

Analysis of the determinants of *bba64* (*P35*) gene expression in *Borrelia burgdorferi* using a *gfp* reporter

Aarti Gautam, Marianne Hathaway, Natalie McClain, Geeta Ramesh and Ramesh Ramamoorthy

Correspondence
Ramesh Ramamoorthy
rramesh@tulane.edu

Division of Bacteriology and Parasitology, Tulane National Primate Research Center, Tulane University Health Sciences Center, Covington, LA 70433, USA

The *bba64* (*P35*) gene of *Borrelia burgdorferi*, the agent of Lyme disease, encodes a surface-exposed lipoprotein. The expression of *bba64* *in vitro* is tightly regulated and dependent on several environmental factors. In nature, its expression is induced in the tick vector during feeding and maintained during infection of the vertebrate host. The pattern of expression of *bba64* suggests that it imparts a critical function to the pathogen. A previous study has shown that the expression of *bba64* is down-regulated in the absence of RpoS, suggesting that the alternative sigma factor may be involved in its expression. A DNA-binding protein has also been shown to specifically recognize a sequence in the 5' regulatory region of the gene. Therefore, the contribution of these putative determinants to the differential expression of *bba64* was investigated. The role of RpoS was critically evaluated by genetic complementation of the *rpoS* mutant using a chromosomally targeted copy of the wild-type gene. The results confirm that RpoS is indeed required for the expression of *bba64*. The role of the upstream DNA-binding site was examined using *bba64* promoter–*gfp* transcriptional fusions in a shuttle vector. The DNA-binding site was studied by targeting mutations to an inverted repeat sequence (IRS), the most prominent feature within the binding site, as well as by deletion of the entire sequence upstream of the basal promoter. Quantitative assessment of gene expression demonstrated that neither the IRS nor the sequence upstream of the promoter was essential for expression. Moreover, the expression of the reporter (GFP) appeared to remain RpoS-dependent in all cases, based on the co-expression of GFP and OspC in a subpopulation of spirochaetes and the selective expression of GFP in the stationary phase. Collectively, the data indicate that RpoS is the sole determinant of differential *bba64* expression in cultured spirochaetes.

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INTRODUCTION

Borrelia burgdorferi, the spirochaetal agent of Lyme disease, is maintained in nature via a complex enzootic life cycle involving *Ixodes* ticks and small rodents. To survive in this enzootic cycle, *B. burgdorferi* must adapt physiologically to diverse environments. Central to its adaptation process is the differential expression of proteins in response to changes in the environment, especially as this organism traverses from its tick vector to the mammalian host and vice versa.

The genome of *B. burgdorferi* strain B31 is composed of a linear chromosome, nine circular plasmids and 12 linear plasmids (Casjens *et al.*, 2000; Fraser *et al.*, 1997). One of the genetic elements that display prolific differential

expression in response to environmental signals is linear plasmid 54 (lp54) (Brooks *et al.*, 2003; Carroll *et al.*, 2000; Clifton *et al.*, 2006; Ojaimi *et al.*, 2003; Revel *et al.*, 2002; Tokarz *et al.*, 2004). lp54 of *B. burgdorferi* B31 consists of 76 ORFs that include lipoproteins such as OspA and OspB (Barbour & Garon, 1987) and decorin-binding proteins A (DbpA) and B (DbpB) (Hagman *et al.*, 1998). In addition to these immunogenic proteins, lp54 also carries eight out of the 14 members of gene family 54. Paralogues of this gene family exhibit significant intrafamily sequence divergence, with amino acid similarity and identity values as low as 7.35 and 5.4%, respectively (McDowell *et al.*, 2005). Two members of this family, BBA64 (Gilmore *et al.*, 1997) and BBA66, have been localized to the surface of the spirochaete (Brooks *et al.*, 2006).

Members of gene family 54 display distinct expression patterns. Some members (*bba64* and *bba66*) of the family are silent during the unfed-tick phase (Gilmore *et al.*, 2001; Tokarz *et al.*, 2004) but are turned on during tick feeding

Abbreviations: Ab, antibody; IRS, inverted repeat sequence.

A supplementary table listing the reagents and settings used for confocal microscopy is available with the online version of this paper.

(Tokarz *et al.*, 2004). Several members (*bba64*, *bba65*, *bba66*, *bba73* and *bbi36/38*) are expressed in the vertebrate host (Gilmore *et al.*, 1997, 2007; Anguita *et al.*, 2000; Liang *et al.*, 2002; Brooks *et al.*, 2006; Clifton *et al.*, 2006; Nowalk *et al.*, 2006). Although the functions of most of the paralogues remain unknown, one member, *bba68*, is known to bind to human factor H (Kraiczky *et al.*, 2004; Wallich *et al.*, 2005) and impart resistance (Brooks *et al.*, 2005). Recent data indicate that *bba68* is not expressed during infection, as inferred from real-time RT-PCR analyses and the absence of an antibody (Ab) response to the protein in infected animals. Moreover, *bba68* expression is not dependent on RpoS (McDowell *et al.*, 2006). The differential expression of these genes may be reproduced in culture under conditions that mimic the unfed tick (pH 8.0, 23 °C) or the feeding tick (pH 7.0, 35 °C). In general, the expression of *bba64*, *bba65*, *bba66*, *bba71* and *bba73* is upregulated while that of *bba69*, *bba70*, *bbi36/38* and *bbi39/41* is down-regulated under culture conditions that resemble the tick feeding process (Carroll *et al.*, 2000; Clifton *et al.*, 2006; Ojaimi *et al.*, 2003; Ramamoorthy & Scholl-Meeker, 2001; Revel *et al.*, 2002). The effect of inclusion of blood in the culture medium was largely similar to the effect observed under feeding-tick-like conditions (Tokarz *et al.*, 2004). However, with respect to gene family 54, spirochaetes cultured in implanted dialysis membrane chambers (DMCs) display an expression pattern that resembles neither the flat (unfed) nor the feeding tick (Revel *et al.*, 2002; Brooks *et al.*, 2003).

The regulation of expression of two members of the *gbb54* family, *bba64* and *bba66*, has recently been investigated. The expression of *bba66* was shown to require the presence of a sequence motif that is the binding site for a sequence-specific DNA-binding protein (Clifton *et al.*, 2006). The expression of *bba64* has also been shown to be associated with a sequence-specific DNA-binding activity (Indest & Philipp, 2000). However, based on their sequence specificities, these two paralogues appear to recruit distinct DNA-binding proteins (Clifton *et al.*, 2006). In the case of *bba64*, the binding site has been localized to a 43 nt region (designated *k2*) immediately upstream of the -35 element (Indest & Philipp, 2000). The *k2* region harbours two features that may comprise the DNA-binding site, an inverted repeat sequence (IRS) and a downstream poly-T tract. Poly-T tracts have been speculated to be involved in regulating gene expression in *B. burgdorferi* (Sohaskey *et al.*, 1999; Caimano *et al.*, 2005). In a recent study, the expression of *bba64* was found to be down-regulated in an *rpoS* mutant as compared to its isogenic wild-type parent (Fisher *et al.*, 2005). However, a subsequent study found the expression of *bba64* to be uniquely constitutive as compared to other paralogues of this gene family with respect to both culture temperature and culture medium pH (Clifton *et al.*, 2006). Therefore, the role of RpoS in the expression of *bba64* remains somewhat uncertain.

