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Identification and function of the RNA chaperone Hfq in the Lyme disease spirochete *Borrelia burgdorferi*

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Summary

Hfq is a global regulatory RNA-binding protein. We have identified and characterized an atypical Hfq required for gene regulation and infectivity in the Lyme disease spirochete *Borrelia burgdorferi*. Sequence analyses of the putative *B. burgdorferi* Hfq protein revealed only a modest level of similarity with the Hfq from *Escherichia coli*, although a few key residues are retained and the predicted tertiary structure is similar. Several lines of evidence suggest that the *B. burgdorferi bb0268* gene encodes a functional Hfq homolog. First, the hfq_{Bb} gene (*bb0268*) restores the efficient translation of an *rpoS::lacZ* fusion in an *E. coli hfq* null mutant. Second, the Hfq from *B. burgdorferi* binds to the small RNA $DsrA_{Bb}$ and the *rpoS* mRNA. Third, a *B. burgdorferi hfq* null mutant was generated and has a pleiotropic phenotype that includes increased cell length and decreased growth rate, as found in *hfq* mutants in other bacteria. The hfq_{Bb} mutant phenotype is complemented in *trans* with the *hfq* gene from either *B. burgdorferi* or, surprisingly, *E. coli*. This is the first example of a heterologous bacterial gene complementing a *B. burgdorferi* mutant. The alternative sigma factor RpoS and the outer membrane lipoprotein OspC, which are induced by increased temperature and required for mammalian infection, are not upregulated in the *hfq* mutant. Consequently, the *hfq* mutant is not infectious by needle inoculation in the murine model. These data suggest that Hfq plays a key role in the regulation of pathogenicity factors in *B. burgdorferi* and we hypothesize that the spirochete has a complex Hfq-dependent sRNA network.

Keywords

Hfq; RpoS; DsrA; OspC; sRNA

Introduction

Borrelia burgdorferi is the causative agent of Lyme disease (Burgdorfer *et al.*, 1982; Benach *et al.*, 1983; Steere *et al.*, 1983; Radolf *et al.*, 2010). The spirochete cycles between a tick vector and a vertebrate host (Lane *et al.*, 1991; Spielman, 1994; Piesman and Schwan, 2010), altering its gene expression to transition between and survive within these two vastly different environments (Singh and Girschick, 2004; Samuels and Radolf, 2009; Skare *et al.*, 2010). The increase in temperature associated with transmission from the tick to the mammal is one signal known to increase synthesis of outer surface lipoprotein C (OspC) and other virulence factors (Schwan *et al.*, 1995; Stevenson *et al.*, 1995; Fingerle *et al.*, 2000; Yang *et al.*, 2000; Revel *et al.*, 2002; Alverson *et al.*, 2003; Ojaimi *et al.*, 2003). OspC synthesis depends on the alternative sigma factor RpoS (σ^S or σ^{38}), which has emerged as a

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global regulator of the enzootic cycle (Hübner *et al.*, 2001; Caimano *et al.*, 2004; Burtnick *et al.*, 2007; Caimano *et al.*, 2007) and is required for infection in the murine model of Lyme disease (Caimano *et al.*, 2004; Blevins *et al.*, 2009). The response regulator Rrp2 and the alternative sigma factor RpoN regulate the transcription of *rpoS* in response to an unknown environmental signal(s) (Hübner *et al.*, 2001; Yang *et al.*, 2003), while post-transcriptional temperature-dependent RpoS synthesis is regulated by the small RNA (sRNA) $DsrA_{Bb}$ (Lybecker and Samuels, 2007), the only sRNA identified to date in *B. burgdorferi*.

Recently, sRNAs have emerged as major regulators in the expression of many bacterial genes, including those involved in virulence and the stress response (Lease and Belfort, 2000; Hengge-Aronis, 2002; Repoila *et al.*, 2003; Gottesman, 2004; Storz *et al.*, 2004; Majdalani *et al.*, 2005; Narberhaus *et al.*, 2006; Serganov and Patel, 2007; Fröhlich and Vogel, 2009; Klinkert and Narberhaus, 2009). Many sRNAs post-transcriptionally regulate gene expression by base pairing with target *trans*-encoded mRNAs, affecting either their translation or stability (Repoila *et al.*, 2003; Gottesman, 2004; Majdalani *et al.*, 2005). In *Escherichia coli*, the sRNA DsrA base pairs with the *rpoS* mRNA upstream region, releasing the Shine-Dalgarno sequence and start site from a stem-loop that inhibits translation. In *B. burgdorferi*, $DsrA_{Bb}$ and the upstream region of the *rpoS* transcript have extensive complementarity, suggesting a similar mechanism of translational regulation (Lybecker and Samuels, 2007). Most *trans*-acting antisense sRNAs require the RNA chaperone Hfq (Massé *et al.*, 2003b; Gottesman, 2004; Storz *et al.*, 2004; Valentin-Hansen *et al.*, 2004; Majdalani *et al.*, 2005; Romby *et al.*, 2006; Brennan and Link, 2007; Waters and Storz, 2009). Hfq is a highly conserved RNA-binding protein that was first identified in *E. coli* as a host factor required for Qβ bacteriophage replication (Franze de Fernandez *et al.*, 1968). Hfq is the bacterial homolog of the eukaryotic and archaeal Sm and LSm proteins (Arluison *et al.*, 2002; Möller *et al.*, 2002; Schumacher *et al.*, 2002; Sun *et al.*, 2002; Zhang *et al.*, 2002; Sauter *et al.*, 2003). Sm and LSm proteins are involved in various aspects of RNA metabolism, including processing of nuclear RNA, RNA localization, and mRNA decay. The Sm and LSm proteins are characterized by two highly conserved motifs, Sm1 and Sm2, and form cyclic ring-shaped oligomers (Achsel *et al.*, 1999; Kambach *et al.*, 1999; Achsel *et al.*, 2001). The Hfq protein is an oligomeric torus, like Sm and LSm (Törö *et al.*, 2001; Törö *et al.*, 2002). Whereas Sm and LSm proteins form heptameric structures that are homomeric in archaea and heteromeric in eukaryotes, most Hfq homologs are found as homohexamers (reviewed in Brennan and Link, 2007).

Hfq hexamers have two independent RNA-binding surfaces that have different specificities. The proximal surface was defined based on the *Staphylococcus aureus* crystal structure and includes the Sm2 motif (Schumacher *et al.*, 2002). RNAs that bind this surface typically have AU-rich single-stranded sequences either just 3′ or just 5′ of a stem-loop (Brescia *et al.*, 2003; Moll *et al.*, 2003b; Geissmann and Touati, 2004; Večerek *et al.*, 2005). These RNAs bind by circumnavigating the proximal face of the central cavity. The distal face of Hfq, on the opposite side of the torus, also binds RNAs (Mikulecky *et al.*, 2004). This surface, which has been characterized in a recent X-ray crystal structure, is typically associated with poly(A) and A-rich structures (Link *et al.*, 2009). The crystal structure predicts that the distal surface has specificity for purine-rich repeats with the sequence $(ARN)_{2–5}$. However, several other secondary structural regions have been identified as Hfq-binding sites, including hairpin motifs, a loop region connecting two stems in a pseudoknot structure, and the T- and D-stems of tRNA (Antal *et al.*, 2005; Lee and Feig, 2008).

