**Borrelia burgdorferi** Complement Regulator-Acquiring Surface Protein 2 Does Not Contribute to Complement Resistance or Host Infectivity

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**Abstract**

*Borrelia burgdorferi*, the pathogen of Lyme disease, cycles in nature through ixodes ticks and mammalian hosts. At least five Complement Regulator-Acquiring Surface Proteins (BbCRASPs) are produced by *B. burgdorferi*, which are thought to assist spirochetes in host immune evasion. Recent studies established that BbCRASP-2 is preferentially expressed in mammals, and elicits robust antibody response in infected hosts, including humans. We show that BbCRASP-2 is ubiquitously expressed in diverse murine tissues, but not in ticks, reinforcing a role of BbCRASP-2 in conferring *B. burgdorferi* defense against persistent host immune threats, such as complement. BbCRASP-2 immunization, however, fails to protect mice from *B. burgdorferi* infection and does not modify disease, as reflected by the development of arthritis. An infectious BbCRASP-2 mutant was generated, therefore, to examine the precise role of the gene product in spirochete infectivity. Similar to wild type *B. burgdorferi*, BbCRASP-2 mutants remain insensitive to complement-mediated killing *in vitro*, retain full murine infectivity and induce arthritis. Quantitative RT-PCR assessment indicates that survivability of BbCRASP-2-deficient *B. burgdorferi* is not due to altered expression of other BbCRASPs. Together, these results suggest that the function of a selectively expressed *B. burgdorferi* gene, BbCRASP-2, is not essential for complement resistance or infectivity in the murine host.

**Introduction**

*Borrelia burgdorferi* is the causative agent of Lyme disease, the most prevalent vector-borne disease in the United States and Europe [1,2]. In nature, *B. burgdorferi* cycles between rodent reservoirs and *Ixodes scapularis* ticks. This complex enzootic life cycle requires successful colonization and coordinated transmission between strikingly different host and vector environments. It is thought that differential gene expression plays an important role in allowing the spirochete to navigate the transitions between hosts and in establishing persistent infection [3-6]. Due to the availability of excellent murine models of Lyme disease, *B. burgdorferi* gene expression through the tick-rodent transmission cycle can be examined in the laboratory [7-11]. These efforts may provide important clues for understanding functional aspects of microbial gene products that support *B. burgdorferi* persistence in nature [3-6,12,13].

The genes encoding the Complement Regulator-Acquiring Surface Proteins (BbCRASPs) of *B. burgdorferi* are differentially expressed in the pathogen life cycle [14,15]. As many as five BbCRASPs were identified that bind host proteins of the factor H (FH) family, and possibly contribute to the spirochete defense against host complement-mediating killing [16-18]. BbCRASP-1 (also known as *cspA* or *bba608* and BbCRASP-2 (also called *cspC* or *bba606*), located on linear plasmids *lp*54 and *lp*28-3 respectively, share little sequence homology with other BbCRASP sequences. In contrast, BbCRASP-3, -4 and -5 are sequentially similar and belong to the *erp* paralog family and are known as *erpP* (*bbn3B*), *erpC* and *erpA* (*bhp3B* and *bb139*), respectively. Collectively these *erp* genes are also known as *ospE*, and are encoded on multiple cp32 plasmids [19-22]. The gene *erpC* (located on cp32-2) and one of the three *erpA* genes (located on cp32-5) currently lack TIGR database annotations, as the sequenced B31 M1 isolate lost these plasmids. Of all the BbCRASP genes, BbCRASP-2 is the only gene without paralogous family members, and is therefore unique in *B. burgdorferi* [17].