In this study, we critically examined the role of RpoS in the expression of *bba64* by complementing the B31 A3*rpoS*

mutant with a wild-type copy of the *rpoS* gene inserted into the chromosome. We also investigated the role of the upstream sequence, specifically the IRS and the poly-T tract within the *k2* region, in the expression of *bba64* using *gfp* as a reporter. The importance of the *k2* region in *bba64* expression was examined using a combination of mutations and deletion.

METHODS

Bacterial strains and culture conditions. Low-passage, infectious *B. burgdorferi* clones B31 A3 (Elias *et al.*, 2002) and B31 5A4NP1 (*cp9⁻ bbe02::kan^r*) (Kawabata *et al.*, 2004), as well as the B31 A3 *rpoS* mutant (Elias *et al.*, 2002) were used in the current study. The *Escherichia coli* strains Top 10 (Invitrogen) and XL1 Blue MR (Stratagene) were used in the generation of constructs and for the preparation of plasmids for the transformation of *B. burgdorferi*. *E. coli* transformants were selected by plating on Luria agar (1.3%) supplemented with 100 µg ampicillin ml⁻¹, 10 µg gentamicin ml⁻¹ or 100 µg spectinomycin ml⁻¹. *B. burgdorferi* strains and transformants were grown in BSK II + 6% rabbit serum (Sigma) or in BSK-H complete media (Sigma). Spirochaetes were cultured in 5% CO₂, 3% O₂ and 92% N₂ at 34 °C. The cultures were set up at an initial density of 1 × 10⁵ organisms ml⁻¹ and harvested at stationary phase (1–2 × 10⁸ organisms ml⁻¹). Spirochaetal cultures for confocal microscopy were harvested at late exponential phase. Enumeration of cells in culture was performed by dark-field microscopy.

Generation of B31 A3*rpoS*/*rpoS*⁺ clones. The *rpoS* mutant was complemented with a wild-type copy of strain B31 *rpoS* that was targeted to the chromosome at the BB0472–BB0473 intergenic site simply because this presented a large region of sequence with no known function. The first step in the assembly of the complementation construct was the construction of a hybrid *bmpA promoter–aadA* gene for positive selection of transformants in *B. burgdorferi*. The *bmpA* promoter (*bmpAp*) region was amplified with primers T79 and B83 and cloned into pQE30. The *aadA* coding sequence (conferring streptomycin resistance in *B. burgdorferi*) (Frank *et al.*, 2003) was amplified from plasmid pAM34 [American Type Culture Collection (ATCC) catalogue no. 77185] using primers T227 and B237, and cloned downstream of the *bmpAp*. The *bmpAp–aadA* gene was then transferred to pBR322 by PCR using primers T228 and B237. Next, a 1.3 kb DNA fragment containing the wild-type *rpoS* gene and 5' flanking sequence, including the RpoN promoter, was amplified from B31 by PCR using primers T267 and B274. This fragment was cloned downstream of the *bmpAp–aadA* sequence. To target the *rpoS* gene to the BB0472–BB0473 intergenic locus on the chromosome, BB0472 and BB0473 sequences were cloned upstream of *bmpAp–aadA* and downstream of *rpoS*, respectively. The primers are all listed in Table 1. The resulting plasmid, designated p472ApSrpoS473, was used to transform the B31 A3*rpoS* mutant by electroporation, as described elsewhere (Samuels, 1995). After overnight recovery, the electroporated spirochaetes were plated on semisolid BSK-H containing streptomycin (50 µg ml⁻¹) and kanamycin (100 µg ml⁻¹) (Sung *et al.*, 2000). The plates were incubated at 35 °C in a candle jar container. Colonies usually appeared 2 weeks after plating. The colonies were transferred to liquid media and subsequently expanded. The integration of the wild-type *rpoS* was confirmed by Western blotting and PCR analysis. Two clones were chosen for further characterization.

***bba64 promoter–gfp* transcriptional fusion constructs.** DNA inserts for cloning were derived in most cases by PCR using

Table 1. Primers used in this study

Primer	Sequence*	Location†	Gene(s)/plasmid	Use
T79	agtcgatgactcgagatcgatTAAACAATAGGTTGGTTGATGAAG	-130 to -105	<i>bmpAp</i>	<i>bmpA</i> promoter
T81	agtcgatgactcgagatcgatACTTCACTTAGACACATTATTTAA	-177 to -153	<i>A64p</i>	pQE30-A64p
T82	AAAGAGAGCGCATGCTCTCTT	-117 to -96	<i>A64p5' m</i>	pQE30-A64p5' m
T88	CAGCTATGACCATGATTACG	+4246 to +4266	pBSV	Sequencing
T188	cggggtaccACTTCACTTAGACACATTATTTAA	-177 to -153	<i>A64p</i>	pBSV2G-A64p-gfp
T227	cgtgaagaattcATGAGGGAAGCGGTGATCGT	+1 to +20	<i>StrR</i>	<i>rpoS</i>
T228	tgtagatctctcgagatcgatTAAACAATAGGTTGGTTGAT	-130 to -110	<i>bmpAp</i>	<i>rpoS</i>
T265	CTGTTACAGCCACAGAAAAT	+528 to +548	<i>bb0472</i>	<i>rpoS</i>
T266	agttcagttgtcgacCTCTGCTTTAATTCGTAGAA	Intergenic region	<i>bb0473</i>	<i>rpoS</i>
T267	gatcagatagctagcGGAGGAAATTGATGGAAACC	-478 to -498	<i>bb0771</i>	<i>rpoS</i>
T239	cggggtaccTTCTTGTAACAATAACAATTTTG	-83 to -61	<i>A64p</i>	pBSV2G-A64pmin-gfp
T253	AGGTTACCCCTGGAAAGAT	+381 to +362	<i>A64p</i>	RT-PCR
T306	GCATGTATTAACGTGTGCTAA	+51 to +70	<i>A64p</i>	RT-PCR
B83	gtagcatgagaattcTTACAAACAAGCTATATTTAAGTAGTT	-15 to -44	<i>bmpA 5'</i>	<i>bmpA</i> promoter
B86	aaaaagagatcgatTAATCTCGTGGGTACAAGGTCTTATTTT	-137 to -109	<i>A64p5' m</i>	pQE30-A64p5' m
B87	gaagatgagcaattcAAAATGTTACCCCTCAATA	+3 to -17	<i>A64p</i>	pQE30-A64p
B121	gaattcAAAAAATCGTATGGCATGCTAATCTAGTGGG	-90 to -122	<i>A64p5' 3' m</i>	pQE30-A64p5' 3' m
B199	tcgcatcgGTTCTTTACGATGCCATTGGGATAT	+406 to +382	pQE30	pBSV2G-A64p-gfp
B237	actagctagcTTATTTGCCGACTACCTTGGTG	+772 to +792	<i>StrR</i>	<i>rpoS</i>
B272	gaattcctactcgagATCAGAAATACCTCTCTGCA	Intergenic region	<i>bb0472</i>	<i>rpoS</i>
B273	TTAAGCTCCCCTTTTACACC	+382 to +402	<i>bb0473</i>	<i>rpoS</i>
B274	tgttgaacctcgacAACATGTCATGAATGTCAATG	+798 to +818	<i>bb0771</i>	<i>rpoS</i>
FlaBF	ACAGCTGAAGAGCTTGAAT	+439 to +458	<i>flaB</i>	RT-PCR
FlaBR	TTGCTCCAACATGAACTCTT	+538 to +519	<i>flaB</i>	RT-PCR