Hfq functions as an RNA chaperone by facilitating the interaction of an sRNA with its target mRNA via colocalizing the RNAs and/or altering the structure of the RNAs (Sledjeski *et al.*, 2001; Massé and Gottesman, 2002; Møller *et al.*, 2002; Zhang *et al.*, 2002; Kawamoto *et al.*, 2006; Večerek *et al.*, 2007). Hfq protects sRNAs from degradation likely by blocking the

RNase E binding site on the sRNA (Massé *et al.*, 2003a; Moll *et al.*, 2003a). Hfq also functions in mRNA turnover by stabilizing an mRNA, stimulating turnover of the mRNA by promoting polyadenylation, or facilitating base pairing between an sRNA and its target RNA resulting in cleavage of both RNAs by RNase E (Tsui *et al.*, 1997; Vytvytska *et al.*, 2000; Folichon *et al.*, 2003).

An *E. coli hfq* null mutant has a pleiotropic phenotype that includes decreased growth rate, increased cell length, and increased sensitivity to stress conditions (Tsui *et al.*, 1994; Muffler *et al.*, 1997). Hfq is required for post-transcriptional regulation of *rpoS* and, therefore, the syntheses of more than 50 proteins are affected by *hfq* mutations (Muffler *et al.*, 1996; Brown and Elliott, 1997). Moreover, Hfq plays an important role in several pathogenic bacteria: *hfq* mutations either attenuate or abolish virulence (reviewed in Chao and Vogel, 2010). Hfq is largely conserved in bacteria (Sun *et al.*, 2002); however, *B. burgdorferi* lacks an annotated *hfq* gene in its genome (Fraser *et al.*, 1997) and BLAST searches using sequences of conserved *hfq* genes do not reveal a homolog. We now identify and characterize an unusual Hfq in *B. burgdorferi* that regulates RpoS and is required for murine infection by needle inoculation.

Results

Identification of Hfq in *B. burgdorferi*

The RNA chaperone Hfq is typically required for RNA-RNA interactions between small regulatory RNAs and their cognate target mRNAs. After identifying and characterizing the first sRNA, $DsrA_{Bb}$, in *B. burgdorferi*, we hypothesized that the spirochete has an Hfq despite the lack of an annotated, or readily identifiable, homolog in its genome. This was supported by an MFOLD (Zuker, 2003) secondary structure prediction of $DsrA_{Bb}$ that revealed a putative Hfq-binding site, an AU stretch of residues situated between two stable stem loops (Fig. 1). BLAST searches of the *B. burdorferi* genome were performed using as queries the Hfq sequences from *E. coli* and *S. aureus*, as well as just the highly conserved N-terminal 68 amino acids of *E. coli* Hfq. No homologous sequences were found in *B. burgdorferi* (data not shown). We next searched the genome for small ORFs that had the potential to form Sm1 and Sm2 motifs, and we identified a conserved hypothetical ORF encoded by the *bb0268* gene, which was predicted to have 33% similarity and 12% identity with *E. coli* Hfq. A ClustalW alignment revealed that several important amino acids are conserved and a Phyre prediction suggested that this ORF could fold into an Hfq-like structure (Fig. 2). The residues comprising the nucleotide-binding pocket of Hfq, in the *S. aureus* Hfq-RNA complex (Schumacher *et al.*, 2002), are conserved or similar in the predicted BB0268 protein. Other Hfq motifs (Sm1 NG and Sm2 F/YKHA) are partially conserved in BB0268. However, the BB0268 protein (159 amino acids) is considerably larger than most Hfq proteins (typically 77 to 110 amino acids). The N-terminal 68 amino acids of known Hfq homologs are highly conserved and include the Sm motifs, while the C-terminal portions of the proteins vary in length and sequence (Sauter *et al.*, 2003). The ClustalW alignment demonstrated that the BB0268 sequence is most similar to the N-terminal domain of the canonical *E. coli* Hfq protein, but BB0268 has a longer C-terminal tail with a divergent sequence (Fig. 2A).

B. burgdorferi bb0268 complements an *E. coli hfq* mutant

In *E. coli*, Hfq is required for DsrA to stimulate translation of *rpoS* in response to a decrease in temperature and entrance into stationary phase (Sledjeski *et al.*, 2001). Evidence suggests that Hfq colocalizes *rpoS* and DsrA, presenting the two RNAs in favorable positions for complex formation (Brescia *et al.*, 2003; Lease and Woodson, 2004). To determine if BB0268 functions as an RNA chaperone, similar to Hfq in *E. coli*, we assayed its ability to

trans-complement an *hfq* null mutant of *E. coli* (Sledjeski *et al.*, 2001). The *bb0268* gene was fused to an arabinose-inducible promoter in the vector pBAD24. The empty pBAD24 vector and conserved hypothetical ORF *bb0260*, which shares sequence similarity with *bb0268*, were used as negative controls; *E. coli hfq* was used as a positive control. Hfq is needed for growth-phase related regulation of *rpoS::lacZ* at 30°C in *E. coli*. The efficient translation of an *rpoS::lacZ* fusion, in response to changes in cell density, was measured with a β-galactosidase assay. *bb0268* partially restored the efficient translation of *rpoS::lacZ* at 30°C (Fig. 3), suggesting that it has an Hfq-like RNA chaperone activity in *E. coli*. In contrast, neither *bb0260* nor the empty vector complemented the *hfq* null mutant in *E. coli*. Five independent experiments with *bb0268* yielded similar results. Therefore, we now refer to BB0268 as Hfq_{Bb}.

Recombinant Hfq_{Bb} binds *rpoS* mRNA and DsrA

The Hfq and Sm/Lsm family of proteins form homohexameric and heteroheptameric oligomers (Tsui *et al.*, 1994; Sun *et al.*, 2002; Valentin-Hansen *et al.*, 2004). We purified recombinant Hfq_{Bb} using an N-terminal His-tag. The predicted molecular mass of Hfq_{Bb} is ~20 kDa (with the His-tag) and gel filtration chromatography indicated that it has a molecular mass of ~34 kDa in solution (Fig. 4), suggesting that Hfq_{Bb} forms a dimer or a monomer, rather than the characteristic hexamer, under these *in vitro* conditions. In contrast, *E. coli* Hfq was primarily a hexamer as analyzed by gel filtration chromatography under the same conditions, although monomer was also observed in this experiment (Fig. 4C).

Electrophoretic mobility shift assays (EMSAs) were performed using recombinant Hfq_{Bb} protein and *in vitro*-transcribed *rpoS* and $DsrA_{Bb}$ RNAs (Fig. 5). The RNAs were heated to 65°C for 10 min and slow-cooled to room temperature. The RNAs were then incubated with increasing amounts of Hfq_{Bb} protein. The Hfq_{Bb} protein bound both *rpoS* and $DsrA_{Bb}$ as seen by the decreased migration of the RNAs in the EMSA (Fig. 5).

Construction and complementation of the *hfq_{Bb}* mutant

To determine the function of Hfq_{Bb} in *B. burgdorferi*, we deleted the *hfq_{Bb}* gene in a low-passage infectious clone of strain 297 (Fig. 6A). Two different plasmids were constructed to complement the *hfq_{Bb}* mutation. The *hfq_{Bb}* (*bb0268*) and the *E. coli hfq* ORFs were fused to the inducible *flac* promoter and inserted into the shuttle vector pKFSS1-*lacI*, which carries the *lacI* gene fused to the constitutive *flgB* promoter (Fig. 6B). The two complementing plasmids and the parental shuttle vector were transformed into the *hfq_{Bb}* mutant strain. RT-PCR analysis demonstrated that the wild-type and *hfq_{Bb}*-complemented strains, but not the *hfq_{Bb}* mutant strain, expressed *hfq_{Bb}* transcript (Fig. 6C).

