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Borrelia burgdorferi Adhesins Identified Using *In Vivo* Phage Display

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Summary

Borrelia burgdorferi, the agent of Lyme disease, disseminates from the site of deposition by *Ixodes* ticks to cause systemic infection. Dissemination occurs through the circulation and through tissue matrices, but the *B. burgdorferi* molecules that mediate interactions with the endothelium *in vivo* have not yet been identified. *In vivo* selection of filamentous phage expressing *B. burgdorferi* protein fragments on the phage surface identified several new candidate adhesins, and verified the activity of one adhesin that had been previously characterized *in vitro*. P66, a *B. burgdorferi* ligand for β_3-chain integrins, OspC, a protein that is essential for the establishment of infection in mammals, and Vls, a protein that undergoes antigenic variation in the mammal, were all selected for binding to the murine endothelium *in vivo*. Additional *B. burgdorferi* proteins for which no functions have been identified, including all four members of the OspF family and BmpD, were identified as candidate adhesins. The use of *in vivo* phage display is one approach to the identification of adhesins in pathogenic bacteria that are not easily grown in the laboratory, or for which genetic manipulations are not straightforward.

Introduction

Phage display is a technology that allows the identification of a gene or random nucleotide sequence of interest through the selection of particular properties of the protein or peptide encoded. The DNA encoding the peptide sequence selected is packaged within the same bacteriophage particle, allowing rapid isolation of the portion of the gene encoding the activity of interest. This approach has proven to be a powerful tool for the identification of peptides with particular binding characteristics, and of recombinant antibodies that recognize a particular antigen of interest (examples include Clackson *et al*., 1991, Coburn *et al*., 1999, Healy *et al*., 1995, Koivunen *et al*., 1994, Lowman *et al*., 1991, McCafferty *et al*., 1990). The technology has also allowed the identification of small peptide sequences that bind to the vascular endothelium in particular tissues in a living animal. This "*in vivo*" phage display approach has demonstrated that the endothelium in different tissues bears distinctive surface markers, and consequently, can be targeted specifically (Pasqualini & Ruoslahti, 1996, Arap *et al*., 1998, Rajotte *et al*., 1998). The identification of a peptide that bound to the endothelium in tumor tissue allowed the conjugation of a synthetic form of that peptide to the chemotherapeutic agent doxyrubicin, resulting in the delivery of the toxic drug specifically to the tumor (Arap et al., 1998). These studies therefore lay the groundwork for the application

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of phage display technology to analyses of protein-receptor interactions in other fields, including infectious diseases.

Borrelia burgdorferi is the causative agent of Lyme disease, currently the most common arthropod-borne infection in the United States and some other countries in the northern hemisphere. A key feature of *B. burgdorferi* is its ability to cause persistent infection in multiple tissues, which is essential to its maintenance in the tick-rodent cycle in nature, but has unfortunate consequences for accidental hosts outside this cycle, including humans. The ability to cause disseminated infection is facilitated by the ability of *B. burgdorferi* to invade, and then migrate out of, the vascular system. In early, disseminated infection, *B. burgdorferi* can be found in blood, but is never abundant (Barbour & Hayes, 1986, Wormser *et al.*, 2005). The organism is thought to be cleared from the blood by the host immune response and by the organism's propensity for invasion of vascular walls and colonization of perivascular connective tissues. In infected laboratory mice, the bacteria are most commonly found in or around arterial vessel walls (Barthold *et al.*, 1991, Barthold *et al.*, 1993).

Although tremendous strides have recently been made in the genetic manipulation of, and generation of mutants in, *B. burgdorferi* (reviewed in Cabello *et al.*, 2001, Elias *et al.*, 2003), there are still a number of challenges to the identification of genes important to the virulence of this organism using genetic approaches such as signature-tagged mutagenesis (STM) or *in vivo* expression technology (IVET). The transformation of infectious *B. burgdorferi* is hindered by a relatively low efficiency due to the presence of two putative restriction/modification systems encoded by the loci *bbe02* and *bbq67* (Kawabata *et al.*, 2004), by the segmented genome comprised of a linear chromosome plus numerous linear and circular plasmids (Fraser *et al.*, 1997, Casjens *et al.*, 2000), by the frequent loss of plasmids required for infectivity during *in vitro* culture, and by the requirement that each transformant clone to be tested for infectivity first be screened to ensure that the full complement of plasmids is still present (Elias *et al.*, 2002, Grimm *et al.*, 2003, Purser & Norris, 2000, Lawrenz *et al.*, 2002). Furthermore, the efficiency of recovery of *B. burgdorferi* from infected animal tissues appears to be low, and the infection persists in immunocompetent animals with relatively low numbers of organisms present in infected tissues as compared to organisms that, for example, colonize and cause disease in the intestinal tract.

Phage display was previously used *in vitro* to identify the *B. burgdorferi* protein, P66, that binds β_3-chain integrins (Coburn et al., 1999). Other approaches have been used to identify *B. burgdorferi* proteins that bind to fibronectin (Probert & Johnson, 1998), glycosaminoglycans (Parveen & Leong, 2000), and decorin (Guo *et al.*, 1995). However, the specific role of P66 during animal infection remains unknown, and Bgp (the *Borrelia* glycosaminoglycan-binding protein) is not required for mammalian infection (Parveen *et al.*, 2006). Two recent reports tested the role of the fibronectin-binding protein, BBK32, in the life cycle of *B. burgdorferi*. One study found that, at a single relatively high infectious dose, there was no significant difference between the wild-type and knockout strains in murine infection, or in bacterial acquisition by or transmission by feeding ticks (Li *et al.*, 2006). In contrast, the other study found that at lower doses, the mutants were attenuated in mice (Seshu *et al.*, 2006). The role of binding to decorin has been explored using decorin-deficient mice, in which arthritis severity, and survival of the bacteria in the skin and joint, are diminished (Brown *et al.*, 2001, Liang *et al.*, 2004a). Recent work suggested that the decorin binding proteins (DbpA and DbpB) are not required for infectivity in mice (Shi *et al.*, 2006), but one single dose of bacteria was used to infect the mice, so whether the *dbp*- strains might be attenuated could not be determined, and in fact the *dbp*- mutants were recovered from mouse tissues less efficiently than was the wild type. The fibronectin binding protein, BBK32, also binds to glycosaminoglycans (Fischer *et al.*, 2006), and so may functionally overlap with the DbpA, DbpB, and Bgp proteins. *B. burgdorferi* also binds to type I collagen in the native lattice form

(Zambrano *et al.*, 2004), but the protein responsible is not yet known. Since *B. burgdorferi* expresses several different adhesion activities that functionally overlap *in vitro*, it may be difficult to establish the pathogenic role of any one particular adhesin during infection. In addition, cell lines in culture do not necessarily reflect the properties of the cells from which they derive in the physiologically relevant setting of a living animal. To determine whether any of the known adhesins might be functional *in vivo*, and to identify additional candidate adhesins that might mediate these interactions but are not expressed by laboratory-cultivated *B. burgdorferi*, we selected for *B. burgdorferi* proteins that bind to the endothelium in three different tissues using *in vivo* phage display.