Evasion of host complement is especially important for *B. burgdorferi*, as it establishes an extracellular and disseminated infection in many tissue environments where the complement system is readily available through host vasculature or body fluids. The complement system includes soluble membrane binding proteins which, upon contact with foreign cells, become activated, and are then capable of direct chemical lysis via membrane disruption [23,24]. Specific regulatory proteins, such as FH family proteins, protect the host from self-inflicted damage by preventing...
unwarranted complement activation. Pathogens such as *Candida albicans*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* have been shown to bind host FH, and that FH binding in *N. gonorrhoea* and *B. burgdorferi* provides protection against complement killing in *vitro* [25–29]. BbCRASPs have been identified according to their ability to bind proteins of the FH family, although individual BbCRASPs vary in their affinities for particular FH family proteins. For example, only BbCRASP-1 and -2 preferentially bind factor H-like protein (FHL)-1, while BbCRASP-3, -4 and -5 selectively bind factor H-related protein (FHR)-1 [17,30,31]. BbCRASPs also vary in their interaction with uncharacterized serum proteins [14,32]. Though the binding affinities and the expression profiles of the BbCRASPs have been studied, the independent role of each BbCRASP in *B. burgdorferi* infectivity is not clear. Recently studies using a non-infectious mutant demonstrated that the loss of BbCRASP-1 sensitized the *B. burgdorferi* to complement-mediated lysis in human serum, an effect that can be rescued with gene complementation [25]. While there is some disagreement as to the expression of BbCRASP-1 during *B. burgdorferi* infection, RT-PCR analysis indicate that it is only expressed transiently at the tick bite site and in ticks [14], but not expressed in mice [33]. BbCRASP-1 therefore, may not play an essential role in mammalian infection [33], but could be important in spirochete survival in feeding ticks. Although the above set of studies suggest an important role for BbCRASPs in spirochete immune evasion, the precise role of individual BbCRASPs, or their orchestrated role in the *B. burgdorferi* infection cycle is not clear, largely because infectious BbCRASP-deficient *B. burgdorferi* have not yet been successfully generated [14].

BbCRASP-2 is expressed by *B. burgdorferi* during murine infection [14,34], and infected hosts, including human patients, readily generate BbCRASP-2-specific antibodies [17,35]. This protein is conserved among *B. burgdorferi* isolates [22], reported to be localized on the spirochete surface [17] and has recently been suggested as a possible target for a second generation Lyme disease vaccine [17,35]. The previous studies also suggest a possible functional role for BbCRASP-2 in immune evasion and pathogen survival [17,22]. In order to test this hypothesis, we sought to determine whether BbCRASP-2 is consistently produced in diverse murine tissues throughout the infection, and whether BbCRASP-2 immunization could provide host immunity and influence disease outcome. To explore the precise role of BbCRASP-2 in *B. burgdorferi* infectivity of a mammalian host, we assessed how targeted deletion of BbCRASP-2 in an infectious isolate influences *B. burgdorferi* infection in the murine model of Lyme borreliosis. Functional characterization of microbial ligands that are differentially expressed in the complex enzootic cycle of *B. burgdorferi* is critical to understanding the adaptive strategies of a pathogen that has evolved to persist in diverse tissue environments resulting in multi-system disorders.

**Materials and Methods**

**Bacteria, Mice and Ticks**

*Borrelia burgdorferi* infectious isolate A3 [36], a clonal derivative of B31 M1, was used throughout the study. Female C3H/HeN mice between 4 and 6 weeks old purchased from the National Cancer Institute. Mice were inoculated with a single subcutaneous injection of 10^7 spirochetes per mouse. All animal procedures were in compliance with the guidelines set by the Institutional Animal Care and Use Committee. The ticks used in this study belong to a colony that is reared and maintained in the laboratory as described [37].

**PCR**

Mice were sacrificed following infection, and the heart, tibiotarsal joint, and skin samples were removed and frozen in liquid nitrogen. *B. burgdorferi*-infected ticks were isolated by allowing ticks to feed on an infected murine host as described [37]. RNA was extracted using TRIzol reagent (Invitrogen) and further treated with DNase I (Invitrogen), and finally purified using the RNeasy kit (Qiagen). RNA was used as a template for reverse-transcriptase polymerase chain reaction (RT-PCR) using the AffinityScript cDNA synthesis kit (Stratagene). The primers used for PCR reactions are indicated in Supplementary Table S1. Quantitative PCR analysis was performed using iTQ SybrGreen Supermix (BioRad) as previously described [13]. For quantitative analysis of gene expression, the target transcripts were normalized to the number of *fluB* transcripts, whereas for quantitative measurement of *B. burgdorferi* burden in infected tissues, *fluB* transcripts were normalized to mouse or tick *β-actin* levels. All quantitative PCR results were checked for specificity by melting curve analysis.

