Borrelia burgdorferi basic membrane proteins A and B participate in the genesis of Lyme arthritis

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Lyme arthritis results from colonization of joints by *Borrelia burgdorferi* and the ensuing host response. Using gene array–based differential analysis of *B. burgdorferi* gene expression and quantitative reverse trancription–polymerase chain reaction, we identified two paralogous spirochete genes, *bmpA* and *bmpB,* that are preferentially up-regulated in mouse joints compared with other organs. Transfer of affinity–purified antibodies against either BmpA or BmpB into *B. burgdorferi*–infected mice selectively reduced spirochete numbers and inflammation in the joints. *B. burgdorferi* lacking *bmpA/B* were therefore generated to further explore the role of these proteins in the pathogenesis of Lyme disease. *B. burgdorferi* lacking *bmpA/B* were infectious in mice, but unable to persist in the joints, and they failed to induce severe arthritis. Complementation of the mutant spirochetes with a wild–type copy of the *bmpA* and *bmpB* genes partially restored the original phenotype. These data delineate a role for differentially produced *B. burgdorferi* antigens in spirochete colonization of mouse joints, and suggest new strategies for the treatment of Lyme arthritis.

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Abbreviations used: DECAL, differential expression analysis using a custom-amplified library; GST, glutathione *S*-transferase; q, quantitative.

Lyme arthritis results from infection with a tick-borne spirochete, *Borrelia burgdorferi* (1). Spirochetes are deposited into the skin when *Ixodes scapularis* feed on mice or humans. *B. burgdorferi* replicate in the dermis, and then disseminate to distant cutaneous sites and other organs, including the joints (2). Clinical manifestations of Lyme disease may involve a characteristic skin rash called *erythema migrans*, arthritis, carditis, or neurological symptoms (3, 4). Lyme arthritis occurs in a substantial number of untreated patients (1), often several weeks to months after the tick bite, and is associated with spirochete invasion of the joints (3). In most cases, antibiotic therapy is curative; however, some patients develop a form of antibiotic-resistant arthritis that is thought to be unrelated to persistent infection (5).

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C3H/HeN mice infected with *B. burgdorferi* develop joint swelling that partially mimics human disease and has been helpful in understanding the pathogenesis of Lyme arthritis (6–9). Intradermally inoculated spirochetes reside in the mouse skin for ∼1 wk, after which they disseminate to many tissues. Arthritis begins to develop at ∼10 d and is prominent at 2 or 3 wk (2, 10). At 4 wk, the disease may begin to regress, and by 8 wk the arthritis has resolved (2, 6, 7, 11–13). The presence of *B. burgdorferi* in mouse joints, and the innate and adaptive host responses to the pathogen, contributes to the development of inflammation (1). Spirochete genes are also implicated in mouse Lyme arthritis, as *B. burgdorferi* lacking certain plasmids are less arthritogenic (14). Indeed, *B. burgdorferi* Arp is expressed in many tissues during infection, and antisera against Arp can attenuate mouse Lyme arthritis (15, 16). Specific spirochete antigens that are selectively induced in the joints and causally associated with the genesis of arthritis have, however, not yet been identified.

The preferential up-regulation of specific *B. burgdorferi* genes throughout the spirochete

life cycle, both in the vector and mammalian host, plays an important role in pathogen survival (17–19). It is likely that the diverse metabolic or immune microenvironments within mammalian tissues may influence the ability of *B. burgdorferi* to persist in different organs (20–22). We determined, therefore, whether particular *B. burgdorferi* genes are selectively expressed in mouse joints, and if the gene products contribute to spirochete colonization of the joints and the development of arthritis. Characterization of microbial ligands that are expressed in a tissue-specific manner is critical for understanding the pathogenesis of complex infectious diseases.

RESULTS

Identification of *B. burgdorferi* genes up–regulated in mouse joints

We identified a subset of *B. burgdorferi* genes that are preferentially up-regulated in mouse joints using a microarray-based analysis to compare spirochete transcriptomes from different tissues. The differential expression analysis using a custom-amplified library (DECAL) technique (22, 23) specifically amplified spirochete mRNAs from infected skin, joints, heart, and bladder. To accomplish this, C3H/HeN mice were challenged with *B. burgdorferi*. At day 15, when arthritis was evident (14, 24) and was reflected by swelling of the tibiotarsal joints, total RNA was isolated from the skin, joints, heart, and bladder tissue. *B. burgdorferi*–specific mRNAs were selected and amplified using the DECAL technique and hybridized onto a *B. burgdorferi* genomic array, as detailed in the Materials and methods. Analysis of array data revealed that the expression of *B. burgdorferi* genes varied in different host tissues, and that the *B. burgdorferi* *bmpA/B* gene operon displayed the most significant up-regulation in joints.

The temporal expression of *bmpA/B* coincides with *B. burgdorferi* infection of mouse joints and the induction of Lyme arthritis

We used the gene array data as a guide to perform more detailed quantitative RT-PCR (qRT-PCR) analyses on the genes of interest. The *B. burgdorferi* bmp gene family has four paralogous members, *bmpA*, *bmpB*, *bmpC*, and *bmpD* (25), where *bmpA* and *bmpB* are on a bicistronic operon and may be controlled by a common promoter (26, 27). We assessed the independent expression of each bmp gene throughout the first few weeks of *B. burgdorferi* infection in multiple tissues using gene-specific qRT-PCR analysis. Groups of C3H/HeN mice were infected with *B. burgdorferi*, and tissues were isolated at 6, 9, 12, and 15 d. Isolated mRNA, without the DECAL enrichment, was converted to cDNA and subjected to qRT-PCR to measure the individual *B. burgdorferi* bmp transcripts. Expression of *bmpA* and *bmpB* in joints was dramatically and selectively up-regulated at 12 and 15 d compared with the skin, heart, and bladder (Fig. 1). *bmpC* and *bmpD* expression showed no differences between joint or other tissues and remained unchanged in the joints at 12 and 15 d; at earlier time points, spirochetes were not readily detectable in the joints, heart, or bladder (unpublished data). Furthermore, expression of both *bmpA* and *bmpB* was
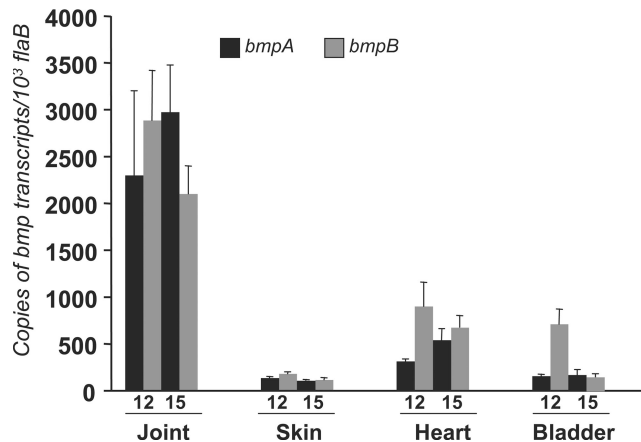