Results and Discussion

The subset of B. burgdorferi proteins (and genes) selected in vivo

To identify *B. burgdorferi* proteins that mediate interactions of the bacteria with the endothelium in different tissues during infection, filamentous bacteriophage clones encoding *B. burgdorferi* proteins fused to the phage protein III were selected in three tissues that are known to be colonized by the organism. Although many other tissues are infected by *B. burgdorferi*, limitation to this subset facilitated the prompt processing required to recover phage particles. A schematic representation of the protocol is shown in Figure 1. Not all of the phage pools that had been through selection demonstrated the highest titers in the tissues in which they were selected (i.e. for a tibiotarsus-selected pool, titer was not highest in the tibiotarsus); those that did not were not further analyzed. The latter set is nevertheless likely to contain tissue selected clones, as in most cases the titers were highest in the spleen, followed by the tissue of selection. A total of nine heart-selected pools (six of which were randomly chosen for further analysis), three tibiotarsus-selected (hereafter referred to as joint-selected) pools and one bladder-selected phage pool were obtained. These pools demonstrated enrichment of bacteriophage particles from the library on the basis of phage titer relative to titers in the other tissues analyzed. It is unclear why the different tissues appeared to have different selective potentials, e.g. why we did not obtain 9 tibiotarsus-selected pools with the highest titers in the tibiotarsus, as was the case with the heart. The 9 heart-selected phage pools were derived from all three unselected library pools, the three joint-selected pools derived from two of the three input pools, and the bladder-selected pool represented one starting pool. All of the tissue-selected phage pools contained single clones encoding fragments of many *B. burgdorferi* proteins, representing the background “noise” of this system. For a protein (and its corresponding gene) to be considered to be selected above the background, it had to have been enriched from multiple input library pools, or represented by multiple overlapping clones derived from the same pool. Only a small subset of the *B. burgdorferi* proteome (and genome) was represented among the selected clones that fit these criteria.

Of the 842 predicted genes (769 of >300 bp, 73 of ≤300 bp (Casjens et al., 2000)) in the *B. burgdorferi* chromosome, we obtained at least two independent isolates of 43 (5.1%). Of the 665 predicted genes (535 >300 bp, 130 ≤300 bp (Casjens et al., 2000)) contained in the plasmids of *B. burgdorferi* strain B31 clone M1, we obtained at least two independent phage clones representing 16 (2.4%). This calculation does not include the predicted pseudogenes in the *B. burgdorferi* strain B31 clone M1 plasmid genome, which are almost entirely plasmid-borne. All pools yielded single isolations of additional genes that were not further analyzed.

Proteins known or predicted to be on the B. burgdorferi surface and encoded on the chromosome

The genes encoding proteins that are known to be, or are predicted to be, localized on the surface of *B. burgdorferi* are of greatest interest in terms of how the bacteria might interact with the mammalian host. The surface proteins selected by *in vivo* phage display are listed in

Table 1. Three genes encoded on the chromosome and eight encoded by plasmids were selected *in vivo*. The three chromosomal genes, BB0210, BB0385, and BB0603, each have features of interest.

The first chromosomally encoded candidate adhesin gene identified, BB0210, encodes a very large protein that is predicted to be localized in the outer membrane. The localization of the protein, however, has not been experimentally demonstrated, and the mature protein is not predicted to contain any transmembrane helices. The function of this protein has not been determined in *B. burgdorferi*, and it is not clear whether the protein is expressed by the bacteria in the tick host or in laboratory culture, but serum antibodies from *B. burgdorferi*-infected mice do recognize the selected fragment of BB0210 in recombinant form, suggesting that the protein is expressed by the bacteria during mammalian infection (M.L. and S.A., unpublished data). Of the predicted 1,119 amino acids, all clones selected *in vivo* in heart, joint, and bladder shared a common region of 49 amino acids. This sequence comprises most of the repeat module predicted in the *B. burgdorferi* B31 M1 sequence, and the B31 protein is predicted to contain 7 repeats (Figure S1 in Online Supplemental Material).

The second chromosomal locus selected by *in vivo* phage display, *bb0385*, encodes a putative lipoprotein termed BmpD. This is one member of a family of four *B. burgdorferi* proteins that are expressed during mammalian infection, but whose functions are not yet known (Ramamoorthy *et al.*, 1996, Bryksin *et al.*, 2005). The *in vivo* selected clones encode the carboxyl-terminal half of BmpD (Figure S2 in Online Supplemental Material) plus an extension that appears to be encoded by strain N40 clone D10E9 but not by B31 M1 (the sequenced strain). Localization of BmpD on the surface of *B. burgdorferi* has not been definitively demonstrated, but the protein is recognized by antibodies from human patients (Bryksin et al., 2005).

BB0603, which was present in heart- and joint-selected pools, encodes P66, which was previously shown to bind to the β_3-chain integrins, $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ (Coburn et al., 1999). P66 also binds to $\alpha_3\beta_1$ and weakly to $\alpha_5\beta_1$ (J.C., unpublished, and (Coburn et al., 1999)). The portion common to the clones selected *in vivo* overlaps with the portion of the protein previously shown to be required for integrin binding (Figure 2) (Coburn et al., 1999, Defoe & Coburn, 2001), suggesting that the integrin binding domain is required for binding to the endothelium. Integrin $\alpha_{IIb}\beta_3$ is expressed exclusively by platelets and megakaryocytes. Platelets adherent to the endothelium in regions of microvascular damage (e.g. around the heart valves) might therefore serve as sites of attachment for P66, and intact *B. burgdorferi* cells have been noted in perivascular connective tissue in regions of vascular damage or stresses (Johnston *et al.*, 1985, Barthold et al., 1991, Armstrong *et al.*, 1992, Barthold et al., 1993). Integrins $\alpha_v\beta_3$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$ are expressed by diverse cell types. Although only two clones encoding P66 fragments were selected *in vivo*, they were selected independently from different starting pools and from different tissues. These observations, together with the previously characterized integrin binding activity of P66, support the hypothesis that binding to integrins might play a role during the course of *B. burgdorferi* infection and association with vessel walls.

Proteins known or predicted to be on the B. burgdorferi surface and encoded on the plasmids

Several *B. burgdorferi* plasmid-encoded proteins were also selected by *in vivo* phage display for attachment to the endothelium *in vivo*. Two plasmid lp-54-encoded proteins (BBA52 and BBA66) of unknown function were selected (Table 1). Both were present only in heart-selected pools. BBA52 is predicted to be a non-lipidated outer membrane protein that is down regulated in *B. burgdorferi* in the “host-adapted” dialysis membrane chamber model in comparison to *B. burgdorferi* grown *in vitro* (Brooks *et al.*, 2003), so the significance of BBA52 in mammalian infection is dubious. In contrast, BBA66 is predicted to be a lipoprotein of paralogous family 54, and was recently demonstrated to be surface-exposed and expressed during mammalian

infection (Brooks *et al.*, 2006,Clifton *et al.*, 2006). Neither BBA52 nor BBA66 has significant homology to proteins outside the *Borrelia* genus.

Four of the plasmid-encoded genes listed in Table 1, namely BBM38 (ErpK), BBO39 (ErpL), BBK2.10, and BBS41 (OspG), are the members of the OspF family of proteins (paralogous family 164) encoded on circular plasmids. The sequences that we list as BBK2.10 are most related to this protein, which was originally identified in *B. burgdorferi* strain 297 as an OspF homolog (Akins *et al.*, 1995). In the *B. burgdorferi* strain B31 M1 sequence the closest match is to the locus BBR42 (OspF) (Fraser et al., 1997,Casjens et al., 2000). Although some of these proteins have been previously studied and are known by other names, their functions have remained unclear. Our selection of the four OspF family members by *in vivo* phage display suggests that this family may mediate adhesion to host molecules. The OspE subfamily of the Erp family of proteins, in contrast to the OspF proteins, comprises a group that has been shown to bind complement regulatory factor H. The four proteins that we identified as candidate endothelial adhesins have not been found to bind factor H (Alitalo *et al.*, 2002).

Alignment of the sequences of selected clones representing BBM38 (ErpK), BBO39 (ErpL), BBK2.10, and BBS41 suggests that two distinct regions of these proteins may have adhesin activity (Figure S3 in Online Supplemental Material). OspG and BBK2.10 have both regions, while ErpK and ErpL have one each. It is apparent that our clone of N40 contains not only the *ospG* gene, but also *bbK2.10*, which has not previously been noted in other N40 clones. It is possible that the original tick isolate designated “N40” in fact represents a population of at least two distinct *B. burgdorferi* clones.