**Production of recombinant BbCRASP-2 protein and BbCRASP-2 antibodies**

Recombinant BbCRASP-2 protein was produced in *E. coli* using the pET303/CT-His Champion vector (Invitrogen) using specific primers (Supplementary Table S1). Recombinant BbCRASP-2 was fused with a C-terminal 6-histidine tag for purification, and lacked the peptides encoding the lipopeptide signal. Polyclonal antibodies against recombinant BbCRASP-2 were generated in mice as described earlier [37,38]. Briefly, recombinant BbCRASP-2 (10 μg/animal) was emulsified in complete Freund's adjuvant (Sigma) and injected into groups of 10 mice. The animals were boosted twice at 10 days intervals with the same dose of antigen in incomplete Freund's adjuvant (Sigma) and the sera were collected two weeks following the second boost. Titer and specificity of the serum was tested by ELISA and immunoblot blot as described previously [39].

**Proteinase K accessibility assay**

Proteinase K accessibility assays were performed as described [40]. Briefly, *B. burgdorferi* (2 × 10^6) were gently washed three times in 1 ml of PBS (pH 7.4) and collected by centrifugation at 4,000 g for 4 min. Washed spirochetes were then gently resuspended in 1 ml of PBS and split into two equal 500 μl volumes. One aliquot received 200 μg of proteinase K (PK) (Sigma) while the other aliquot received an equal volume of PBS without PK. Both aliquots were incubated for 1 h at room temperature before the addition of 10 μl of phenylmethylsulfonyl-fluoride (Sigma) to stop PK activity. Spirochete suspensions were subsequently pelleted by centrifugation at 10,000 g for 10 min and resuspended in PBS for immunoblot analysis using antibodies against BbCRASP-2, *F. tularensis* FLA-B, or Ospa A.

**Active immunization and infection studies**

Groups of mice (6 animals/group) were immunized with adjuvant containing either recombinant BbCRASP-2, or adjuvant containing the same volume of phosphate buffered saline (PBS) in similar fashion as described in above paragraph. Ten days after the final boost, mice were infected with a subcutaneous injection of *B. burgdorferi* (10^7 spirochetes/mouse). Mice were sacrificed after 7, 12, 15 and 30 days of infection. Heart, tibiotarsal joint, and skin samples were collected and frozen in liquid nitrogen. RNA was isolated from infected tissues and *B. burgdorferi* burden was measured using quantitative PCR. *B. burgdorferi*-infected mice were examined for swelling and histology of the tibiotarsal joints as detailed [13,41,42].
Generation and phenotypic analysis of BbCRASP-2 mutant

BbCRASP-2-deficient B. burgdorferi was generated by homologous recombination replacing the entire open reading frame of the BbCRASP-2 gene with a kanamycin resistance cassette [13,38,39,43,44] using primers as indicated in Supplementary Table S1. DNA fragments flanking the BbCRASP-2 open reading frame on the 5’ and 3’ sides were PCR-amplified and inserted into the plasmid pXL10061. This plasmid was sequenced to confirm identity and electroporated into wild-type B. burgdorferi A3, and transformants were selected for growth in the presence of kanamycin. PCR analysis was performed to confirm the intended recombination event using primers P5-P12. Immunoassay analysis using BbCRASP-2 antibodies was performed to confirm the loss of BbCRASP-2. The plasmid profile of the mutant B. burgdorferi was assessed to confirm no loss of wild type plasmids [13,39,44].