Figure 1. Joint–specific up–regulation of *B. burgdorferi* bmpA and bmpB in infected mice. The relative expression levels of *bmpA* (black bar) and *bmpB* (gray bar) are represented as copies of bmp transcript per 1,000 copies of *flaB* transcript. Total RNA was isolated from multiple tissues of *B. burgdorferi*–infected mice (10 mice/group) and converted to cDNA for measuring bmp transcripts using qPCR 12 and 15 d after challenge. Bars represent the mean ± the SEM from three independent experiments. The transcript levels in joints were significantly higher than those in skin, heart, or bladder tissue. $n = 3$. $P < 0.01$–0.001.

also minimal in spirochetes isolated from infected ticks; their expression levels were ~8–10-fold lower than the corresponding levels in spirochetes isolated from joints at day 15. As *bmpA* and *bmpB* showed the greatest degree of selective up-regulation in joints, these gene products were further examined to explore the paradigm that tissue-specific gene expression contributes to arthritis.

Antibodies against BmpA or BmpB interfere with *B. burgdorferi* persistence most dramatically in joints

To explore the physiological relevance of the joint-specific up-regulation of *bmpA* and *bmpB*, we assessed whether specific antibodies were capable of interfering with *B. burgdorferi* persistence in the joints. To achieve this goal, we produced recombinant BmpA and BmpB in *Escherichia coli* and generated antisera against the individual proteins in rabbits. As antisera against BmpA may cross-react with BmpB, or vice versa (28), monospecific BmpA and BmpB antibodies were isolated by affinity purification, as detailed in the Materials and methods. We then characterized the specificity of the purified BmpA or BmpB antibodies and their capacity to bind to the spirochete surface. Immunoblots demonstrated substantial binding of purified BmpA or BmpB antibodies to their respective proteins, with minimal cross-reactivity (Fig. 2 A). In addition, immunofluorescence microscopy showed that both of the Bmp antibodies were able to bind to the surface of unfixed cultured *B. burgdorferi* (Fig. 2 B) and had partial borreliacidal activity in vitro (Fig. 2 C).

We next performed passive immunization studies to assess whether purified BmpA or BmpB antibodies interfered with selective *B. burgdorferi* persistence in joints. To accomplish
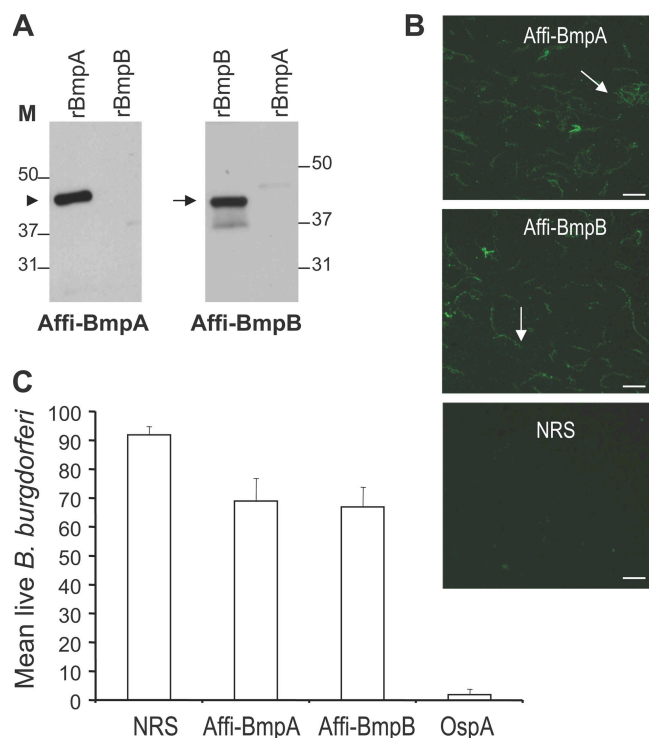
Figure 2. Characterization of affinity–purified BmpA and BmpB antibodies. (A) 1 ng of recombinant Bmp proteins (rBmpA and rBmpB) was probed with either affinity-purified BmpA (Affi-BmpA) or BmpB (Affi-BmpB) antibodies. The arrowhead and arrow indicate the positions of BmpA and BmpB, respectively. (B) BmpA or BmpB antibodies bind the surface of intact unfixed *B. burgdorferi* (arrows). Spirochetes were immobilized on glass slides and probed with affinity-purified BmpA (Affi-BmpA), BmpB (Affi-BmpB) antibodies, or normal rabbit sera (NRS). Images were acquired using the 40× objective lens of an epifluorescence microscope (Axiovert 200M; Carl Zeiss, Inc.). Bars, 10 μm. (C) Borreliacidal activities of BmpA or BmpB antibodies in vitro. Spirochetes were incubated with normal rabbit sera (NRS), affinity-purified BmpA (Affi-BmpA), BmpB (Affi-BmpB), or rabbit OspA antibodies (OspA). The number of viable spirochetes was assessed by fluorescent microscopy after labeling the spirochete preparations with vital dyes at 48 h of incubation. Data represent the mean number of viable spirochetes/microscopic field after antibody incubation $\pm$ the SEM from three independent experiments. The number of viable spirochetes exposed to the affinity-purified BmpA or BmpB antibodies were significantly diminished compared with spirochetes that received NRS. $n = 3$. $P < 0.01$. As expected, OspA antisera resulted in the complete killing of *B. burgdorferi*.

