In Vivo Expression Technology Identifies a Novel Virulence Factor Critical for Borrelia burgdorferi Persistence in Mice

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**In Vivo Expression Technology Identifies a Novel Virulence Factor Critical for *Borrelia burgdorferi* Persistence in Mice**

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**Abstract**

Analysis of the transcriptome of *Borrelia burgdorferi*, the causative agent of Lyme disease, during infection has proven difficult due to the low spirochete loads in the mammalian tissues. To overcome this challenge, we have developed an *In Vivo Expression Technology* (IVET) system for identification of *B. burgdorferi* genes expressed during an active murine infection. Spirochetes lacking linear plasmid (lp) 25 are non-infectious yet highly transformable. Mouse infection can be restored to these spirochetes by expression of the essential lp25-encoded *pncA* gene alone. Therefore, this IVET-based approach selects for *in vivo*-expressed promoters that drive expression of *pncA* resulting in the recovery of infectious spirochetes lacking lp25 following a three week infection in mice. Screening of approximately 15,000 clones in mice identified 289 unique *in vivo*-expressed DNA fragments from across all 22 replicons of the *B. burgdorferi* B31 genome. The *in vivo*-expressed candidate genes putatively encode proteins in various functional categories including antigenicity, metabolism, motility, nutrient transport and unknown functions. Candidate gene *bbk46* on essential virulence plasmid lp36 was found to be highly induced *in vivo* and to be RpoS-independent. Immunocompetent mice inoculated with spirochetes lacking *bbk46* seroconverted but no spirochetes were recovered from mouse tissues three weeks post inoculation. However, the *bbk46* gene was not required for *B. burgdorferi* infection of immunodeficient mice. Therefore, through an initial IVET screen in *B. burgdorferi* we have identified a novel *in vivo*-induced virulence factor critical for the ability of the spirochete to evade the humoral immune response and persistently infect mice.

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**Introduction**

Lyme disease is a multi-stage inflammatory disease caused by the pathogenic spirochete *Borrelia burgdorferi*, which is transmitted by the bite of an infected tick [1]. *B. burgdorferi* has an enzootic life cycle that requires persistence in two disparate environments, the arthropod vector and the mammalian host. *B. burgdorferi* is well adapted to modulate its expression profile in response to the different conditions encountered throughout its infectious cycle [2]. Although the specific environmental signals that induce changes in spirochete gene expression are not fully defined, it has been reported that changes in temperature, pH, the presence or absence of mammalian blood, as well as changes in bacterial growth rate, can affect patterns of gene expression [2–8]. DNA microarray analysis and proteomics have been used to examine changes in the global expression profile of *B. burgdorferi* grown under *in vitro* conditions that partially mimic the tick and mouse environments [3–5]. A rat dialysis membrane chamber (DMC) implant model, together with microarray technology, has been used to help identify *B. burgdorferi* genes expressed in response to mammalian host-specific signals [7–10]. Although the data reported in these studies provide insight into the molecular mechanisms of gene regulation, they may not fully reflect the patterns of *B. burgdorferi* gene expression during an active mammalian infection. Furthermore, transcriptome analysis of *B. burgdorferi* during murine infection has proven difficult given that spirochete loads in the blood and tissues are too low to recover sufficient spirochete RNA for direct microarray analysis [11].

*In vivo* expression technology (IVET) is a gene discovery method used to identify transcriptionally active portions of a microbial genome during interaction of the microorganism with a particular environment or host organism [12,13]. In this system, the environment itself directly selects for upregulated bacterial loci [14]. The IVET selection system functions on the premise that deletion of a biosynthetic gene can lead to attenuation of growth and persistence of a pathogen in the host environment. This attenuation can be complemented by expression of the biosynthetic gene driven by promoters that are transcriptionally active *in vivo*. Thus, in the environment of interest, *in vivo* transcriptionally active promoters can be selected from a genomic library of DNA fragments cloned upstream of the essential biosynthetic gene [12–
Author Summary

Lyme disease is caused by tick-bite transmission of the pathogenic spirochete Borrelia burgdorferi. An increased understanding of how B. burgdorferi survives throughout its infectious cycle is critical for the development of innovative diagnostic and therapeutic protocols to reduce the incidence of Lyme disease. One of the major difficulties blocking this effort has been genome-wide identification of the B. burgdorferi genes that are expressed in the mammalian host environment. Using in vivo expression technology (IVET) in B. burgdorferi for the first time, we have identified B. burgdorferi genes that are expressed during an active murine infection. We demonstrate that candidate gene bkb46, encoded on essential linear plasmid 36, is highly expressed in vivo and, unlike some other known B. burgdorferi in vivo-induced genes, is not RpoS regulated. Spirochetes lacking bkb46 establish an infection in mice and elicit an antibody response but are undetectable in mouse tissues three weeks post inoculation. The bkb46 is not required for spirochete infection of mice lacking a functional immune system. In sum, development of an IVET-based approach in B. burgdorferi has identified a novel virulence gene critical for the spirochete’s ability to evade the mammalian adaptive immune response.

While B. burgdorferi varies depending on the environment from which the spirochetes are derived. For example, the 50% infectious dose (ID50) of spirochetes derived from partially fed ticks has been found to be two orders of magnitude lower than that of log phase in vitro grown B. burgdorferi [19]. In order to quantitatively assess the impact that adaptation to the mammalian environment has on B. burgdorferi infectivity the 50% infectious dose (ID50) of spirochetes derived directly from the mammalian host was determined and compared to that of log phase in vitro grown spirochetes. B. burgdorferi are only transiently present in the blood of immunocompetent mice [20], whereas spirochetes persist longer in the blood of immunocompromised mice [21]. Therefore, the blood of severe combined immunodeficiency (scid) mice infected with B. burgdorferi was used as a source of spirochetes adapted to the mammalian environment. Strikingly, an inoculum containing approximately eight in vivo-derived spirochetes was able to infect five out of six mice, whereas, 5,000 in vitro grown spirochetes were required to obtain this level of infectivity (Table 1). The ID50 for in vivo-derived spirochetes was found to be less than eight organisms. In contrast, the ID50 for in vitro grown spirochetes was calculated to be 660 organisms. These data indicate that mammalian host-adapted spirochetes are 100-fold more infectious than in vitro grown spirochetes, likely due to appropriate coordinate expression of in vivo-expressed genes important for murine infectivity.

The B. burgdorferi IVET system is a robust method for selection of B. burgdorferi sequences that are expressed during murine infection

We have developed a genome-wide genetic screening method to identify B. burgdorferi genes that are expressed during murine infection using an in vivo expression technology (IVET) approach [12,13]. The in vivo expression technology vector, pBbIVET, carries the B. burgdorferi bmpB Rho-independent transcription terminator sequence [22] repeated in triplicate (3XTT), to prevent any read-through promoter activity from the pBSV2* Borrelia shuttle vector backbone, followed by the promoter-less pncA essential gene (Figure 1) [23,24]. Spirochetes lacking linear plasmid lp25, which is non-infectious in mice and severely compromised in the tick vector [25–31]. The pncA gene, located on lp25, encodes a nicotinamidase that is sufficient to restore murine infectivity to B. burgdorferi lacking the entire lp25 plasmid [24]. Genetic transformation of low-passage, infectious B. burgdorferi occurs at low frequency and efficiency hampering introduction of

Results

Mammalian host-adapted spirochetes demonstrate a 100-fold decrease in ID50 relative to in vitro grown spirochetes

It is clear that B. burgdorferi modulates its gene expression profile at different stages of the infectious cycle [2]. The infectious dose of

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Table 1. In vivo-adapted B. burgdorferi are highly infectious.

| In vivo grown spirochetes | In vitro grown spirochetes |
|--------------------------|---------------------------|
| Spirochete grown spirochetes | Number of mice infected*/number of mice analyzed | Spirochete dose | Number of mice infected*/number of mice analyzed |
| 8 × 10⁸ | 6/6 | 5 × 10⁶ | 6/6 |
| 8 × 10⁷ | 6/6 | 5 × 10⁵ | 6/6 |
| 8 × 10⁴ | 5/6 | 5 × 10² | 2/6 |
| 8 × 10² | 1/6 | 5 × 10¹ | 0/6 |
| ID₅₀ | < 8 spirochetes | ID₅₀ | 660 spirochetes |

*Mouse infection was determined 3 weeks post inoculation by serological response to B. burgdorferi proteins and reisolation of spirochetes from ear, bladder and joint tissues.

The ID₅₀ was calculated according to method of Reed and Muench [83].

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a complex DNA library into an infectious background [32,33].

Because B. burgdorferi clones lacking lp25 and lp56 demonstrate increased transformability [34], we isolated a clonal derivative of the low-passage infectious clone A3 that lacks both lp25 and lp56. This clone was designated A3 68-1 [35]. Clones A3 and A3 68-1 were transformed by electroporation with 20 µg of a B. burgdorferi plasmid DNA library, in front of the promoterless pncA gene (Figure 1), creating plasmid pBbIVET-<i>pncA</i><sub>−</sub>. This plasmid, along with pBbIVET alone, was transformed into the non-infectious, low-passage, highly transformable B. burgdorferi clone A3 68-1. All clones were tested for their infectivity for different groups of C3H/HeN mice at an infectious dose 100 (ID<sub>100</sub>) of 1 x 10<sup>6</sup> spirochetes [37], indicating the presence or absence of an active promoter sufficient to drive expression of <i>pncA</i> thereby restoring infectivity. Spirochetes were reisolated from the ear, bladder and joint tissues of 5 of 6 mice infected with <i>B. burgdorferi</i> harboring pBbIVET-<i>pncA</i><sub>−</sub>. No spirochetes were reisolated from mice (0/6) infected with <i>B. burgdorferi</i> carrying the promoter-less pBbIVET alone. Together these data demonstrated that our promoter trap system functioned with a known in vivo active promoter.

