Published in final edited form as:
Mol Microbiol. 2009 March ; 71(6): 1551–1573. doi:10.1111/j.1365-2958.2009.06621.x.

Rrp1, a cyclic-di-GMP-producing response regulator, is an important regulator of *Borrelia burgdorferi* core cellular functions

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Summary

Two-component systems (TCS) are universal among bacteria and play critical roles in gene regulation. Our understanding of the contributions of TCS in the biology of the *Borrelia* is just now beginning to develop. *Borrelia burgdorferi*, a causative agent of Lyme disease, harbours a TCS comprised of open reading frames (ORFs) BB0419 and BB0420. BB0419 encodes a response regulator designated Rrp1, and BB0420 encodes a hybrid histidine kinase–response regulator designated Hpk1. Rrp1, which contains a conserved GGDEF domain, undergoes phosphorylation and produces the secondary messenger, cyclic diguanylate (c-di-GMP), a critical signaling molecule in numerous organisms. However, the regulatory role of the Rrp1–Hpk1 TCS and c-di-GMP signaling in *Borrelia* biology are unexplored. In this study, the distribution, conservation, expression and potential global regulatory capability of Rrp1 were assessed. *rrp1* was found to be universal and highly conserved among isolates, co-transcribed with *hpk1*, constitutively expressed during *in vitro* cultivation, and significantly upregulated upon tick feeding. Allelic exchange replacement and microarray analyses revealed that the Rrp1 regulon consists of a large number of genes encoded by the core *Borrelia* genome (linear chromosome, linear plasmid 54 and circular plasmid 26) that encode for proteins involved in central metabolic processes and virulence mechanisms including immune evasion.

Introduction

Borrelia burgdorferi, *B. garinii* and *B. afzelii*, causative agents of Lyme disease (Burgdorfer *et al*., 1982; Benach *et al*., 1983; Steere *et al*., 1983), are maintained in an enzootic cycle involving *Ixodes* ticks and diverse mammalian reservoirs (Anderson and Magnarelli, 2008). During the transmission cycle, the spirochetes must adapt to changing environmental parameters including temperature, pH, nutrient availability, host-derived factors and immune pressures. Differential expression of *Borrelia* genes in response to environmental conditions

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has been demonstrated (Revel *et al*., 2002; Roberts *et al*., 2002; Brooks *et al*., 2003; Ojaimi *et al*., 2003; Caimano *et al*., 2007; Lybecker and Samuels, 2007); however, many aspects of the molecular basis of differential regulation of *Borrelia* gene expression are unknown.

Two-component systems (TCS), typically consisting of a histidine kinase and response regulator, play important roles in the adaptive responses of bacteria (Stock *et al*., 2000; West and Stock, 2001). Signal transduction begins when the histidine kinase senses an environmental signal, leading to autophosphorylation at a conserved histidine residue. The phosphoryl group is then transferred to an aspartate in the receiver domain of the response regulator, allowing for activation of the output domain which may have DNA-binding, RNA-binding, protein-binding or enzymatic functions (Galperin *et al*., 2001; Shu and Zhulin, 2002; Galperin, 2006). Almost all bacteria possess TCS, with most species containing 20–30 response regulator–histidine kinase pairs (Galperin *et al*., 2001; Skerker *et al*., 2008). In contrast, the genome of *B. burgdorferi* has only two gene pairs that encode for TCS that are likely to have global regulatory capabilities. Of these, the Hpk2–Rrp2 (histidine protein kinase 2–response regulatory protein 2) TCS was the first to be studied (Yang *et al*., 2003a,b; Burtnick *et al*., 2007; He *et al*., 2007; Boardman *et al*., 2008; Ouyang *et al*., 2008). Initial analyses demonstrated that Rrp2 influences the expression of several *B. burgdorferi* virulence factors. The DNA-binding output domain of Rrp2 binds to the −24/−12 region upstream of *rpoS* (σ^S) to enhance transcription with the help of RpoN (σ^{54}). The Rrp2–RpoN–RpoS regulatory pathway positively regulates mainly plasmid-carried genes including *ospC* (outer surface protein C), *dbpA* (decorin-binding protein A) and the *mlp* proteins (multi-copy lipoproteins) (Brown *et al*., 1999; Hubner *et al*., 2001; Pikas *et al*., 2003; Yang *et al*., 2003b). It has recently been demonstrated that Rrp2 regulates genes independent of RpoS, the majority of which are plasmid-encoded proteins that have no known function (Boardman *et al*., 2008).

In this study, we investigate the regulatory potential of the *B. burgdorferi* Rrp1 (BB0419, a response regulator) and Hpk1 (BB0420, a hybrid sensory transduction histidine kinase-response regulator) TCS. Rrp1 differs from Rrp2 in that it lacks a DNA-binding output domain, but carries an enzymatic GGDEF domain with diguanylate cyclase activity. Diguanylate cyclase enzymes convert two molecules of GTP, via the linear intermediate diguanosine tetraphosphate (pppG3′p5′G) to cyclic dimeric guanosine monophosphate (cyclic diguanylate or c-di-GMP) (Ausmees *et al*., 2001; Galperin *et al*., 2001; Simm *et al*., 2004; Jenal and Malone, 2006). The ability of recombinant Rrp1 to produce c-di-GMP has been demonstrated to be dependent on phosphorylation (Ryjenkov *et al*., 2005). Rrp1 is the only *B. burgdorferi* protein that harbours a GGDEF domain and thus appears to be the only *B. burgdorferi* protein capable of c-di-GMP production. C-di-GMP is a secondary messenger molecule that influences cellular processes including motility, cell differentiation, phage and heavy metal resistance, expression of virulence genes, cell–cell signaling, fimbrial synthesis, exopolysaccharide production, and the transition between biofilm and planktonic modes of growth (Garcia *et al*., 2004; Simm *et al*., 2004; Tischler and Camilli, 2004; Karaolis *et al*., 2005; Romling *et al*., 2005; Beyhan *et al*., 2006; Galperin, 2006; Mendez-Ortiz *et al*., 2006; Romling and Amikam, 2006; Bobrov *et al*., 2007; Cotter and Stibitz, 2007; Kim and McCarter, 2007; Lee *et al*., 2007; Tamayo *et al*., 2007; Wolfe and Visick, 2008). The intracellular pool of c-di-GMP is controlled by the opposing activities of diguanylate cyclase and phosphodiesterase enzymes (EAL or HD-GYP domain-containing proteins). The *B. burgdorferi* genome harbours one EAL (BB0363) and one HD-GYP (BB0374) domain-containing protein. The identification of GGDEF (in Rrp1/BB0419), EAL (in BB0363) and HD-GYP (in BB0374) domains indicates that *B. burgdorferi* has the full set of proteins required for regulating c-di-GMP levels within the cell. The mechanisms by which c-di-GMP exerts its regulatory effects are largely unknown; however, c-di-GMP has been shown to bind to proteins harbouring the PilZ domain (Amikam and Galperin, 2006; Ryjenkov *et al*., 2006; Benach *et al*., 2007). *B. burgdorferi* BB0733 harbours this domain, although its ability to bind c-di-GMP has not been demonstrated.

Additionally, c-di-GMP riboswitches with GEMM domains have been implicated as a possible mechanism for translational control (Sudarsan *et al*., 2008).

In this report, we demonstrate that *rrp1* and *hpk1* are conserved and universal among Lyme disease isolates. Deletion of *rrp1* through allelic exchange replacement and subsequent microarray analyses revealed that Rrp1 influences the expression of a large and functionally diverse set of genes. The majority of these genes are present on the chromosome and code for proteins with basic cellular functions. This study is the first to explore the regulatory capability of a protein with diguanylate cyclase activity in any spirochete and the first to identify a central regulator of the core genome. The data presented shed significant new light on the global regulatory mechanisms of *B. burgdorferi* and indirectly suggest that c-di-GMP may be a key secondary messenger molecule in the *Borrelia.*

Results

Polymerase chain reaction and Southern hybridization analyses of *rrp1* and *hpk1*

To assess the distribution of *rrp1–hpk1* in a diverse panel of *Borrelia* species and strains, polymerase chain reaction (PCR) analyses were performed on 30 Lyme disease isolates (*B. burgdorferi*, *B. garinii* and *B. afzelii*), 4 relapsing fever isolates (*Borrelia hermsii* and *B. turicatae*) and 1 *Borrelia coriaceae* isolate (a causative agent of epizootic bovine abortion). Representative data are presented in Fig. 1A. All Lyme disease isolates were PCR positive with an internal *rrp1* primer set (primers 5 and 6) and with a primer set designed to amplify from the 3′ end of *hpk1* into the 5′ end of *rrp1* (primers 3 and 4). Amplification with internal *hpk1* primers yielded more variable results (primers 1 and 2) suggesting sequence variation within this gene. *rrp1–hpk1*-derived amplification products were also obtained from the relapsing fever spirochetes *B. hermsii* and *B. turicatae* (data not shown). Hybridization analyses were also performed to screen for *rrp1*. The probe was generated by radiolabeling a PCR product of *rrp1* from *B. burgdorferi* B31MI (Fig. 1B). All Lyme disease spirochete isolates screened ($n = 14$) were hybridization positive while *B. coriaceae* was hybridization negative (relapsing fever spirochetes were not assessed by this approach). Collectively, these data indicate that the *rrp1–hpk1* operon is carried by *Borrelia* species that are pathogenic in humans.

Analysis of the transcriptional expression patterns of *rrp1* and *hpk1*

Most histidine kinase–response regulator pairs are co-transcribed as a polycistronic operon. The separation of *rrp1* and *hpk1* by a 3 bp spacer is indicative of co-transcription. To assess co-transcription, reverse transcription PCR (RT-PCR) was performed using primers that span the intergenic spacer (3 and 4). Co-transcription of these genes was detected in *B. burgdorferi* grown at 25°C, 33°C and 37°C (Fig. 1C). Transcript levels were determined using real-time reverse transcription PCR (qRT-PCR) with an internal *rrp1* primer set (primers 9 and 10). In these specific qRT-PCR experiments, transcript levels were normalized against *flaB* transcript. *rrp1* was expressed at equivalent levels in spirochetes grown at the different temperatures tested (Fig. 1D).

To determine if *rrp1* is expressed in infected ticks, transcript levels were determined in flat (unfed) and fed *Ixodes scapularis* nymphs. *rrp1* expression was sixfold higher in fed nymphs as compared with unfed nymphs. The transcriptional analyses demonstrate that *rrp1* and *hpk1* are co-transcribed, expressed *in vitro* in a temperature-independent manner, and induced upon tick feeding.

Rrp1 sequence analyses

To determine if residues of Rrp1 thought to be involved in diguanylate cyclase activity (i.e. c-di-GMP production) and response regulator function are conserved across species lines, *rrp1* amplicons from Lyme disease and relapsing fever isolates were cloned and sequenced (Fig. 1E). Residues within the receiver and GGDEF domains that have been identified as functionally important in other bacteria were conserved in all sequences (Ausmees *et al*., 2001;Romling *et al*., 2005;Simm *et al*., 2005;Gjermansen *et al*., 2006;Cotter and Stibitz, 2007). Within the Lyme spirochetes, at the intraspecies level, the amino acid identity values were 97.7–100%. The relapsing fever spirochetes *Borrelia hermsii*, *B. turicatae*, *B. duttonii*, and *B. recurrentis* also carry *rrp1* and the residues associated with diguanylate cyclase activity are conserved (Lescot *et al*., 2008). In summary, *rrp1* and *hpk1* are widely distributed among the *Borrelia* and have conserved genetic organization and sequence. It is likely that the Rrp1 regulons in the Lyme disease and relapsing fever spirochetes have significant overlap and play important roles in the pathophysiology of the *Borrelia*.

Analysis of Rrp1 cellular location and production during *in vitro* cultivation

To determine if Rrp1 is produced during cultivation, antiserum was generated against r-Rrp1 derived from *B. burgdorferi* B31MI. The antiserum was used to screen immunoblots of cell lysates from 17 *Borrelia* strains (Fig. 2A). Rrp1 (35 kDa) was detected in all Lyme disease strains tested. The antiserum also detected a protein of ~48 kDa in some isolates; however, the identity of this protein is unknown. *B. coriaceae*, which was PCR and Southern hybridization negative for *rrp1*, did not produce a protein that was detected with the Rrp1 antiserum.