*Sequences homologous to genes are in upper-case type and unrelated sequences are in lower-case type. Functional restriction enzyme sites added to the primers are underlined.

†Locations are numbered with respect to the coding sequences of the corresponding genes.

ProofStart enzyme (Qiagen). All inserts were purified from low-temperature-gelling agarose gels using gel extraction kits (Qiagen). Plasmids for analysis were prepared using Plasmid Miniprep columns (Qiagen) and those used for electroporation were prepared under sterile conditions using Tip100 columns (Qiagen). The *bba64* promoter region was amplified by PCR with primers T81 and B87 and cloned into pQE30-gfp (Ramamoorthy *et al.*, 2005) to yield pQE30-A64p-gfp. To generate pQE30-A64p5' m, the *bba64* promoter region was amplified in two parts using primer sets T81 and B86 and T82 and B87. The T81/B86 and T82/B87 amplicons were assembled in pQE30/*XhoI/EcoRI* in a three-way ligation to yield pQE30-A64p5' m-gfp. To generate pQE30-A64p5' 3' m-gfp, the promoter region was amplified from pQE30-A64p5' m using primers T81 and B121 and cloned into the *XhoI/EcoRI* sites of pQE30-A64p5' m-gfp.

The shuttle vector derivatives of these constructs were generated as follows. For cloning into pBSV2G (Elias *et al.*, 2003), the fragments (*A64p-gfp*, *A64p5' m-gfp* and *A64p5' 3' m-gfp*) were amplified using primers T188 and B199. A minimal promoter construct, *A64pmin-gfp*, was generated from pQE30-A64p-gfp using primers T239 and B199. These fragments were all cloned into pBSV2G at the *KpnI/PvuI* sites, resulting in plasmids pBSV2G-A64p-gfp, pBSV2G-A64p5' m-gfp, pBSV2G-A64p5' 3' m-gfp and pBSV2G-A64pmin-gfp. Finally, a promoterless *gfp* construct was also generated for use as a control. The promoter sequences of all constructs were confirmed by sequencing using the T88 primer.

Transformation of *B. burgdorferi*. Plasmid DNAs for electroporation were produced under sterile conditions using Qiagen Tip100 columns. The cells were prepared for electroporation as described

elsewhere (Samuels, 1995). Electrocompetent *B. burgdorferi* was transformed as described elsewhere (Samuels, 1995) with the different promoter-gfp fusion plasmids, with a minor modification. Ten micrograms of DNA was electroporated into 90 µl of cells. Immediately following electroporation, the cells were resuspended in 10 ml liquid BSK-H media and incubated overnight at 34 °C to allow the cells to recover. The transformants were selected according to the limiting-dilution method (Yang *et al.*, 2004). After overnight recovery, the cultures were supplemented with 40 ml fresh BSK-H containing gentamicin (40 µg ml⁻¹) and kanamycin (100 µg ml⁻¹), and distributed into 96-well tissue-culture plates (200 µl per well). Two to three weeks after plating, wells that were positive for dividing spirochaetes were identified by a colour change in the medium, and the presence of viable spirochaetes was verified by dark-field microscopy. The antibiotic-resistant clones were inoculated into 1 ml complete BSK-H medium containing the relevant antibiotics. After 3 days, the transformants were expanded into 15 ml BSK-H complete media. The 15 ml culture was used for the preparation of freezer stocks and to inoculate fresh cultures for analysis of gene expression.

Generation of rat polyclonal anti-RpoS Ab. To assess RpoS expression, a rat polyclonal anti-RpoS Ab was generated. Briefly, *B. burgdorferi rpoS* was cloned into the pQE30 expression vector (Qiagen) and expressed as a hexahistidine fusion protein in *E. coli*. Overexpression resulted in an insoluble fusion protein that was purified under denaturing conditions, dialysed to remove urea and then used for the preparation of rat anti-RpoS Ab (Genemed Synthesis). The specificity of the anti-RpoS Ab was verified in *E. coli*

using whole-cell lysates prepared from uninduced and IPTG-induced cells carrying the pQE30-*his₆rpoS^{Bb}* plasmid. Whereas the Ab showed strong reactivity to a band of ~33 kDa in the induced sample, consistent with the expected size of the fusion protein, there was no reactivity with the uninduced sample (data not shown). The Ab was then titrated to determine the highest dilution of the Ab that provided the best signal in Western blots (data not shown). A dilution of 1 : 200 provided the best signal.

RNA isolation and RT-PCR. DNA-free RNA was isolated from B31 A3, B31 A3*rpoS* and B31 A3*rpoS/rpoS⁺* as previously described (Ramamoorthy *et al.*, 1996). Furthermore, the integrity and concentration of each RNA sample were verified as described previously (Ramamoorthy *et al.*, 1996). About 200 ng total RNA was converted to cDNA in a 10 µl volume using Taqman reverse transcription reagents (Applied Biosystems) following the manufacturer's instructions. cDNA synthesis was primed with random hexamers and carried out under the following conditions: 26 °C for 10 min followed by 48 °C for 30 min. The enzyme was inactivated at 95 °C for 5 min prior to PCR. PCR was performed with 2 ng of each cDNA using ProofStart polymerase (Qiagen) in a volume of 30 µl. To rule out amplification from DNA, reactions containing RNA without reverse transcriptase were also included with the *bba64* primer set. The primers used were as follows: T253 and T306 (*bba64*), and FlaBF and FlaBR (*flaB*). The reaction conditions consisted of a 5 min, 95 °C denaturation step, followed by 40 cycles of 95 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min, and then a final extension step at 72 °C for 10 min.