The *hfq_{Bb}* mutant has a decreased growth rate and increased cell length

We assayed growth rate using a wild-type low-passage infectious clone of 297, the *hfq_{Bb}* mutant, and the two complemented strains. *B. burgdorferi* cultures were grown at 23°C to a low cell density ($1–3 \times 10^7$ cells ml^{-1}) and then inoculated into a culture at 37°C at a cell density of 1×10^3 cells ml^{-1}. Cell densities were determined daily until cultures reached stationary phase. The *hfq_{Bb}* mutant grew slower than the wild type; both the *B. burgdorferi* and *E. coli hfq* genes complemented the growth phenotype of the *hfq_{Bb}* mutant (Fig. 7). The *hfq_{Bb}* mutant strain also was significantly longer than wild type. The cell length phenotype was fully complemented by the *hfq_{Bb}* gene expressed in *trans* and, somewhat unexpectedly, partially complemented by the *E. coli hfq* gene (Fig. 8).

RpoS and OspC are regulated by Hfq_{Bb}

$DsrA_{Bb}$ post-transcriptionally regulates RpoS levels in response to increased temperature at low cell density (Lybecker and Samuels, 2007). This sRNA has extensive complementarity to the upstream region of the *rpoS* mRNA and our data suggest that DsrA stimulates efficient translation of the *rpoS* mRNA through base pairing with the upstream region of the mRNA. Hfq in other bacteria is required for regulating *trans*-encoded sRNAs and often post-transcriptionally regulates RpoS levels, so we expected that the hfq_{Bb} mutant strain would show reduced levels of RpoS and OspC proteins in response to a temperature shift at a low cell density, as seen in the $dsrA_{Bb}$ mutant strains. *B. burgdorferi* low-passage wild-type, hfq_{Bb} mutant, and complemented strains were temperature-shifted to 37°C and grown to low and high cell densities ($1–3 \times 10^7$ and 1×10^8 cells ml^{-1}, respectively). Western blot analyses of whole cell lysates demonstrated that the wild-type and complemented strains displayed an increase in RpoS and OspC levels after a temperature shift, while the hfq_{Bb} mutant strain showed reduced levels of RpoS and OspC (Fig. 9A). Surprisingly, the *E. coli hfq* also restored the temperature-dependent increase in RpoS and OspC. The inducible promoter fusions are leaky in these *trans*-acting configurations (Fig. 6C and data not shown), so IPTG was not required for transcription of the complementing *hfq* genes. Adding high levels of IPTG caused decreases in RpoS and OspC levels, presumably due to the overabundance of Hfq inhibiting translation (data not shown).

Transcription of *rpoS* requires RpoN and Rrp2 (Hübner *et al.*, 2001; Yang *et al.*, 2003). To further understand the mechanism by which Hfq_{Bb} regulates RpoS, the steady-state levels of *rpoS, rpoN*, and *rrp2* mRNA were analyzed by Northern blot analyses (Fig. 9B). The constitutively expressed *flaA* transcript was used as a gel loading control. The amounts of the *rpoN* and *rrp2* transcripts were similar between the wild-type, mutant and complemented strains demonstrating that Hfq does not regulate the levels of *rpoN* or *rrp2* mRNA. However, the levels of *rpoS* transcript were 1.5-fold higher in the hfq_{Bb} mutant strain compared to the wild-type and complemented strains; this pattern of regulation was also observed in the $dsrA_{Bb}$ mutant strain (Lybecker and Samuels, 2007). These data, taken together, suggest that Hfq_{Bb} regulates RpoS synthesis post-transcriptionally and does not stabilize the *rpoS* mRNA.

As previously seen with the $dsrA_{Bb}$ mutant (Lybecker and Samuels, 2007), serial passage of the hfq_{Bb} mutant results in a reversal of the phenotype. *B. burgdorferi* cultures were serially passaged at 23°C and then temperature-shifted to 37°C and grown to a low cell density. Western blot analyses demonstrated that both RpoS and OspC syntheses were increased, rather than decreased, in the mutant compared to the wild type after as few as six passages (data not shown). The mechanism by which the phenotype switches is not yet understood.

Hfq_{Bb} is required for mouse infection by needle inoculation

Several bacterial species require Hfq for virulence (Chao and Vogel, 2010). We hypothesized that the hfq_{Bb} mutant strain would not be infectious in mice due to the absence of RpoS and OspC. We assayed the infectivity of the hfq_{Bb} mutant in the mouse model of Lyme disease by needle inoculation (Barthold *et al.*, 2010). *B. burgdorferi* cultures were temperature-shifted to 37°C and grown to a low cell density ($1–3 \times 10^7$ cells ml^{-1}); C3H-HeJ female mice were needle inoculated with 5×10^3 cells i.p. Tissue samples were cultured and assayed for the presence of *B. burgdorferi* by dark-field microscopy; cultures that were negative by this method were also screened by PCR (Wang *et al.*, 2010). The hfq_{Bb} mutant was unable to infect mice and complementation with *hfq* from *B. burgdorferi*, but not *E. coli*, restored infectivity (Table 1). Thus, hfq_{Bb} is essential for *B. burgdorferi* infection of mice by needle inoculation.

The high-passage *hfq*$_{Bb}$ mutant retained the plasmids essential for infectivity and was used to needle inoculate mice. RpoS and OspC are highly expressed in the high-passage *hfq*$_{Bb}$ mutant, so we expected it to be infectious, but the strain did not infect the mice (Table 1). These data and the finding that the *hfq*$_{Bb}$ mutant complemented with *hfq* from *E. coli* was avirulent, even though it expressed wild-type levels of RpoS, suggest Hfq$_{Bb}$ may play additional roles in mammalian infection besides regulation of RpoS.

Discussion

The RNA chaperone Hfq has emerged as an important global post-transcriptional regulator in mRNA translational control by sRNAs (Valentin-Hansen *et al.*, 2004). In this study, we identified and characterized an unusual Hfq in *B. burgdorferi*. The Hfq$_{Bb}$ primary sequence does not have a high degree of similarity or identity to annotated Hfq homologs, but does retain a few key residues and was predicted to have a superimposable tertiary structure (Fig. 2). A BLAST search using full-length Hfq$_{Bb}$, or the more highly conserved N-terminal region of Hfq$_{Bb}$, as the query only identified hypothetical open reading frames within the spirochete phylum (data not shown). Hfq is characterized as a part of the Sm/Lsm protein family and these proteins form ring-shaped oligomeric structures that are homohexameric in bacteria and heteroheptameric in eukaryotes. Despite these differences, the Hfq and Sm/Lsm monomers fold into a similar structure, consisting of an N-terminal α-helix followed by a curved five-stranded β-sheet (Schumacher *et al.*, 2002; Sauter *et al.*, 2003). Our gel filtration data suggested that recombinant Hfq$_{Bb}$ does not form a stable hexamer in solution (Fig. 4), which is unlike most other characterized Hfq homologs. However, we hypothesize that the functional quaternary structure of Hfq$_{Bb}$ *in vivo* is a homohexamer, which is unstable under our experimental conditions. Possibly, our inability to observe a hexamer *in vitro* is due to the presence of a lysine residue rather than the conserved histidine at position 57 (in the *E. coli* sequence), which is known to be involved in stabilizing the hexameric structure (Moskaleva *et al.*, 2010). Despite the differences in primary and quaternary structure between Hfq$_{Bb}$ and the canonical Hfq, our results clearly establish that Hfq$_{Bb}$ functions as an RNA chaperone as demonstrated by *hfq*$_{Bb}$ complementing an *rpoS* translational defect in an *hfq* null mutant of *E. coli* (Fig. 3). Moreover, *E. coli hfq* complemented the *hfq*$_{Bb}$ mutant phenotype with regard to growth rate (Fig. 7), cell length (Fig. 8), and RpoS and OspC induction (Fig. 9). This is the first time, to our knowledge, that a heterologous bacterial gene has complemented a *B. burgdorferi* mutant.