To assess the basic properties of Rrp1, Triton X-114 extraction and phase partitioning was performed as described in *Experimental procedures*. The resulting fractions and the whole-cell lysate were screened by immunoblotting (Fig. 2B). The anti-Rrp1 antiserum detected Rrp1 in the whole-cell lysate and in the fraction containing the protoplasmic cylinder which consists of the inner membrane and cytoplasmic proteins. As controls, additional blots were screened with antiserum against DbpA (a known outer membrane protein) and BBG31 (a periplasmic protein). The control proteins partitioned as expected. These analyses, coupled with the lack of export signals or predicted transmembrane-spanning domains, indicate that Rrp1 is most likely a cytoplasmic protein. This putative cellular location is consistent with that of other response regulators (Galperin *et al*., 2001;Guvener and Harwood, 2007).

Construction and characterization of an *rrp1* deletion mutant

To determine the physiological role of Rrp1, the gene was deleted by allelic exchange replacement from the *B. burgdorferi* 5A13 background. Numerous clonal populations were obtained, and deletion of *rrp1* was confirmed using PCR (Fig. 3A), Southern hybridization (Fig. 3B) and immunoblotting (Fig. 3C). Attempts at deleting *rrp1* from the infectious *B. burgdorferi* 5A4 strain, which has been reported to have an inherently low transformation efficiency (Purser and Norris, 2000), were not successful. However, with the recovery of stable 5A13 transformants, it was possible to assess the potential global regulatory capability of Rrp1 *in vitro*.

Electroporation commonly results in the loss of some of plasmids. To determine the plasmid content of clonal populations of the *rrp1* knockout (*Bb*5A13-Δ*rrp1*), a PCR-based approach using plasmid-specific primer sets was employed. Clonal population 9, which lost the fewest plasmids (cp9, lp28-1 and lp21; plasmids C, F and U respectively) relative to 5A13, was selected for all future analyses. cp9, lp21 and lp28-1 are relatively unstable plasmids that can be lost upon cultivation and/or passage through mice and are not essential for *in vitro* growth (Xu *et al*., 1996; 2003; Palmer *et al*., 2000; McDowell *et al*., 2001; Lawrenz *et al*., 2002; Iyer *et al*., 2003; Stewart *et al*., 2005; Embers *et al*., 2008). It is noteworthy that lp21 is comprised

largely of repeat elements and pseudogenes (Casjens *et al*., 2000; Palmer *et al*., 2000). In addition, earlier microarray analyses and the microarray data presented below demonstrate that the overwhelming majority of genes encoded by these plasmids are either poorly expressed or not expressed at all *in vitro* (Table S1) (Brooks *et al*., 2003; Ojaimi *et al*., 2003; 2005; Tokarz *et al*., 2004; Caimano *et al*., 2007; Boardman *et al*., 2008; Ouyang *et al*., 2008). Furthermore, none of the open reading frames (ORFs) present on these plasmids encodes proteins that are putative transcriptional regulators or intermediaries in metabolic pathways. Consistent with this, these plasmids do not influence infective potential or *in vitro* growth characteristics, and hence their absence in clone 9 does not compromise this initial and preliminary analysis of the potential global regulatory influence of Rrp1 during *in vitro* cultivation.

To examine potential growth defects due to the inactivation of Rrp1, cultures of *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* were diluted to equal cell densities in fresh media and incubated at 25°C, 33°C and 37°C. The average number of spirochetes in 10 high-powered fields was determined every day for 19 days (Fig. 3D). All *Bb*5A13-Δ*rrp1* cultures displayed a growth defect compared with *Bb*5A13-wt. *Bb*5A13-Δ*rrp1* cultures grown at 33°C and 37°C were found to undergo an extended (1 week) lag phase. This is in contrast to *Bb*5A13-wt, which entered exponential phase within 2 days. Growth of *Bb*5A13-Δ*rrp1* at 25°C was severely impaired, and while the spirochetes remained viable, the population never entered exponential phase over the 19-day period of analysis. These data demonstrate that while Rrp1 is not essential for survival *in vitro*, it does influence growth characteristics. The inability of the *Bb*5A13-Δ*rrp1* mutant to grow at lower temperatures, coupled with its upregulation in fed ticks (Fig. 1D), suggests that Rrp1 may be important in environmentally induced adaptive responses in the tick.

Deletion of *rrp1* results in wide-scale changes in the *B. burgdorferi* transcriptome

To identify genes regulated by Rrp1, gene-specific mRNA levels were compared between *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* by microarray analyses. RNA was isolated from three independent cultures of each strain, converted to cDNA, labeled, and applied to oligonucleotide microarrays that represent the entire genome of *B. burgdorferi* B31MI. The arrays have been previously described (Terekhova *et al*., 2006). Hybridization to oligonucleotides representing 1140 ORFs (65.5% of the total ORFs) was above background in at least one of the strains. ORFs for which hybridization was below background in both strains are listed in Table S1.

The expression of 140 genes (8% of the genome) was influenced by Rrp1 (Table 1). The majority of these genes are encoded by the chromosome (102 genes), lp54 (plasmid A; 10 genes) and cp26 (plasmid B; 7 genes) (Fig. 4A). These genetic elements are the most conserved components of the Lyme disease spirochete genome and comprise the core *Borrelia* genome (Terekhova *et al*., 2006). Of the genes regulated by Rrp1, 131 had lower expression in *Bb*5A13-Δ*rrp1* while only 9 genes had elevated expression (Table 1). Most genes with lower mRNA levels in *Bb*5A13-Δ*rrp1* (80 out of 131) have predicted functions and span almost all functional categories except cell division, translation factors, heat shock and haemolysis (Fig. 4B). The functional categories most affected were: cell envelope (21 genes), transport (12 genes), intermediary metabolism (10 genes), nucleotide metabolism (6 genes), chemotaxis (6 genes) and flagellar biosynthesis (5 genes). Of the nine genes negatively regulated by Rrp1 (i.e. with elevated expression in the *rrp1* knockout), only two have predicted functions: *ruvB* helicase (BB0022) and *groES* (BB0741). Since the deletion of *rrp1* resulted predominantly in lower expression of the affected genes, it can be concluded that Rrp1 exerts its regulatory effects primarily through gene activation (directly or indirectly).

Cell envelope protein genes with lower mRNA levels in *Bb*5A13-Δ*rrp1* belong to several highly studied paralogous protein families including the OspE (PF162), OspF (PF164), Mlp (PF113) and Bdr (PF80) families. As noted above, the majority of Rrp1-regulated genes are

chromosomal; however, all of the cell envelope-encoding genes listed above are carried on the cp32 plasmids. The OspE proteins have been implicated as important virulence factors that facilitate complement evasion by binding negative regulators of the complement system such as factor H (FH) (Hellwage *et al*., 2001; McDowell *et al*., 2003; Alitalo *et al*., 2004; Kraiczy and Würzner, 2005; Hovis *et al*., 2006a). There is also evidence that members of this protein family bind to additional serum proteins and that this may influence the host–pathogen interaction (Hovis *et al*., 2006b; Rogers and Marconi, 2007). The microarray analyses revealed that the FH-binding OspE paralogue, BBN38, had significantly lower expression in *Bb*5A13-Δ*rrp1*. In addition, the two other members of this gene family, BBP38 and BBL39 (identical paralogues), also had lower transcript levels in *Bb*5A13-Δ*rrp1*, but the difference was below the cut-off value set for significance. However, it is important to note that the dependence of this gene family on Rrp1 as inferred from the arrays is strongly supported by western blot and immunofluorescence assay (IFA) analysis (see below Fig. 6A and B). The expression of several members of the OspF protein family was also lower in *Bb*5A13-Δ*rrp1*. Several of the *ospF* genes are co-transcribed with *ospE* gene family members or are located just downstream of *ospE* genes (Lam *et al*., 1994). The OspF proteins have been postulated to serve as adhesins (Antonara *et al*., 2007), and earlier work demonstrated that they might carry out stage-specific functions during infection in mice (McDowell *et al*., 2004). The microarrays demonstrated that the OspF paralogue, BBR42, has significantly lower expression in *Bb*5A13-Δ*rrp1*. This observation is supported by immunoblot analyses (Fig. 5).

The expression of the eight-member *mlp* gene family (PF113) (Yang *et al*., 1999; Porcella *et al*., 2000) was also influenced by Rrp1. The function(s) of this cp32-carried gene family have not yet been defined. Three *mlp* paralogues (BBL28, BBO28 and BBR28) had lower expression in *Bb*5A13-Δ*rrp1*. The *mlp* paralogues BBN28 and BBP28 were also repressed in the mutant but missed the stringent statistical criteria set for significance. The data suggest that this family as a whole is activated by Rrp1.

The transcription of select members of the *bdr* gene family (PF80) (Zuckert *et al*., 1999; Carlyon *et al*., 2000a,b) was also influenced by deletion of *rrp1*. Expression of $BdrF_2$ (BBG33) and $BdrE_3$ (BBO27) was significantly reduced in *Bb*5A13-Δ*rrp1*. The expression of $BdrE_4$ (BBR27), Bdr frameshift (BBR35), and $BdrE_5$ (BBS29) were similarly affected in *Bb*5A13-Δ*rrp1*, but missed the significance cut-off. The Rrp1 dependence of some Bdr proteins was also demonstrated by western blot (Fig. 5C). The varying influence of the *rrp1* deletion on the expression of individual Bdr paralogues is consistent with earlier analyses demonstrating that the individual *bdr* paralogues respond differently to environmental variables and thus may be regulated through different mechanisms (Roberts *et al*., 2002).

Genes encoding proteins involved in the transport of ions, carbohydrates, amino acids, and oligopeptides were affected by the deletion of Rrp1. The Na^+/H^+ antiporters *nhaC-1* (BB0637) and *nhaC-2* (BB0638) had lower mRNA levels in *Bb*5A13-Δ*rrp1*. The Na^+/Ca^+ exchange protein, BB0164, also appears to be regulated by Rrp1. Carbohydrate transporter genes with lower mRNA levels in *Bb*5A13-Δ*rrp1* included *lctP*, a L-lactate permease (BB0604), *fruA-2*, a fructose-specific IIABC component (BB0629), and proteins of the glucose, maltose, glucosamine, and *N*-acetylglucosamine transporter systems [PTS system, maltose- and glucose-specific IIABC component *malX* (BBB29), and glucose-specific IIBC component *ptsG* (BB0645)]. Thus, expression of almost all carbohydrate transport genes, with the exception of those specifically involved in chitobiose, ribose and galactose transport, was affected in *Bb*5A13-Δ*rrp1*. Transcript levels for numerous carbohydrate metabolism genes were also significantly reduced in *Bb*5A13-Δ*rrp1*. These include *N*-acetylglucosamine-6-phosphate deacetylase (*nagA*; BB0151), glucosamine-6-phosphate isomerase (*nagB*; BB0152), glycerol kinase (*glpK*; BB0241), glycerol-3-phosphate dehydrogenase (*glpA*; BB0243), mannose-6-phosphate isomerase (*manA*; BB0407), phosphate acetyltransferase

(*pta*; BB0589), beta-glucosidase (BB0620), 1-phosphofructokinase (*fruK*; BB0630), glucose-6-phosphate 1-dehydrogenase (*zwf*; BB0636), and ornithine carbamoyltransferase (*arcB*; BB0842). Strikingly, most of the affected carbohydrate metabolism genes code for enzymes that generate substrates for glycolysis. Beta-glucosidase (BB0620) results in generation of glucose-6-phosphate; *manA*, *nagA* and *nagB* result in production of fructose-6-phosphate; *fruK* converts fructose-1-phosphate into fructose-1,6-bisphosphate and *glpK* and *glpA* produce dihydroxyacetone phosphate.

The expression of almost all genes involved in amino acid and oligopeptide transport was affected in *Bb*5A13-Δ*rrp1*. Lower mRNA levels were observed in *Bb*5A13-Δ*rrp1* for the glycine/proline transporter [glycine betaine, L-proline ABC transporter, ATP-binding protein *proV* (BB0146)], the spermidine/putrescine transporter (*potA*, BB0642) and the oligopeptide transporter [oligopeptide ABC transporter, periplasmic oligopeptide-binding protein *oppAIV* (BBB16)].

Some genes involved in nucleotide biosynthesis were found to have reduced expression in *Bb*5A13-Δ*rrp1*. These include uridylate kinase (*smbA*; BB0571), CTP synthase (*pyrG*; BB0575), 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (*pfs-2*; BB0588), IMP dehydrogenase (*guaB*; BBB17) and GMP synthase (*guaA*; BBB18). Only *pfs-2* had been previously shown to be regulated by RpoS (Caimano *et al*., 2007). Thus, the data suggest that Rrp1 regulates a unique set of nucleotide metabolism genes.