**Screening for B. burgdorferi genes expressed during murine infection**

A B. burgdorferi genomic DNA library using an average DNA fragment size of approximately 200 bps was constructed upstream of the promoter-less <i>pncA</i> gene (Figure 1) in the pBbIVET vector in E. coli, yielding approximately 30,000 independent clones. A small subset of individuals from the 30,000 clone library in <i>E. coli</i> was analyzed by PCR and DNA sequencing and determined to carry non-identical <i>B. burgdorferi</i> DNA fragments. The strategy used to construct the pBbIVET library allowed the DNA fragments to be cloned in either the forward or reverse orientation relative to the <i>pncA</i> gene. Therefore, a library of 30,000 clones each harboring a unique 200 bp DNA fragment represented approximately 2 x coverage of the 1.5 Mb genome of <i>B. burgdorferi</i>. Although the initial analysis of the transformation efficiency of <i>B. burgdorferi</i> clone A3 68-1 demonstrated that each transformation of 20 µg of a single purified plasmid into this genetic background yielded approximately 10,000 transformants, this transformation efficiency was not achieved when 20 µg of complex library plasmid DNA was transformed into A3 68-1. Forty four transformations of the library plasmid DNA resulted in recovery of approximately 15,000 individual clones in <i>B. burgdorferi</i> A3 68-1, representing an IVET library in <i>B. burgdorferi</i> with approximately 1 x coverage of the spirochete genome. As described for the pBbIVET library in <i>E. coli</i>, a subset of individuals from 15,000 clone library in <i>B. burgdorferi</i> were analyzed and found to carry non-identical <i>B. burgdorferi</i> DNA fragments.

Like the BbIVET system described here, many IVET strategies are based upon complementation of auxotrophy. For microorganisms other than <i>B. burgdorferi</i> these strategies have allowed negative selection against “promoter-less” clones in minimal medium in which the auxotroph mutants are unable to grow [38]. <i>B. burgdorferi</i> lacking <i>pncA</i> are not attenuated for growth in the complex, undefined <i>B. burgdorferi</i> medium, BSKII. Moreover, there is currently no minimal medium available that supports the growth of wild-type <i>B. burgdorferi</i>. Therefore, the BbIVET system did not include negative selection against “promoter-less” clones in vitro. 179 mice were infected with pools from the <i>B. burgdorferi</i> IVET library of approximately 100 clones each, with each clone at a dose of 1 x 10<sup>6</sup> spirochetes resulting in a total dose of 1 x 10<sup>6</sup> spirochetes per mouse. Three weeks post inoculation, mice were sacrificed and ear, heart, bladder and joint tissues were harvested for reisolation of infectious spirochetes. 175 out of 179 mice became infected with <i>B. burgdorferi</i> as determined by reisolation of spirochetes from at least two or more of the tissue sites analyzed (Table 2). However, due to the potentially stochastic nature of the kinetics of infection [39] and/or tissue-specific promoter activity of distinct <i>B. burgdorferi</i> genomic fragments not all four tissue sites from all 175 infected mice were found to be positive for spirochete reisolation. Nonetheless, the recovery of live spirochetes from infected mouse tissues suggested that these spirochetes harbored in-vivo active promoter(s) in the pBbIVET plasmid sufficient to drive expression of the in-vivo essential <i>pncA</i> gene to restore spirochete mouse infectivity. Total genomic DNA was isolated from each pool of reisolated spirochetes from each of the four mouse tissues and the pBbIVET plasmid DNA rescued in <i>E. coli</i>. Colony PCR using primers targeting the genomic DNA insert region of the pBbIVET vector was performed on 24 of the resulting <i>E. coli</i> colonies from each plasmid rescue transformation. No reisolated spirochetes were found to harbor a pBbIVET plasmid lacking a genomic DNA fragment insert. The amplified inserts were analyzed by restriction digest using a cocktail of the A/T-rich restriction enzymes to identify those DNA fragments with distinct restriction patterns, suggesting that these fragments represent different in-vivo active promoters. Up to eleven non-identical restriction digest patterns were detected for every subset of 24 <i>E. coli</i> transformants carrying pBbIVET DNA that were analyzed (Figure S1). The DNA fragments corresponding to each distinct restriction digest pattern were further analyzed by DNA sequencing and the identities of the sequences determined by microbial genome BLAST analysis. Screening of approximately 15,000 BbIVET insertions identified 2,000 unique DNA fragments.
clones through mice resulted in the identification of 289 non-
identical *B. burgdorferi* in vivo-expressed (*Bbive*) DNA fragments from
across the chromosome and all 21 plasmid replicons of the *B.
burgdorferi* B31 segmented genome (Table 2). Although the 1:1
molar ratio of insert to vector used to generate the pbBIVET
library did not preclude insertion of more than one fragment into
each clone, only 20 out of the 289 clones were found to harbor two
distinct DNA fragments. Of these clones the 3′ DNA fragment,
proximal to the *pncA* ORF, was assumed to be the active promoter
and was included in the subsequent analyses.

*B. burgdorferi in vivo* expressed promoters map to
distinct classes of putative regulatory sequences across the
genome

Genomic mapping of the 289 unique *Bbive* promoters identified
in this genetic screen demonstrated that 67% of the sequences
mapped to sense DNA in the same direction as annotated open
reading frames, 27% mapped to antisense DNA in the opposite
direction to annotated open reading frames and 6% mapped to
intergenic regions lacking annotated open reading frames. Of the
large percentage of sense sequences, 41%, which represented
28% of the total *Bbive* sequences, mapped to regions just
upstream of and in the same orientation to annotated open
reading frames, suggesting that these sequences are promoters for
the associated open reading frames and that these open reading
frames are candidate in vivo-expressed genes. The remaining 59%
the other overlapping sequence pairs harbored distinct 3′ ends, suggesting that each
sequence contained a unique promoter. The 71 in vivo-expressed
candidate genes have been annotated to encode proteins in
various functional categories including: cell division, cell enve-
lope, replication, metabolism, motility, protein synthesis, trans-
port and unknown functions (Table 3).

**IVET identified candidate gene bbk46 on virulence
plasmid lp36**

Linear plasmid 36 is required for *B. burgdorferi* mouse infection;
however, the genetic elements on lp36 that contribute to this
phenotype have not been fully defined [37]. IVET identified a
candidate in vivo-expressed promoter sequence, *Bbive162*, which
mapped to lp36. This sequence was found to be 60 bp long, with
48 bp immediately upstream of and in the same direction as the
BBK46 open reading frame (Figure 2), suggesting that the *bbk46*
may be expressed during mammalian infection and may
contribute to the essential role of lp36 in *B. burgdorferi* infectivity.
Therefore, the *bbk46* gene was selected for further analysis.

**Expression of the *bbk46* gene is induced during murine
infection**

Our BbIVET screen identified gene *bbk46* as a putative in vivo-
expressed gene. The BbIVET screen was designed to identify *B.
burgdorferi* DNA fragments that are expressed in *vivo* and did not
discriminate between those promoters that are specifically induced
in *vivo* and those promoters that are expressed both in *vivo* and in
*vitro*. Therefore, quantitative reverse transcription PCR (qRT-
PCR) was used to validate the expression of *bbk46* in *vivo* and to
determine whether *bbk46* expression was upregulated in *vivo*
compared to *in vitro*. Total RNA was isolated from bladder tissue
collected from mice infected with 1×10⁷ wild-type *B. burgdorferi*
three weeks post-inoculation as well as log phase *in vitro* grown
spirochetes. RNA was converted to cDNA using random hexamer
primers and the mRNA level of each target gene was measured
relative to the constitutive *recA* gene using quantitative PCR. The
gene expression levels of flaB and ospC were also measured as
control constitutively-expressed and in *vivo*-induced genes, respec-
atively. These data demonstrated that although *bbk46* was expressed
during *in vitro* growth, expression of this gene was increased more
than 100-fold during mammalian infection (Figure 3A). Consistent
with their known patterns of gene regulation, flaB expression was
relatively unchanged in *vivo* compared to *in vitro* whereas, ospC

demonstrated a nearly 1000-fold increase in expression in *vivo*
compared to *in vitro* (Figure 3A). Moreover, the relative amount of
*bbk46* expression during *in vitro* growth was found to be
approximately 10-fold more than that of ospC. Whereas, the *in
vivo* expression levels of genes *bbk46*, *ospC* and *flaB* were similar.