Western blotting. Whole-cell lysates were prepared from stationary-phase cultures and normalized to an OD₆₀₀ of 5, as described previously (Ramamoorthy & Philipp, 1998). For the analysis of protein expression, 10 µl (unless specified otherwise) of each sample was electrophoresed through a 12.5% SDS-polyacrylamide gel and the proteins were transferred to nitrocellulose. Following Ab incubations, protein bands were visualized using the chromogen 4-chloro-1-naphthol. The following Abs were used: mAb specific for BBA64 (Indest *et al.*, 1997), rabbit polyclonal anti-GFP Ab (Santa-Cruz Biotechnology), anti-FlaB mAb H9724 (University of Texas Health Sciences Center, San Antonio), anti-OspC mAb B5 mAb (Mbow *et al.*, 1999) and rat polyclonal anti-RpoS Ab (this study). For quantitative analysis of protein expression, the Western blots were digitized and the intensity of individual bands was quantified by densitometry using Kodak Molecular Imaging Software, version 4.0. All experiments were repeated at least once and the analyses of the pooled data are presented.

Immunofluorescence staining and confocal microscopy. The spirochaetal cultures were spun down and the resulting pellets were washed twice with PBS (Invitrogen) to remove the culture medium. The pellets were resuspended in PBS at a density of ~2 × 10⁸ cells ml⁻¹. A 50 µl volume of borrelial suspension containing ~1 × 10⁷ cells was applied to Superfrost Plus slides (Fisher). Smears were air-dried, taking care to protect them from exposure to direct light. Slides were fixed in methanol for 10 min. Bacterial smears were blocked for 1 h in blocking buffer [PBS containing 10% normal goat serum (Invitrogen), 0.2% fish skin gelatin (FSG; Sigma) and 0.02% sodium azide (Sigma)]. The blocking solution was removed by gently flicking the slides before addition of the primary Abs. Primary Abs were diluted to the desired concentration in a PBS-FSG buffer (PBS, 0.2% FSG, 0.02% sodium azide) (see Supplementary Table S1). Isotype Ab controls (Dako) in combination with the corresponding secondary-Ab-fluorochrome conjugates were also included in the analyses. The slides were washed with PBS buffer after the application of each Ab. All incubations were performed in a dark humidified slide chamber at room temperature. Finally, slides were mounted in anti-queching medium (Sigma) with premium coverslips (Surgipath) and sealed.

The stained and mounted slides were stored in the dark at 4 °C until imaging. Imaging was performed using a Leica TCS SP2 true confocal laser-scanning microscope, DMIRE2 (Leica), equipped with three lasers (Ar, Ar-Kr, He-Ne) that span from the visible to the far-red region of the spectrum. Using Leica software, the fluorescence of individual fluorochromes was captured separately in sequential mode after optimization to reduce bleed through between the channels (photomultiplier tubes). Images of individual channels were also merged to obtain composite images containing all channels.

RESULTS

The expression of *bba64* is dependent on RpoS

One of our first objectives was to rigorously examine the dependence of *bba64* expression on the alternative sigma factor RpoS. Although an earlier study demonstrated that the expression of *bba64* was down-regulated in an *rpoS* mutant, that study did not employ complementation to confirm this dependence of gene expression on RpoS (Fisher *et al.*, 2005). Therefore, we set out to complement the *rpoS* mutation to definitively ascertain the dependence of *bba64* expression on RpoS. For complementation, a construct containing a wild-type copy of the *rpoS* (*bb0771*) gene inserted into the *bb0472*-*bb0473* intergenic site (Fig. 1) was used to transform B31 A3*rpoS* (Elias *et al.*, 2002), the same strain used in the earlier study. The complemented clone, B31 A3*rpoS/rpoS⁺*, was characterized by PCR using total DNA. The PCR amplification patterns were consistent with the expected genotype (data not shown). Plasmid profile analysis confirmed the presence of all plasmids that were present in the parental strain, B31A3 (Elias *et al.*, 2002). Moreover, the complemented clones displayed normal growth kinetics in BSK-H and BSK II media.

The expression of RpoS in the complemented strain was examined by Western blotting using a rat monospecific anti-RpoS Ab. Both clones exhibited a band similar in size to the wild-type band and consistent with the expected size of the protein (31 kDa). This band was absent in the *rpoS* mutant strain (Fig. 2a, RpoS). To further confirm RpoS expression in the complemented clones, we also tested the samples for the presence of OspC, a known RpoS-dependent protein (Hübner *et al.*, 2001). As expected, the presence of RpoS in the complemented clones restored the expression of OspC (Fig. 2a, panel OspC). Finally, we examined the samples for the presence of BBA64 using an anti-BBA64 mAb (Gilmore *et al.*, 1997). Whereas no BBA64 expression was detected in the absence of RpoS (Fig. 2a, panel BBA64, lane 2), this protein was clearly present in the two complemented clones at a level similar to the wild-type level (Fig. 2a, panel BBA64, lanes 3 and 4). The dependence of *bba64* expression on RpoS was further verified at the mRNA level by RT-PCR. The *bba64* sequence could be amplified from RNA derived from the wild-type and the complemented strains, but not from the *rpoS* mutant (Fig. 2b, panel *bba64*). In contrast, the constitutively expressed *flaB* transcript was present in all samples examined (Fig. 2b, panel *flaB*). These results

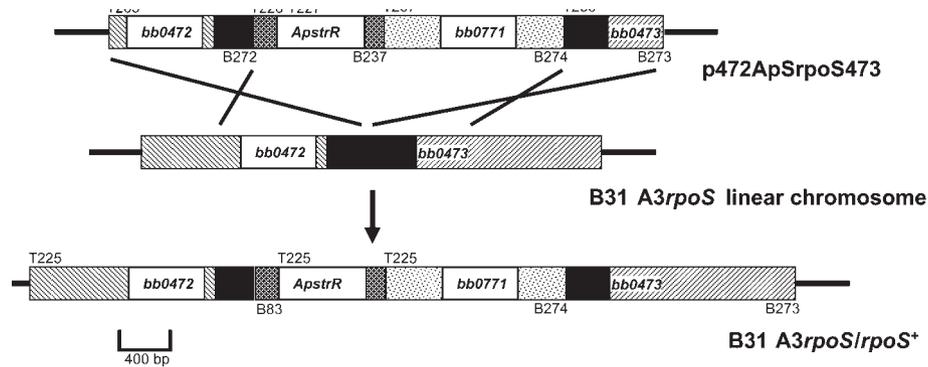


Fig. 1. Strategy for the complementation of the B31 A3 *rpoS*::Kan mutant. Primers used in the construction of p472ApSrpoS473 are shown. Also shown are the primers used for confirming the genotype of the B31 A3*rpoS*/*rpoS*⁺ strain. For more information on the T and B primers shown here, please refer to Table 1. The *bb0472*–*bb0473* intergenic region, defined as the sequence between the stop codon of *bb0472* and the start codon of *bb0473*, is shown as a filled box. The *bmpA* promoter–*aadA* hybrid gene is designated *ApstrR* in the figure.

conclusively establish the dependence of BBA64 expression on the alternative sigma factor RpoS under conditions of high cell density.