Two of the plasmid-encoded proteins that were identified in our *in vivo* phage selection are of considerable interest in the pathogenesis of *B. burgdorferi* infection. BBF32 encodes the *vls* locus, which has been demonstrated to encode a single expression site plus 15 silent cassettes (in the sequenced strain B31 M1) that undergo segmental recombination into the expression site “variable regions”, generating an enormous number of potential variants (Zhang *et al.*, 1997, Zhang & Norris, 1998a). Recombination occurs during murine infection but not during *in vitro* cultivation (Zhang & Norris, 1998b), and the plasmid encoding the *vls* is required for long term infection but not for the initial colonization of mammals (Labandeira-Rey *et al.*, 2003, Grimm *et al.*, 2004a), suggesting a critical role for the *vls* locus in persistent infection of mammals. The segment of *vls* common to all our selected clones (KAIVDAA; Figure S4 in Online Supplemental Material) is similar to a portion of invariable region two (IR2: KEIVEAA) and a portion of invariable region four (IR4: SAIVTAA) of the *B. burgdorferi* strain B31 M1 sequence (Eicken *et al.*, 2002, Zhang et al., 1997). The sequences flanking the sequence common to all the clones differ, which would be expected after passage of the bacteria through an animal. This variation makes precise alignments of the selected Vls sequences from N40 D10E9 to those of B31 M1 difficult. It appears that the selected clones fall into different subgroups based on the alignments (group 1: clones 42D and 971G, group 2: clones 116A and 196Q), so it is possible that both IR2 and IR4 are represented. The clone of N40 used to generate the phage library had been passaged through a mouse to demonstrate infectivity when it was isolated in the early 1990s (J.M. Leong, personal communication). The crystal structure and antibody accessibility studies of VlsE show that the second and fourth invariable regions are at least partially exposed on the surface of the protein and are recognized by the immune system (Eicken et al., 2002, Liang *et al.*, 2000, Liang & Philipp, 1999). These regions do not appear to be accessible to antibodies in *in vitro* cultivated bacteria, but it is possible that they are more accessible when the bacteria are in a mammal, a condition in which the abundant OspA protein is not expressed.

BBB19 encodes OspC, which has been demonstrated to be essential for mammalian infection (Grimm *et al.*, 2004b). Recent work demonstrated that OspC is required exclusively early in

infection, as when an *ospC* null mutant is complemented with *ospC* on a shuttle vector, that plasmid can be lost during murine infection (Tilly *et al.*, 2006). However, the bacteria remain viable and can be acquired by ticks, in which they survive the molt between blood meals and migrate to the salivary glands, but cannot establish infection in mice. Our results are consistent with the hypothesis that OspC may facilitate the initial colonization of the mammal by serving as an adhesin. OspC-encoding phage were selected most frequently in the heart, and, given that the protein appears to be essential in the early stages of infection, OspC may bind to a receptor, or a modification thereof, that is expressed by cells of the innate immune system and/or the skin, and in the cardiac endothelium. Two groups that determined the crystal structure of OspC noted that the protein forms a dimer and appears to have a binding site for an unknown ligand (Eicken *et al*., 2001, Kumaran *et al.*, 2001). All of our clones share 75 amino acids that include the segment from loop two through alpha helix four (Figure 3A) (Eicken et al., 2001, Kumaran et al., 2001), which comprises much of the surface exposed portion of the protein (Figure 3B). The helices involved in dimerization were not included in our selected clones.

Proteins not known or predicted to be localized on the surface of B. burgdorferi

A number of *B. burgdorferi* genes that are not predicted to encode surface-exposed proteins were also identified using *in vivo* phage display (Table 2). Some of these encode proteins that encode secretion signals but that are expected to be in the periplasm, e.g. the oligopeptide permease A2 and A4 subunits, and the flagellar subunit FlaB. Most are predicted to be cytoplasmic enzymes involved in the essential cell functions such as glycolysis, transcription, and translation. Several of the latter group, including BB0337 (enolase), BB0388 (RpoC), and BBG21 (hypothetical protein) have been found to have a non-specific “stickiness”, as they bind to plastic wells (L.T. Hu, personal communication). The others have not been tested, but some may display the same property. One interesting note, however, is that enolase has been reported to be localized on the surface of streptococci, where it binds plasmin(ogen) (Pancholi & Fischetti, 1998,Bergmann *et al.*, 2003).

Cell binding activities of selected phage clones and MBP fusion proteins

To verify that selected clones encoding fragments of known or predicted surface proteins were not non-specifically “sticky” we assessed attachment of representative phages to a number of mammalian endothelial and epithelial cell lines cultured *in vitro.* Cell lines were used because of the difficulties in obtaining primary murine endothelial cells in quantities sufficient for our experiments, and because primary cells are often not stable in *in vitro* culture. Phage clones containing *in vivo* selected sequences from OspC, ErpK, and Vls, bound to several mammalian cell lines (Figure 4 and data not shown), as did P66-containing phage ((Coburn et al., 1999) and data not shown). In multiple experiments, OspC, Vls, and ErpK containing phage bound most efficiently to the A549 and LA4 cells, which are human and murine lung epithelial cell lines, respectively. However, while OspC and ErpK containing phage did not bind to Ea.hy926 cells, the Vls containing phage did, supporting the hypothesis that different receptors are recognized. The vector phage, fdDog, also bound efficiently to the Ea.hy926 and 293 cells, but not to the A549 and LA4 cell lines, demonstrating that binding to the latter two cell lines is not simply conferred by the phage vector sequences. It is important to note here that binding to epithelial cells *in vitro* does not necessarily suggest that binding to endothelial cells *in vivo* is artifactual. Some classes of receptors, e.g. glycosaminoglycans, are expressed by multiple cell types, and not all receptors expressed by a particular cell type *in vivo* may be expressed by derivatives cultured *in vitro*.

To further ensure that the attachment of the *B. burgdorferi*-encoded protein fragments was not an artifact of expression in the bacteriophage particle, MBP fusions to ErpK and OspC were generated and tested for cell binding activity. These fusions also bound most efficiently to the A549 and LA4 cell lines (Figure 5 and data not shown). Surprisingly, the BB0210 phage did

not reproducibly or efficiently bind to any cell line tested, suggesting that either the apparent selection *in vivo* was an artifact, possibly due to the presence of several copies of the repeat sequence in close proximity on the phage particle, or perhaps because the receptor for BB0210 is not widely expressed by cultured cell lines.

Titering of individual phage clones in mouse tissues

Some of the *in vivo* selected phage clones were enriched in particular tissues. To determine whether this was a result of tissue-specific homing of particular *B. burgdorferi* protein fragments, or was the result of a bias in the first round of selection that was amplified in the second and third rounds, we established tissue-specific titers of particular phage clones after injection into the tail veins of mice. Not surprisingly, all phage clones tested, including the vector, showed high titers in the spleen (Figure 6). This is most likely a reflection of the circulatory system in that organ, as the vector and unselected library pools had the highest phage titers in the spleen (Table S1). In the remaining tissues, however, the titers of all clones were lower, and the vector control phage was clearly less efficient at binding to the endothelium than was any other phage clone. This result in itself is remarkable, as the vector control phage are more stable *in vitro* and *in vivo* than are phage containing large inserts, and are approximately 23 fold more infectious for *E. coli* than are the library phage (data not shown). In all tissues, the P66 phage bound significantly more efficiently than did the vector phage (Figure 10), and ErpK and Vls bound significantly more efficiently than the vector control in the ear (skin). Surprisingly, neither the BB0210 nor the OspC phage clone showed more efficient binding than did the vector phage in any tissue. This result, however, cannot be attributed to artifactual amplification in the first round of selection in any starting pool, because both were selected from all three starting pools of the phage library. While these results were somewhat different from the frequencies in which these clones appeared in the tissue-selected pools, they support the hypothesis that several particular *B. burgdorferi* protein fragments may have roles in binding of the bacteria to different mammalian receptors.

