Borrelia burgdorferi surface-localized proteins expressed during persistent murine infection and the importance of BBA66 during infection of C3H/HeJ mice

by

Jessica Lynn Hughes

Bachelor of Science, University of Washington, 2001

Submitted to the Graduate Faculty of

the School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2008

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Jessica Lynn Hughes

It was defended on

April 3, 2008

and approved by

Saleem A. Khan, Ph.D., Microbiology and Molecular Genetics

Jeffrey G. Lawrence, Ph.D., Biological Sciences

Bruce A. McClane, Ph.D., Microbiology and Molecular Genetics

Ted M. Ross, Ph.D., Microbiology and Molecular Genetics

Dissertation Advisor: James A. Carroll, Ph.D., Microbiology and Molecular Genetics

Borrelia burgdorferi surface-localized proteins expressed during persistent murine infection and the importance of BBA66 during infection of C3H/HeJ mice

Jessica Lynn Hughes, PhD

University of Pittsburgh, 2008

Select members of the group *Borrelia burgdorferi sensu lato* are the causative agents of Lyme disease (LD), a multisystem, potentially chronic disorder with debilitating clinical manifestations including Lyme arthritis, carditis, and neuroborreliosis. Current knowledge regarding the expression of virulence factors encoded by *B. burgdorferi* and the breadth of their distribution amongst *Borrelia* species within or beyond the *sensu lato* group is limited. Some genes historically categorized into paralogous gene family (pgf) 54 have been suggested to be important during transmission to and/or infection of mammalian hosts. By studying the factors affecting the expression of this gene family and its encoded proteins, their distribution, and the disease profile of a *bba66* deletion isolate, we aimed to determine the importance of pgf 54 genes in Lyme disease and their conservation amongst diverse *Borrelia* species. The culmination of the studies discussed in this thesis describe the association of select genes historically categorized into pgf 54 with infectious phenotypes, with the borrelial sigma factor cascade, and the localization of their encoded proteins to the outer surface of the bacterial cell. Together, the expression profiles and localization of these genes/proteins demonstrates that they are regulated by the σ^N-σ^S cascade, similarly to the known virulence factor, OspC, and that they are found on the outer surface of the cell where they would have the potential to interact with or sense host factors. Moreover, putative orthologs of these genes were detected by Southern blotting and PCR in diverse *Borrelia* species associated with both Lyme disease and relapsing fever, some of

which expressed pH-responsive proteins that were cross-reactive with antibodies specific for orthologs expressed by *B. burgdorferi* isolate B31. Finally, an insertion-deletion of one of these genes, *bba66*, was examined *in vivo* and was found to be infectious in C3H/HeJ mice. Though *bba66* was not found to be absolutely required for murine infection in the study presented here, we and other groups hypothesize that *bba66* may instead be important during dissemination or adherence to murine cardiac tissue. Thus, future studies are aimed to determine the function and putative importance of BBA66 beyond the establishment of murine infection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

"I would thank you from the bottom of my heart, but for you my heart has no bottom."

Author Unknown

Potentially the most important part of my thesis is giving thanks to all those who have positively impacted and influenced my Ph.D. experience. This process has been both an academic and a personal challenge for me that many have contributed to. For your time, efforts, and belief in me, I can not thank you enough. However, for those who stood by me at the frontlines, I would like to recognize you in some small way.

To those who have contributed to my scientific and professional success:

The Carroll laboratory – Dr. James Carroll for your guidance, your critique, and your boundless energy and passion for science; Christi Nolder, Dr. Dawn Clifton, and Dr. Andrew Nowalk, for your support, sharing your scientific knowledge, and your friendship both in and out of the laboratory. Data contributed by lab members: J. Carroll – Table 1.6.1; C. Nolder – Figure 3.1.1A; D. Clifton – plasmid rescue from *Borrelia* recovered from murine tissues (Section 3.3.3) and Figure 3.3.7; Table 3.3.1 – C. Nolder, D. Clifton, and J. Hughes.

My thesis committee – Drs. Khan, McClane, Lawrence, Ross, and Chad Steele (former committee member), for your scientific expertise and critical assessment of my work and for broadening my scientific perspective.

Departmental mentors – Drs. Joanne Flynn and Mike Parniak for your scientific and professional advice and expertise. Your guidance has been much appreciated.

For providing materials used in these studies – Drs. Mohamed A. Motaleb and Nyles Charon for the monoclonal α-FlaB, Dr. Linden T. Hu for the polyclonal α-OppA1 rabbit serum, Dr. Kit Tilly for B. burgdorferi B31 A-34, Drs. Patricia Rosa and Tom Schwan for various RML *Borrelia* isolates, and Drs. Mark A. Fisher and Frank C. Gherardini for the strains A3-Gm, A3ntrA-Gm, A3ntrA-comp, and A3rpoS. Drs. Sherwood R. Casjens, John J. Dunn, Benjamin J. Luft, Claire M. Fraser, Weigang Qiu, and Steven E. Schutzer, working under grants from the Lyme Disease Association and National Institutes of Health (AI37256, and AI49003), for access to unpublished sequence information. This research was supported in part by CDC cooperative agreement number CI000181. J. Hughes supported by NIH predoctoral training grant number AI060525. Dr. Andrew Nowalk was supported by NIH training grant number HD042987.

To my friends and family:

To my parents and my fiancé: you've applauded me at my best and cheered me on at my worst. You've sent care packages, taken late-night phone calls, and given me advice when I've needed it most. For you my love and gratitude are endless.

To my family: for not always understanding what my work was about, but always asking about it, always supporting it, and always lending your support.

To my friends: you have sustained my sanity, been there for both scientific and personal advice, and you have been fantastic champions of my success.

1.0 INTRODUCTION

1.1 DISCOVERY, ISOLATION AND DESCRIPTION OF *B. BURGDORFERI*

The earliest description of erythema migrans (EM), the bull's eye-shaped rash associated with the acute phase of Lyme disease, was reported in 1908 during a lecture given by a Swedish dermatologist, Arvid Afzelius. After further observations, Afzelius hypothesized that EM developed in patients after the bite of a tick (2). EM was first reported in the United States in 1970 (395) and was followed in 1977 and 1978 with reports that identified a cluster of children and adults near Lyme, Connecticut, that suffered from arthritic manifestations, some of whom suffered from a preceding EM skin lesion; importantly, some patients who developed EM could also recall being bitten by a tick (416, 420-422). This "new clinical entity" was dubbed Lyme arthritis (420-422). Given the correlation between ticks, EM, and Lyme arthritis, Steere and colleagues suggested that the infectious agent of Lyme arthritis was transmitted through the bite of ticks, specifically ticks of the *Ixodes* complex which were prevalent in and around Lyme, Connecticut (420, 422). In line with this hypothesis, Dr. Willy Burgdorfer observed spirochetal organisms of approximately 10 to 30 nm in length and 0.18 to 0.25 nm in diameter in the midgut and select other tissues from *I. dammini* ticks collected from Shelter Island, New York (64). The spirochetes were noted to stain well with Giemsa and could be visualized under darkfield and by indirect immunofluorescence. Drawing from epidemiological data, Burgdorfer suggested that

the organism observed in *Ixodes* ticks may be the causative agent of Lyme arthritis. In a collaborative study, Steere, Burgdorfer, and others conclusively identified the spirochete isolated by Burgdorfer as the causative agent of Lyme disease (419). In their report, serum from Lyme arthritis patients was immunoreactive with the spirochete isolated by Burgdorfer; moreover, spirochetes isolated from patient EM, cerebrospinal fluid, or blood were found to have a similar morphology to the isolate obtained by Burgdorfer. Through genetic and phenotypic analyses, the Lyme disease spirochete was shown to be more closely related to relapsing fever *Borrelia* species and less so to the treponemal or leptospiral spirochete species (203, 209, 384). Thus, in 1984, the spirochete was named *Borrelia burgdorferi* in honor if its discoverer, Dr. Willi Burgdorfer (210).

In the twenty-seven years following the identification of *B. burgdorferi*, 14 *Borrelia* species have been categorized into the group collectively referred to as *B. burgdorferi sensu lato* (i.e., in the broad sense; *sl*) (22, 144, 216, 250, 276, 337, 338, 364, 461). Though it is unknown if the majority of *B. burgdorferi sl* species are able to infect humans, three genospecies, including *B. burgdorferi sensu stricto* (i.e., in the strict sense, *ss*), *B. garinii*, and group VS461 (now referred to as *B. afzelii* (76)), have been shown to be definitively associated with Lyme disease in human infections (16, 17). *B. valaisiana* has also been associated with human infection, albeit infrequently (374, 375, 382). Several distinctions can be made between the species that infect humans including their geographic ranges and the disease manifestations with which they are most commonly associated. The geographical range each *Borrelia* species is associated with is based primarily upon the range of their cognate tick vectors; *B. burgdorferi ss* in the United States is found in the mid-West and Northeast in association with *I. scapularis* and in the Northern Californian coastal region with *I. pacificus*. *B. afzelii* and *B. garinii* meanwhile,

as well as *B. burgdorferi ss*, are found throughout Europe in association with *I. ricinus* and in Asia with *I. persulcatus* (4, 5, 63). Within the Midwestern and Northeastern United States, 10 % - 20 % of nymphs and 20 % - 30 % of adult ticks are colonized with *B. burgdorferi* while only 1 % - 3 % of *I. pacificus* ticks are colonized. In addition to the differences between *Borrelia* species and the specificity of the tick vector each is associated with, there are also differences in the predominant disease manifestations associated with each species. While the acute stage of disease is similar between species, manifestations during the later stages of Lyme disease are often, though not exclusively, associated with a single species: Lyme arthritis and cardiac complications with *B. burgdorferi ss*, neuroborreliosis with *B. garinii*, and acrodermatitis chronica atrophicans (ACA) with *B. afzelii* (18, 462). The course of Lyme disease and its associated clinical manifestations are discussed below in detail (Section 1.2).

In the United States, 64,682 new cases of Lyme disease were reported between 2003 and 2005 to the Centers for Disease Control (CDC); of these, 59,770 (93%) cases originated in the 10 reference states endemic for Lyme disease, which includes Connecticut, Delaware, Maryland, Massachusetts, Minnesota, New Jersey, New York, Pennsylvania, Rhode Island, and Wisconsin (70). Importantly, estimates predict that the reported incidence of LD may be under-reported by as much as 6- to 12-fold in these endemic areas (84, 94, 160, 288). Given the growing incidence of Lyme disease in the United States and abroad, *B. burgdorferi* has been recognized as an emerging pathogen (273, 324, 325, 410, 417, 457). Though people of any age and sex are susceptible to the bite of tick, LD is reported more frequently in boys between the ages of 5 and 19 and in persons of either sex beyond the age of 30 (70, 84-86, 316). The likelihood of developing Lyme disease also correlates with outdoor activities and travel, especially in wooded

(63, 105, 164-166, 243, 279, 290), high-shrub (155) and grassland (247) areas where tick populations tend to be greatest.

1.2 CLINICAL MANIFESTATIONS

Lyme disease is a multi-stage and multi-system disorder affecting people of all ages, races, and genders. Of the three species belonging to the *B. burgdorferi sl* group known to cause Lyme disease and to infect humans (see Section 1.1), only *B. burgdorferi ss* has been linked to ticks and disease in North America (263, 277) and it is the main species discussed and utilized in this body of work. *B. burgdorferi* is transmitted by the bite of a *B. burgdorferi*-colonized *Ixodes* species tick and is the most common reportable vector-borne disease in North America (70, 84-86). The tick lifecycle and mode of *B. burgdorferi* transmission is discussed in greater detail in Section 1.3. Antibiotic treatment can successfully clear borrelial infections, especially when treatment is administered early. Of individuals who remain untreated, however, clinical manifestations affecting the joints, heart, and/or nervous system may develop. Whether *B. burgdorferi* is transmitted transplacentally in pregnant women is a controversial topic, but it is considered to be a rare phenomenon. Furthermore, the damage caused to the fetus due to borrelial infection is believed to be minimal but remains to be fully characterized (407, 434, 471, 478).

1.2.1 Early/localized stage

The early/localized stage of LD generally occurs during the first days or weeks of infection. The hallmark of early of Lyme disease is the presence of erythema migrans (EM) at the site of the tick bite (37, 292, 300, 418). The rash may be rapidly expanding and have zones of clearing which give it a bulls-eye appearance. These zones of clearance are more often associated with *B. burgdorferi ss* infection and less so with *B. afzelii* or *B. garinii*; EM associated with these later species is often slower to spread and demonstrates less intense inflammation (433). The estimated number of patients that present with EM range from 60 % to 80 % (112, 119, 423). However, those who do not develop EM may have no other outward signs of infection at this early stage and may not seek treatment unless they recall having been bit by a tick in a region endemic for Lyme disease. While EM alone is often considered suggestive of borrelial infection during this early stage of infection, diagnosis during this early stage can be difficult in the absence of EM. Serology is weak within the first weeks of infection and seroconversion toward borrelial antigens may be difficult to detect (Section 1.2.4).

1.2.2 Early disseminated stage

After the appearance of the initial EM, secondary lesions may appear days to weeks later at distal sites. These lesions are an indicator of borrelial dissemination (473) and are more commonly described among patients in the United States (418). Patients may experience additional symptoms such as fever and chills, malaise, fatigue, headache, myalgia, and general body aching (414, 418). Occurring weeks to months later, some untreated patients in the United States may develop acute neurological complications, also referred to as neuroborreliosis (317, 358), and/or

Lyme carditis (415). Neurological manifestations may include Bells palsy or meningitis-like symptoms. Bells palsy involves paralysis of the seventh cranial nerve causing flaccid facial paralysis that is typically unilateral; in one study, 87.5% of untreated patients (14/16) presenting with Bells palsy went on to develop Lyme arthritis (212). Symptoms resembling meningitis include severe headaches, neck stiffness, and confusion (212). Lyme carditis is caused by inflammation of the cardiac tissues and can lead to dizziness or fainting, shortness of breath, and heart palpitations (476); some patients may also present with heart block (221, 281, 282, 332, 362, 452). There are numerous causes, including viral and bacterial agents as well as autoimmunity arising from molecular mimicry, that may cause carditis (31, 109, 110, 124, 172, 177, 283); thus, without a previous diagnosis of Lyme disease, Lyme carditis may be misdiagnosed and the appropriate treatment regimen delayed. Furthermore, patients may have long-lasting damage or continuing heart manifestations even after the clearance of infection with antibiotic treatment (281, 282, 299, 452).

1.2.3 Late disseminated phase

Manifestations of the late disseminated phase may occur months to years after the initial infection. During this phase, arthritic, cardiac, or neurological abnormalities may persist and/or worsen and skin involvement may develop. Interestingly, human-pathogenic species of the *B. burgdorferi sl* complex appear to be more strongly associated with specific Lyme disease manifestations: *B. burgdorferi ss* with Lyme arthritis, *B. garinii* with neurological complications, and *B. afzelii* with skin abnormalities (17, 76, 451, 462); it is important to note, however, that there is considerable overlap among all of these species and the clinical symptoms they may cause (462). Late Lyme arthritis is defined as recurring joint inflammation and pain that

involves sporadic joint swelling that may last for weeks or months before spontaneously resolving (476). The condition usually manifests unilaterally in larger joints, especially in the knees. In the United States, recent reports estimate that somewhere between 10 % and 33 % of untreated patients may develop Lyme arthritis (70, 86, 476). Meanwhile, neurological complications from late Lyme disease may range from encephalomyelitis, which can cause fever, headache, and confusion, to Lyme disease-associated encephalopathy, a degenerative brain disorder which can cause an altered mental state (179, 180, 214, 265). Finally, acrodermatitis chronica atrophicans (ACA) is characterized by a papery-thin skin lesion and swelling that may present uni- or bi-laterally; ACA is typically observed on the feet and hands and sensory loss in the affected extremity may also occur (222, 236). Though neurological and skin manifestations of late Lyme disease have been reported in the United States, they are relatively rare (70, 86, 298) and occur more often in Europe. Given the geographical distribution of these species (Section 1.1), Lyme arthritis is more often observed in the United States while neurological and skin manifestations are more often reported in Europe. Though serology remains the best method for diagnosis during this phase, α-borrelial antibodies can be long lived and may reflect a past infection that has been cleared (213); thus, positive serology is not conclusive for an active infection.

1.2.4 Post-Lyme disease syndrome

Though controversial, some believe a fourth phase of disease, dubbed post-Lyme syndrome, may occur in a small but distinct set of patients. Unlike patients in the chronic stages of Lyme disease, individuals with post-Lyme syndrome are non-responsive to antibiotic treatment (468) and experience clinical manifestations including non-localized myalgia and arthralgia, as well as

tiredness. This lack of responsiveness to antibiotic would seemingly suggest that spirochetes are no longer present in these patients and that the chronic symptoms might be caused by autoimmunity caused by molecular mimicry (Section 1.2.7); however, this remains a controversial topic within the field, with some suggesting that *Borrelia* might well still be present in post-Lyme syndrome patients (328). It should be noted, though, that the very existence of post-Lyme syndrome is hotly contested with some researchers believing that the initial diagnosis of Lyme disease in those patients later identified to suffer from post-Lyme syndrome is questionable at best (425).

1.2.5 Diagnosis of Lyme disease

The guidelines recommended by the CDC and others for the diagnosis of Lyme disease involves a two-tier approach comprised of an enzyme-linked immunosorbant assay (EIA) followed by Western blotting (71, 115, 125). The first tier uses a polyvalent EIA and tests serial dilutions of patient sera. If the patient sera is found to be positive by EIA, Western blotting is performed to assess for the presence of specific IgM and IgG α-*B. burgdorferi* antibodies. At least two out of three known bands for IgM and/or five out of ten known bands for IgG must be detected for a test to be considered positive. Because α-*B. burgdorferi* antibodies may be detectable in patient serum months after an active infection has resolved (3, 15, 156, 181, 182), positive EIA and/or Western blotting are only indicative that a patient has been infected with the spirochete and not that they are actively infected.

Alternative techniques for the diagnosis of Lyme disease have been used with varying levels of success. Culturing spirochetes from EM biopsies (264, 307), patient blood (38, 474, 477, 479), and occasionally from whole blood (477), plasma (474, 475, 477) or cerebral spinal

fluid (CSF) (215, 293) have demonstrated marginal success. PCR from patient EM biopsies, plasma, or synovial fluid, on the other hand, is relatively sensitive and may indicate the presence of an active infection (108, 161, 175, 259, 304, 394, 456). Moreover, in a study by Nowakowski and colleagues (307), major laboratory diagnostic techniques were compared and quantitative PCR (qPCR) of skin biopsies was found to be the most sensitive diagnostic method, followed by two-tier immunological analysis of disseminated (early or late) phase patient samples, skin culture, and blood culture (307). Given the invasive nature of skin biopsies and the expected 10 to 14 days for the production of α-*B. burgdorferi* specific antibodies, antibiotic treatment is routinely administered immediately and without further testing if a patient presents with EM and/or has a history suggesting exposure to *Borrelia*-colonized ticks; however, EM is not considered a definitive diagnostic marker of early Lyme disease (307, 476). To definitively diagnose Lyme disease the gold standard is the two-tier test comprised of EIA and Western blotting of patient sera (307, 476). Intrathecal antibodies, however, are the best diagnostic marker for neurological manifestations (293) as PCR (87, 219, 251) and culture (215, 373) of CSF may demonstrate varied levels of sensitivity.

1.2.6 Treatment of Lyme disease

Treatment recommendations were recently reviewed and updated by panel of experts belonging to the Infectious Diseases Society of America (476). During the early stage of LD, it is suggested that oral antibiotics be prescribed from 10 to 21 days. In order of recommended use, effective antibiotics include doxycycline (halts microbial protein synthesis), amoxicillin (blocks bacterial cell wall synthesis by inhibiting peptidoglycan cross-linking; recommended for children and pregnant women), as well as cefuroxime axetil (blocks cell wall synthesis; recommended for

patients allergic to doxycycline and amoxicillin) and erythromycin (inhibits protein synthesis; recommended only if patients are unable to take the aforementioned antibiotics).

In the absence of neurological or cardiac manifestations, treatment recommendations during early disseminated phase remain the same as for the early/acute phase. In the presence of neurological or cardiac involvement, however, the treatment regimen often requires a longer course of antibiotics and may necessitate intravenous delivery. The following are recommended: adults and children with acute neurological manifestations should receive daily intravenous injections of ceftriaxone (inhibits bacterial cell was synthesis) or oral doxycycline if β-lactam antibiotics are not tolerated; doxycycline is not recommended for patients who are pregnant.

Similar to the early disseminated phase, late disseminated phase antibiotic treatment regimens are often longer and may require intravenous delivery during this stage. Patients presenting with Lyme arthritis alone can be successfully treated with oral antibiotics such as doxycycline, amoxicillin, or cefuroxime axetil; the later two are also recommended for children (476). If neurological complications alone or in addition to Lyme arthritis are present, intravenous ceftriaxone is recommended. Though not a major concern in the United States, acrodermatitis chronica atrophicans (ACA) can be successfully treated using the same regimen as suggested for EM (see above) (476). Antibiotic therapy is not suggested for patients with post-Lyme disease syndromes if the appropriate treatment recommendations have been followed and there is no evidence of persistent infection (476).

1.2.7 The Lyme vaccine

In 1998, GlaxoSmithKline (GSK) released the Lyme vaccine, LYMErix®. Based on a recombinant outer surface protein (Osp) A peptide, the vaccine demonstrated the greatest

efficacy after three injections over a one year period (76 % efficacy) relative to two injections given within one month of each another (49 % efficacy) (424). In the initial clinical study, a limited number of participants developed arthritis; however, both vaccinated and placebo groups had a similar incidence of arthritis and thus this manifestation was not linked to the vaccine (424). A second, smaller study confirmed the dosing regimen and safety of the vaccine (385). Unfortunately, by the time the second article was published, the vaccine had been removed from the market by GSK amid poor sales and a public outcry that claimed the vaccine could cause arthritis in some of its recipients (1, 41, 171, 287, 303, 344, 352, 371, 449). These protests stemmed from reports that suggested OspA could trigger autoimmunity and arthritis via molecular mimicry to human proteins such as leukocyte function-associated antigen-1 (hLFA-1) (170, 187, 449), but the results are considered by many to be inconclusive (1, 287, 303, 344) or to require additional clinical evidence (224). Protests even continued after the Food and Drug Administration's Vaccine Adverse Event Reporting System published a report in which no significant adverse effects were found to be conclusively linked to LYMErix® (127). There are currently no commercially available vaccines against Lyme disease.

1.3 THE TICK-MAMMAL TRANSMISSION CYCLE

The transmission cycle of all *Borrelia* species involves either a tick or louse vector and typically a mammalian or avian host. The geographic range of borrelial tick vectors are well-defined and are dependent upon both the environment and the hosts that are available to feed upon. Moreover, *Borrelia* species are associated with a single genus or species of tick. Fittingly, Lyme disease is prevalent in temperate climates where hard-bodied tick species responsible for the

transmission of Lyme-associated *Borrelia* species reside. Tick-borne relapsing fever (TBRF), meanwhile, is more common in warmer regions where soft-bodied ticks are found. Louse-borne relapsing fever (LBRF), however, is the exception; lice are responsible for the transmission of LBRF (caused by *B. recurrentis*) and are prevalent worldwide where ever humans are present and the living conditions of their hosts are conducive to their survival. Details regarding the relationship between lice and humans and soft-bodied ticks that transmit TBRF are provided in Section 1.4.2. The following sections will focus on the lifecycle of hard-bodied, *Ixodes* species ticks and their role in the transmission of Lyme-associated *Borrelia* species to humans and other mammals.

1.3.1 *Ixodes* species ticks and their lifecycle

The primary vectors of *B. burgdorferi sl* are ticks belonging to the *Ixodes* complex. The acquisition of *B. burgdorferi* by suitable mammalian hosts is closely associated with the geographical range of *Ixodes* ticks which are generally found in temperate climates of the Northern hemisphere (62, 69, 383), especially in wooded and grassy areas. Regions of the United States known to harbor *Ixodes* species ticks include the Northeastern states, the Great Lakes region, and the Northern California coast. Beyond the United States, ticks of this genus are found throughout the United Kingdom and much of Northern Eurasia and Japan; many of these regions are also endemic for Lyme disease. Within the United States, *Ixodes* ticks will commonly feed on small mammals such as the white footed-mouse, *Peromyscus leucopus* (254), during the larval and nymphal stages and larger mammals, such as deer, during the nymphal and adult stages. In nature, mice and deer do not demonstrate clinical signs of disease (114, 440).

In general, *Ixodes* ticks have a two year lifecycle during which they will typically feed three times, once during each of their life stages: larval, nymph, and adult (246, 316). Uninfected larvae hatch from eggs in the spring that measure less than a millimeter wide and are difficult to see with the naked eye. Their first blood meal is typically acquired during the summer months from small mammals such as *Peromyscus leucopus*, the major reservoir of *B. burgdorferi*; in the United States other major reservoirs include chipmunks, and shrews (51). Birds, squirrels, and deer have also been suggested as reservoirs, but do not appear to have the same reservoir potential as those species listed above (51). Because *B. burgdorferi* is not transmitted transovarially in ticks (198, 370), the first blood meal is the earliest opportunity for ticks to become colonized with the spirochete. Following the initial blood meal, larvae enter into the first instar stage and molt into nymphs. Ticks at this stage are morphologically distinct from larvae both in size (typically measuring 1 mm wide and ~2 mm long) and with the addition of a fourth set of legs. Nymphs remain dormant throughout the fall and winter until they begin to quest for their next blood meal in the spring or summer. Small mammals may still serve as the source for this meal, but larger mammals are also a common source. Due to the increased likelihood that they will feed on larger hosts as well as their minute size, nymphs are most commonly associated with transmission of *B. burgdorferi* to humans (246, 278). The second blood meal is followed by the second molt and it is at this stage that the ticks become full adults capable of reproducing. Adult ticks feed during the fall and prefer blood meals from larger mammals such as deer, giving *Ixodes scapularis* its common name, the deer tick. It is during this final meal that males (approx. 1.5 mm wide and 3.0 mm long) will seek out and mate with females (approx. 2 mm wide and 4 mm long). A final period of dormancy ensues over the winter months until spring when the eggs are laid, the adults die, and the cycle repeats. In correlation

with the *Ixodes* lifecycle, the incidence of Lyme disease is greatest during the summer and fall months when ticks actively seek blood meals and human outdoor activities and their exposure to ticks are high.

1.3.2 Interactions with mammalian hosts and transmission of *B. burgdorferi*

Ixodes species ticks have a chitinous dorsal plate and are often described as 'hard-bodied'. Because of their rigid encasement, these ticks required five to seven days to feed to repletion as they concentrate nutrients from the blood meal and regurgitate unused material. After a tick has found a suitable host, it attaches by inserting its hypostome into the host dermis. The hypostome is covered in barb-like projections, called denticles, which make it difficult to remove the tick once it has punctured the skin. Removal is made more difficult by a cementing agent secreted by the tick that anchors the palp – structures flanking the hypostome – to the skin surface. Once the tick has successfully attached to its host, it begins to feed and to regurgitate its saliva into the bite site; tick saliva has numerous anti-inflammatory and immune-suppressing functions which are believed to aid in protecting the tick from detection by the host and from the potentially harmful effects of the host's immune system (48, 198, 226-228, 252, 280, 294, 363, 380, 402, 470). Importantly, these attributes are also believed to aid in the initial survival of *B. burgdorferi* once it is transferred into the mammalian host; transmission becomes efficient after approximately 48 hours of attachment and feeding of nymphal ticks (113, 329, 470).

Within the unfed tick, *B. burgdorferi* resides within the midgut where it undergoes minimal replication. Once the tick begins to feed and a blood meal enters the midgut, however, the spirochete senses changing environmental cues such as increased temperature and decreased pH. In response to these cues, *B. burgdorferi* begins to rapidly divide and multiply and to

change the expression of genes and proteins, thereby altering the composition of its surface in preparation for transmission. Two outer surface proteins (Osps), OspA and OspC, represent the paradigm of *B. burgdorferi* protein expression during the tick to mammal transmission cycle. OspA, which anchors *B. burgdorferi* to the tick midgut via TROSPA, tick receptor for OspA, is expressed and readily detectable on the surface of spirochetes during colonization of the unfed tick; conversely, OspC expression is difficult to detect in this environment (134, 295, 312, 389, 390). As the tick begins to feed, the surface protein profile of *B. burgdorferi* changes with OspA expression decreasing and OspC expression becoming detectable (312, 330, 389, 390). In correlation with the changing borrelial surface composition, spirochetes begin to migrate via the hemolymph to the salivary glands where $OspA^{-}/OspC^{+}$ spirochetes can be detected (312, 330). Once in the salivary glands, *B. burgdorferi* is transmitted via the tick's saliva into the host dermis from which point the spirochete disseminates to distal tissues. While it has been determined that OspC is required for *B. burgdorferi* transmission from the tick to mammalian host (390) and for the bacterium's survival during the early stages of mammalian infection (443, 445), efforts to determine the function of OspC are ongoing (431).