A minimal promoter is sufficient for optimal expression of *bba64* in culture

The presence of any functional sequence elements in the 5' regulatory sequence of *bba64* was assessed by a combination of deletions and mutations. A previous study identified a 43 nt region (designated *k2*) immediately upstream of the –35 element as the binding site for a *bba64*-specific DNA-binding activity (Indest & Philipp, 2000). This region is characterized by an IRS terminating in a poly-T tract. Presumably, the IRS is the site of interaction with the DNA-binding protein. Therefore, the IRSs were mutated, either singly (*A64p5' m–gfp*) or in combination (*A64p5' 3' m–gfp*) (Fig. 3). To further assess the importance of the *k2* region and any other potential regulatory sequences upstream of the promoter, another construct was generated in which the sequence upstream of the –35 was entirely deleted (*A64pmin–gfp*) (Fig. 3). The expression

of the marker *gfp* gene from these constructs was compared to the expression of *gfp* from a wild-type construct (*A64p–gfp*) and a promoterless construct (*gfp*) (Fig. 3). For the assay of gene expression, the promoter–*gfp* fusion constructs were assembled in the *E. coli*–*B. burgdorferi* shuttle vector pBSV2G. The shuttle vector constructs were introduced into *B. burgdorferi* B31 5A4NP1, a highly transformable and infectious strain (Kawabata *et al.*, 2004). Moreover, the presence of a kanamycin-resistance determinant on linear plasmid 25 (lp25) provides positive selection for the presence of this plasmid in transformants. Determinants on the lp25 plasmid have been shown to be essential for virulence (Grimm *et al.*, 2004; Labandeira-Rey & Skare, 2001; Purser & Norris, 2000). Therefore, all transformants were selected with kanamycin to ensure the presence of lp25.

For each construct, two transformants were examined. The expression of GFP was estimated by quantitative Western blotting. The samples were probed with Abs specific for GFP, BBA64, FlaB and RpoS. The GFP bands in individual samples were quantified by densitometry (Fig. 4a, panel GFP) and normalized to the corresponding FlaB bands (Fig. 4a, panel FlaB). The normalized values were then

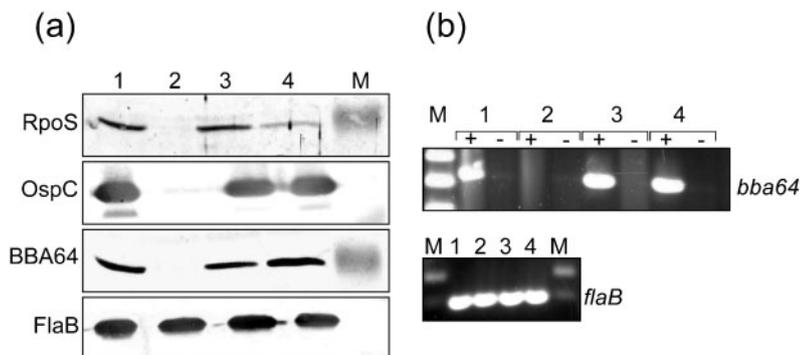


Fig. 2. BBA64 expression is dependent on RpoS. (a) Expression of the RpoS, BBA64, FlaB and OspC proteins was examined by Western blotting. (b) Expression of *bba64* and *flaB* mRNAs was examined by RT-PCR. In the case of *bba64*, the RT-PCRs for the test samples (+ lanes) as well as negative control reactions in which the reverse transcriptase was omitted (– lanes) are shown. Lanes: 1, B31 A3 wild-type; 2, B31 A3*rpoS*; 3 and 4, B31 A3*rpoS*/*rpoS*⁺ clones 1 and 2, respectively. M, protein (a) and DNA (b) markers.

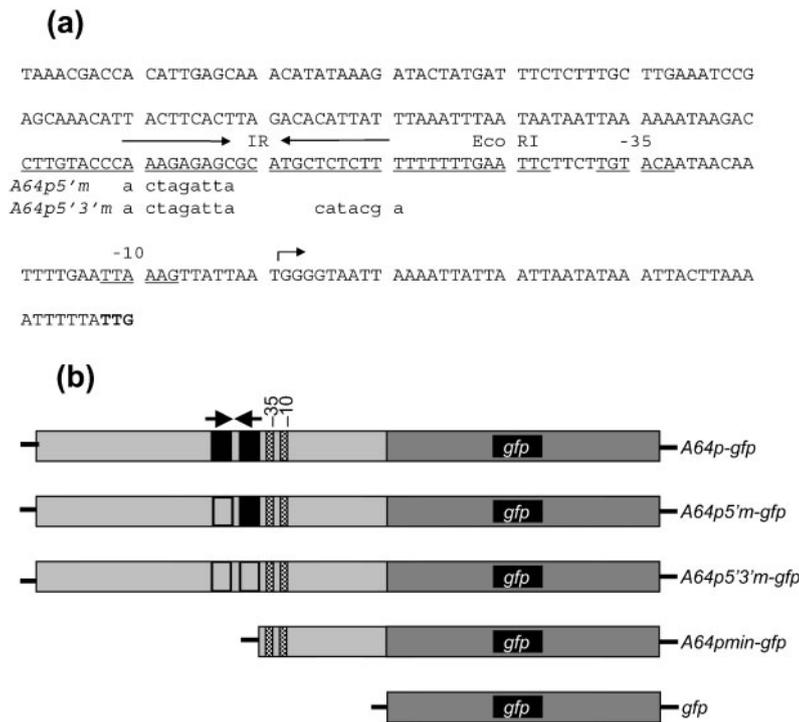


Fig. 3. (a) Sequence and features of the *bba64* upstream region. The transcription start site (bent arrow) (Indest *et al.*, 1997), the associated -10 and -35 elements (underlined), and the translation start codon (indicated in bold type) are shown. The IRSs are indicated by arrows above the *k2* DNA-binding region (underlined). The mutations in the two promoter variants *A64p5'm* and *A64p5'3'm* are shown below the corresponding wild-type sequence. The *A64pmin* promoter variant contains the sequence downstream of the *EcoRI* site (GAATTC) and includes the *EcoRI* site. (b) A diagrammatic representation of the different promoter constructs used in the study. The wild-type IRSs are indicated by filled boxes, whereas the open boxes indicate that these sites have been mutated.

expressed relative to the wild-type promoter construct (*A64p-gfp*) (Fig. 4b). As expected, no GFP expression was detectable in the absence of the *bba64* promoter (Fig. 4a, panel GFP, lane 1). In contrast, the GFP band was evident for all of the promoter constructs (lanes 2–4). However, surprisingly, the expression of GFP from the two mutant constructs *A64p5'm-gfp* and *A64p5'3'm-gfp*, as well as from the minimal promoter construct *A64pmin-gfp*, was similar to the level of expression derived from the wild-type promoter construct (*A64p-gfp*) (Fig. 4b). To further ensure that the levels reflected the true transcription potential of these fusions and were not the consequence of other determinants, these samples were also screened with Abs specific for BBA64 and RpoS. All samples were positive for both proteins, and more importantly, with the exception of the wild-type construct, which exhibited

slightly lower levels (75 % of that of the other samples) of both RpoS and BBA64, the levels of these two proteins were similar in all other samples, including the promoterless *gfp* fusion (Fig. 4a, panels RpoS and BBA64).