this, groups of C3H/HeN mice (15 animals/group) were infected with *B. burgdorferi*, and 3 d after spirochete challenge, mice were administered 100 μl of BmpA, BmpB, or control antibodies. Skin, bladder, heart, and joints were collected on day 12, 15, and 18. *B. burgdorferi* levels in each tissue were measured by qPCR. The passive transfer of either of the Bmp antibodies dramatically reduced *B. burgdorferi* levels in the joints, and to a lesser extent in skin, but not in other tissues, such as heart and bladder, at all time points. The data from days 15 and 18 are presented in Fig. 3. Because Lyme arthritis has been directly attributed to the presence of *B. burgdorferi* in joint tissue (1),

we determined whether BmpA or BmpB antibodies influenced disease. Development of joint swelling in *B. burgdorferi*–infected mice immunized with glutathione *S*-transferase (GST) antisera was significantly higher (1.36 ± 0.25 mm; $P < 0.02$) than in groups of mice administered either BmpA (0.47 ± 0.13) or BmpB (0.65 ± 0.1) antibodies, suggesting a role for these proteins in the genesis of arthritis.

bmpA/B–deficient *B. burgdorferi* persist poorly in joints and fail to induce arthritis

To directly study the importance of BmpA and BmpB in the spirochete persistence within joints, we created *bmpA/B*-deficient ($bmpA/B^-$) *B. burgdorferi*. The mutant was constructed by exchanging a DNA fragment within the *bmpAB* gene with a kanamycin–resistance cassette via homologous recombination (Fig. 4 A). A suicide plasmid for recombination in the intended chromosomal locus was constructed and transformed into *B. burgdorferi*, and transformants were screened as detailed in the Materials and methods. PCR analysis indicated that the antibiotic cassette was appropriately inserted into the intended chromosomal locus and that the plasmid profile of the mutant spirochetes was similar to that of the wild-type organisms (unpublished data). RT-PCR showed that *bmpA* or *bmpB* mRNA was absent in the mutant, and, as expected, the mutant expressed both *bmpC* and *bmpD* mRNA (Fig. 4 B). In addition, qRT-PCR showed that both wild-type spirochetes and $bmpA/B^-$ *B. burgdorferi* produced similar levels of transcripts expressed
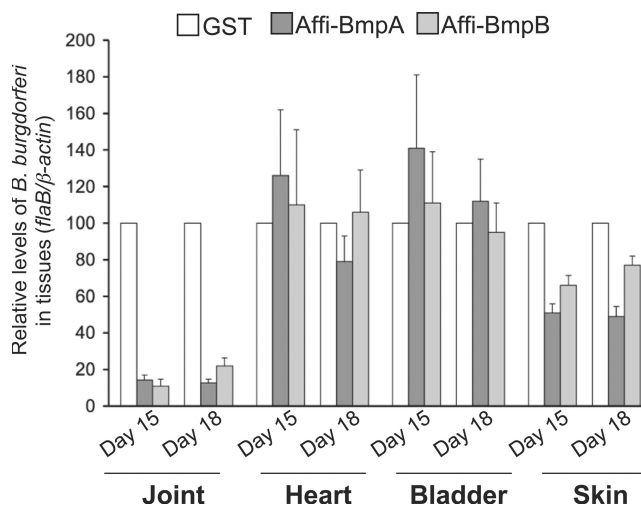

Figure 3. Affinity–purified Bmp antibodies most dramatically affect *B. burgdorferi* persistence in mouse joints. Affinity-purified BmpA or B antibodies interfere with the persistence of *B. burgdorferi* in joints. Mice (15 animals/group) were infected with *B. burgdorferi* and administered affinity-purified BmpA (Affi-BmpA), BmpB (Affi-BmpB), or control GST antibodies. The *B. burgdorferi* level in mouse tissues was assessed by measuring *flaB* copies at days 15 and 18 after *B. burgdorferi* infections, and it is expressed as *flaB*/mouse *b-actin*. The *B. burgdorferi* load in joints and skin of mice receiving BmpA or BmpB antibodies was significantly lower than in mice receiving control GST sera. $n = 3$. $P < 0.01$–0.001. Bars represent the mean $\pm$ the SEM from two independent experiments and the spirochete burden of the control mice was considered as the 100% value.
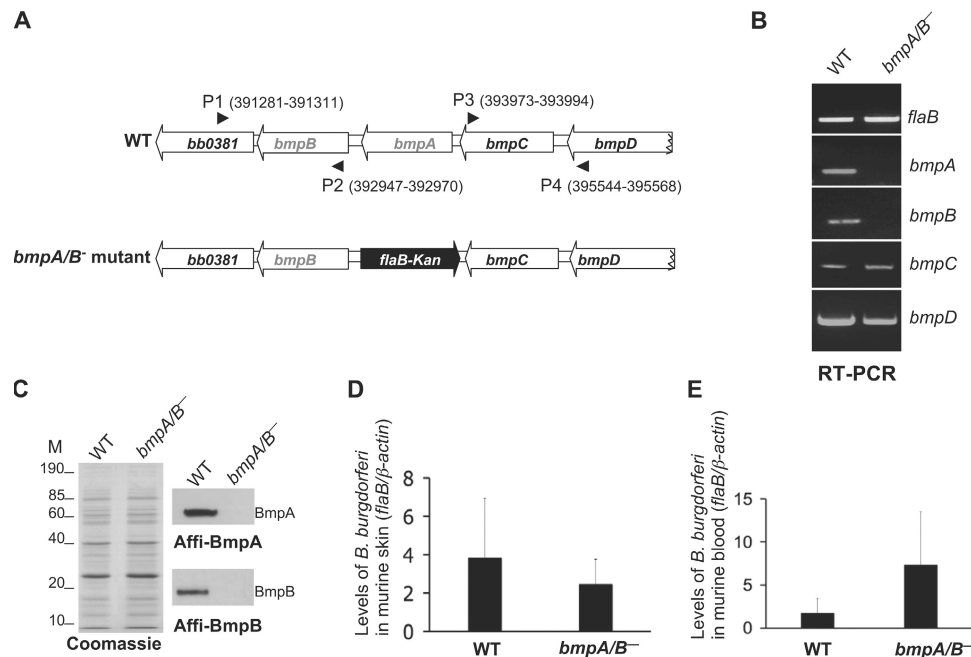
Figure 4. Construction and analysis of the *bmpA/B⁻ B. burgdorferi*. (A) Schematic drawings of the wild-type isolate (WT) and the *bmpA/B⁻* mutant at the *bmpAB* locus. Genes *bb0381*, *bmpB*, *bmpA*, *bmpC*, and *bmpD* (white box-arrows) and the kanamycin-resistance cassette driven by the *B. burgdorferi flaB* promoter (flaB-Kan, black box-arrow) are indicated. Nucleotide positions of primers P1-P4 in the *B. burgdorferi* genomic database (www.tigr.org) are indicated within parentheses. The 5′ and the 3′ arms for homologous recombination, flanking up- and downstream of the *bmpAB* locus were amplified using primers P1-P2 and P3-P4 and ligated to the flaB-Kan cassette, as detailed in the Materials and methods. (B) RT-PCR analysis of the *bmpA* and *B* transcripts. Total RNA were isolated from either the wild-type (WT) or mutant (*bmpA/B⁻*) *B. burgdorferi*, converted to cDNA, and subjected to PCR analysis with *flaB*, *bmpA*, *bmpB*, *bmpC*, or *bmpD* primers and analyzed on a 2% agarose gel. (C) Protein analysis of wild-type (WT) or *bmpA/B⁻* spirochetes. Equal amounts of proteins from wild-type or *bmpA/B⁻ B. burgdorferi* were separated on a SDS-PAGE gel, and either stained with Coomassie blue (left) or transferred onto a nitrocellulose membrane and probed with the affinity-purified BmpA (top right) or affinity-purified BmpB (bottom right) antibody. Migration of protein standards is shown to the left in kilodaltons. (D) Comparable levels of wild-type and *bmpA/B⁻ B. burgdorferi* in the dermis of infected mice. Total DNA was isolated from the skin of mice 1 wk after *B. burgdorferi* challenge. The *B. burgdorferi* burden was analyzed by qPCR measurement of *flaB* copies and expressed as *flaB*/mouse *b-actin*. Wild-type (WT) and *bmpA/B⁻ B. burgdorferi* were evident in similar numbers. *n* = 3. P > 0.5. (E) The *bmpA/B⁻ B. burgdorferi* were capable of dissemination from the dermis of infected mice. Blood was collected from mice 1 wk after *B. burgdorferi* challenge, and spirochete burden was analyzed by qPCR measurement of *flaB* copies and expressed as *flaB*/mouse *b-actin*. Similar levels of wild-type (WT) and *bmpA/B⁻ B. burgdorferi* were detected. *n* = 3. P > 0.5.