The sensitivity of the BbCRASP-2 mutant was measured using a published procedure [16]. Briefly, triplicate samples of wild type B. burgdorferi or isogenic BbCRASP-2 and BbCRASP-1 mutant [25] were seeded into 1 ml tubes at a final concentration of 5×10^6 bacteria per ml. These aliquots were incubated in either 50% normal human serum or 50% heat-inactivated human serum. At 0, 1, 4, and 16 hours after the addition of serum, spirochete viability was examined using dark-field microscopy. Normal human serum (Sigma) collected from healthy, anonymous donors with no reactivity to B. burgdorferi after chemiluminescent immunoblot analyses were used in the assay.

To examine the phenotype of the BbCRASP-2 mutant in vivo, the mutant and wild type B. burgdorferi were separately inoculated into 2 groups of mice (6 animals/group, 10^5 spirochetes/mouse). A skin sample, the heart and the joints from infected mice were isolated at day 7, 12 and 18 after challenge, and B. burgdorferi burdens were measured by quantitative RT-PCR analysis. Before sacrifice, ear and spleen tissues from the mice were cultured in BSK medium for the presence of viable B. burgdorferi. Rear ankle joints of individual mice were measured before infection and at 7, 12 and 18 days after infection until sacrifice. Acquisition of wild type and mutant B. burgdorferi by nymphal I. scapularis ticks was performed as described earlier [37]. Briefly, groups of C3H mice (6 animals/group) were infected with wild type or BbCRASP-2 mutant B. burgdorferi (10^5 spirochetes/mouse). Following 12 days of infection, 25 I. scapularis nymphs were placed on each mouse. The ticks were forcibly detached from the mice following replation and immediately stored in liquid nitrogen. B. burgdorferi burdens in each tick sample were measured as described earlier by quantitative RT-PCR analysis.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). The significance of the difference between the mean values of the groups was evaluated by two-tailed Student’s t test.

Results

BbCRASP-2 is ubiquitously expressed during murine infection

To understand role of BbCRASP-2 in B. burgdorferi infectivity, we first assessed the transcript levels of BbCRASP-2 in multiple murine tissue locations where B. burgdorferi persists during infection, and in various stages of infected ticks. C3H mice were infected with B. burgdorferi, and heart, joints and skin samples were collected at days 5, 7, 12, 16, and 24 following infection. Larval and nymphal ticks were fed on parallel groups of 15 day-infected mice (25 ticks/mouse) and fully engorged ticks were isolated following 3 days of replation. Batches of infected fed larvae were allowed to molt and then collected as unfed nymphs. Total RNA was prepared from murine and tick samples, and subjected to quantitative RT-PCR to measure the B. burgdorferi BbCRASP-2 transcript levels. BbCRASP-2 is ubiquitously and consistently expressed throughout infection (Fig. 1A), but was undetectable in larval or nymphal ticks. As reported earlier [17], the infected mice developed a specific antibody response against BbCRASP-2 (data not shown).

BbCRASP-2 immunization failed to evoke protective immunity in mice

Because BbCRASP-2 is surface-exposed [17], immunogenic, and expressed throughout the murine infection, we next assessed if immunization of mice using recombinant BbCRASP-2 could elicit protective immunity and influence the outcome of Lyme disease. To accomplish this, we produced recombinant BbCRASP-2 in E. coli and immunized the murine host with purified BbCRASP-2. Separate groups of C3H mice (6 animals/group) were immunized with either BbCRASP-2 or PBS (control) mixed with similar volume of adjuvant. ELISA (data not shown) and immunoblotting performed after final boosting indicated that immunized mice had developed strong antibody titer that specifically recognized recombinant and native BbCRASP-2 (Fig. 2A). Although previous immunofluorescence studies indicated that BbCRASP-2 antibodies recognized native antigen on the surface of the intact spirochetes [17], our proteinase K accessibility assay indicated that BbCRASP-2 is not significantly exposed on the spirochete surface (Fig. 2B). Ten days after the final immunization mice were needle-inoculated with B. burgdorferi (10^5 spirochetes/mouse). B. burgdorferi levels were measured by quantitative PCR from skin, heart and joint samples collected at 7, 12, 15 and 30 days after infection. Results indicated no significant difference in spirochete burden between mice immunized with BbCRASP-2 or the control at any time points (Fig. 2C). Quantitative RT-PCR analysis further showed no difference between the transcript levels of BbCRASP-2 in immunized and control groups (data not shown).