**RpoS is a global regulator that controls expression of genes
expressed during mammalian infection, including *ospC* [2]. Because
*bbk46* expression was induced in *vivo* in a manner similar
to that of *ospC*, we sought to determine whether, like *ospC*, *bbk46* is an
RpoS-regulated gene. RNA was isolated from stationary phase
temperature-shifted wild-type and *ΔrpoS* mutant spirochetes, a
growth condition previously shown to induce expression of *rpoS*
and *rpoS*-regulated genes [42]. Quantitative RT-PCR was then
performed for genes *bbk46*, *flaB*, *ospC* and *recA*, as described above.
As expected, *ospC* expression was increased approximately 20
times in the presence compared to the absence of *rpoS* (Figure 3B).
In contrast, *bbk46* expression was RpoS-independent under these
growth conditions (Figure 3B). Likewise, no RpoS-dependent
change in gene expression was detected for *flaB*. Interestingly, the
Table 3. *B. burgdorferi* in vivo-expressed candidate genes organized by functional category.

| **Bbive clone** | **Replicon** | **ORF** | **Protein designation, Annotated function** |
|-----------------|--------------|---------|-------------------------------------------|
| **Cell division** |              |         |                                           |
| 289             | chromosome   | BB0715  | FtsA cell division protein                |
| **Cell envelope** |              |         |                                           |
| 15              | chromosome   | BB0213  | Putative lipoprotein                      |
| 94              | chromosome   | BB0760  | Gp37 protein                              |
| 175             | lp54         | BBA36   | Lipoprotein                               |
| 271             | lp54         | BBA57   | Lipoprotein                               |
| 297             | lp25         | BBE16   | RptA                                      |
| 151             | lp28-2       | BBG01   | Putative lipoprotein                      |
| 267             | lp38         | BBJ34   | Putative lipoprotein                      |
| 269             | lp38         | BBJ51   | ViE paralog, pseudogene                   |
| 162             | lp36         | BBK46   | Immunogenic protein P37, authentic frameshift |
| 77              | cp32-8, cp32-3, cp32-7, cp32-9, cp32-4, cp32-6, cp32-1 | BBB28, BBS30, BBO28, BBN28, BBQ35, BBR28, BBM28, BBP28 | Mlp lipoprotein family |
| **DNA replication** |              |         |                                           |
| 274             | chromosome   | BB0111  | DnaB replicative helicase                 |
| 226             | chromosome   | BB0632  | RecD exodeoxyribonuclease V, alpha chain  |
| 152             | lp28-3       | BBH13   | RepU replication machinery                |
| **Energy metabolism** |              |         |                                           |
| 62              | chromosome   | BB0057  | Gap glyceraldehyde-3-phosphate dehydrogenase, type 1 |
| 34              | chromosome   | BB0327  | Glycerol-3-phosphate O acyltransferase    |
| 44              | chromosome   | BB0368  | NAD(P)H-dependent glycerol-3-phosphate dehydrogenase |
| 47              | chromosome   | BB0381  | Trehalase                                 |
| 81              | chromosome   | BB0676  | Phosphoglycolate phosphate                |
| **Fatty acid and phospholipid metabolism** |              |         |                                           |
| 85              | chromosome   | BB0704  | AcpP acyl carrier protein                |
| **Motility and chemotaxis** |              |         |                                           |
| 14              | chromosome   | BB0181  | FlbF putative flagellar protein          |
| 29              | chromosome   | BB0293  | FlgB flagellar basal body rod            |
| 290             | chromosome   | BB0755  | Flagellar hook-basal body complex protein |
| 65              | chromosome   | BB0551  | CheY-1 chemotaxis response regulator     |
| 222             | chromosome   | BB0568  | Chemotaxis response regulator protein-glutamate methylesterase |
| **Prophage function** |              |         |                                           |
| 295             | cp32-8, cp32-7, cp32-1, cp32-3, cp32-6, cp32-4 | BBB23, BBO23, BBP23, BBS23, BBM23, BBR23 | Holin BlyA family |
| **Protein fate** |              |         |                                           |
| 193             | chromosome   | BB0031  | LepB signal peptidase I                  |
| **Protein synthesis** |              |         |                                           |
| 202             | chromosome   | BB0113  | RpsR ribosomal protein S18               |
| 216, 217        | chromosome   | BB0485  | RplP ribosomal protein L16               |
| 58              | chromosome   | BB0495  | RpsE 305 ribosomal protein S5             |
| 59              | chromosome   | BB0496  | 50S ribosomal protein L30                |
| 219             | chromosome   | BB0503  | RplQ ribosomal protein L17               |
| 232             | chromosome   | BB0660  | GTP-binding Era protein                  |
| 288             | chromosome   | BB0682  | TrmU tRNA (5'-methylaminomethyl-2-thiouridylate)-methyltransferase |
| **Regulation** |              |         |                                           |
| 208             | chromosome   | BB0379  | Protein kinase C1 inhibitor              |
| 50              | chromosome   | BB0420  | Hk1 histidine kinase                     |
amount of *flaB* expression detected in the stationary phase temperature-shifted spirochetes (Figure 3B) was dramatically decreased compared to the amount of *flaB* expression detected in log phase and *in vivo* grown spirochetes (Figure 3A), suggesting that *flaB* is not expressed at the same level under all growth conditions. Together these data demonstrated that *bbk46* was highly induced during murine infection and *bbk46* expression was not controlled by RpoS during *in vitro* growth.

| Nucleoside salvage | Replicon | ORF<sup>a</sup> | Protein designation, Annotated function<sup>c</sup> |
|--------------------|----------|----------------|--------------------------------------------------|
| 148                | lp25     | BBE07          | Pf5 protein, pseudogene                          |
| Transcription      | 1        | chromosome     | BB0389 RpoB DNA-directed RNA polymerase, beta subunit |
|                    | 287      | Chromosome     | BB0607 PcrA ATP-dependent DNA helicase           |
|                    | 84       | chromosome     | BB0697 RimM 16S rRNA processing protein          |
| Transport          | 204      | chromosome     | BB0318 MglA methylgalactoside ABC transporter ATP-binding protein |
|                    | 46       | chromosome     | BB0380 Mg<sup>2+</sup> transport protein         |
| Unknown            | 56       | chromosome     | BB0049 Hypothetical protein                      |
|                    | 69       | chromosome     | BB0063 Pasta domain protein                      |
|                    | 2        | chromosome     | BB0102 Conserved hypothetical                    |
|                    | 8        | chromosome     | BB0138 Conserved hypothetical                    |
|                    | 13       | chromosome     | BB0176 ATPase family associated with various cellular activities |
|                    | 23       | chromosome     | BB0265 Conserved hypothetical                    |
|                    | 212      | chromosome     | BB0428 Conserved hypothetical                    |
|                    | 52, 53   | chromosome     | BB0429 Conserved hypothetical                    |
|                    | 220      | chromosome     | BB0504 Conserved hypothetical                    |
|                    | 67       | chromosome     | BB0562 Conserved hypothetical                    |
|                    | 223      | chromosome     | BB0577 Conserved hypothetical                    |
|                    | 71       | chromosome     | BB0592 Caax amino protease family                |
|                    | 73       | chromosome     | BB0619 DHH family phosphoesterase function       |
|                    | 96       | chromosome     | BB0799 Conserved hypothetical                    |
|                    | 240      | cp26           | BBB27 Unknown essential protein                  |
|                    | 145, 146 | lp25           | BBE0036 Hypothetical protein                     |
|                    | 147      | lp25           | BBE01 Conserved hypothetical                     |
|                    | 265, 266 | lp38           | BB130 Conserved hypothetical                     |
|                    | 171      | lp38           | BB136 Conserved hypothetical                     |
|                    | 173      | lp38           | BB146 Conserved hypothetical                     |
|                    | 129, 296 | cp32-8, cp32-7, lp56, cp32-9, cp32-4 | BBL41, BBP40, BBO42, BBQ48, BBN41 Conserved hypothetical |
|                    | 130, 151 | cp32-8, cp32-1, cp32-6 | BBL42, BBP41, BBM41 Conserved hypothetical |
|                    | 117      | cp32-6, lp56, cp32-9, cp32-8, cp32-3, cp32-4 | BBM18, BBQ25, BBN18, BBL18, BBS18, BBP18, BBR18 Conserved hypothetical |
|                    | 182, 183 | lp56           | BBQ41 PF-49 protein                              |
|                    | 188      | lp56           | BBQ84.1 Conserved hypothetical                   |
|                    | 189      | lp56, lp28-3, lp17 | BBQ89, BBH01, BBD01 Conserved domain protein |
|                    | 244      | cp32-4, cp32-3, cp32-6, lp56, cp32-8, cp32-9 | BBRO5, BBNO5, BMM05, BBQ12, BBL05, BBO05, BPP05 Lyme disease protein of unknown function |
|                    | 157      | lp28-4         | BB007 Conserved hypothetical                     |

<sup>a</sup>In some cases two *Bbive* clones shared overlapping, non-identical sequence, as indicated by two *Bbive* clone numbers.

<sup>b</sup>ORF, open reading frame that maps just downstream and in the same orientation to the *Bbive* sequence.

<sup>c</sup>Annotation described by Fraser et al. [45].

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**Table 3. Cont.**

| *Bbive* clone<sup>a</sup> | Replicon | ORF<sup>b</sup> | Protein designation, Annotated function<sup>c</sup> |
|---------------------------|----------|----------------|--------------------------------------------------|

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The *bbk46* open reading frame fails to produce detectable amounts of protein during *in vitro* growth.

