissue cultivation was performed. Tissues and organ biopsies were immediately inserted into complete BSK-H media containing *Borrelia* antibiotic cocktail (phosphomycin, rifampicin, and amphotericin B) and incubated at 33° for 2–3 weeks followed by dark-field microscopy.

Tick studies

Naïve larval stage *Ixodes scapularis* ticks (Oklahoma State University Tick Rearing Facility) were brushed onto mice 4 weeks after the mice had been inoculated with each of the strains described above. The ticks were fed to repletion, collected and DNA isolated using the DNeasy Blood and Tissue kit (Qiagen). qPCR was performed using a *B. burgdorferi flaB* primer set and the data were normalized against tick rDNA using the RIB-3 and RIB-4 primers (Zahler *et al.*, 1995). Ticks were also infected using immersion methods (Policastro and Schwan, 2003). Larval ticks were submerged in *B. burgdorferi* cultures (10^8 cells/ml; 33°C; 2 hr), washed, dried, and then fed on naive mice. One week after drop off, DNA was isolated and analyzed by qPCR.

Motility analyses

Motility in BSK-H complete media with and without 1% methyl cellulose was assessed using dark-field and DIC microscopy. Timelapse movies were recorded and analyzed using Slidebook 5 (Intelligent Imaging Inovations) motion-tracking software. Velocity

measurements were calculated from twenty tracks per strain. Microscopy was performed at the VCU Department of Anatomy and Neurobiology Microscopy Facility.

Swarm plate and capillary tube chemotaxis assays

Swarm assays were adapted from Li et al (Li *et al.*, 2002) and Motaleb et al (Motaleb *et al.*, 2000). Plates containing 0.35% (wt/vol) Seakem GTG agarose in BSK-H were punched 3 times with a 1 ml pipette tip. 5×10^5 spirochetes were resuspended in 5 μl of a 1:10 BSK-H dilution in dPBS, and then placed in the punched holes. Plates were incubated at 33°C and colony diameters assessed at 2, 4, and 6 days. Statistics were generated using a one-way ANOVA on the means from 3 independent experiments.

The capillary assay was performed using a 96 well format as previously described (Motaleb *et al.*, 2005) with some modifications. All experiments were conducted in triplicate. In brief, cells were harvested from liquid cultures by centrifugation and suspended at 10^7 cells ml^{-1} in motility buffer containing 1% (wt/vol) BSA and 1% (wt/vol) methylcellulose (Shi *et al.*, 1998). The cells were placed in the wells of a 96 well plate. Capillary tubes, filled with motility buffer plus or minus chemoattractant (0.1 M N-acetyl-D-glucosamine), were inserted into the wells and the open end was sealed with Critoseal (Leica). The plates were incubated at 33° for 2h and then the contents of the capillary tubes were expelled into microcentrifuge tubes. The average number of spirochetes per field using dark-field microscopy was determined by averaging 10 fields of view. One-way ANOVA statistical analysis was performed on the means from 3 separate experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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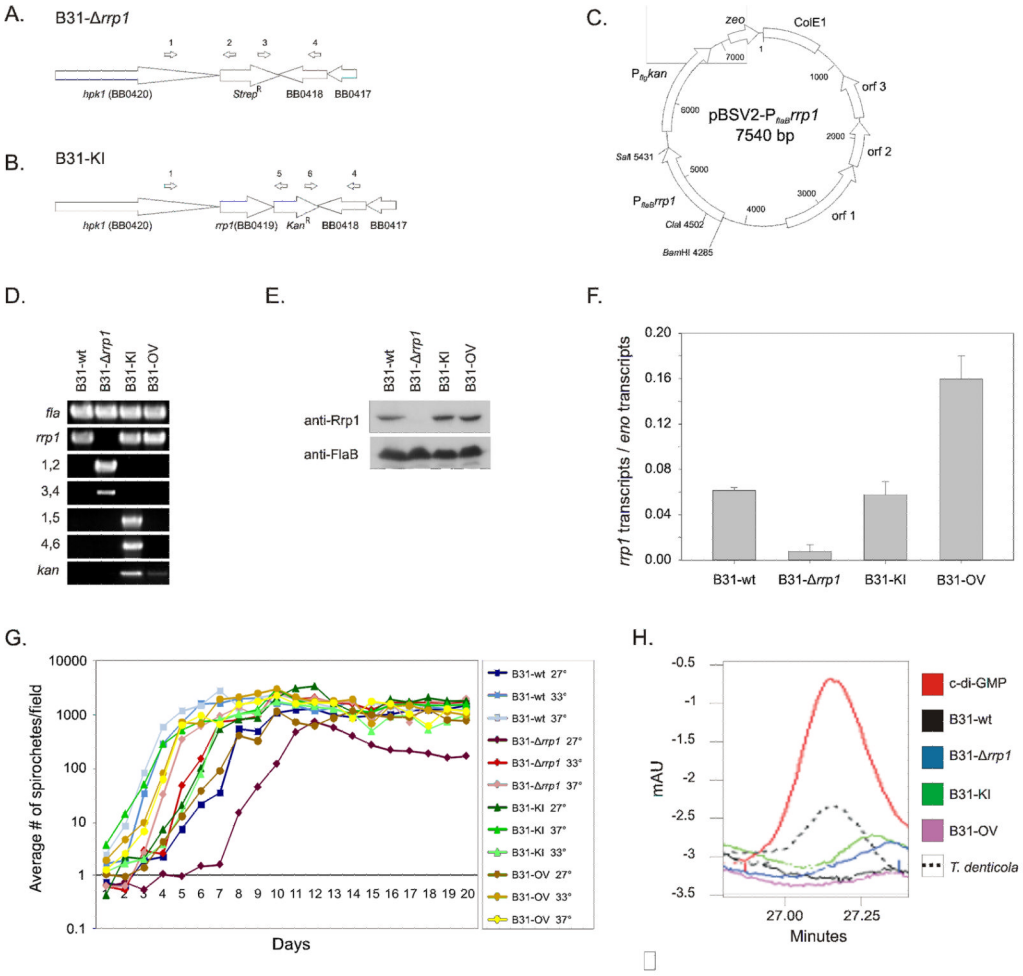


