Regulation of Expression of the Fibronectin-Binding Protein BBK32 in *Borrelia burgdorferi*[▽]

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The BBK32 protein binds to host extracellular ligand fibronectin and contributes to the pathogenesis of *Borrelia burgdorferi*. Here we showed that expression of the BBK32 gene is influenced by multiple environmental factors and that its regulation is governed by the response regulator Rrp2 and RpoN-RpoS (σ^{54}-σ^{S}) sigma cascade in *B. burgdorferi*.

Borrelia burgdorferi, a spirochetal pathogen that causes Lyme disease, contains genes encoding numerous lipoproteins in its genome. Emerging evidence has shown that coordinated regulation of many of these lipoproteins is critical to *B. burgdorferi*'s successful maintenance in the enzootic life cycle involving *Ixodes* ticks and mammals (28, 37, 38, 46). This is highlighted by the finding that two of the key virulence factors, outer surface lipoproteins OspA and OspC, undergo reciprocal alteration of their expression in *B. burgdorferi* during tick feeding (for a review, see reference 40). In unfed ticks (dormant state), spirochetes express primarily OspA, an adhesion molecule that is indispensable for *B. burgdorferi* colonization of the tick midgut (29, 30, 51). When the tick feeds, OspA is downregulated, while OspC, a protein required for the early phase of mammalian infection and also possibly for the transmission within the tick vector (16, 20, 31, 36, 45), is upregulated. OspC may also play a role within ticks, although this hypothesis is controversial. Thus, elucidating the mechanisms underlying differential expression of lipoproteins is critical to our understanding of *B. burgdorferi* pathogenesis.

Lipoprotein BBK32 was first identified as an antigen that elicits an antibody response in infected mice as well as in Lyme disease patients (14, 44), and it is a potential agent for serological test and vaccine development (2, 24). The BBK32 protein was also identified independently as a fibronectin-binding adhesin of *B. burgdorferi* (33). Binding to host extracellular matrix molecules is one of the common strategies that bacterial pathogens employ for adhesion and invasion of host tissues. *B. burgdorferi* is capable of binding to a variety of host extracellular matrix molecules (for reviews, see references 5 and 12). Although the fibronectin-binding feature of the BBK32 protein has been recognized for a number of years and the biochemical mechanism of the binding has been elucidated (23, 34, 35), biological roles of this protein have only recently been elucidated due to advances in genetic manipulation of *B. burgdorferi* (38). Overexpression of the BBK32 protein in a high-passage *B. burgdorferi* strain that lacks this protein enhances *B. burgdorferi*'s binding to fibronectin, as well as to glycosaminogly-

cans (17). Furthermore, inactivation of the BBK32 gene in infectious strains of *B. burgdorferi* reduced spirochetal binding to fibronectin, as well as its infectivity in mice (42), although the mutants had no apparent defect in tick vectors (25). The biological function of the BBK32 protein is consistent with the finding that expression of this protein in *B. burgdorferi* is induced during tick feeding and during mammalian host infection (15, 25, 26). However, little is known about the molecular mechanism that governs the differential expression of the BBK32 protein.

Influence of temperature and pH on BBK32 protein expression. To study the regulation of the BBK32 protein, we first investigated the effects on BBK32 protein expression of culture temperature and pH, two of the well-studied environmental cues that affect *B. burgdorferi* gene expression (1, 9, 28, 37, 40, 41, 43). BbAH130, an infectious clone of *B. burgdorferi* strain 297, was cultivated at either 23 or 37°C in BSK-H medium (32) or at 37°C in BSK-H medium adjusted to pH 8.0 with 1 M NaOH. Whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then subjected to immunoblotting with a monoclonal antibody against the BBK32 protein (kindly provided by Seppo Meri, University of Helsinki, Finland). The specificity of the monoclonal antibody against the BBK32 protein of *B. burgdorferi* strain 297 was demonstrated by immunoblotting against whole-cell lysates from BbAH130 and an isogenic BBK32 gene knockout mutant (Fig. 1A). Inactivation of the BBK32 gene in strain 297 was achieved by inserting an *aadA* marker (which confers streptomycin resistance in *B. burgdorferi* [19]) at the XbaI restriction site located 210 bp downstream of the ATG start codon of the BBK32 gene. As shown in Fig. 1B, spirochetes cultivated at 23°C did not display any appreciable amount of the BBK32 protein. However, expression of the BBK32 protein was greatly induced at 37°C. Furthermore, temperature-induced BBK32 protein expression was abolished in spirochetes cultivated at pH 8.0 (Fig. 1B). This pattern of temperature- and pH-dependent expression of the BBK32 protein is similar to the pattern for a number of *B. burgdorferi* lipoproteins, including OspC, DbpA/B, Mlp, OspF, BBA66, and RevA (10, 11, 13, 47, 49). The proteins sharing this pattern of expression have been previously designated the group I proteins (47). Therefore, the BBK32 protein is an additional group I protein. Interestingly, Skare and his coworkers recently showed that the BBK32 pro-