GFP from all promoter constructs is co-expressed with OspC in a subpopulation of spirochaetes

We showed that the expression of the *bba64* gene is RpoS dependent and that the expression of the endogenous *rpoS* and *bba64* genes in the transformants harbouring the various fusion constructs is similar. These results are consistent with RpoS also being involved in the expression of GFP from the fusion constructs. To further examine the nature of *gfp* expression from the various pBSV2G *bba64* promoter constructs, we resorted to confocal microscopy.

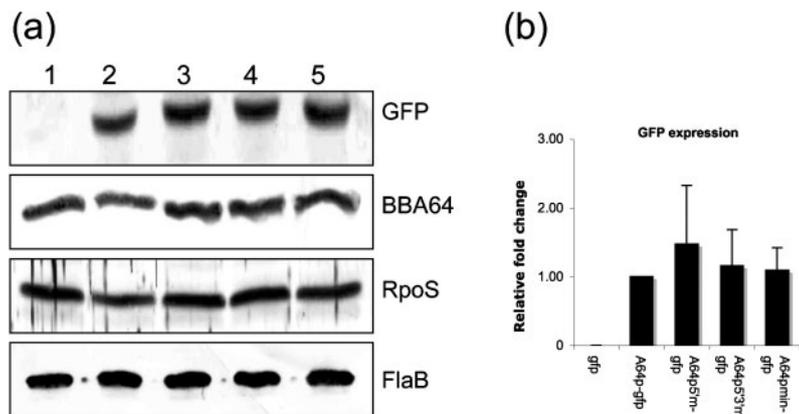


Fig. 4. Influence of the *bba64* upstream elements on GFP expression. (a) Expression of FlaB, GFP, BBA64 and RpoS was detected by Western blotting using specific Abs. For the detection of GFP, BBA64 and RpoS, sixfold greater volumes were loaded than the volume of sample used for the detection of FlaB. Lanes: 1, pBSV2G-gfp; 2, pBSV2G-A64p-gfp; 3, pBSV2G-A64p5'm-gfp; 4, pBSV2G-A64p5'3'm-gfp; 5, pBSV2G-A64pmin-gfp. (b) Relative expression of GFP from the different constructs. The SEM for each sample is indicated by the error bars.

The rationale for using confocal microscopy was based on the following observations. First, the expression of both *ospC* and *bba64* is dependent on RpoS (Hübner *et al.*, 2001; Fisher *et al.*, 2005; Yang *et al.*, 2005; this study). Second, only a proportion of cultured spirochaetes stain positive for expression from an *ospC* promoter (Carroll *et al.*, 2003) or OspC (our unpublished observations). We speculated that the expression of RpoS in cultured spirochaetes, for unknown reasons, is limited to a subpopulation, and consequently results in the selective expression of OspC. We therefore examined the populations of transformed spirochaetes for the expression of GFP, OspC and BBA64 proteins by confocal microscopy.

We first examined the relationship between BBA64, GFP and OspC expression at the population level using pBSV2G-A64p-gfp-transformed B31 5A4NP1 spirochaetes. Slides containing these spirochaetes were stained with an

anti-OspC mAb (Mbow *et al.*, 1999) followed by a rabbit polyclonal anti-*B. burgdorferi* Ab, and subjected to confocal microscopy. The Abs, dilutions and wavelengths used are listed in Supplementary Table S1. The expression of both GFP and OspC was found to be limited to a subpopulation of cells. The green fluorescence of GFP was noticeable in only some spirochaetes (Fig. 5, compare panel GFP and panel Bb) against a teeming background of spirochaetes that appeared negative for GFP [panel Bb+GFP; the overlap of GFP (green) and Bb (blue) appears as sea green]. Similarly, the expression of OspC was also restricted [panels OspC (red) versus Bb (blue), and Bb+OspC; overlap appears pink]. Most notably, cells with the OspC⁺ phenotype congregated with cells that exhibited a GFP⁺ phenotype (panel Bb+GFP+OspC; the overlap of the three colours appears as yellow staining). In the second experiment, we examined the relationship between OspC