from neighboring gene loci, such as *bb0381*, *bmpC*, and *bmpD*. The protein profiles of the *bmpA/B⁻* spirochetes and the wild-type organisms were identical (Fig. 4 C, left), except that the *bmpA/B⁻ B. burgdorferi* did not produce BmpA (Fig. 4 C, top right) or BmpB protein (Fig. 4 C, bottom right).

We then compared the infectivity between *bmpA/B⁻ B. burgdorferi* and the wild-type spirochete in vivo using the mouse model of Lyme borreliosis. In nature, ticks transmit *B. burgdorferi* infection to mammals; however, to ensure an identical inoculation dose of wild-type and mutant spirochetes in the mouse host, we infected mice with a needle inoculum containing a specific amount of *B. burgdorferi*. Groups of 10 C3H/HeN mice were inoculated intradermally on the hind legs with 10⁴ wild-type or *bmpA/B⁻ B. burgdorferi*. Both wild-type and mutant spirochetes were readily cultured from skin, and qPCR of mouse skin biopsy (ear) specimens or blood (Fig. 4, D and E) collected 1 wk after infection showed similar pathogen levels. When ticks were allowed to engorge on mice infected with *bmpA/B⁻ B. burgdorferi*, the mutant spirochetes were able to migrate into fed ticks, albeit at a lower level than

the wild-type spirochetes (unpublished data). Because *bmpA/B⁻ B. burgdorferi* were as infectious as the isogenic wild-type spirochetes in the mice, we assessed whether BmpA and BmpB were required for *B. burgdorferi* to effectively colonize mouse joint tissue. The spirochete loads were analyzed in joints or in control tissue locations (skin and bladder) 14 and 18 d after *B. burgdorferi* infection. qPCR indicated that *bmpA/B⁻ B. burgdorferi* were not detected in joints, but were evident in the skin and bladder tissue at all time points, such as day 14 or 18 (unpublished data) after infection. Severe joint swelling developed in mice infected with wild-type *B. burgdorferi*, but not in animals infected with *bmpA/B⁻ B. burgdorferi*. Collectively, these data suggest that BmpA and BmpB may be essential for *B. burgdorferi* to colonize joints and induce Lyme arthritis.

Complementation restores the ability of *bmpA/B⁻ B. burgdorferi* to colonize joints and cause arthritis

To exclude the possibility that the failure of the *bmpA/B⁻ B. burgdorferi* to persist within joints was caused by an anomalous effect of transformation, we complemented, in trans, the

bmpA/B⁻ spirochetes with a wild-type copy of the *bmpA* and *bmpB* gene under the control of native promoter. To construct complemented isolates of *B. burgdorferi*, we transformed the mutant with the recombinant shuttle plasmid pKFSS1 carrying a wild-type copy of the *bmpA* and *bmpB* gene (Fig. 5 A) placed upstream of a streptomycin-resistance gene. As controls for the complementation experiments, additional mock-complemented isolates were also constructed by transforming the wild-type spirochetes and *bmpA/B⁻ B. burgdorferi* with the empty pKFSS1vector. PCR analysis confirmed that, similar to the wild-type *B. burgdorferi*, the mock-complemented and *bmpAB*-complemented spirochetes retained all of the *B. burgdorferi* plasmids (unpublished data). Immunoblotting showed that the *bmpAB*-complemented mutant, but not the mock-complemented mutant, produced both BmpA and BmpB protein (Fig. 5 B).