In several bacterial systems, intracellular c-di-GMP levels influence chemotaxis and motility (Wolfe and Visick, 2008). In that Rrp1 is the sole protein of *B. burgdorferi* likely to have diguanylate cyclase activity, it was predicted that deletion of *rrp1* would influence the expression of some chemotaxis and motility genes. Consistent with this, numerous genes involved in chemotaxis and motility had reduced expression in *Bb*5A13-Δ*rrp1*. Examples include *cheA-1* (BB0567), *cheB-2* (BB0568), *cheY-2* (BB0570), *cheY-3* (BB0672), *mcp-1* (BB0578), and *mcp-2* (BB0596). Expression of *mcp-3* (BB0597) and *mcp-4* (BB0680) was also reduced in the *Bb*5A13-Δ*rrp1*, but missed the cut-off for statistical significance. The flagellar hook-associated proteins *fliD* (BB0149) and *flgL* (BB0182), the flagellar motor switch protein *fliG-1* (BB0221), and *flaB* (BB0285) had lower expression levels in *Bb*5A13-Δ*rrp1*. Thus, Rrp1 appears to be involved in the positive regulation of chemotaxis and motility in *B. burgdorferi*.

Validation of microarray analyses by qRT-PCR, immunoblot, IFA, and FH binding assays

To validate the microarray data, several approaches were applied including qRT-PCR, immunoblotting, and IFA. The RNA used for the microarray and qRT-PCR analyses and the cell lysates used for the immunoblot studies were derived from the same log-phase cultures. For the qRT-PCR analyses, relative transcripts levels of 20 of the differentially expressed genes were assessed (Table 2). In general, the trends in differential expression for these genes (i.e. either elevated or reduced expression in wild-type and mutant spirochetes) are similar. It should be noted that for Lyme disease spirochetes, qRT-PCR data are typically normalized against the levels of *flaB*, which is considered to be constitutively expressed under most conditions. However, the array data indicate that *flaB*, as well as several other genes involved in motility and chemotaxis, are regulated to varying degrees either directly or indirectly by Rrp1. This may have a modest effect on the fold changes determined by qRT-PCR. As an additional means of validating the microarray analyses, a series of identical immunoblots were generated and screened with antiserum to a number of *B. burgdorferi* proteins including OspA, DbpA, OspE (BBL39 and BBN38), OspF (BBO39 and BBR42) and the Bdr protein family in order to assess protein expression (Figs 5 and 6A). Consistent with the array analysis, production of OspA and DbpA were not affected by the absence of Rrp1, whereas production of certain Bdr, OspF, and OspE proteins was abrogated. Furthermore, the OspE protein, BBL39, was not detected

at the cell surface by IFA analysis in *Bb*5A13-Δ*rrp1* (Fig. 6B). Since OspE proteins function as FH-binding proteins, the ability of *Bb*5A13-Δ*rrp1* to bind FH was also assessed (Fig. 6A). Consistent with the expression data, FH binding to proteins in the OspE size range (15–20 kDa) was not observed; FH binding to the CspZ and CspA proteins (25–27 kDa) was detected.

To determine if the Rrp1 regulon includes proteins that are antigenic during infection, whole-cell lysates of *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* strains were screened with serum recovered from mice infected with *B. burgdorferi* B31 (Fig. 5D). While the identity of individual antigens was not determined in this particular experiment, the data demonstrate that inactivation of Rrp1 eliminated or decreased production of several proteins that elicit an antibody response during murine infection with *B. burgdorferi* B31.

Increase in serum sensitivity of the *rrp1* knockout

The FH binding capabilities of the Lyme spirochetes have been correlated with serum resistance (McDowell *et al*., 2003; Kraiczy *et al*., 2004; Brooks *et al*., 2005). While the Rrp1 regulon appears to be comprised primarily of housekeeping genes, the regulation of several FH-binding proteins by Rrp1 indicates a connection between this specific immune evasion/virulence mechanism and Rrp1-mediated transcriptional regulation. To determine if the inactivation of Rrp1 influences serum resistance, a serum sensitivity assay was performed on the *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* strains (Fig. 6C). Significant differences in serum sensitivity were evident by 6 h. After a 24 h incubation with normal human serum, less than 40% of the *Bb*5A13-Δ*rrp1* cells were alive compared with cells incubated with heat-inactivated serum. This is in contrast to the *Bb*5A13-wt cells, in which 90% of cells survived in normal human serum after 24 h. The serum sensitivity of the *Bb*5A13-Δ*rrp1* cells proved to be equivalent to that of *B. garinii*, which has been demonstrated to be highly sensitive to complement-mediated killing (Brooks *et al*., 2005). It is noteworthy that the highly serum-sensitive *Bb*5A13-Δ*rrp1* strain still produces the FH-binding proteins, CspA and CspZ. While the dramatic increase in serum sensitivity of the *Bb*5A13-Δ*rrp1* strain suggests that OspE proteins are key players in serum resistance, it is possible that this effect could be indirect. Nonetheless, it can be concluded that the significant changes in overall protein production in the Rrp1 mutant results in a highly serum-sensitive phenotype.

Discussion

Borrelia burgdorferi encodes only two TCS with global regulatory capability. The Rrp2–Hpk2 system controls a regulon that consists primarily of plasmid-carried genes (94% of its regulon) (Boardman *et al*., 2008; Ouyang *et al*., 2008). The majority of genes regulated by Rrp2 encode proteins of unknown function and many have been demonstrated in earlier analyses to be environmentally regulated (Revel *et al*., 2002; Brooks *et al*., 2003; Ojaimi *et al*., 2003; Yang *et al*., 2003a; Burtnick *et al*., 2007; Caimano *et al*., 2007; He *et al*., 2007; Lybecker and Samuels, 2007; Boardman *et al*., 2008). This study is the first to report on the regulatory capability of Rrp1, a unique response regulator with diguanylate cyclase activity (Ryjenkov *et al*., 2005). Rrp1 is the only *Borrelia* protein that carries a GGDEF domain, which is associated with c-di-GMP production. C-di-GMP is an important secondary messenger molecule that influences the expression of proteins involved in a wide range of core biological functions (Cotter and Stibitz, 2007). Recombinant Rrp1 converts GTP to c-di-GMP and this diguanylate cyclase activity is dependent on phosphorylation of Rrp1 (Ryjenkov *et al*., 2005). It is important to note that the *B. burgdorferi* genome possesses the full set of proteins that are required for the control of c-di-GMP levels including GGDEF, EAL, HD-GYP, and PilZ domain-containing proteins. To date, only the enzymatic activity of the GGDEF-containing protein, Rrp1, has been assessed. It remains to be determined if *B. burgdorferi* also harbours a GEMM riboswitch motif, an RNA domain that interacts with c-di-GMP (Weinberg

et al., 2007; Sudarsan *et al*., 2008). Although such a domain has not been detected in *B. burgdorferi*, this could simply be a reflection of sequence divergence in GEMM domains consistent with the deep phylogenetic divergence of the *Borrelia* from other eubacteria.

As a first step in investigating the potential biological importance of Rrp1, and by extension of c-di-GMP, we assessed the distribution of Rrp1 and conservation of its critical functional residues. Using PCR and Southern hybridization, *rrp1* was shown to be widely distributed among *Borrelia* as it was detected in all analysed species that are associated with human disease (Lyme disease and relapsing fever spirochetes). Although not analysed as part of this report, recent genome sequence analyses of *B. duttonii* and *B. recurrentis* have revealed that these species also possess genes that encode for the Rrp1–Hpk1 TCS (Lescot *et al*., 2008). The residues thought to be essential for diguanylate cyclase and response regulator activity were also found to be absolutely conserved in all *Borrelia* species. While this study is focused primarily on Rrp1, the functional link between *rrp1* and *hpk1* is supported by the conservation of their gene organization and co-transcription. Using RT-PCR and immunoblot analyses, we further demonstrate that all Lyme disease spirochetes produce Rrp1 and Hpk1 during *in vitro* cultivation under all temperatures assessed. Importantly, qRT-PCR demonstrated that *rrp1* expression is detectable in unfed nymphal ticks, but is upregulated sixfold upon tick feeding, indicating that Rrp1 may play a role in regulation of genes required for the transition from the tick to mammalian environment.

To identify the Rrp1 regulon, *rrp1* was deleted from the genome of *B. burgdorferi* by allelic exchange replacement and comparative microarray analyses were conducted. *Bb*5A13, which is non-infectious, is widely used in *B. burgdorferi* studies that involve genetic manipulation because it is highly transformable (Purser *et al*., 2003). Attempts to inactivate *rrp1* in the infectious *Bb*5A4 background were not successful. Nonetheless, the successful deletion of *rrp1* from the *Bb*5A13 background allowed us to assess the overall global regulatory capability of this uncharacterized TCS. The microarray analyses revealed that Rrp1 is a key regulator of the core genome of *B. burgdorferi* which consists of the linear chromosome, lp54, and cp26 (Terekhova *et al*., 2006). Of the 140 genes differentially regulated by Rrp1, 85% are encoded on these three genetic elements (102 genes on the chromosome, 10 on lp54 and 7 on cp26). In contrast only 5.6% of the genes regulated by Rrp2 map to the chromosome (Boardman *et al*., 2008). Hence it is evident that there are fundamental differences in the Rrp1 and Rrp2 regulons.

As a first step in assessing the influence of Rrp1 on *Borrelia* biology, the growth rates of *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* strains maintained at different temperatures were compared. The *rrp1* knockout achieved equivalent cell densities during *in vitro* cultivation in standard BSK-H media when the spirochetes were grown at 33°C and 37°C. However, there was a significantly longer lag phase observed in *Bb*5A13-Δ*rrp1* at these temperatures than in the wild type and overall growth was significantly impaired at 25°C. While the *Bb*5A13-Δ*rrp1* spirochetes remained motile over the course of the growth curve analyses at 25°C, the culture densities remained low, suggesting that Rrp1 is required for spirochete replication at the lower temperature. These analyses suggest that Rrp1 either directly or indirectly controls genes involved in the transition to exponential growth and for overall growth at lower temperatures (such as that which might be encountered in the tick environment).

The microarray analyses revealed that the genes that are regulated by Rrp1 span a wide range of functional categories. Rrp1-activated genes encode for proteins that are involved in transport pathways, carbohydrate and nucleotide metabolism, chemotaxis, and flagellar biosynthesis. Additionally, the loss of Rrp1 resulted in pronounced effects on the expression of genes involved in carbohydrate and amino acid transport. These processes may be particularly important in *B. burgdorferi* in its naturally encountered environments because *B. burgdorferi* cannot synthesize nucleosides and amino acids *de novo* (Fraser *et al*., 1997). The

ability of *Bb*5A13-Δ*rrp1* to grow, at least in the nutrient-rich BSK-H media under optimal laboratory conditions, possibly indicates that other, not yet identified, transporters may complement the functions of transporters affected in *Bb*5A13-Δ*rrp1*. It is important to note that the spirochetes, as a whole, represent a deep phylogenetic branch and may have evolved many unique biological strategies for their core metabolic or physiological functions.

Chemotaxis and motility are likely key components of successful completion of the *B. burgdorferi* transmission cycle. As an example, it has been demonstrated that tick salivary gland extracts are a potent chemoattractant that may facilitate spirochete acquisition (Shih *et al.*, 2002). The microarray analyses revealed that expression of a number of flagellar and chemotaxis genes including *fliD*, *flgL*, *fliG-1*, *cheA-1*, and *mcp-1* are activated by Rrp1. Some of these genes are also regulated, at least in part, by RpoS and have been shown to be induced by exposure to blood (Tokarz *et al*., 2004; Fisher *et al*., 2005; Caimano *et al*., 2007). In other bacteria, c-di-GMP levels have been demonstrated to be a key regulator of chemotaxis and motility (Simm *et al*., 2004; Wolfe and Visick, 2008). The general paradigm for c-di-GMP-mediated signaling is that increases in c-di-GMP lead to decreases in motility and a transition to biofilm formation (Wolfe and Visick, 2008). In *B. burgdorferi*, inactivation of Rrp1, which presumably results in decreased intracellular c-di-GMP, resulted in repression of some motility and chemotaxis genes. However, comparative dark-field microscopic analyses of the *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* strains did not reveal an obvious motility defect in the mutant. It is worth noting that c-di-GMP signaling has only been studied in bacteria that have distinct motile and non-motile stages during the infection process (including species of *Salmonella*, *Yersinia*, *Pseudomonas*, *Escherichia* and *Vibrio*) (Simm *et al*., 2004; Tischler and Camilli, 2004; Meissner *et al*., 2007; Tamayo *et al*., 2007; Wolfe and Visick, 2008). In contrast, there is no evidence that the life cycle of *Borrelia* includes a non-motile stage. Thus, *B. burgdorferi* may have developed a novel use for c-di-GMP signaling in the infection process. The number of key chemotaxis and motility genes that are regulated by Rrp1 serves as an additional example of the central regulatory contributions of Rrp1 to the core biology of *B. burgdorferi*.