1.4 RELAPSING FEVER *BORRELIA*

Predating the discovery of *B. burgdorferi sl*, known *Borrelia* species were recognized as the causative agents of both louse-borne (LBRF) and tick-borne relapsing fever (TBRF). *B. recurrentis* was the first to be described and was identified as the causative agent of LBRF in "Russia, the Balkan Peninsula, Turkey, Persia and India, and known by sporadic cases over the entire world" (465). Throughout the first half of the 1900s a worldwide LBRF epidemic

occurred that affected approximately 50 million people and was reported to have a 10% to 40% mortality rate (59). Including *B. recurrentis*, at least 16 relapsing fever *Borrelia* species have been identified to date. The remaining relapsing fever *Borrelia* species cause TBRF and are primarily transmitted by soft-bodied ticks of the genus *Ornithodoros*. Notable exceptions are *B. anserina*, which causes avian spirochetemia and is transmitted by *Argas persicus*, and *B. lonestarii*, which is transmitted by *Amblyomma americanum*. The predominant species transmitted within the United States include *B. hermsii*, *B. turicatae*, and less frequently *B. parkeri* (412). Similar to Lyme disease-associated *Borrelia* species, TBRF species may be transmitted via tick saliva and additionally through coxal secretions (413). Many TBRF *Borrelia* can also be passed transovarially in both soft-bodied *Ornithodoros* species ticks (65, 325) and occasionally transplacentally in the human host (143). *B. recurrentis*, on the other hand, multiplies in the hemolymph but does not move into the louse salivary glands. Thus, *B. recurrentis* is transmitted after an infected louse has been crushed by its human host and the louse hemolymph or feces containing spirochetes is rubbed into cracks or wounds in the skin (194, 348, 413).

1.4.1 Disease course and treatment of relapsing fever

Both LBRF and TBRF are described as episodic febrile illnesses lasting 2 to 3 days and interspersed with 7 to 10 days of well-being before the recurrence of fever. Patients may experience normal febrile symptoms, including joint and muscle aches, headaches, and chills. What distinguishes relapsing fever from fevers caused by other infectious agents is the ‘crisis’ period which occurs near the end of the first febrile episode and is strongly associated with patient mortality. During the crisis period, patients may experience rigors, sweats, and severe

dehydration followed by hypotension, excessive sweating, and a drop in temperature from the peak of the febrile episode. If untreated, 10 % to 40 % of infected individuals may die; with antibiotic treatment, the mortality rate becomes <1 % to 4 % (411, 448, 454). Following crisis, patients may experience one or two relapses, but symptoms are much less severe during these subsequent episodes.

Diagnosis of TBRF and LBRF is typically made by microscopic observation of spirochetes in thick blood smears. During a febrile episode, spirochetes reach an average of 10^5 cells/ml of blood (59, 432) can multiply as high as $10^6 – 10^8$ cells/ml (54). These corresponding peaks in fever and spirochete number are associated with distinct serological reactivity to the variable major proteins (VMPs) expressed by relapsing fever *Borrelia* (24, 455). In a classic illustration of antigenic variation, VMPs vary by the recombination of one of many *vmp* sequences, each encoding a protein sequence of a specific serotype, into a single expression cassette. Thus, as one serotype is cleared by the host's immune system, a second clone expressing a distinct VMP will expand and cause the succeeding relapse.

Both TBRF and LBRF are commonly treated with the antibiotics doxycycline and tetracycline, but erythromycin may also be used in the treatment of pregnant and/or nursing women (19, 151, 396). In untreated individuals, LBRF has an increased mortality rate of 10 – 70 % relative to TBRF, which has a 4 % - 10 % mortality rate (19, 353). The chances of spontaneous abortion is also greatly increased in pregnant women with LBRF (59). A high incidence of neurological manifestations is associated with TBRF (162, 448).

1.4.2 Epidemiology of relapsing fever

In the past, relapsing fever was recognized as a global disease; today, however, it occurs primarily in Sub-Saharan Africa and the Middle East (101). In a longitudinal study, subjects from 30 West African villages were followed for 14 years and the incidence of TBRF was reported to occur on average in 11 out of 100 people, a rate higher than any other bacterial infection in the region (454); other reports in Africa have reported a similarly high incidence of the disease (117, 223). In Israel, reports spanning from 1971 to 2003 revealed that on average <1 in 100,000 of its civilians and 6.4 in 100,000 of its military personnel developed TBRF (406). Importantly, some believe that the incidence of TBRF is under-reported in these endemic areas, similar to what has been suggested for LD (284). In the United States, approximately 25 cases of TBRF are reported each year (68), the majority of which occur in states West of the Mississippi River; these states include those required to report the incidence of relapsing fever, namely Arizona, California, Colorado, Idaho, Nevada, New Mexico, Oregon, Texas, Utah, Washington, and Wyoming (http://www.cdc.gov/ncidod/dvbid/RelapsingFever/RF_Epidemiology.htm, last updated November 12, 2004). In total, 450 cases were reported in the United States between 1977 and 2000 (118) and the mortality rate, which varies with geographic locale, was within the expected 0 – 8% range (19, 162, 411).

LBRF has been reported in Europe, Russia, and Africa and caused millions of deaths in these regions throughout the early half of the twentieth century (59). While no recent epidemics have been reported, outbreaks have been reported in Ethiopia (7, 46, 47, 58, 291, 347, 437) and Sudan (376); LBRF has also been tied to the homeless in France (55). The inhabitants of these areas are often found to practice poor hygiene, to change their clothing infrequently, and to wear

multiple layers of clothing. These habits provide an ideal environment for the human body louse, *Pediculus humanus corporis*, which lives in and lays its eggs in the clothing fibers of its host between taking multiple daily blood meals. Given these factors, LBRF is primarily associated with individuals of a lower socioeconomic status (104).

1.4.3 Biology and genetics of relapsing fever *Borrelia*

Similar to the Lyme disease spirochetes, relapsing fever *Borrelia* species are fastidious and cultivatable *in vitro* in normal BSK-H media containing 6% [final] rabbit serum (102, 103, 349, 432). Some species, however, show improved growth when rabbit serum is supplemented to 10 % or 12 % [final] (196, 340, 391). Relapsing fever species are morphologically indistinguishable from Lyme disease species by darkfield microscopy or staining, both groups being similar in dimension and the range in the number of coils each species typically harbors. Using electron microscopy, however, relapsing fever species have been shown to contain an average of 15 to 30 flagellar bundles (195) as compared to the 7 to 10 normally found in Lyme disease *Borrelia* (64, 195).

The genome structure of relapsing fever spirochetes is thought to be highly similar to Lyme-associated species, containing a linear chromosome of approximately 1 Mb and multiple linear and circular plasmids. Relapsing fever *Borrelia* circular plasmids range in size from approximately 8 to 40 kb and linear plasmids from 10 to 180 kb (19, 132, 203, 333, 428). The sizes of these molecules are relatively similar to the circular and liner plasmids found in Lyme-associated *Borrelia* species which range from 9 to 32 kb and 5 to 60 kb, respectively (82, 140).

While the relapsing fever and Lyme-associated *Borrelia* species are highly similar in GC content (103, 146), the two groups represent distinct branches of the *Borrelia* genus. Sequencing of the *rrs* gene encoding 16s rRNA from genomic DNA has demonstrated between 98.6 % and 89.4 % similarity between all *Borrelia* species at this locus; Lyme disease species alone were shown to share approximately 96 % identity and relapsing fever species an average of 94 % identity (146, 349). In other reports, the gene encoding the major flagellar structural unit, *flaB*, was utilized to show Lyme-associated *Borrelia* share between 100 % and 92.8 % identity while relapsing fever *Borrelia* share between 100 % and 88 % identity; relative to one another, the two groups shared no more than 85.6 % sequence identity at the *flaB* locus (145). Sequence analysis using the gene encoding a histone-like protein, *hbb*, demonstrated that no less than 93.0 % identity was shared among Lyme-associated *Borrelia*, but no more than 87.2 % identity was shared between these species and relapsing fever *Borrelia*; 96.6 % sequence identity was shared between the two relapsing fever *Borrelia* species analyzed in the study (450). From these reports, phylogenetic analyses have demonstrated that the relapsing fever *Borrelia* comprise a clade distinct from the Lyme disease *Borrelia*.

1.5 *B. BURGDORFERI*

1.5.1 Microbiological characteristics

Borrelia is fastidious, microaerophilic spirochete with a Gram negative-like membrane structure that includes an inner and outer membrane divided by the periplasmic space. *Borrelia* is difficult to visualize by light microscopy, averaging 0.2 μm in width and 20 – 25 μm in length; darkfield

microscopy is instead routinely used. *Borrelia* harbors between 7 and 30 flagellar bundles that radiate from either end of the bacteria and are contained within the periplasmic space (64, 195). Thus, the flagella are not exposed to the host milieu. The sub-membranous localization of the flagella lends *Borrelia* both its corkscrew structure and its planar wave motility.

In vitro culture of *Borrelia* species has proved a challenge that is still being tackled today. The liquid medium first formulated by Dr. Richard Kelly in 1971 for the cultivation of *Borrelia* (220) was modified in 1982 by Stoenner (26) and in 1983 by Barbour (20) who coined the name of the medium commonly used today, BSK (Barbour-Stoenner-Kelly). BSK is a complex medium containing, among numerous other components, 20 mg/ml bovine serum albumin fraction V, 6 % rabbit serum, and N-acetylglucosamine which is required for the growth and survival of *Borrelia* in culture (444). Though *Borrelia* can be successfully expanded in BSK medium, there are numerous differences observed between *in vivo* versus *in vitro* grown *Borrelia* including gene expression, protein synthesis, infectivity, and plasmid content. Changes in the expression of certain genes in cultured spirochetes has been shown to be reduced relative to spirochete gene expression in mice (153) (& Hughes *et al*. 2008, *Infect Immun*, in press) and to differ between cultured versus tick-borne *Borrelia* (350). Normal protein synthesis of OspA is increased during tick colonization, reduced during tick feeding, and undetectable in mammals during natural transmission and infection (106, 134, 192, 330, 389, 390). During cultivation in BSK, however, OspA is constitutively expressed (28, 237, 295, 311, 365, 494). The expression of still other genes and proteins is lot-dependent in the commercially available BSK-H medium from Sigma (89, 121); this alteration in gene expression has been demonstrated to adversely affect the infectivity potential of low-passage patient isolates in certain BSK-H lots (460). Finally, serial passage of *Borrelia* in BSK *in vitro* has been shown to decrease isolate infectivity

either by the loss of plasmids required for infection (167, 240, 241, 249, 343, 388) and/or by the decrease in expression of specific genes required for infection (79, 388). Genetic instability of *B. burgdorferi* appears to be limited to *in vitro* cultivation as the spirochete has been demonstrated to be genetically stable over one year or more of persistent murine infection (153, 327).

In part due to its fastidious nature and in part due to the difficulty of maintaining infectious isolates in culture, genetic manipulation of *B. burgdorferi* has been demonstrated to be far more difficult and time consuming than that of other Gram-negative bacteria such as *E. coli* and *S. typhimurium*. Advances within the past decade, however, have allowed for the transformation of *B. burgdorferi* with stable reporter constructs (40, 80, 381), for mutagenesis of borrelial genes via homologous recombination with suicide plasmids (42, 122, 368, 369), and complementation of mutants using *B. burgdorferi*-derived vectors (137, 183, 249, 322, 342, 360, 404, 430). Restriction modification systems harbored on lp56 and lp25 decrease electroporation efficiency and offer an additional level of difficulty when transforming *B. burgdorferi* with DNA (208, 217). Furthermore, lp25 is required for murine infection (240, 241, 343) and thus must be maintained or complemented with the minimal region required for infection (342). Minimal complementation of lp25, however, may not fully recover the parental phenotypes (discussed in Section 4.3.1).

A second linear plasmid, lp28-1, has also been demonstrated to be required for murine infection (167, 240, 241, 343). lp28-1 harbors the gene encoding VlsE which is involved in antigenic variation (Sections 1.4.1 and 1.5.3), a process which plays a major role in the survival and virulence of Lyme disease- and relapsing fever-associated *Borrelia* species during persistent infection. Studies utilizing *B. burgdorferi* isolates that had lost lp28-1 but were complemented with the *vlsE* gene demonstrated that either an intact *vls* variation locus and/or additional factors

present on lp28-1 were required to restore full infectivity in mice (249). Some of these additional factors may include genes required for the establishment of arthritis; this was demonstrated by Xu *et al* in a comparison between *B. burgdorferi* isolates that did or did not harbor lp28-1 which showed that SCID mice infected with isolates containing lp28-1 developed severe arthritis while those infected with isolates that had lost lp28-1 did not develop arthritis (481).

1.5.2 Sequencing of the B31 MI genome

Though *B. burgdorferi* was isolated in 1982 (64) and cultivated in 1983 (20, 64), the *B. burgdorferi* genome was not fully sequenced until 1997. This was due in part to the difficulty of sequencing the spirochete's fragmented genome which is comprised of a linear chromosome of less than 1 Mb and multiple linear and circular plasmids. Utilizing the whole-genome random sequencing technique first reported in 1995 (139), Fraser and colleagues were able to publish the nearly complete sequence of *B. burgdorferi* isolate B31 MI in 1997 (140). Casjens *et al* completed the sequencing project by 2000 (82) and determined that *B. burgdorferi* harbored 12 linear and 9 circular plasmids ranging in size from approximately 5 kb to 56 kb and 9 kb to 32 kb, respectively. Annotation by The Institute for Genomic Research (TIGR) assigned a role to 60% of chromosomal genes, but to only 15 % of plasmid-encoded genes. Interestingly, over 161 paralogous gene families were initially identified that were comprised of substantially more plasmid genes than chromosomal genes (82). The following nomenclature was derived for *B. burgdorferi* gene and protein designations: BBX##. BB is an abbreviation for *B. burgdorferi*, the X refers to the letter designation of plasmid that the corresponding gene is harbored on, and the gene number, ##, indicates the position of the open reading frame on that plasmid (140).

Among all of the genes that were assigned a role, relatively few were found to be involved in biosynthetic pathways and metabolism (140). This was not unexpected given the fastidious nature of the bacterium and its requirement for either a tick or mammalian host. Similar findings have been reported in other fastidious and parasitic bacteria including *Treponema pallidum* (142) and *Mycoplasma genitalium* (141). Unlike many other Gram-negative bacteria, *B. burgdorferi* lacks both the structural components and the mechanical components required to synthesize lipopolysaccharide (LPS). LPS can serve as a mechanical barrier to host defenses such as opsonization and phagocytosis (133, 261), it can contribute to antigenic variation (356), and can aid in resistance to complement (351). Also of interest, nearly 8 % of the *B. burgdorferi* genome is predicted to encode lipoproteins (401), lipid-modified proteins that are often membrane-associated. Though the spirochete *T. denticola* encodes predicted lipoproteins with a frequency of 6.0% (401), it is *B. burgdorferi* that encodes more lipoproteins than an other bacterial genome. A growing number of borrelial lipoproteins have been characterized and are present on the borrelial outer surface (23, 25, 53, 121, 136, 178, 242, 305, 326, 360, 472, 491). Borrelial lipoproteins serve both structural and protective functions and play a role in host colonization. The functions and importance of some of these lipoproteins are reviewed in Section 1.5.3.

1.5.3 *B. burgdorferi* lipoproteins

Bacterial lipoproteins have a wide array of functions including structural stability of the bacterial membrane (81, 334); protein transport (92, 453); antibacterial drug resistance (44, 489); adherence to extracellular matrix factors (56, 163, 340); escape from the host immune system (186, 231, 233, 427); interacting with the host environment (436); and assisting in pathogenesis

(39, 185, 301, 339, 357, 372, 386, 403, 442, 466, 490). Lipoproteins are lipid-modified proteins that are often membrane-associated, anchoring to the inner or outer leaflet of a lipid bilayer via their lipid moiety. In *B. burgdorferi*, lipoproteins typically contain an N-terminal L-X-X-C motif that is post-translationally cleaved at the cysteine residue which is then modified into a tripalmitoyl-S-glyceryl-cysteine (Pam3Cys) moiety (34, 49). According to a publication by Schulze and Zuckert (387), borrelial lipoproteins are transported to the outer surface of the spirochete by default. Fittingly, the functions of borrelial lipoproteins have been associated with the interaction between the bacterial lipoprotein and host factors; these functions include immune evasion by binding to host immune factors or antigenic variation (24, 186, 230, 234, 235, 289, 427, 432, 491-493), to adhesion of *B. burgdorferi* to host extracellular matrix or host cell surfaces (90, 91, 163, 173, 174, 326, 340, 341), to host-specific adaptation and survival (10, 12, 312, 390, 443, 445, 458, 488). Also of importance, numerous borrelial lipoproteins are up-regulated during tick colonization (389), mammalian infection (11, 153, 258, 493), and/or under *in vitro* culture conditions mimicking these environments (77, 78, 89, 204, 205, 314, 361, 446) suggesting that the function of many lipoproteins are host-specific.

One of the most well-characterized lipoproteins is variable major protein (Vmp) -like sequence (Vls) E (491) which is orthalogous to the Vmps first identified in *B. hermsii* (24, 289, 432). VlsE utilizes alternative splicing of fifteen silent cassettes into a single expression site, allowing *B. burgdorferi* to alter its surface antigen profile (491-493) to escape immune detection (256, 493). The lipoproteins BbCRASP-2 through BbCRASP-5, and OspE also aid in immune evasion (6, 186, 197, 233, 234). These proteins bind to factor H (fH) and/or fH-like protein (FHL) -1, regulators of the alternative complement cascade which is a component of the host innate immune response. fH and FHL-1 increase the enzymatic degradation of factors necessary

to complete complement-mediated lysis of target, non-self cells via the membrane attack complex (129, 207); therefore, it is possible that *B. burgdorferi* may prevent its own lysis or opsonization by binding fH and FHL-1 to its surface and inhibiting progression of the alternative complement cascade. A similar mechanism has been described for group A streptococci which binds to fH via protein M, reducing the deposition of a key mediator of the alternative pathway on the surface of the bacterium (193).

B. burgdorferi also expresses lipoproteins capable of binding to the mammalian host extracellular matrix and select tissues via host molecules such as β_3-chain integrins, decorin, glycosaminoglycans, and fibronectin; these include P66, DbpAB, Bgp, and BBK32 (90, 91, 163, 173, 174, 326, 340, 341). It is presumed that these proteins aid in the dissemination of and/or the extracellular lifecycle of *B. burgdorferi* with in the mammalian host. Lipoproteins have also been identified which play vital roles in the adaptation and survival of *B. burgdorferi* in the distinct environments of its arthropod vector and mammalian hosts (10, 12, 312, 390, 443, 445, 458, 488). The best characterized example is a pair of reciprocally expressed outer-surface membrane proteins, OspA and OspC. As discussed in Section 1.3.2, OspA is expressed during tick colonization (312, 390) and is required for persistence through molting (488). OspC, on the other hand, is expressed in (390) and required for the early stages of acute infection (443, 445) in mammalian hosts. Still other lipoproteins are believed to be involved in dissemination to specific host tissues (10, 12, 458). However, few published examples exist that link borrelial proteins to dissemination of the spirochete to mammalian tissues known to exhibit manifestations of Lyme disease (98, 131, 321, 482).

1.6 PARALOGOUS GENE FAMILY 54

1.6.1 Overview of TIGR's paralogous gene families

From the original annotation of the *B. burgdorferi* genome, TIGR identified 161 unique paralogous gene families (pgfs) (82, 140). Many of these families are comprised entirely of genes that are harbored on plasmids and have no known function (82, 140). With completion of the B31 sequence, it became possible to identify genes by microarray and proteins by mass spectrometry that were differentially expressed during the tick-mammal infection cycle. What was observed was that many of the differentially regulated borrelial genes belonged to pgfs and were encoded on plasmids (52, 361, 446). Among these plasmid-encoded genes, members of pgf 54 were consistently found to be the most dynamically regulated in response to changing environmental cues. Intriguingly, family 54 genes were split into two groups based on their regulation to environmental cues, the first comprised of *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* and the second comprised of the remaining seven genes (*bba68*, *bba69*, *bba70*, *bbi36*, *bbi38*, *bbi39*, and *bbj41*). While the first group was typically upregulated under conditions that mimicked the mammalian host, the second group was either unaffected or downregulated under mammalian-like conditions (52, 361, 446). The criteria set by TIGR for categorizing genes into pgfs is unknown and inspection of the pgf 54 gene sequences showed that while some shared as much as 99 % identity, others shared as little as 27 % identity (89)(Hughes *et al*. 2008, *Infect Immun*, in press).

Recently, TIGR redefined many of its pgfs and eliminated others, including pgf 54 (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=gbb). After this reorganization, *bba64*, *bba65*, *bba66*, and *bba73* were not grouped into any pgf while *bba71* and the seven

Table 1.6.1 Sequence distances from Clustal V analysis

Panels are arranged as follows: p. 28, upper left, p.29 upper right, p. 30 lower left, p. 31 lower right. Table contributed by J. Carroll.

	Bb 297 bba66 homolog	Bb 297 bbl36 homolog	Bb 297 bbl38 homolog	Ba PKo 2061 (B31 bba65 homolog)	Bb B31 bba64	Bb B31 bba65	Bb B31 bba66	Bb B31 bba68 (cspA)	Bb B31 bba69	Bb B31 bba70	Bb B31 bba71	Bb B31 bba73	Bb B31 bbl36
Bb 297 *bba66* homolog	********	28.9	28.3	29.9	27.3	32.9	99.3	28.3	28.1	31.6	29.7	27.4	28.1
Bb 297 *bbl36* homolog	67.1	********	88.5	29.6	34.6	29.7	28	53.9	52.3	49.8	32.8	28.9	99.5
Bb 297 *bbl38* homolog	71.5	9.7	********	30.3	30.1	31.7	28.3	53.3	49	47	35.3	30.3	87.4
Ba PKo *2061* (B31 *bba65* homolog)	52.9	84.3	84.2	********	31.8	81.7	29.7	29.6	31.7	30	32.4	31	31.2
Bb B31 *bba64*	110.9	86.5	84.9	106.2	********	33.1	26.6	32.9	31.7	30.8	29.7	31	34.3
Bb B31 *bba65*	54.3	84.5	87.9	17.7	112.2	********	33.7	30.3	29.3	32.1	30.7	29.9	28.1
Bb B31 *bba66*	0.7	67.6	72.1	52.5	110.7	53.6	********	27.4	28.4	31.9	30.2	27.3	28.1
Bb B31 *bba68* (*cspA*)	87.3	46.5	49.8	93.6	92.1	105.1	86.7	********	67.3	46.9	36.3	30.5	53.9
Bb B31 *bba69*	86.3	47.4	50.9	93.6	97	108	85.7	24.9	********	49.5	37.7	28.4	52.2
Bb B31 *bba70*	81	48.9	52.6	95.7	104	97	81.7	56.8	53.3	********	47.4	32.4	50
Bb B31 *bba71*	96.5	41.2	39	84.2	93.2	91.3	98	74.7	68.8	60.4	********	29.2	33.6
Bb B31 *bba73*	109.1	81.6	82.3	114.7	89.2	114	109.4	99.4	106.4	85.6	91.6	********	30

Percent Identity

Bb B31 bbi38	Bb B31 bbi39	Bb B31 bbj41	Bg PBi bga63 (B31 bba64 homolog)	Bg PBi bga64 (B31 bba65 homolog)	Bg PBi bga65 (B31 bba66 homolog)	Bg PBi *bga66* (B31 *bba68* homolog)	Bg PBi *bga67* (B31 *bba69* homolog)	Bg PBi *bga68* (B31 *bba70* homolog)	Bg PBi *bga72* (B31 *bba71* homolog)	Bg PBi *bga73* (B31 *bba73* homolog)	Bb JD1 *bba66* homolog	Bb JD1 *bbl36* homolog	Bb JD1 *bbl38* homolog	Bb N40 *bba66* homolog	Bb N40 *bbl36* homolog	Bb N40 *bbl38* homolog	Ba MMS *p46* (B31 *bba66* homolog)	Ba PKo *p46* (B31 *bba66* homolog)	Bb ZS7 *p46* (B31 *bba66* homolog)	Bg ZQ1 *p46* (B31 *bba66* homolog)
27.7	28.5	28.5	28.9	30.8	57.2	28.4	28.2	29.1	29.1	27.4	99.3	27.9	27.9	99.5	28.9	28.3	60	59.8	98.8	57.9
98.8	82.3	81.9	31.8	30.7	29.6	58.7	54.9	55.6	56.5	27.7	28.9	99.9	87.4	29.2	100	88.4	29.6	30.6	28.1	28.9
87.9	97.2	96.9	31.5	30.7	28.2	56.6	54.7	54.8	57.1	30.5	29.6	88.4	98.9	28.3	88.5	99.9	28.5	28.7	28.3	28.2
30.3	31.1	30.5	34.3	84.1	29	32.9	34.1	30.6	31.3	30.7	30	29.4	30.9	29.7	29.6	30.3	29.6	29.1	31.3	30.9
33.8	30.8	30.4	74.6	30.1	26.8	33.6	35.8	31.9	35.1	32.2	28.1	34.6	29.9	28.4	32.8	29.9	26.3	25.8	28.8	25.7
28.3	31.4	31.2	33.1	77.5	29	31.5	31	29.4	30.1	27.4	33.3	29.6	31.6	33.1	29.7	31.8	31	30.9	32.2	32.5
27.7	28.6	28.6	28.9	31	59.1	27.8	30.4	29.2	28.8	27.3	99.4	28	28.1	99.3	28	28.3	60.6	60.5	99.3	60
54.1	53.3	53.3	32.7	32.1	28.3	65.6	59.5	61.3	52.3	31.6	27.9	53.9	53	28.2	53.9	53.3	27.4	30.1	28	27.2
52.7	48.7	48.4	33.1	29.4	25.6	56.7	54	55.4	49.1	30.5	29.7	52.3	48.8	28	52.3	49	26.2	25.7	28.5	27.5
50.5	46.9	46.2	34.4	31.3	30.5	51.6	49.4	48.6	50	36	31.3	49.8	47	31.8	49.8	47	31.9	32.2	31.3	29.4
33.6	33.1	33.1	29.4	32.4	30.9	39.4	38.7	42.3	31.9	33.3	28.5	33.1	34.8	28.5	32.8	35.3	32.6	34.1	29.9	32.6
29.9	30.9	30.8	32.1	30.9	27.3	31.6	31.4	32.1	29.6	69.2	27.8	28.9	30.7	27.7	28.9	30.9	29.3	29.4	27.8	24

Divergence

Bb B31 *bbl36*	69.1	0.5	10.7	85.6	86.3	85.8	68.7	46.6	47.4	48.6	41.3	82.5	********
Bb B31 *bbl38*	69.7	1.1	10.2	85.1	87.1	86.1	69.3	46.5	47.1	48.7	42.3	82.5	0.6
Bb B31 *bbl39*	75.5	12.8	2.2	84.1	83.9	88.2	74.1	49.8	52.2	53.7	39	84.2	13.4
Bb B31 *bbj41*	74.9	12.8	2.2	84.4	84.3	88.5	73.6	49.7	52.1	53.5	39.5	84.2	13.4
Bg PBi *bga63* (B31 *bba64* homolog)	105.5	83.1	83.3	101.4	24.1	111.7	105.5	84.6	87	90.8	89.4	88.2	82.5
Bg PBI *bga64* (B31 *bba65* homolog)	54.3	86.1	85.2	14	108	21.3	53.9	96.6	95.8	91.3	93.2	116.2	87.5
Bg PBi *bga65* (B31 *bba66* homolog)	38.9	74.9	74.8	69.1	109	68.2	38.8	92.8	94.5	87.1	98.3	120.5	76.3
Bg PBi *bga66* (B31 *bba68* homolog)	81.8	39.1	41.4	105.6	97.6	110.6	82.9	35.6	46.2	53.4	68	86.2	39.2
Bg PBi *bga67* (B31 *bba69* homolog)	82	42.7	45.1	102.5	88.7	103.1	82.5	43	47.9	48.7	71.4	87.1	42.9
Bg PBi *bga68* (B31 *bba70* homolog)	84.6	43.7	45.9	95.5	93.1	101.1	85.2	41.3	44.2	49.3	54	91.1	43.7
Bg PBi *bga72* (B31 *bba71* homolog)	90	47.4	46	100.8	87.9	104.4	93	52.6	59.4	72.9	67.2	91.2	48.2
Bg PBi *bga73* (B31 *bba73* homolog)	113.3	84.2	81.8	109.3	99	103.8	113.1	92.3	99.8	78.9	90.4	27.7	84.7
Bb JD1 *bba66* homolog	0.7	67.4	71.8	52.9	110.2	53.7	0.7	86.1	84.1	80.3	93.8	107.7	69.1
Bb JD1 *bbl36* homolog	67.5	0.1	9.8	85	86.1	85.2	68	46.5	47.7	49.3	41.2	82.1	0.6
Bb JD1 *bbl38* homolog	71.9	10.8	0.9	82.8	84.9	86.4	72.6	50.1	51.8	53.4	39	83.3	11.8
Bb N40 *bba66* homolog	0.5	67.1	71.5	52.5	109.5	53.9	0.7	86.7	86.3	81.6	96.5	107.7	69.1
Bb N40 *bbl36* homolog	67.1	0	9.7	84.3	86.5	84.5	67.6	46.5	47.4	48.9	41.2	81.6	0.5
Bb N40 *bbl38* homolog	71.2	9.8	0.1	84.4	84.9	87.3	71.8	49.9	51	52.5	39	82.2	10.8
Ba MMS *p46* (B31 *bba66* homolog)	37.6	73.8	77.4	63.4	117.6	67.7	36.9	94.2	87.8	85.5	95.3	118.6	75.4
Ba PKo *p46* (B31 *bba66* homolog)	37	73.9	77.5	62.5	117.5	66.7	36.7	95.3	88.7	84.9	94	118	75.5
Bb ZS7 *p46* (B31 *bba66* homolog)	0.8	67.6	72.1	53.2	110	54.2	0.2	86.7	86.3	82.3	98	109	68.7
Bg ZQ1 *p46* (B31 *bba66* homolog)	41.9	75.9	80.6	68.3	122.5	72.4	41.4	97.9	98.4	95	100.3	120.2	77.1