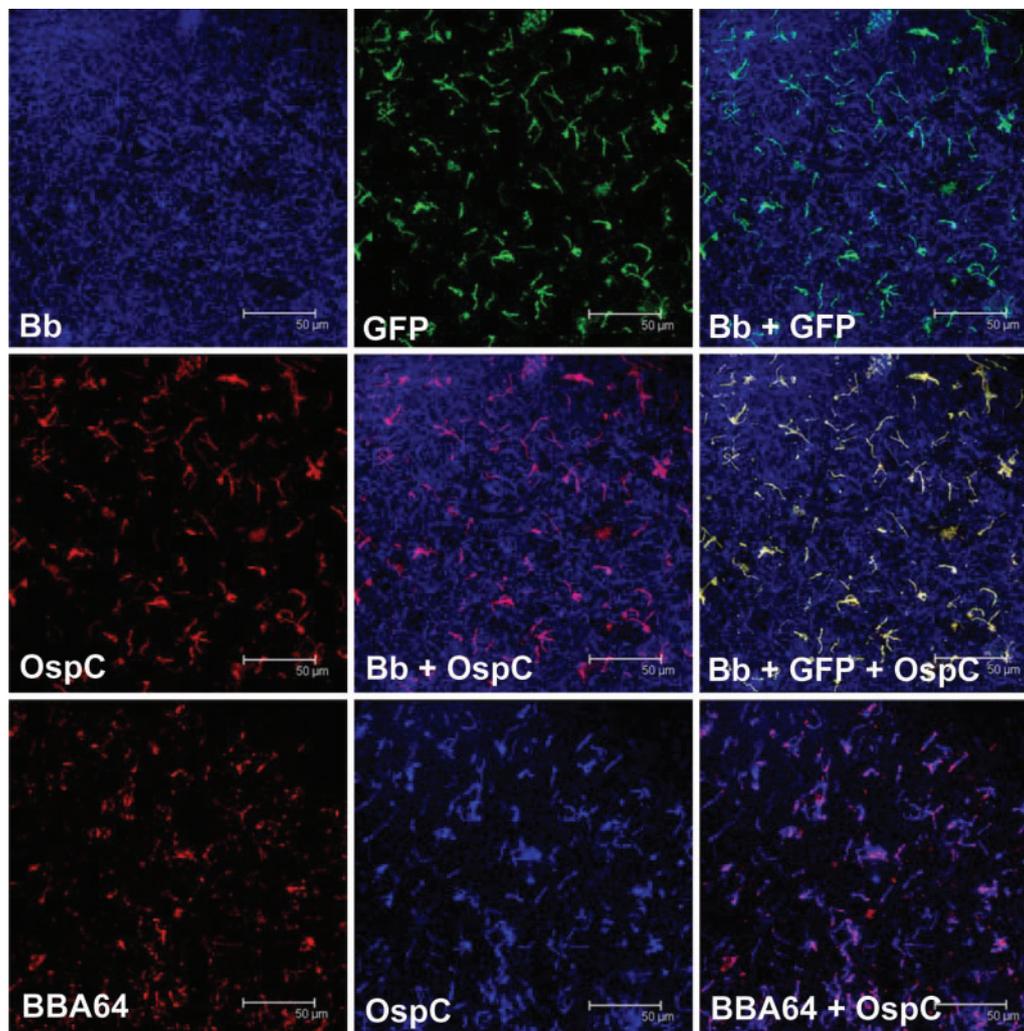


Fig. 5. Confocal microscopic imaging of spirochaetal populations expressing GFP, OspC and BBA64 proteins. Slides containing B31 5A4NP1/pBSV2G-A64p-gfp spirochaetes were stained with Abs specific for OspC (red) and *B. burgdorferi* (blue), or with Abs specific for BBA64 (red) and OspC (blue), and analysed by confocal microscopy. The individual images were merged to obtain composite images to visualize the co-expression of the proteins in individual cells.

and BBA64 in spirochaetal populations. Slides were stained first with the mouse anti-BBA64 Ab followed by the anti-OspC Ab. Again, only a limited number of spirochaetes appeared positive for BBA64 (panel BBA64, red) or OspC (panel OspC, blue), but more importantly, these two subpopulations were the same (panel BBA64 + OspC; the overlap appears as pink staining). Taken together, these results indicate that the same subpopulation of spirochaetes express all three proteins, GFP, BBA64 and OspC.

We next analysed spirochaetes harbouring the other promoter constructs to determine if the co-expression of GFP and OspC seen in B31 5A4NP1/A64p-gfp spirochaetes extended to the other spirochaetes as well. Slides were prepared from each of the five transformed clonal populations: promoterless *gfp*, *A64p-gfp*, *A64p5'm-gfp*, *A64p5'3'm-gfp* and *A64pmin-gfp*, and stained for OspC. As expected, GFP fluorescence was not detected in spirochaetes harbouring the promoterless *gfp* construct (Fig. 6, column 1 GFP or 1GFP + OspC). In contrast, GFP fluorescence was clearly visible in numerous spirochaetes transformed with all of the other *bba64* promoter constructs (Fig. 6, columns 2–5). Most notably, in all four cases, the same subpopulations stained positive for both

GFP and OspC (panels GFP + OspC). These results suggest that the expression of GFP and OspC shares a common feature that is maintained in all the GFP-expressing clones analysed in this study.

DISCUSSION

In this study, we characterized the expression of *bba64*, one of the well-known members of a family of genes that exhibit prolific differential expression in culture under different conditions that are meant to simulate the natural history of this organism. We examined the requirement for RpoS as well as that for an upstream region previously determined to be the site of binding of a *bba64*-specific DNA-binding protein. The requirement for RpoS was critically evaluated by complementing the B31 A3*rpoS* mutant with a wild-type copy of the gene targeted to the chromosome. Our study complements a recent comparison of global gene expression in strain B31 A3 and its isogenic *rpoS* mutant in which it was demonstrated that *bba64* gene expression is down-regulated in the *rpoS* mutant relative to the wild-type (Fisher *et al.*, 2005). A subsequent report found *bba64* to be constitutively