Figure 1. Ubiquitous expression of BbCRASP-2 in infected mice.

The relative expression levels of BbCRASP-2 in the murine host, and in representative life stages of ticks, are analyzed and presented as copies of BbCRASP-2 transcript per 1000 copies of flaB transcripts. Total RNA was isolated from multiple tissues of B. burgdorferi-infected mice (6 mice/group) at day 5, 7, 12, 16 and 24 following challenge, from infected fed larva (FL) after 3 days of feeding, unfed nymphs following larval molting (UN) and fed nymphs (FN) after 3 days of feeding. BbCRASP-2 transcripts were measured using quantitative RT-PCR. Error bars represent the mean ± SEM from four quantitative PCR analyses of two independent murine infection experiments. BbCRASP-2 transcripts were abundant in all murine tissues tested but were not detected in any stages of the ticks.

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These results indicated that BbCRASP-2 immunization did not influence the ability of B. burgdorferi to establish infection. Development of ankle swelling (Fig. 2D) or histopathological changes in the joint tissue (data not shown) in B. burgdorferi-infected mice immunized with BbCRASP-2 did not differ from the control, suggesting that host BbCRASP-2 antibodies fail to influence the ability of B. burgdorferi to induce arthritis in the murine host.

Generation of an infectious isolate of BbCRASP-2-deficient B. burgdorferi

Since BbCRASP-2 immunization did not influence spirochete infection, we created a BbCRASP-2-deficient B. burgdorferi to more directly assess the role of the gene product in B. burgdorferi survival and infectivity. An infectious isogenic mutant was created by replacing the BbCRASP-2 open reading frame with a kanamycin resistance cassette via homologous recombination (Fig. 3A). PCR analysis was performed to ensure that the antibiotic cassette was appropriately inserted into the intended chromosomal locus (Fig. 3D), and that the plasmid profile of the mutant was unchanged. Out of 4 transformed clones that grew in antibiotic-containing media, 2 clones contained the desired integration of the antibiotic cassette and retained the same set of plasmid as in the parental isolate. One of the mutant clones was chosen for further study. RT-PCR analysis showed that BbCRASP-2 mRNA was absent in the mutant (Fig. 3C), and that BbCRASP-2 mutagenesis

Figure 2. BbCRASP-2 immunization does not interfere with B. burgdorferi infectivity. A, Recognition of recombinant and native BbCRASP-2 proteins by immunized murine serum, as assessed by immunoblotting. Recombinant BbCRASP-2 protein (50 ng) or B. burgdorferi lysates (200 ng) were probed with BbCRASP-2 antiserum or normal mouse serum (NMS). The arrow indicates the position of BbCRASP-2. Migration of protein standards is shown to the left. B, BbCRASP-2 is not sensitive to proteinase K-mediated degradation of B. burgdorferi surface proteins. Viable spirochetes (2×10^8 cells) were incubated with (+) or without (−) proteinase K for removal of protease sensitive surface proteins and processed for immunoblot analysis using BbCRASP-2 antibodies. B. burgdorferi OspA and FlaB antibodies were utilized as controls for surface-exposed and subsurface proteins, respectively. C, Comparable levels of B. burgdorferi in rBbCRASP-2-immunized or control mice. Groups of mice (6 animals/group) were immunized with either rBbCRASP-2 or PBS (control) mixed with adjuvant, and 10 days after final immunization mice were infected with B. burgdorferi (10^8 spirochetes/mouse). The spirochete burdens in both groups of mice were assessed by measuring copies of the B. burgdorferi flaB gene at 7, 12 and 30 days following infection. Amounts of murine β-actin were determined in each sample and used to normalize the quantities of spirochete RNA. Bars represent the mean measurements ± SEM from four quantitative PCR analyses from two independent infection experiments. Levels of B. burgdorferi were similar in BbCRASP-2-immunized (black bars) and control mice (white bars). The burdens found in BbCRASP-2-immunized mice were not statistically significant from the control burdens in any tissue or time point (P>0.05, n = 4). D, Severity of joint swelling in BbCRASP-2-immunized and infected mice. Groups of mice (6 animals/group) were immunized with BbCRASP-2 (black bars) or PBS (control, white bars) and infected with B. burgdorferi. Development of joint swelling was assessed after 12 and 30 days of spirochete challenge by measuring the largest diameter of the rear tibiotarsal joints using a digital caliper. doi:10.1371/journal.pone.0003010.g002
did not impose polar effects on the transcription of surrounding genes, \( bbh05 \) and \( bbh07 \) (data not shown). The \( BbCRASP-2 \) mutant spirochetes displayed a similar protein profile to that of the wild type (Fig. 3D, left panel), except that the \( BbCRASP-2 \) mutant failed to produce \( BbCRASP-2 \) protein (Fig. 3D, upper right panel).