99.3	84.5	84.2	34.7	31.2	29.7	58.6	55.2	55.9	50.6	28.2	28.8	99.4	86.2	29	99.5	87.3	30.3	29.1	29.4	28.8
********	85	84.7	31.7	30	30.8	58.2	54.7	55.9	50.8	27.2	28.7	98.7	86.7	29.3	98.8	87.8	29.7	29.4	29.9	28.8
12.9	********	99.5	32.9	30.7	29.9	57.4	54.9	54.1	52.1	30	28.2	82.2	98.4	28.7	82.3	97.1	29.6	29.5	29.5	29.1
12.9	0.2	********	33.1	30	29.1	57.2	54.2	53.2	51.4	30.1	28.2	81.8	97.8	28.7	81.9	96.8	29.6	29.5	29.4	28.8
83.7	85.1	85.5	********	32.3	27.6	34	36.8	33.8	34.5	34	29.5	31.8	31.1	28.9	31.8	32.3	30.9	30.6	28.9	28.6
87.8	85.5	85.8	101.6	********	29.8	31.7	31.8	30.6	29.5	28.6	30.9	30.6	30.4	30.9	30.7	30.7	32	31.9	30.1	28.5
75.2	76.8	76.9	102.8	68.4	********	27.5	28.2	29.5	29.7	30.3	57.4	29.6	28.2	57.2	29.6	28.2	64.6	64.7	60	88.9
39.9	40.9	40.5	97	98.5	92.3	********	76.5	66.5	64.5	34.4	29	58.6	56.6	29.5	58.7	56.6	28.6	27.8	29.2	28
43.7	45.3	45.2	87.1	95.9	87.3	24.4	********	67.7	62.7	33.5	30.3	55.4	54.8	30.3	55.4	54.7	30.4	30.5	30.8	28.6
43.7	47.3	47.2	88.6	101.7	88.3	33.1	30.3	********	59.9	31.6	28.4	55.6	54.5	29.2	55.6	54.8	29.7	29.4	30.9	28.4
48.2	48.3	48.2	94.2	99.3	92.4	35.5	36.1	41.6	********	31.3	28.8	56.5	52.1	28.9	56.5	57.1	27.9	29.3	29.2	27.3
83.1	81.9	81.9	88.8	107.1	110.1	87.4	88.5	90.5	93.9	********	27.5	27.7	30.3	27.5	27.7	30.4	28.7	28.7	27.9	27.7
69.7	75.5	74.9	105.5	54.7	39	82.4	82	84.6	91.1	111	********	28.8	28.1	99.4	28.9	29.6	60.3	59.9	99	58.3
1.2	12.9	13	83.1	86.9	74.9	39.4	43	44	47.6	84.2	67.8	********	87.3	29.3	99.9	88.3	29.6	30.6	28.1	28.9
11.4	1.3	1.5	84.9	83.7	75.2	41.6	45.4	46.1	46.8	81.3	72.2	11	********	28.1	87.4	98.8	30.5	28.6	28.5	28.4
69.7	75.5	74.9	104.9	54.6	38.4	81.8	82	84	90.6	111.1	0.6	67.5	71.9	********	29.2	30.1	60.1	60	98.9	58.2
1.1	12.8	12.8	83.1	86.1	74.9	39.1	42.7	43.7	47.4	84.2	67.4	0.1	10.8	67.1	********	88.4	29.6	30.6	28.1	28.9
10.4	2.4	2.4	83.3	85.4	75.1	41.4	45.1	45.9	46	81.7	71.5	10	1	71.2	9.8	********	30.6	30.9	28.3	28.2
74.9	79.4	79	110.5	63.6	27.6	87.3	88.1	84.6	87.7	115.8	37	73.8	77.4	37.5	73.8	77.8	********	99.5	61.3	66.3
75	80.2	79.8	111	62.6	27.1	88.2	88	84.5	88.6	116.3	36.7	73.9	77.5	37.2	73.9	77.9	0.5	********	61.2	66.7
69.3	74.1	73.6	105.7	54.7	38.5	82.9	83.1	85.2	93	113.1	0.6	68	72.6	0.7	67.6	71.8	36.9	36.7	********	60.8
77.8	83.8	83.9	111.7	67.4	7.8	93.4	86.3	87.4	93.5	115.5	41.9	75.9	81.1	41.7	75.9	81	28.8	28.4	41.1	********

remaining genes historically grouped into pgf 54 were classified as a new pgf, gbb fam_b_burgdorferi_b31.pep_35; little difference in the overall range of sequence identities shared between the genes of pep_35 (33.1 % to 99.5 %) from the original pgf 54 family (97 % to 27 %) (Table 1.6.1). Despite this rearrangement of borrelial pgfs, the regulation and expression of *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* suggests that they are related in their ability to respond to environmental cues, in their regulation during mammalian infection, and in their close genetic arrangement to one another on lp54 (plasmid A). These observations and those discussed in subsequent sections make a compelling argument that these genes may have similar or complementary functions that aid in the survival or pathogenicity of *B. burgdorferi* during mammalian infection.

1.6.2 Characteristics of *bba64, bba65, bba66, bba71,* and *bba73* and their encoded proteins

Analyses of the *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* sequences have demonstrated that these genes and their known homologs in *B. afzelii* and *B. garinii* are unique to the *Borrelia* genus. Moreover, these genes follow suit with the *B. burgdorferi* genome and have a low % GC content that ranges from 26.81 % to 33.66 % (Table 1.6.2). The proteins encoded by *bba64*, *bba65*, *bba66*, and *bba73* all have a basic pI and each is predicted to be lipidated. Only BBA71 has an acidic pI and is not predicted to be lipidated. Interestingly, BBA71 has an apparent molecular weight by SDS-PAGE and Western blotting analysis of 27.5 kiloDaltons which differs from its predicted molecular weight of 16.4 kiloDaltons. The reason for the aberrant migration of BBA71 by SDS-PAGE is unknown; however, it may be possible that BBA71 migration is affected either by its predicted strong negative charge at neutral pH (-8.563) or by possible post-translational modification. At least two other borrelial proteins are known to be post-

Table 1.6.2 *bba64, bba65, bba66, bba71,* and *bba73* gene and protein characteristics

Gene & Protein Characteristics					
Locus	Base pairs	%GC	Predicted MW (kDa)[1]	pI[1]	Lipidation[2]
bba64	924	31.60	34.9	9.176	Yes
bba65	849	29.56	32.5	8.241	Yes
bba66	1,236	33.66	45.8	9.186	Yes
bba71	414	26.81	16.4	4.849	No
bba73	891	28.40	34.6	8.907	Yes

[1] kiloDalton (kDa) & pI based on full amino acid sequence without post-translational processing.

[2] As predicted by the SpLip algorithm (401).

translationally modified by glycosylation, OspA and OspB (378, 379), and both have apparent molecular weight values by SDS-PAGE analysis (approximately 31 kDa and 34 kDa, respectively, Figures 3.1.3, 3.1.4, and 3.1.5, silver-stained gels) that are greater than their predicted molecular weights (29.6 kDa and 31.7 kDa, respectively). Whether BBA71 is glycosylated remains to be tested experimentally.

1.6.3 Expression *in vivo* and antigenicity of *bba64, bba65, bba66, bba71,* and *bba73*

Several genes clustering to the right end of lp54 belonging to the family formerly known as pgf 54 (comprised of *bba64*, *bba65*, *bba66*, *bba68* (*cspA*), *bba69*, *bba70*, *bba71*, *bba73*) were consistently shown in multiple studies and DNA microarray analyses to be the most highly regulated group of genes in response to environmental cues mimicking the mammalian host

environment (77, 78, 89, 204, 205, 314, 361, 446). Importantly, many of these same genes localizing to lp54 are also up-regulated *in vivo* (153, 258) and are stable throughout murine infection (153). Recently, quantitative real time-PCR (qRT-PCR) demonstrated that *bba64*, *bba65*, and *bba66* are transcriptionally regulated in *B. burgdorferi* B31 in ear tissue during persistent infection in mice (153), and microarray analysis of *B. burgdorferi* 297 indicated that *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* are highly expressed when grown in dialysis membrane chamber (DMC) implants (74). Furthermore, qRT-PCR has also revealed similar expression profiles for *bba65*, *bba66*, *bba71* and *bba73* when *B. burgdorferi* was grown under the combined *in vitro* mammalian-like culture conditions of pH 7.0 and 35 °C versus tick-like conditions of pH 8.0 and 23 °C (25).

In addition to evidence of gene expression *in vivo*, antibodies specific for BBA64 (P35), BBA65, and BBA66 are also detectable over the course of persistent infection in mice (153); moreover, these proteins are immunogenic in humans during early- and late-disseminated disease, in rabbits, and/or in mice (78, 89, 153, 154, 308, 309, 458). Proteins BBA64 (P35), BBA66, and BBA69 have also been shown to localize to the borrelial outer surface (53). Taken together, these data suggest that a subset of these former gene family members encode proteins that are exposed to direct interaction with the mammalian host environment and may, therefore, play an important role during mammalian infection and/or pathogenesis. This is supported by evidence that BBA68 (BbCRASP-1 encoded by *cspA*) binds host factor H (233, 285) and that a fragment of BBA66 isolated from a phage display library was capable of interacting with mouse heart tissue (12).

1.6.4 Expression & regulation of *bba64, bba65, bba66, bba71,* and *bba73* genes/proteins

Advances in culturing methods for *B. burgdorferi* have allowed for the identification and characterization of environmental signals and molecular mechanisms involved in *B. burgdorferi* gene regulation. Environmental signals or cues are indicators utilized by an organism such as *B. burgdorferi* to sense its location so that it can appropriately control gene and protein expression to survive in the given environment. For bacteria like *B. burgdorferi* this is particularly important given the multitude of distinct environments and mammalian species it is able to survive in and/or cause disease in. The environmental signals described for *B. burgdorferi* include culture density, the addition of mammalian blood, reducing dissolved oxygen and CO_2 levels, pH, and temperature (77, 78, 204, 205, 345, 399, 429, 446, 484). The later two conditions are used frequently in culture systems to simulate tick- (pH 8.0/23 °C) and mammalian-like (pH 7.0/35 °C) growth conditions; the downstream sigma subunit of the borrelial sigma factor regulatory cascade has been shown to alter its expression in response to pH and temperature, demonstrating an increase under mammalian- relative to tick-like conditions (73, 120, 484). This regulatory cascade controls numerous lipoproteins, some of which have been associated with survival and/or virulence within the tick-mammalian transmission cycle (72, 74, 138, 149, 183, 200, 486). Moreover, the expression of *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* has been linked to these sigma factors both *in vitro* using DNA microarray and in artificial infection models (74, 89, 138).

1.6.4.1 Environmental signals

Schwan was the first to report upon temperature as a signal that could affect the synthesis of *B. burgdorferi* proteins (390). In his report, Schwan demonstrated that synthesis of OspC was

induced at 35 °C relative to 23 °C, temperatures akin to the mammalian host and tick environments, respectively. This was confirmed in a later report and demonstrated for the outer surface proteins OspE, and OspE by Stevenson, Schwan, and colleagues (426, 429). The affect of temperature upon *B. burgdorferi* gene and protein regulation has since been investigated by numerous groups who found that a large proportion of the *B. burgdorferi* genome is differentially regulated in response to changing *in vitro* culture temperatures and that changes were observed at the levels of both gene transcription and protein synthesis. In a microarray study by Ojaimi and colleagues (314), *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* were shown to be up-regulated 2.10- to 6.26-fold at 35 °C relative to 23 °C. Recently, our laboratory utilized the more dynamic and quantitative method of quantitative real-time (qRT) PCR to corroborate that *bba65*, *bba66*, *bba71*, and *bba73* were up-regulated when cultured at a mammalian-like temperature relative to a tick-like temperature (89); notably, while our study was in agreement with the microarray analysis, indicating that the transcript levels of these genes were increased, the transcript levels measure in our analyses were significantly greater, 4.11 to 28.6-fold, relative to those levels measured by the microarray analysis (361).

Another important environmental cue, pH, was identified in a study by Carroll *et al* (78) that demonstrated alterations in culture pH led to changes in both target gene transcript levels and in the synthesis of proteins known to be involved in the *B. burgdorferi* infectious cycle (77). Notably, *bba64* and *bba66*, and the virulence factor *ospC* were shown to be up-regulated at pH 6.0 and pH 7.0 (mammalian-like) relative to pH 8.0 (tick-like). In another study, the fold induction of *bba65*, *bba66*, *bba71*, and *bba73 in vitro* was measured using qRT-PCR and demonstrated an increase in gene transcripts between 6.45- and 22.3-fold at pH 7.0 relative to pH 8.0 (89). In combination, pH and temperature have an even greater affect on *B. burgdorferi* gene

transcription (89, 361). A DNA microarray study employed by Revel *et al* that analyzed the global effects of changing pH upon the transcriptome of *B. burgdorferi* showed *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* transcripts to be increased under mammalian-like culture conditions (35 °C/pH 7.0) relative to tick-like culture conditions (23 °C/pH 8.0) (361); in this study, the transcription of these genes was increased 3.48- to 75.2-fold. These values were in close agreement with a second study that utilized qRT-PCR in which the comparison of similar combined culture conditions of pH and temperature demonstrated the transcripts of each of these genes, with the exception of *bba64*, to increase between 9.99- and 61.4-fold (89).

Additional environmental cues affecting *B. burgdorferi* gene transcription or protein synthesis include dissolved CO_2 or O_2 levels, the addition of mammalian blood, and culture density (204, 205, 399, 446). Reports investigating the affect of dissolved O_2 (399) and culture density (205) on transcript and/or protein expression examined a relatively small set of target genes; however, P35 (BBA64) transcript and protein levels were shown to be increased upon entry into and the early hours of stationary phase of *B. burgdorferi in vitro*. In a study examining gene expression under anaerobic conditions with increased dissolved CO_2 levels relative to microaerophilic conditions, the authors detected an increase in expression of σ^S which has been shown by microarray to affect *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* transcription (204). In combination with temperature, the addition of blood to *in vitro B. burgdorferi* cultures significantly increased the expression of *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* relative to cultures that lacked blood (446).

1.6.4.2 σ^N/σ^S regulatory cascade

Sigma factors are DNA sequence-specific binding proteins that are required for and give specificity to the binding of transcription machinery to promoter sequences; once bound, transcription is initiated. These regulatory proteins typically bind to sequences that are specific for individual sigma factors and lie approximately -10 and -35 nucleotides upstream of the start codon. The promoter specificities differ for σ^{70}-like sigma factors, which bind to -35/-10 promoter sequences, versus σ^N, which binds to -24/-12 promoter sequences (218). The general sigma factor, σ^{70}, has been identified in many bacterial species, such as *E. coli* and *S. typhimurium*, and has been shown to be constitutively expressed and involved primarily in general transcription of housekeeping genes (320). Other sigma factors are upregulated in response to specific stimuli and are responsible for the increased transcription of specific genes that may be involved in bacterial adaptation to the conditions detected; these sigma factors include RpoN (σ^N, σ^{54}), and RpoS (σ^S, σ^{38}), both of which are involved in transcriptional regulation of genes involved in surviving various environmental stresses. Initial observations in other bacteria showed σ^N to be involved in the response to nitrogen (238, 359) while σ^S was shown to be involved in the stationary phase response (189, 190, 315). Additionally, these sigma factors have been shown to respond to heat (467), osmotic stress (189, 248, 297, 435, 467), and acid stress (32, 33, 188, 253, 323, 409). Importantly, both σ^N and σ^S have been demonstrated to be important for virulence in multiple bacterial species, including *S. typhimurium* (128, 184), *Y. enterocolitica* (206), *V. vulnificus* (323), *V. cholerae* (225), *E. coli* (150), and *L. pneumophila* (14).

B. burgdorferi encodes a unique sigma factor cascade comprised of σ^N and σ^S, shown schematically in Figure 1.6.1. The cascade is activated when environmental signals are sensed

via a two component regulatory system involving a sensor kinase and the response regulator, Rrp2; it has been suggested that the sensor kinase Hk2 may be responsible for phosphorylation of Rrp2 (485), but it does not appear to be the only kinase capable of this role (66). Once phosphorylated, Rrp2 interacts with the σ^N and promotes binding to σ^N-dependent promoters and subsequent recruitment of RNA polymerase to initiate gene transcription. σ^N controls the expression of numerous borrelial genes, including σ^S. Both σ^N and σ^S are required for murine infection (138) and σ^S has been shown to directly control the expression of *ospC* (120, 487), a *B. burgdorferi* virulence factor required for murine infection (169, 322, 443, 445). σ^S has also been implicated in the regulation of other genes involved in borrelial infection and/or pathogenicity (73, 138, 200, 487). Furthermore, microarray analysis of *B. burgdorferi* strain B31 *ntrA*$^-$ and *rpoS*$^-$ mutants established that the transcription of numerous genes is influenced by the sigma factor cascade, including *bba64*, *bba65*, *bba66*, and *bba71*; relative to wildtype, these genes demonstrated 1.5- to 3.8-fold decreases in transcript expression while *ospC* showed a decrease in transcripts between 2.3- and 2.5-fold (138). *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* transcripts were also shown by microarray to be significantly decreased in an *rpoS*$^-$ mutant grown in DMCs implanted in either rats or rabbits when compared to its wildtype parent, isolate 297 (74).

Finally, an in depth analysis of *bba66* suggests that the expression of this gene may be controlled indirectly by σ^S in conjunction with an as yet unidentified regulatory protein that binds to a 29-base pair inverted repeat upstream of the -10/-35 region of the mapped promoter (89). Additional studies performed in our laboratory using competitive gel-shift assays have demonstrated that *bba65*, *bba71*, and *bba73* promoter sequences can compete for binding of the same protein that binds to the *bba66* promoter, suggesting that these genes may also be indirectly regulated by σ^S (unpublished data, Dawn R. Clifton and James A. Carroll). Since our 2006

publication, a similar mechanism of regulation in the form of a transcription repressor has been proposed for *bba64* (149). An added level of transcriptional control further supports the hypothesis that *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* are important for borrelial infection and/or pathogenicity.

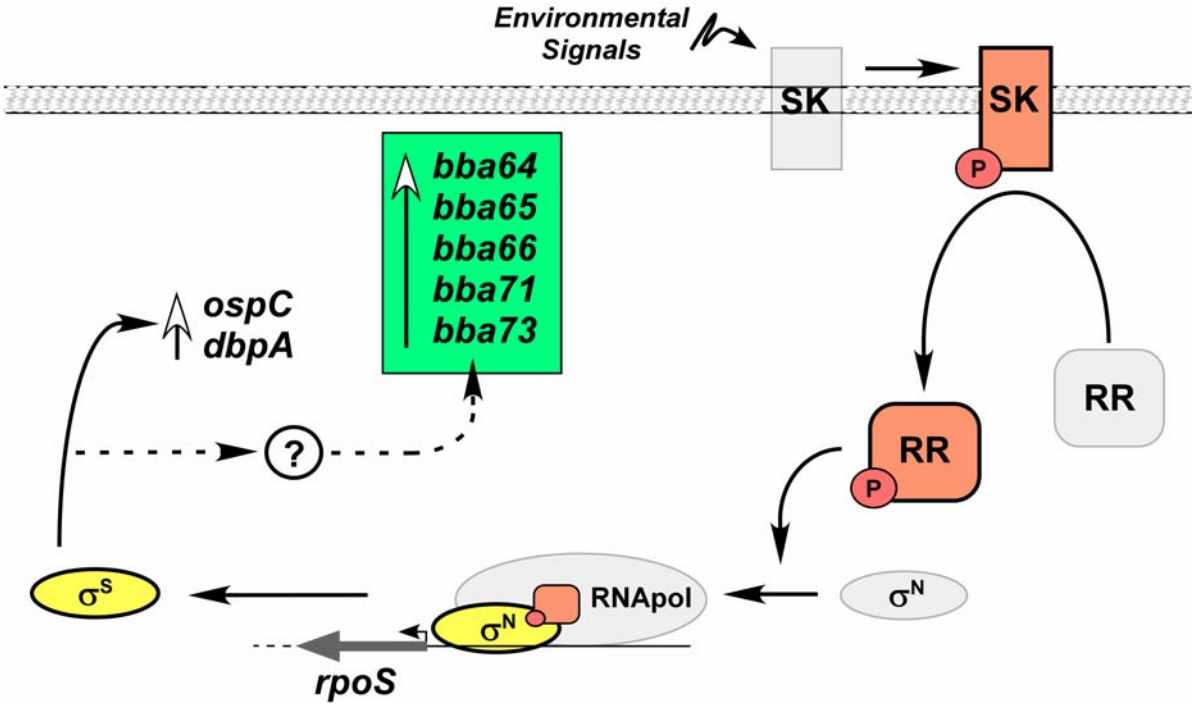


Figure 1.6.1 Schematic representation of the borrelial sigma factor regulatory cascade.

The sigma factors σ^N and σ^S are uniquely regulated by *B. burgdorferi* in a cascade of gene activation that involves a two-component regulatory system (orange boxes) and two sigma factors (yellow ovals). Undefined environmental signals that may include pH and temperature are detected by a membrane-associated sensor kinase (SK) which undergoes autophosphorylation and subsequently activates a response regulator (RR), Rrp2, by transfer of a phosphate group. RR-P then oligomerizes (not shown for simplicity) and activates σ^N to upregulate the expression of σ^N-dependent genes, including *rpoS* which encodes σ^S. σ^S directly upregulates the expression of genes encoding the decorin binding protein A (*dbpA*) and the known virulence factor Outer surface protein C, (*ospC*) (200). Studies have also shown that σ^S is involved in the expression of *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* (green box), though the regulation of these genes may also require the assistance of an activator (89) or repressor (149), represented by the encircled '?'.

1.6.5 Putative roles in infection and/or pathogenicity

The expression of *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* during persistent murine infection and the immunogenicity of their encoded proteins during infection are highly suggestive that these genes play an important role during murine infection and/or pathogenicity. Moreover, each of these genes is expressed in response to various environmental signals mimicking the mammalian host and is associated with the borrelial σ^N/σ^S regulatory cascade. The sigma factors that comprise this cascade have been shown in numerous Gram-negative bacteria to control the expression of genes required for survival and/or virulence and in *B. burgdorferi* has been demonstrated to directly control the expression of at least one virulence factor, *ospC*. The analyses performed by two separate groups have shown that the BBA66 protein is potentially important in binding to murine cardiac tissue (12, 458). These data have compelled us to further examine and characterize *i*) the expression, regulation, and localization of these genes and their encoded proteins to further our understanding of how they are controlled during infection and whether they are localized on the bacterial cell in a position where they may interact with mammalian host tissues or the host environment; *ii*) whether these genes are conserved by many or a few borrelial species which could suggest they play a role that is important in species beyond those that cause Lyme disease; *iii*) the importance of *bba66* during murine infection and/or pathogenesis.

1.7 STATEMENT OF HYPOTHESES ADDRESSED IN THIS THESIS

While microarray studies have allowed general assumptions on the potential importance of the genes historically categorized in pgf 54 during mammalian infection, few studies have described these genes in detail. The hypotheses stated in this thesis and the techniques utilized to answer these questions aimed to address the correlations noted in other studies regarding *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* in greater detail. This includes their association with the σ^N/σ^S regulatory cascade, their association with infectious phenotypes, and the localization of their encoded proteins on the borrelial cell. Furthermore, we undertook to assess the conservation of putative gene orthologs and cross-reaction of antibodies to these proteins from diverse *Borrelia* species associated with Lyme disease or relapsing fever. Finally, a *bba66* mutant was created and analyzed to determine whether it remained infectious and pathogenic in a murine model of Lyme disease.

1.7.1 Hypothesis #1 – BBA65, BBA71, and BBA73 are associated with the borrelial sigma factor regulatory cascade and pathogenic isolates while BBA65 and BBA73, but not BBA71, are localized to the outer surface of B31

Expression of virulence factors and proteins involved in *B. burgdorferi* infectivity and/or pathogenicity have been shown to be regulated in association with the borrelial σ^N/σ^S sigma factor regulatory cascade (72, 73, 138, 183, 200). Gene array analyses have demonstrated that the expression of *bba65*, *bba71*, and *bba73* is influenced by the σ^N/σ^S cascade (74, 138). However, more sensitive techniques have not been used to confirm these findings nor has the influence of the regulatory cascade upon the synthesis of these proteins been assessed. Using

qRT-PCR and immunoblotting, we hypothesized that transcript levels of *bba65*, *bba71*, and *bba73* would be significantly increased in wild-type and a complemented sigma factor mutant relative to σ^N and σ^S mutants and that these findings will be reflected at the protein level. Because the σ^N/σ^S cascade has been linked with borrelial virulence (73, 138), we further hypothesized that BBA65, BBA71, and BBA73 would be expressed by infectious, but not non-infectious, *B. burgdorferi ss* isolates.

Lipoproteins, as discussed above, are lipid-modified proteins that associate with cellular membranes via their covalently attached lipid moieties. Lipoproteins encoded by *B. burgdorferi* typically contain the N-terminal signal sequence L-X-X-C that is post-translationally processed and covalently-modified by the addition of a lipid at the cysteine residue (Section 1.5.3). In a study by Schulze and colleagues (387), *B. burgdorferi* lipoproteins were shown to be localized to the outer surface by default. OspA, amongst other known lipoproteins, has been proven to be localized to the borrelial outer-surface (93), a characteristic that is dependent upon the presence of its N-terminal signal sequence (116). Additionally, BBA66 was shown to be extracted from whole cells with Triton X-114 and to be expressed on the outer-surface by Brooks, *et al* (53). Given that BBA65 and BBA73 contain the N-terminal L-X-X-C motif, we hypothesized these proteins were also extractable with Triton X-114; we did not predict BBA71 to share these characteristics given that it does not contain the L-X-X-C motif and is not predicted by available programs to be an integral membrane protein. For similar reasons, we also hypothesized that BBA65 and BBA73 would be degraded by protease treatment of intact borrelial cells while BBA71 would not.

We expected that the outcome of these studies would further the body of knowledge linking BBA65, BBA71, and BBA73 to mammalian infection and/or pathogenicity. Current data

shows that BBA65, BBA71, and BBA73 are expressed early during murine infection (258) and antibodies to BBA66 arise during the early disseminated phase of human infection (89, 308). Therefore, if our hypotheses hold true, our findings along with current knowledge of these proteins would suggest that they may serve as virulence determinants and diagnostic markers during the early stages of Lyme disease.

1.7.2 Hypothesis #2 – *bba64, bba65, bba66, bba71,* and *bba73* are conserved among species belonging to the *B. burgdorferi sl* group and antibodies specific for these proteins will cross-react with protein orthologs expressed by *B. burgdorferi sl* group species

As discussed in Section 1.4.3, phylogenetic analyses of *Borrelia* species based on the flagellin (145) and *hhb* (450) genes show close relatedness amongst *B. burgdorferi ss*, *B. garinii*, *B. afzelii*, *B. andersonii*, and *B. japonica* while *B. parkerii*, *B. hermsii*, and *B. turicatae* are more distantly related. Furthermore, *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* orthologs have been identified in the *B. afzelii* isolate PKo and *B. garinii* isolate PBi genomes; analysis of BBA66 aligned with orthologs from PKo and PBi shows high identity between the C-terminal regions of these proteins. Moreover, orthologs to the *bba66* gene in *B. burgdorferi ss* isolate ZS7, ZS7.A66, have been identified by Southern blot in *B. afzelii* isolate MMS and *B. garinii* isolate ZQ1 (458). Given the relatedness of these species based on phylogenetic analyses, we hypothesized that *bba66* and other similarly regulated genes localized to the far end of lp54, namely *bba64*, *bba65*, *bba71*, and *bba73*, would be conserved in *B. burgdorferi sl* species as assessed by Southern blotting and PCR. Furthermore, we hypothesized that antibodies specific for the *B. burgdorferi ss* pH-responsive proteins BBA65, BBA66, BBA71, and BBA73 would be cross-reactive with pH-responsive proteins expressed by *B. burgdorferi sl* species when assessed

by immunoblotting. Conservation of these genes and their encoded proteins throughout the *sl* group would suggest that these genes/proteins are potentially important beyond *B. burgdorferi ss*, *B. afzelii*, and *B. garinii*.

1.7.3 Hypothesis #3 – Mutagenesis of *bba66* will result in reduced infectivity of *B. burgdorferi* strain A3 in C3H/H3J mice

BBA66 has been demonstrated in numerous reports to be expressed during mammalian infection. Microarray analysis of *B. burgdorferi* isolated from ear tissue of C3H/HeN mice that had been infected by transplant of infected murine tissue showed an increase in *bba66* expression within the first 11 days of infection and a decrease in expression after 3 weeks of infection (258). Moreover, in a study of persistent murine infection, *bba66* transcripts were detected throughout infection up to 17 months post infection (153). It has also been shown that anti-BBA66 antibodies are produced during infection of humans, mice, and rabbits (153, 308). Thus, BBA66 is expressed throughout infection, despite the fact that it is targeted by antibody. In two studies, BBA66 has been implicated in dissemination of spirochetes to the heart (12, 458), but no studies have examined whether this protein is required for infection. Utilizing a murine model of *B. burgdorferi* infection, we hypothesized that a *bba66* knockout mutant constructed in an infectious background would remain infectious given that the only one borrelial lipoprotein, OspC, has been mutated and shown to be required for murine infection (445). Moreover, evidence suggests that BBA66 may be involved in binding to and/or dissemination to heart tissue which, given that *B. burgdorferi* is able to disseminate to and survive in other tissues, it is unlikely that reducing the spirochete's ability to bind to heart tissue will cause it to be non-infectious. While future studies will focus on whether BBA66 is involved in pathogenicity, the

goal of this study was to *i*) create a *bba66* knockout mutant and *ii*) to determine its role in the establishment of infection.