We then characterized the ability of the complemented spirochetes to establish infection and induce joint inflammation in mice. Groups of 10 C3H/HeN mice were inoculated intradermally in the hind legs with 10^4 wild-type spirochetes, mock-complemented *bmpA/B⁻ B. burgdorferi*, or *bmpAB*-complemented *bmpA/B⁻ B. burgdorferi*. The spirochete burden in the joint, bladder, or skin (ear) tissues and the joint swelling were evaluated 14, 21, and 28 d after *B. burgdorferi* infection. The *bmpA/B⁻* mutant and the complemented isolates all readily colonized the bladder and skin tissues. Although the *bmpA/B⁻* mutant was not detected in the joints, the *bmpAB*-complemented *B. burgdorferi* were able to persist in the joints at all times during peak infection, between days 14–21, albeit at lower levels than wild-type isolates (Fig. 5 C). At day 28, as the host immune response controlled the spirochete load, the *B. burgdorferi* levels in all tissues declined significantly. Joint swelling was
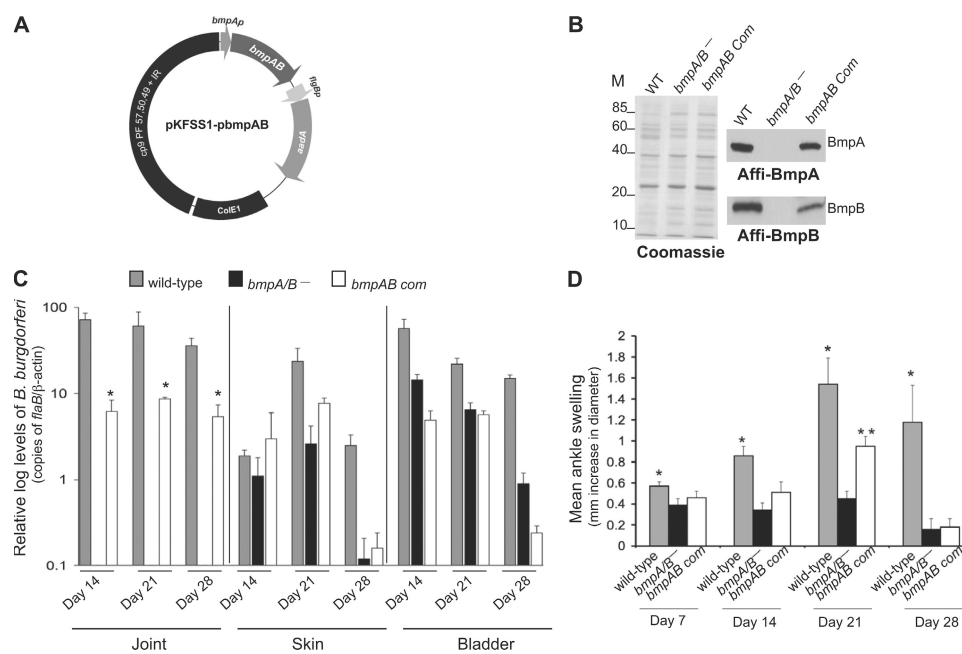

Figure 5. Complementation of *bmpA/B⁻ B. burgdorferi* with *bmpA* and *bmpB* restores the ability of spirochetes to survive in joints and induces greater levels of arthritis. (A) The *bmpA* and *B* gene, including its native promoter region was amplified and cloned into the shuttle vector pKFSS1 and transformed into the *bmpA/B⁻* spirochetes. (B) Western blot demonstrates production of the BmpA and BmpB proteins by the complemented *B. burgdorferi*. Equal amounts of proteins from the mock-complemented *bmpA/B⁻* spirochetes (*bmpA/B⁻*), mock-complemented wild-type (WT), or complemented spirochetes (*bmpAB* Com) were separated on a SDS-PAGE gel, which was either stained with Coomassie blue (left) or transferred to nitrocellulose membrane. Individual Bmp proteins were probed with the affinity-purified BmpA (top right) or BmpB (bottom right) antibodies. (C) The *B. burgdorferi* burden in mice infected with mock-complemented wild-type (gray bar), mock complemented *bmpA/B⁻* mutant (black bar), and *bmpA/B⁻* mutant complemented with *bmpA* and *bmpB* (white bar). Groups of mice (10 animals/group) were infected with wild-type or genetically manipulated isolates of *B. burgdorferi*, and the spirochete burden was analyzed at day 14, 21, and 28 by measuring copies of the *B. burgdorferi* flaB gene. Amounts of mouse *b-actin* were determined in each sample and used to normalize the quantities of spirochete DNA. Mutant or complemented spirochete levels in the bladder and skin (ear) tissue were similar. *n* = 3. P > 0.5. Levels of *bmpAB* complemented isolates were significantly higher in the joints than *bmpA/B⁻* mutants. Error bars represent the mean ± the SEM of relative tissue levels of *B. burgdorferi*. *n* = 3. *, P < 0.05. (D) Severity of joint swelling in *B. burgdorferi*–infected mice. Groups of mice (10 animals/group) were separately infected with mock complemented wild-type *B. burgdorferi* (gray bar), mock complemented *bmpA/B⁻* mutant (black bar), and *bmpA/B⁻* spirochetes complemented with *bmpA* and *bmpB* gene (white bar). Arthritis was evaluated by the assessment of development of joint swelling after 14, 21, and 28 d of spirochete challenge using a digital caliper. Bars represent the mean measurements (± the SEM) from three independent experiments. Significant differences in the inflammation level were noticed between groups of mice infected with wild-type isolates with the *bmpA/B⁻* mutant at all time points. **, P < 0.001 - 0.04. Highly significant differences between joint inflammation was also noted in groups of mice infected with *bmpA/B⁻* spirochetes and *bmpA/B⁻* spirochetes complemented with *bmpAB* at day 21. **, P < 0.001.

minimal in mice infected with the *bmpA/B⁻ B. burgdorferi* at 7, 14, 21, or 28 d, demonstrating that the kinetics of joint disease development was abrogated, and not merely delayed, in mice infected with the mutant. In agreement with earlier observations (14, 24), wild-type *B. burgdorferi* induced severe ankle swelling that is most dramatic at day 21 and is consistent with histopathological signs of arthritis (Fig. 5 D [P < 0.001] and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070962/DC1). Joint inflammation in *bmpAB*-complemented *bmpA/B⁻ B. burgdorferi*–infected mice were lower than that induced by the wild-type spirochetes, however, at the peak period of joint inflammation, day 21, the *bmpAB*-complemented isolates induced significantly higher swelling of the joints compared with *bmpA/B⁻ B. burgdorferi* (Fig. 5 D [P < 0.001] and Fig. S1).