The *hfq*$_{Bb}$ mutant has a phenotype that includes decreased growth rate, increased cell length, altered RpoS regulation, and loss of infectivity. The *B. burgdorferi* and *E. coli hfq* genes complemented the *in vitro* mutant phenotype demonstrating that the phenotype is due to the loss of *hfq*$_{Bb}$; these phenotypes have been observed in several *hfq* mutants of other bacteria (Brennan and Link, 2007; Chao and Vogel, 2010). RpoS is required for mammalian infection by *B. burgdorferi*, and, therefore, the *hfq*$_{Bb}$ mutant was not expected to be infectious in the mouse model. Our findings established that Hfq$_{Bb}$ is required for mammalian infectivity, but that the mechanism is not solely due to the loss of RpoS induction. A high-passage *hfq*$_{Bb}$ mutant strain that produces RpoS and OspC was still not infectious in mice (Table 1). These data demonstrated that Hfq$_{Bb}$ is required for infectivity in mice independent of RpoS and OspC expression. We hypothesize that there are other Hfq-dependent sRNAs in *B. burgdorferi* that regulate expression of virulence factors. In *E. coli* and *Salmonella typhimurium*, Hfq has other regulatory functions independent of its effects on RpoS expression (Muffler *et al.*, 1997; Sittka *et al.*, 2007). Hfq in the pathogenic bacteria *S. typhimurium*, *Vibrio cholerae*, and *Brucella abortus* is required for full virulence, but the mechanism is independent of the requirement of Hfq for RpoS expression (Robertson and Roop, 1999; Ding *et al.*, 2004; Sittka *et al.*, 2007).

We hypothesized that Hfq_{Bb} regulates RpoS synthesis via the sRNA $DsrA_{Bb}$ and, thus, we expected the RpoS defect would be similar in the *dsrA*$_{Bb}$ mutant and *hfq*$_{Bb}$ mutant strains. Our data demonstrated that Hfq_{Bb} post-transcriptionally regulates the temperature-dependent increase in RpoS. Surprisingly, unlike the *dsrA*$_{Bb}$ mutant strain, which only shows reduced levels of RpoS at a low cell density (log-phase growth), the *hfq*$_{Bb}$ mutant strain had reduced levels of RpoS at both low and high cell density (log and stationary growth phase, respectively), suggesting that Hfq_{Bb} also plays a $DsrA_{Bb}$-independent role in RpoS regulation.

In conclusion, we have identified and characterized an unusual Hfq protein in the Lyme disease spirochete *B. burgdorferi* that is essential for infectivity. Although only one sRNA has been identified to date in *B. burgdorferi*, the pleiotropic phenotype of the *hfq*$_{Bb}$ mutant suggests other sRNAs exist and likely play a role in the enzootic life cycle.

Experimental procedures

B. burgdorferi strains and culture conditions

B. burgdorferi low-passage virulent strain 297 (clone BbAH130) (Hübner *et al.*, 2001) and all derivatives used in this study were cultivated in Barbour-Stoenner-Kelly II (BSK-II) complete medium (Barbour, 1984) at either 23°C or 37°C. Cultures were passaged to 1×10^5 cells ml^{-1} from 23°C to 37°C and grown to either $1–3 \times 10^7$ cells ml^{-1} (low density), which corresponds to mid-log phase, or about 1×10^8 cells ml^{-1} (high density), which corresponds to stationary phase. For growth curves, cultures were grown at 23°C to $1–3 \times 10^7$ cells ml^{-1} and passaged to 1×10^3 cells ml^{-1} at 37°C. Cell density was determined using a Petroff Hausser counting chamber (Hausser Scientific Partnership) as previously described (Samuels, 1995). All cultures were counted in triplicate and at least three independent experiments were performed.

Sequence analysis and structural modeling

The ClustalW sequence alignment was performed with NPS@: Network Protein Sequence Analysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html) (Combet *et al.*, 2000). A computational prediction of the Hfq_{Bb} fold was performed using Protein Homology/analogY Recognition Engine (Phyre) (http://www.sbg.bio.ic.ac.uk/~phyre/) (Kelley and Sternberg, 2009). The resulting PDB coordinate file covering residues 6–71 was then visualized and overlaid with the structures of Hfq from *E. coli* (PDB 1HK9) (Sauter *et al.*, 2003) and Hfq from *S. aureus* (PDB 1KQ2) (Schumacher *et al.*, 2002) using Chimera (http://www.cgl.ucsf.edu/chimera/) (Pettersen *et al.*, 2004).

E. coli strains and plasmid construction for *hfq* complementation

E. coli strain DDS1631 has an *hfq* null mutation and an *rpoS::lacZ* translational fusion (Sledjeski *et al.*, 2001). The pBADHfq plasmid (pDDS400) carries the *E. coli hfq* gene cloned into the EcoRI and PstI sites of the arabinose-inducible vector pBAD24 (Sledjeski *et al.*, 2001). The *bb0268* and *bb0260* genes were PCR-amplified using the proofreading enzyme KOD with engineered MfeI and PstI sites at the 5′ and 3′ ends, respectively (Table 2). After poly(A) tailing with Taq, the PCR products were cloned into pCR®2.1-TOPO. Positive transformants were screened by PCR and confirmed by DNA sequencing. The pBAD260 and pBAD268 plasmids were generated by directionally cloning the MfeI-PstI excised *bb0268* and *bb0260* fragments into the EcoRI and PstI sites of pBAD24.