2.0 MATERIALS AND METHODS

2.1 BACTERIAL STRAINS AND GROWTH

2.1.1 *Borrelia* strains and growth conditions

For cloning and *in vitro* characterization, *Borrelia* were grown in BSK-H media (Sigma, St. Louis, MO) at 35 °C and an atmosphere of 5% CO_2. BSK-H lots #045K8412 and #057K8413 were supplemented with an additional 4% rabbit serum (Invitrogen, Carlsbad, CA) unless otherwise noted. All strains are listed in Table 2.1.1. Kanamycin and gentamycin were used at 200 μg/ml and 40 μg/ml, respectively. pH shift experiments were carried out as previously described (78). Briefly, *Borrelia* were grown to mid-log phase (5×10^7 cells/ml), centrifuged for 15 min at 8,000 × *g*, and resuspended in BSK-H buffered with 25 mM HEPES and adjusted to either pH 7.0 or pH 8.0. pH 7.0 cultures were always grown at 35 ºC while pH 8.0 cultures were grown at either 35 ºC or 23 ºC. To determine growth kinetics, clones were grown to mid-log phase, collected by centrifugation, and resuspended to 5 x 10^6 in fresh BSK-H media. Spirochetes were counted in triplicate at various time points up to 96 h after cultures were diluted. These experiments were completed twice. *Borrelia* species that did not grow well in BSK-H were grown in 1X BSK media. All *Borrelia* were enumerated under darkfield microscopy using a Petroff-Hauser counting chamber.

2.1.2 Transformation of *Borrelia* and plating

B. burgdorferi clones were transformed as previously described (80). In brief, electrocompetent cells were prepared from mid-log phase cultures. Cells were centrifuged 15 min at 7,000 rpm, washed twice with 0.5 vol then once with 0.1 vol EPS (0.27 M sucrose, 15% [v/v] glycerol), then resuspended in 50 µl EPS. DNA was added in the amounts indicated and incubated with cells for 15 to 30 min on ice. Cells were electroporated using settings of 2.5 kV, 25 µF, and 200 Ω and then resuspended in 3 ml pre-warmed BSK-H and allowed to recover overnight at 35 ºC. Transformants and mutant clones obtained by subsurface agarose plating were isolated after 8 to 15 days of growth and subsequently cultured in BSK-H media for the expansion of individual isolates.

2.1.3 *Escherichia coli* strains and growth conditions

Escherichia coli were grown at 37 °C in Luria-Bertani (LB; Fisher, Pittsburgh, PA) media unless otherwise noted. For plating, *E. coli* were grown on solid LB agarose supplemented with 40 µg/ml kanamycin, 15 µg/ml gentamycin, 30 µg/ml spectinomycin, or 100 µg/ml chloramphenicol. All strains are listed in Table 2.1.1.

Table 2.1.1 Bacterial strains used in these studies

Isolate	Description	Reference
***Borrelia* isolates**		
B31 p5*	non-clonal, infectious isolate; all plasmids present	(64)
clone A	clonal, non-infectious isolate derived from B31; missing cp9, lp25, lp28-1, lp28-4, lp36, cp32-6, lp5, lp21	(42, 331) †
A-34	clonal, non-infectious isolate derived from clone A; also missing lp56	(89) #
A3	clonal, infectious isolate derived from B31 MI; missing cp9	(123)
A3-Gm	A3 containing lp25::P*flaB-aacC1*	(138) §
A3ntrA-Gm	A3 with *ntrA* deletion	(138) §
A3ntrA-comp	A3ntrA-Gm complemented with *ntrA in trans*	(138) §
A3rpoS	A3 with *rpoS* deletion	(138) §
B. californiensis	Isolate CA8, isolated from *I. pacificus*	(245) †
B. afzelii	Isolate PGau, isolated from skin	(201) †
B. garinii	Isolate G1, isolated from human CSF	(50) †
B. japonica	Isolate HO14, isolated from *I. persulcatus*	(275) †
B. japonica	Isolate IKA2, isolated from *I. ovatus*	(275) †
B. parkeri	From the Rocky Mountain Laboratories collection	(393) †
B. crocidurae	Isolate CR2A, from the Rocky Mountain Laboratories collection	(393) †
B. andersonii	Isolate 21038, isolated from *I. dentatus*	(9) †
B. valaisiana	Isolate VS116, isolated from *I. ricinus*	(250) †
B. anserina	From the Rocky Mountain Laboratories collection	(393) †
B. turicatae	From the Rocky Mountain Laboratories collection	(393) †
B. hermsii	Isolate DAH, isolated from human blood	(191) †
***Escherichia coli* strains**		
TOP10	F⁻ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
ER2508	F⁻ *ara-14 leuB6 fhuA2* Δ(*argF-lac*)*U169 lacY1 lon*::*miniTn*10 (Tet^R) *glnV44 galK2 rpsL20*(Str^R) *xyl-5 mtl-5* Δ(*malB*) *zjc*::*Tn*5 (Kan^R) Δ(*mcrC-mrr*)$_{HB101}$	NEB

† Provided by Dr. Patricia Rosa, Rocky Mountain Laboratories, NIH
# Provided by Dr. Kit Tilly, Rocky Mountain Laboratories, NIH
§ Provided by Drs. Mark Fisher and Frank Gherardini, Rocky Mountain Laboratories, NIH
¥ Provided by Dr. Tom Schwan, Rocky Mountain Laboratories, NIH

2.2 NUCLEIC ACID PURIFICATION AND ANALYSIS

2.2.1 Genomic DNA purification

For Southern blotting and PCR analysis of various *Borrelia* isolates, genomic DNA was prepared as described previously (289), with slight modification. *Borrelia* isolates grown to high cell density (approx. 1 x 10^8 cells/ml) were pelleted and washed once in saline-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0). Cells were treated with 3 mg/ml lysozyme in saline-EDTA then fractured by rapid freezing in a dry ice/ethanol bath and thawing with the addition of 1 vol Tris-SDS (0.1 M Tris-Cl [pH 9.0], 0.1 M NaCl, 1 % SDS). Protein was removed by phenol/chloroform/isoamyl alcohol (25:24:1) (Fisher) phase separation and the upper aqueous phase was treated with 50 μg/ml (final) RNase A (ABgene Inc., Rochester, NY) to remove contaminating RNA. Samples were re-extracted by phenol/chloroform/isoamyl alcohol and genomic DNA was precipitated with 0.1 vol 5 M ammonium acetate and 1 vol 100 % isopropanol. For other genomic DNA preparations, Promega's Wizard kit was utilized (Madison, WI). DNA was resuspended and stored at 4 °C in TE, pH 8.0 (10 mM Tris•Cl [pH 8.0], 1 mM EDTA [pH 8.0]). Concentrations were determined by OD_{260} readings.

2.2.2 RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from *B. burgdorferi* grown at pH 7.0 using the Ultraspec II RNA Isolation System (Biotecx, Houston, TX). Contaminating DNA was removed using Ambion's DNA-free kit per the manufacturer's instructions. RNA quantities were measured at OD_{260} and purity was assessed using the Access RT-PCR kit (Promega) and by the Agilent Bioanalyzer

using the RNA chip (Santa Clara, CA). qRT-PCR analyses were performed as outlined by Clifton, *et al* (89). Briefly, 25 µl reactions were performed at least twice in triplicate using PCR conditions as follows: 1 cycle at 50 °C for 30 min, 1 cycle at 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Melting curves were generated by a cycle of 95 °C for 1 min, 50 °C for 1 min and 80 × 0.5 °C increments every 10 s beginning at 50 °C. Primers used are listed in Table 2.3.1. The comparative C_T method was used and values were normalized to the constitutively expressed *flaB* gene. Changes in fluorescence were monitored with the MyiQ single color real-time PCR detection system using SYBR green (Bio-Rad, Hercules, CA).

2.2.3 PCR conditions

Unless otherwise noted, all PCR reactions were carried out using Sigma's JumpStart TAQ PCR kit with appropriate primers added to 0.5 µM (final) as follows: 3 min at 97 °C, 40 cycles of 1 min at 93 °C, 1 min at 60 °C, and 2 min at 72 ºC, and a final extension at 72 ºC for 7 min. When necessary, alternate annealing temperatures were used and are noted. For plasmid profiles, the primer set of Elias, *et al* was used (123).

2.2.4 Southern blotting

One microgram of genomic DNA was digested for 1 h with EcoRI (Promega) and separated on a 0.8 % agarose gel in Tris/acetate/EDTA (TAE; 40 mM Tris•acetate, 2 mM EDTA) electrophoresis buffer. Bands were visualized by staining with ethidium bromide (Bio-Rad) and the DNA transferred to Hybond XL membranes (Amersham Biosciences, Piscataway, NJ) as described by Marconi, *et al* (268, 269), except that depurination, denaturation, and neutralization

steps were increased to 20 min each. Transfers were completed with a 1 h treatment with 20 X sodium chloride/sodium citrate (SSC; 3 M NaCl, 0.3 M Na•citrate•$2H_2O$ [pH 7.0]). Transferred material was cross-linked to membranes using the auto cross link function on Stratagene's UV Stratalinker 2400 (La Jolla, CA) and DNA was visualized by staining membranes with 0.03 % methylene blue in 1 % acetic acid. Blots were dried and stored at room temperature until use. Probes were made using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Indianapolis, IN) with *B. burgdorferi* isolate B31 MI gene-specific primers for *bba64*, *bba65*, *bba66*, *bba71*, *bba73*, and *flaB* (Table 2.3.1). Membranes were prehybridized at 42 ºC overnight in hybridization buffer (43) and hybridized with DIG-labeled probes diluted 1:500 (*bba65*, *bba66*, *bba71*, *bba73*, and *flaB*) or 1:1,000 (*bba64*) in hybridization buffer at 42 °C overnight. Membranes were then washed under moderate stringency conditions for 10 min at room temperature with 2 X SSC, 0.1 % (w/v) SDS followed by 30 min at 65 °C with 0.5 X SSC, 0.1 % (w/v) SDS. Bound probes were detected using anti-DIG-POD antibody from Roche Diagnostics and bands were visualized with ECL reagents and exposure to film. Band sizes were approximated by calculating the linear regression equation based on the mobility of known standards (lambda / λ HindIII DNA standards; Promega) for individual gels.

2.2.5 Plasmid rescue

To determine whether pBSV2G-*rpoS*comp and pBSV2G-*bba66*comp were maintained *in trans* once transformed into *Borrelia*, 30 ng of genomic DNA from individual clones that had been chosen for further study was transformed into TOP10 *E. coli* and colonies were selected with gentamycin. Isolated colonies were assessed by PCR for the presence of each construct with plasmid-specific primers (Table 2.3.1). Genomic DNA isolated from A3-*bba66*comp-infected

mouse tissue outgrowth cultures (see below) was also assessed. PCR reactions were performed with a 60 ºC annealing temperature.

2.2.6 Phylogenetic analysis

Clustal V analysis was performed using a PAM250 table for the mutation probability matrix for the evolutionary distance (Table 1.6.1) on thirty-four DNA sequences using the MegAlign module of the Lasergene v.4.06 software package (DNASTAR, Inc., Madison, WI). Sequences (with accession numbers in parentheses) of *B. burgdorferi* B31 *bba64*, *bba65*, *bba66*, *bba68*, *bba69*, *bba70*, *bba71*, *bba73*, *bbi36*, *bbi38*, *bbi39*, *bbj41* (NC_001857), and their orthologs in *B. garinii* PBi (CP000015) were downloaded from the Comprehensive Microbial Resource (CMR) website provided by TIGR (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi). Sequence data for p46 from *B. garinii* ZQ1 (AJ430851), p46 from *B. burgdorferi* ZS7 (AJ430850), p46 from *B. afzelii* PKo (NC_008564), p46 from *B. afzelii* MMS (AJ430849), and BAPKO_*2061* from *B. afzelii* PKo (CP000395) were downloaded from the NCBI website (http://www.ncbi.nlm.nih.gov/). Sequences for *bba66*, *bbi36*, *bbi38*, and *bbi39* orthologs from *B. burgdorferi* isolates 297, JD1, and N40 were kindly provided by the *Borrelia* sequencing team composed of Drs. Sherwood R. Casjens, John J. Dunn, Benjamin J. Luft, Claire M. Fraser, Weigang Qiu, and Steven E. Schutzer.

2.3 CLONING AND GENETIC MANIPULATIONS

2.3.1 Construction of the *rpoS* complementing plasmid

The gene encoding the RpoS homologue (*rpoS*) and the *flaB* promoter were amplified using specific engineered primers by PCR from *B. burgdorferi* B31 genomic DNA and independently cloned into pCR2.1-TOPO creating pCR2.1-*rpoS* and pCR2.1-*flaB*p, respectively. The *flaB* promoter was excised from pCR2.1*flaB*p by digestion with NotI and NdeI and ligated into compatible sites within pCR2.1-*rpoS*, placing *rpoS* under the transcriptional control of the constitutive *flaB* promoter (pCR2.1-*rpoS*comp) similar to that of Hübner, *et al* (200). The transcriptional fusion was excised by digestion of pCR2.1-*rpoS*comp with NotI and KpnI and ligated into compatible sites within pBSV2G (122), creating pBSV2G-*rpoS*comp. All constructs were sequenced to verify that the sequences were correct and that the fusion was in frame. *B. burgdorferi* clones A, A34, and the A3 *rpoS* deletion mutant (Table 2.1.1) were transformed by electroporation using 40 μg pBSV2G-*rpoS*comp as described elsewhere (80). Transformants were plated into semisoft BSK plating media supplemented with 40 μg/ml gentamycin and incubated for up to 35 days at 35 °C under a humidified atmosphere of 5.0 % CO_2. No clones harboring pBSV2G-*rpoS*comp were confirmed by PCR screening.

2.3.2 Construction of the *bba66* knockout plasmid

A 3,262 bp region encompassing *bba66* and partial sequences of flanking genes (*bba65* and *bba68*) was amplified by PCR from *B. burgdorferi* isolate B31 genomic DNA using primers bba65RT.R and bba68RT.F (Table 2.3.1). The resulting product was cloned into pBAD-TOPO

(Invitrogen) to create pBAD*bba68*RT-*bba65*RT. The kanamycin resistance cassette was amplified by PCR from the pBSV2 shuttle vector (430) using primers pflg.1+NheI and Kan+BamHI (Table 2.3.1) and cloned into pCR2.1-TOPO, yielding pCR2.1p*flg*-Kan. pBADbba68RT-bba65RT was digested with BglII and NheI (Promega), removing 757 bp of internal *bba66* sequence, purified by gel extraction (Qiagen, Valencia, CA), and de-phosphorylated with shrimp alkaline phosphatase (Promega). pCR2.1p*flg*-Kan was digested with NheI and BamHI (Promega) and the 1,243 bp kanamycin resistance cassette purified by gel extraction. The cassette was then ligated into pBAD*bba68*RT-*bba65*RT with T4 ligase (Invitrogen) to yield pBAD-*bba66*::Kan. All plasmids were transformed into TOP10 *E. coli* and clones selected with appropriate antibiotics. For mutation of *bba66*, pBAD-*bba66*::Kan was electroporated (Section 2.1.2) into *B. burgdorferi* clone A3 and the *bba66*::Kan construct replaced the native gene by homologous recombination (Figure 3.3.1C). Plasmid constructs and A3-*bba66*::Kan clones were confirmed by PCR, restriction endonuclease digest, and sequencing.

2.3.3 Construction of the *bba66* complementing plasmid

bba66 and its native promoter were amplified by PCR from *B. burgdorferi* isolate B31 genomic DNA using primers proA66.1 and BBA66FL.6 (Table 2.3.1) and cloned into the Gateway entry vector, pCR8/GW/TOPO (Invitrogen), to create pCR8-*bba66*comp. The shuttle vector pBSV2G (122, 168) was converted into a Gateway destination vector using cassette RfB following the manufacturer's protocol (Invitrogen). pCR8-*bba66*comp and pBSV2G-GDV-B were recombined using the Gateway system LP recombination reaction per the manufacturer's instructions (Invitrogen), producing pBSV2G-*bba66*comp. pCR8-*bba66*comp and pBSV2G-*bba66*comp were maintained in TOP10 *E. coli* and pBSV2G-GDV-B in *ccdB* survival *E. coli*

(Invitrogen), each under appropriate antibiotic selection. pBSV2G-*bba66*comp was electroporated (Section 2.1.2) into A3-*bba66*::Kan to complement the *bba66* mutation in trans. Plasmid constructs and A3-*bba66*comp clones were confirmed by PCR (conditions as described above), restriction endonuclease digest, and/or sequencing.

2.3.4 Construction of a plasmid for the constitutive expression of *bba66*

bba66, including its native stop codon, and the *flaB* promoter (p*flaB*) were PCR amplified from *B. burgdorferi* isolate B31 genomic DNA using primers BBA66FL.1 with BBA66FL.6 and FlaB5'prom+NotI with FlaB3'prom+NdeI, respectively (Table 2.3.1). PCR products were subsequently cloned into pCR2.1-TOPO or pCRT7-TOPO, creating pCR2.1-*bba66* and pCRT7-p*flaB*. Both plasmids were next digested with NdeI in combination with either SpeI or XbaI, respectively. After gel purification (Qiagen) p*flaB* was ligated into de-phosphorylated (shrimp alkaline phosphatase, Promega) pCR2.1-*bba66*. p*flaB*-*bba66* was then PCR amplified from the resultant plasmid, pCR2.1-p*flaB*-*bba66*, and cloned into the pCR8/GW/TOPO vector producing pCR8-p*flaB*-*bba66*. The shuttle vector pBSV2*flaB*p-*gfp* (80) was converted into a Gateway destination vector using cassette RfC.1 (Invitrogen). pCR8-p*flaB*-*bba66* and pBSV2*flaB*p-*gfp*-GDV-C.1 were recombined using the Gateway system LP recombination reaction (Invitrogen), producing pBSV2-gfp^{+}-$bba66^{+}$. All constructs were maintained in TOP10 *E. coli*, except for pCR8-p*flaB*-*bba66* which was maintained in *ccdB* survival *E. coli*, and all were confirmed by PCR, restriction endonuclease digest, and sequencing. All TOPO vectors were purchased from Invitrogen. pBSV2-gfp^{+}-$bba66^{+}$ was electroporated (Section 2.1.2) into *B. burgdorferi* clones A, A-34, A313, and A3, but no positive clones were obtained after multiple attempts (data not shown).

2.3.5 Construction of MalE-fusion protein plasmids

This work was a collaborative effort of all members of the Carroll laboratory. Cloning and over-expression of BBA64, BBA65, and BBA66 was described previously (89, 153) and similar methods were used for the remaining genes. To summarize, genes encoding either a full length protein (*bba65*, *bba69* and *bba70*) or a N-terminal truncated protein (*bba66*, *bba68* [*cspA*], *bba71* and *bba73*) were amplified by PCR using engineered specific primers in Table 2.3.1, and products were digested with appropriate restriction endonuclease and ligated using T4 DNA ligase (Promega) into similarly digested pMAL-c2x vector (New England Biolabs [NEB], Beverly, MA). The resulting vectors, pMAL-*bba65*FL, pMAL-*bba66*TR, pMAL-*bba68*TR, pMAL-*bba69*FL, pMAL-*bba70*FL, pMAL-*bba71*TR, and pMAL-*bba73*TR, were electroporated into the *E. coli* strain ER2508 (NEB). ER2508 lacks the genes encoding the Lon protease and MalE (maltose binding protein) and therefore has a reduced capacity for protein degradation and negates competition between endogenous MalE and recombinant MalE-fusion proteins for binding to amylose columns during protein purification. Clones were selected on LB agar supplemented with ampicillin and positive colonies were screened for by PCR using gene-specific primers (Table 2.3.1), restriction endonuclease digest of plasmids, and sequencing.

Table 2.3.1 Primers used in these studies

Primer name [#]	Sequence	Use*
BBA64 FL.1	TTGAAGGATAACATTTTGAAAAAT	SB, PCR
BBA64 FL.2	CTGAATTGGAGCAAGAATATTGG	SB, PCR
BBA64F RT.3	CAGAGGCTTTAGCATTCAAT	qRT-PCR, PCR
BBA64R RT.4	TGTACTCGTCAATAAGGTCATCT	qRT-PCR, PCR
BBA65 FL.1	TTGAATAAAATAAAATTATCAATA	SB, cloning, PCR
BBA65 FL.2	ATTAAATTTAAATAATGTGTC	SB, cloning, PCR
BBA65 RT.F	AGAATGGCTTAGCACAATAGA	qRT-PCR, PCR
BBA65 RT.R [5]	TCAAGCAAAGAGAAATCATAGTA	qRT-PCR, PCR
BBA66 FL.1 [3]	TTGAAAATCAAACCATTAATAC	SB, PCR

BBA66 FL.2 [4]	TTGAGTTTGTATCAGCACTTGTTG	SB, PCR
BBA66 RT.F	ATACCTATTCAAGCCGTTAC	qRT-PCR, PCR
BBA66 RT.R	ATTTACTGCCAGAAGATGTTGTTG	qRT-PCR, PCR
proA66.1	CTTGTCGCAAAAATAGAG	Cloning, PCR
BBA68.3 TR FWD	ACCTCATGCGCACCTTTT	Cloning
BBA68.2 REV+XbaI	TTTTTCTCTAGATTAGTAAAAGGCAGGTTT	Cloning
BBA68 RT.F [8]	CTAAAAGCAATTGGTAAGGAACTG	qRT-PCR
BBA68 RT.R	TCAATAAGATCGTAAGGACCAACT	qRT-PCR
BBA69 FL.1 FWD	ATTTTGAAAAAAGCCAAACTAAAT	Cloning
BBA69 FL.2 REV+XbaI	TTTTATCTAGATTAATAAAAGGCAGATTG	Cloning
BBA69 RT.F	AAAAAGAAAACATAGAGACATT	qRT-PCR
BBA69 RT.R	AAGTGCTTTTCCAGTTTTA	qRT-PCR
BBA70.1 FWD	ATGGCTCCAGAAGTAAACA	Cloning
BBA70.2 REV+SalI	TGGCTTATGTCGACTTATTTTATATTAGT	Cloning
BBA70 RT.F	TACACCAATACAAAAGAAGACACC	qRT-PCR
BBA70 RT.R	TTTGCATTTTTACATTATTTTTAG	qRT-PCR
BBA71 FL.1	ATGAATAAATTAAAAGAAATTCTTG	SB, PCR
BBA71 FL.2	GTTTTGTTTAAATTCAACACTG	SB, cloning, PCR
BBA71 TR.3	ATTATAGGACTACTTTATCACAAAGC	Cloning
BBA71 RT.F	AAAGGCAGCCCCGATTAC	qRT-PCR, PCR
BBA71 RT.R	AACTTTTCTTTTAGCATTAGGTCA	qRT-PCR, PCR
BBA73 FL.1	TTGAAAAGAAACAAAATTTGGAAAAC	SB, PCR
BBA73 FL.2	GTAGTGTATGTGGTCACAACAGG	SB, cloning, PCR
BBA73 TR.3	TTTTATTCTAAATCAAACAACAC	Cloning
BBA73 RT.F	ATGACAAAAATACGGGAGGAT	qRT-PCR, PCR
BBA73 RT.R	CAGGTTTTTAGCGGTGTT	qRT-PCR, PCR
BBI36/38 RT.F	GTGAAAAAGAAGCCGAAAAGT	qRT-PCR
BBI36/38 RT.R	TAGTAGATAAAGAGGATGGGAATA	qRT-PCR
BBI39/J41 RT.F	GTGAAAAAGAAGCCGAAAAGT	qRT-PCR
BBI39/J41 RT.R	TAGTAGATAAAGAGGATGGGAATA	qRT-PCR
3'Kan [6]	CGAAAAACTCATCGAGCATCAA	Cloning, PCR
pflg.1+NheI [7]	GCTAGCTACCCGAGCTTCAA	Cloning, PCR
pBSV seq F	CCCAATACGCAAACCGCCTCTCCC	PCR
FlaB5'prom+NotI [1]	TGTCTGTCGCCTCTTGCGGCCGCCGGAGGAG	Cloning, PCR
FlaB3'prom+NdeI	GATTGATAATCATATGTCATTCCTCCATG	Cloning
flaB.RT For	TGTTGCAAATCTTTTCTCTGGTGA	SB
flaB.RT Rev	CCTTCCTGTTGAACACCCTCTT	SB
OspC.RT For	TACGGATTCTAATGCGGTTTTAC	qRT-PCR
OspC.RT Rev	GTGATTATTTTCGGTATCCAAACCA	qRT-PCR
rpoS.2 [2]	TTAATTTATTTCTTCTTTTAATTT	Cloning, PCR

*SB = Southern blotting probe, qRT-PCR = quantitative real time-PCR, PCR = confirmatory PCR
= refers to primer numbering used in Figures 3.1.2 and 3.3.1.

2.3.6 *Borrelia* cloning

Plasmids were electroporated into *B. burgdorferi* clone A3 (80) and plated into semi-soft BSK media, with modification as described by Rosa, *et al* (369), and supplemented with antibiotics. The following amounts of DNA were used: 40 μg pBSV2G-*rpoS*comp; 40 μg pBAD-*bba66*::Kan; 50 μg pBSV2G-*bba66*comp. Colonies appeared within eight to fifteen days and were inoculated into BSK-H media with antibiotics. Individual isolates were stored in 50% glycerol at -80 ºC until used. Genomic DNA was isolated using Promega's Wizard kit and the plasmid content of each clone was determined by PCR amplification of target genes using the primer set of Elias, *et al* (123) and an annealing temperature of 55 ºC.

2.4 PROTEIN PURIFICATION AND ANTIBODY PRODUCTION

2.4.1 Protein over-expression and purification

For protein over-expression of MalE fusion proteins, clones were grown to mid-log at 37 °C, except for ER2508-pMAL-*bba64*TR, in LB supplemented with 0.2 % glucose and ampicillin and then induced with 0.3 mM (final) isopropyl β-D-1-thiogalactopyranoside (IPTG; Fisher) for 2 h at 37 °C. ER2508-pMAL-*bba64*TR was grown and induced at 16 °C to reduce protein degradation. Cells were collected by centrifugation and resuspended in column buffer (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1 mM DTT and complete protease inhibitor, EDTA-free). Cells were lysed by French pressure cell by three passes at 16,000 lbs per in^2. Lysate was clarified by ultracentrifugation for 1 h at 45,000 × *g* and 4 °C and supernatants were

stored at -20 °C until use. Recombinant proteins were purified from clarified cell lysates using an amylose resin (NEB) column following the manufacturer's protocol using the AKTA Prime system (Amersham Biosciences). Purified fusion proteins were stored at -80 °C and protein concentrations determined by modified Lowry assay using BSA as a standard.

2.4.2 Antibody production

Polyclonal anti-MalE-BBA66TR and anti-MalE-BBA71TR antibodies were produced at Aves Labs (Tigard, OR) in hens and used at 1:8,000 or 1:4,000, respectively. Additional antibodies to BBA65, BBA66, and BBA73 were produced through immunization of 8 week old female C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) as follows. Fifty micrograms of purified and concentrated MalE-fusion proteins and 2.5 mg Imject alum (Pierce, Rockford, IL) were injected subcutaneously in the back at days 0 and 14. Mice immunized with BBA65 received an extra 50 μg protein boost at day 28 to improve seroconversion. Mice were bled via the dorsal tail vein and sera was collected and stored at 4 °C.

2.5 PROTEIN ANALYSES

2.5.1 SDS-PAGE and immunoblotting

Cells and proteins resuspended in Laemmli buffer were heated to 100 °C for 5 min and equal amounts were loaded to 12.5 % polyacrylamide SDS gels for separation by electrophoresis. Gels were stained with either the Silver Stain Plus kit (Bio-Rad) or transferred to 0.45 μm Transblot

nitrocellulose membrane (Bio-Rad) by the method of Towbin, *et al* (447). Standards were visualized and marked after staining with 0.1 % naphthol blue black (Sigma) in 1 % acetic acid. Membranes probed with either mouse or rabbit antibodies were blocked in 5 % non-fat dry milk in Tris-buffered saline/Tween 20 (TBS-T_{20}; 10 mM Tris-HCl [pH 8], 150 mM NaCl, 0.1 % Tween 20 [Fisher]) and those probed with hen antibodies were blocked in 2 % bovine serum albumin (BSA) fraction V (Sigma) in TBS-T_{20}. Membranes were incubated with primary antibodies for 1 h at room temperature or overnight at 4 ºC in blocking buffer, washed in TBS-T_{20}, incubated for 1 h with secondary antibodies in blocking buffer, and washes were repeated. Reactive bands were detected using enhanced chemiluminescence (ECL) (Amersham Biosciences) or Super Signal reagents (Pierce) and subsequent exposure to film. Densitometric analyses were performed using the Bio-Rad Gel Doc XR system and values calculated as a percentage of total densitometry of all bands assessed for a given protein using Quantity One 1-D Analysis software (Bio-Rad). Background was accounted for using the global background subtraction option.

2.5.2 Antibodies

Primary antibodies. Polyclonal chicken anti-MalE-BBA66TR and anti-MalE-BBA71TR antibodies were used at 1:8,000 or 1:4,000, respectively. Murine antibodies to BBA65, BBA66, BBA73, FlaB (monoclonal antibody H9724 provided by Drs. Nyles Charon and Mohamed A. Motaleb (21, 296)), and serum from RML white mice infected by needle inoculation with B31 clone A3 (polyclonal antibody from Dr. Patricia Rosa (308)), along with polyclonal rabbit anti-OppAI (provided by Dr. Linden T. Hu (199)) sera, were all diluted 1:1,000 for immunoblots. All murine sera used for EIA were diluted 1:100. *Secondary antibodies*. HRP-conjugated protein A

(Sigma) was used in conjunction with the anti-FlaB antibody at 1:10,000. For the remaining antibodies, appropriate HRP-conjugated secondary antibodies were purchased from Sigma and diluted 1:5,000. AP-conjugated goat-anti-mouse IgG was used for EIA at 1:10,000.