DISCUSSION

B. burgdorferi alters its gene expression profile as it cycles between arthropods and mammals (29, 30), and specific genes help maintain the spirochete in nature (20, 24, 31–35). *I. scapularis* ticks, while feeding on a mammalian host, deposit a heterogeneous population of *B. burgdorferi* in the dermis (36), which eventually spread to multiple organs (2). Selected *B. burgdorferi* genes may be preferentially expressed in different locations (22); however, it is not known if the gene products that are induced in tissues can directly contribute to disease. We show that both genes of the *B. burgdorferi bmpA/B* operon undergo selective up-regulation in mouse joints (37), and that the targeted deletion of *bmpA/B* specifically decreases *B. burgdorferi* persistence in this tissue.

Immune sera from a *B. burgdorferi*–infected host (38, 39), or specific antisera (40–42), is protective when administered before spirochete challenge. The same antisera does not eradicate *B. burgdorferi* when transferred after challenge, but can modulate arthritis (39), showing that the borreliacidal and disease-modifying components in immune sera are different. Antisera against ubiquitously expressed spirochetal antigens, such as Arp (15, 16, 43) and DbpA (43), can modify arthritis with no effect on spirochete levels in tissues. Functions of BmpA and BmpB proteins in the *B. burgdorferi* natural cycle, and their correlation with the induction of arthritis, are currently unknown. In our study, both BmpA and BmpB antisera most dramatically inhibited the colonization of spirochetes in the joints, and to a lesser extent, in the skin, but not in other tissues. Although BmpA, DbpA, OspC, and FlaB antibodies have been identified in immune sera that has arthritis-resolving properties (12, 39), the passive transfer of BmpA antisera did not previously influence the genesis of arthritis (39). Differences in study design, spirochete isolates, such as N40 versus B31, used in the current experiments, or the quality of the antisera can potentially influence such results. Although the N40 isolate has been well known for its capability to induce arthritis in mice (12, 39), the limited genomic information on this isolate compared with B31 (25), and more importantly, the extreme difficulties in the genetic transformation of N40 (44), makes B31 preferable for these genetic

studies. The current experiments conclusively demonstrate that loss of both *bmpA* and *bmpB* influence the selective survival of spirochetes in joints and severely impeded the development of inflammation. Complementation of the *bmpA/B⁻ B. burgdorferi* with both *bmpA* and *bmpB* partially reconstituted the wild-type arthritis phenotype, further supporting a role for both BmpA and BmpB in Lyme arthritis. Differences between the complemented and wild-type spirochetes could be caused by the expression of *bmpA/B* on a shuttle vector in the complemented isolate, rather than from their native position on the *B. burgdorferi* chromosome, among other possibilities.

We have also examined several human samples to extend our observations beyond mice. *bmpA* and *bmpB* levels were measured in skin biopsies from five patients with early Lyme disease and *erythema migrans,* and synovial fluid from four patients with acute Lyme arthritis. qRT-PCR showed significantly higher levels (P < 0.03–0.009) of *bmpA* and *bmpB* expression per spirochete in the synovial fluid compared with the skin. In the joints, 21.4 ± 10.8 ng *bmpA*/ng *flaB* was noted compared with 0.36 ± 0.2 ng *bmpA*/ng *flaB* in the skin. Similarly, 11.0 ± 3.0 ng *bmpB*/ng *flaB* was present in the joints, compared with 0.34 ± 0.17 ng *bmpB*/ng *flaB* in the skin. We recognize that the skin and joint samples come from different patients; however, simultaneous skin and joint specimens from the same patient are rarely, if ever, obtained. Moreover, the spirochete in each sample may not be identical, even though the vast majority of organisms in the New York/Connecticut region are *B. burgdorferi* sensu stricto. Nevertheless, these data provide a first suggestion that our mouse data may be applicable to human disease as well.

In nature, *B. burgdorferi* survives in an enzootic cycle where immature ticks acquire the pathogen from wild rodents, and then maintain *B. burgdorferi* and subsequently transmit the spirochetes back to mice (3). As the tick bite site is primarily located in the mouse dermis, the population of *B. burgdorferi* that persistently resides in skin is most likely to complete the tick–mouse–tick life cycle, and should have a selective advantage in nature. The adaptive significance of dissemination of spirochetes to deeper host organs (37), such as the joints, where they are unlikely to be acquired by ticks, is not clear, although certain sites may afford survival advantages because of protection from host responses, binding ligands, or nutrients present in the microenvironment.

Lyme arthritis is the direct result of a localized host inflammatory response (1), presumably against antigens that are abundant on the surface of the invading spirochete. Joint-induced, surface-exposed, and highly antigenic *B. burgdorferi* proteins, such as BmpA and B could potentially initiate the complex cascade of host inflammatory responses that lead to arthritis. However, as the spirochete is coated with many proinflammatory lipoproteins, it is more likely that BmpA and B have a role in spirochete survival in the joints, and that the lack of arthritis in the *bmpA/B⁻ B. burgdorferi* is caused by the inability of these spirochetes to colonize and persist in joint tissue. In summary, we present direct evidence for a dynamic transcriptome of *B. burgdorferi* in vivo and show that differentially

up-regulated genes, such as *bmpA* and *bmpB* in the joints, play a role in spirochete persistence in a specific host environment. This information contributes to our understanding of the adaptive strategies of a pathogen that is able to survive in numerous tissues, and may lead to new ways to modulate Lyme arthritis.

METERIALS AND METHODS

B. burgdorferi and mice. A low passage (passage 3) virulent isolate of *B. burgdorferi* B31, clone 5A11, was used throughout this study (45). Mice were infected with a single inoculum of $10^4–10^5$ spirochetes/mouse by intradermal injection to the hind leg. 4–6-wk-old C3H/HeN mice were purchased from the National Institutes of Health. All animal procedures were performed in compliance with the guidelines and with the approval of the Institutional Animal Care and Use Committee.