Numerous genes encoding cell envelope-associated proteins and *in vivo* expressed antigens are also regulated by Rrp1. While the majority of the genes encoding these proteins reside on the chromosome, those located on extrachromosomal elements reside primarily on the cp32 plasmids. Based on the homology of many cp32-encoded proteins to phage proteins and on the transcriptional properties of the cp32s, it has been postulated that these genetic elements are prophage (Eggers and Samuels, 1999; Eggers *et al*., 2000; 2001; Casjens, 2003; Canchaya *et al*., 2004; Zhang and Marconi, 2005). Interestingly, all of the cp32-carried genes that are regulated by Rrp1 (*ospE*, *bdr*, *ospF*, and *mlp* gene families, for example) reside outside of the phage operon and appear to be of bacterial origin (Zhang and Marconi, 2005). Hence the genes of phage origin on the cp32 plasmids appear to be regulated by different mechanisms that are Rrp1-independent. The functional roles of the OspE and OspF proteins and their expression during infection suggest that the genes encoding these proteins represent ‘morons’ which have been defined as phage-carried genes of bacterial origin that enhance the fitness of the lysogen (Brussow *et al*., 2004). It is important to note that consistent with the upregulation of Rrp1 upon tick feeding reported here, the *ospE* and *ospF* proteins are also upregulated upon tick feeding (Hefty *et al*., 2001; 2002; El-Hage and Stevenson, 2002; Eggers *et al*., 2004; 2006). The functions of other cp32-carried, Rrp1-regulated genes, such as the *mlp* and *bdr* families, have not yet been determined. The Bdr protein family is large, with individual isolates possessing as many as 18 different Bdr paralogues (Carlyon *et al*., 2000a). This gene family is universal among the *Borrelia*, and these proteins possess putative serine/threonine phosphorylation motifs (Carlyon *et al*., 2000b; Roberts *et al*., 2000; Lescot *et al*., 2008). At least some members of the Bdr family have been demonstrated to be upregulated in spirochetes cultivated in implanted dialysis membrane chambers suggesting an important role in the

pathogenic process (Roberts *et al*., 2002). Consistent with the potential importance of the OspE, OspF, and Bdr proteins in the host environment, a recent analysis of the *B. burgdorferi* proteome revealed that members of these protein families are among the dominant antigens recognized during murine infection (Barbour *et al*., 2008).

mlp expression has previously been demonstrated to be activated by the Rrp2–RpoN–RpoS regulatory pathway (Yang *et al*., 2003b; Boardman *et al*., 2008). However, it has been postulated that at least one additional layer of gene regulation contributes to *mlp* gene expression (Yang *et al*., 2003b). Consistent with this, we demonstrate that Rrp1 also serves as an activator of certain members of the *mlp* gene family as their expression was repressed in *Bb*5A13-Δ*rrp1*. Other genes encoding cell membrane-associated proteins whose expression was affected in *Bb*5A13-Δ*rrp1* include BBA03, BBA52, BBA59 and BBA74. Several of the plasmid-encoded genes that we demonstrate in this report to be regulated by Rrp1 have previously been shown to be regulated by environmental conditions (Carroll *et al*., 2000; Revel *et al*., 2002; Roberts *et al*., 2002; Brooks *et al*., 2003; Ojaimi *et al*., 2003; Seshu *et al*., 2004; Tokarz *et al*., 2004; Hyde *et al*., 2007). Hence, for these specific genes, stimuli associated with their expression may be transduced by both the Rrp1 and Rrp2 regulatory networks (Yang *et al*., 2003a; Fisher *et al*., 2005; Caimano *et al*., 2007). It is now apparent that the regulation of the expression of cell membrane-associated protein genes in *B. burgdorferi* is complex with several contributing regulatory mechanisms.

In many bacteria, the regulons of individual TCS are often interconnected. However, in *B. burgdorferi* the two main response regulators do not appear to influence the expression of each other. The expression of *rrp1* was not significantly affected by inactivation of *rrp2* (Boardman *et al*., 2008), and as demonstrated here, inactivation of *rrp1* did not influence *rrp2* expression. Interestingly, *rpoS* expression does appear to be controlled at least in part by Rrp1 as inactivation of *rrp1* led to reduced expression of *rpoS* (2.6-fold as measured by qRT-PCR). The converse regulation does not appear to occur (i.e. inactivation of *rpoS* do not influence *rrp1* expression) (Fisher *et al*., 2005; Caimano *et al*., 2007; Boardman *et al*., 2008). Consistent with co-regulation of *rpoS* by Rrp1 and Rrp2, *ospC* expression is lower in both *rrp1* and *rrp2* mutants (Boardman *et al*., 2008). Other genes regulated by both Rrp1 and Rrp2 include *ospE* (BBL39/BBP38), *ospF* (BBO39 and BBR42), *cspZ* (BBH06), and a gene encoding a hypothetical protein (BBR36) (Table 3). However, the overall set of genes that require signals from both Rrp1 and Rrp2 for their activation appears to be small. When the Rrp1 and Rrp2 regulons are compared, one of the most striking differences observed lies in the genomic location of the genes that they regulate. Rrp1 primarily regulates genes from the core genome, while Rrp2 primarily regulates plasmid genes. Earlier studies suggest that the Rrp2 regulon may be particularly important in transmission and dissemination and that Rrp2-regulated genes may be repressed in ticks (Caimano *et al*., 2007; Boardman *et al*., 2008). The Rrp1 regulon may be important in controlling metabolic processes that are required for adapting to changes in nutrient availability, acquisition by and/or survival of spirochetes in ticks. The qRT-PCR analyses presented here indicate a sixfold induction of Rrp1 upon tick feeding. Consistent with this, the expression of *hpk1*, the gene that encodes for the sensory transduction histidine kinase, was previously shown to have elevated expression in fed ticks compared with flat ticks (Narasimhan *et al*., 2002). As the Rrp1–Hpk1 TCS is activated, genes that encode for proteins involved in the transport of ions, carbohydrates and amino acids are expressed. This may be followed by an increase in the expression of genes that encode proteins involved in carbohydrate and nucleotide metabolism, thus leading to activation of energy metabolism and nucleotide and protein synthesis. Additionally, Rrp1 would activate expression of motility and chemotaxis genes, as well as cell envelope genes and genes involved in immune evasion (examples include *ospC, ospE, ospF* and *cspZ*) to prepare the spirochetes for entry into the host. Hence, Rrp1 is likely a critical regulator during spirochete transmission and adaptation

to the mammalian host. Further studies using an infectious Rrp1 knockout are required to examine this possibility.

In summary, the data presented within demonstrate that Rrp1 plays a central role in the global regulation of core metabolic functions in pathogenic *Borrelia* species. The Rrp1 regulon is unique and exhibits only minimal overlap with other regulatory networks. The data presented in this report provide important information that enhances our understanding of the global regulatory networks of the *Borrelia* and the ability of these bacteria to adapt to rapidly changing environmental conditions. While it remains to be determined if Rrp1 directly or indirectly influences the transcription of the Rrp1 regulon, the production c-di-GMP by Rrp1 (Ryjenkov *et al*., 2005) suggests that this secondary messenger is an important contributing factor. As detailed above, *Borrelia* possess the necessary machinery to regulate c-di-GMP levels. The presence of single proteins with the domains known to be associated with the formation and degradation of c-di-GMP in *B. burgdorferi* suggests that this important human pathogen may be an excellent model system to study the regulatory effects of c-di-GMP and c-di-GMP-producing TCS.

Experimental procedures

Bacterial isolates, cultivation, and ticks

Table 4 lists and describes the *Borrelia* isolates analysed in this report. All *Borrelia* isolates were cultivated in BSK-H complete media (Sigma-Aldrich) at 25°C, 33°C, or 37°C in sealed bottles under 5% CO_2 and harvested by centrifugation (14 000 *g*; 4°C; 20 min). Infected *I. scapularis* nymphal stage ticks were kindly provided by Melissa Caimano and Justin Radolf (University Connecticut Health Sciences Center). All methods used to propagate and infect the ticks have been previously described (Caimano *et al*., 2007).

PCR, cloning, and DNA sequencing

Polymerase chain reaction was performed using the Bio-Rad DNAEngine Peltier Thermal Cycler-200 and the following cycle conditions: one cycle at 95°C for 2 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 7 min. Each PCR reaction included 0.75 units of *Taq* polymerase (Promega), 1× polymerase buffer including 1.5 mM $MgCl_2$, 0.75 pmol μl^{-1} of each primer, 200 μM each of dATP, dGTP, dCTP and dTTP, and 1–10 ng of genomic DNA for template. Primers are listed in Table S2 and were designed based on the genome sequence of *B. burgdorferi* B31MI. PCR amplicons were analysed by electrophoresis in 1% agarose gels with Tris-acetate-EDTA (TAE) buffer and visualized by staining with ethidium bromide. For sequence analyses, the PCR amplicons were cloned into the pCR2.1-TOPO vector (Invitrogen). DNA sequencing was performed on a fee-for-service basis (MWG Biotech). The sequence accession numbers for Rrp1 sequences determined or analysed as part of this report or in earlier genome sequence analyses are as follows: *B. burgdorferi* N40 –FJ643425, B348 – FJ643424, BL224 – FJ643423; *B. garinii* PBi – YP_072864, *B. afzelii* PKo – ABH01689, ECM1 –FJ345410, VS461 – FJ345411; *B. spielmanii* A14S –ZP_03099004; *B. valaisiana* VS116 – ZP_02368043; *B. turicatae* 91E135 – YP_945417, PE1926 – FJ263942, OZ1 – FJ263941; *B. hermsii* DAH – YP_001883847, MAN –FJ643426; *B. duttonii* Ly – ACH93363; and *B. recurrentis* A1 – ACH94659.

Southern blot hybridization analysis

For hybridization analyses, DNA was isolated using the MasturePure DNA purification kit (Epicentre), digested with *Eco*RI (New England Biolabs), and fractionated by agarose gel electrophoresis in 0.7% gels with TAE buffer. The DNA was transferred to Hybond-N membranes (Amersham Biosciences) by vacuum blotting, and the DNA was fixed to the membrane by UV irradiation. An *rrp1* gene probe was generated by PCR (primers 7 + 8; Table

S2) using purified *B. burgdorferi* B31 DNA as the template. Probe radiolabeling and all hybridization conditions were as previously described (Rogers and Marconi, 2007).

RNA isolation, RT-PCR, and qRT-PCR

RNA was isolated from ticks as previously described (Caimano *et al*., 2007). To isolate RNA from *Borrelia* cultures, cells were lysed with diethylpyrocarbonate-treated 1% SDS and RNA was recovered using the RNeasy Midi kit as instructed by the manufacturer (Qiagen) and with modifications that have been previously described (Zhang *et al*., 2005). Residual DNA was removed by treatment with DNase I (Invitrogen). RT-PCR analyses were performed as described (Zhang *et al*., 2005). qRT-PCR was performed using the DNA Engine Opticon 2 System (MJ Research) and SYBR Green PCR Master Mix (Applied Biosystems). Primer pairs (Table S2) were designed to amplify a 100–150 bp fragment of each target and a standard curve was generated using serial dilutions of a known amount of genomic DNA as template. qRT-PCR was performed in triplicate and error bars in Fig. 1D represent standard deviation. The limited quantity of RNA isolated from ticks prevented qRT-PCR analysis in triplicate and as a result there are no error bars. qRT-PCR was performed using the following cycling parameters: one cycle of 10 min at 95°C followed by 40 cycles of 10 s at 94°C, 20 s at 60°C and 20 s at 72°C. Melting curves were generated over a temperature range of 55–95°C. PCR product was quantified and the data analysed using software provided by the thermocycler manufacturer (MJ Research).