Figure 1. Generation and verification of *rrp1* deletion, complementation, and overexpression strains and comparative analyses of their properties

Schematics in panel A and B represent the chromosome of the B31-Δ*rrp1* and B31-KI complement mutants after allelic replacement, respectively. Schematic C depicts the pBSV2-P$_{flaB}$*rrp1* plasmid construct utilized in this study for the B31-OV mutant. Successful allelic exchange mutagenesis deleting and reinserting *rrp1* was confirmed by PCR (Panel D), western blot (Panel E), and qRT-PCR analysis (Panel F). Verification of pBSV2-based overexpression of Rrp1 was likewise performed by PCR (Panel D), western blot (Panel E), and qRT-PCR (Panel F). Primers used for validation of proper integration are indicated by numbers above the schematic arrows and are listed to the left of the respective PCR panels. All primers used are listed in Table 1. The error bars in panel F indicate the standard deviation. Growth of each strain was assessed at 27, 33 and 37° over 20 days in BSK-H complete media. In panel H, HPLC chromatograms of nucleotide extracts from each strain are presented. The elution time of purified c-di-GMP is shown for reference. *T. denticola* nucleotide extracts were assessed as a positive control. All methods were as described in the text.

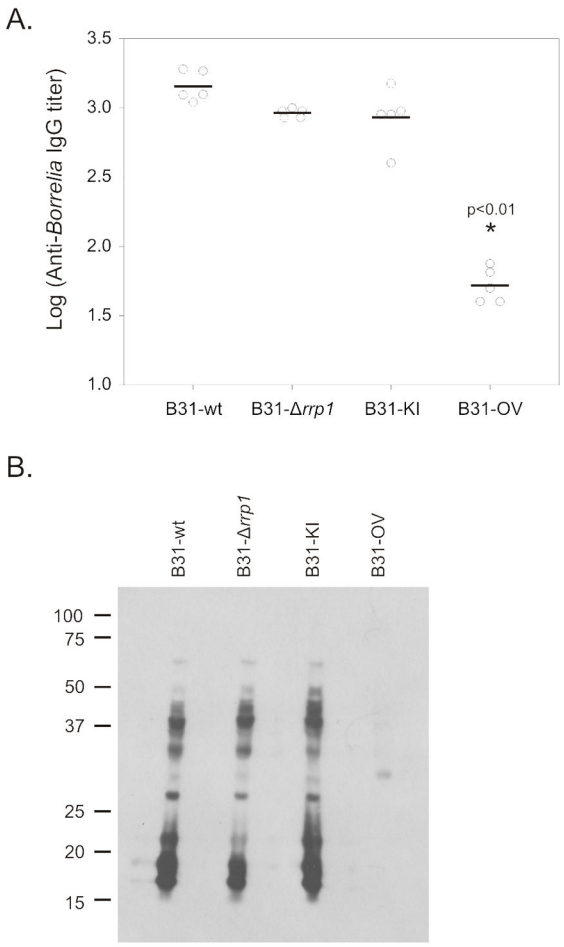
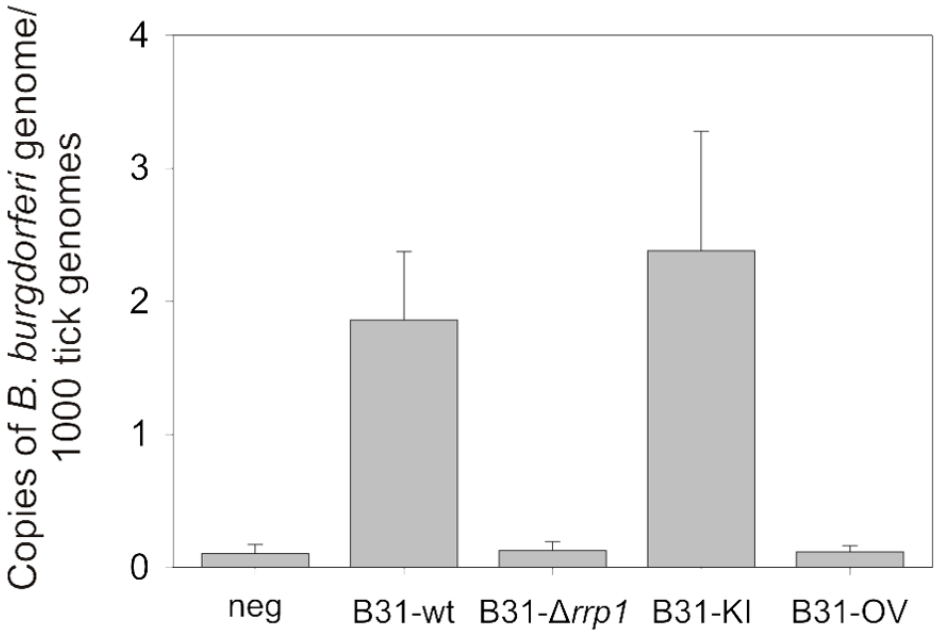