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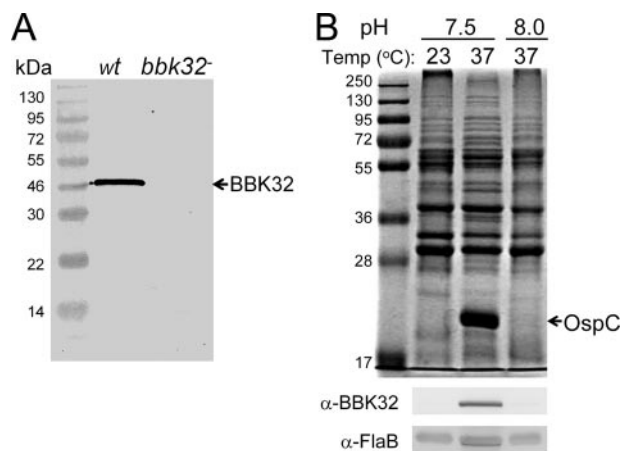
FIG. 1. Temperature and pH influence BBK32 protein expression in *B. burgdorferi*. Infectious clone BbAH130 (*wt*) or the isogenic BBK32 gene mutant (*bbk32⁻*) was cultivated in BSK-H medium at various temperatures or pHs. Spirochetes were harvested at the late logarithmic phase of growth ($\sim5 \times 10^7$ spirochetes/ml), and whole-cell lysates (5×10^7 spirochetes/gel lane) were subjected to immunoblot assays. (A) Specificity of monoclonal antibody directed against the BBK32 protein. Spirochetes were cultivated at 37°C and pH 7.5. The band corresponding to the BBK32 protein is indicated on the right. (B) (Top panel) Coomassie blue-stained gel. The band corresponding to OspC is indicated by an arrow. (Bottom panels) Immunoblots with monoclonal antibodies against the BBK32 protein or FlaB (loading control).

tein, along with several other group I proteins, including OspC and DbpA, is influenced by CO_2 levels in a similar fashion (22), providing further evidence that group I proteins are coregulated.

Control of BBK32 protein expression by RpoN and RpoS. In the past few years, advances in *Borrelia* genetics (38) led to the identification of a novel regulatory pathway, the RpoN-RpoS pathway, that governs differential expression of numerous *Borrelia* genes, including many of the group I genes, such as *ospC*, *dbpA/B*, *mlp*, *ospF*, and the BBA66 gene (11, 13, 18, 21, 48–50). This pathway consists of the response regulator Rrp2 and two alternative sigma factors, RpoN (σ^{54}; encoded by *rpoN* or *ntrA*) and RpoS (σ^s; encoded by *rpoS*) (21, 48). It was shown that Rrp2, an enhancer-binding protein (EBP) (48), in conjunction with RpoN, controls the transcription of *rpoS*. RpoS, a global regulator, further activates transcription of many genes in *B. burgdorferi*. In addition, RpoS was recently shown to be indispensable for the repression of OspA and other proteins when spirochetes were cultivated in a host-adapted model (6, 8). Accumulated evidence has emerged that supports the hypothesis that the RpoN-RpoS pathway functions as a central regulatory system that modulates expression of many *Borrelia* genes essential for spirochetal transmission in ticks and infection in mammals (4, 7, 8, 18, 20, 21, 27, 48, 49, 51).