**BbCRASP-2-deficiency did not affect serum resistance of \( B. burgdorferi \) in vitro**

\( B. burgdorferi \) is known to be resistant to complement-mediated lysis in serum, and deficiency of \( BbCRASP-1 \) has been shown to render \( B. burgdorferi \) highly susceptible to serum-mediated killing in vitro [25]. Because \( BbCRASP-2 \) is expressed by wild type spirochetes grown in culture, we assessed whether \( BbCRASP-2 \) deficiency affects the serum resistance of the spirochetes. Equal concentrations of wild type and \( BbCRASP-2 \) mutant \( B. burgdorferi \) were separately incubated with human serum containing active complement and the serum sensitivity of each isolate was assessed.

While the isogenic \( BbCRASP-1 \) mutant were readily killed within 1 hour of serum exposure, the viability of the \( BbCRASP-2 \) mutant did not differ significantly from that of the wild type (Fig. 4) indicating that \( BbCRASP-2 \) is not essential for \( B. burgdorferi \) resistance to complement-mediated lysis in serum.

**BbCRASP-2-deficient \( B. burgdorferi \) retain full murine infectivity**

To examine whether the lack of \( BbCRASP-2 \) influences \( B. burgdorferi \) infectivity in a mammalian host, \( \text{C3H} \) mice were infected with wild type or \( BbCRASP-2 \) mutant \( B. burgdorferi \). Both the mutant and wild type spirochetes were readily cultured from ear and spleen tissues taken from mice 12 days after the inoculum (data not shown). When nymphal ticks were allowed to feed on infected mice, \( BbCRASP-2 \) mutant \( B. burgdorferi \) were able to migrate into fed ticks at a similar level to the wild type spirochetes (data not shown). Quantitative RT-PCR further showed that the

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**Figure 3. Construction and analysis of the \( BbCRASP-2 \) mutant \( B. burgdorferi \).**

- **A.** Schematic drawings of the wild type isolate (WT) and the \( BbCRASP-2 \) mutant (M) at the \( BbCRASP-2 \) (\( bbh06 \)) locus. Genes \( bbh02, bbh03, bbh04, bbh05, bbh07, bbh08 \) and \( bbh09 \) (white box arrows) and the kanamycin-resistance cassette driven by the \( B. burgdorferi \) flaB promoter (\( flaB-Kan \), black box arrow) are indicated. Primers P1-P4 (black arrow-heads) amplified 5’ and 3’ arms for homologous recombination, regions flanking up- and down-stream of the \( BbCRASP-2 \) locus and ligated on either side of the \( flaB-Kan \) cassette as detailed in the text.
- **B.** Integration of the mutagenic construct, \( flaB-Kan \), in the intended genomic locus. Primers 5–12 (gray arrow-heads, positions indicated in Fig. 3A) were used for PCR analysis using isolated DNA from wild type (WT) or \( BbCRASP-2 \) mutant \( B. burgdorferi \) (M) and subjected to gel electrophoresis. The combination of primers used for PCR is indicated at the top. Migration of DNA ladder is shown on the left.
- **C.** RT-PCR analysis of \( BbCRASP-2 \) transcripts. Total RNA was isolated from either the wild type (WT) or \( BbCRASP-2 \) mutant (M) \( B. burgdorferi \), converted to cDNA and used to PCR-amplify regions within \( BbCRASP-2 \) and \( flaB \), and these amplicons visualized on a gel.
- **D.** Protein analysis of wild type (WT) or \( BbCRASP-2 \) mutant (M) \( B. burgdorferi \). Equal amounts of protein from wild type or \( BbCRASP-2 \) mutant spirochetes were separated on an SDS-PAGE gel, and either stained with coomassie blue (left panel) or transferred onto a nitrocellulose membrane and probed with \( BbCRASP-2 \) or FlaB antibodies. Migration of protein standards is shown to the left in kDa.