The *bbk46* gene is a member of paralogous gene family 75, which also includes *lp36*-encoded genes *bbk45*, *bbk48* and *bbk50*, all of which are annotated to encode putative P37 immunogenic lipoproteins [43–45] (Figure 4). These genes are located on the right arm of *lp36* in *B. burgdorferi* clone B31, which is a highly variable region among distinct *B. burgdorferi* isolates [44]. The members of paralogous gene family 75 are conserved within *B. burgdorferi* isolates but are not present in the relapsing fever spirochetes. The *B. burgdorferi* clone B31 *BBK45*, *BBK48* and *BBK50* proteins are predicted to be 301, 288 and 332 amino acids, respectively. However, *B. burgdorferi* clone B31 *bbk46* is annotated as a pseudogene as a result of an authentic frame shift resulting in a TAA stop codon at nucleotide 625 [43–45], thereby producing a putative 209 amino acid protein. In contrast, the *BBK46* homolog...
BBK46 protein production was assessed in both E. coli and B. burgdorferi. Immunoblot analyses using αFLAG and αMyc monoclonal antibodies resulted in detection of a FLAG-epitope tagged protein of an approximate molecular mass of 23 kDa, which is the predicted size of the 209 amino acid BBK46 protein, in the E. coli clones carrying both the flaB-driven and the bbbk46p-driven constructs (Figure 6). Surprisingly, no FLAG epitope tagged protein was detected in either B. burgdorferi clone (Figure 6), although bbbk46 gene expression was observed in these clones (data not shown), indicating that the lack of detectable BBK46 protein was not likely the result of a transcription defect. Furthermore, no cMyc epitope tagged protein was detected in either E. coli or B. burgdorferi. Together these data suggested that although the bbbk46 ORF is competent to produce a 23 kDa protein in E. coli and the transcript is expressed in B. burgdorferi during in vitro growth, the protein is either not produced or is rapidly turned over in log phase in vitro grown B. burgdorferi.

As a putative member of the P37 immunogenic lipoprotein family, BBK46 is predicted to localize to the spirochete outer surface and to be immunogenic during mammalian infection. Therefore, recombinant BBK46, lacking the first 32 amino acids that are predicted to comprise the signal sequence for the lipoprotein, was produced in E. coli as an N-terminal fusion to glutathione S-transferase (GST). To assess the immunogenicity of the BBK46 protein, immunoblot analysis was performed using purified rGST-BBK46 probed with mouse immune serum collected 21 days post inoculation with 1×10^6 wild-type B. burgdorferi. The rGST-BBK46 protein was found to be non-immunoreactive with mouse immune serum, in contrast to the control antigen BmpA (Figure S2). These data suggest that, if produced in B. burgdorferi, BBK46 is not an immunoreactive antigen. However, these data do not rule out the possibility that the immunogenic epitope is not present or available in the recombinant protein produced in E. coli.

The bbbk46 gene is required for B. burgdorferi persistence in immunocompetent mice

In vitro growth analysis demonstrated that the bbbk46 mutant and complemented clones had no detectable in vitro phenotypes (Figure 5C). Therefore, to examine the role of bbbk46 in mouse infectivity, groups of five C3H/HeN female mice were needle inoculated intradermally under the skin of the upper back with 1×10^6 wild-type, Δbbk46/vector or Δbbk46/bbk46 spirochetes. Three weeks post inoculation, mice were assessed for B. burgdorferi infection by serology and reisolation of spirochetes from the inoculation site, ear, bladder and joint tissues. All five mice from each infection group were seropositive for anti-B. burgdorferi antibodies (Figure 7, Table 4). Surprisingly however, no spirochetes were reisolated from all tissues examined from the five mice inoculated with the Δbbk46/vector clone (Table 4), whereas, all five mice inoculated with the wild-type or the Δbbk46/bbk46 clone resulted in reisolation of spirochetes from all tissues analyzed (Table 4). Together these data demonstrated that spirochetes lacking the bbbk46 gene transiently infected and elicited a humoral response in mice, but were unable to maintain a persistent infection in mouse tissues. To further define the contribution of the host immune response to the inability of spirochetes lacking the bbbk46 genes to cause a persistent infection, groups of five severe combined immunodeficiency (scid) mice were inoculated with 1×10^6 wild-type, Δbbk46/vector or Δbbk46/bbk46 spirochetes. Three weeks post inoculation the animals were assessed for infection by reisolation of spirochetes from the ear, bladder and joint tissues. Consistent with the hypothesized role of

Figure 3. Expression of the bbbk46 gene is upregulated during murine infection and is RpoS-independent. Total RNA was isolated from bladder tissue collected from (A) mice infected with 1×10^6 wild-type B. burgdorferi three weeks post-inoculation (in vivo, gray bars) and from log phase in vitro grown spirochetes (in vitro, white bars) or (B) stationary phase temperature-shifted stationary phase in vitro grown wild-type (white bars) or ΔrpoS (gray bars) B. burgdorferi. RNA was reverse transcribed to cDNA using random hexamer primers. The expression of bbbk46, flaB and ospC were quantified using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and a standard curve analysis method. The mRNA levels of the bbbk46, flaB and ospC gene transcripts were normalized to that of the constitutive recA gene. The data are expressed as the gene transcript/recA transcript. The data represent the average of triplicate qRT-PCR analyses of 3 biological replicates. Error bars represent the standard deviation from the mean.

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in clone N40, BD04, harbors a CAA codon at nucleotide 625, resulting in a glutamic acid residue at amino acid 209 and producing a putative 273 amino acid protein [46]. Sequence analysis of the cloned bbbk46 open reading frame confirmed the presence of the TAA stop codon at nucleotide 625 (Figure 2). To experimentally determine the size of the BBK46 protein produced in B. burgdorferi B31 the bbbk46 ORF along with a FLAG epitope tag sequence prior to the stop codon at nucleotide 625 and a cMyc epitope tag sequence prior to the stop codon at nucleotide 620 (Figure 2) was cloned into the B. burgdorferi shuttle vector pBSV2G under the control of either the constitutive flaB promoter or the putative endogenous bbbk46 promoter. A mutant clone lacking the entire BBK46 open reading frame was constructed by allelic exchange and verified by PCR analysis (Figure 5A and 5B). The bbbk46 mutant clone was transformed with the shuttle vectors carrying the epitope tagged bbbk46 constructs. All transformants were verified to contain the plasmid content of the parent clone.
bbk46 in immune evasion and persistence, four out of five immunodeficient mice inoculated with the Δbbk46 mutant were positive for spirochete reisolation from all tissues examined (Table 4). These data demonstrated that a functional host immune response is required for the clearance of spirochetes lacking bbk46 from mouse tissues 3 weeks post infection, indicating that bbk46 is essential for the ability of B. burgdorferi to avoid killing by the host immune system in order to establish a persistent infection.

**Discussion**

In this study we have successfully adapted and applied for the first time an IVET-based genetic screen for use in B. burgdorferi for the purpose of identifying spirochete genes that are expressed during mammalian infection. Historically, genetic manipulation of low passage, infectious B. burgdorferi has been challenged by the low transformation frequencies of these spirochetes, preventing application of classic in vivo genetic screening techniques such as in vivo expression technology (IVET) and signature-tagged mutagenesis (STM) [47] to identify B. burgdorferi genetic elements important for pathogenicity. However, advances in the understanding of the B. burgdorferi restriction modification systems that inhibit transformation [34,48–51] have recently allowed construction and characterization of a comprehensive STM mutant library in infectious B. burgdorferi [52]. The foundation for our strategy for development of IVET in B. burgdorferi was based upon the spirochete’s requirement of lp25 for both restriction modification and virulence functions. Spirochetes lacking lp25 are highly transformable but non-

![Amino acid alignment of the putative members of the immunogenic protein P37 family encoded on Ip36.](doi:10.1371/journal.ppat.1003567.g004)
infectious in mice [27–29,34]. Restoration of the lp25-encoded \textit{pncA} gene to lp25 \textit{spirochetes} restores wild-type infectivity [24] but maintains high transformation frequency. At the time of the development of the pBbIVET system the true start codon of the \textit{pncA} gene was not defined; therefore, the promoter-less \textit{pncA} gene construct in the pBbIVET plasmid used an engineered AUG start codon and was missing the first 24 nucleotides of the now defined \textit{pncA} ORF [23]. Furthermore, this construct was purposefully

**Figure 5.** Generation of the \( \Delta \text{bbk46} \) mutant and genetic complemented clones in \textit{B. burgdorferi}. (A) Schematic representation of the wild-type (WT) and \( \Delta \text{bbk46} \) loci on lp36. The sequence of the entire \( \text{bbk46} \) open reading frame was replaced with a \textit{flaB}_{p}-aadA antibiotic resistance cassette [37,82]. Locations of primers for analysis of the mutant clones are indicated with small arrows and labels P7–P12, P19 and P20. Primer sequences are listed in Table 5. (B) PCR analysis of the \( \Delta \text{bbk46} \) mutant clone. Genomic DNA isolated from WT and \( \Delta \text{bbk46} \) vector \textit{spirochetes} served as the template DNA for PCR analyses. DNA templates are indicated across the bottom of the gel image. The primer pairs used to amplify specific DNA sequences are indicated at the top of the gel image and correspond to target sequences as shown in A. Migration of the DNA ladder in base pairs is shown to the left of each image. (C) \textit{In vitro} growth analysis of mutant clones. A3-68\textit{BBE02} (WT), \( \text{bbk46}:\text{flaB}_{p}-\text{aadA}/p8SV2G (\Delta \text{bbk46}/\text{vector}) \) and \( \text{bbk46}:\text{flaB}_{p}-\text{aadA}/p8SV2G-\text{bbk46} (\Delta \text{bbk46}/\text{bbk46}^{+}) \) \textit{spirochetes} were inoculated in triplicate at a density of \( 1 \times 10^{9} \) spirochetes/ml in 5 ml of BSKII medium. Spirochete densities were determined every 24 hours under dark field microscopy using a Petroff-Hausser chamber over the course of 96 hours. The data are represented as the number of spirochetes per ml over time (hours) and is expressed as the average of 3 biological replicates. Error bars indicate the standard deviation from the mean.