E. coli Hfq complementation

β-galactosidase units were assayed using the Zhang and Bremer (1995) modification of the Miller assay (1972). Briefly, cells were grown with shaking at 30°C in lysogeny broth medium supplied with kanamycin and carbomycin in the presence or absence of 150 μM arabinose. The cultures were inoculated 1:100 from overnight cultures and samples were taken at an OD_{600} ranging from 0.4 to 1.0 (approximately log phase). The OD_{600} of each culture was measured and 30μl aliquots were removed and mixed with 70 μl of permeabilization solution [100 mM Na_2HPO_4, 20 mM KCl, 2 mM $MgSO_4$, 0.8 mg ml^{-1} CTAB (hexadecyltrimethylammonium), 0.4 mg ml^{-1} sodium deoxycholate and 5.4 μl ml^{-1} 2- mercaptoethanol] and stored at room temperature. The samples were incubated at 30°C for 30 min. The substrate solution [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg ml^{-1} *o*-nitrophenyl-β-D-galactoside (ONPG) and 2.7 μl ml^{-1} 2-mercaptoethanol] was also incubated at 30°C for 30 min and 600 μl of substrate solution was added to each sample. After sufficient color developed, 700 μl of stop solution (1 M Na_2CO_3) was added to each sample and the time was recorded. The samples were centrifuged at 14,000 rpm in an Eppendorf 5417R for 5 min to remove cell particulates. The OD_{420} (between 0.05 and 1.0) was measured for each sample and the total β-galactosidase units were calculated from the formula:

$$1000 \times (OD_{420}) \div [(OD_{600} \text{ of culture}) \times (\text{volume of culture}) \times (\text{reaction time})]$$

The volumes of permeabilization solution and cell culture varied between experiments and were appropriately changed in the calculation of total β-galactosidase units.

Cloning and protein purification of Hfq

The hfq_{Bb} gene was amplified by PCR with the proofreading enzyme KOD, poly(A) tailed, and cloned into pCR®2.1-TOPO (Table 2). The hfq_{Bb} gene was directionally cloned into a derivative of pET28b with an N-terminal, tobacco etch virus protease-cleavable His_6 tag (Corbett *et al.*, 2004) using an engineered NdeI site at the 5′ end of the gene and the pCR®2.1-TOPO vector BamHI site. The resulting plasmid pET28b-TEV::Hfq was sequenced and transformed into *E. coli* Rosetta (DE3) cells (Novagen). Cell cultures were grown at 37°C to an OD_{600} of 0.4–0.6, induced with 1 mM of IPTG and grown overnight at 11°C and again overnight at 18°C. Cells were lysed using Bugbuster (Novagen), filtered through a 0.45 μm filter and loaded directly onto 1 ml Ni^{2+} column (HisTrap HP, Amersham Biosciences). The resin was washed with ten column volumes of 20 mM NaH_2PO_4, 500 mM NaCl and 5 mM imidazole, pH 7.4, five column volumes of 1M NH_4Cl, and five column volumes of 1M urea, and finally eluted with a 10-ml linear gradient of 10 to 500 mM imidazole, pH 7.4, in 20 mM NaH_2PO_4 and 500 mM NaCl. The fractions containing Hfq were dialyzed into a storage buffer (20 mM NaH_2PO_4 and 125 mM NaCl, pH 7.4) and concentrated in Amicon Ultra-4 spin columns. The concentration of the purified recombinant protein was determined with the BCA protein assay (Pierce). The *E. coli* Hfq protein was purified as previously described (Mikulecky *et al.*, 2004).

Gel filtration

Recombinant purified Hfq_{Bb} and *E. coli* Hfq were dialyzed overnight in 50 mM Tris-HCl, pH 7.5 and 100 mM KCl. Sigma gel filtration molecular weight markers were run on a Superdex 75 HR 10/30 column and a standard curve was plotted. The Hfq proteins were run on the column and the molecular mass was calculated using the standard curve.

Template preparation for *in vitro* transcription

B. burgdorferi strain 297 total DNA was used as a template in PCR with the T7 bacteriophage promoter added to the 5′ end of either the forward primer to generate sense RNAs for the EMSA or the reverse primer to generate antisense RNAs for probes in Northern blots (Table 2). The PCR products were visualized on an agarose gel stained with ethidium bromide. If a single product was visualized, the PCR product was precipitated with ammonium acetate and resuspended in DEPC-treated water. If more than one product was visible, the appropriate band was purified with the Qiagen gel purification kit following the manufacturer's protocol and resuspended in DEPC-treated water.

In vitro synthesized RNA

$DsrA_{Bb}$ and *rpoS* RNAs for EMSAs were synthesized and radioactively labeled by incorporation of [α-^{32}P]UTP during *in vitro* transcription using the T7-Megascript In Vitro Transcription kit (Ambion) according to the protocol suggested by the manufacturer. PCR products (described above) were used as templates for the *in vitro* transcription reactions. Reaction time was increased to overnight at 37°C to increase the yield of short products. Following *in vitro* transcription, the full-length RNA transcripts were resolved on a 6% polyacrylamide-7M urea gel and eluted from gel slices by incubating in elution buffer (20 mM Tris-HCL pH 7.5, 0.5 M ammonium acetate, 10 mM EDTA, 0.1% SDS) at 37°C overnight or 65°C for 2 h. The RNAs were purified and concentrated by an ammonium acetate precipitation and resuspended in 60 μl of DEPC-treated water. OD_{260} was used to determine the concentration of RNAs.

Electrophoretic mobility shift assay (EMSA)

The purified *in vitro* synthesized $DsrA_{Bb}$ and *rpoS* RNAs were incubated with increasing amounts of purified recombinant Hfq_{Bb} in binding buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 50 mM KCl). 0.17 pmoles of $DsrA_{Bb}$ or *rpoS* RNA was mixed with 7 μl of the binding buffer, heated to 65°C for 10 min, and slowly cooled to room temperature. Two μl of serially diluted Hfq_{Bb} was added to the RNA and incubated for 5 min at room temperature. The samples were mixed with 10 μl gel loading buffer II (Ambion), fractionated on a Novex® Pre-Cast 6% DNA retardation gel (Invitrogen) in 1X TBE at 100V for 2 h. The gel was dried, exposed to a phosphorimager screen, and visualized using a Fujifilm FLA-3000G PhosphorImager.

Gene disruption and complementation of hfq_{Bb}

The hfq_{Bb} (*bb0268*) gene was deleted and replaced with a kanamycin resistance cassette (Bono *et al.*, 2000). Regions flanking the hfq_{Bb} ORF were amplified by PCR (Table 2). The 3′ end of the upstream flanking sequence and the 5′ end of the downstream flanking sequence were engineered with AatII sites for insertion of the kanamycin resistance cassette. The two flanking regions were cloned into pCR®2. 1-TOPO and ligated together to form a 2.1 kb target DNA fragment. The antibiotic resistance cassette was cloned into the synthetic AatII site. The plasmid was linearized with AhdI and electroporated into competent *B. burgdorferi* as previously described (Samuels, 1995). *B. burgdorferi* transformants were cloned in liquid BSK-II medium (Yang *et al.*, 2004) containing kanamycin (200 μg ml^{-1}) at 34°C and a 1.5% CO_2 atmosphere. Transformants were screened by PCR with primers that flank the insertion site.

Two different plasmids were constructed to complement the hfq_{Bb} mutant strain. The *E. coli* or *B. burgdorferi hfq* genes were fused to the inducible *flac* promoter (Gilbert *et al.*, 2007) and inserted into a modified pKFSS1 vector (Frank *et al.*, 2003) carrying the *lacI* repressor gene fused to the constitutive *flgB* promoter in the multiple cloning site. The recombinant

plasmids were electroporated into the kanamycin-resistant hfq_{Bb} mutant and selected in both streptomycin and kanamycin. The resulting transformants were assayed for the hfq_{Bb} expression plasmid by both PCR and xenodiagnosis (transformation of plasmid DNA from *B. burgdorferi* into *E. coli*).