Selective amplification of *B. burgdorferi* RNA and microarray analysis. Whole-genome nylon membrane arrays containing 1,697 putative ORFs encoded by *B. burgdorferi* B31 were used in this study (46). Selective amplification of *B. burgdorferi* transcripts in the infected mouse host was performed using the DECAL approach, as previously detailed (22, 47). Groups of C3H/HeN mice (10 mice/group) were injected with *B. burgdorferi* ($10^4–10^5$ spirochetes/mouse), and spirochetal infection was confirmed by PCR and culture (35). The mice were killed 15 d after spirochete inoculation, and total RNA was isolated from distal skin, joints (tibiotarsal), heart, and urinary bladder using the RNeasy RNA isolation kit (QIAGEN) as described by the manufacturer. 1 mg of total RNA was reverse transcribed into biotinylated cDNA using random hexamer primers, biotin-dNTP, and the SuperScript First Strand synthesis system (Invitrogen). Positive selection and amplification of spirochete transcripts was performed according to a published method (22), and the PCR products representing amplified *B. burgdorferi* mRNAs (skin-DECAL, joint-DECAL, heart-DECAL, and bladder-DECAL) were normalized using *flaB*-specific primers and labeled with a random priming method using FITC-labeled dNTPs (GE Healthcare). The labeled probes were further normalized based on their hybridization intensity to equal amounts of *flaB* PCR products blotted onto nylon membrane (unpublished data). The normalized probes were then used to hybridize duplicate microarrays using RapidHyb buffer (GE Healthcare) according to the manufacturer's instructions. After probing the arrays with DECAL-labeled cDNAs, the hybridization was scored visually, and spots were given a score ranging from 0 to 3 (in increments of 0.5) based on the intensity of hybridization, as previously described (22). A spot was considered positive when a minimum of twofold increase in hybridizations was noted (in joint), in comparison to similar spot hybridizations in other tissues (in skin, heart, and bladder).

PCR. The nucleotide sequence of various primers used in specific PCR applications is indicated in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20070962/DC1). RT-PCR or qRT-PCR analysis was performed as previously described (48) using iQ SYBR Green Supermix (Bio-Rad Laboratories) or using TaqMan probes synthesized by Applied Biosystems. All probes contained a 5′ reporter (FAM) and a 3′ quencher (TAMRA). The amounts of *B. burgdorferi bmp* transcripts in each sample were normalized to levels of *flaB* transcripts in qRT-PCR reactions. For quantitative detection of *B. burgdorferi* burdens within mouse tissue samples, qPCR was performed on an equal amount of isolated DNA using flagellin (*flaB*) as a surrogate marker (35). The mouse β-*actin* gene was amplified to normalize the amount of *B. burgdorferi* cDNA in the qPCR reaction. Skin biopsy specimens from five patients with *erythema migrans*, and synovial fluid from four patients with acute Lyme arthritis, were obtained at New York Medical College and Yale University School of Medicine. Skin biopsy specimens were immediately placed in RNALater (Ambion) and stored at −80°C; synovial fluid samples were rapidly frozen and stored at −20°C. Human samples were processed for cDNA synthesis and used for qRT-PCR, as described in this section.

Protein expression and preparation of affinity-purified polyclonal antibody. Recombinant BmpA and BmpB proteins were produced in *E. coli* using the bacterial expression vector pGEX-6P1 (GE Healthcare). The list of primers used for directional cloning of Bmp proteins (without the N-terminal leader sequence) is indicated in Table S1. Expression, purification, and enzymatic cleavage of the GST fusion proteins were performed as previously described (48). To generate polyclonal antisera, BmpA or BmpB (without the GST tag) produced in *E. coli* was emulsified in complete Freund's adjuvant and injected into groups of 2–3 rabbits (100 µg/animal). The animals were boosted twice at 3 wk intervals with the same dose of antigen in incomplete Freund's adjuvant, and the sera were collected 2 wk after the second boost. Respective cross-reactivity of the BmpA or BmpB polyclonal antibodies against BmpB or BmpA were removed using the previously described affinity purification approach (28), with the following modifications. Recombinant BmpB (rBmpB) was expressed with a GST fusion protein, as described in this section, and bound to a Sephadex 4B column (GE Healthcare) according to the manufacturer's instructions. The column was washed with 10 bed volumes of PBS. To purify BmpA antibody, the antisera was diluted fivefold in PBS and cycled over the rBmpB-bound column by gravity flow for 5 cycles of 10 passages each, for a total of 50 passages. BmpB antibody was also purified in a similar fashion by passing the diluted antisera through rBmpA-bound column.

Characterization of antibody in vitro. Affinity-purified BmpA and BmpB antibodies were tested for their bactericidal activity using a combination of two vital stains that specifically label live and dead spirochetes, as previously described (49). In brief, spirochetes (5×10^7/ml) were incubated in 15 µl BSK-H medium (Sigma-Aldrich) with the addition of equal volume of normal rabbit sera or purified Bmp antibodies for 48 h at 33°C. Spirochetes were labeled with the live/dead *Bac*Light Viability kit (Invitrogen) according to manufacturer's instructions. Bactericidal activities of the antiserum were determined by counting the number of live and dead spirochetes from 10 random microscopic fields under high magnification, as previously described (49). Bactericidal rabbit antiserum against *B. burgdorferi* OspA was used as a control in the assay.

Antibody binding to unfixed *B. burgdorferi* was assessed by immunofluorescence microscopy, as previously described (32). Spirochetes (10^7/ml) were spotted on silylated glass slides (PGC Scientific), blocked with PST (0.05% Tween-20, 5% normal goat sera in PBS), and incubated with normal rabbit sera or affinity-purified BmpA or BmpB antibodies (1:500 dilution in PST). Samples were similarly incubated with secondary FITC-labeled goat anti–rabbit antibody (KPL) at a dilution of 1:100, and spirochetes were visualized using an epifluorescence microscope (Carl Zeiss, Inc.).

Western blotting was performed as previously described (48) using 1:5,000 dilutions of either of the affinity-purified BmpA or BmpB antibody and HRP-conjugated anti–rabbit secondary antibody (Sigma-Aldrich).