Figure 2. Rrp1 is not essential to mammalian infection, but overproduction inhibits disease establishment

C3H-HeJ mice were needle inoculated subcutaneously with 10^4 spirochetes (5 mice per strain). Four weeks post-infection, mice were bled and sera collected. Seroconversion was evaluated using whole-cell ELISA (Panel A) and immunoblot (Panel B).

A.



B.

Infection method	B31-wt	B31-Δ*rrp1*	B31-KI	B31-OV
Natural infection	10/10	0/10	10/10	0/10
Immersion	4/10	0/10	2/10	4/10

Figure 3. The diguanylate cyclase, Rrp1, is necessary for *Ixodes* infection

Evaluation of spirochete acquisition by *Ixodes* ticks was assessed by qPCR. DNA was isolated from naive ticks fed on infected mice till repletion (Panel A & B). Larval ticks were also immersed into spirochete culture, washed, fed, and assessed for spirochete uptake (Panel B).

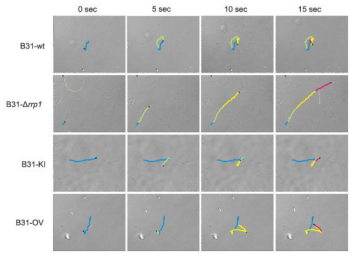


Figure 4. Translational motion patterns requires Rrp1

Movement patterns of the B31-wt, B31-Δ*rrp1*, B31-KI, and B31-OV strains were visualized using DIC microscopy. Images were captured at 5 sec intervals are shown. Motion tracks were manually recorded and overlaid on the images using motion tracking software. The tracks are colored according to motion achieved during each timelapse (blue - 0 sec, green - 5 sec, yellow - 10 sec, pink - 15 sec). Note that the patterns shown are representative of the entire population of cells.