RT-PCR

Total RNA was isolated from 100-ml cultures using TRIzol™ (Gibco BRL) as previously described (Lybecker and Samuels, 2007). RNA samples were treated with TurboDNase (Ambion) and RT-PCR was performed using the RETROscript™ kit (Ambion) according to manufacturer's instructions, except a gene-specific primer was used in the reverse transcription reaction instead of random decamers (Table 2).

Microscopic analysis of *B. burgdorferi*

Cell cultures were grown at 37°C to a low cell density (1–3× 10^7 cells ml^{-1}) and centrifuged at 5800 × *g*. Cell pellets were resuspended in 100 μl of water. Samples were visualized by differential interference contrast (DIC) microscopy using a Nikon E800 microscope at 100× . *B. burgdorferi* length was measured using ImageJ software. The length of hfq_{Bb} mutant cells do not display a normal distribution, so significance was evaluated using both parametric (one-way ANOVA with a Tukey's post-hoc test and a two-tailed unpaired *t*-test) and non-parametric (Kruskal–Wallis one-way ANOVA and a Mann Whitney *U* test) methods.

SDS-PAGE and immunoblotting

B. burgdorferi protein extracts were prepared as previously described (Lybecker and Samuels, 2007). Protein extracts were fractionated in Novex® Pre-Cast 4–20% Tris-Glycine gels (Invitrogen) and transferred to Immobilon-P PVDF membranes (Millipore) by electroblotting. Proteins were detected with either an anti-RpoS antiserum (Yang *et al.*, 2000) or anti-OspC antiserum (Yang *et al.*, 2005). The membranes were developed by chemiluminescence using the ECL Plus Western Blotting detection system (Amersham Biosciences) on a Fujifilm LAS-3000 (Yang *et al.*, 2005; Gilbert *et al.*, 2007).

Northern blot analysis

Total RNA was isolated from 100-ml cultures using TRIzol™ (Gibco BRL) as previously described (Lybecker and Samuels, 2007). RNA samples were treated with TurboDNase (Ambion) and fractionated in a Novex® Pre-Cast 6% polyacrylamide 7 M urea gel (Invitrogen) according to the manufacturers' instructions. The RNA was transferred to a BrightStar-Plus™ membrane by electroblotting using the Xcell SureLock™ Mini-Cell (Invitrogen) following the manufacturer's specifications. Northern blot hybridizations were performed using the NorthernMax™ kit (Ambion) according to the manufacturer's instructions. Non-isotopic RNA probes were generated from *in vitro* transcription of PCR-generated DNA templates using the MAXIscript® *in vitro* transcription kit (Ambion). Biotin-16-UTP (Roche) was used in the *in vitro* transcription reaction. Northern blot membranes were developed by chemiluminescence using the BrightStar® Biodetect™ nonisotopic detection kit on a Fujifilm LAS-3000.

Mouse infectivity studies

C3H-HeJ female mice were needle inoculated intraperitoneally (i.p) with 5 × 10^3 cells of wild-type 297 (BbAh130), hfq_{Bb} mutant, or complemented strains. All strains used in mouse infectivity studies were screened for the presence of plasmids essential for infectivity (lp28-1, lp25, and lp54) (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001). Three weeks post injection, ear punches were taken and cultured in BSK-II. Five weeks post

injection, mice were sacrificed and ear punches, tibiotarsal joints and bladders were collected and cultivated in BSK-II. Cultures were screened for the presence and growth of *B. burgdorferi* by darkfield microscopy. In addition, PCR was used to screen the cultures that appeared negative by dark-field microscopy. The University of Montana Institutional Animal Care and Use Committee approved all experimental procedures involving mice (protocol number 038-08SSDBS-081208).

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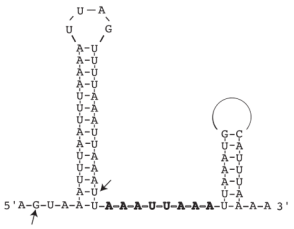

Fig. 1.

Predicted secondary structure of $DsrA_{Bb}$. The arrows flank the *rpoS* complementary region (34 nucleotides). The sequence predicted to be the Hfq-binding site is in bold. The open circle represents a loop of 32 nucleotides.

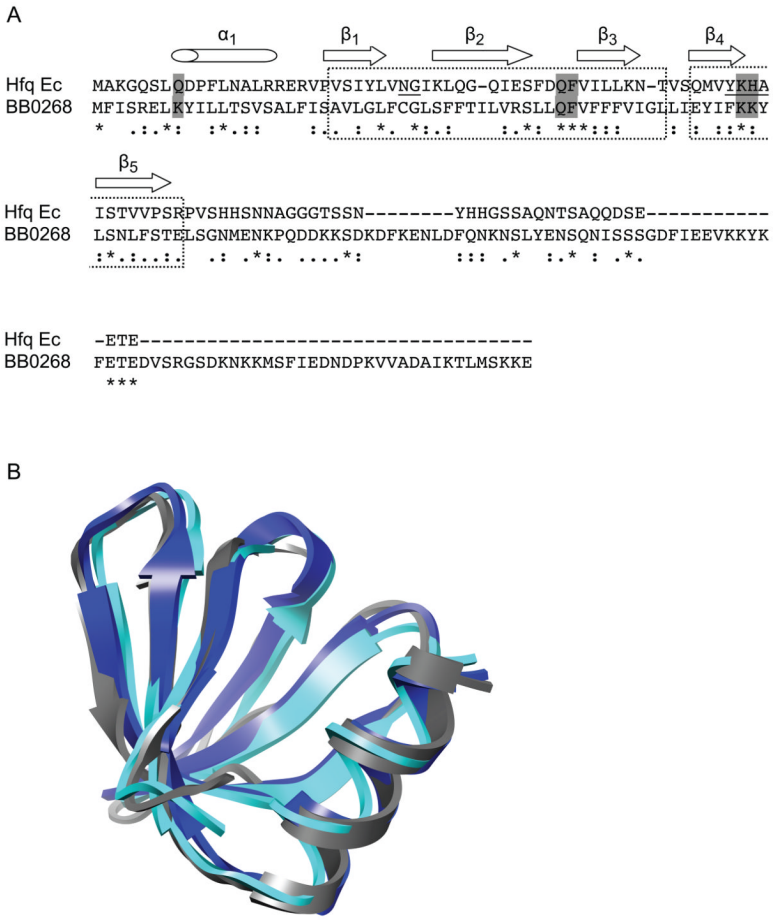

Fig. 2.

Hfq structural homology. (A) ClustalW alignment of the BB0268 conserved hypothetical protein with the *E. coli* (Ec) Hfq protein. Amino acids denoted by asterisks (*) are identical, colons (:) strongly similar and dots (.) weakly similar. The dashed boxes indicate the Sm1 and Sm2 motifs and the secondary structure of the *E. coli* Hfq is indicated above the sequence. Two of the signature motifs of Hfq, the Sm1 NG and the Sm2 F(Y)KHA, are underlined. The residues that are important in RNA binding by the *S. aureus* Hfq are shown in gray boxes. This figure panel is modified from Nielsen *et al.* (2007). (B) Modeling of Hfq structures. The structure of Hfq_{Bb} (gray) was predicted using Phyre and superimposed onto the experimentally determined *S. aureus* Hfq (blue) and *E. coli* Hfq (cyan) structures using Chimera. Hfq homologs from many organisms have a variable C-terminal extension that has not been structurally characterized; therefore, we have no information on the folded structure of the C-terminal region of Hfq_{Bb} and it is not shown.