Summary and Perspectives

The use of *in vivo* phage display has confirmed the adhesion activity of P66, the *B. burgdorferi* ligand for β_3-chain integrins, in a physiologically relevant setting. *In vivo* phage display also identified several new candidate adhesins of the Lyme disease agent: OspC, Vls, certain Erp proteins, and BmpD. One curious result of our *in vivo* phage display selection is the preponderance of *B. burgdorferi* proteins that were selected in the heart more frequently than in the other two tissues (Table 1), including BmpD and OspC. A trivial explanation is that, for whatever reason, more phage are randomly trapped within the heart than within other tissues, perhaps the result of the heart being the first organ harvested in this study in which the phage are exposed to arterial circulation after injection through the tail vein. This speculation is supported by our observation that the heart is efficient at trapping the vector and the unselected library pools (Table S1). However, because we required multiple independent isolations for any gene to be considered “selected”, and we did not deliver the phage library pools directly into the arterial circulation by intracardiac injection, other explanations are worthy of consideration. Although no other data are available for BmpD, our results for OspC are consistent with those published by other groups, which showed that *ospC* transcripts are more abundant in the heart than in any other tissue in *B. burgdorferi* infected mice, although the adaptive immune response reduces *ospC* mRNA levels (Hodzic *et al.*, 2003,Liang *et al.*, 2004b). One model that is consistent with these results and others (Grimm et al., 2004b) is that OspC serves as an adhesin that is required for initial colonization of the mammal, and that *B. burgdorferi* cells that continue to express OspC after inoculation by the tick bind more efficiently to the endothelium in heart tissues during the dissemination of the bacteria. OspC has also been shown to bind to the tick salivary protein Salp15, which is believed to facilitate survival of the bacteria after inoculation into the mammal (Anguita *et al.*, 2002). Since Salp15

is predicted to be heavily glycosylated (Anguita et al., 2002), it is possible that OspC recognizes glycoproteins of both tick and mammalian origin.

A second curious aspect of this work is that, aside from P66, we did not obtain any *in vivo*-selected clones encoding *B. burgdorferi* adhesins that have been characterized *in vitro*. These include the *Borrelia* glycosaminoglycan binding protein Bgp (BB0588), the fibronectin binding protein (BBK32), and the two decorin-binding proteins, DbpA and DbpB (BBA24 and BBA25). It is likely that, within the lumen of a blood vessel, decorin is not available, as this proteoglycan is primarily associated with collagen fibrils in connective tissue. It is also entirely possible that serum proteins, e.g. fibronectin, removed the BBK32-containing phage from the pools, rendering them unavailable to adhere to the endothelium due to competition from the soluble protein. Another possibility is that *dbpA/B* and *bbk32* were absent from, or underrepresented in, our library. To address the possibilities of under-representation or absence, we performed quantitative PCR on the starting library and on the selected pools (Figure 7 and data not shown). While *dbpA* sequences were easily detectable in the starting library pools, this locus was undetectable in the *in vivo*-selected pools. We were unable to find a primer set that reliably amplified *bbk32* from the genomic DNA of our starting *B. burgdorferi* clone, N40 D10E9. It is possible that this gene might be under-represented in the DNA from which the library was made, as *bbk32* is not absolutely required for mammalian infection (Li et al., 2006 claimed that *bbk32* is not required, while Seshu et al., 2006 showed that *bbk32* mutants are attenuated). Most but not all of the bacterial cells in the population may have lost the plasmid carrying the gene. A more likely possibility is that the *bbk32* sequence in our N40 clone D10E9 may differ from published sequences from strain B31 and a different clone of N40 (Probert & Johnson, 1998), and therefore was inefficiently amplified by the PCR. That clone of N40 differs considerably from the one used in this work, as judged by the *ospC* and *bbk2.10/ospF* sequences.

The portion of the Bgp gene required for binding has not yet been identified, and the full-length gene is unlikely to be either efficiently amplified by quantitative PCR or to be present in the library, due to the nature of filamentous phage libraries (see below). There was evidence of selection of OspC, BB0210, and P66 in the selected pools, but we saw no evidence that *bb0210* and *ospC* (*bbb19*) were over represented in the library, although *p66* (*bb0603*) was over represented (data not shown). Analyses of the tissue-selected pools indicated that *bb0210, bbb19* (*ospC*), and *bb0603* (*p66*) were present at somewhat different levels in the selected pools (Figure 7). These data demonstrate that the *in vivo* phage display approach was selective for particular genes in particular tissues, as *ospC* was more abundant in five of the six heart-selected pools than in two of the three joint-selected pools. In contrast, *p66* was more abundant in the bladder-selected pool and two of the three joint selected pools than in any of the heart-selected pools. It is apparent, however, that selections in the same tissues were not always reproducible, a result that is likely a reflection of both selective and stochastic events that may occur in any selection. This emphasizes the importance of starting with multiple library pools and with multiple replicate selective media, in this case, mice.

There are a number of caveats to any filamentous phage display selection that are inherent to the biology of the bacteriophage itself. For example, the fusion of foreign sequences with those of phage gene III, as in the case of our library of *B. burgdorferi* DNA, requires that the open reading frame continue from the 5′ end of gene III through inserted sequence and through to the 3′ end of gene III. This would preclude the identification of proteins (and their corresponding genes) whose activities reside near or at the C-terminus of the protein. It is also possible that expression on the surface of a bacteriophage particle results in improper folding of the fusion protein, resulting in loss of adhesion activity. Furthermore, this approach is not applicable to the identification of proteins that are important for bacterial dissemination through

tissue matrices, colonization of extravascular sites, or persistence in the host. This is due to the limited lifetime and tissue penetration of bacteriophage particles circulating in animals.

Phage display has previously been used to identify peptides that bind to the endothelium in living animals, and has more recently been used for the same purpose in humans (Pasqualini & Ruoslahti, 1996, Rajotte et al., 1998, Arap *et al.*, 2002). This approach has demonstrated that the endothelium differs between tissues. To our knowledge, however, *in vivo* phage display selection from bacterial genomic libraries for proteins or peptides that serve as adhesins in a living animal has not been reported previously. The ease of genetic manipulations in some bacteria would obviate the need for approaches such as *in vivo* phage display, and for many pathogens the sites of colonization would render this approach untenable or irrelevant. Even for *B. burgdorferi*, which does disseminate through the vascular system, *in vivo* phage display would not identify the bacterial molecules that are involved only in extravascular interactions, which is likely to be the case for the decorin binding proteins. Nevertheless, this approach should be amenable to use in the identification of pathogen adhesins that mediate interactions with mammalian receptors expressed *in vivo*, particularly when the pathogen cannot be grown in the laboratory or genetically manipulated with ease or confidence.

Experimental Procedures

Bacterial strains, fd library, culture conditions

The filamentous phage display library of *B. burgdorferi* strain N40 DNA and associated *E. coli* growth and infection protocols were described previously (Coburn et al., 1999). The total genomic *B. burgdorferi* DNA used to make the library was isolated from an infectious clone (D10E9) of the tick isolate N40. The DNA was digested with *Mse*1 and *Csp*61, both of which leave 5′TA overhangs, under conditions that result in a light partial digestion of the intact DNA. Fragments between 200 and 1000 bp were purified from an agarose gel and ligated to adaptor oligonucleotides to allow ligation into the phage vector. The filamentous phage vector was derived from fd-tet (McCafferty et al., 1990, Hoogenboom *et al.*, 1991, Clackson et al., 1991). The library host *E. coli* strain was MC1061 (F^- *araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL* (Str^R) *hsdR2* (r_k-m_k+) *mcrA mcrB1)*. The *E. coli* strain TG1 (F^+ *Δ(lac-pro) supE thi hsd5/F'traD36 proA+B+* $lacI^q$ *lacZΔM15*), which expresses the F pilus required for phage fd infection, was used to amplify selected phage. The library was originally frozen down in six independent pools, but for the *in vivo* selections the original pools were combined in sets of two (1+2, 3+4, 5+6), so that selections were performed with three independent starting library pools. Where required, media were supplemented with 12.5 μg/ml tetracycline to select for the presence of the phage genome. Aprotinin was added to 10^{-2} TIU/ml (trypsin inhibitory units/ml) and benzamidine HCL was added to 1 mM to liquid cultures from which phages were harvested. After overnight growth of cultures at 37°C with agitation, phage particles were precipitated from 0.45 μm-filtered culture supernatants with PEG/NaCl, as described (Coburn et al., 1999). For injection into mice, phages from 60 ml culture supernatant were resuspended in 200 μl HEPES-buffered saline (25 mM HEPES pH 7.8, 150 mM NaCl) supplemented with aprotinin and benzamidine (HBSAB).