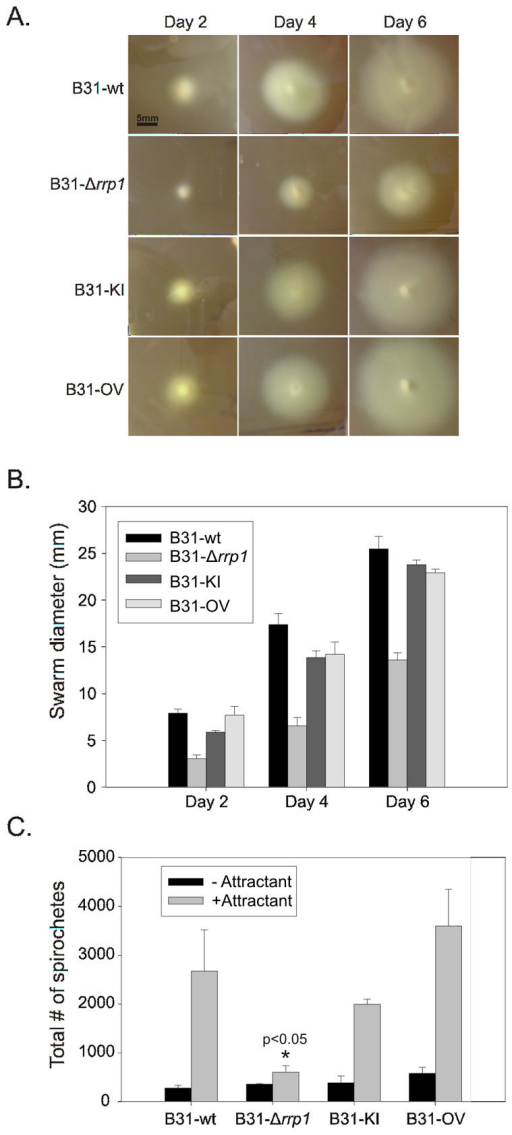


Figure 5. Rrp1 positively regulates motility and chemotaxis

The B31-wt, B31-Δ*rrp1*, B31-KI, and B31-OV strain motility and chemotaxis abilities were assessed by swarming assay and capillary chemotaxis assay. *Borrelia* were spotted into punched wells and diameters were measured at day 2, 4 and 6 (Panel B) from the respective swarms (Panel A). Mutant movement toward N-acetyl-D-glucosamine was analyzed by capillary assay. Spirochetes in capillary tubes with and without chemoattractant were counted after a 2h incubation at 33° (Panel C). The error bars indicate the standard deviation.