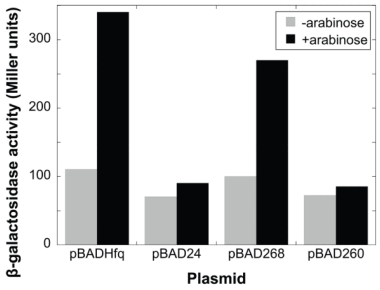

Fig. 3.

BB0268 has Hfq-like activity in *E. coli*. *hfq*-1 mutant strains containing an *rpoS::lacZ* translational fusion and carrying the indicated plasmids were assayed for total β-galactosidase activity. pBADHfq expresses *E. coli* Hfq under control of the *araBAD* promoter. pBAD24 is the empty *araBAD* vector. pBAD268 and pBAD260 have the *B. burgdorferi bb0268* and *bb0260* ORFs under the control of the *araBAD* promoter. Cultures were grown at 30 C either with (black bars) or without (gray bars) 150 μM arabinose and samples were taken at various times during logarithmic growth. Total β-galactosidase activity was determined as described by Miller (1972). Three independent experiments with BB0260 and five independent experiments with BB0268 were performed and yielded similar results.

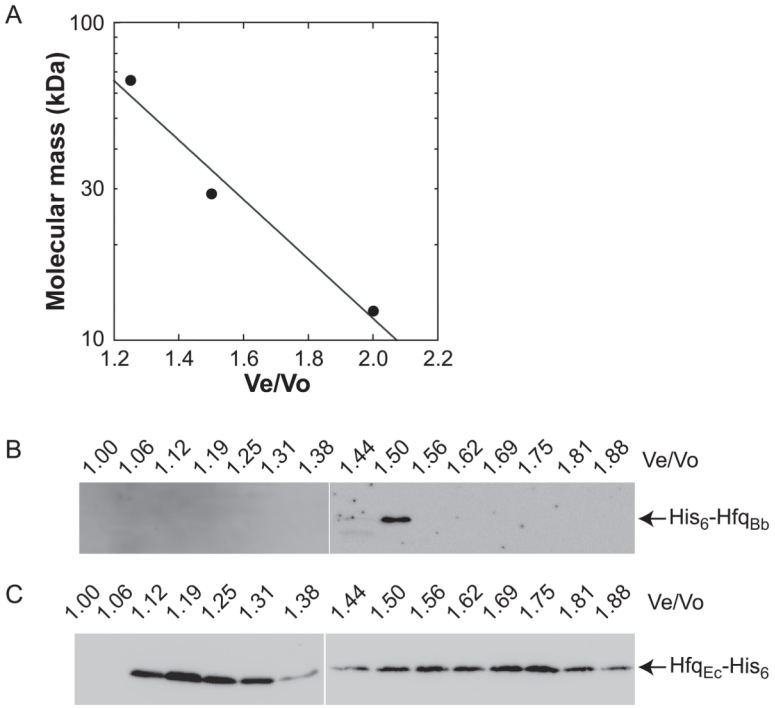

Fig. 4.

Hfq_{Bb} is not a stable hexamer in solution. (A) Standard curve of the Sigma gel filtration molecular weight markers resolved on a Superdex 75 HR 10/30 column. Regression analysis yielded the equation: $y = 869.58 \times e^{(-2.1536x)}$, which was used to calculate molecular mass. Recombinant Hfq_{Bb} (B) and *E. coli* Hfq (C) were resolved on a Superdex 75 HR 10/30 column and fractions analyzed using a HisProbe-HRP.

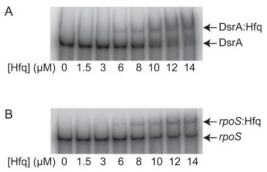

Fig. 5.

The Hfq_{Bb} protein binds RNA. Gel mobility shift analyses of Hfq_{Bb} binding to $DsrA_{Bb}$ (A) and *rpoS* (B). The RNAs were labeled with [α-^{32}P]UTP during *in vitro* transcription, gel-purified, and incubated with increasing concentrations of purified recombinant Hfq_{Bb}. The binding reactions were fractionated on a native Novex® Pre-Cast 6% DNA retardation gel.

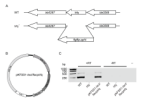

Fig. 6.

Mutation and complementation of hfq_{Bb}. (A) Strategy for disrupting the hfq_{Bb} gene (*bb0268*). The hfq_{Bb} gene was replaced with the kanamycin resistant cassette (*flgB*p-*aphI*). Transformation into wild-type (WT) *B. burgdorferi* and homologous recombination generated an hfq_{Bb} mutant strain (hfq^{-}). (B) Plasmid for *trans*-complementing the hfq_{Bb} mutant strain. The *B. burgdorferi* or *E. coli hfq* genes were fused to the inducible promoter *flac*p and inserted into the modified shuttle vector pKFSS1-*lacI*, which carries the *lacI* repressor gene fused to the constitutive *flgB* promoter in the multiple cloning site. The plasmid also carries both a *B. burgdorferi* replication origin (cp9 rep) from the 9-kb circular plasmid and an *E. coli* replication origin (ColE1) from pCR®-XL-TOPO. (C) RT-PCR of *hfq* mRNA in the wild type (WT), the hfq_{Bb} mutant (hfq^{-}), and the hfq_{Bb} mutant complemented with hfq_{Bb} (hfq^{-} pKFSS1-*lacI flac*phfq_{Bb}). The RT-PCR (+RT), the PCR on samples that were not treated with reverse transcriptase (−RT), and the PCR without template (−) were fractionated on a 2% agarose gel and stained with ethidium bromide.

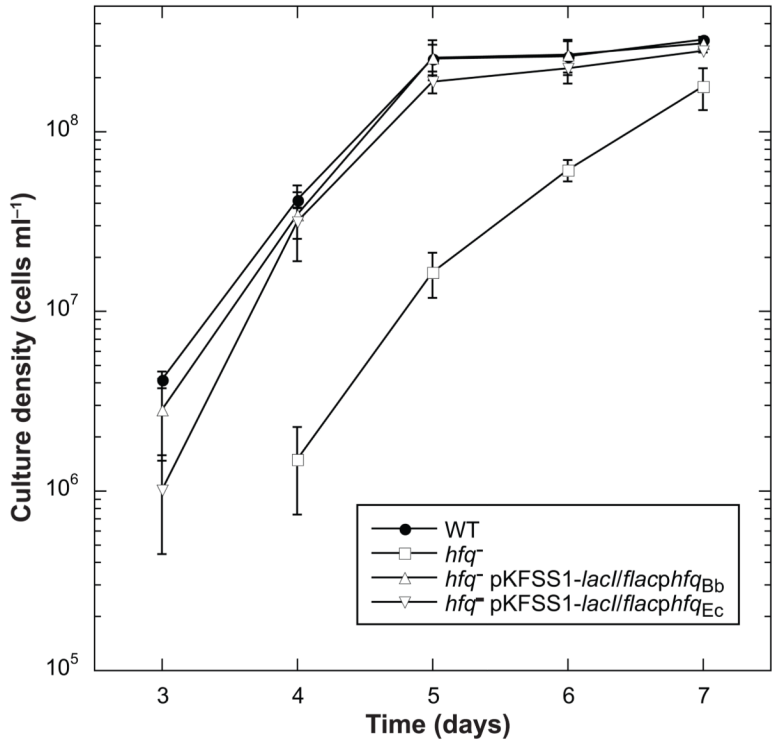

Fig. 7.