Because the BBK32 protein has an expression pattern similar to that of the group I proteins, it is reasonable to hypothesize that the BBK32 gene is also regulated by the RpoN-RpoS pathway. However, previous microarray analyses showed that there is no significant difference between the BBK32 gene mRNA levels of the wild-type strain and an *rpoN* or *rpoS* mutant when the organisms are cultivated in vitro (18). To
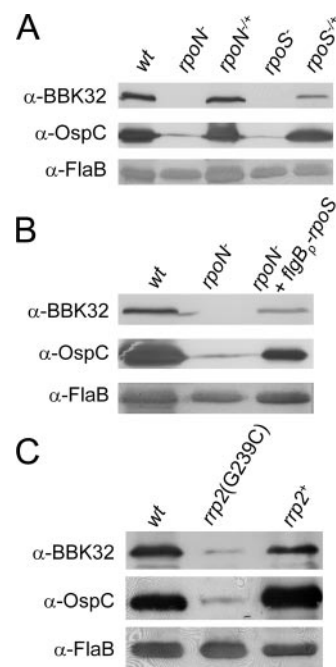

FIG. 2. Effects of Rrp2, RpoN, and RpoS on BBK32 protein expression in *B. burgdorferi*. Wild-type and various mutant strains of *B. burgdorferi* were cultivated at 37°C either in BSK-H medium at pH 7.5 (A and C) or in BSK-H medium adjusted to pH 6.8 (B). Spirochetes were harvested at late logarithmic phase ($\sim5 \times 10^7$ spirochetes/ml) and subjected to immunoblotting. Each gel lane was loaded with approximately 5×10^7 spirochetes. *wt*, wild-type *B. burgdorferi* strain BbAH130; *rpoN⁻*, RpoN-deficient mutant; *rpoN⁻/⁺*, RpoN-deficient mutant complemented with a wild-type copy of *rpoN*; *rpoS⁻*, RpoS-deficient mutant; *rpoS⁻/⁺*, RpoS-deficient mutant complemented with a wild-type copy of *rpoS*; *rpoN⁻* + *flgB*ₚ-*rpoS*, RpoN-deficient mutant complemented with a constitutively expressed wild-type copy of *rpoS*; *rrp2*(G239C), *rrp2* mutant; *rrp2⁺*, a wild-type *rrp2* allele was restored in the *rrp2* mutant. Monoclonal antibodies used for the immunoblots are indicated on the left.

examine if the RpoN-RpoS pathway plays a role in BBK32 protein expression, wild-type *B. burgdorferi* 297 (BbAH130) and various isogenic *B. burgdorferi* strains were cultivated in BSK-H medium at 37°C and whole-cell lysates were subjected to immunoblotting. As shown in Fig. 2A, inactivation of either *rpoN* or *rpoS* (21) abolished BBK32 protein expression, whereas the *cis*-complemented *rpoN* strain (21) or the *trans*-complemented *rpoS* strain rescued the mutants' defect. Of note, complementation of the *rpoS* mutant in *trans* was achieved by transforming a pBSV2 shuttle vector carrying a wild-type *rpoS* gene with 4 kb of flanking sequence (data not shown), a strategy similar to that used by Caimano et al. (6). These results strongly suggest that BBK32 protein expression is controlled by RpoN and RpoS.

Control of BBK32 protein by RpoN via RpoS. Results of recent microarray analyses suggested that in addition to the genes governed by the RpoN-RpoS pathway, RpoN and RpoS each may independently control additional sets of genes. To test the possibility that BBK32 protein expression might be modulated by RpoN and RpoS independently, we further examined BBK32 protein expression in BbAH64, an *rpoN* mutant that harbors a pGK12-based shuttle vector (39) carrying

an *rpoS* gene under the control of a constitutive *flgB* promoter (*flgB$_p$-rpoS*) of *B. burgdorferi* (21). It is noteworthy that we observed that BbAH64 expresses a much higher level of RpoS when it is cultivated at pH 6.8 than when it is cultivated at pH 7.5. Therefore, the wild type, the *rpoN* mutant, and BbAH64 were cultivated at 37°C and pH 6.8, and whole-cell lysates were subjected to immunoblotting. Unlike expression in the *rpoN* mutant, expression of OspC and expression of the BBK32 protein were readily detected in BbAH64 (Fig. 2B). These data indicate that RpoN-independent RpoS expression could overcome the RpoN deficiency in BBK32 protein expression and that RpoN controls BBK32 protein expression via RpoS.

Control of BBK32 protein expression by Rrp2. RpoN requires an EBP to activate gene transcription from a unique −24/−12 σ^{54} promoter (3). Rrp2 is the only EBP present in the *B. burgdorferi* genome. We previously showed that a point mutation (G239C) in the activation domain of Rrp2 abolishes the RpoN-dependent transcriptional activation of *rpoS*, which in turn diminishes the expression of OspC and other RpoS-controlled genes in *B. burgdorferi* (48). Thus, the *rrp2(G239)* mutant should have a similar defect in BBK32 protein expression. Indeed, the BBK32 protein level was greatly diminished in the *rrp2* mutant cultivated at 37°C (Fig. 2C), which further supports the notion that the BBK32 protein is governed by the RpoN-RpoS regulatory pathway.