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BbCRASP-2 mutant established infection in mice in comparable levels to the parental isolate. No significant differences in the burdens of BbCRASP-2 mutant and wild type isolates were detected in murine skin, heart and joint samples isolated after 7, 12 and 18 days of infection (Fig. 5). Development of swelling in the murine joints infected with either the BbCRASP-2 mutant or the wild type B. burgdorferi was also similar (data not shown). Overall, these results suggest that BbCRASP-2 is not essential for establishment of B. burgdorferi infection in the mouse model of Lyme disease.

Loss of BbCRASP-2 function is not compensated by augmented or new expression of other BbCRASPs

BbCRASP-2 deficiency did not affect the ability of the BbCRASP-2 mutant to survive complement-mediated lysis in vitro or establish infection in a mammalian host in vivo. Since B. burgdorferi encodes multiple BbCRASPs that are capable of binding host complement regulators, we explored the possibility that the loss of BbCRASP-2 could be compensated by altered expression of other potential BbCRASP genes, such as BbCRASP-1, -3 and -5. We did not examine the expression of BbCRASP-4, as the parental B. burgdorferi isolate A3[36] lacks the non-essential cp32-2 plasmid housing the gene. To examine BbCRASP expression, groups of 6 C3H mice were needle-inoculated with wild type or BbCRASP-2-deficient B. burgdorferi (10⁶ spirochetes/mouse). Infected murine skin and heart samples were isolated 7, 12 and 18 days after infection, and expression of each BbCRASP was measured by quantitative RT-PCR. In vitro expression of BbCRASP genes was also assessed by growing wild type and mutant B. burgdorferi in BSK medium to various cell densities (10⁶–10⁸ spirochetes/ml) and analyzed by quantitative RT-PCR. The expression profiles of BbCRASP-1, -3, and -5 remained unaltered in the BbCRASP-2 mutant when compared to the wild type spirochetes, both in vitro and in vivo, such as in the murine skin and heart tissues at all time points. BbCRASP expression in cultured spirochetes grown in vitro to a density of 10⁷/ml and in infected murine skin and heart tissue samples at 12 days of infection is presented (Fig. 6). These results suggest that the loss of BbCRASP-2 function is not compensated by alteration or new expression of other BbCRASP genes (Fig. 6).

Discussion

B. burgdorferi express up to five BbCRASPs that are either structurally unique, such as BbCRASP-1 and -2, or closely related, BbCRASP-3, -4 and -5 [17,18,21,45]. These BbCRASPs are differentially expressed and are postulated to confer defense against host-derived complements via specific interaction with FH family proteins [16,17,31,32]. The precise role of individual BbCRASPs in the B. burgdorferi infection cycle, however, is currently unclear. BbCRASP-2 is specifically produced in the mammalian host including humans, and is immunogenic, and thus, is thought to be important in spirochete pathogenesis and may be useful in a future Lyme disease vaccine [17,35]. Here, we show that BbCRASP-2 is ubiquitously expressed throughout murine infection, evoking a detectable antibody response. However, BbCRASP-2 immunization fails to protect the host against B. burgdorferi infection or influence the genesis of disease. Targeted deletion of BbCRASP-2 did not impair the ability of the mutants to resist serum-mediated killing in vitro, establish infectivity in vivo, or the severity of disease. Deficiency of BbCRASP-2 expression in mutants is not functionally compensated by the enhanced expression of other BbCRASP genes. BbCRASP-2, therefore, is not essential for B. burgdorferi survivability in vitro, and based on the time periods covered in the present study, we conclude that BbCRASP-2 function is dispensable for B. burgdorferi infectivity mice and in feeding ticks.