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**Figure 6.** BBK46 protein production is detectable in \textit{E. coli} but not in \textit{B. burgdorferi}. Immunoblot analysis of total protein lysate prepared from \( 1.5 \times 10^{8} \) \textit{B. burgdorferi} \( \text{bbk46} \) (Bb) or \textit{E. coli} harboring either pBSV2G \( \text{flaB}_{p}-\text{bbk46}-\text{FLAG-cMyc} (\text{flaB}_{p}) \) or pBSV2G \( \text{bbk46}_{p}-\text{bbk46}-\text{FLAG-cMyc} (\text{bbk46}_{p}) \). Protein lysates were separated by SDS-PAGE and immunoblots performed using anti-FLAG monoclonal antibodies (α FLAG) and anti-cMyc monoclonal antibodies (α cMyc). 300 ng of purified PncA-FLAG [23] and GST-BmpA-cMyc [77] proteins served as positive controls (+) for each antibody. The positions of markers to the left of the panel depict protein standard molecular masses in kilodaltons.

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**Figure 7.** Spirochetes lacking \( \text{bbk46} \) retain seroreactivity in mice. Immunoblot analysis of sera collected three weeks post inoculation from groups of five C3H/HeN mice inoculated with clone A3-68\textit{BBE02} (WT), \( \text{bbk46}:\text{flaB}_{p}-\text{aadA}/p8SV2G (\Delta \text{bbk46}/\text{vector}) \) and \( \text{bbk46}:\text{flaB}_{p}-\text{aadA}/p8SV2G-\text{bbk46} (\Delta \text{bbk46}/\text{bbk46}^{+}) \) \textit{spirochetes} per mouse. (A) Total protein lysate from \textit{B. burgdorferi} clone B31 A3 was probed with the serum from each individual mouse (1–5). (B) Purified recombinant GST-OspC protein was probed with pooled sera from the five mice in each infection group or αOspC polyclonal antibodies. The positions of markers to the left of the panel depict protein standard molecular masses in kilodaltons.

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The B. burgdorferi genome. Unexpectedly, the well characterized in vivo-expressed ospC promotor was not among these sequences. However, the ospC\textsubscript{p} was successfully recovered in our functional validation of the BbIVET system, suggesting that the BbIVET screen had not reached complete saturation of the genome and with further screening of the BbIVET library the \textit{ospC\textsubscript{p}} sequence may be recovered. Alternatively, given that \textit{ospC} expression is known to be down-regulated after the initial stages of infection [11,53–56] it is possible that in the context of a mixed infection individual pBbIVET clones carrying the \textit{ospC\textsubscript{p}} lack a fitness advantage due to decreased expression three weeks post inoculation and may not be recovered in our screen. This explanation may appear to conflict with the findings reported herein that \textit{ospC} expression is high at three weeks post inoculation and the \textit{ospC\textsubscript{p}} served as a robust positive control promotor for the BbIVET system. However, down-regulation of \textit{ospC} expression at this time point in infection is a stochastic process that occurs at the level of the individual spirochete and does not occur simultaneously across the entire population [55]. Although at the population level the \textit{ospC\textsubscript{p}} is expressed at this time point in our studies, in the context of the BbIVET screen individual clones carrying the \textit{ospC\textsubscript{p}} may express reduced amount of \textit{pncA} and may be out competed by other BbIVET clones carrying stronger promoters.

A subset of the genes identified in the BbIVET screen included known in vivo-expressed genes, which provided validation that our genetic system was working as expected and was sufficiently powerful. The screen recovered the promotor for genes \textit{bba36 (Bbive175)}, \textit{bba57 (Bbive271)}, \textit{bhh27 (Bbive240)}, \textit{bhh34 (Bbive267)}, \textit{bhh36 (Bbive171)}, \textit{bhh51 (Bbive269)}, \textit{bb0213 (Bbive15)} and \textit{bb0760 (Bbive94)}, all of which have been shown previously to be expressed during mammalian infection [11]. Furthermore, \textit{bba57} was recently reported to be up-regulated in vivo and to contribute to pathogenesis in the mouse [57]. The \textit{bptA} gene encodes a function that has been shown to be required for \textit{B. burgdorferi} survival in the tick and to contribute to mouse infectivity [30,31]. In addition, \textit{Bbive144, 38, 232, 84, 269, 293} and \textit{77} are associated with genes that have been shown to be up-regulated in in vivo-like conditions and/or gene products that are immunogenic in humans and mice [5,8,38,59]. Notably, few in vivo-expressed candidate genes identified using BbIVET were previously observed to be up-regulated in mammalian host-adapted spirochetes derived from growth within rat dialysis membrane chambers (DMCs). Genes identified in our analyses that have also been detected by microarray analysis of DMC grown spirochetes include \textit{bba36}.
specifically selects for promoters that are capable of driving during an active infection. Finally, the BbIVET system DMCs may not fully reflect those experienced by candidate genes. Similarly, only one putative BosR-regulated in vitro bb0592 by the BbIVET screen. Rp oS-regulated genes RpoS-regulated promoters were recovered, the recently identified expression of pncA allowing the spirochetes to survive throughout a three week mouse infection. Together, these technical and biological differences between the DMC microarray and BbIVET screen likely contributed to the distinct results obtained from the two methods of gene expression analysis. In addition, few genes that have been previously established to be RpoS-regulated in vitro and/or within DMCs [10,60] were identified by the BbIVET screen. RpoS-regulated genes bha36, bha57, bb0263 and bb0601 [10,60] were among the in vivo-expressed Bbivc candidate genes. Similarly, only one putative BosR-regulated gene, bb0592 [61], was identified in the BbIVET screen. Although it is unclear why only a small number of know RpoS-regulated promoters were recovered, the recently identified AT-rich BosR binding site [61] contains the restriction site for the Tsp509I restriction enzyme used to generate the BbIVET library. Therefore, it is possible that the BosR binding sites were subject to cleavage by Tsp509I, perhaps resulting in a limited number of DNA fragments that contained BosR-dependent promoters.

The BbIVET screen was carried out in such a way that both DNA fragments that are expressed in vitro and in vivo, as well as those fragments that are specifically induced in vivo, could be recovered. Therefore, it was not surprising that genes encoding cell division, DNA replication, energy metabolism, protein synthesis and transcription functions were identified, all of which are likely functions essential for spirochete growth under all condition. These findings were consistent with those categories of genes not recovered by genome-wide transposon mutagenesis, suggesting that these genes encode essential functions [52]. The BbIVET screen identified genes that encode proteins in functional categories that may contribute to B. burgdorferi infectivity and pathogenesis including, putative lipoproteins, motility and chemotaxis proteins, transport proteins and proteins of unknown function. Similarly, transposon mutagenesis analysis indicated that motility and chemotaxis genes as well as transport genes are important for B. burgdorferi survival in the mouse [52].

Linear plasmid 36 is known to be critical for B. burgdorferi survival in the mouse; however, the genes on lp36 that contribute to this requirement have not been fully characterized [37]. The recently published comprehensive STIM study suggests that many of the genes encoded on lp36 participate in B. burgdorferi infectivity [37,52]. BbIVET identified gene bbb46 on lp36. We found that bbb46 was expressed both in vitro and in vivo. However, bbb46 expression was dramatically induced in spirochetes isolated from infected mouse tissues as compared to spirochetes grown in vitro, suggesting a possible role for this gene in B. burgdorferi infectivity. Moreover, consistent with lack of identification of bbb46 as an RpoS-regulated genes in previous studies of the RpoS regulon [10,62], control of bbb46 expression was found to be RpoS-independent under in vitro growth conditions that typically induce expression of rpoS regulated genes [4,8,42,60]. These findings highlight the power and uniqueness of the IVET-based approach for identification of B. burgdorferi in vivo-expressed genes, which might not be discovered using other genome-wide gene expression methods. Surprisingly, BBK46 protein was not detected in spirochetes expressing FLAG epitope tagged bbb46 under the control of the putative native promoter or the constitutive flaB promoter. Moreover, sera from B. burgdorferi infected mice were non-immunoreactive against recombinant Bbb46 protein. In support of these data, no peptide corresponding to BBK46 has been detected in genome-wide proteome analysis of B. burgdorferi under different environmental conditions [63]. Our findings suggest that despite high gene expression, the encoded Bbb46 protein is produced at low levels in the spirochete and/or BBK46 is rapidly turned over in the cell. Alternatively, bbb46 may function as an RNA. The molecular nature of the functional product of bbb46 is currently under investigation.