Generation of recombinant proteins, protein-specific antiserum, and infection serum

Recombinant (r-) proteins were generated by PCR amplification of the gene of interest with primers harbouring tail sequences that allow for cloning into the pET-46 Ek/LIC vector (Novagen). All methods were as previously described (Rogers and Marconi, 2007). Antiserum to r-proteins was generated in C3H/HeJ mice (Jackson Laboratories) by injection of 50 μg of each r-protein in Freund's complete adjuvant (Pierce), followed by two injections of 50 μg of r-protein in Freund's incomplete adjuvant at weeks 2 and 4. At week 6, the mice were euthanized and blood was collected. Serum was recovered from blood by centrifugation (5 min; 14 000 *g*) after a 3 h incubation at room temperature. To generate infection serum, C3H/HeJ mice were infected subcutaneously with 10^5*B. burgdorferi* B31, and infection was confirmed using ear punch biopsies.

Triton X-114 extraction and phase partitioning, SDS-PAGE, and immunoblotting

Triton X-114 extraction and phase partitioning was performed as described by Cunningham *et al*. (1988) with modifications (Zhang *et al*., 2005). A 1% Triton X-114 solution removes the outer membrane of the *Borrelia*, leaving the protoplasmic cylinder intact. The protoplasmic cylinder, consisting of the inner membrane and cytoplasmic proteins, is pelleted by centrifugation. The remaining outer membrane and periplasmic proteins are separated based on their hydrophobic/hydrophilic properties. For SDS-PAGE and immunoblotting, proteins or cell lysates were fractionated using 12.5% Criterion Precast Gels (Bio-Rad) and transferred to PVDF membranes (Millipore), blocked (5% non-fat dry milk, 0.2% Tween, 1× PBS), and screened with various antisera including mouse anti-BBO39 (1:500), mouse anti-BBR42 (1:500), mouse anti-BdrF2 (BBG31; 1:1000), mouse anti-Rrp1 (BB0419; 1:5000), mouse anti-BBN38 (1:5000), mouse anti-BBL39 (1:1000), rabbit anti-Bdr (1:40 000), rabbit anti-DbpA (BBA24; 1:400 000), rat anti-OspA (BBA15; 1:600 000) and mouse anti-*B. burgdorferi* B31 infection serum (1:1000). Detection was through the use of the appropriate horseradish peroxidase-conjugated secondary antibody (1:40 000; Pierce). All antibodies were diluted in blocking buffer, and in all cases, antibody binding was detected by chemiluminescence using the SuperSignal West Pico chemiluminescence substrate (Pierce) and exposure to X-ray film (Phenix).

Allelic exchange mutagenesis

Allelic exchange gene inactivation was accomplished using procedures described by Samuels *et al*. (1994). In separate PCR reactions, 1000 bp segments of DNA that reside immediately upstream and downstream of *rrp1* (chromosomal co-ordinates 431 616–430 616 and 429 693–428 693 respectively) were PCR amplified and cloned into the pCR2.1-TOPO vector to yield pCR2.1-up and pCR2.1-dn respectively. Where appropriate, the primers were constructed with restriction sites to facilitate subsequent cloning steps. Both pCR2.1-up and pCR2.1-dn were digested with *Aat*II and *Age*I. The downstream fragment was excised from pCR2.1-dn and ligated into the linearized pCR2.1-up to yield pCR2.1-updn. The spectinomycin resistance cassette (1203 bp), derived from pKFSS1-*Aat*II (Frank *et al*., 2003), was inserted between the upstream and downstream fragments at an *Aat*II site to generate pΔBB0419. The plasmid was propagated, purified, linearized with *Bam*HI, and electroporated into *B. burgdorferi* clones 5A13 and 5A4. Clonal populations were obtained by subsurface plating and plasmid profiles were assessed by PCR using plasmid-specific primers (Table S2).

Growth curve analysis

To compare the growth rates of the *Bb*5A13-wt and *Bb*5A13-Δ*rrp1*, equal numbers of actively growing spirochetes were seeded into fresh media and incubated at 25°C, 33°C, and 37°C. Aliquots were removed once a day for 19 days and spirochete numbers determined by counting under dark-field microscopy. Ten fields were counted at each time point and the average number of spirochetes per field was determined.

Microarray hybridization, scanning, and data analysis

RNA isolated from three independent stationary-phase cultures of *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* strains grown at 33°C was used for microarray hybridizations. RNA was labeled indirectly with cy3 and cy5 fluorescent dyes and co-hybridized onto the same microarray slide as previously described (Caimano *et al*., 2007). To avoid dye bias, two hybridizations were performed for each of the three biological replicates: in the first hybridization RNA from *Bb*5A13-wt was labeled with cy3 fluorescent dye and RNA from *Bb*5A13-Δ*rrp1* was labeled with cy5 fluorescent dye. In a second hybridization, RNA from *Bb*5A13-wt was labeled with cy5 and RNA from *Bb*5A13-Δ*rrp1* was labeled with cy3. The custom glass slide microarrays employed in this study consist of 1741 70-mer oligonucleotides representing all annotated ORFs and pseudogenes of *B. burgdorferi* B31MI. These arrays and all associated methods for hybridization and array scanning have been previously described (Terekhova *et al*., 2006; Caimano *et al*., 2007).

Data analysis was conducted as detailed previously. In brief, mean and standard deviation of fluorescence intensities were calculated for all blank control spots in cy3 and cy5 channels separately; the mean + 2SD was considered to be background and was subtracted from each intensity value in the appropriate channel. Background correction was performed separately for each slide. Spots yielding signals below background in both cy3 and cy5 channels were considered undetected. The background adjusted intensity values were normalized across all arrays. The resultant values were used to calculate $\log_2$(*Bb*5A13-Δ*rrp1*/*Bb*5A13-wt) for each spot in each slide, and 18 $\log_2$(*Bb*5A13-Δ*rrp1*/*Bb*5A13-wt) values for each ORF from two separate microarrays and three biological replicates were averaged yielding a final value. Two-tailed, two-sample unequal variance *t*-test between 18 background-subtracted normalized *Bb*5A13-Δ*rrp1* and 18 background-subtracted normalized *Bb*5A13-wt hybridization intensity values was used to determine which ORFs had statistically significant expression differences between wild-type and *rrp1* knockout strains. ORFs with $P < 0.05$ and $\log_2$(*Bb*5A13-Δ*rrp1*/*Bb*5A13-wt) > 2 or < $^{-}$2 were considered induced or repressed in the *Bb*5A13-Δ*rrp1*.

Factor H affinity ligand binding immunoblot (FH ALBI) assays

Factor H binding to membrane-immobilized proteins obtained from *Bb*5A13-Δ*rrp1* and *Bb*5A13-wt was assessed using the affinity ligand binding immunoblot (ALBI) assay approach (referred to as the FH ALBI assay) as previously described (Rogers and Marconi, 2007). In brief, the membranes were blocked, incubated with purified human FH (5 ng μl^{-1}; Calbiochem), washed with PBS containing 0.2% Tween (PBS-T) and incubated with goat anti-human FH (1:1000; Calbiochem) in blocking buffer (1 h each). Detection was through HRP-conjugated rabbit anti-goat IgG (1:40 000; Pierce) and chemiluminescence.

Indirect IFA

To generate slides for IFA analysis, ~5–7 × 10^7 *B. burgdorferi* cells (either *Bb*5A13-Δ*rrp1* or *Bb*5A13-wt) grown at 37°C were collected by centrifugation (7000 *g*; 10 min), washed three times with PBS and re-suspended in 1 ml of PBS. One hundred microlitres of this cell suspension was spread over a 2 cm^2 area on Superfrost Plus charged slides (Fisher) and air dried. For screening, all steps were performed in a dark, humidified chamber. The slides were blocked (5% BSA in PBS-T) for 1 h and then screened simultaneously with a 1:100 dilution of mouse anti-BBL39 and rabbit anti-DbpA (1 h). The slides were washed and incubated with 1:200 dilutions of both Alexafluor 488-conjugated goat anti-mouse IgG and Alexafluor 555-conjugated goat anti-rabbit IgG (1 h). The slides were mounted with fluoromount-G (Electron Microscopy Sciences) and visualized by fluorescence and dark-field microscopy.

Serum sensitivity

To assess spirochetes for serum sensitivity, cells (*Bb*5A13-Δ*rrp1*, *Bb*5A13-wt and *B. garinii* FRG) were incubated in 50% normal human serum (NHS) or heat-inactivated serum (HIS). Serum was heat-inactivated by incubation at 56°C for 20–30 min. Cell viability was assessed after 1.5, 3, 6, and 24 h using the BacLight LIVE/DEAD kit (Invitrogen) and viewed by fluorescent microscopy. At each time point, 10 fields were counted, averaged and graphed as a percentage of average number of cells in NHS divided by the average number of cells in HIS. The graph shows the average of three independent experiments. The error bars represent standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank members of the Marconi and Schwartz labs for their contributions to this study and Melissa Caimano and Justin Radolf for providing *B. burgdorferi*-infected ticks. This work was supported in part by funding from the National Institutes of Health (AI45801 to I. Schwartz) and by support from the Department of Microbiology and Immunology, Virginia Commonwealth University.

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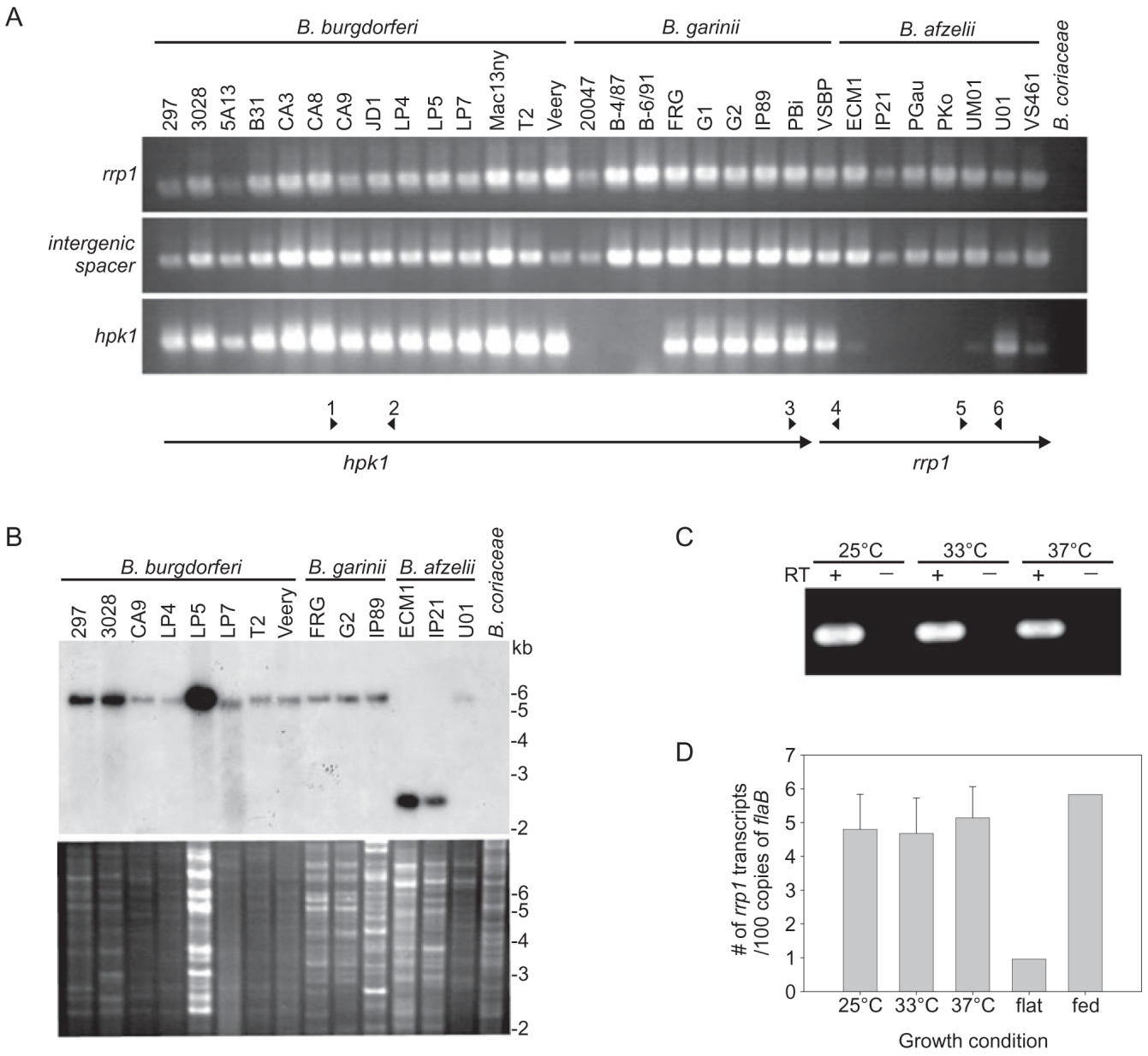