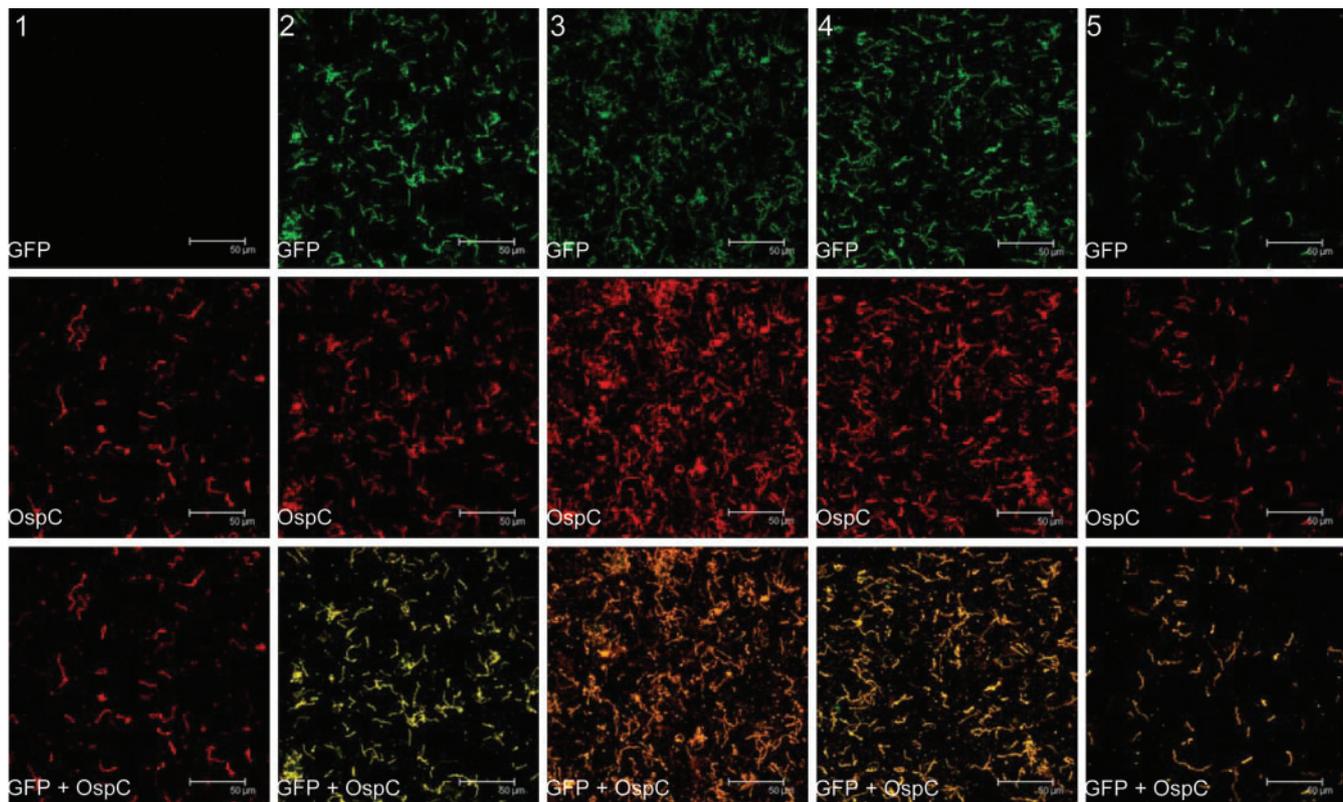


Fig. 6. Co-expression of GFP and OspC in subpopulations of spirochaetes transformed with the various promoter constructs. Slides containing the transformants listed below were stained with an anti-OspC Ab and imaged by confocal microscopy. The GFP and OspC images were merged to assess the expression of these proteins in individual cells. Columns: 1, pBSV2G-gfp; 2, pBSV2G-A64p-gfp; 3, pBSV2G-A64p5'm-gfp; 4, pBSV2G-A64p5'3'm-gfp; 5, pBSV2G-A64pmin-gfp.

expressed in the same strain with respect to the two culture variables tested, pH and temperature (Clifton *et al.*, 2006), both of which conditions influence the expression of RpoS (Hübner *et al.*, 2001; Yang *et al.*, 2000, 2003). Therefore, it was crucial to complement the RpoS defect for an unambiguous assessment of its role in the expression of *bba64*. Complementation of the *rpoS* mutant restored the expression of *bba64* to a level comparable to the wild-type level, thereby definitively establishing a requirement for RpoS for expression. Incidentally, to our knowledge, this is the first report of complementation of a *B. burgdorferi* *rpoS* mutant with a chromosomal copy of the wild-type gene. Using this strategy, we were fortunate to restore RpoS to nearly the same level as that observed in the wild-type parental strain. Although chromosomal integration may be challenging as compared to shuttle-vector transformation, it may be the ideal choice in certain cases by circumventing problems associated with plasmid maintenance and/or copy numbers. Finally, the *bb0472–bb0473* intergenic chromosomal target should prove useful for targeting other genes for complementation studies.

In addition to RpoS, one other factor may be involved in the expression of *bba64*. This factor is the putative DNA-binding protein previously demonstrated to specifically bind to the *k2* sequence upstream of the gene (Indest & Philipp, 2000). Surprisingly, however, mutations of the IRS, the most prominent feature within the *k2* region, failed to evoke any response *vis-à-vis* protein expression. Similarly, deletion of the entire upstream sequence beginning with the *k2* region also proved to have no effect. Therefore, the expression of GFP in culture appears to utilize just the *bba64* basal promoter. It is essential to note that in all cases, the expression of GFP was limited to the same subpopulation of cells that also expressed OspC. Phenotypic heterogeneity of OspC has been previously observed in spirochaetal populations during tick feeding (Schwan *et al.*, 1995; Schwan & Piesman, 2000) and in culture (Earnhart *et al.*, 2007). Since both OspC and BBA64 require RpoS for expression, it is tantalizing to speculate that in culture only a limited number of spirochaetes express RpoS, or alternatively express higher levels of RpoS, resulting in the observed phenotypic heterogeneity at the population level.

The passivity of the sequence upstream of the *bba64* basal promoter in cultured spirochaetes is similar to that reported for the *ospC* gene. In the case of *ospC*, a deletion of the sequence upstream of the promoter, which features an IRS, results in no effect on gene expression *in vitro* (Yang *et al.*, 2005; Xu *et al.*, 2007). Nonetheless, the IRS, subsequently dubbed the operator, assumes functional significance *in vivo*, wherein its presence is crucial for the suppression of OspC expression post-infection (Xu *et al.*, 2007). It is very likely that a DNA-binding protein is responsible for this suppression of *ospC*, although no such protein has yet been reported. In contrast to *ospC*, a DNA-binding protein specific to *bba64* has been shown to be present in cultured spirochaetes (Indest & Philipp, 2000).

However, the lack of any response from the *k2* region suggests that the reported *bba64*-specific DNA-binding protein is inactive in cultured spirochaetes under the conditions tested. Alternatively, the expression of the *bba64*-specific DNA-binding protein may be very low or absent in strain B31 5A4NP1, the focus of this study. Notwithstanding, based on its location downstream of the stop codon of *bba65*, it is very likely that the *k2* region with its IR element functions as a transcription terminator for *bba65*.

Two reports that are pertinent to the discussion of *bba64* regulation must be highlighted. Anguita *et al.* (2000) noted that the high-passage but infectious strain N40-P75 failed to express *bba64* and several other genes now known to be RpoS-regulated *in vivo* (Fisher *et al.*, 2005), despite a vigorous synthesis of OspC (Anguita *et al.*, 2000) and BBA64 (our unpublished observations) *in vitro*. The failure to induce gene expression appears to be unrelated to any gross loss of genetic material (Anguita *et al.*, 2000). Therefore, the simplest explanation for these observations is that the *in vivo* expression of RpoS or some other common factor is defective in this high-passage variant, leading to a broader loss of gene expression. A more recent investigation of gene expression during persistent infection of mice has revealed the down-regulation of *bba64* mRNA expression in the ear relative to that in cultured spirochaetes at all time points tested (Gilmore *et al.*, 2007), although importantly, unlike N40-P75, the down-regulation of *bba64* mRNA appears in this case to be specific, as the same tissue sample(s) exhibited an upregulation of *bba65* and *bba66*, two other RpoS-dependent genes (Fisher *et al.*, 2005). However, this loss of expression in the ear was countered by the expression of *bba64* elsewhere in the body, as these mice continued to harbour anti-BBA64 Abs throughout the course of infection. If these observations hold true, it suggests that *bba64* expression in the ear, and perhaps other organs, is repressed. Such repression may well involve the *k2* region and the putative *bba64*-specific DNA-binding protein.

The pattern of expression of *bba64* in culture in response to different environmental conditions and during infection of the vertebrate host points to a complex mode of regulation of *bba64*. Moreover, its expression pattern suggests an important function in establishing and maintaining infection in the vertebrate host. Given this importance, it is crucial to continue to explore the function and regulation of *bba64* expression and assess its role in virulence and pathogenesis. Finally, understanding the function and regulation of this molecule may also shed light on the orchestration of regulation of the other members of the *gbb54* gene family and their contribution to the overall molecular strategies of this pathogen.

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