In vivo selections of phage

A schematic depiction of the protocol is shown in Figure 1. All protocols were approved by the institutional IACUC, and the mice were housed in the laboratory animal facility at Tufts-New England Medical Center. Female C3H/HeJ mice were used for all *in vivo* phage selections. This mouse strain was chosen because it is non-responsive to lipopolysaccharide (LPS) and therefore would not suffer unintended consequences in response to any LPS that night be present in the phage preparations. The mice were anesthetized with 2.5 % avertin (2,2,2-tribromoethanol dissolved in one part tert-amyl alcohol (Papaioannou & Fox, 1993)) in

phosphate-buffered saline. Aliquots of the phage preparations were taken to determine the input titers. The mice were injected through the tail vein with 50–100 μl phage (at minimum, 1–5 × 10^{10} total phage), which were allowed to circulate for 4 minutes (Rajotte et al., 1998), after which they were sacrificed by cervical dislocation. The mice were then perfused through the left ventricle of the heart with 4 ml HBSAB. The outlet for perfusion was the right ventricle of the heart (the heart tissue would have been perfused because the coronary arteries branch off the aorta immediately after it emerges from the left ventricle). Note that for all phage preparations, the numbers of phage particles actually used could not be determined in advance of the experiment. This was due to the differential stability of different phage clones (JC, unpublished data), combined with the requirement for infection of *E. coli* followed by overnight growth of the transduced *E. coli* cells on selective medium. In the initial round of selection, the heart, tibiotarsal joints, and urinary bladder were harvested from each mouse, rinsed with HBSAB, transferred to preweighed tubes containing 0.5 ml HBSAB and placed on dry ice. Although *B. burgdorferi* is known to infect skin as well, selection of phage in this tissue is problematic (Rajotte et al., 1998) and so was not performed. In subsequent rounds of selection, only the tissue from which the input phage pool had been selected in the previous round was harvested. After weighing, the tissues were homogenized by first chopping on dry ice, then grinding with glass mortars and pestles or disposable pestles in microfuge tubes, depending on the tissue. The tissue homogenates or dilutions thereof were used to infect naïve *E. coli* TG1, followed by growth on plates containing tetracycline. Phage pools were analyzed after three rounds of selection in the same tissue. After the third round, the pools were injected into mice from which the heart, tibiotarsal joints, bladder, spleen, and kidney were harvested to measure phage titers in the tissue in which that pool of phage had been selected vs. other tissues. For a phage pool to be considered “tissue-selected” the phage titer in the selective tissue had to be higher than those in all other tissues, including the spleen, which entraps phage efficiently. For example, for a pool to be considered “heart-selected”, it must have been selected in heart three times, with the final selection round yielding a higher phage titer in the heart than in all other tissues measured. Only the “tissue-selected” phage pools were analyzed further.

Analysis of tissue-selected phage clones

Each tissue-selected pool was plated to obtain single colonies, which were restreaked to isolate pure clones. Each clone was first analyzed by colony PCR to ensure the presence of *B. burgdorferi* DNA inserts, as described previously (Coburn et al., 1999) using fd primers listed in Table S2 (Online Supplemental Material). The PCR products that contained *B. burgdorferi* DNA were sequenced by the Tufts University DNA/Protein core facility. The sequences were then matched to the *B. burgdorferi* strain B31 M1 sequence on the TIGR website (http://www.tigr.org) (Fraser et al., 1997, Casjens et al., 2000). For the sake of simplicity and universality, genes identified in this work are referred to by the TIGR designation, with apologies to the authors who have named the same loci or the proteins they encode differently. Alignments were performed using CLUSTAL.

Several criteria were used to identify *B. burgdorferi* genes and their corresponding proteins that were selected for binding to the endothelium *in vivo*. First, evidence of multiple independent selections was required. Multiple independent selections of a particular gene were determined to be either those obtained from different starting (input) library pools, or those containing overlapping but non-identical fragments of the same gene, regardless of the input library pool. In addition, for a protein to serve as an adhesin, that protein must be localized on the bacterial surface, so clones representing surface proteins were of greatest interest in this study.

Titering phage clones in mouse tissues

Individual phage clones expressing a representative sequence of *bb0210, vls, p66, erpK* or *ospC* and the empty vector control phage fdDog, were prepared as described above for the library stocks. Female C3H/HeJ mice were anesthetized with isofluorane, and injected through the tail vein with 50–100ul of the phage preparations (at least 10^8 phages). The phages were allowed to circulate for 4 min., after which the mice were perfused through the heart (left ventricle) with 30ml of perfusion buffer under physiologic pressure (the outlet was the right ventricle). The heart, bladder, ear, tibiotarsus, and spleen were collected from each mouse at the end of the perfusion and were placed in preweighed tubes and put on dry ice immediately. After weighing, the tissues were minced and homogenized with plastic disposable pestles (or, for tibiotarsi, glass in glass homogenizers) in perfusion buffer supplemented with 100mM PMSF. The homogenates were diluted in L broth, then added to *E. coli* TG1 cells. Phage infected cells were selected on 12.5 μg/ml tetracycline plates. The preference of the individual phages for specific tissues was calculated as the log of the transducing units (TU) of output phage per gm of tissue/TU of input phage in the individual experiment. The experiments for each phage clone were repeated at least three times. GraphPad Prism software was used to graph and calculate the statistical significance of the phage per gram tissue. The non-parametric Mann-Whitney test was used to calculate the statistical significance of the binding of each phage clone in each tissue compared to the control phage, fdDog.

Generation of MBP fusion proteins

The *in vivo*-selected fragments of *ospC* (*bbB19*) and *erpK* (*bbM38*) were amplified by PCR using the primer sets listed in Table S2 (Online Supplemental Material). The gene fragments were digested with BamH1 and Sal1, then cloned in pMalC2 (New England Biolabs) that had been digested with the same enzymes. Clones containing the correct sequence were used to generate recombinant MBP fusion proteins as directed by the manufacturer's protocol and as previously described (Coburn et al., 1999). All of the proteins were present in the soluble fraction of *E. coli* and were stable during incubations with cells (data not shown).

Binding of phage clones and MBP fusion proteins to mammalian cells in culture

Most mammalian cell lines were purchased from the ATCC and cultured in the ATCC-recommended medium. The Ea.hy926 cell line was a generous gift from Dr. Cora Jean Edgell, and was cultured as described previously (Edgell *et al.*, 1983). For phage binding assays, the cells were plated in 96 well tissue culture-treated plates and allowed to grow to confluence. Phage stocks prepared as described above were suspended in HBSC + BSA buffer (25mM HEPES pH 7.5, 150mM NaCl, 1mM $MgCl_2$, 1mM $MnCl_2$, 0.25mM $CaCl_2$, 0.01 TIU/ml (trypsin inhibitory units/ml) aprotinin and 1mM benzamidine) plus 1% BSA (Coburn et al., 1999), and added to the cells, or to control wells without cells. To quantify the phage added to the wells, equal amounts were added to wells without cells and immediately fixed, then processed in parallel. After 3 hours at ambient temperature, the cell layers were washed with HBSC, then fixed with paraformaldehyde. Binding was quantified by ELISA using a rabbit anti-M13 antiserum (Abcam, Cambridge, MA), essentially as described (Coburn et al., 1999). A similar protocol was used to quantify binding of MBP fusion proteins to cultured mammalian cells, with the exception that incubations were carried out for 1.5 hours and the primary antibody used was anti-MBP (New England Biolabs).