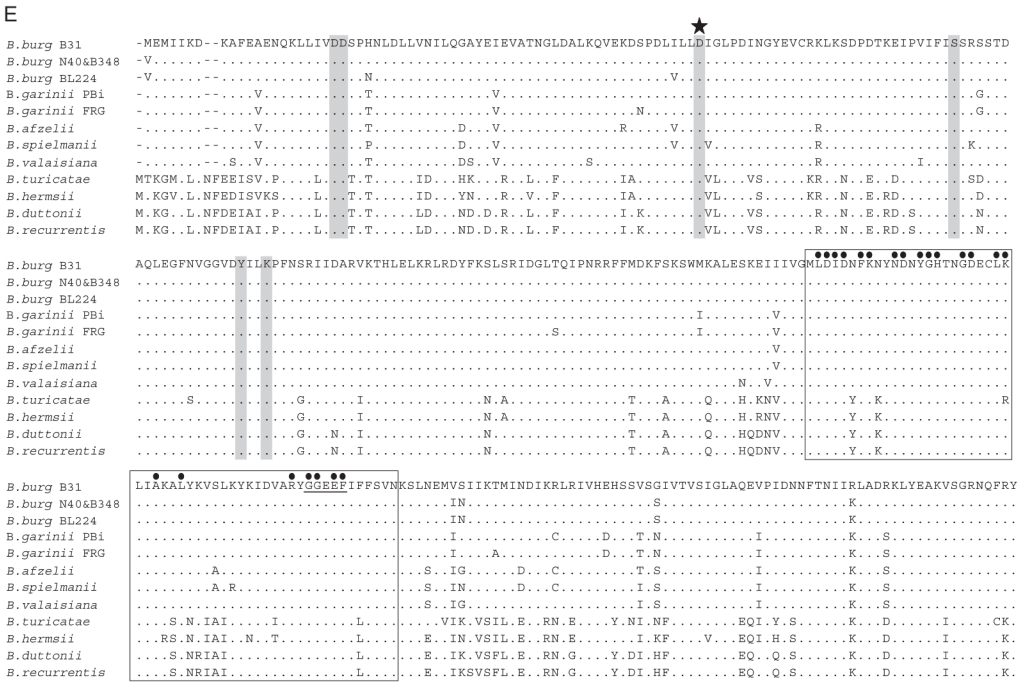

Fig. 1.

rrp1 and *hpk1* are universally distributed, highly conserved, co-transcribed, and constitutively expressed *in vitro*. (A) presents PCR analyses conducted with primers designed to amplify an internal region of *rrp1* (primers 5 + 6), across the *rrp1–hpk1* intergenic spacer (primers 3 + 4), and an internal region of *hpk1* (primers 1 + 2). The isolates analysed are indicated above each lane with the species names above the isolates. The PCR results for a select group of isolates were confirmed through Southern hybridization using a radiolabeled *rrp1* probe and *Eco*RI-restricted genomic DNA from the isolates listed above each lane (B). The top and bottom portions of (B) present the hybridization results and ethidium bromide-stained gel of the restricted DNA respectively. Molecular weight markers are shown to the right in kilobases (kb). (C) presents the results of RT-PCR analyses conducted using RNA isolated from *B. burgdorferi* B31 grown at 25°C, 33°C or 37°C. The plus (+) and minus (−) signs indicate reactions that were run with or without reverse transcriptase (RT) enzyme, respectively. Using the same RNA, transcripts of *rrp1* were quantified using qRT-PCR and are graphed as number of *rrp1* transcripts per 100 copies of the *flaB* gene (D). (E) presents an amino acid sequence alignment of deduced Rrp1 sequences. Dashes indicate gaps in the sequence and dots indicate an identical amino acid to the reference sequence (*Bb*B31). Species and isolate names are shown on the left. The sequence shown for *B. afzelii* represents three isolates with identical amino acid sequences (PKo, ECM1, and VS461), *B. turicatae* represents three isolates with identical sequences (91E135, PE1926, and OZ1), and *B. hermsii* represents two isolates with identical sequences (DAH and MAN). The six critical residues associated with receiver domain function of response regulators are indicated by shaded boxes with the phosphorylated aspartate (D) residue indicated with a star. The box indicates the location of the GGDEF domain and the filled circles above each amino acid indicate the residues that are most conserved among proteins with GGDEF domains. The active site in this domain is underlined in the reference sequence.

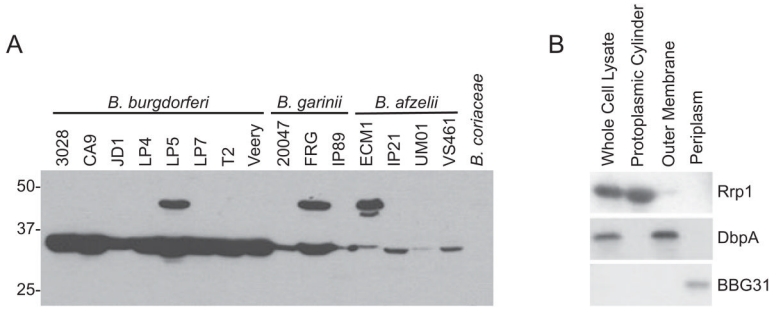

Fig. 2.

Demonstration of the universal production of Rrp1 by Lyme disease isolates and analysis of its Triton X-114 extraction and phase partitioning properties. A panel of Lyme disease isolates was cultivated under standard conditions and then cell lysates were fractionated, immunblotted and screened with Rrp1 antiserum (A). Species and isolate names are indicated above each lane and molecular weight markers are indicated to the left. (B) presents the results of immunoblot analyses of *B. burgdorferi* B31 fractions obtained from Triton X-114 extraction and phase partitioning. Identical blots of the fractionated proteins were screened with antiserum to Rrp1, DbpA (an established lipidated outer membrane protein) and BBG31 (a member of the inner membrane-localized Bdr protein family).

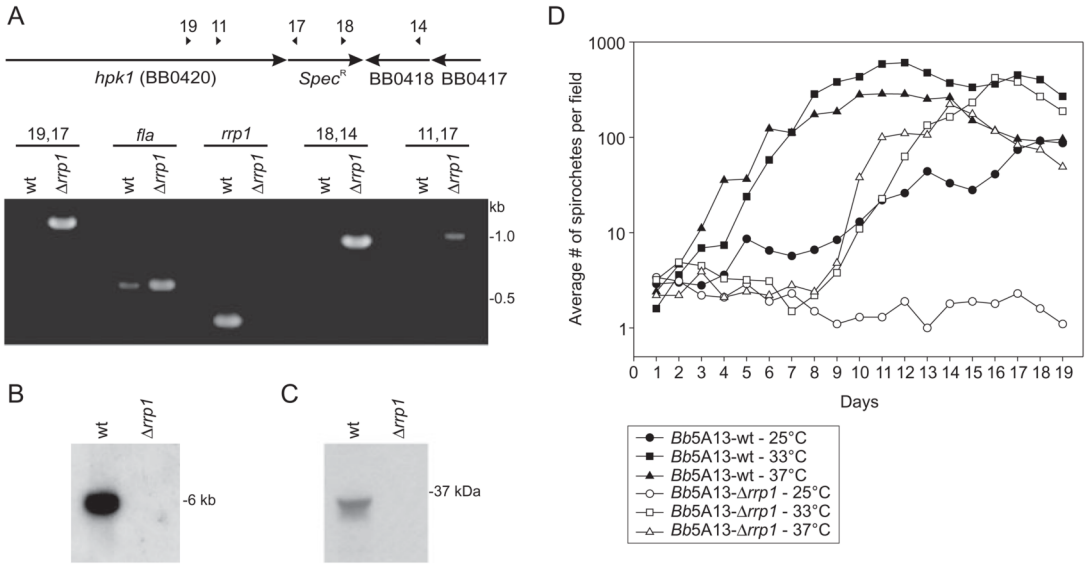

Fig. 3.

Verification of *rrp1* deletion by allelic exchange replacement and analysis of growth rate of *Bb5*A13-wt and *Bb*5A13-Δ*rrp1*. To confirm allelic exchange replacement was successful, PCR (A), Southern hybridization (B) and immunoblot analyses (C) were performed. (A) shows a schematic of the chromosome after allelic exchange, in which *rrp1* (BB0419) has been replaced by the spectinomycin/streptomycin resistance cassette (*Spec*^{R}). Primers are indicated by numbers and listed in Table S2. Numbers above each lane in the ethidium bromide-stained gel indicate primer combinations. In (B), *Eco*RI-restricted genomic DNA was fractionated, blotted and screened with a radiolabeled *rrp1* probe. In (C), cell lysates were screened with Rrp1 antiserum. In (D), *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* cells were cultivated at 25°C, 33°C and 37° C in BSK-H complete media. All methods were as described in the text.

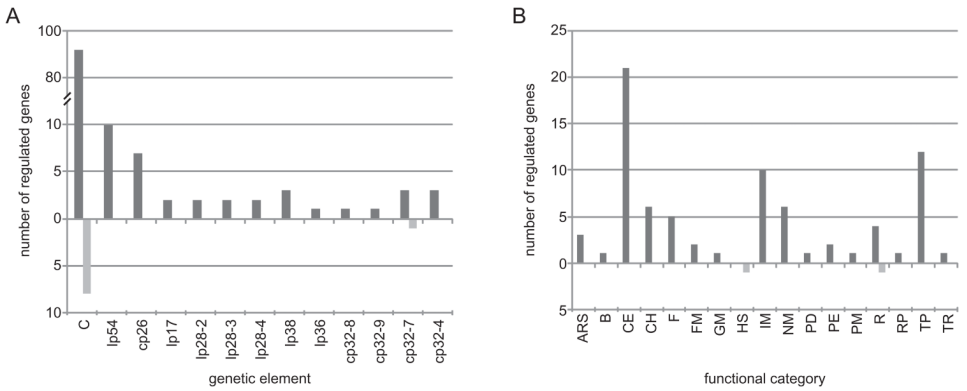

Fig. 4.

Rrp1 is a central regulator of the core *Borrelia* genome. Genes differentially expressed in *Bb*5A13-Δ*rrp1* relative to *Bb*5A13-wt were identified by microarray analysis as described in *Experimental procedures*. Numbers of genes with lower or higher mRNA levels (dark grey and light grey bars respectively) in *Bb*5A13-Δ*rrp1* relative to *Bb*5A13-wt are organized by genetic location (A) and by functional category (B). Abbreviations: C, chromosome; lp, linear plasmid; cp, circular plasmid; ARS, amino acid biosynthesis; B, biosynthesis; CE, cell envelope; CH, chemotaxis; F, flagellar biosynthesis; FM, fatty acid metabolism; GM, general metabolism; HS, heat-shock proteins; IM, intermediary metabolism; NM, nucleotide metabolism; PD, protein degradation; PE, protein export; PM, protein metabolism; R, replication; RP, ribosomal proteins; TP, transporter proteins; TR, transcription.

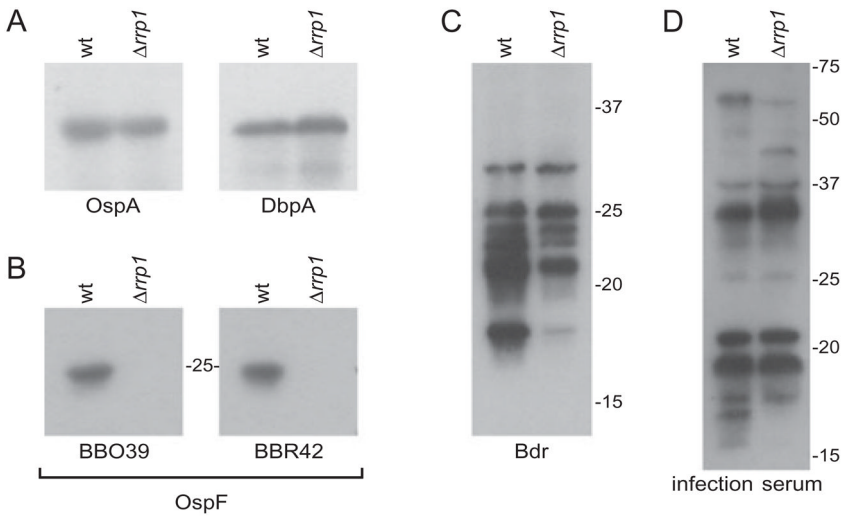

Fig. 5.