To confirm that regulation of the BBK32 gene occurs at the mRNA level, quantitative reverse transcription PCR (qRT-PCR) was performed with RNA isolated from wild-type *B. burgdorferi* cultivated under different temperature conditions and from various *B. burgdforferi* mutants. Total RNA was extracted using an RNeasy mini kit (QIAGEN). PCR was first performed with the RNA samples to ensure that there was no detectable DNA contamination. cDNA was then synthesized using the ThermoScript reverse transcription system (Invitrogen). Quantitative PCR was performed in triplicate with an ABI 7000 sequence detection system using Platinum SYBR green quantitative PCR SuperMix (Invitrogen). Similar to the protein level, the BBK32 gene transcript was upregulated more than 30-fold by an elevated culture temperature (Fig. 3A). This result was consistent with the results of previous microarray (28, 37, 46) and qRT-PCR analyses (25), which showed that there was a 1.7- to 28-fold increase in the transcript level of the BBK32 gene when an elevated culture temperature was used. Similar to results observed for the BBK32 protein, a mutation in *rrp2* or inactivation of either *rpoN* or *rpoS* greatly diminished the BBK32 gene mRNA level, supporting the notion that regulation of the BBK32 gene by Rrp2, RpoN, and RpoS occurs at the transcriptional level (Fig. 3B). These data are also consistent with the results of a very recent microarray analysis by Caimano et al., which demonstrated that the BBK32 gene is among the more than 40 *B. burgdorferi* genes whose transcription is dependent on RpoS under both in vitro and host-adapted conditions (8).

In summary, the data presented here, along with previous findings of other workers (8, 22, 25, 28, 37, 46), demonstrate that the BBK32 gene is coregulated with *ospC* and other group I genes by multiple environmental factors and that its expression is modulated by the Rrp2-dependent RpoN-RpoS regulatory pathway. Such a mechanism of activation ensures that there is coordinated expression of the BBK32 protein, OspC,
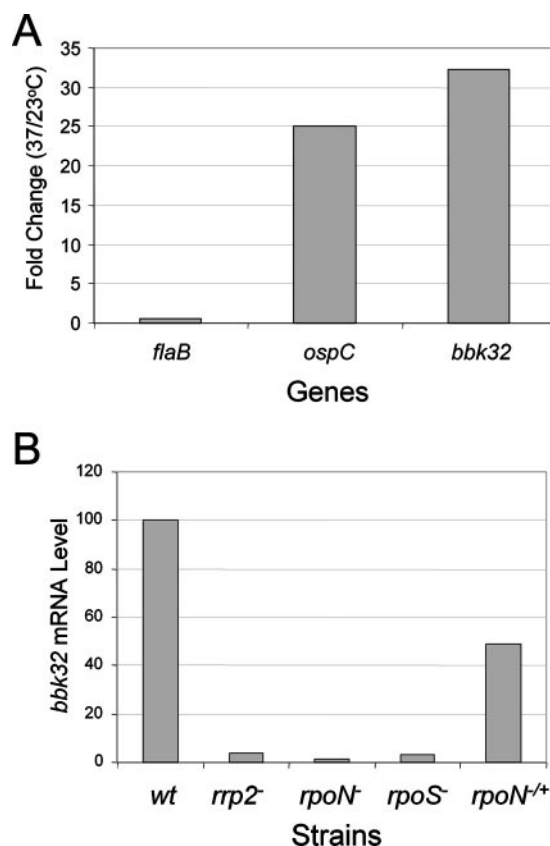
FIG. 3. qRT-PCR analyses of the BBK32 gene transcript. (A) Fold change in the BBK32 gene mRNA levels in wild-type strain BbAH130 cultivated at 37°C compared to the levels at 23°C. (B) BBK32 gene mRNA levels in various mutants relative to the level in wild-type strain BbAH130 (grown at 37°C). The level of 16S rRNA was used for normalization of the relative mRNA level of each gene. Data were calculated from experiments done in triplicate.

and other group I proteins prior to *B. burgdorferi* transmission from the tick vector to the mammalian host. Further research is warranted to delineate whether the BBK32 gene is directly controlled by RpoS, as demonstrated for *ospC*, or is indirectly controlled via an undefined transcriptional regulator.

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