In vivo antibody-blocking studies. In brief, C3H/HeN mice were infected with *B. burgdorferi* B31 (10^5 spirochetes/mouse) for 3 d and affinity purified Bmp or control (GST) antibodies were administered to groups of mice (15 animals/group, 100 µl antibody/mouse). To maintain effective concentrations of the antibody, the animals were treated with the respective antibody every 3 d for 2 wk. Parallel control studies confirmed that similar treatment of naive C3H mice with rabbit anti-BmpA/B sera do not result in joint pathology, as indicated by the assessment of arthritis at 2 and 4 wk after the initiation of antibody treatment (unpublished data). The mice were killed 12, 15, and 18 d after *B. burgdorferi* infections, and skin, heart, joint, and bladder samples were isolated and stored at −80°C. *B. burgdorferi* burdens in tissues samples were measured by qPCR analysis, as described in PCR.

Evaluation of joint inflammation. *B. burgdorferi*–infected mice were examined for swelling of the tibiotarsal joints, as previously detailed (12, 50, 51). Ankle joints from each of the rear legs of each mouse were measured using a precision metric caliper in blinded fashion. Thickest diameters of the tibiotarsal joints were measured in each mouse before *B. burgdorferi* infection, and the development of inflammation was monitored and tabulated on a weekly basis until the sacrifice of the mice. At least 5 ankle joints from each group of mice

(10 animal/group) infected with the different strains were subjected to histological analyses. 20 randomly chosen sections from each of the mouse groups were assessed for histopathological comparisons. For histology, joints were fixed in 10% formalin, decalcified, and processed for hematoxylin and eosin staining. Overall signs of arthritis represented a combined assessment of histological parameters of *B. burgdorferi*–induced inflammation, such as exudation of fibrin and inflammatory cells into the joints, alteration in the thickness of tendons or ligament sheaths, and hypertrophy and hyperlexia of the synovium.

Generation and phenotypic analysis of *bmpA/B* mutant and genetic complementation of *B. burgdorferi*. The oligonucleotide primers used for genetic manipulation of *B. burgdorferi* are indicated in Table S1. The *bmpA/B* mutant was constructed by exchanging the upstream of *bmpA/B* operon with a kanamycin-resistance cassette from the plasmid pXLF10601 (45) via homologous recombination. First, the 5′ and the 3′ arms flanking *bmpA* ORF (open reading frame) were PCR-amplified using primers P1-P4 and cloned into two multiple-cloning sites flanking the kanAn cassette in plasmid pXLF10601. Plasmid DNA of pXLF10601-bmpAB (30 μg) was electroporated into *B. burgdorferi* B31 clone 5A11 using the previously described protocol (45). Transformants were selected in BSK-H complete medium (Sigma-Aldrich) containing kanamycin (350 μg/ml). Spirochetes that survived antibiotic selection were further analyzed by PCR to confirm the desired integration of kanAn cassette, and all known endogenous plasmids that are present in parental B31 isolate were also assessed in the mutant clones, as previously described (32). One of the *bmpA/B* knockout clones that retained the same complete set of plasmids as the wild-type isolate was used in further experiments.

For genetic complementation of the *bmpA/B* mutant, we used the *B. burgdorferi* shuttle plasmid pKFSS1 (52) to deliver the wild-type copy of the *bmpA* and *bmpB* gene. The DNA fragment encompassing *bmpA* and *bmpB* gene and the native promoter (26) were PCR-amplified using primers (Table S1) and cloned into the *BamHI* and *SalI* sites of pKFSS1. 20 μg of the recombinant plasmid, designated pKFSS1-pbmpAB, were electroporated into the *bmpA/B*⁻ *B. burgdorferi*, and complemented clones were selected using 50 μg/ml of streptomycin in the growth medium. As appropriate controls for complementation studies, additional mock-complemented isolates were also generated by transformation of the wild-type *B. burgdorferi* and *bmpA/B*⁻ *B. burgdorferi* with the empty vector pKFSS1. PCR analyses were further performed to confirm that the mock or the *bmpAB*-complemented clones contained the respective plasmids, either the empty vector or pKFSS1-pbmpAB and also retained the same plasmid profile as the wild-type as described in the previous paragraph.

For phenotypic analysis of mock-complemented *bmpA/B* mutants and *bmpAB*-complemented isolates in vivo, *B. burgdorferi* isolates were injected into groups of mice (10 animals/group) via needle inoculation (10⁴ spirochetes/mouse) intradermally on the hind legs. For efficient maintenance of the shuttle plasmid in vivo, the mice were injected with streptomycin (300 μg/animal) every 2 d after the spirochetal inoculum and until the sacrifice of the animals. Separate experiments indicated that the injected doses of streptomycin do not interfere with *B. burgdorferi* pathogenicity or infectivity in mice (unpublished data). For tick acquisition studies, groups of mice (5 animals/group, 20 ticks/mouse) were allowed to be fed by naive ticks after 1 wk of *B. burgdorferi* infection, as previously described (48). The ticks were allowed to feed to repletion and detach from the mice, which usually occurred between 72–96 h. Immediately after engorgement, fed ticks were analyzed for qRT-PCR measurement of *bmp* expression or *B. burgdorferi* burden, as detailed earlier. Guts from parallel group of nymphs were also dissected under a microscope in PBS (20 μl/gut) after engorgement, and analyzed for BmpA and BmpB localization in wild-type spirochetes using immunofluorescence studies with specific Bmp antibodies as described above. For mutagenesis studies, mice were killed at day 14 and 18, and for complementation experimentation the mice were killed 14, 21, and 28 d after the initial inoculum. Joint, skin (ear), and bladder samples were isolated from infected mice, and *B. burgdorferi* burdens in tissue samples were measured by qPCR analysis, as described in PCR. Development of joint swelling in the infected mice was also evaluated on day 7, 14, 21, and 28.

Statistical analysis. Results are expressed as the mean ± the SEM. The significance of the difference between the mean values of the groups was evaluated by Student's *t* test with StatView software (SAS Institute).

Online supplemental material. Histological images of the ankle joints of mice infected with the wild-type *B. burgdorferi*, *bmpA/B* mutant and *bmpAB* complemented isolates are shown in Fig. S1. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20070962/DC1.

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