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

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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...
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 5AANP1 (cp9 bba02:: kan) (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 10 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 × 10⁸ organisms ml⁻¹ and harvested at stationary phase (1–2 × 10⁹ 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 A3rpoS/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. The integration of the wild-type rpoS gene into the chromosome resulted in a hybrid bnpA promoter–aadA construct. The bnpA promoter (bnpAp) region was amplified with primers T79 and B25 and cloned into pQE30. The bnpAp region 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 bnpAp–aadA sequence. To target the rpoS gene to the BB0472–BB0473 intergenic locus on the chromosome, BB0472 and BB0473 sequences were cloned upstream of bnpAp–aadA and downstream of rpoS, respectively. The primers are listed in Table 1. The resulting plasmid, designated p472A2rpoS4573, was used to transform the B31 A3rpoS mutant by electroporation, as described elsewhere (Samuels, 1995). After overnight recovery, the electroporated spirochaetes were plated on semisolid BSK-H containing spectinomycin (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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>Location†</th>
<th>Gene(s)/plasmid</th>
<th>Use</th>
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<td>agtcgtagctcgagatcgatTAACAATGGTGGTGATGAGG</td>
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<td>+1 to +10</td>
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*Sequences homologous to genes are in upper-case type and unrelated sequences are in lower-case type. Functional restriction enzyme sites added elsewhere (Samuels, 1995).
†Locations are numbered with respect to the coding sequences of the corresponding genes.

Table 1. Primers used in this study

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-his-rpoS<sup>+</sup> 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 A3rpoS<sup>−</sup> and B31 A3rpoSrpoS<sup>−</sup> 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 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 OD<sub>600</sub> of 5, as described (Ramamoorthy et al., 1996). 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 A3rpoS (Elias et al., 2002), the same strain used in the earlier study. The complemented clone, B31 A3rpoSrpoS<sup>−</sup>, 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
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 (A64p59m–gfp) or in combination (A64p59m–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...
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 A64p5’9m–gfp and A64p5’3’9m–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.
The rationale for using confocal microscopy was based on the following observations. First, the expression of both \textit{ospC} and \textit{bba64} is dependent on RpoS (Hübner \textit{et al.}, 2001; Fisher \textit{et al.}, 2005; Yang \textit{et al.}, 2005; this study). Second, only a proportion of cultured spirochaetes stain positive for expression from an \textit{ospC} promoter (Carroll \textit{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 \textit{et al.}, 1999) followed by a rabbit polyclonal anti-\textit{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\textsuperscript{+} phenotype congregated with cells that exhibited a GFP\textsuperscript{+} 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 \textit{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.](http://mic.sgmjournals.org)
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

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**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 bbo472–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 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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