Comparative immunoblot analysis of *Bb*5A13-wt and *Bb5*A13-Δ*rrp1*. The results of the microarray analyses were confirmed by western blot analysis of a select group of proteins. Cell lysates of the *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* were separated by SDS-PAGE, immunoblotted, and screened with the antiserum indicated below each blot. (A) represents proteins not affected in the Rrp1 knockout, (B) represents an example of two members of a protein family that are coordinately regulated by Rrp1, and (C) represents family members that are differentially regulated by Rrp1. In (D), cell lysates were screened with serum collected from mice infected with *B. burgdorferi* B31MI.

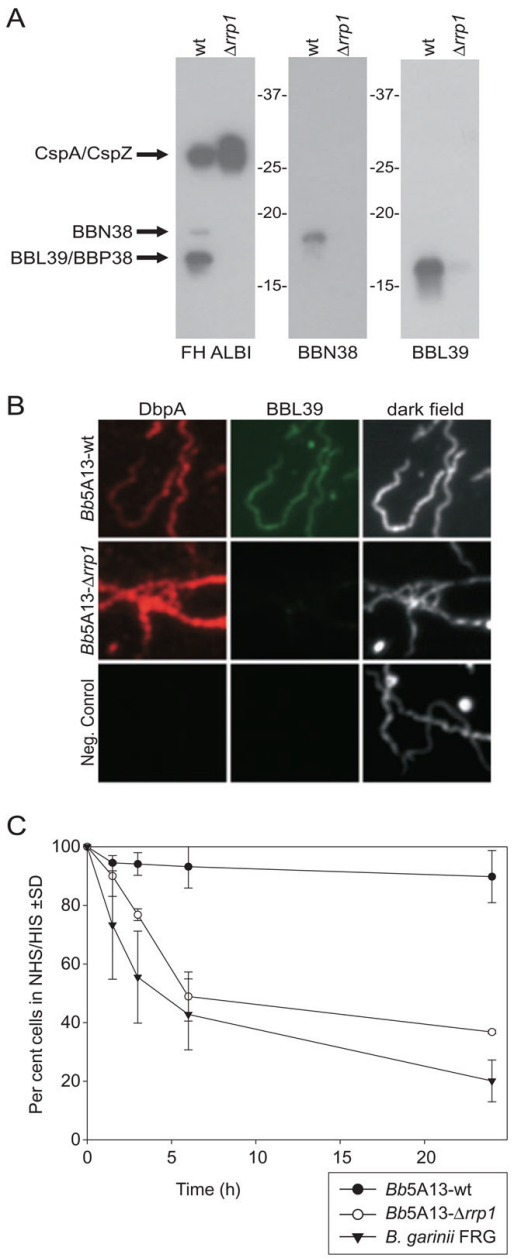

Fig. 6.

Rrp1 regulation of the OspE factor H binding proteins and analysis of *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* cells for serum sensitivity. Western blots of the *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* cell lysates were screened for factor H binding ability and with anti-BBN38 and anti-BBL39 (A). For the IFA analysis in (B), *Bb*5A13-wt and *Bb*5A13-Δ*rrp1* cells were washed, dried onto slides, and screened simultaneously with anti-DbpA and anti-BBL39. The negative control slide was screened with secondary antibodies only. For (C), *Bb*5A13-wt, *Bb*5A13-Δ*rrp1* and *B. garinii* FRG cells were incubated in 50% normal human serum (NHS) or in 50% heat-inactivated serum (HIS) for 1.5, 3, 6, and 24 h. At each time, the number of cells in 10 high-powered fields was counted and averaged. The graph shows the number of cells in NHS divided by the number of cells in HIS, expressed as a percentage. The error bars represent standard deviation (SD) from three independent experiments.

Table 1

Borrelia burgdorferi genes (a) activated and (b) repressed by Rrp1.

Gene number and annotation[a,b]	Functional category[a]	WT/M[c]	*P*-value	Relevant citation[d]
(a) *Borrelia burgdorferi* genes activated by Rrp1				
BB0014 primosomal protein N′ (*priA*)	R	9.55	0.0117	
BB0021 *S*-adenosylmethionine: tRNA ribosyltransferase-isomerase	ARS	8.36	0.00924	Caimano *et al*. (2007)
BB0047 conserved hypothetical protein	HX	11.0	0.0275	Ojaimi *et al*. (2003); Hyde *et al*. (2006)
BB0048 hypothetical protein	U	122	0.0350	Ojaimi *et al*. (2003)
BB0084 nifS protein (*nifS*)	ARS	5.79	0.0269	
BB0100 glutamate racemase (*murI*)	CE	94.0	0.0232	
BB0107 N-utilization substance protein B (*nusB*)	TR	4.02	0.00995	
BB0110 hypothetical protein	U	6.13	0.0209	
BB0114 single-stranded DNA-binding protein (*ssb*)	R	4.15	0.00559	
BB0129 conserved hypothetical protein	HX	37.1	0.0203	Narasimhan *et al*. (2002)
BB0140 acriflavine resistance protein (*acrB*)	X	11.6	0.0136	
BB0146 glycine betaine, L-proline ABC transporter, ATP-binding protein (*proV*)	TP	80.1	0.0132	Narasimhan *et al*. (2002)
BB0149 flagellar hook-associated protein 2 (*fliD*)	F	7.87	0.0300	
BB0151 *N*-acetylglucosamine-6-phosphate deacetylase (*nagA*)	IM	12.3	0.0163	Narasimhan *et al*. (2002)
BB0152 glucosamine-6-phosphate isomerase (*nagB*)	IM	66.7	0.0260	Brooks *et al*. (2003)
BB0153 superoxide dismutase (*sodA*)	X	129	0.0484	Hyde *et al*. (2006)
BB0156 hypothetical protein	U	9.78	0.0183	
BB0160 alanine racemase (*alr*)	CE	10.8	0.0277	
BB0162 hypothetical protein	U	8.86	0.00235	Ojaimi *et al*. (2003); Tokarz *et al*. (2004)
BB0167 outer membrane protein (*tpn50*)[b]	**CE**	**29.8**	**0.00587**	
BB0170 hypothetical protein	U	136	0.0207	
BB0173 hypothetical protein	U	131	0.0142	
BB0174 hypothetical protein	U	27.0	0.0112	
BB0175 conserved hypothetical protein	HX	4.49	0.00218	Ojaimi *et al*. (2003); Tokarz *et al*. (2004)
BB0178 glucose inhibited division protein A (*gidA*)	R	4.15	0.00934	
BB0179 thiophene and furan oxidation protein (*thdF*)	X	30.5	0.0193	
BB0180 flagellar protein, putative	F	30.9	0.00809	
BB0182 flagellar hook-associated protein 3 (*flgL*)	F	10.8	0.00882	
BB0185 conserved hypothetical protein	HX	5.65	0.00311	
BB0186 conserved hypothetical protein	HX	4.98	0.0338	
BB0221 flagellar motor switch protein (*fliG-1*)	F	9.67	0.0130	
BB0241 glycerol kinase (*glpK*)[b]	**IM**	**5.43**	**0.000391**	Narasimhan *et al*. (2002); Ojaimi *et*

Gene number and annotation[a,b]	Functional category[a]	WT/M[c]	*P*-value	Relevant citation[d]
				al. (2003); Hyde *et al*. (2006); Caimano *et al*. (2007)
BB0242 hypothetical protein	U	367	0.00274	Ojaimi *et al*. (2003); Tokarz *et al*. (2004); Hyde *et al*. (2006); Caimano *et al*. (2007)
BB0243 glycerol-3-phosphate dehydrogenase, anaerobic (*glpA*)[b]	**IM**	**21.9**	**0.00201**	Ojaimi *et al*. (2003); Hyde *et al*. (2006); Caimano *et al*. (2007)
BB0250 dedA protein (*dedA*)	X	14.9	0.0363	
BB0261 hypothetical protein	U	14.9	0.0310	Tokarz *et al*. (2004)
BB0287 flagellar protein (*flbA*)	F	11.6	0.0233	
BB0319 exported protein (*tpn38b*)	CE	53.9	0.0114	
BB0349 hypothetical protein	U	57.0	0.00831	
BB0358 conserved hypothetical protein	HX	29.6	0.0376	
BB0367 hypothetical protein[b]	**U**	**18.8**	**0.00123**	Tokarz *et al*. (2004)
BB0407 mannose-6-phosphate isomerase (*manA*)	IM	59.0	0.0397	
BB0420 sensory transduction histidine kinase/ response regulator (*hpk1*)	**GM**	**236**	**0.0343**	Narasimhan *et al*. (2002)
BB0451 chromate transport protein, putative	TP	63.0	0.0475	
BB0466 ABC transporter, ATP-binding protein	TP	12.4	0.0404	
BB0475 lipoprotein, putative	CE	4.66	0.0231	
BB0530 hypothetical protein	U	7.66	0.0172	
BB0531 hypothetical protein	U	11.0	0.0387	
BB0553 hypothetical protein	U	6.61	0.0438	
BB0567 chemotaxis histidine kinase (*cheA-1*)	CH	45.1	0.0348	Tokarz *et al*. (2004); Fisher *et al*. (2005)
BB0568 protein-glutamate methylesterase (*cheB-2*)	CH	6.50	0.0198	
BB0570 chemotaxis response regulator (*cheY-2*)	CH	4.79	0.0322	
BB0571 uridylate kinase (*smbA*)	NM	99.7	0.0177	
BB0572 glycosyl transferase (*lgtD*)	CE	67.5	0.0367	
BB0575 CTP synthase (*pyrG*)	NM	15.5	0.0130	
BB0578 methyl-accepting chemotaxis protein (*mcp-1*)	CH	31.8	0.0163	Caimano *et al*. (2007)
BB0583 MATE efflux family protein	TP	264	0.0382	
BB0584 conserved hypothetical integral membrane protein	CE	7.78	0.0173	
BB0586 femA protein (*femA*)	X	6.43	0.0153	
BB0587 methionyl-tRNA synthetase (*metG*)	ARS	19.5	0.0125	
BB0588 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase, putative (*pfs-2*)	NM	14.2	0.0104	Caimano *et al*. (2007)
BB0589 phosphate acetyltransferase (*pta*)	IM	26.6	0.0149	
BB0590 dimethyladenosine transferase (*ksgA*)	NM	198	0.0166	

Gene number and annotation[a,b]	Functional category[a]	WT/M[c]	*P*-value	Relevant citation[d]
BB0596 methyl-accepting chemotaxis protein (*mcp-2*)	**CH**	**240**	**0.0114**	
BB0598 UDP-*N*-acetylmuramate dehydrogenase (*murB*)	CE	104	0.0475	
BB0600 hypothetical protein	U	13.6	0.0181	Narasimhan *et al*. (2002)
BB0604 L-lactate permease (*lctP*)	TP	4.63	0.0297	
BB0617 hypothetical protein	U	33.9	0.0146	
BB0620 beta-glucosidase, putative	IM	17.8	0.0142	
BB0625 *N*-acetylmuramoyl-L-alanine amidase, putative	CE	6.25	0.00591	
BB0629 PTS system, fructose-specific IIABC component (*fruA-2*)[b]	**TP**	**20.1**	**0.0128**	
BB0630 1-phosphofructokinase (*fruK*)	IM	15.1	0.0326	
BB0635 conserved hypothetical protein	HX	5.02	0.0102	Narasimhan *et al*. (2002)
BB0636 glucose-6-phosphate 1-dehydrogenase (*zwf*)	IM	60.9	0.0230	
BB0637 Na+/H+ antiporter (*nhaC-1*)	TP	33. 7	0.0154	
BB0638 Na+/H+ antiporter (*nhaC-2*)	TP	6.14	0.00977	
BB0642 spermidine/putrescine ABC transporter, ATP-binding protein (*potA*)	TP	17.4	0.0158	
BB0645 PTS system, glucose-specific IIBC component (*ptsG*)	TP	30.5	0.0481	
BB0648 serine/threonine kinase, putative	PM	4.11	0.0253	
BB0650 hypothetical protein	U	22.0	0.0229	
BB0652 protein-export membrane protein (*secD*)	PE	37.7	0.0136	Brooks *et al*. (2003)
BB0653 protein-export membrane protein (*secF*)	PE	55.9	0.00618	
BB0656 oxygen-independent coproporphyrinogen III oxidase, putative	B	147	0.00662	
BB0664 hypothetical protein	U	108	0.0394	
BB0672 chemotaxis response regulator (*cheY-3*)	CH	7.63	0.0141	
BB0675 hypothetical protein	U	4.45	0.0137	
BB0683 3-hydroxy-3-methylglutaryl-CoA synthase[b]	**FM**	**14.7**	**0.00139**	
BB0687 phosphomevalonate kinase, putative	**FM**	**5.34**	**0.0126**	
BB0702 lipopolysaccharide biosynthesis-related protein (*kdtB*)	X	17.5	0.0497	
BB0769 sialoglycoprotease (*gcp*)	**PD**	**84.2**	**0.0117**	
BB0785 stage V sporulation protein G	**X**	**11.2**	**0.0176**	Tokarz *et al*. (2004); Caimano *et al*. (2007)
BB0804 ribosomal protein S15 (*rpsO*)	RP	8.54	0.0263	
BB0837 excinuclease ABC, subunit A (*uvrA*)	R	11.8	0.0212	
BB0842 ornithine carbamoyltransferase, catabolic (*arcB*)	IM	72.1	0.0359	
BBA03 outer membrane protein	CE	247	0.00704	Carroll *et al*. (2000); Tokarz *et al*. (2004); Hyde *et al*. (2006)