2.5.3 Preparation of membrane fractions

Membrane fractions were prepared as described (79). Briefly, spirochetes were shifted to pH 7.0 and membrane-associated proteins were separated from soluble proteins by ultracentrifugation for 1 h at 435,700 × *g* and 4 °C in a Sorvall Discovery M150 SE Ultracentrifuge (Milford, MA). Supernatant containing the soluble fraction was removed and pellets containing membrane-associated proteins were rinsed and resuspended in HN buffer (10 mM HEPES, pH 8.0, 50 mM NaCl) supplemented with complete protease inhibitor, EDTA-free (Roche Diagnostics) using a Teflon-coated pestle. Protein concentrations were determined by modified Lowry assay using BSA as a standard (270).

2.5.4 Triton X-114 phase partitioning

Detergent extraction of lipidated and integral membrane proteins from cell lysate or intact cells was performed as previously described (45, 49, 100) with slight modification. For cell lysates, 500 µg cell lysate was brought to 500 µl in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4•7H2O, 1.4 mM KH2PO4) and treated with 2% (v/v) Triton X-114 (TX-114; Sigma) with agitation on ice for 30 min. Centrifugation for 30 min at 10,000 × *g* and 10 °C yielded the insoluble cold pellet. Supernatant containing TX-114 soluble material was heated to 37 °C for 10 min to bring the detergent to its cloud point. The aqueous and detergent

phases were subsequently resolved by centrifugation at 14,000 × *g* and 37 °C for 10 min. The aqueous phase was washed 3 times with 100 µl 10 % cold TX-114 while the detergent phase was washed 3 times with 500 µl 1 % TX-114 and incubated 10 min at 37 °C prior to re-centrifugation. Proteins from the aqueous and detergent phases were precipitated with 10 vol cold 100 % acetone. For treatment of intact cells, *Borrelia* were resuspended to 5×10^9 cells/ml in PBS and rotated overnight at 4 °C with 2 % (v/v) TX-114. Dithiothreitol (DTT; EMD Biosciences, San Diego, CA) was used at 50 mM (final) where indicated. The cold pellet, containing the protoplasmic cylinder, was obtained by centrifugation as above and the supernatant was heated for 10 min at 37 °C and centrifuged for 10 min at 14,000 × *g* to yield the warm pellet and the aqueous and detergent phases. Aqueous and detergent phases were washed and precipitated, as described above. All pellets were resuspended in 3 X Laemmli buffer (Bio-Rad) to a final concentration of 1×10^7 cell equivalents/µl.

2.5.5 Protease treatment of intact *B. burgdorferi* isolate B31

Protease experiments were performed according to the methods of El-Hage *et al* (121), with slight modification. Briefly, infectious *B. burgdorferi* strain B31 cells shifted to pH 7.0 were collected by centrifugation at 8,000 × *g* and 4 ºC for 15 min, washed once, and resuspended in HN buffer. Visualization by darkfield microscopy was used to ensure spirochetes were intact and to enumerate cells. Cells diluted to 2×10^9 cells/ml were treated for 1 h at room temperature with 400 µg/ml proteinase K (Promega), trypsin (Sigma), pronase SC (Roche Diagnostics), or buffer as a control. Reactions were stopped by the addition of phenylmethylsulfonyl fluoride (Fisher) to a final concentration of 1.6 µg/ml to all treatments, pefabloc (Fisher) to a final

concentration of 0.3 μg/ml to trypsin, pronase, and buffer treated cells, and EDTA to a final concentration of 0.5 μg/ml (EMD Biosciences) to pronase and buffer treated cells. Samples were centrifuged, resuspended in 3 X Laemmli buffer to a final concentration of 5×10^6 cells/μl, and stored at -20 °C until analyzed. Densitometric analyses were performed using the Bio-Rad Gel Doc XR system and values calculated as a percentage of total densitometry of all bands assessed for a given protein using Quantity One 1-D Analysis software (Bio-Rad).

2.6 MURINE INFECTIONS AND ANALYSES

2.6.1 Experimental mouse infections

All protocols were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee and meet the requirements of the National Institutes of Health. Six to eight week-old female C3H/HeJ mice purchased from The Jackson Laboratories (Bar Harbor, ME) were used in for all infections. Three groups of eight mice were infected subcutaneously via needle-inoculation with 1×10^4 of the *B. burgdorferi* clones B31 clone A3, A3-*bba66*::Kan (*bba66* mutant), or A3-*bba66*comp (complemented *bba66* mutant). Blood was collected from the tail vein on the day of infection prior to inoculation (pre-immune serum) and weekly thereafter. Blood was allowed to clot at 37 ºC for 1 h then serum was collected by centrifugation for 10 min at 3,000 × *g* and stored at -20 ºC until use. Swelling was used as a gross indicator of infection and was measured laterally across rear tibiotarsal joints using digital calipers on a bi-weekly basis. Mice were sacrificed after 35 days and the heart, spleen, bladder, tibiotarsal joints, and ears were aseptically removed. Half of each heart, spleen, and bladder and one joint and ear

from each mouse was minced and resuspended in BSK-H medium supplemented with RPF (Sigma) for outgrowth of spirochetes. Cultures were monitored for the presence spirochetes for four to six weeks by darkfield microscopy. The remaining tissues were stored at -80 ºC until they were processed for the isolation of total genomic DNA.

2.6.2 Genomic DNA isolation from murine tissues and plasmid rescue

Total genomic DNA (murine and *Borrelia* DNA) was isolated from mouse tissues using the methods of Seiler, *et al* (398). *Borrelia* genomic DNA alone was isolated as described above from cultured spirochetes (Section 2.2.1). All DNA was stored at 4 ºC until use. For plasmid rescues, 100 ng of genomic DNA from infected mouse tissues was transformed into chemically competent TOP10 *E. coli* (Table 2.1.1). *E. coli* were plated to LB agar plates supplemented with gentamycin to select for the complementing plasmid, pBSV2G-*bba66*comp.

2.6.3 Enzyme-linked Immunosorbant Assay (EIA) analysis of mouse sera

For EIA, equal parts of non-denatured and heat-denatured protein (B31 total membrane fraction, purified MalE, or purified recombinant MalE-BBA66TR) suspended in binding buffer (0.1 M $NaHCO_3$, pH 8.6) were mixed and 200 ng total protein was bound to wells of a 96-well plate overnight at 4 ºC. All further incubations were conducted at room temperature. Unbound material was rinsed away with deionized water and all wells were blocked for 1 h with blocking buffer (5% non-fat dry milk in PBS-T_{20} [150 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.02 % Tween 20]). Mouse sera diluted 1:100 in blocking buffer were added in triplicate to the plate and incubated for 1 h. Unbound material was removed by rinsing with

PBS-T_{20} and AP-conjugated goat-anti-mouse IgG (Sigma) diluted 1:10,000 in blocking buffer was added and incubated for 1 h. Wells were washed with PBS-T_{20}, incubated for 30 min with PNPP substrate (Pierce), and then reactions stopped by the addition of 2 N NaOH. Absorbance was measured at OD_{405}.

3.0 RESULTS

3.1 *IN VITRO* EXPRESSION AND CHARACTERIZATION OF SELECT GENES LOCALIZING TO THE RIGHT END OF LINEAR PLASMID 54

3.1.1 Influence of the σ^N-σ^S regulatory cascade upon *bba65, bba66, bba71,* and *bba73* transcript and protein expression

DNA microarray analyses have demonstrated the σ^N-σ^S regulatory cascade is involved in the transcriptional regulation of numerous outer-surface localized lipoproteins (74, 138) and in the expression of borrelial virulence factors (73). To validate whether the σ^N-σ^S regulatory cascade influenced the expression genes historically categorized to pgf 54, we measured transcript levels of *ntrA*$^-$ and *rpoS*$^-$ mutants *in vitro* using qRT-PCR which has a broader dynamic range of detection than previous DNA microarray studies. Given that mRNA levels and protein amount do not always correlate (176), we also assessed changes at the protein level for those genes that appeared to be influenced by the σ^N-σ^S cascade in our qRT-PCR analyses. RNA and membrane-associated proteins were isolated from B31 isolates A3-Gm (wildtype control), A3ntrA-Gm (σ^N deletion mutant), A3ntrA-comp (complemented σ^N deletion mutant), and A3rpoS (σ^S deletion mutant) (Table 2.1.1) as described in Sections 2.2.2 and 2.5.3, respectively. Transcript (Figure 3.1.1A) and protein amounts (Figure 3.1.1B) of *bba65*, *bba66*, *bba71*, and *bba73* were decreased

in both sigma factor mutants relative to A3-Gm, in agreement with previous microarray studies (74, 138). Complementation of the *ntrA* mutation restored transcript (Figure 3.1.1A) and protein (Figure 3.1.1B) expression of these genes to levels comparable to A3-Gm. *ospC* transcript and protein amounts were substantially decreased in both sigma factor mutants and restored with complementation of the *ntrA*$^{-}$ mutant (Figure 3.1.1), as expected based on previous studies (120, 138, 487). Transcription of the remaining eight genes examined, including *bba64*, *bba68*, *bba69*, *bba70*, *bbi36*/*38*, and *bbi39*/*j41* were minimally affected by the loss of either sigma factor (Figure 3.1.1).

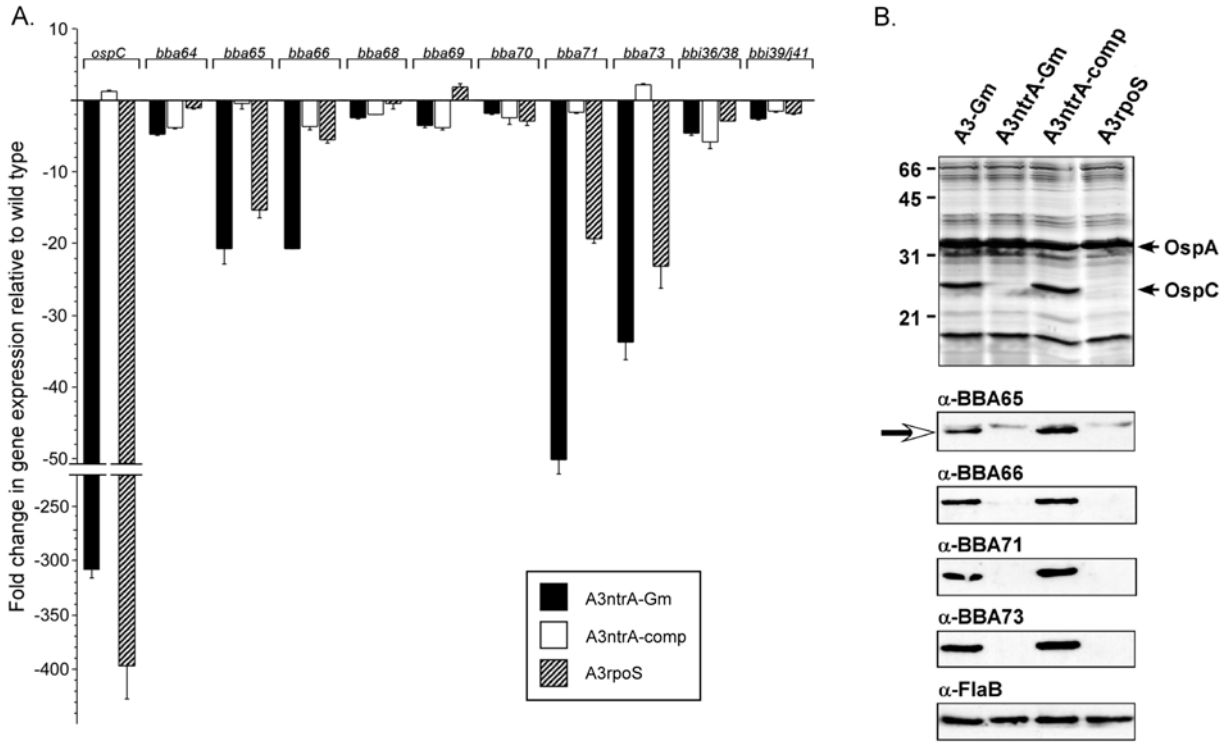


Figure 3.1.1 Analysis of transcript levels and protein synthesis of genes formally categorized in pgf 54 in *ntrA* and *rpoS* mutants.

(A) qRT-PCR was performed using RNA isolated from A3-Gm, A3ntrA-comp, A3ntrA-Gm, and A3rpoS with gene specific primers (Table 2.3.1). Average C_T values were normalized to *flaB* and the fold differences were

calculated using the comparative C_T method ($2^{-\Delta\Delta}$ C_T). *ospC* served as a control. Gene pairs with greater than 99% sequence identity cannot be discerned by this method and are therefore referred to as *bbi36/38* and *bbi39/j41*. Error bars: standard error of the mean (SEM). Data contributed by C. Nolder. (B) Total membrane fractions prepared from each isolate were separated by SDS-PAGE (7.5 μg/lane) and gels were either stained with silver (upper panel) or immunoblotted with BBA65, BBA66, BBA71, BBA73, or FlaB specific antibodies (lower panels). An open arrow points to BBA65, the lower of the two bands recognized by our polyclonal serum (see text). FlaB served as a loading control. Relative molecular masses are indicated in kiloDaltons to the left of the gel.

3.1.2 Complementation of the A3*rpoS* mutant *in trans*

In the study reported by Fisher *et al* (138) for which A3*rpoS* was created, the mutation was not complemented and an explanation for its absence was not discussed. To demonstrate that the observed phenotype of A3rpoS was due solely to the loss of *rpoS* expression, we attempted to complement the *rpoS* mutant *in trans*. *rpoS* was cloned under the control of the constitutively expressed *flaB* promoter into the pBSV2G shuttle vector (Figure 3.1.2A). The *flaB* promoter (p*flaB*) was chosen so that *rpoS* could be expressed constitutively in the *ntrA* mutant for the purpose of identifying genes that were influenced by σ^S but not by σ^N. Intended studies included qRT-PCR and immunoblotting to measure gene transcripts and protein synthesis of *bba64*, *bba65*, *bba66*, *bba71*, and *bba73*. p*flaB-rpoS* was successfully constructed in *E. coli* as demonstrated by PCR amplification (Figure 3.1.2B), restriction endonuclease digests (Figure 3.1.2C), and nucleotide sequencing (data not shown). Our attempts to introduce the pBSV2G-*rpoS*comp construct into A3*rpoS* and the readily transformable high-passage clones A and A-34, however, were unsuccessful. Though a limited number of colonies were obtained from each transformation, these either did not expand in liquid media, did not produce PCR products of the expected sizes, or did not yield PCR products at all when primers specific for the p*flaB-rpoS*

construct were used (data not shown). Where plasmid purification from *Borrelia* clones was successful, sequencing revealed nonsense or mutated sequences (data not shown). We conclude that complementation *in trans* and the over-expression of RpoS was not well tolerated in *Borrelia*. Interestingly, in a study by Gilbert *et al* (152), *rpoS* expressed under the control of an inducible *lac* promoter and recombined by knock-in into the *rpoS* chromosomal locus was tolerated by *Borrelia* and induced synthesis of a protein directly controlled by σ^S, OspC. Thus, we may in future construct an *rpoS* knock-in for the complementation of the *rpoS* mutant.

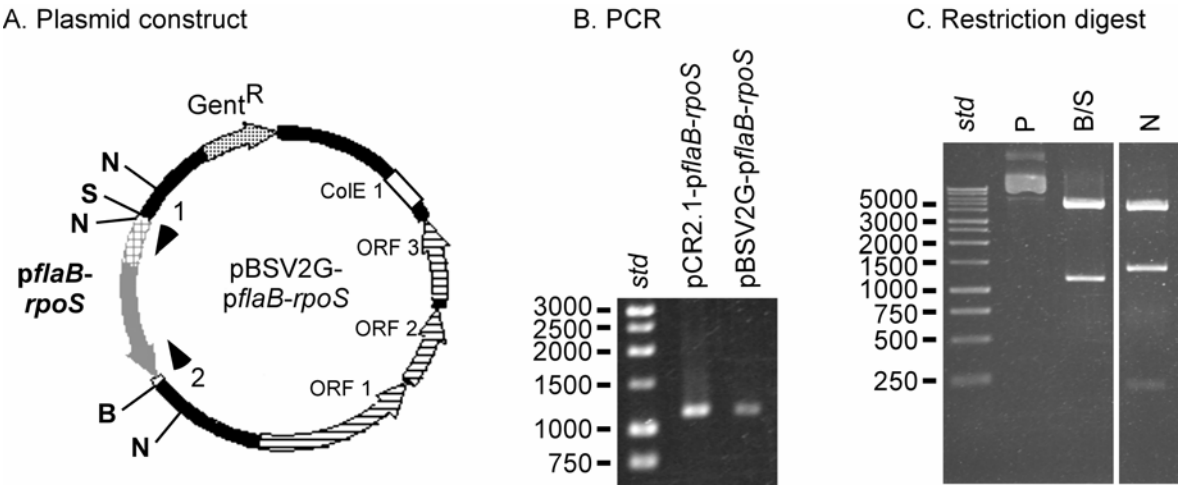


Figure 3.1.2 Cloning pBSV2G-p*flaB-rpoS*.

(A) Schematic representation of pBSV2G-p*flaB-rpoS*. Primers utilized for PCR analysis are depicted by filled arrow heads. Restriction enzymes and their recognition sites are denoted by single letters: N – NotI, B – BamHI, S – SphI; the *E. coli* origin of replication, ColE, is indicated by an open box; open reading frames (ORFs) 1 – 3 from cp9, the plasmid from which pBSV2G was derived, are shown as striped arrows. (B) PCR amplification of the p*flaB-rpoS* construct from pCR2.1-p*flaB-rpoS* and pBSV2G-p*flaB-rpoS* plasmids isolated from *E. coli* clones. Primers are noted in Table 2.3.1 and in (A) by arrowheads 1 and 2. (C) Restriction endonuclease digests of pBSV2G-p*flaB-rpoS* plasmid purified from an *E. coli* clone (P). Two-hundred nanograms of P were digested with BamHI + SphI (B/S) or NotI (N) restriction endonucleases. Expected sizes were obtained after digest: B/S – 5445 bp

and 1191 bp; N – 5020 bp, 1431 bp, and 185 bp. For (B) and (C), DNA was separated on 0.8% agarose gels, stained with ethidium bromide, and visualized under UV; standards (*std*) are shown to the left in bp.

3.1.3 Association of BBA65, BBA66, BBA71, and BBA73 production with infectious phenotypes

We have shown using qRT-PCR and immunoblotting that transcript and protein expression of BBA66 is influenced by the σ^N-σ^S regulatory cascade (Figure 3.1.1), which is also known to regulate the expression of borrelial virulence factors. Additionally, we demonstrated in a previous study that synthesis of BBA66 *in vitro* is associated with infectious phenotypes (89). Because the expression of BBA65, BBA71, and BBA73 was also influenced by the σ^N-σ^S regulatory cascade (Figure 3.1.1), we hypothesized that their expression was also linked to infectious phenotypes. To test this, membrane-associated proteins were prepared from infectious isolates and compared to non-infectious isolates (Table 2.1.1). As demonstrated in Figure 3.1.3, BBA65, BBA71, and BBA73 expression was detected in infectious isolates but not in the non-infectious clones, similar to what was reported for BBA66 (89). We noted that a second, slightly higher molecular weight band was commonly detected with our BBA65-specific antibodies, however, the presence of this weakly immunoreactive band did not correlate with expected *bba65* expression and is believed to be a cross-reactive band. OspC, previously linked to infectious phenotypes (10, 79, 89, 274, 313, 445), was also produced in infectious but not non-infectious isolates. The presence of OspA indicated that lp54, which carries *bba65*, *bba66*, *bba71*, and *bba73*, is harbored by all four isolates.

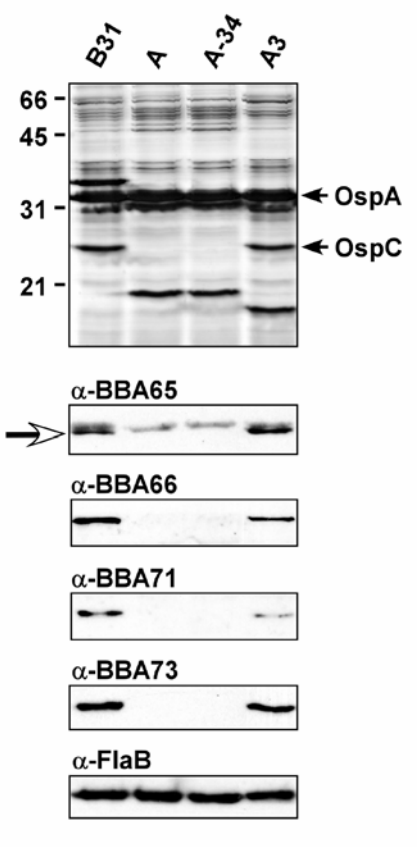


Figure 3.1.3 *In vitro* protein expression of BBA65, BBA66, BBA71, and BBA73 in association with infectious *B. burgdorferi* isolates.

Total membrane fractions were prepared from infectious isolates (B31 and A3) and non-infectious isolates (A and A-34) and 7.5 μg of protein was loaded per lane. Proteins were separated by SDS-PAGE and either stained with silver (upper panel) or immunoblotted with antibodies specific for BBA65, BBA66, BBA71, BBA73, or FlaB (lower panels). OspA and OspC are indicated to the right and molecular weight markers, in kiloDaltons, to the left of the silver-stained gel. Open arrow points to BBA65, the lower of two bands recognized by our α-BBA65 polyclonal serum (see text).

3.1.4 Triton X-114 detergent phase partitioning of BBA65, BBA66, BBA71, and BBA73

In addition to demonstrating the association of BBA65, BBA71, and BBA73 with infectious phenotypes, our immunoblot analyses of total membrane fractions in Figure 3.1.3 also indicated

that these proteins are membrane associated. To corroborate this finding, we used the non-ionic detergent Triton X-114 (TX-114) which solubilizes lipidated and integral membrane proteins (45, 49, 100) and has been used to fractionate borrelial lipoproteins (49, 408). Protein fractions from intact B31 cells treated with TX-114 were examined by immunoblotting and showed that both BBA65 and BBA71 partitioned completely into the detergent phase while 19 % of the total amount of BBA73 partitioned into the detergent phase, the remainder fractioning to the cold pellet (Figure 3.1.4 and data not shown). As expected, BBA66 separated completely into the detergent phase (53) while non-lipidated FlaB remained in the cold pellet. Moreover, the well-characterized lipoproteins OspA and OspC separated as expected into the detergent phase (Figure 3.1.4, upper panel) Oligopeptide permease (Opp) A1, which is hypothesized to be lipidated (43, 401), was used as an additional control and partitioned into both the aqueous and detergent phases. TX-114 treatment of B31 cell lysates demonstrated similar results with BBA65, BBA66, and BBA71 partitioning fully into the detergent phase and 48% of the total amount of BBA73 protein partitioning into the detergent phase (data not shown). FlaB from cell lysates partitioned solely into the insoluble cold pellet while OspA and OspC were only detected in the detergent phase (data not shown). Together, these data indicate that BBA65, BBA71, and BBA73 are lipidated or are integral membrane proteins, similar to BBA64 and BBA66 (53).

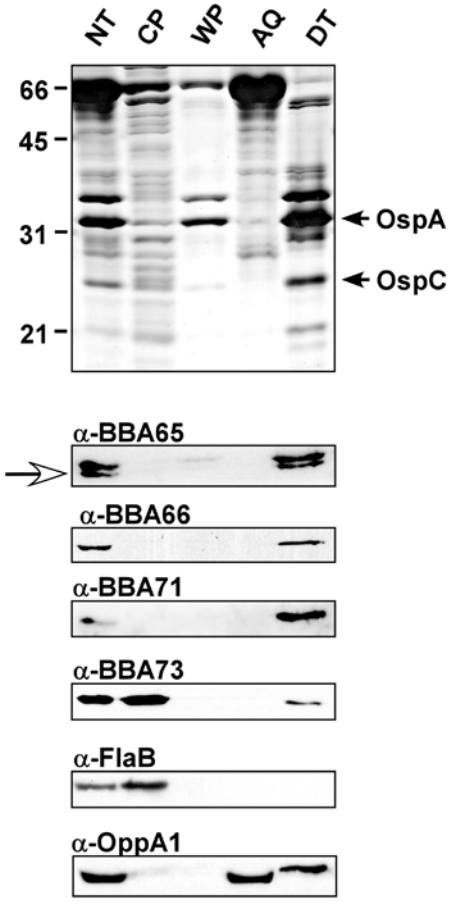
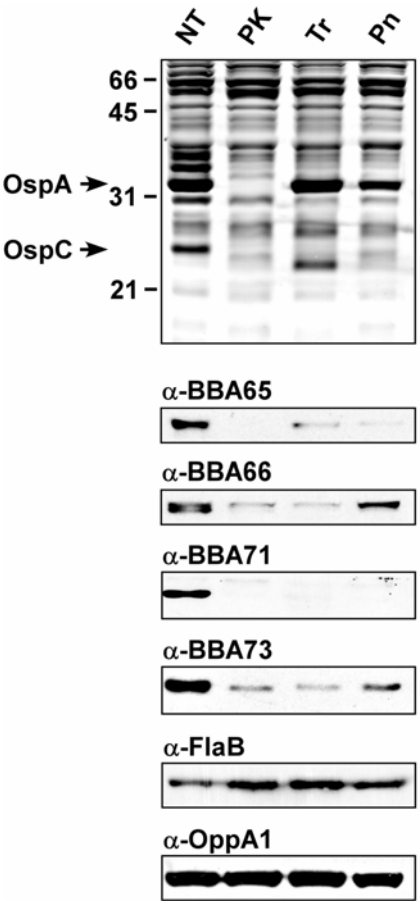


Figure 3.1.4 Membrane association of BBA65, BBA66, BBA71, and BBA73.

Intact B31 cells were treated with TX-114 and an equivalent of 1 x 10^8 cells of the no treatment (NT) control was loaded to each lane and separated by SDS-PAGE. Membranes were either stained with silver (upper panel) or immunoblotted with antibodies specific for BBA65, BBA66, BBA71, BBA73, FlaB, or OppAI (lower panels). OspA and OspC are indicated to the right and molecular weight markers, in kiloDaltons, to the left of the silver-stained gel. The open arrow points to BBA65, the lower of two bands recognized by our α-BBA65 polyclonal serum (see text). CP = insoluble cold pellet; WP = insoluble warm pellet; AQ = aqueous phase; DT = detergent phase. FlaB and OppAI served as controls and appropriately partitioned to the expected fractions.

3.1.5 Outer surface localization of BBA65, BBA66, BBA71, and BBA73

To address whether membrane-associated proteins BBA65, BBA71, and BBA73 were also localized to the outer surface, intact infectious B31 cells were treated with proteinase K (PK), trypsin (Tr), or pronase (Pn) and examined by immunoblot (Figure 3.1.5). Detectable levels of BBA65 and BBA73 were decreased by all treatments and to the greatest extent by PK and Tr, respectively. BBA71 was undetectable following treatment with all proteases utilized. Agreeing with previous data (53), detectable BBA66 was also decreased following protease treatment of intact cells. These observations were supported by densitometry analysis (Figure 3.1.5, table). Levels of known outer surface proteins OspA and OspC were also decreased as expected by protease treatment (Figure 3.1.5, upper panel); the incomplete degradation of OspA can be attributed to its interaction with the integral membrane protein P66 which has been demonstrated in the past to obstruct full protease digest of OspA (60). Cell integrity was maintained throughout the experiment as demonstrated by constant levels of periplasmically-localized FlaB and inner membrane-localized OppA1 (Figure 3.1.5). This analysis in combination with TX-114 treatments (Section 3.1.4) shows that BBA65, BBA73, and BBA71, along with BBA64 and BBA66 (53), are membrane-associated, outer surface-localized proteins.



Densitometry of protein bands (left)

	NT	PK	Tr	Pn
BBA65	1.00	-372	-5.50	-31.5
BBA66	1.00	-5.53	-10.2	-1.28
BBA71	1.00	-126	-40.0	-77.0
BBA73	1.00	-5.08	-9.36	-3.11
FlaB	1.00	1.95	2.10	1.65
OppAI	1.00	1.04	1.09	-1.21

Values normalized to no treatment (NT) sample for each protein.

Figure 3.1.5 Association of BBA65, BBA66, BBA71, and BBA73 with the borrelial outer surface.

Intact B31 cells were treated with 200 μg/ml of either proteinase K (PK), trypsin (Tr) or pronase (Pn) or received no treatment (NT) for 1 h. Proteins were separated by SDS-PAGE and gels were either stained with silver (upper panel) or immunoblotted with BBA65-, BBA66-, BBA71-, BBA73-, FlaB-, or OppAI-specific antibodies (lower panels). OspA and OspC are indicated to the right with arrows and molecular weight markers are indicated to the left in kiloDaltons (upper panel). FlaB and OppAI served as controls. Fold-change in densitometry values shown in the table to the right were determined relative to NT and with the background removed for each protein.

3.1.6 Section 3.1 summary

The data presented in this section reveal substantial evidence to suggest that BBA65, BBA66, BBA71, and BBA73 are involved in *B. burgdorferi* infection and/or pathogenicity. Each of

these genes/proteins is affected by the σ^N-σ^S regulatory cascade in a manner similar to the virulence factor OspC. The expression of these four proteins was also found to be associated with infectious phenotypes under culture conditions mimicking the mammalian host environment. Moreover, while BBA66 had been demonstrated to be a membrane-associated outer-surface protein, the localization of the remaining three proteins had not been determined. Using Western blotting to analyze total membrane fractions, fractions obtained through TX-114 phase partitioning of intact cells, and lysates from whole cells treated with a panel of proteases, we demonstrated that BBA65, BBA71, and BBA73 were also membrane associated and localized to the outer surface of *Borrelia*. Outer surface localization is a key prerequisite for interaction with the host environment and has been demonstrated for numerous lipoproteins involved in attachment, adaptation to the host environment, and immune evasion (23, 25, 53, 61, 174, 233, 234, 326, 340, 390, 491).