Deletion of bbb46 from low-passage, infectious B. burgdorferi resulted in no observable in vivo growth defect. Immunocompetent mice needle inoculated with spirochetes lacking bbb46 were found to be seropositive for B. burgdorferi antibodies three weeks post-infection, although the serological responses appeared to be slightly diminished relative to those of mice infected with the wild-type and complemented clones. Surprisingly, however, no live spirochetes were reisolated from all tissues examined from the mutant infected mice at this same time point. Conversely, all mice infected with the wild-type or complemented clone were both seropositive and reisolation positive. Furthermore, bbb46 was not required for spirochete survival in immunocompromised mice. These data indicate that bbb46 is dispensable for the initial stages of B. burgdorferi murine infection but this gene is essential for B. burgdorferi persistence in mouse tissues and may contribute to a mechanism of spirochete evasion of host-acquired immune defenses.

B. burgdorferi survival in the mammalian host requires diverse mechanisms that allow the spirochete to resist and evade the host’s immune responses. However, the genetic components of these important properties of the pathogen have yet to be well defined. Here we demonstrate that spirochetes lacking bbb46 establish an initial infection and are seroreactive but are unable to persist in murine tissues following host antibody production. To our knowledge a similar phenotype has been documented for only two other B. burgdorferi genes, the lp28-1 encoded vls antigenic variation locus [27,29,64–66] and the chromosomally encoded lmp-1(bb0210) gene [67]. Moreover, analogous to the bbb46 mutant, the phenotypes of spirochetes lacking a functional vls locus as well as spirochetes lacking lmp-1 have been shown to be dependent on the host immune response as these mutants demonstrate wild-type survival under immune privileged growth conditions and in immunocompromised mice [27,29,65–67]. Although it is clear that the antigenic switching mechanism conferred by the vls locus is essential for B. burgdorferi persistence in the host [65,66], the precise mechanism of vls-dependent immune evasion remains unknown. Similarly the mechanism of lmp-1-dependent protection of B. burgdorferi against the host’s humoral immune response is unknown [67]. VlsE and Lmp-1 are highly antigenic proteins present on the outer surface of the spirochete [67–69]. The BbK46 open reading frame appears to encode a lipoprotein with a predicted signal sequence for outer cell surface localization; however, recombinant BBK46 protein produced in E. coli was not found to be seroreactive when analyzed by immunoblot using immune sera collected from mice infected with wild-type B. burgdorferi. Future studies are focused on elucidation of the role of bbb46 in the pathogenesis of B. burgdorferi.

In conclusion, we have developed and applied the IVET technology to B. burgdorferi to identify spirochete genes expressed
IVET in *B. burgdorferi*

during mammalian infection. This represents the first use of this system in *B. burgdorferi*. The power of this system was validated by identification of a subset of genes that have been demonstrated previously to be upregulated *in vivo*. Furthermore, IVET identified *bbk46*, a novel, uncharacterized gene located on essential virulence plasmid lp36. We have presented evidence that *bbk46* is highly upregulated during *B. burgdorferi* murine infection and is critical for the spirochete’s ability to persistently infect immunocompetent mouse tissues. Further analysis of the molecular mechanism of *bbk46*-promoted survival, as well as identification and characterization of other putative virulence factors identified by BbIVET, will contribute to advancing understanding of *in vivo* persistence and pathogenicity of *B. burgdorferi*.

### Materials and Methods

#### Ethics statement

The University of Central Florida is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Protocols for all animal experiments were prepared according to the guidelines of the National Institutes of Health and were reviewed and approved by the University of Central Florida Institutional Animal Care and Use Committee (Protocol numbers 09-38 and 12-42).

**Bacteria clones and growth conditions**

All *B. burgdorferi* clones used were derived from clone B31 A3. Clone A3 68-1, which lacks lp25 and lp56 [55] was used for the pBbIVET library. The B31 A3 wild-type and *rpoS*::kan *B. burgdorferi* clones [70] were used for gene expression experiments. All low-passage *B. burgdorferi* mutant and complemented clones generated herein were derived from infectious clone A3-68ABBE02, which lacks cp9, lp56 and gene *bbf02* on lp25 [51]. *B. burgdorferi* was grown in liquid Barbour-Stoenner-Kelly (BSK) II medium supplemented with gelatin and 6% rabbit serum [71] and plated in solid BSK medium as previously described [72,73]. All spirochete cultures were grown at 35°C supplemented with 2.5% CO2. Kanamycin was used at 200 μg/ml, streptomycin

### Table 5. List of primers used in this study.

| Primer number | Designation          | Sequence (5’ – 3’)a |
|---------------|----------------------|---------------------|
| 1             | pncA 5’ EcoRi A      | cggaattcatgGCCTATATTAAAATAGATATAC |
| 2             | pncA 3’ XbaI         | gctctagatTTATATAAAGCTTACTTGGTCGTG |
| 3             | ospC prom 5’ EcoRi   | cggaattctTCTTTTTTCATAAATTGGCTCC |
| 4             | ospC prom 3’ EcoRi   | cggaattctTTATTTAGCACATATTTGGCTTATGTCG |
| 5             | pUC18R BSV2          | AGGGATAAACATTTTCAACAG |
| 6             | pncA prom 3’ seq     | ACTGTATGATACTGGCAAAAGTGCC |
| 7             | bbbk46Fup500         | GTTCTTTTTTAGGACAGAACAATTA |
| 8             | bbbk46Rup500         | CGGGAGCACAAAGAGGGAGACGACATCTCAGCTCTAGATGTCG |
| 9             | bbbk46Fdown500       | GCCGAGATCACCAGGTAGTCGGAATAATAATACATATCAGATAGCTACGTTT |
| 10            | bbbk46Rdown500       | CTAGAAGCCTACTTGGTCGTG |
| 11            | flaBpaadA F          | TGTCTGTGGCTTCTTTTGTTG |
| 12            | flaBpaadA R          | TTATTTGCCGACTAATTTGGTG |
| 13            | K46’5’kpn1fwd        | cgggtaccTTTCTCAGTTGATCTTTAGTTT |
| 14            | K46’3’FLAGrev        | TTATTTTATCTTTTTATAATGTCG |
| 15            | K46’5’FLAGfwd        | gattataagatgatgatgataaaaAAATTAGCTTTAAAAGGAAATTGATGTT |
| 16            | K46’3’C-mycSalrev    | acgcttgatcTTTCGCGATTTTCGTTTTTATTTTTTTTTTTTTTTTTTTTTTTT |
| 17            | K46’3’PCR3fwd        | CCGGGATCCCTCTCAGTTGAG |
| 18            | K46’3’PCR3rev        | CCGGGATCCCTCAGCTTCAATGTAC |
| 19            | Lp3629018F           | AGCATTATTGTTTCTAGGCT |
| 20            | Lp3629013R           | ACATACTAGACCAAAACAGTC |
| 21            | flaBF3               | GCATTACGCGTCTAATCTTTAG |
| 22            | flaBR3               | GCATTACGCGTCTAATCTTTAG |
| 23            | recA F               | AATAAGGATGGAGATTGTTG |
| 24            | recA R               | GAACCTCAACGTTAAGAGATG |
| 25            | ospC1 F              | AGCGGATTCTAATGCGTCTT |
| 26            | ospC1 R              | CAATAGCTATAGCAATGTTCTAG |
| 27            | flaBp 5’ KpnI        | gggggatcTTTGCTGTCCGGCCTTTGTTGGCT |
| 28            | flaBp 3’ BamHI       | gggggatccTTTGCTGTCCGGCCTTTGTTGGCT |
| 29            | bbbk46+5’ BamHI      | cggggattcTTTGCTGTCCGGCCTTTGTTGGCT |
| 30            | bbbk46-5’ BamHI      | cggggattcTTTGCTGTCCGGCCTTTGTTGGCT |
| 31            | bbbk46 3’ XhoI R     | ccggcttcgagTTAATAACGAGCCTTCAATGTATGTTTATAG |

*aLowercase indicates all non-*B. burgdorferi* sequence.

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was used at 50 μg/ml and gentamicin was used at 40 μg/ml, when appropriate. All cloning steps were carried out using DH5α E. coli, which were grown in LB broth or on LB agar plates containing 50 μg/ml kanamycin, 300 μg/ml spectinomycin or 10 μg/ml gentamicin.

Generation of the pBbIVET plasmid

The promoterless pncA gene was amplified from B. burgdorferi B31 genomic DNA using primers 1 and 2 (Table 5) and Taq DNA polymerase (New England Biolabs). The EcoRI/XbaI-digested pncA fragment was cloned into EcoRI/XbaI-linearized plasmid pBSV29TT [23], creating plasmid pBbIVET. The in vivo-expressed ospC promoter with EcoRI ends was amplified from B. burgdorferi B31 genomic DNA using primers 3 and 4 (Table 5) and cloned into the EcoRI-cut, Antarctie phosphatase-treated (New England Biolabs) pBbIVET plasmid in front of the promoterless pncA gene, resulting in plasmid pBbIVET ospC. All plasmids were analyzed and verified by restriction digest and sequence analysis. The pBbIVET and pBbIVET ospC plasmids were each transformed by electroporation into A3 68-1 [35] as described [37] and transformants selected in solid BSK medium containing kanamycin, resulting in approximately 30,000 independent clones. Thirty-four transformants were chosen at random from each plasmid rescue and colony PCR performed using primers 5 and 6 (Table 5) to amplify the in vivo-expressed DNA fragment. PCR products were subsequently digested with a cocktail of restriction enzymes (DraI, SspI and AseI) and visualized on a 1% agarose gel. Approximately 14,000 E. coli clones were analyzed in this manner. All unique BbIVET fragments, as determined by the restriction digest pattern (Figure S1), were analyzed by direct sequencing of the PCR product using primer 5. Each individual sequence was identified by blastn analysis and mapped to its location in the B. burgdorferi B31 genome.