Gene number and annotation[a,b]	Functional category[a]	WT/M[c]	*P*-value	Relevant citation[d]
BBA19 conserved hypothetical protein	HX	26.9	0.00915	
BBA30 hypothetical protein[b]	**U**	**157**	**0.00375**	
BBA41 conserved hypothetical protein	HX	3.42	0.0218	Ojaimi *et al*. 2003)
BBA52 outer membrane protein	CE	575	0.0216	Brooks *et al*. (2003); Tokarz *et al*. (2004)
BBA59 lipoprotein	CE	741	0.00397	Brooks *et al*. (2003); Tokarz *et al*. 2004)
BBA61 conserved hypothetical protein	HX	5.32	0.0331	Brooks *et al*. (2003)
BBA69 hypothetical protein	U	5.77	0.0460	Brooks *et al*. (2003); Tokarz *et al*. (2004); Caimano *et al*. (2007)
BBA74 outer membrane-associated periplasmic protein	**CE**	**159**	**7.76E-05**	Brooks *et al*. (2003); Ojaimi *et al*. (2003); Tokarz *et al*. (2004); Caimano *et al*. (2007)
BBA76 thymidylate synthase-complementing protein (*thy1*)	X	324	0.0335	
BBB14 hypothetical protein	U	206	0.0261	Ojaimi *et al*. (2003)
BBB16 oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (*oppAIV*)[b]	**TP**	**17.0**	**7.26E-05**	
BBB17 IMP dehydrogenase (*guaB*)[b]	**NM**	**6.91**	**0.0307**	
BBB18 GMP synthase (*guaA*)[b]	**NM**	**8.30**	**0.000847**	
BBB19 outer surface protein C (*ospC*)[b]	**CE**	**33.9**	**0.0476**	Brooks *et al*. (2003); Ojaimi *et al*. (2003); Yang *et al*. (2003a); Tokarz *et al*. (2004); Fisher *et al*. (2005); Caimano *et al*. (2007)
BBB22 conserved hypothetical protein	HX	366	0.0182	
BBB29 PTS system, maltose and glucose-specific IIABC component (*malX*)	**TP**	**25.7**	**0.0154**	
BBD09 hypothetical protein	U	10.6	0.0130	Hyde *et al*. (2006)
BBD10 hypothetical protein	U	47.2	0.00975	
BBG06 conserved hypothetical protein	HX	6.09	0.0103	Tokarz *et al*. (2004)
BBG33 *Borrelia* direct repeat protein (*bdrF2*)	CE	27.5	0.0151	
BBH06 factor H-binding protein (*cpsZ*)[b]	**U**	**159**	**0.0140**	
BBH27 conserved hypothetical protein	HX	34.9	0.00127	
BBI29 hypothetical protein	U	223	0.0241	Ojaimi *et al*. (2003); Caimano *et al*. (2007)
BBI38 hypothetical protein	U	224	0.0302	Brooks *et al*. (2003); Caimano *et al*. (2007)
BBJ08 hypothetical protein	U	11.6	0.0340	Brooks *et al*. (2003); Ojaimi *et*

Gene number and annotation[a,b]	Functional category[a]	WT/M[c]	*P*-value	Relevant citation[d]
				al. (2003); Tokarz *et al*. (2004)
BBJ09 outer surface protein D (*ospD*)	CE	7.13	0.0146	Brooks *et al*. (2003); Ojaimi *et al*. (2003); Tokarz *et al*. (2004)
BBJ34 hypothetical protein	U	4.68	0.0432	Ojaimi *et al*. (2003)
BBK19 hypothetical protein	U	15.4	0.0131	Caimano *et al*. (2007)
BBL28 lipoprotein	CE	111	0.047	Brooks *et al*. (2003); Ojaimi *et al*. (2003); Tokarz *et al*. (2004)
BBN38 factor H-binding protein (*ospE*)[b]	**CE**	**5.36**	**0.0126**	
BBO21 conserved hypothetical protein	HX	45.0	0.0102	
BBO27 *Borrelia* direct repeat protein (*bdrE3*)	HX	484	0.00972	Brooks *et al*. (2003)
BBO28 lipoprotein (*lp*)	CE	292	0.0131	Tokarz *et al*. (2004)
BBR28 lipoprotein (*lp*)	**CE**	**9.63**	**0.00599**	Brooks *et al*. (2003); Tokarz *et al*. (2004)
BBR36 conserved hypothetical protein	HX	11.1	0.0475	Tokarz *et al*. (2004)
BBR42 outer surface protein F (*ospF*)[b]	**CE**	**151**	**0.0336**	Ojaimi *et al*., 2003; Yang *et al*., 2003a; Caimano *et al*. (2007)
(b) *Borrelia burgdorferi* genes repressed by Rrp1				
BB0022 Holliday junction DNA helicase (*ruvB*)	R	15.0	0.0108	
BB0340 hypothetical protein	U	16.0	0.0192	
BB0398 lipoprotein, putative	U	12.3	0.0239	
BB0470 hypothetical protein	U	14.4	0.0124	
BB0509 hypothetical protein	U	4.98	3.73E-05	
BB0510 hypothetical protein	U	126	0.0129	
BB0741 chaperonin (*groES*)	HS	4.96	0.0440	
BB0794 hypothetical protein	U	82.1	0.0410	
BBO05 hypothetical protein	U	4.06	0.0254	

[a] As published in TIGR *B. burgdorferi* B31MI genome database and in Fraser *et al*. (1997).

[b] Genes differentially expressed that were validated by qRT-PCR and/or western blotting are bolded.

[c] M, mutant mRNA hybridization intensity values; WT, wild-type mRNA hybridization intensity values.

[d] Only genes that pass the statistical criteria of $\log_2(M/WT) < -2$ or > 2 and $P < 0.05$ are listed.

Table 2

Quantitative RT-PCR analysis of select genes.

Gene	Annotation	Array	qRT-PCR
BB0167	Outer membrane protein (*tnp50*)	29.8	6.2
BB0241	Glycerol kinase (*glpK*)	5.4	2.9
BB0243	Glycerol-3-phosphate dehydrogenase (*glpA*)	21.9	11.3
BB0367	Hypothetical protein	18.8	10.0
BB0420	Sensory transduction histidine kinase (*hpk1*)	236.0	1.3
BB0596	Methyl-accepting chemotaxis protein (*mcp-2*)	240.0	1.3
BB0629	Fructose-specific IIABC component (*fruA-2*)	20.1	2.9
BB0683	3-Hydroxyl-3-methylglutaryl-CoA synthase	14.7	2.7
BB0687	Phosphomevalonate kinase	5.3	1.1
BB0769	Sialoglycoprotease (*gcp*)	84.2	1.5
BB0785	Stage V sporulation protein G	11.2	3.5
BBA30	Hypothetical protein	157.0	4.9
BBA74	Periplasmic protein	159.0	4.5
BBB16	Oligopeptide ABC transporter (*oppAIV*)	17.0	6.0
BBB17	IMP dehydrogenase (*guaB*)	6.9	2.1
BBB18	GMP synthase (*guaA*)	8.3	2.1
BBB19	Outer surface protein C (*ospC*)	33.9	3.7
BBB29	Maltose-glucose-specific IIABC component	25.7	3.2
BBH06	Factor H-binding protein (*cspZ*)	159.0	3.4
BBR28	Lipoprotein	9.6	2.1

Values expressed as a ratio of expression for the given gene in the *Bb*5A13-wt relative to *Bb*5A13-Δ*rrp1*.

Table 3

Genes regulated by Rrp1, Rrp2 and RpoS.[a]

Genes induced by Rrp1, Rrp2 and RpoS	
BBB19	Outer surface protein C (*ospC*)
BBR36	Conserved hypothetical protein
BBR42	Outer surface protein F (*ospF*)
Genes induced by Rrp1 and Rrp2	
BBH06	Factor H-binding protein (*cspZ*)
BBL39/BBP38	Factor H-binding protein (*ospE*)[b]
BBO39	Outer surface protein F (*ospF*)[b]
Genes induced by Rrp1 and RpoS	
BB0021	*S*-adenosylmethionine: tRNA ribosyltransferase-isomerase
BB0250	dedA protein (*dedA*)
BB0567	Chemotaxis histidine kinase (*cheA*-1)[c]
BB0578	Methyl-accepting chemotaxis protein (*mcp-1*)[c]
BB0588	5′-Methylthioadenosine/*S*-adenosylhomocysteine nucleosidase
BB0648	Serine/threonine kinase, putative
BBB14	Hypothetical protein
BBN38	Factor H-binding protein (*ospE*)[c]
BBO28	Mlp lipoprotein[c]
Genes induced by Rrp1 but repressed by RpoS	
BB0241	Glycerol kinase (*glpK*)
BB0242	Hypothetical protein
BB0243	Glycerol-3-phosphate dehydrogenase, anaerobic (*glpA*)
BB0785	Stage V sporulation protein G
BBA69	Hypothetical protein
BBA74	Membrane-associated periplasmic protein
BBI29	Hypothetical protein
BBI38	Hypothetical protein
BBK19	Hypothetical protein
Genes induced by Rrp1 but repressed by Rrp2	
BB0048	Hypothetical protein
BB0160	Alanine racemase
BB0180	Flagellar protein, putative

[a] Based on Caimano *et al*. (2007) and Boardman *et al*. (2008).

[b] These genes did not appear in the Rrp1 arrays, but protein levels were downregulated in the *rrp1* knockout as shown by western blot analysis.

[c] Genes in these protein families are also regulated by Rrp2 [ex: OspE (BBL39), Mlp (BBM28 and BBP28), chemotaxis (*mcp-4*, *mcp-5*, *cheR-1*)].

Table 4

Borrelia isolates used in this study.

Species/isolate	Geographic origin	Biological source
B. burgdorferi		
297	Connecticut	Human CSF
3028	Texas	Human skin
5A4	New York	B31 derivative, infectious
5A13	New York	B31 derivative, non-infectious
B31	New York	*Ixodes scapularis*
B348	New York	Human skin
BL224	New York	Human blood
CA3, CA8, CA9	California	*Ixodes pacificus*
JD1	Massachusetts	*Ixodes scapularis*
LP4, LP5, LP7	Connecticut	Human EM
Mac13NY, N40	New York	Human EM
T2	USA	*Ixodes scapularis*
Veery	Connecticut	Veery bird
B. afzelii		
ECM1	Sweden	Human skin
IP21	Russia	*Ixodes persulcatus*
PGau	Germany	Human ACA
PKo	Germany	Human skin
UM01, U01	Sweden	Human skin
VS461	Switzerland	*Ixodes scapularis*
B. garinii		
20047	France	*Ixodes ricinus*
B-4/87, B-6/91	Norway	*Ixodes ricinus*
FRG	Germany	*Ixodes ricinus*
G1, G2	Germany	Human CSF
IP89	Russia	*Ixodes persulcatus*
PBi	Germany	*Ixodes ricinus*
VSBP	Switzerland	*Ixodes ricinus*
B. turicatae		
OZ1, PE-926	Texas	*Ornithodoros turicata*
B. hermsii		
MAN	California	Human blood
DAH	Washington	Human blood
B. coriaceae	California	*Ornithodoros coriaceus*

CSF, cerebral spinal fluid; EM, erythema migrans; ACA, acrodermatitis chronica atrophicans.