3.2 IDENTIFICATION OF PUTATIVE BBA64, BBA65, BBA66, BBA71, AND BBA73 ORTHOLOGS IN DIVERSE *BORRELIA* SPECIES

3.2.1 Southern blotting analysis of *B. burgdorferi sl* and relapsing fever *Borrelia* species for the presence of *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* orthologs

Previous studies (52, 89, 153, 257, 314, 361, 446, 458) and the data presented thus far suggested that *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* may be important factors for borrelial pathogenesis that are expressed by infectious *B. burgdorferi sensu stricto* (*ss*) isolates throughout murine infection. Though it has been shown that orthologs to these genes are harbored by *B. burgdorferi*,

B. afzelii, and *B. garinii* (154, 157, 158, 232, 458), it has not been demonstrated experimentally in other *sensu lato* (*sl*) species. Thus, to determine if these genes are conserved in *Borrelia* species in and beyond the *sl* group, genomic DNA from various *B. burgdorferi sl* and relapsing fever *Borrelia* species (Table 2.1.1) was analyzed for the presence of gene orthologs by Southern blotting with *B. burgdorferi* isolate B31 gene-specific probes (Figure 3.2.1). Moderate stringency wash conditions were used to increase our ability to identify putative orthologs in more distantly related species. A representative agarose gel stained with ethidium bromide shows equal loading and the expected laddering of genomic DNA following restriction endonuclease digestion (Figure 3.2.1A). When hybridized with *bba64*, *bba65*, and *bba66* gene probes (Figure 3.2.1B–D) intense bands were apparent in the *B. burgdorferi* B31 control, *B. californiensis*, *B. japonica* (HO14), *B. andersonii*, *B. valaisiana*, and *B. anserina* as indicated by the asterisks. Hybridizing bands were also evident with *bba71* and *bba73* gene probes in *B. burgdorferi*, *B. californiensis*, *B. andersonii*, and *B. valaisiana* (Figure 3.2.1E and F, asterisks). Weakly hybridizing bands detected in *B. afzelii* PGau and *B. garinii* isolate G1 with *bba64*, *bba65*, and *bba66* gene probes corresponded to the expected restriction fragment sizes of the sequenced *B. afzelii* isolate PKo (accession # CP000395) or *B. garinii* isolate PBi (accession # CP000015); the intensities of these bands, though not increased over background (Figure 3.2.1B–D, open arrows), did correlate with findings for *bba64* by Gilmore, *et al* (154). *flaB* was used as a hybridization control and demonstrated definitive hybridization to genomic DNA from species containing orthologs with 84 % sequence identity or higher to the *B. burgdorferi* isolate B31 *flaB* sequence (Figure 3.2.1G). Southern blotting results are summarized in Table 3.2.1.

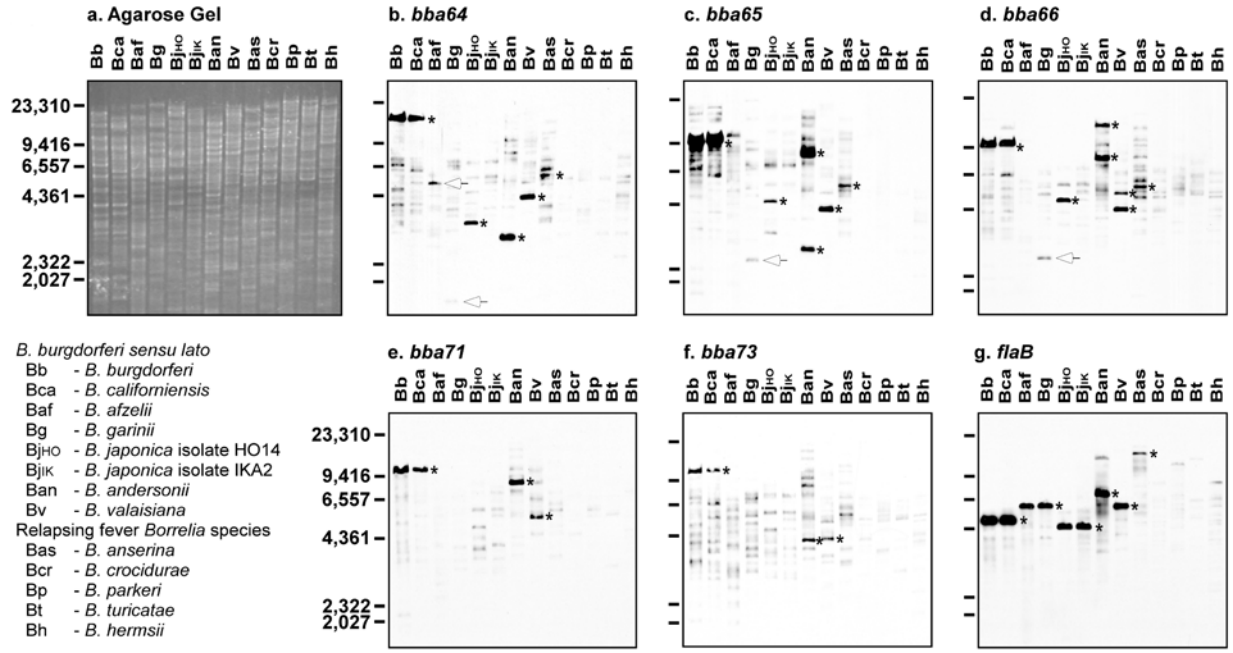


Figure 3.2.1 Southern blot analyses of *B. burgdorferi sl* and relapsing fever spirochetes for *bba64, bba65, bba66, bba71,* and *bba73* gene orthologs.

Genomic DNA prepared from various *Borrelia* species (lower left panel and Table 2.1.1) was digested with EcoRI and separated on 0.8% agarose gels (1 μg/lane). A representative ethidium bromide-stained gel is shown in panel a. In panels b – f, DNA was transferred to nylon membranes and probed with DIG-labeled, gene-specific probes based on B31 type strain sequences. Wash conditions are noted in the Materials and Methods. DNA standard values are given in base pairs to left of the leftmost panels and are indicated by hash marks to the left of the remaining panels. The asterisks denote regions of strong hybridization. Open arrows indicate weak hybridization corresponding to the predicted restriction digest DNA fragment according to the genome sequence of known gene orthologs.

3.2.2 PCR amplification from the genomic DNA of diverse *Borrelia* species using *B. burgdorferi ss*-specific *bba64, bba65, bba66, bba71,* and *bba73* PCR primers

To corroborate data from the Southern blotting analyses, we examined whether we could amplify products from diverse *Borrelia* species using *B. burgdorferi* isolate B31-specific PCR primers,

and if so, whether those PCR products were of a similar size to those produced using *B. burgdorferi sensu stricto* (*ss*) isolate B31 genomic DNA. All species examined by Southern blotting were analyzed and three pairings of primers for each gene (Table 2.3.1); this is demonstrated schematically in Figure 3.2.2A. Products of a relatively similar size were obtained using each of the three *bba64* primers sets from *B. burgdorferi*, *B. californiensis*, *B. afzelii*, *B. garinii*, *B. japonica* isolate HO14, *B. andersonii*, and *B. valaisiana* (Figure 3.2.2B). Excluding *B. californiensis*, PCR product sizes were slightly larger for each of these species using both primer sets 1 and 2. This finding was anticipated for *B. afzelii* and *B. garinii* based on sequence data of known *bba64* orthologs harbored by these species. PCR products of a similar size to *B. burgdorferi* B31 were obtained from *B. parkeri*, *B. turicatae*, and *B. hermsii* using both primer sets 2 and 3, in addition to *B. anserina* with primer set 1, *B. crocidurae* with primer set 2, and *B. japonica* isolate IKA2 with primer set 3.

Using primers specific for *bba65*, *B. burgdorferi* B31, *B. californiensis* and *B. afzelii* yielded definitive PCR products with all three primer sets (Figure 3.2.2C). *B. japonica* isolate HO14, *B. andersonii*, and *B. valaisiana* yielded definitive products with primer sets 1 and 3 while faint products were detected for *B. japonica* and *B. valaisiana* with primer set 2. All three *bba66* primers yielded products for *B. burgdorferi* B31, and *B. californiensis*; additional definitive PCR products were produced only from *B. japonica* isolate HO14 and *B. andersonii* using primer set 2 (Figure 3.2.2D). For *bba71*, definitive PCR products were obtained from *B. burgdorferi ss* and *B. californiensis* using all three primer sets, from *B. andersonii* using primer sets 1 and 2, and from *B. crocidurae* and *B. parkeri* using primer set 1 (Figure 3.2.2E). Faint products relative to *B. burgdorferi* B31 were obtained from the following reactions: *B. afzelii* with all three *bba71*-specific primer sets; *B. garinii*, *B. japonica* isolate HO14, *B. valaisiana*, and

B. hermsii using primer set 1; *B. garinii*, *B. japonica* isolate IKA2, and *B. hermsii* with primer set 2; *B. andersonii*, *B. crocidurae*, and *B. parkeri* using primer set 3. Lastly, definitive PCR products were obtained with all three *bba73*-specific primer sets from *B. burgdorferi* B31, *B. californiensis*, and *B. andersonii*, though the product using primer set 2 with *B. andersonii* was less intense than those obtained from the other two species (Figure 3.2.2F). Additional PCR products were detected from *B. afzelii*, *B. garinii*, both *B. japonica* isolates, *B. anserina*, *B. crocidurae*, and *B. hermsii* using primer set 3 and faint products were detected from *B. afzelii* and *B. garinii* using primer set 2. PCR results are summarized in Table 3.2.1.

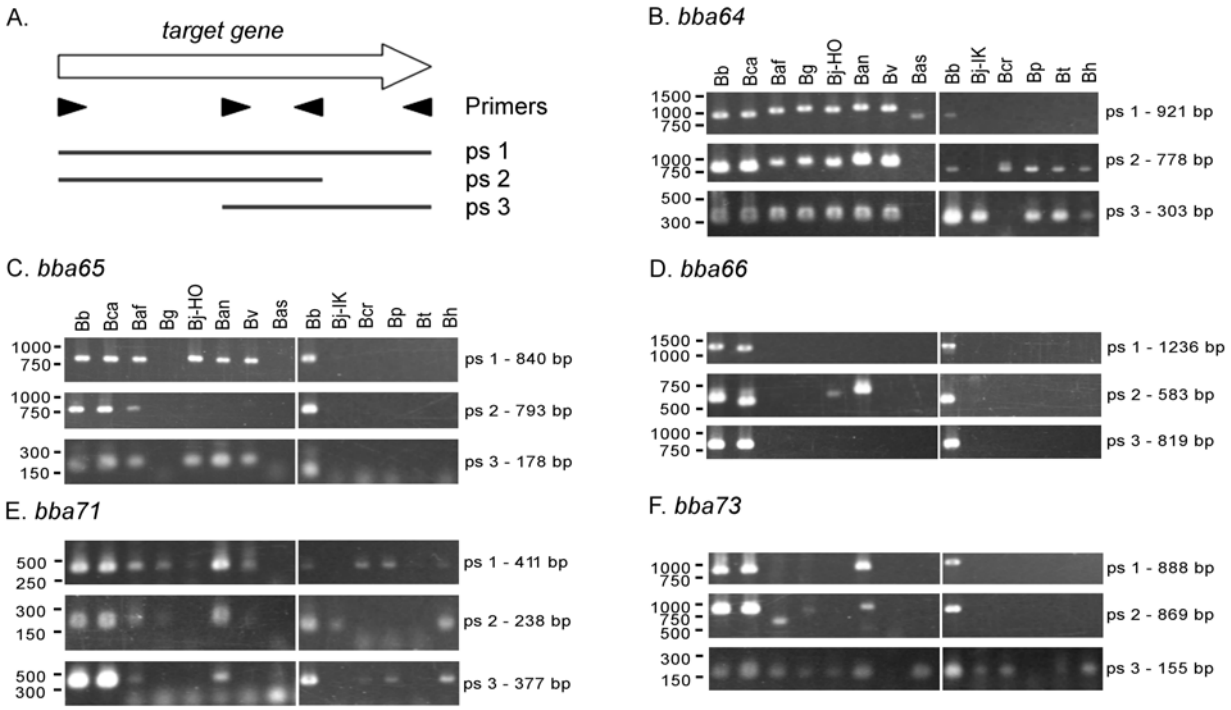


Figure 3.2.2 PCR analysis of *B. burgdorferi sl* and relapsing fever *Borrelia* with *B. burgdorferi sensu stricto*-specific primers for *bba64, bba65, bba66, bba71,* and *bba73*.

(A) A schematic representation of the primer sets (ps) used for each target gene. (B – F) Products amplified from genomic DNA by PCR were separated on agarose gels, stained with ethidium bromide, and photographed under UV light. Standards are shown in basepairs to the left of each panel. The ps used and the

expected size from *B. burgdorferi* B31 genomic DNA (Bb) is indicated to the right of each agarose gel. ps 1: both full-length (FL) primers specific for the target gene; ps 2: the forward FL primer and reverse, reverse transcription (rt) primer specific for the target gene; ps 3: the forward rt primer and reverse FL primer specific for the target gene. Species abbreviations shown above panels (B) and (C) are defined in the lower left panel of Figure 3.2.1.

3.2.3 Immunoblot analysis of *B. burgdorferi sl* and relapsing fever *Borrelia* species for the presence of α-BBA64, -BBA65, -BBA66, -BBA71, and -BBA73 antibody cross-reactive proteins

Immunoblotting was utilized to assess the cross-reactivity of BBA64, BBA65, BBA66, BBA71, and BBA73 *B. burgdorferi*-specific antibodies with seven of the *Borrelia* species that either demonstrated hybridization with our gene probes by Southern blotting (Figure 3.2.1) or were known to harbor target gene orthologs. *B. japonica* isolate HO14 was not able to survive in the current BSK-H lot available to us (lot # 057K8413) and was thus eliminated from this analysis. Total membrane fractions were prepared from each of the remaining seven species and from the high-passage *B. burgdorferi*-derived clone A which, due to repeated *in vitro* propagation, does not express the proteins under examination (discussed in Section 4.2). Total membrane fractions were examined for the presence of cross-reactive proteins recognized by α-BBA64, -BBA65, -BBA66, -BBA71, and -BBA73 antibodies. BBA66, BBA71, and BBA73 were detected in *B. burgdorferi* B31 total membranes (Figure 3.2.3, lanes 1 and 2) while none of these were detected in clone A total membranes (Figure 3.2.3, lanes 3 and 4). Cross-reactivity was observed for *B. californiensis* with BBA66 and BBA73 antibodies, for *B. afzelii* with BBA66 and BBA71 antibodies, and for *B. garinii* with BBA71 antibodies (Figure 3.2.3, lower panels). BBA64 and

BBA65 protein synthesis was not detected in any of the species examined (data not shown). Given that *B. burgdorferi* protein expression has been demonstrated to be BSK-H lot-dependent (13, 89), it is likely that we did not detect BBA64 protein synthesis due to the specific lot of BSK-H used for these studies. All species examined, including clone A, were cross-reactive with a polyclonal rabbit antibody specific for the inner membrane-localized oligopeptide permease protein, OppAI; OppAI may be well conserved due to its role in peptide transport and *B. burgdorferi*'s inability to synthesize amino acids *de novo* (140, 260). These results are summarized in Table 3.2.1.

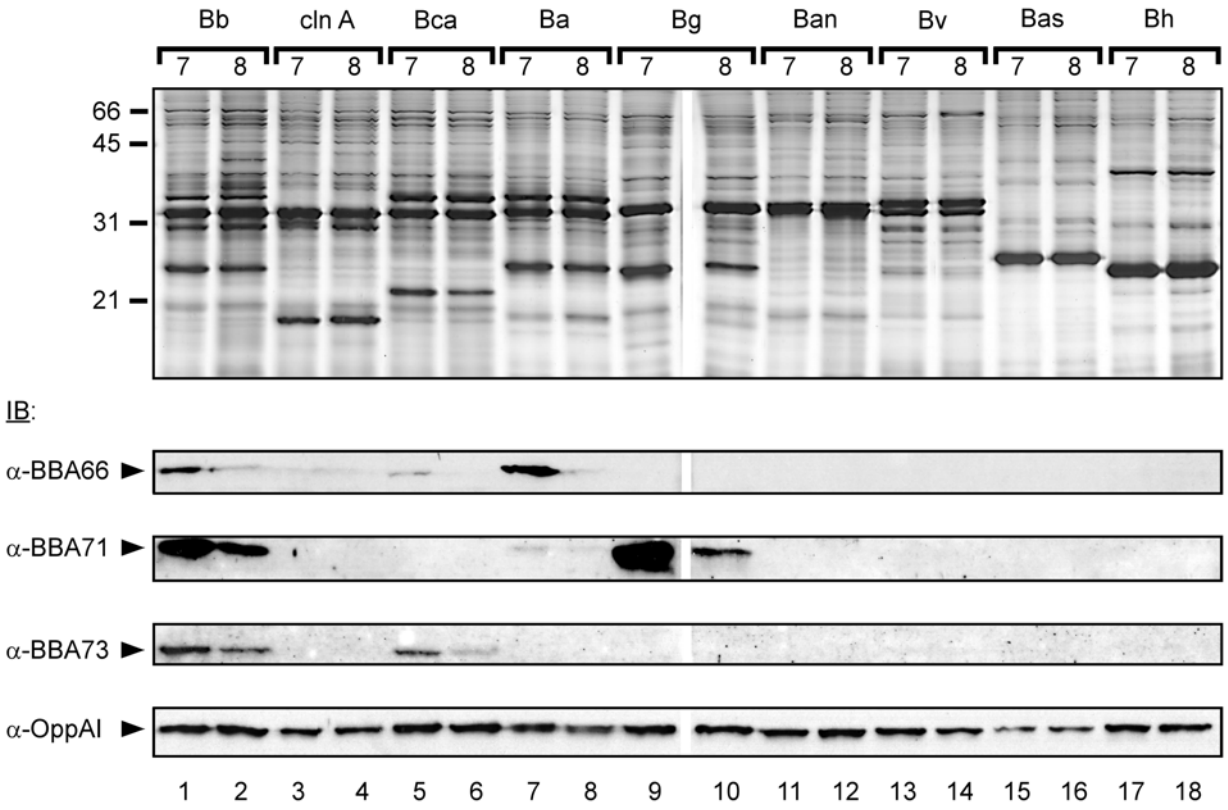


Figure 3.2.3 BBA66, BBA71, and BBA73 antibody cross-reactivity with total cell membranes from diverse *Borrelia* species.

Borrelia were grown at pH 7.0 or pH 8.0 and total cell membranes were prepared as described in Materials and Methods. Five micrograms of protein was separated per lane by SDS-PAGE and either stained with silver

(upper panel) or transferred for immunoblotting (IB) with α-BBA64, -BBA65, -BBA66, -BBA71, -BBA73, or -OppAI specific antibodies (indicated to the left of each lower panel). α-BBA64 and BBA65 immunoblots are not shown (see text). Molecular weight standards are indicated in kiloDaltons to the left of the upper panel. Species abbreviations and a summary of findings are listed in Figure 3.2.1. OppAI served as a loading control.

3.2.4 pH regulation of α-BBA64, BBA65, BBA66, BBA71, and BBA73 antibody cross-reactive proteins in diverse *Borrelia* species

pH has been identified as a key environmental cue in the regulation of numerous outer-surface lipoproteins expressed by *B. burgdorferi* (67, 77, 78, 89, 346, 361), including BBA65, BBA66, BBA71, and BBA73 (77, 78, 89, 346, 361). pH regulation of protein synthesis in species beyond *B. burgdorferi ss* has not been addressed; however, given that all known *Borrelia* species share a similar arthropod-mammalian transmission cycle and must sense changes in the environment in order to adapt and survive in each of these distinct environments, we hypothesized that cues pH would be utilized by *Borrelia* species beyond *B. burgdorferi ss*. To address whether the synthesis of cross-reactive proteins detected with B31-specific antibodies was responsive to changes in environmental pH, total membrane fractions were prepared from the seven species cultivatable in BSK-H (discussed in Section 3.2.2) after growth in HEPES buffered pH 7.0 or pH 8.0 BSK-H media. All cross-reactive proteins detected with anti-BBA66, -BBA71, and -BBA73 antibodies (Figure 3.2.3) were synthesized at greater levels under pH 7.0 relative to pH 8.0 *in vitro* growth conditions. Our findings correlated with expression patterns described for these outer-surface lipoproteins in *B. burgdorferi* B31 (77, 78, 89, 346, 361). Cross-reactive OppAI protein levels were not detectably different in any of the species at either of the pH conditions

tested (Figure 3.2.3, bottom panel) suggesting that these cross-reactive proteins are not affected by changes in pH, similar to what has been described for OppAI in *B. burgdorferi* B31 (463). These results are summarized in Table 3.2.1.

3.2.5 Section 3.2 summary

Using Southern blotting that targeted diverse *Borrelia* species with B31 sequence-specific whole-gene probes, we demonstrated that putative *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* gene orthologs are present in both Lyme-associated and relapsing fever-associated *Borrelia* species. To substantiate these findings and to compensate for the possibility that gene orthologs in some species may have been too fragmented by EcoRI digestion to detect by Southern blotting, we used PCR to amplify putative orthologs using B31 gene-specific primers (Figure 3.2.2). In addition to amplifying products from species that orthologs were detected in by Southern blotting, we also amplified products from additional *B. burgdorferi sl* and relapsing fever *Borrelia* (Figure 3.2.2). Finally, immunoblotting demonstrated that α-BBA66, -BBA71, and -BBA73 cross-reactivity was limited to pH-responsive proteins expressed by *B. burgdorferi sl* species known to infect and cause disease in humans. The cumulative data from these studies are summarized in Table 3.2.1.

Table 3.2.1 Summary of Southern blotting, PCR, and antibody cross-reactivity analyses

	bba64				*bba65*				*bba66*				*bba71*				*bba73*			
		PCR				PCR				PCR				PCR				PCR		
spp	SB	ps 1	ps 2	ps 3	pH	SB	ps 1	ps 2	ps 3	pH	SB	ps 1	ps 2	ps 3	pH	SB	ps 1	ps 2	ps 3	pH	SB	ps 1	ps 2	ps 3	pH
Bb	+	+	+	+	nd	+	+	+	nd	nd	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bca	+	+	+	+	nd	+	+	+	+	nd	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-
Ba	+/-	+	+	+	nd	-	+	+	+	nd	-	-	-	+	-	-	+	+	+	-	-	+	+	+	+
Bg	+/-	+	+	+	nd	+/-	-	-	-	nd	+/-	-	-	-	-	-	+	+	+	+	-	-	+	+	+
Bl_{MO}	+	+	+	+	nd	+	+	-	+	nd	+	-	+	-	-	-	+	-	-	+	-	-	-	-	+
Bl_{IK}	-	-	-	+	nd	-	-	-	-	nd	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
Ban	+	+	+	+	nd	+	+	-	+	nd	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-
Bv	+	+	+	+	nd	+	+	-	+	nd	+	-	-	-	-	+	+	+	+	-	+	-	-	-	-
Bas	+	+	-	-	nd	+	-	-	-	nd	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-
Bcr	-	-	+	-	nd	-	-	-	-	nd	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
Bp	-	-	+	+	nd	-	-	-	-	nd	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
Bt	-	-	+	+	nd	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bh	-	-	+	+	nd	-	-	-	-	nd	-	-	-	-	-	-	+	+	-	-	-	-	+	+	-

Abbreviations: spp, species; SB, Southern blotting (Figure 3.2.1); ps, primer set (Figure 3.2.2); pH, pH-responsive & antibody cross-reactive proteins detected (Figure 3.2.3); nd, not detected.
Spp. abbreviations listed in leftmost column are defined in Figure 3.2.1.
Scoring: +, detected; +/-, not definitive; - not detected.

3.3 BBA66 KNOCKOUT MUTAGENESIS AND THE EFFECT UPON MURINE INFECTION AND PATHOGENICITY

To determine whether BBA66 is required for murine infectivity and/or pathogenesis during the early stages of disease, a *bba66* deletion mutant was constructed in the infectious isolate, A3. We assessed gross joint swelling, seroreactivity, and spirochete burden in select tissues of C3H/HeJ mice infected with either wildtype, *bba66* mutant, or *bba66* complemented mutant *Borrelia*. Genetic characteristics of inbred mouse strains have been demonstrated to play an important role in the severity of arthritis that develops during infection with *B. burgdorferi* (469). During *B. burgdorferi* infection, C3H/HeJ mice demonstrate acute arthritis and swelling that is more severe than in other murine models (27, 30, 267, 483).

3.3.1 Construction and *in vitro* characterization of *bba66* mutant and complemented clones

The suicide plasmid pBAD-*bba66*::Kan and complementing plasmid pBSV2G-*bba66*comp were constructed as described in Sections 2.3.2 and 2.3.3, respectively, and are depicted schematically in Figure 3.3.1. PCR (Figure 3.3.1D and E), restriction endonuclease digest (data not shown), and sequence analyses (data not shown) were used to verify plasmid constructs. Mutation of *bba66* in the infectious isolate B31 clone A3 was accomplished by allelic exchange using pBAD-*bba66*::Kan whereby 757 base pairs of the structural gene were replaced with a kanamycin resistance cassette under the control of the constitutive borrelial *flgB* promoter (Figure 3.3.1). The *bba66* mutant, A3-*bba66*::Kan, was complemented *in trans* with pBSV2G-*bba66*comp (Figure 3.3.1) yielding A3-*bba66*comp. Genomic DNA was isolated from A3, A3-bba66::Kan,

and A3-bba66comp and subjected to PCR amplification of the *bba66* coding region using primers specific for the 5' and 3' ends of the target gene (Table 2.3.2). The plasmid constructs pBAD-*bba66*::Kan and pBSV2G-*bba66*comp were used as controls. The expected 1,266 bp full-length *bba66* product was amplified from pBSV2G-*bba66*comp and from WT and A3-*bba66*comp genomic DNA (Figure 3.3.1D). A 1,709 bp product resulting from the insertion of the kanamycin resistance cassette into *bba66* was amplified from pBAD-*bba66*::Kan and from A3-*bba66*::Kan and A3-*bba66*comp genomic DNA (Figure 3.3.1D). PCR amplification from the kanamycin-resistance gene to either *bba65* or *bba68* was used for confirmation of homologous recombination into lp54 (Figure 3.3.1E).

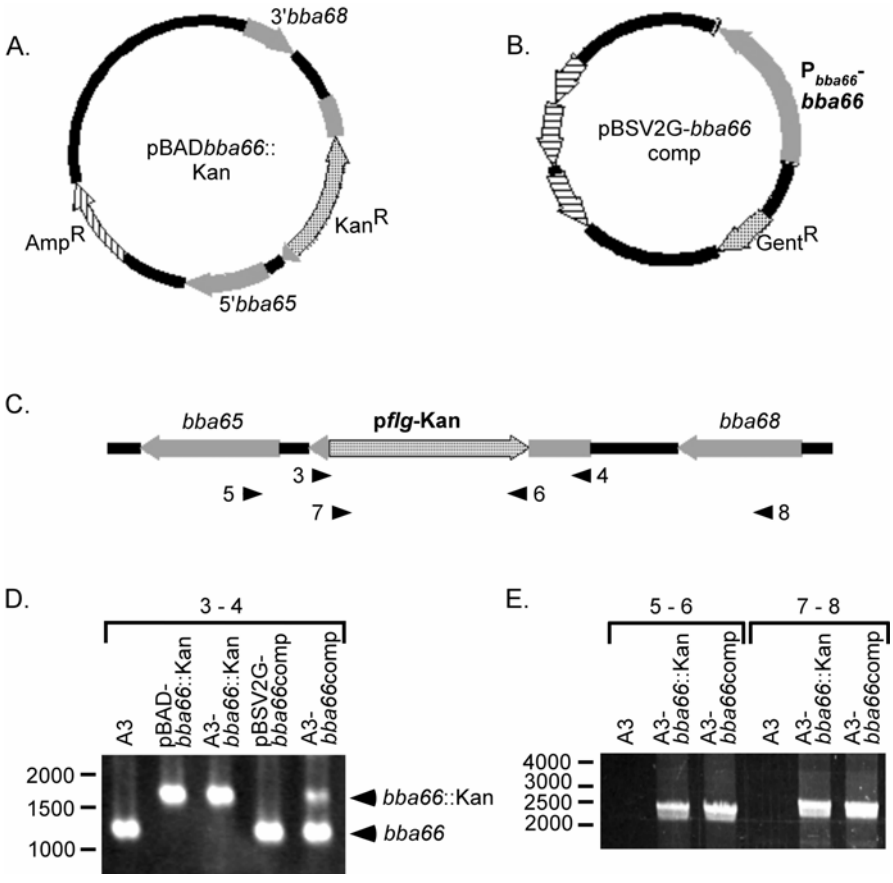


Figure 3.3.1 Plasmid constructs and characterization of *Borrelia* clones by PCR.

(A – B) Schematic representations of the plasmid constructs used for knockout mutagenesis of the *bba66* locus, pBAD-*bba66*::Kan, (A) and complementation of the *bba66* mutation *in trans*, pBSV2G-*bba66*comp (B). (C)

Schematic of the *bba66* locus on lp54 after homologous recombination with pBAD-*bba66*::Kan. (D – E) Assessment of *bba66* mutagenesis and complementation by PCR amplification. *bba66* (D) or *bba65*-kan and kan-*bba68* (E) were amplified by PCR from either the A3 parent, pBAD-*bba66*::Kan, A3-*bba66*::Kan, pBSV2G-*bba66*comp, or A3-*bba66*comp. Products were separated on agarose gels, stained with ethidium bromide, and DNA was visualized under UV light. The primers used for PCR are indicated in (C) and listed in Table 2.3.1; primer pairings are noted above each agarose gel. Size standards are shown to the left in basepairs and the positions of *bba66* and *bba66*::Kan PCR products are indicated to the right with arrow heads (D).