Generation of the BbIVET library

Total genomic DNA was isolated from a 250 ml culture of B. burgdorferi B31 clone A3 grown to a density 1 × 10⁸ spirochetes/ml using the Qiagen genomic DNA buffer set and Genomic-tip 500/G, according to the manufacturer’s protocol (Qiagen). A3 genomic DNA was partially digested with Tsp509I (New England Biolabs). The partial digests were electrophoretically separated on a 0.8% agarose gel and the 300 to 500 bp range of DNA fragments extracted and ligated in a 1:1 molar ratio with EcoRI-digested and Antarctie phosphatase-treated pBbIVET. Library ligations were electroporated into E. coli Top10 cells (Life Technologies) and transformants selected on LB agar containing 30 μg/ml kanamycin, resulting in approximately 30,000 independent clones. Plasmid DNA was isolated from these cells and 20 μg aliquots of the plasmid library were transformed by electroporation into B. burgdorferi A3 68-1, as previously described [73]. One fifth of each transformation was plated on solid BSK medium containing kanamycin. B. burgdorferi pBbIVET colonies were verified to contain B. burgdorferi DNA fragments by PCR using primers 5 and 6 (Table 5) and the number of transformants recovered quantitated. The approximately 15,000 B. burgdorferi clones recovered over 40 transformations were stored in aliquots of pools of approximately 100 BbIVET clones each in 25% glycerol at −80°C.

Selection of B. burgdorferi clones having in vivo-expressed DNA fragments

Each BbIVET pool (~100 clones) was grown in 10 ml of fresh BSKII medium to a density of 1 × 10⁸ spirochetes/ml. In groups of approximately 20 animals, 144 6-8 week old C3H/HeN female mice were each inoculated (80% intraperitoneal and 20% subcutaneous) with a dose 1 × 10⁸ spirochetes of a unique pool of ~100 BbIVET clones, under the assumption that each clone was present at dose 1 × 10⁴ spirochetes. A fraction of each inoculum was plated on solid BSK medium and colonies screened for the presence of virulence plasmid lp28-1. Three weeks post inoculation, spirochetes were reisolated from ear, heart, bladder and joint tissues in 10 ml BSKII medium containing 20 μg/ml phosphomycin (Sigma), 50 μg/ml rifampicin (Sigma) and 2.5 mg/ml amphotericin B (Sigma) in 0.2% dimethyl sulfoxide (Sigma). Total genomic DNA was isolated from each spirochete cultures using the Wizard genomic DNA purification kit (Promega) and transformed into chemically competent E. coli DH5α cells and colonies selected on LB agar containing kanamycin to recover the pBbIVET plasmids. Twenty-four transformants were chosen at random from each plasmid rescue and colony PCR performed using primers 5 and 6 (Table 5) to amplify the in vivo-expressed DNA fragment. PCR products were subsequently digested with a cocktail of restriction enzymes (DraI, SspI and AseI) and visualized on a 1% agarose gel. Approximately 14,000 E. coli clones were analyzed in this manner. All unique BbIVET fragments, as determined by the restriction digest pattern (Figure S1), were analyzed by direct sequencing of the PCR product using primer 5. Each individual sequence was identified by blastn analysis and mapped to its location in the B. burgdorferi B31 genome.

Deletion of bbk46

We used a PCR-based overlap extension strategy to delete the bbk46 gene. A spectinomycin/streptomycin resistance cassette, flaBp-aadA [74] with blunt ends, was amplified from genomic DNA isolated from clone ΔguaAB [35] using Phusion High-fidelity DNA polymerase (Thermo Scientific) and primers 11 and 12 (Table 5). The 500 bp flanking region upstream of the bbk46 ORF was amplified from the B. burgdorferi B31 clone A3 genomic DNA using the Phusion High-fidelity DNA polymerase and primers 7 and 8 (Table 5). This introduced a 25 bp sequence at the 3’ end of this fragment that was complementary to the 5’ end of the flaBp-aadA cassette. Similarly, the 500 bp flanking region downstream of the bbk46 ORF was amplified using the primers 9 and 10 (Table 5), which introduced a 5’ sequence of 30 bp that was complementary to the 3’ end of the resistance cassette. The PCR products from the above 3 reactions were mixed in equal volumes and used as a template for a fourth amplification reaction using Phusion High-fidelity DNA polymerase and primers 7 and 10 (Table 5) in order to generate a product containing the resistance cassette flanked by the 500 bp sequences upstream and downstream of the bbk46 ORF. This product was ligated with linear PCR-Blunt using a Zero Blunt PCR cloning Kit (Life technologies), yielding the allelic exchange plasmid PCR-Blunt-Δbbk46-flaBp-aadA. B. burgdorferi A3-68ABBE02 was transformed with 20 μg of PCR-Blunt-Δbbk46-flaBp-aadA purified from E. coli as previously described [37]. Streptomycin-resistant colonies were confirmed to be true transformants by PCR using primer pairs 7 and 10 and 11 and 12 (Table 5). Positive Δbbk46-flaBp-aadA clones were screened with a panel of primers [70] for the presence of all of the B. burgdorferi plasmids of the parent A3-68ABBE02 clone [31], and a single clone was selected for further experiments.

Complementation of the Δbbk46 mutant

A PCR-based overlap extension strategy was used to create a DNA fragment encompassing the bbk46 gene and putative upstream promoter sequence with the introduction of a FLAG epitope tag immediately upstream of the putative premature stop codon and a cMyc epitope tag immediately upstream of the downstream stop codon. This was done by using Phusion High-fidelity DNA polymerase (New England Biolabs) and the primers pairs 13 and 14, 15 and 16, and 17 and 18 (Table 5). A Km resistance site was introduced at the 5’ end of this fragment and a SalI site at the 3’ end. The Km+Sal-digested PCR product was ligated into Km+ Sal-digested B. burgdorferi shuttle vector.
The pBSV2G [75] and cloned in E. coli. The pBSV2G bbk46-hbb46-FLAG-cMyc plasmid structure and sequence were confirmed by restriction digest and DNA sequence analysis. In addition, a 400 bp DNA fragment encompassing the flaB promoter with KpnI and BamHI ends was amplified from B31 A3 genomic DNA using primers 27 and 28 (Table 5). The KpnI-BamHI-digested PCR product was ligated into KpnI-BamHI-digested B. burgdorferi shuttle vector pBSV2G [75]. The bbk46-FLAG-cMyc gene within the putative bbk46 promoter sequence and with BamHI and SalI ends is amplified from pBSV2G bbk46-hbb46-FLAG-cMyc plasmid DNA using Phusion High-fidelity DNA polymerase (New England Biolabs) and primers 29 and 18 (Table 5). The BamHI-SalI-digested PCR product was ligated into BamHI-SalI-digested pBSV2G flaBp and cloned in E. coli. The pBSV2G flaBp-bbk46-FLAG-cMyc plasmid structure and sequence were confirmed by restriction digest and DNA sequence analysis. The bbk46 mutant was transformed with 20 μg of pBSV2G bbk46-hbb46-FLAG-cMyc, pBSV2G flaBp-bbk46-FLAG-cMyc or pBSV2G alone isolated from E. coli and positive transformants selected as previously described [37, 76]. The clones that retained the B. burgdorferi plasmid content of the parent clone were selected for use in further experiments.

**Immunoblot analysis of BBK46-FLAG-cMyc**

Production of the BBK46-FLAG-cMyc protein was examined in both E. coli and B. burgdorferi carrying pBSV2G bbk46-hbb46-FLAG-cMyc or pBSV2G flaBp-bbk46-FLAG-cMyc. Total E. coli protein lysates were prepared from 2 × 10^5 cells harvested following overnight growth in LB medium at 37°C with aeration. E. coli cells were resuspended and lysed in 200 μl B-PER protein extraction reagent (Pierce), followed by the addition of 200 μl 2x Laemmli sample buffer plus 2-mercaptoethanol (Bio-rad). Total B. burgdorferi protein lysates were prepared from 2 × 10^5 spirochetes harvested at mid-log phase. The spirochetes were washed twice in 1 ml cold HN buffer (50 mM Hepes, 50 mM NaCl, pH 7.4) and lysed in 200 μl B-PER protein extraction reagent (Thermo Scientific), followed by the addition of 200 μl 2 x Laemmli sample buffer plus 2-mercaptoethanol (Bio-rad). 30 ml of each protein lysate (~1.5 × 10^6 cells) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. 300 ng of PhnA-FLAG [23] and GST-BmpA-cMyc [77] proteins served as positive controls. Immunoblot analysis was performed using anti-FLAG monoclonal antibody (Sigma Aldrich) and 45 μl of 5 M sodium acetate. The membrane was then stripped using 0.2 M NaOH, reblocked using 5% skim milk in TBST and probed with anti-cMyc primary antibody (Genescript) diluted 1:500 in TBST and goat anti-mouse IgG+IgM-HRP (EMD Millipore) and visualized as described above.