Quantitative PCR

The presence of selected gene fragments in the tissue-selected pools and in the starting library pools was assessed by quantitative PCR using SYBR green PCR master mix (Qiagen, Hilden Germany and Valencia, CA, USA). All *B. burgdorferi* targets were amplified using 4.18×10^7 copies of the phage DNA per reaction (as determined by OD_{260}), and normalized to phage

vector sequences, for which the reactions contained 4.18×10^5 copies of the phage DNA (by OD_{260}). The standard curves consisted of known quantities of plasmid DNA (phage vector) or genomic DNA (*B. burgdorferi* genes) to allow quantification of the copy number in each phage pool. The final primer concentrations used were 0.2 μM. All reactions were performed for 40 cycles in a final volume of 25 μl in 96 well plates in a Stratagene MX3000p real time thermocycler, and the data were analyzed using the accompanying software. The copy number of each target *B. burgdorferi* gene was normalized to the copy number of the vector phage in each pool. Specific primer sequences and reaction conditions for each target are listed in Table 3 (Online Supplemental Material).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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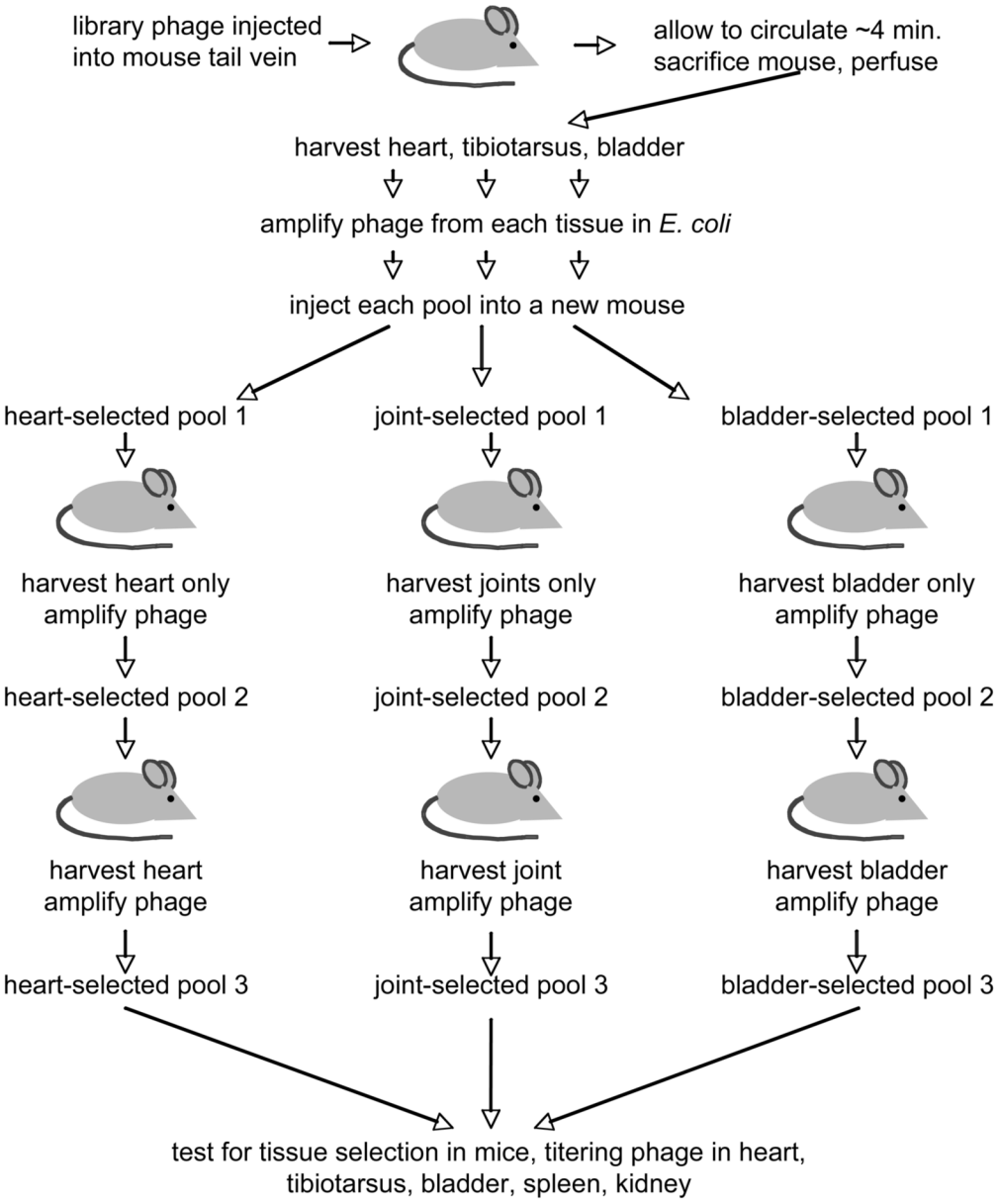

Figure 1.
Schematic representation of the selection for *B. burgdorferi* proteins that mediate interactions with endothelial cells *in vivo*.

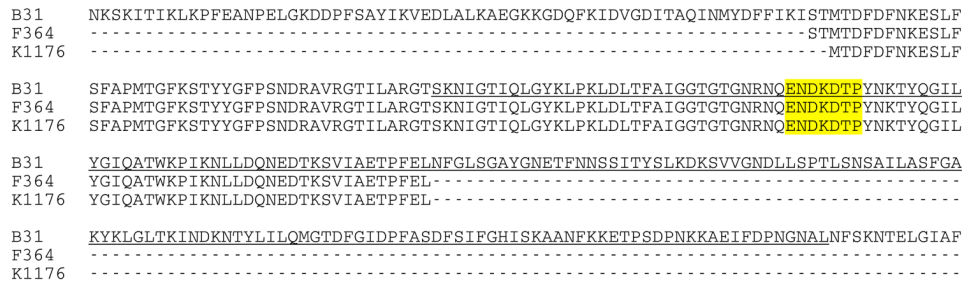

Figure 2.