A subset of the 21 plasmids harbored by *B. burgdorferi*, including lp25 and lp28-1, is required for murine infectivity (167, 240, 241, 342, 343). *B. burgdorferi*, however, is known to lose plasmids with long-term passage in culture or through transformation and subsequent plating in semi-solid BSK medium (159, 306, 388, 441). Thus, to ensure A3-*bba66*::Kan and A3-*bba66*comp isolates harbored these plasmids, PCR amplification of plasmid-specific genes from genomic DNA was performed. Fifteen of the eighteen A3-*bba66*::Kan clones and eight of the ten A3-*bba66*comp clones examined were found to harbor both lp25 and lp28-1 (data not shown). Of these, at least one isolate from each group was found to harbor the full complement of plasmids with the exception of cp9 (Figure 3.3.2), which is also lacking in the parental clone, A3, and is dispensable for murine infection (168, 241, 343).

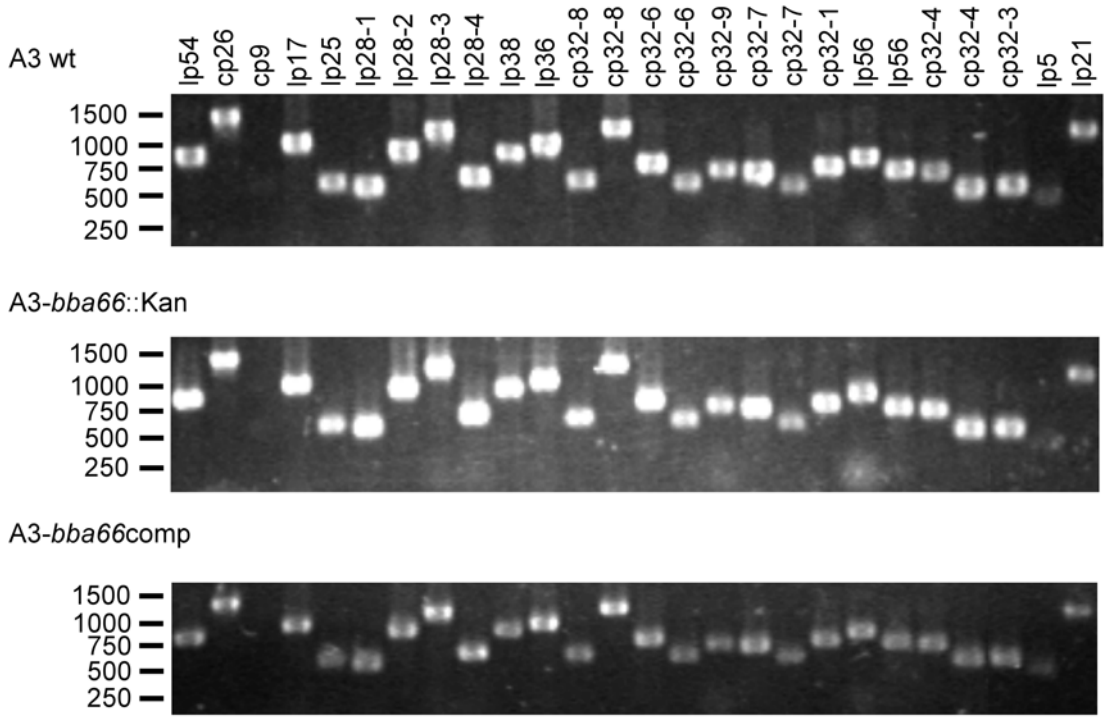


Figure 3.3.2 Plasmid profiles of A3, A3-*bba66*::Kan and A3-*bba66*comp isolates used in murine infection studies.

At least one target gene localized to each of the 21 borrelial plasmids was amplified by PCR using the primer set of Elias, *et al* (123). Products were separated on agarose gels, stained with ethidium bromide, and DNA was visualized under UV light. The isolate examined is noted to the upper left of each gel and size standards are noted to the left in basepairs. The plasmid targeted in each PCR reaction is noted above the uppermost gel.

To demonstrate that the complementing plasmid was maintained *in trans* in the A3-*bba66*comp clone chosen for these studies, plasmid rescue was performed to re-isolate pBSV2G-*bba66*comp from the isolate with a full plasmid complement (Figure 3.3.2). Genomic DNA purified from A3-*bba66*comp was transformed into chemically competent TOP10 *E. coli* and clones were isolated under gentamycin selection. Colonies were too numerous to count on a plate containing 50 λ of a 1:7 dilution of the transformed *E. coli* while 296 colonies were obtained on a second plate containing 50 λ of a 1:70 dilution. Plasmid DNA was purified from

four *E. coli* clones on the 1:70 dilution plate and used for PCR amplification of the 1,444 bp full-length construct (*bba66* and its native promoter) using primers proA66.1 and BBA66 FL.6 (Table 2.3.2). Each of the *E. coli* clones examined yielded PCR products of the expected size that matched the product amplified from the plasmid control (Figure 3.3.3).

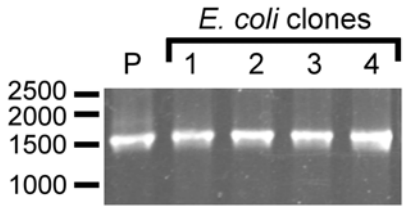


Figure 3.3.3 Plasmid rescue of pBSV2G-*bba66*comp from A3-*bba66*comp.

Plasmid DNA from four *E. coli* clones obtained by transformation of chemically competent TOP10 *E. coli* with A3-*bba66*comp genomic DNA was used for PCR amplification of the *bba66*comp construct using specific primers (see text and Table 2.3.1). Products were separated on an agarose gel and DNA was visualized by staining with ethidium bromide and exposure to UV light. P = plasmid control, pBSV2G-*bba66*comp. Size standards are noted to the left in base pairs.

To determine whether any growth defects arose as a consequence of our mutagenesis strategy, all strains were grown to mid-log phase, diluted to 5×10^6 cells / ml, and counted at various times up to 100 hours. As shown in Figure 3.3.4, all strains grew at similar rates and reached stationary phase at similar times in culture, indicating no loss of fitness *in vitro* as a result of our manipulations. Additionally, we examined whether each strain was able to alter protein expression in response to changes in culture pH and temperature. Total cell lysates from cultures shifted to pH 7.0/35 °C or pH 8.0/23 °C and grown to mid-log phase were analyzed by immunoblotting and demonstrated that BBA66 was expressed as expected by the WT and A3-*bba66*comp isolates (Figure 3.3.5). We noted that BBA66 was detectable at levels that were visibly slightly greater in A3-*bba66*comp relative to A3, a phenomenon that is likely do to

plasmid copy number (445) and has been demonstrated before when using pBSV2G to complement a mutation *in trans* (445). Importantly, BBA66 was not detected in either of the A3-*bba66*::Kan cultures. OspC was differentially regulated in all strains demonstrating that there were no defects in the ability of these isolates to differentially express proteins in response to changing pH (Figure 3.3.5, upper panel). OspA, which is constitutively expressed in culture, served as a loading control.

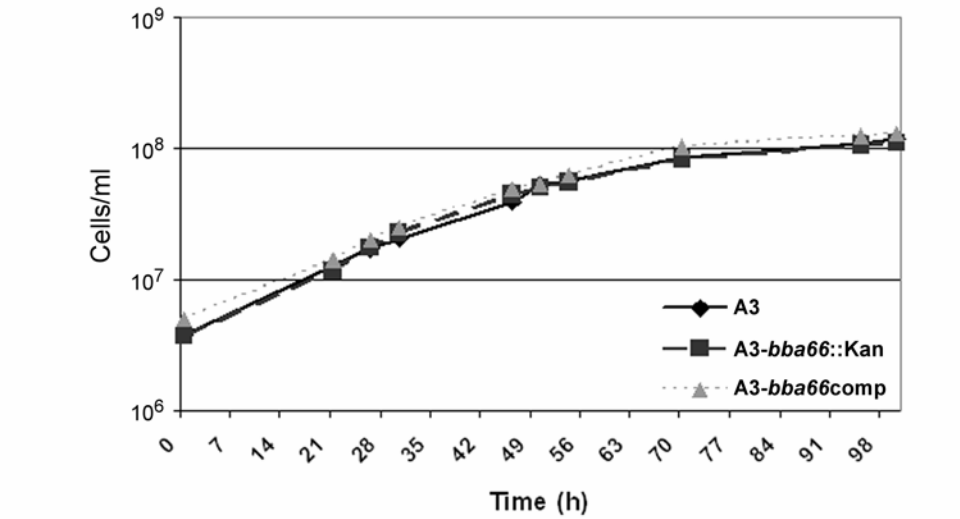


Figure 3.3.4 Growth kinetics of A3, A3-*bba66*::Kan, and A3-*bba66*comp.

A3, A3-*bba66*::Kan, and A3-*bba66*comp were grown to mid-log phase in BSK-H (lot # 104K8411), diluted to 5 x 10^6 cells/ml, then counted at various time points over the course of 100 h post-dilution. Culture densities are reported in cells/ml on a log scale.

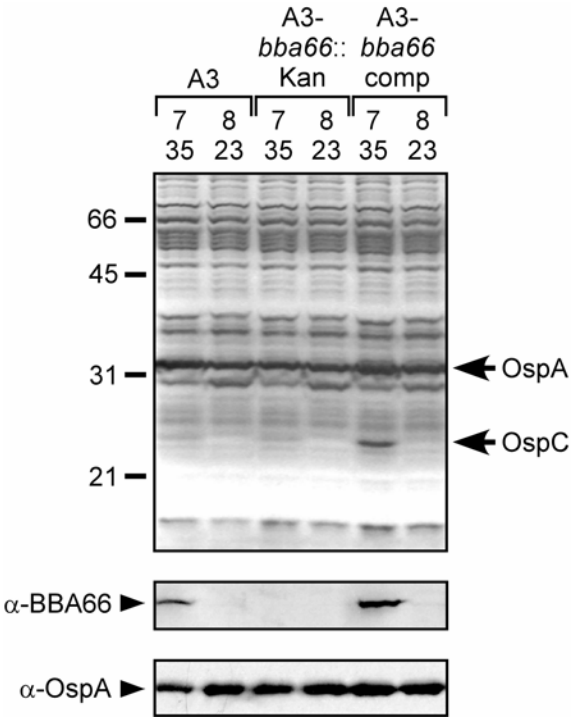


Figure 3.3.5 pH and temperature shift of A3, A3-*bba66*::Kan, and A3-*bba66*comp to assess protein expression of BBA66.

All *Borrelia* were grown to mid-log phase in BSK-H (lot # 045K8411) then diluted into pH 7.0 or pH 8.0 media and grown to mid-log phase at either 35 °C or 23 °C, respectively. Whole cell lysate proteins were separated by SDS-PAGE and either stained with silver (upper panel) or transferred for immunoblot with α-BBA66 or -OspA antibodies (lower panels). Molecular weight markers are shown to the left and OspA and OspC are indicated with arrows to the right of the silver-stained gel. OspA was used as a loading control.

3.3.2 Infectivity of *bba66* mutant and complemented clones in C3H/HeJ mice

To determine if BBA66 was required for infection and/or pathogenicity in mice, 8 week-old female C3H/HeJ mice were inoculated with a low-dose (1×10^4) of spirochetes, either A3, A3-*bba66*::Kan, or A3-*bba66*comp, and observed for 35 days. A low dose of spirochetes of 1×10^3 has been shown to induce serological responses in mice that closely mimic the serology of either laboratory mice infected by tick bite or infected *P. leucopus* from nature (308). A

schematic of the experimental design is shown in Figure 3.3.6. As assessed by ELISA using *B. burgdorferi* cell lysate as the target antigen, all mice became seropositive for anti-borrelial antibodies except for a single A3-*bba66*comp-infeted mouse (Table 3.3.1 and data not shown). In ELISAs using MalE-BBA66TR as the target antigen, serum from all seropositive A3-*bba66*comp-infected mice demonstrated significantly greater levels of α-BBA66 reactivity compared to A3-infected mouse serum (Figure 3.3.7) which correlates to the increased synthesis of BBA66 *in vitro* by A3-*bba66*comp (Figure 3.3.5); the A3-*bba66*::Kan-infected mice demonstrated α-BBA66 seroreactivity at levels significantly decreased relative to A3. During the early stages of infection, C3H mice develop severe swelling of rear tibiotarsal joints; using this as a gross analysis of disease progression, we measured changes in joint diameter relative to the day of infection. Throughout the five week infection period, all seroreactive mice demonstrated joint swelling with peak measurements occurring near day 21 p.i. (data not shown). Furthermore, all mice that were seroreactive toward borrelial cell lysate were also positive for outgrowth of *Borrelia* from tissue (Table 3.3.1). Spirochetes were recovered from the bladders of all infected mice except a single A3-infected mouse, from all hearts except one from a A3-*bba66*::Kan-infected mouse, from 71 % of joints, and from less than half of all ear tissues. No significant differences between isolates and outgrowth from specific tissues were observed.

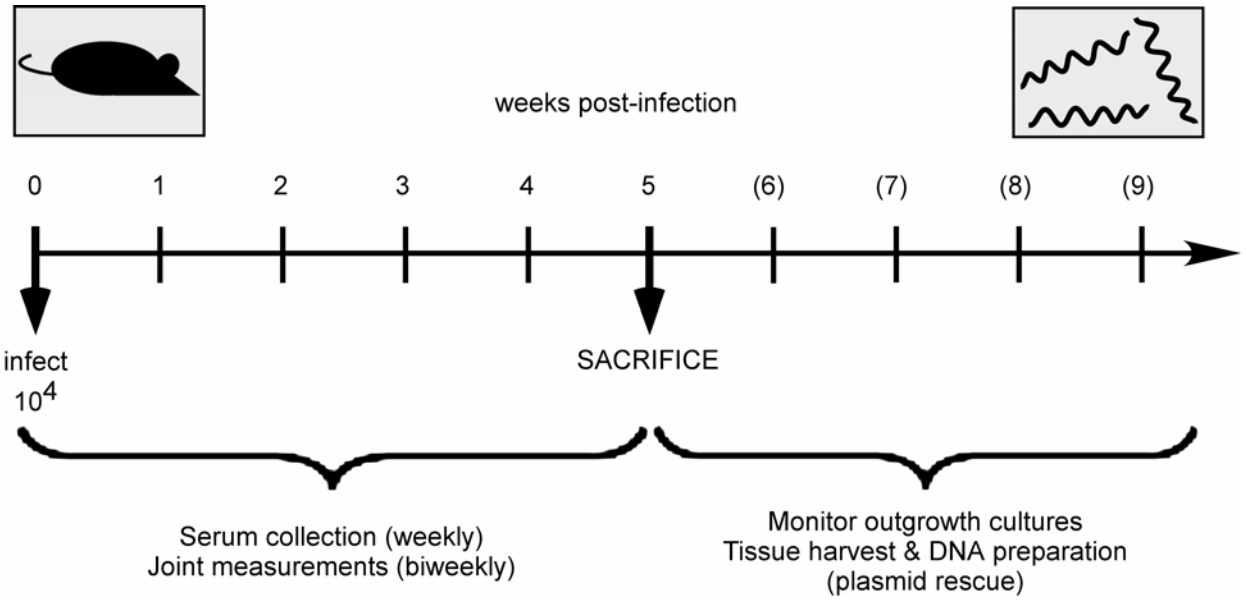


Figure 3.3.6 Schematic of murine infection study.

C3H/HeJ mice infected with 10^4 spirochetes were monitored for five weeks until they were sacrificed at day 35 post-infection; the weeks post-sacrifice are shown in parentheses. Serum and rear-tibiotarsal joint measurements were collected as noted above. Select tissues were harvested (see text) and either inoculated into media for outgrowth or used to prepare genomic DNA to assess for tissue spirochete load by qPCR or for the presence of the complementing plasmid by plasmid rescue.

Table 3.3.1 Outgrowth and seroreactivity of infected mice.

	Heart	Bladder	Joint	Ear	No infected/ No. inoculated	No. positive by serology/ No. inoculated
A3	8/8	7/8	5/8*	4/8	8/8	8/8
A3-*bba66*::Kan	7/8	8/8	6/8	3/8	8/8	8/8
A3-*bba66*comp	7/8	7/8	6/8	3/8	7/8	7/8

* 3 cultures were contaminated
Data contributed by C. Nolder, D. Clifton, and J. Hughes

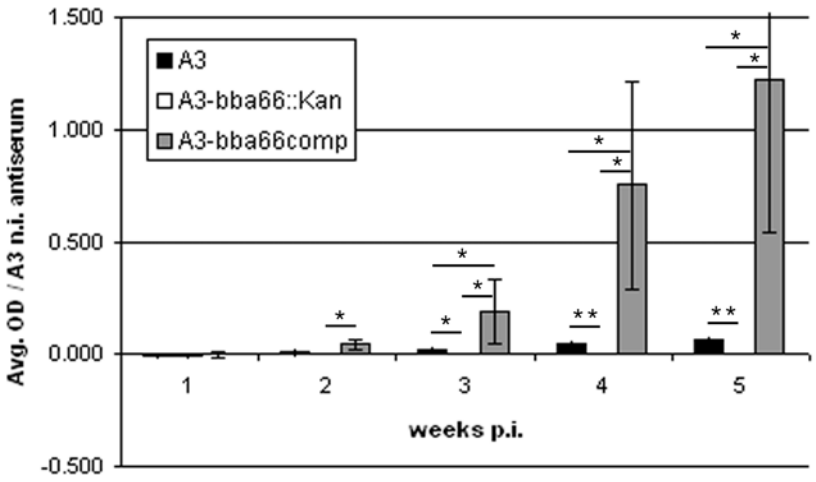


Figure 3.3.7 Assessment of α-BBA66TR reactivity of murine sera by ELISA.

Sera collected on days 7, 14, 21, 28, and 35 post-infection from all groups of mice were diluted 1:100 and tested for reactivity with purified MalE-BBA66TR by ELISA. Values shown are the averages from each day for individual groups of mice with background (week 5 serum with MBP) removed relative to a positive control serum (pooled A3 needle inoculated mouse serum provided by Dr. P. Rosa). Error bars represent the standard deviation. The Student t-test was performed and P values are indicated as follows: * < 0.05; ** < 0.0001. Data contributed by D. Clifton.

3.3.3 Reisolation of pBSV2G-*bba66*comp following murine infection with A3-*bba66*comp

To test the stability of the *bba66* complementing plasmid during murine infection, genomic DNA was isolated from *Borrelia* grown from A3-*bba66*comp-infected mouse tissues (3 bladders, 1 heart, and 1 joint) and 100 ng of DNA was transformed into chemically competent TOP10 *E. coli* that were grown under gentamycin selection. Genomic DNA from A3-*bba66*-::Kan-infected mouse tissues was used as a control (2 bladders, 1 joint). Gentamycin-resistant colonies were obtained from all A3-*bba66*comp transformed *E. coli* while none were recovered from A3-*bba66*::Kan transformed *E. coli* (data not shown). These findings corroborated the MalE-BBA66TR ELISA results which showed reactivity of A3-*bba66*comp-infected mouse sera,

but little to none with A3-*bba66*::Kan-infected serum samples (Figure 3.3.7). These data were contributed by D. Clifton.

3.3.4 Evaluation of the BBA66 amino acid sequence

An association between heart tropism and BBA66 has been described previously in two reports. In one report, Wallich and colleagues found that *bba66* transcripts were consistently upregulated in all SCID mouse heart tissues examined between days 30 and 90 p.i. (458); in joint tissues, however, the expression of *bba66* transcripts was variable and was not detected in all mice. In a second report, Antonara *et al* demonstrated that phage display library clones expressing an N-terminal region of BBA66 (personal communication, Dr. Jennifer Coburn and Figure 4.3.1) were able to bind to murine heart tissue (12). Analysis of the amino acid sequence harbored by these clones revealed a PEST-like sequence that is present in the N-terminal half of BBA66 (Figure 4.3.1). PEST sequences are regions of amino acids that are rich in proline (P), glutamic acid (E), serine (S), and threonine (T) that have been shown to be present in proteins with short half-lives, i.e., two hours or less in eukaryotes (367); however, these sequences may also be involved in protein phosphorylation (354) and/or protein-protein interactions (111). A PEST-like domain harbored by listeriolysin O is required for pathogenesis of *Listeria monocytogenes* (111) and lends credence to the hypothesis that the PEST-like sequence encoded by BBA66 maybe similarly important for its function. Moreover, a conserved domain belonging to pfam 05109 (Herpes_BLLF1) was found that encompasses the PEST-like sequence and overlaps with the sequence identified by phage display (Figure 4.3.1); this domain shares homology with the Herpes virus major outer envelope glycoprotein, gp340/220, which is involved in viral binding to the B cell receptor C2 (135) and to human epithelial cells (271).

Using the Receptor Binding Domain (RBD) finder software (http://nbc11.biologie.uni-kl/de/rbdfinder), three coiled-coil domains were predicted within the C-terminal half of BBA66 (Figure 4.3.1). Coiled-coil domains have been identified in bacterial proteins ranging in function from transcriptional regulation via DNA binding (57, 107, 202) to signal transduction (239, 438, 464). Protein-protein or protein-host factor interactions have also been described (83, 459) and have been demonstrated between host factors and at least three coiled-coil-containing *B. burgdorferi* proteins, CspZ (366), BBA68 (285), and OspE (286). Thus, it is conceivable that the coiled-coil domains found within BBA66 may play a role in binding to host proteins or extracellular matrix factors found within cardiac tissue. In addition to the coiled-coil domains, three amino acids were predicted by RBDfinder to be involved in protein-protein interactions, namely W361, S362, and Y392 based on their hydrophobic and amphipathic characteristics (Figure 4.3.1). Studies to determine the contribution of individual amino acids to protein-protein binding interactions have shown tryptophan (W) to have the highest probability to lie within binding domains and to be conserved within these binding domains; moreover, binding surface interactions are typically due to one or a few amino acids, similar to what was predicted for BBA66 (88, 148, 266). Tryptophan residues have also been linked with bacterial virulence (130, 336, 355, 377). Serine (S) and tyrosine (Y), on the other hand, are common targets for phosphorylation, a process which has been implicated in bacterial virulence; the phosphorylation of either of these residues can cause conformational changes in target proteins that may be important for adherence and/or the activation or repression of protein function (95, 96). Interestingly, S362 was predicted to be phosphorylated using the DISPHOS prediction program (http://core.ist.temple.edu/pred/pred.html) and is found within the serine-rich (20%) C-terminus of BBA66 (Appendix A). Beyond the potential for phosphorylation, serine-rich proteins have

been implicated in adhesion to host cells (36, 335, 405, 439), in biofilm formation (480), and in virulence (310, 397). Though the coiled-coil domains and individual amino acids identified by RBDfinder do not fall within the region identified by Antonara and colleagues (12), they do offer additional amino acids of interest to help identify a putative binding domain(s) that may be involved in adherence of spirochetes to cardiac tissue. While our analyses and those of Antonara *et al* (12) provide some initial guidance for identifying putatively important regions and residues critical to BBA66 function, the true contribution of these sequences will need to be determined experimentally in a murine model of infection using *bba66* mutants.

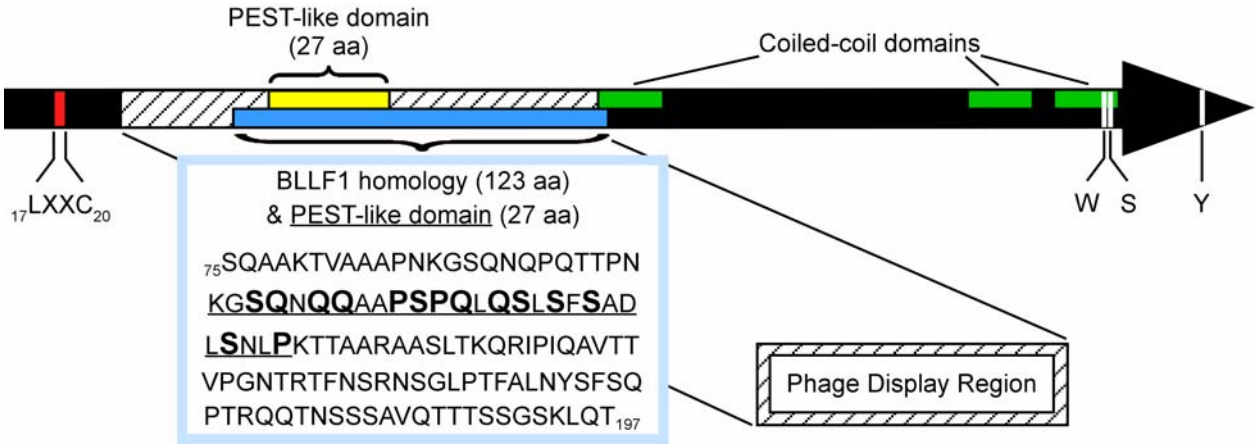


Figure 3.3.8 Schematic of BBA66 sequence features.

A schematic representation of the BBA66 protein sequence highlights regions of interest for future studies. The LXXC signal sequence identified by SpLip is shown in red. The PEST-like domain found using PESTfind is shown in yellow while the overlapping BLLF1 homology domain (pfam 05109) was found using CDD and is shown in blue. The aa sequence of the BLLF1 homology domain is shown below and boxed in blue with the PEST-like domain underlined and P, E/Q, S, and T residues shown in bolded, larger font. The region identified by Antonara and colleagues using phage display is represented by a white box with diagonal lines. Coiled-coil domains shown in

green and individual amino acids (aa) putatively important in binding interactions labeled in white with their single letter aa designations shown below were identified using RBDfinder.

3.3.5 Section 3.3 summary

Using an infectious clone, A3, *bba66* was mutated to assess whether it was required for infection and/or pathogenicity in mice. Characterization of a *bba66* mutant and complemented mutant demonstrated that these clones were isogenic to the parental isolate (Figure 3.3.2), demonstrated similar growth kinetics *in vitro* (Figure 3.3.4), and regulated protein expression appropriately (Figure 3.3.5). When inoculated into C3H/HeJ mice, all clones were infectious, causing both seroconversion (Table 3.3.1) and joint swelling in mice that peaked near day 21 p.i. (Section 3.3.2). Therefore, future studies will utilize the 21 day p.i. time point to assess whether tissue burdens of A3-*bba66*::Kan spirochetes are decreased relative to A3- and A3-*bba66*comp tissue burdens by qPCR.

4.0 DISCUSSION

4.1 THE POTENTIAL OF MEMBRANE-ASSOCIATED, OUTER SURFACE-LOCALIZED LIPOPROTEINS BBA65, BBA66, BBA71, AND BBA73 AS IMPORTANT FACTORS DURING MAMMALIAN INFECTION

4.1.1 The alternative sigma factor cascade influences *bba65, bba66, bba71,* and *bba73* gene transcription and protein synthesis

The alternative sigma factor σ^S has been implicated in the regulation of virulence factors in many diverse bacterial species (128, 229, 435). In *B. burgdorferi* the expression of σ^S is directly controlled by the alternative sigma factor σ^N, and several reports have highlighted the importance of this unique regulatory cascade in *B. burgdorferi* gene expression and infectivity (72-74, 138, 200, 484, 486). Comprehensive transcriptome analyses of *ntrA* (encoding σ^N) and *rpoS* (encoding σ^S) mutants in strains B31 and 297 have demonstrated the pleiotropic effect of knocking out these sigma factors upon transcription in *B. burgdorferi*, and numerous genes appear to be influenced by this cascade, including *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* (74, 138). Using qRT-PCR, a technique with a broader dynamic range than microarray, we too demonstrated the effects upon *in vitro* expression of these genes when either σ^N or σ^S were absent (Figure 3.1.1A). Our results agreed with previous microarray analyses (74, 138) for

bba65, *bba66*, and *bba71*, but our calculated fold-changes in transcript expression were in better agreement with the findings of Caimano *et al* (74), demonstrating 21- to 50-fold decreases in gene transcripts with mutation of *ntrA* or *rpoS* versus 1.5- to 3.8-fold decreases measured by Fisher and colleagues (138). Notably, the first of these reports examined spirochetes grown in dialysis membrane chambers transplanted into rats (74) while the later utilized cultured spirochetes (138). The lack of an appreciable change in *bba64* transcript levels in the absence of either σ^N or σ^S (Figure 3.1.1A) is likely due to the specific lot of BSK-H media used for these experiments as has been well documented (13, 75, 89, 460). Furthermore, these findings suggest factors involved in *bba64* regulation may differ from those that affect *bba65*, *bba66*, *bba71*, and *bba73*. Immunoblotting of total membrane fractions prepared from σ mutant and complemented strains demonstrated that changes observed in gene transcript levels correlated well with BBA65, BBA66, BBA71, and BBA73 protein levels (Figure 3.1.1B).

Though we attempted to complement the σ^S mutant *in trans*, our efforts proved unsuccessful. A review of current literature revealed that two groups have complemented a similar mutation in other *B. burgdorferi sensu stricto* (*ss*) backgrounds using a knock-in method (73, 200). Importantly, however, the first group did not show σ^S protein expression and the expression of two proteins known to be directly controlled by σ^S to be fully restored in the complemented mutant (200); the second group failed to show or to discuss the protein levels of σ^S or any of its downstream targets in one report (73) and in another report demonstrated that both complementation either in trans or by knock-in did not fully restore wildtype expression of σ^S-influenced proteins (72). One explanation for the inherent difficulty of restoring wildtype expression of σ^S and its targets may be that DNA supercoiling, which has been shown to be an important factor affecting the regulation of *Borrelia* gene transcription (8), may be important for

rpoS transcription; complementation either *in trans* or by knock-in may result in supercoiling of *rpoS* that does not allow for wildtype regulation of the gene. An alternative explanation may be that the identification of *rpoS* regulatory elements is incomplete and the upstream DNA cloned along with the *rpoS* structural gene has not been adequate to attain full expression and/or regulation of *rpoS* in this and other reports. These hypotheses, however, have not been tested and further research will be needed to conclude whether supercoiling plays a role in *rpoS* regulation and/or further regulatory elements upstream of *rpoS* are required for wildtype regulation of this sigma factor gene.