Growth phenotype of the hfq_{Bb} mutant. Growth curve of the wild type (WT), hfq_{Bb} mutant (hfq^{-}), and hfq_{Bb} mutant *trans*-complemented with either *hfq* from *B. burgdorferi* (hfq^{-}pKFSS1-*lacI flac*phfq_{Bb}) or *hfq* from *E. coli* (hfq^{-} pKFSS1-*lacI flac*phfq_{Ec}) following a temperature shift to 37°C. The means of the cell densities from three independent experiments are graphed with error bars representing the standard error of the means.

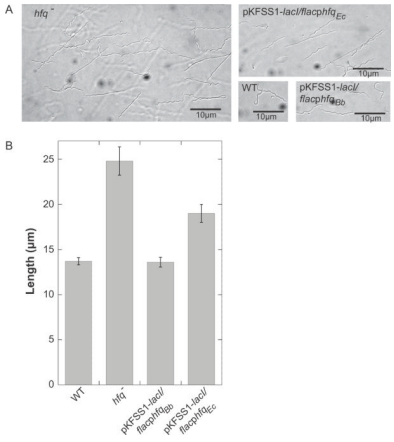

Fig. 8.

Cell morphology phenotype of the *hfq*$_{Bb}$ mutant. Cultures were grown at 37°C to mid-log phase and the cells were visualized using Nomarski microscopy and analyzed using ImageJ. (A) Microscopic images of the wild-type (WT), *hfq*$_{Bb}$ mutant (*hfq*$^{-}$), and complementing strains (pKFSS1-*lacI/flac*p*hfq*$_{Bb\ or\ Ec}$). (B) ImageJ was used to measure the length of fifty spirochetes per strain and the means of the lengths are graphed with error bars representing the standard error of the means. Differences between wild-type and *hfq*$_{Bb}$ mutant strains and between *hfq*$_{Bb}$ mutant and pKFSS1-*lacI/flac*p*hfq*$_{Bb}$-complemented strains are statistically significant ($P < 0.01$) by both a one-way ANOVA with a Tukey's post-hoc test and a Kruskal–Wallis test; differences between *hfq*$_{Bb}$ mutant and pKFSS1-*lacI/flac*p*hfq*$_{Ec}$-complemented strains ($P < 0.01$) and between wild-type and pKFSS1-*lacI/flac*p*hfq*$_{Ec}$-complemented strains ($P < 0.001$) are significant by both a two-tailed unpaired *t*-test and a Mann Whitney *U* test.

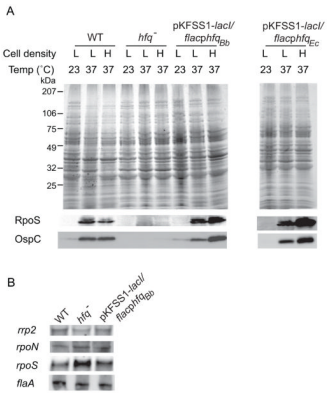

Fig. 9.

Hfq_{Bb} regulates RpoS levels post-transcriptionally in response to a temperature shift. Immunoblot analyses of whole-cell lysates of wild-type, hfq_{Bb} mutant, and *trans*-complemented strains at 23°C and after a temperature shift to 37°C at low (L) and high (H) cell densities (1–3 × 10^7 and 1 × 10^8 cells ml^{-1}, respectively(A). The top panel is a Coomassie brilliant blue-stained SDS-PAGE gel and the bottom panels are immunoblots probed with either anti-RpoS or anti-OspC antibodies. Three independent experiments were performed and representative data are shown. Northern blot analyses of total RNA (15 μg) fractionated on a 6% polyacrylamide 7 M urea gel, blotted to a nylon membrane, and hybridized with a *rpoS*, *rpoN*, *rrp2*, or *flaA* single-stranded RNA probe (B). Two independent experiments were performed and representative data are shown; the signal was quantified on a FujiFilm LAS-3000.

Table 1

Hfq_{Bb} is required for mouse infection by needle inoculation

	# of positive mice by culture		
***B. burgdorferi* strain**	**Ear**	**Joint**	**Bladder**
297 (BbAH130) WT	6/6	6/6	6/6
297 *hfq*$^{-}$	0/6	0/6	0/6
297 *hfq*$^{-}$ high passage	0/3	0/3	0/3
297 *hfq*$^{-}$/*flac*p-*hfq*$_{Bb}$	3/3	3/3	3/3
297 *hfq*$^{-}$/*flac*p-*hfq*$_{Ec}$	0/3	0/3	0/3

Table 2

Oligonucleotides used.

Name	Sequence (5′-3′)	Function
BB268 1F+MfeI	ACAATTGACCATGTTTATAAGCAGGGAATTG	Complementation in *E. coli*
BB268 480R+PstI	ACTGCAGTTATTCCTTCTTGCTCATTAAAG	
BB260 1F+MfeI	ACAATTGACCATGAAAGGGTTTTTAGCG	
BB260 1014R+PstI	ACTGCAGTTAAACATATTGATCTTTTTC	
BB268 1F+NdeI	CATATGTTTATAAGCAGGGAATTG	Complementation, expression, and RT-PCR
BB268 480R+AatII	GACGTCTTATTCCTTCTTGCTCATTAAAG	
BB268 249R+AatII	GACGTCTTAACTCTTCTTATCATCTTG	RT-PCR
Echfq 1F+NdeI	CATATGGCTAAGGGGCAATCTTTAC	Complementation in *B. burgdorferi*
Echfq 310R+AatII	GACGTCTTATTCGGTTTCTTCGCTGTC	
BB268 U1048F	TTTCTTATGTTACGGATGGGC	Mutant construction
BB268 U4R+Aat+Age	ACCGGTCATGACGTCTTATTCCACACCAAAAAATC	
BB268 474F+AatII	GACGTCGGAATAAATATGGATGACAGGGC	
BB268 D1578+AgeI	ACCGGTTACTATATCTCCAGTAGCAGGTCC	
dsrA L1F+T7	TAATACGACTCACTATAGGGAAAGCTAAATTAGAAGAGTTTATTG	Template for *in vitro* transcription
dsrA L275/352R	CATAAAAACTTTTTTTTGAATAG	
rpoS U449F+T7	TAATACGACTCACTATAGGGTCACAAAATCTAAAATTTAAAAATC	
rpoS 128R	GTTTTTTGCTTTTGCATTGC	
rpoS 705R+T7	TAATACGACTCACTATAGGCGGTGCTTTTTTTTGGGACTATTG	Northern blot probes
rpoS 443F	GATTCAACCTATCTCCTGCTCAG	
rrp2 185F	GAGAAAAATTGCTCAAAATA	
rrp2 394R+T7	TAATACGACTCACTATAGGCTTTTGAGTTTTTTCAAGAAG	
rpoN 285F	GCAATTAAGAATTCAAAGAA	
rpoN 572R+T7	TAATACGACTCACTATAGGCTTTTGAGTTTTTTCAAGAAG	
flaA 64F	GCTCAAGAGACTGATGGATTAGC	
flaA 284R+T7	TAATACGACTCACTATAGGCGCAGAAGGAGTAAGTAAAACGCTC	