Alignment of P66 sequences selected *in vivo* with the *B. burgdorferi* strain B31 M1 structural gene sequence, amino acids 61-376. The two clones, F364 and K1176, were obtained from different starting library pools and from different tissues. The portion of P66 identified in an *in vitro* selection for β_3 integrin binding is underlined, the peptide that inhibits *B. burgdorferi* attachment to β_3 integrins is highlighted. Dashes indicate absence of amino acids corresponding to those in the strain B31 M1 protein.

```
B31       81 IHQNNGLDTE NNHNGSLLAG AYAISTLIKQ KLDGLKN-EG LKEKIDAAKK CSETFTNKLK
N40 #1    81 IGN-NGLEAN QSKNTSLLSG AYAISDLIAE KLNVLKN-EE LKEKIDTAKQ CSTEFTNKLK
N40 #2    74 IAQ-NGLNAG ANQNGSLLAG AYVISTLIAE KLDGLKNSEE LKEKIEDAKK CNKAFTDKLK
fd         1 -------NAG ANQNGSLLAG AYVISTLIAE KLDGLKNSEE LKEKIEDAKK CNKAFTDKLK

B31     140 EKHTDLGKEG V--TDADAKE AILKTNGTKT KGAEELGKLF
N40 #1  139 SEHAVLGLDN L--TDDNAQR AILKKHANKD KGAAELEKLF
N40 #2  133 SSHAELGIAN GAATDANAKA AILKTNGTKD KGAQELEKLF
fd       34 SSHAELGIAN GAATDANAKA AIL------- ----------
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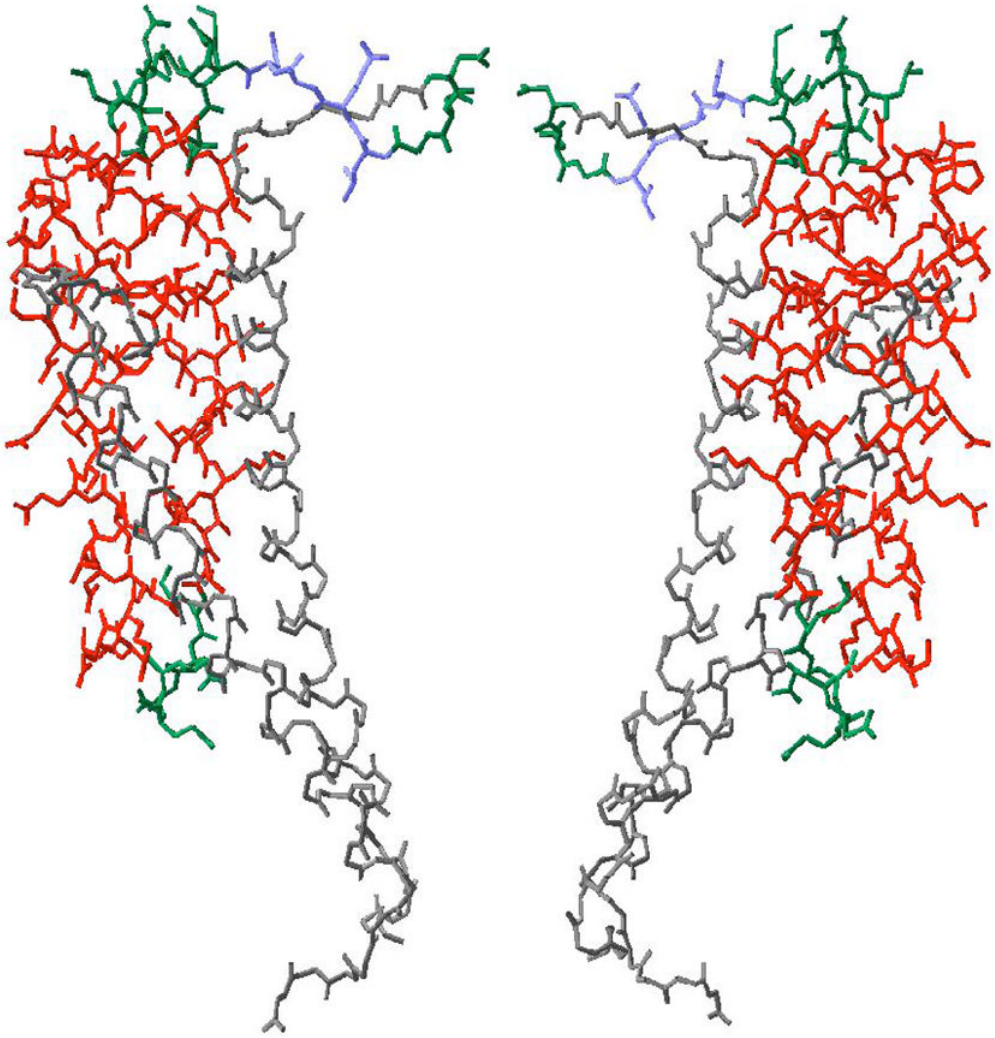

Figure 3.

The *in vivo*-selected portion of OspC. Panel A: the selected sequence aligned to two different sequences previously deposited in the database for *B. burgdorferi* strain N40 (Bunikis *et al.*, 2004, Shih & Chao, 2002), and to the B31 sequence (http://www.tigr.org) (Fraser et al., 1997, Casjens et al., 2000). “fd” denotes the sequence common to our selected clones. Panel B: The regions selected *in vivo* are, in order on the polypeptide chain, loop 2 (green), beta sheet 2 (blue), loop 3 (green), alpha helix 2 (red), loop 4 (green), alpha helix 3 (red), loop 5 (green), and alpha helix 4 (red). The regions shaded gray (alpha helices 1 and 5, beta sheet 1, and loop 6) were not selected *in vivo*. For the sake of clarity, the two monomers have been separated and the side chains are not shown in the non-selected (gray) regions. The image was created using the structural data deposited by Eicken et al. (Eicken et al., 2001) in the Protein Data

bank, Research Collaboratory for Structural Bioinformatics, Rutgers University (http://www.rcsb.org/pdb/Welcome.do), code 1G5Z, and customized for this figure using Swiss Pdb Viewer (http://www.expasy.ch/spdbv/mainpage.html).

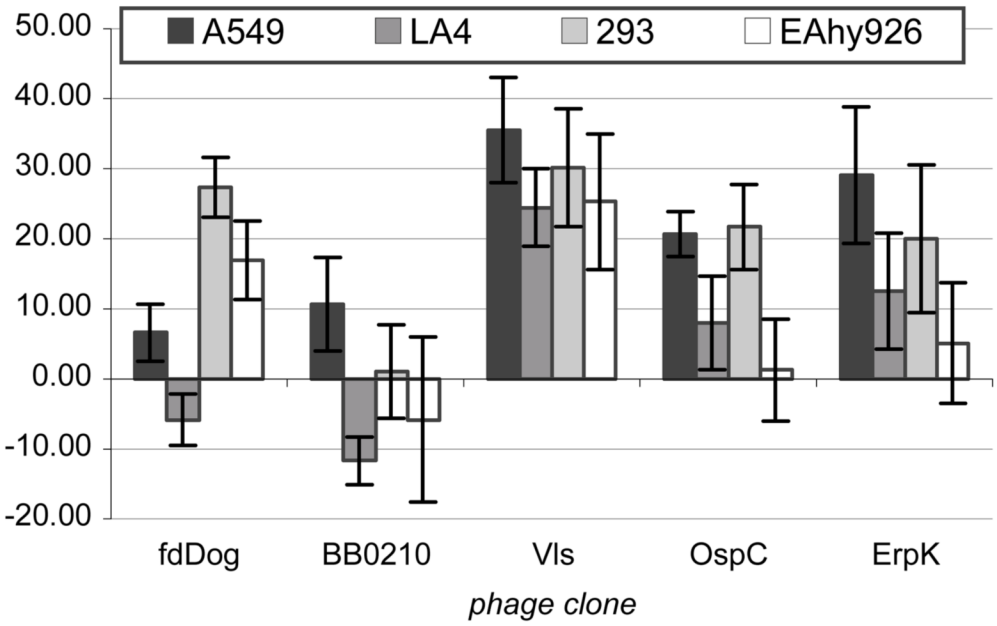

Figure 4.

Binding of selected phage clones to mammalian cells in culture. Individual phage clones containing the common selected *B. burgdorferi* sequences were added to confluent monolayers of cells, or to wells in which the cell culture medium, but no cells, had been plated. Phage binding was quantified by ELISA using an anti-M13 antibody; the inoculum was measured in separate wells in which phage were immediately fixed. Shown are the means + standard deviations of four replicates from a single representative experiment, after subtraction of binding to wells without cells. Each phage clone was tested for binding to at least 6 cell lines in at least four independent experiments.

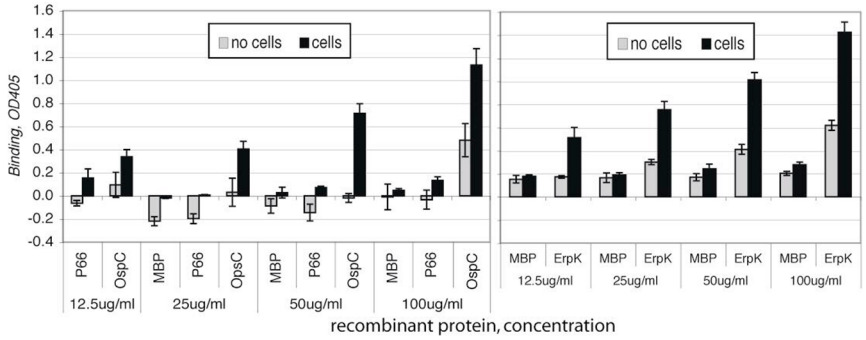

Figure 5.

Binding of ErpK and OspC to mammalian cell lines. The cells were allowed to grow to 90–100% confluence in 96 well plates. Control wells contained the cell culture medium but no cells. Recombinant MBP fusion proteins were added at the concentrations shown and incubated for 1.5 hours. Unbound proteins were removed by washing, then the wells were fixed. Binding of each MBP fusion and the control MBP alone was quantified by ELISA using a commercially available anti-MBP antiserum. Shown are the means ± standard deviations of 4 replicates from one of several (4–6) independent experiments for each panel. Data are shown for a cell line to which a corresponding phage clone binds efficiently.

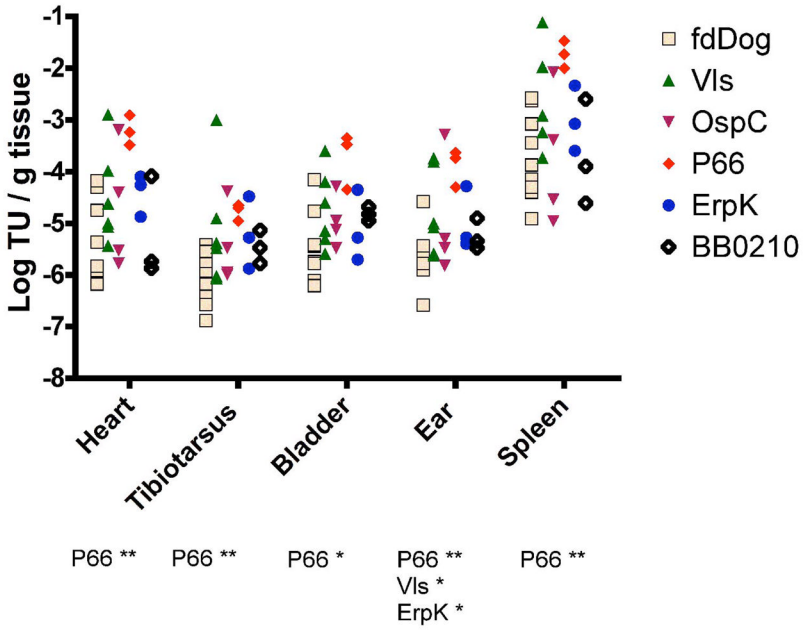

Figure 6.