**Cloning, purification and seroreactivity analysis of rGST-BBK46**

An in-frame glutathione S-transferase (GST)-BBK46 fusion protein lacking the putative BBK46 signal sequence was generated using primers 30 and 31 (Table 5) and purified, as previously described [77]. Approximately 1 μg of GST-BBK46 was separated by SDS-PAGE, transferred to a nitrocellulose membrane and analyzed by immunoblot for seroreactivity using immune serum collected 3 weeks post inoculation from mice infected with wild-type B. burgdorferi as previously described [77]. Controls included 1 μg of GST alone and total protein lysates generated from BL21 E. coli. B. burgdorferi B31 A3 and E. coli expressing B. burgdorferi bmpA [37] prepared as described above. The membrane was stripped as described above and reprobed with anti-GST primary monoclonal antibody (EMD Millipore) diluted 1:1000 in TBST and goat anti-mouse IgG+IgM-HRP (EMD Millipore) and visualized as described above.

**In vitro growth analysis**

Wild-type B31 A3 spirochetes were grown in triplicate in 5 ml of BSK II medium pH 7.5 at 35°C to a density of 3 × 10^7 spirochetes/ml. To obtain stationary phase, temperature-shifted spirochetes, wild-type B31 A3 spirochetes were grown in triplicate in 5 ml of BSKII medium pH 7.5 at 35°C to a density of 3 × 10^7 spirochetes/ml, transferred to 25°C for 48 hours and then returned to 35°C for an additional 24–36 hours to a density of 2 × 10^10 spirochetes/ml. A total of 1×10^8 spirochetes were harvested from each culture and total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. RNA was resuspended in 100 μl DEPC-treated dH2O. RNA was treated with TURBO DNA-free (Life Technologies) to remove any contaminating genomic DNA. 1 μl of Riboguard (40 U/μl) RNAse inhibitor (Epicentre) was added to all samples and RNA stored at −80°C.

**RNA isolation from infected mouse tissue**

B. burgdorferi-infected mouse bladders (see mouse infection experiments below) were manually macerated on ice using sterile scalps and transferred to a 2 ml sterile tube containing lysing Matrix D (MP Biomedicals) 1 ml of RNA pro solution (FastRNA Pro Green kit, MP Biomedicals) was added to each sample on ice. Tissues were homogenized using a PowerGen High-Throughput Homogenizer (Fisher Scientific) following six cycles of beating for 45 sec and 2 minute incubations on ice. Samples were centrifuged at 13,000 rpm for 5 minutes at 4°C. The upper aqueous phase was transferred to new tubes and incubated for 5 minutes at room temperature, 500 μl of 1-bromo-3-chloropropane (Sigma Aldrich) and 45 μl of 5 M sodium acetate were added to each sample and samples were incubated for an additional 5 minutes at room temperature. Samples were centrifuged at 13,000 rpm for 5 minutes at 4°C. The upper aqueous phase was transferred to new tubes and RNA precipitated with the addition of 500 μl of absolute ethanol and 1 μl GlycoBlue (Life technologies). RNA was pelleted by centrifugation at 13,000 rpm for 10 minutes at 4°C. RNA was washed with 70% ethanol in DEPC-treated dH2O and resuspended in 100 μl DEPC-treated dH2O. RNA was treated with TURBO DNA-free (Life Technologies) to remove any contaminating genomic DNA. 1 μl Riboguard (40 U/μl) RNAse inhibitor (Epicentre) was added to all samples and RNA stored at −80°C.

**Gene expression analysis**

cDNA was synthesized from 1.0 μg of each RNA sample using the iScript cDNA synthesis kit (Bio-Rad) with random primers according to the manufacturer’s instructions. Parallel cDNA
reactions were carried out in the absence of reverse transcriptase. Real-time quantitative PCR (qPCR) reactions were prepared using 1 µg of each cDNA and iQ SYBR Green Supermix (Bio-Rad). Using an Applied Biosystems 7500 instrument, samples were assayed for the flaB, recA, ospC and bbk46 transcripts using primers pairs 21 and 22, 23 and 24, 25 and 26, and 19 and 20, respectively (Table 5). Standard curves were generated for each gene target using 100 ng, 10 ng, 1.0 ng, 0.1 ng, and 0.01 ng of B31 A3 B. burgdorferi genomic DNA and the amount of each gene transcript calculated. The recA transcript was used as the endogenous reference to which the transcripts of the other genes were normalized. The bbk46 primers were confirmed to be specific for their gene target. Three biological replicate samples were analyzed in triplicate and normalized to recA mRNA. The data were reported as the average gene transcript/recA transcript for each sample. The amplification of samples lacking reverse transcriptase was similar to that of the no-template control.

Mouse infection experiments

Unless otherwise noted, groups of 6-8 week old C3H/HeN female mice (Harlan) were used for all experiments.

1D50 analysis of mammalian-adapted spirochetes. A single C3H/HeN SCID mouse (Harlan) was inoculated with 2 x 10^6 B. burgdorferi B31 A3. Two weeks post infection the infected blood was harvested and used to inoculate groups of six wild-type C3H/HeN (Harlan) mice with 100 µl of undiluted infected blood or 100 µl of infected blood diluted 1:10 or 1:100 in BSK-H medium. The number of live spirochetes in the infected blood and therefore the actual spirochete dose in the inoculum was determined by plating the blood in solid BSK medium and quantitating the number of colony forming units (Table 1). In addition, groups of six wild-type C3H/HeN mice (Harlan) were inoculated with 5 x 10^2, 5 x 10^3, 5 x 10^4, 5 x 10^5, 5 x 10^6, in vitro grown spirochetes at mid-log phase. The in vitro grown spirochetes were confirmed to harbor all plasmids required for infectivity [70].

Functional validation of the BbIVET system. Groups of 6 mice were needle inoculated as described [76] with 1 x 10^6 spirochetes of clone A3 60-l carrying pBbIVET or pBbIVET-ospC. Mouse infection was assessed 3 weeks post inoculation by reisolation of spirochetes from ear, bladder and joint tissues as previously described [70,73].

Gene expression studies. Three mice were needle-inoculated intradermally under the skin of the upper back with B. burgdorferi clone B31 A3 at a dose of 1 x 10^7 spirochetes. Three weeks post inoculation mouse infection was determined by serology [70,78] and bladders harvested for RNA isolation.

bbk46 mutant infectivity studies. Groups of five mice were needle-inoculated, intradermally under the skin of the upper back, with B. burgdorferi clones wild-type (A3-68 aBBE02 [51]), Δbbk46/ vector or Δbbk46/ bbk46 at a dose of 1 x 10^9 spirochetes. The number of spirochetes inoculated into mice was determined using a Petroff-Hausser counting chamber and verified by colony-forming unit (cfu) counts in solid BSK medium. Twelve colonies per inoculum were verified by PCR for the presence of the virulence plasmids lp25, lp28-1 and lp36 in at least 90% of the individuals in the population. Further, total plasmid content of each inoculum was confirmed to be as expected [37,70,76]. Mouse infection was assessed three weeks post inoculation by serology using total B. burgdorferi lysate, as previously described [37] and 300 ng recombinant GST-OspC [27], as previously described [79]. Spirochetes were reisolated from the inoculation site, ear, bladder and joint tissues, as previously described [37].

Groups of five immunodeficient C3SnSmn.CB17-Prkdc<scid> (Jackson labs stock 001131) were needle-inoculated with B. burgdorferi clones wild-type (A360aABBE02 [51]), Δbbk46/vector or Δbbk46/ bbk46 at a dose of 1 x 10^4 spirochetes. 80% of the inoculum was delivered intraperitoneal and 20% of the inoculum was delivered subcutaneous. The inoculum cultures were analyzed as described above. Mouse infection was assessed three weeks post inoculation by reisolation of spirochetes from ear, bladder and joint tissues [37].

Supporting Information

Figure S1 Representative restriction digest analysis of individual pBBIVET plasmids rescued in E. coli. Colony PCR to amplify the in vivo-expressed DNA fragment was performed on a random subset of twenty four E. coli transformants carrying the rescued pBBIVET plasmids from infected mouse tissues. The PCR products were digested with a cocktail of the restriction enzymes Dral, Spal and Asel and separated on a 1% agarose gel. Numbers above the top of each image identify each non-identical restriction digestion pattern detected for the amplified pBBIVET DNA fragments. Representative data from two mouse tissues (A) and (B) are shown. Migration of the DNA ladders is shown in base pairs on both sides of each image. NTC, PCR no template control. (TIF)

Figure S2 The BBK46 protein is non-immunogenic in mice. Recombinant GST-BBK46 and GST alone produced in and purified from E. coli, along with total protein lysate from E. coli and B. burgdorferi (Bb lysate) and E. coli producing the B. burgdorferi antigen BmpA (+) were separated by SDS-PAGE, and transferred to a nitrocellulose membrane. Immunoblot analysis was performed using immune serum collected from mice infected with wild-type B. burgdorferi and anti-GST monoclonal antibodies (α GST). The positions of markers to the left of the panel depict protein standard molecular masses in kilodaltons. (TIF)

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Author Contributions

Conceived and designed the experiments: TCE MWJ. Performed the experiments: TCE SJ AKL KR AB MH SK MWJ. Analyzed the data: TCE SJ AKL MWJ. Contributed reagents/materials/analysis tools: TCE SJ AKL KR PAR MH SK MWJ. Wrote the paper: TCE MWJ.
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