Table 1

Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′→3′)
General primers	
KOverifyUp F (1)	CTTTAATGGGGCTTGGTATATGC
KOverifyDwn R (2)	GTTGATGCATCTGTTAAAATTG
aad1-5′-R (3)	TCCTTGAAGCTCGGGTATTA
pKFSS1-3′F (4)	GGCGAGATCACCAAGGTAGTC
kan-5′-R (5)	CAGCATCCATGTTGGAATTTAATCGC
kan-3′-F (6)	GATATGAATAAATTGCAGTTTCATTTG
kan pBSV2 F (7)	GCGATTAAATTCCAACATGGATGCTG
kan pBSV2 R (8)	ACTCATCGAGCATCAAATGAAACTGC
flaB F	CAGGTAACGGCACATATTCAGATGC
flaB R	CTTGGTTTGCTCCAACATGAACTC
BB0419F_LIC	GACGACGACAAGATGGAAATGATAATTAAAGATAAAGC
BB0419R_LIC	GAGGAGAAGCCCGGTTTAATATCTAAACTGATTTCTTCCAG
***rrp1* KI construction**	
Rev_419+AatII+AscI	GGCGCGCCTAGGACGTCTTAATATCTAAACTGATTTCTTCCAGAAAC
Fwd_KanR+1kbDNST419	GAGTTTTTCTAAATTTATATTTAATAGACTTTAGTATTTATAAGTTATAGACATTCC
Rev_1kbDNST419+AscI	GGCGCGCCCTTTCCCAAGATCAACAGAACTTAG
Fwd_KanR+AatII	GACGTCCTTTAATAAAACAATATGTTGCGATGATTAAGG
Rev_KanR+1kbDNST419	CTTATAAATACTAAAGTCTATTAAATATAAATTTAGAAAAACTCATCGAGCATCAAATG
***rrp1* OV construction**	
pFlaB+BamHI F	GTGGATCCTGAACTTAATACCTTGG
pFlaB+ClaI+SalI R	GTCGACATAATCGATTCCTCCATGATAAAATTTAAATTTC
419+ClaI F	ATCGATAATGATAATTAAAGATAAAGCTTTTG
419+SalI R	GTCGACCGCTTAATATCTAAACTGATTTCTTCC
qPCR primers	
FlaB BB0147F-RT	CAGGTAACGGCACATATTCAGATGC
FlaB BB0147R-RT	CTTGGTTTGCTCCAACATGAACTC
BB0419F-RT	TTGAGGTTGCAACAAATGGA
BB0419R-RT	CGGGATCGCTTTTTAGCTTT
eno BB0337F-RT	GCTTGAACTTGATGGCACCCCTAC
eno BB0337R-RT	GTACGCTCCAAGATATTGATAAGG
tick RIB-3 (ITS2)	CGGGATCCTTC(A,G)CTCGCCG(C,T)TACT
tick RIB-4 (ITS2)	CCATCGATGTGAA(C,T)TGCAGGACA
BB0002F-RT	GCGGAGCAGACAAAGGGATTGATT
BB0002R-RT	ACATGCTCCATGGCCGGAAA
NagA BB0151F-RT	GCAGCTGGTGGAGTATTTACAGGA
NagA BB0151R-RT	GTGTGTCCCGCTTGAAGGTTTATG
NagB BB0152F-RT	GGGCGGCTAATCATGTAGCACAAA
NagB BB0152R-RT	CAATCGGAGAGCTTCCTGTTGGA

Oligonucleotide	Sequence (5′→3′)
PtsG BB0645F-RT	AGAACTTGCTGCCCAAGGTACAGA
PtsG BB0645R-RT	CCGGGCAAACCAAACATCATGGTA

Table 2

Murine infection study

Strain	# spirochete positive cultures from each tissue or organ biopsy			
	bladder	heart	skin	# of culture positive/# tested
B31-wt	5/5	5/5	5/5	5/5
B31-Δ*rrp1*	3/5 (mice 1 and 3 were negative	0/5	3/5 (mice 2 and 5 were negative)	5/5
B31-KI	5/5	5/5	5/5	5/5
B31-OV	0/5	0/5	0/5	0/5

Table 3

Strain swimming patterns and observations.

Strain	BSK-H	1% methylcellulose
B31-wt	Motile	Translational motion: runs, stops/flexes, reverses Avg run velocity: 4.1426 ± 0.9442 μm/sec Flexes/sec: 0.1738 ± 0.0319
B31-Δ*rrp1*	Motile	Translational motion: runs with very infrequent stops/flexes Avg run velocity: 5.5815 ± 1.3920 μm/sec Flexes/sec: 0.0150 ± 0.0430
B31-KI	Motile	Translational motion; runs, stops stops/flexes, reverses Avg run velocity: 3.9116 ± 0.7613 μm/sec Flexes/sec: 0.1429 ± 0.0679
B31-OV	Motile	Translational motion; runs, stops stops/flexes, reverses Avg run velocity: 4.0359 ± 0.7859 μm/sec Flexes/sec: 0.1593 ± 0.0340

Table 4

Glucosamine metabolism pathway qRT-PCR analysis

Description		Functional Category[a]	Δ*rrp1*/WT	p-value	OV/WT	p-value
BB0002	glycosyl hydrolase family 3 N domain protein (β- – acetylhexosaminidase)	EM	0.2941	0.0536	0.4964	0.0996
BB0151	N-acetylglucosamine-6-phosphate deaminase (*nagA*)	IM	0.4611	0.0029	0.8787	0.2872
BB0152	glucosamine-6-phosphate isomerase (*nagB*)	IM	0.3320	0.0176	0.4331	0.0319
BB0645	PTS system, glucose- specific IIBC component (*ptsG*)	TP	0.4444	0.0921	0.5196	0.1137

[a] As published in TIGR *B. burgdorferi* B31MI genome database.