Immune mobilization of murine hosts against specific B. burgdorferi antigens, such as DbpA [46,47], OspC [48] and OspA [49] can elicit production of borreliacidal antibodies, and thus confer protective host immunity possibly by killing spirochetes in vivo when administered prior to spirochete infection. In contrast, other
B. burgdorferi antigens are also described, such as BmpA/B [13] and Arp [50,51], that fail to protect host against B. burgdorferi infection, but influence pathogenesis either by reduction of the B. burgdorferi burden in selected tissues [13] or by modifying the disease without affecting spirochete load [50,51]. The failure of BbCRASP-2-specific host immunity to influence B. burgdorferi pathogenesis indicates that BbCRASP-2 antibodies lack significant neutralizing effects on spirochetes, possibly due to the lack of significant surface-exposure of the antigen. This is further confirmed by the observation that the BbCRASP-2-deficient B. burgdorferi displayed no phenotypic defects in their ability to infect the murine host or induce disease. Nevertheless, as BbCRASP-2 is abundantly produced by B. burgdorferi throughout infection and evokes development of specific antibody, our data reinforces an earlier contention that BbCRASP-2 could be a reliable marker for the serodiagnosis of Lyme disease [17,35].

B. burgdorferi expresses select lipoproteins [19] in the mammalian host [52], including BbCRASP-2. Owing to its ubiquitous expression in the host and known affinity for FH family proteins [17], BbCRASP-2 appears to be important for B. burgdorferi protection against persistent host immune threats, such as complement system. BbCRASP-2 is conserved among infectious B. burgdorferi isolates, which also suggests an important role for this protein in the infectivity of B. burgdorferi [22]. The plasmid lp28-3 that houses BbCRASP-2 is retained in most of the B. burgdorferi clones isolated from experimentally infected hosts [53–56]. Previous studies attempting to identify specific plasmids required for B. burgdorferi infectivity indicate that lp28-3 plasmid may not be strictly necessary for spirochete infectivity [55] while other studies suggest that several plasmids, including lp28-3, in the correct combinations, may be required to mediate B. burgdorferi infection of the mammals [54]. Nevertheless, our data conclusively show that the function of BbCRASP-2 is not essential for B. burgdorferi survival against serum-mediated killing in vitro or host infectivity. The BbCRASP-2 mutant fails to produce both BbCRASP-1 and BbCRASP-2 in the murine host, yet still retains full infectivity, which suggests that complement evasion in the host, if relevant, could be mediated by BbCRASP-3 and -5, which remain fully expressed by the mutant. Alternatively, binding to certain FH family proteins may not be essential to the spirochetes, as previous studies indicate that B. burgdorferi are able to infect and cause disease in FH-deficient mice [57].

In summary, we present direct evidence that B. burgdorferi adapts for the loss of a differentially expressed and abundant lipoprotein during mammalian infection. Past studies also identified additional B. burgdorferi genes encoding potential lipoproteins, such as BBA64 [58,59] or OspD [60,61] that display highly regulated expression in vivo but lack an essential role in B. burgdorferi persistence in tick-mouse infection cycle. Here we show that BbCRASP-2 function is also dispensable for infectivity of the murine hosts or in feeding ticks. Together, these results highlight B. burgdorferi as a unique pathogen that has evolved versatile adaptive strategies to survive and establish infection in a diverse array of host species, including humans.

**Supporting Information**

**Table S1**

| Found at: doi:10.1371/journal.pone.0003010.s001 (0.05 MB DOC) |

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Author Contributions

Conceived and designed the experiments: ASC UP. Performed the experiments: ASC XY MK ZP MRK. Analyzed the data: ASC DA UP. Contributed reagents/materials/analysis tools: DS JA DA. Wrote the paper: ASC UP.

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