Titers of individual phage clones in mouse tissues. Individual representative phage clones containing the common *B. burgdorferi* sequences selected *in vivo* were injected through the tail vein into mice and allowed to circulate. After perfusion of the mouse circulation through the heart, the tissues were harvested, weighed, homogenized, and diluted serially 10^{-1} to 10^{-8}. Each dilution was used to infect *E. coli*, and resulting colonies were enumerated the following day. Shown are the log transducing units (TU)/gm tissue normalized to the input phage titers for at least three independent experiments for each clone. P66 bound significantly more efficiently than the vector control phage fdDog with one star denoting a p value < 0.05 and two stars indicating a p value < 0.01. Vls and ErpK show statistically significant differences compared to fdDog in the ear, with p values of 0.0176 and 0.0364, respectively. For Vls the p values for heart and tibiotarsus are 0.08, and for bladder 0.06. Note that the value plotted do not take into account 23 fold greater efficiency of fdDog over the library phage in infection of *E. coli* cells (unpublished observation).

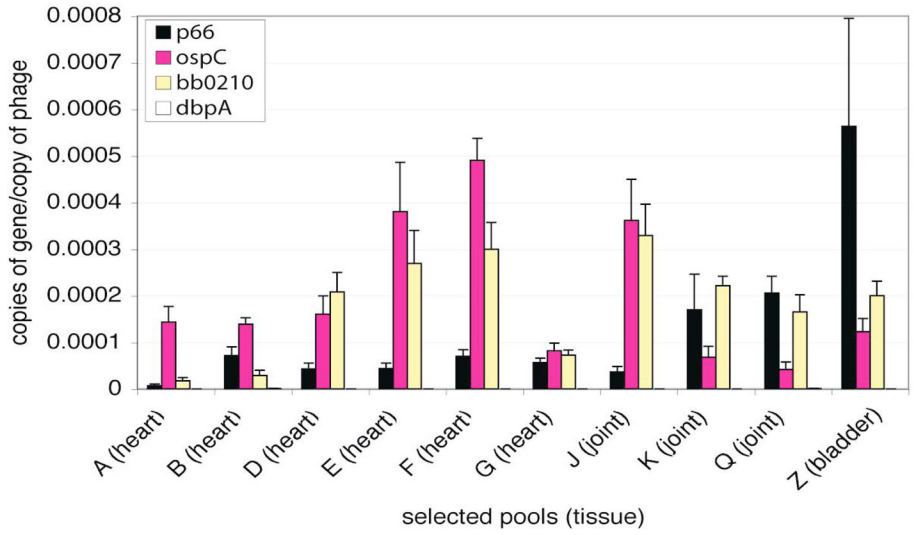

Figure 7.

Analysis of tissue-selected phage pools by quantitative PCR. Plasmid DNA (the replicative form of the phage genome) was purified from each tissue-selected pool, and genes of interest were amplified using real-time PCR. The means and standard deviations of 6–8 replicate experiments, each done in triplicate, are shown after conversion of C_t to copy number using standard curves consisting of phage genomic DNA. Although *dbpA* was not detected in the selected pools, it was easily detectable in the unselected library.

Table 1

Known or predicted outer surface proteins of *B. burgdorferi* selected by *in vivo* phage display.

B31 genome designation[1]	name[2]	library pool(s)	number & tissue of independent isolates
BB0210	Lmp-1	1, 2, 3	4 heart, 3 joint, 1 bladder
BB0385	BmpD	1, 3	3 heart
BB0603	P66	1, 2	1 heart, 1 joint
BBA52	outer membrane protein	1	2 heart
BBA66	putative P35 antigen	1	2 heart
BBB19	OspC	1, 2, 3	7 heart, 2 joint
BBF32	Vls	1, 2, 3	4 heart, 3 joint
BBM38	ErpK	1	4 joint
BBO39	ErpL	1	1 heart, 1 joint
BBK2.10[3]	OspF homolog	3	3 heart
BBS41	OspG	1, 2, 3	3 bladder, 5 heart

[1] The genome designations generally refer to the *B. burgdorferi* strain B31 sequence (Fraser et al., 1997, Casjens et al., 2000) accessible through the TIGR website http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gbb. An exception was made for BBK2.10 for the sake of clarity.

[2] The name appears as listed in the genome site at TIGR, with apologies to the authors who have given some of these proteins other names.

[3] BBK2.10 was originally identified in *B. burgdorferi* strain 297 as a homolog of OspF of strain N40 (Akins et al., 1995). The clones obtained in our *in vivo* selection are clearly more related to BBK2.10 than to the originally identified OspF.

Table 2

Proteins not known or predicted to be normally localized on the outer surface of *B. burgdorferi* selected >4 times by *in vivo* phage display.

B31 genome designation[1]	name[2]	library pool(s)	number & tissue of independent isolates
BB0056	phosphoglycerate kinase	1, 2, 3	4 heart, 3 joint, 1 bladder
BB0057	glyceraldehyde-3-phosphate dehydrogenase	1, 2, 3	13 heart, 7 joint
BB0147	FlaB	1, 2	5 heart, 2 joint
BB0220	alanyl-tRNA synthetase	1, 2	5 heart, 2 joint
BB0329	oligopeptide permease A2	1, 2, 3	6 heart, 3 joint
BB0337	enolase	1, 2, 3	8 heart, 3 joint
BB0388	RpoC	1, 2, 3	7 heart, 6 joint, 1 bladder
BB0540	EF-G	1, 2, 3	3 heart, 2 joint
BB0630	1-phosphofructinase	1, 2, 3	5 heart, 1 joint
BB0681	MCP-5	1, 2, 3	8 heart, 1 joint
BB0805	polyribonucleotide nucleotidyltransferase	1, 2, 3	4 heart, 3 joint
BBA34	oligopeptide permease A4	1, 2	2 heart, 3 joint
BBB23	conserved hypothetical protein	1, 3	5 heart
BBG21	hypothetical protein	1, 2, 3	4 heart, 2 joint
BBG29	conserved hypothetical protein	1, 3	5 heart

[1]The genome designation refers to the *B. burgdorferi* strain B31 sequence (Fraser et al., 1997, Casjens et al., 2000) accessible through the TIGR website http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gbb.

[2]The name appears as annotated in the genome site at TIGR, but the functions of most of these proteins have not been demonstrated experimentally in *B. burgdorferi*.