4.1.2 In vitro expression of BBA65, BBA66, BBA71, and BBA73 is associated with the outer-surface infectious isolate outer membranes

Our comparison of infectious and non-infectious *B. burgdorferi* isolates demonstrated that expression of BBA65, BBA71, and BBA73 was associated with infectious phenotypes (Figure 3.1.2A), similar to what we previously reported for the expression of BBA66 (89). While it is likely that differences in plasmid content or gene expression are responsible for the loss of infectivity, comparing and contrasting infectious and non-infectious *B. burgdorferi* isolates has proven advantageous in detecting antigens associated with infectious phenotypes (10, 79, 305, 388, 389, 392). Moreover, such comparisons have helped to identify proteins involved in strain-specific pathogenicity, including OspC which is required to establish murine infection (431, 445). Importantly, while lp54 was not lost in any of the strains examined as was apparent by the expression of OspA (Figure 3.1.2A, upper panel), BBA65, BBA66, BBA71, and BBA73 protein synthesis was detectable and associated only with infectious isolates.

Our results also indicate that BBA65, BBA66, BBA71, and BBA73 are either lipidated or integral membrane proteins that localize to the outer surface of *B. burgdorferi* B31, as all partitioned to the TX-114 detergent phase (Figure 3.1.4) and were sensitive to protease treatment (Figure 3.1.5) of intact spirochetes. Our findings for BBA66 were in agreement with those of Brooks *et al*, where they reported similar partitioning and surface localization for BBA64, BBA66, and BBA69 (53). It was surprising to find that BBA73 did not demonstrate complete partitioning into the detergent phase, given the presence of a spirochete-specific lipidation motif (401). It is, however, possible that BBA73 may possess biochemical characteristics that do not allow it to be easily solubilized in TX-114. Our findings for BBA71 were also interesting as this protein was dissimilar from others examined in this study. BBA71 is not predicted to contain a recognizable signal sequence or lipidation motif by either the SignalP (35, 302) or LipoP algorithms (211) nor is it predicted to be lipidated by the SpLip algorithm (401) (data not shown). Using the dense alignment surface (DAS) method (99), however, we identified a putative transmembrane domain ($_{20}$I-I-I-G-L-L-Y-H-K$_{28}$) that was recognized using the "loose" cutoff setting (Figure 4.1.1). Thus, unlike the lipoproteins BBA64, BBA65, BBA66, and BBA73, it appears that BBA71 may be a transmembrane protein.

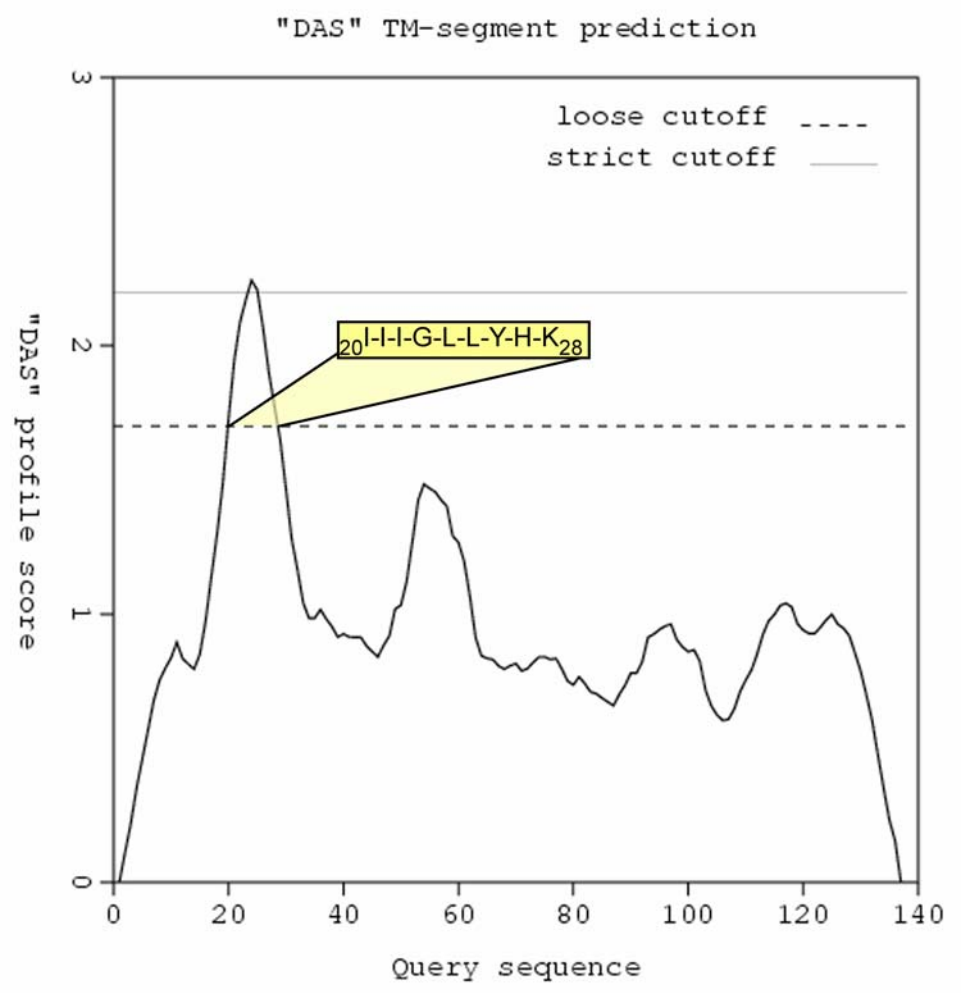


Figure 4.1.1 Graphical representation of DAS transmembrane prediction for BBA71.

The BBA71 protein sequence was analyzed using the DAS algorithm (Section 4.1.2). The segment identified by the loose cutoff is indicative of the predicted transmembrane region (99) indicated in the yellow box.

4.1.3 BBA65, BBA66, BBA71, and BBA73 are potentially important for tissue dissemination during murine infection and/or pathogenicity

In collaboration with Dr. Robert Gilmore, we reported upon the temporal expression of *bba64*, *bba65*, and *bba66*, *bba71*, and *bba73* in ear tissue throughout persistent infection of immunocompetent mice (153) (& Hughes *et al*. 2008, *Infect Immun*, in press). *bba65*, and

bba66, and *bba73* were each expressed in murine ear tissue with a peak at either 41 (*bba73*) or 100 (*bba65* and *bba66*) days post infection (p.i.), and a continual rise in transcript levels between days 180 and 270 p.i.. Strikingly, these genes continued to be expressed at increasing levels throughout persistent infection even after serological evidence indicated that BBA65-, BBA66-, and BBA73-specific antibodies were produced. While BBA64- and BBA71-specific antibodies were detected as early as day 20 p.i. and throughout the remainder of the infection period, increases in *bba64* and *bba71* transcript levels in ear tissue relative to culture were not observed (153) (& Hughes *et al*. 2008, *Infect Immun*, in press); these data suggest that *bba64* and *bba71* may be expressed in greater amounts in murine tissues other than ear/skin during persistence. Differential expression of *B. burgdorferi* proteins in various animal tissues has been previously demonstrated for other surface localized proteins (97, 98), and is likely important in pathogenesis and tissue tropism. Differential expression of BBA64, BBA65, BBA66, BBA71, and BBA73 in different tissue environments is supported by our findings (153) (& Hughes *et al*. 2008, *Infect Immun*, in press) and ongoing analyses (Dr. Robert Gilmore, unpublished data). Moreover, a recent publication by Livengood *et al* (262) demonstrated by DNA microarray that *bba71* is the most highly induced gene transcript when *B. burgdorferi* is exposed to the human neuroglial H4 cell line; *bba64* and *bba73* were the fourth and fifth most highly upregulated, respectively. The data described above in combined with observations from our *in vitro* analyses (Section 3.1) suggests that BBA64, BBA65, BBA66, BBA71, and BBA73 may be involved the long-term survival or pathogenesis of *B. burgdorferi* in mice and more specifically, may be important for dissemination to specific tissues.

4.2 BBA64, BBA65, BBA66, BBA71, AND BBA73 ORTHOLOGS MAY BE HARBORED BY DIVERSE *BORRELIA* SPECIES AND ARE SUBJECT TO ALTERED LEVELS OF EXPRESSION IN RESPONSE TO pH

4.2.1 Putative *bba64, bba65, bba66, bba71,* and *bba73* are detectable using a combination of Southern blotting and PCR analyses

Partial genome sequencing of *B. afzelii* PKo and *B. garinii* PBi indicate that orthologs of varying identity to *bba64*, *bba65*, *bba66*, *bba71* and *bba73* are present in these isolates and found on homologous plasmids. Southern blotting (Figure 3.2.1) and PCR (Figure 3.2.2) experiments performed to assess whether these genes were conserved by other diverse *Borrelia* species demonstrated that putative orthologs may indeed be present in *Borrelia burgdorferi ss* species *B. californiensis*, *B. andersonii*, and *B. valaisiana* as well as in the relapsing fever-causing *Borrelia* species *B. anserina*. Intriguingly, none of the remaining relapsing fever spirochetes displayed hybridization to our gene probes (Figure 3.2.1); however, PCR amplification of select genes using B31 sequence-specific primers (Figure 3.2.1) was successful with at least two gene primer sets for each relapsing fever spirochete that did not demonstrate hybridization by Southern blotting with any of our gene probes. The two hybridizing bands detected in *B. andersonii* with the *bba66* gene probe were also interesting and could suggest either a gene duplication event or an EcoRI restriction endonuclease site is present within the ortholog. Further investigation is required to determine if either of these events has occurred.

Hybridization results were varied for the remaining *B. burgdorferi sensu lato* (*sl*) species, and the appearance of weak hybridization bands or non-definitive bands in genomic DNA from *B. afzelii* and *B. garinii* was not unexpected. The sequence identity of the gene orthologs

between these species ranges from 82 % to 32 % in comparison to *B. burgdorferi* B31, decreasing the likelihood of hybridization under our experimental conditions. Notably, our level of detection of *bba66* orthologs was similar to that of a previous study (458), but *bba64* orthologs were more readily detected by Gilmore *et al* in *B. afzelii* and *B. garinii* genomic DNA and might reflect differences in gene probes or stains used in that study (154) and those used in the present study. Ultimately, the lack of an observable hybridizing band in several *Borrelia* species is not indicative of the absence of an ortholog to our genes of interest, as there may be enough divergence in the sequences that our *B. burgdorferi* B31-derived gene-specific probes were unable to hybridize. This point is highlighted by the lack of hybridization of our *flaB* probe in all *Borrelia* whose *flaB* was less than 84% similar to that of *B. burgdorferi* B31. However, we can not rule out that genomic DNA from some species may have been too fragmented by endonuclease digest to detect with our probes. Furthermore, it is also possible that the plasmids harboring putative orthologs have been lost in some of the diverse species analyzed; however, our PCR results would refute this hypothesis for at least some of those species which we did not observe detectable hybridization with in our Southern blotting analysis.

Using PCR we were able to detect putative orthologs that shared an adequate amount of sequence identity to the B31 primers to allow for their amplification; the results obtained from this study correlated well with our Southern blotting analyses and expanded the number of putative orthologs that were initially identified by Southern blotting. Interestingly, some PCR products varied slightly in size and intensity when visualized under UV, indicating that some species harbored putative orthologs that differed in size and/or their ability to hybridize to our PCR primers, respectively. These findings also indicate that DNA preparations were not contaminated with B31 genomic DNA. From our analyses we noted that PCR products were

amplified from the highly similar species *B. californiensis* using all primer sets and that the products demonstrated little variation in size from any of the *B. burgdorferi ss* products (Figure 3.2.2B-F). Slightly larger products, however, were amplified from *B. afzelii* and *B. garinii* than from *B. burgdorferi* using *bba64*-specific primers Figure 3.2.2B); these data agree with previously published sequence information (157, 458) and southern blotting data (154) (Figure 3.2.1). Amplification using *bba64* primers was successful with each of the remaining *B. burgdorferi sl* species examined, but yielded only a faint product from the relapsing fever spirochete *B. anserina* (Figure 3.2.2B). The lack of detectable PCR products from nearly all of the primer sets used with *B. anserina* genomic DNA suggest that the B31 sequence-specific primers may share too little sequence identity with putative *B. anserina* orthologs to effectively prime PCR amplification; this was in contrast to the B31 whole gene probes which shared enough sequence identity with putative *B. anserina* orthologs to yield definitive hybridization results by Southern blotting. Finally, the amplification of products from the remaining relapsing fever species that were similar in size to products obtained with *B. burgdorferi* B31 DNA with select *bba64*, *bba71*, and *bba73* primer sets suggests that orthologs to these genes may also be present in relapsing fever isolates.

4.2.2 α-BBA66, -BBA71, and -BBA73 cross-reactive proteins are detectable in human-pathogenic *B. burgdorferi sl* species and the closely related *B. californiensis*

Immunoblotting with α-BBA66, -BBA71, and -BBA73 antibodies revealed that not all orthologs detected by either Southern blotting or PCR were detectable with our antibodies (Figure 3.2.3). Instead, antibody cross-reactivity was limited to *B. burgdorferi sl* species known to be pathogenic in humans and the closely related species *B. californiensis*. Possible explanations for

the limited detection of cross-reactive proteins include, but are not limited to: *i*) putative protein orthologs are expressed by various *Borrelia* species, but they lack epitopes that are detectable with the B31 protein-specific polyclonal antibodies used in these studies; *ii*) repeated *in vitro* passage of *B. burgdorferi* has been shown to reduce the expression of BBA66, BBA71 and BBA73 below the levels of detection by immunoblotting (10, 89), a phenomenon which may be occurring in some of the *Borrelia* species examined here. Importantly, detection of α-BBA66, -BBA71, and -BBA73 cross-reactive proteins in the human-pathogenic species *B. afzelii* and *B. garinii* correlates with the association of these proteins with infectious *B. burgdorferi ss* strains B31 and A3 (Section 3.1.3), further strengthening the argument that these proteins are somehow involved and important during mammalian infection and/or pathogenesis.

It was interesting to find that α-BBA66 and -BBA73 pH-responsive, cross-reactive proteins were also expressed by *B. californiensis* isolate CA8. At the time of these analyses, there was no known connection between *B. californiensis* and human infection. However, *B. californiensis* has been shown to be more similar to *B. burgdorferi ss* than to other known *B. burgdorferi sl* species based on the analyses of seven independent DNA loci (337). Based on their relatedness, it may be possible that *B. burgdorferi ss* and *B. californiensis* diverged from one another relatively recently, but *B. californiensis* may have lost factors necessary for human infection and/or pathogenicity while retaining the genes encoding the α-BBA66 and –BBA73 cross-reactive proteins detected in Figure 3.2.3. Alternatively, it may be that *B. californiensis* isolate CA8 is infectious in humans, but it has not been associated with human disease yet because it is transmitted in the Western United States by *I. pacificus*, a vector that has been shown to be less competent than *I. scapularis* (126, 244).

4.2.3 Synthesis of α-BBA66, -BBA71, and BBA73 cross-reactive proteins is responsive to changes in pH *in vitro*

The affect of changing culture pH upon *Borrelia* species beyond a few *B. burgdorferi ss* isolates has not been addressed. Thus, we undertook to determine whether alterations in culture pH caused changes in the protein profiles of those *Borrelia* species examined for antibody cross-reactivity. In *B. burgdorferi* B31, expression of BBA66, BBA71, and BBA73 is increased at pH 7.0 relative to pH 8.0 at the levels of transcription (78, 89) and of protein synthesis (Figure 3.2.3). Correspondingly, each of the cross-reactive proteins detected was responsive to changes in culture pH in a manner that mirrored protein expression in *B. burgdorferi* B31. OppAI has not been demonstrated to be affected by pH in *B. burgdorferi* B31 and fittingly, the expression of α-OppAI cross-reactive proteins detected in other species did not change with alterations in culture pH (Figure 3.2.3). Moreover, OppAI is involved in transporting oligopeptides into the spirochetal cell, helping to compensate for the limited metabolic and biosynthetic capabilities of *B. burgdorferi*. A protein of such importance is unlikely to be significantly altered and as would be expected from such a protein, putative OppAI orthologs expressed by each of the diverse *Borrelia* species examined are cross-reactive with *B. burgdorferi* B31-specific α-OppAI polyclonal antibodies. Therefore, we were able to utilize this protein as a loading control for the pH 7.0 versus pH 8.0 samples from individual species. Combined with Southern blotting (Figure 3.2.1) and PCR (Figure 2.3.2), the observations that cross-reactive proteins were similar in size and pH-responsiveness to B31 proteins increases the likelihood that they are orthalogous to their *B. burgdorferi* B31 counterparts.

4.3 BBA66 IS NOT REQUIRED FOR INFECTION OF MICE

BBA66 has been recognized as an antigenic outer surface-localized lipoprotein that is regulated in response to numerous environmental cues including temperature (361, 446), pH (361), mammalian host factors (361), and mammalian blood (446). Under the influence of these cues, BBA66 is upregulated under conditions mimicking the mammalian host. Moreover, *bba66* transcripts are upregulated during murine infection (153, 258) and α-BBA66 antibodies are present in sera from *B. burgdorferi*-infected mice, rabbits, and humans (89, 308). The known *B. burgdorferi* virulence factor, OspC, is upregulated under similar environmental conditions (78, 308, 346, 361, 390, 446, 484) and is also upregulated and antigenic during murine infection (147, 318, 319). However, while *ospC* transcription has been shown to be directly regulated by the alternative sigma factor RpoS (487), *bba66* transcription is believed to be indirectly influenced by RpoS or require additional accessory regulators (89). Thus, we hypothesized that BBA66 may also be important, though not required (Section 1.7.3) during mammalian infection and/or pathogenesis.

4.3.1 *bba66* mutants retain the ability to infect mice

Using the infectious clone A3, we were able to isolate an infectious *bba66* mutant isogenic to the parental background; this differed from other knockout mutagenesis studies in which other groups used an lp25^{-} background that required complementation of the minimal lp25 region required for murine infection (272, 400). We were careful to utilize an lp25^{+} background as conflicting data has arisen in the literature when comparing knockout mutants that were cloned either in lp25^{+} or lp25^{-} backgrounds (255, 400). In the instance of BBK32, a surface-localized

fibronectin binding lipoprotein (340), two separate studies examined the effect of *bbk32* knockout mutants upon infectivity and pathogenesis in mice. The first of these studies demonstrated that lp25$^-$ *bbk32* mutants, complemented with the minimal region of lp25 required for murine infectivity, were significantly attenuated in mice (400); however, in the second study, a *bbk32* mutant in an lp25$^+$ background retained its full infection and pathogenicity potential (255). Thus, it appears that the loss of pathogenicity in the *bbk32* mutant in the first study could be due to insufficient complementation of lp25 and not the *bbk32* gene mutation. Fittingly, using an lp25$^+$ *bba66* knockout mutant in a murine model of Lyme disease, we demonstrated that *bba66* is not required for murine infection. In our analysis of an lp25$^+$ *bba66* mutant, all groups of mice became infected as demonstrated by ELISA (Table 3.3.1) over a 35 day infection course. *Borrelia* were reisolated from all but one A3-*bba66*comp-infected mouse which demonstrated no signs of infection (Table 3.3.1). Given the similarity shared between *bba66* and *bba64*, *bba65*, and *bba73* (Table 1.6.1), it is possible that these genes and their encoded proteins may be complementing any defects caused by mutation of *bba66*. However, the role of each of these proteins and whether their functions overlap remains to be determined. Regardless, it is not surprising that mutation of *bba66* caused no gross changes in murine infectivity given that only a single, highly-expressed outer surface-localized protein, OspC, has been shown to be required for the establishment of murine infection (445). More telling will be future studies which aim to determine whether spirochete loads are decreased in the cardiac tissue of mice infected with the *bba66* mutant isolate relative to the parental and complementing controls (Section 4.4.2).

4.3.2 pBSV2G-*bba66*comp is maintained throughout acute murine infection

To ensure that our *bba66* mutant strain had neither reverted nor been contaminated with the pBSV2G-*bba66*comp plasmid and to assess whether the complementing plasmid was maintained *in vivo*, we used plasmid rescue from genomic DNA of infected mice (Section 3.3.3). Complementing plasmid was not present in A3-*bba66*::Kan-infected mouse DNA as demonstrated by the inability to isolate gentamycin-resistant *E. coli*. Numerous colonies, however, were recovered from A3-*bba66*comp-infected genomic DNA. This suggests that the complementing plasmid is maintained in a large enough proportion of the spirochetes recovered p.i. to be able to reisolate the plasmid. This also suggests that *B. burgdorferi* would rather maintain the presence of *bba66* as opposed to losing the plasmid, even in the absence of antibiotic selection, during murine infection. This points to a potential importance of BBA66 throughout murine infection which is similarly hypothesized from findings that *bba66* continues to be expressed throughout persistent infection (153), despite the specific immune response toward BBA66 that is elicited during murine infection (89, 153, 308).

4.4 CONCLUDING REMARKS AND FUTURE WORK

4.4.1 Final summary and contributions to the field

The culmination of the information to date from this study and other reports indicate that many of the genes that cluster to the far end of lp54 (i.e., *bba64*, *bba65*, *bba66*, *bba69*, *bba71*, and *bba73*) are influenced by the σ^N-σ^S regulatory cascade in response to environmental signals, are

expressed during persistent infection, and the encoded proteins are exposed on the spirochetal cell surface. Furthermore, putative orthologs to *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* were detected in select *B. burgdorferi sl* strains and relapsing fever *Borrelia* species based on Southern blotting and PCR results (summarized in Table 3.2.1). This indicates that this subset of genes historically categorized into pgf 54, are conserved in both Lyme-associated and relapsing fever *Borrelia* species. Prior to our findings, only *B. afzelii* and *B. garinii* had been shown to harbor *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* gene orthologs. Though the function of these proteins during the infection process, pathogenesis, or tissue tropism is currently undefined, studies suggest that BBA64, BBA65, BBA66, BBA71, and BBA73 may play a role in *Borrelia* tissue-specific pathogenicity in mammalian hosts . Here, however, we demonstrated for the first time that BBA65, BBA71, and BBA73 are expressed by and localized to outer surface of infectious isolates (Figures 3.1.5 and 3.1.3, respectively) which potentially positions them to be able to interact with the mammalian host environment. Moreover, our collaboration with Dr. Robert Gilmore demonstrated that these genes are expressed throughout persistent murine infection (Section 4.1.3) indicating that any putative role these proteins might play in host-pathogen interactions have the potential to do so for the duration of *B. burgdorferi*'s survival within the host. While it is tempting to surmise that BBA64, BBA65, BBA66, BBA71, and BBA73 play redundant or complementary roles during mammalian infection, similar to what has been demonstrated for the CRASPs (6, 186, 197, 233, 234), a specific role has only been suggested for BBA66 (12, 458). Finally, the analyses of a *bba66* knockout in a murine model of Lyme disease demonstrated that *bba66* is not absolutely required for the infection of C3H/HeJ mice. This finding is in line with our original hypothesis and the analysis of a *bba64* mutant (272). While others in the lab did not detect significantly different spirochete loads in tissues at

day 35 p.i., they did note two peaks in joint swelling of mice infected with the parental isolate, A3. Thus, the peak in spirochetemia may be occurring near day 21 p.i. and examination of spirochete load at this time point may better represent early dissemination of *bba66*$^{+}$ versus *bba66*$^{-}$ clones as the clearance of spirochetes by day 35 p.i. may mask potential difference in tissue load. However, studies by Wallich *et al* (458) and Antonara *et al* (12) suggest that BBA66 plays a role in dissemination and/or adhesion of *B. burgdorferi* to murine cardiac tissue. Thus, one of the future studies proposed in the following sections is to examine the effect of *bba66* mutagenesis upon murine infection and tissue burden at earlier time points. Additionally, we will aim to examine *i*) the effects upon infection and/or pathogenicity when *bba65*, *bba71*, and *bba73* are mutated, and *ii*) to sequence putative *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* orthologs to confirm their identity.

4.4.2 Future Study 1: Further analysis of a *bba66* knockout mutant in a murine model of Lyme disease

Though others in the lab observed a trend toward decreased A3-*bba66*::Kan spirochete numbers in heart tissue relative to A3 and A3-*bba66*comp, they did not find these differences to be significant. Spirochete numbers in tissues of infected C3H mice have been shown to be measurably higher than in other mouse models at weeks two through five p.i. (29, 483); additionally, mice infected with our wildtype control, A3, demonstrated an initial peak in joint swelling at day 15 – 20 p.i.. Thus, in a second set of experimental infections, mice will again be infected with either A3, A3-*bba66*::Kan or A3-*bba66*comp but mice will be sacrificed at day 21 p.i. to examine tissue spirochete loads by qPCR at the peak of spirochetemia and cardiac inflammation. Because we showed in our initial study that serology and organ culture for

outgrowth of spirochetes are equally reliable indications of infection (Table 3.3.1), half of each tissue type will be used for histology in the proposed study as opposed to outgrowth. Combined with joint measurements, histology will demonstrate whether mutation of *bba66* has an effect on cellular infiltrates or inflammation within joint tissues.

4.4.3 Future Study 2: Knockout mutagenesis of *bba65, bba71,* and *bba73* for assessment of infectivity and pathogenesis in a murine model of Lyme disease

Further investigation of *bba65*, *bba71*, and *bba73* is clearly warranted to assess their role(s) during the tick-mouse infection cycle. Thus, deletion mutagenesis of *bba65*, *bba71*, and *bba73* will be utilized to determine the effect of these genes upon murine infection and/or pathogenicity. Mutational analysis and murine infections will be carried out in a similar fashion to what was described for *bba66* (Section 3.3). While previous reports linked *bba66* with localization of *Borrelia* to murine heart tissue, only *bba65* has been suggested to be involved in dissemination to or survival within specific tissues (458); the contribution of *bba71* and *bba73* to infection and/or pathogenicity has not been hypothesized upon and thus it is difficult to suggest what the effect of mutating these genes will be. However, given that BBA65, BBA71, and BBA73 are localized to the outer surface of the cell (Figure 3.1.5), it is reasonable to hypothesize that they may be involved in host-pathogen interactions that will become apparent after mutagenesis and infection in a murine model of Lyme disease.

4.4.4 Future Study 3: Sequencing and sequence analysis of *bba64, bba65, bba66, bba71,* and *bba73* orthologs harbored by diverse *Borrelia* species

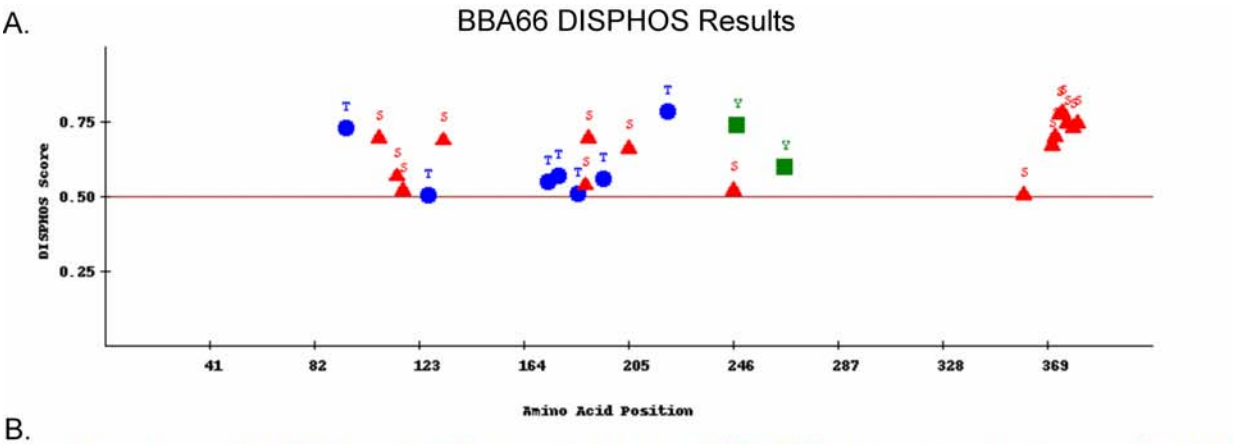
The PCR analysis of Lyme arthritis- and relapsing fever-associated *Borrelia* species presented in Section 3.3.2 revealed that the full-length 3' *bba64* and 5' *bba73* primers were used in at least one successful PCR reaction with all species except *B. valaisiana* and *B. parkeri*. If the orthologs detected either by Southern blotting or by PCR are contiguous in other *Borrelia* species as they are on lp54 in *B. burgdorferi* B31, we may be able to use these primers in combination to amplify, clone, and sequence the regions encoding these genes from the remaining *Borrelia* species. Importantly, each of the three species for which *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* orthologs have been sequenced show that these genes are contiguous on linear plasmids. Sequencing and/or Southern blotting of borrelial genomic DNA separated by contour-clamped homogenous electric field (CHEF) electrophoresis will be utilized to determine whether putative orthologs are similarly harbored on a single plasmid. Alternatively, if long-range PCR of these putatively contiguous regions is not successful, the BBA64FL.1 or BB64rt.F primers can be used to yield the initial sequence data from which species-specific primers will be designed for subsequent sequencing reactions. Completed ortholog sequences will be i) examined for their organization and whether they are harbored on a single linear plasmid, similar to B31, ii) the sequence identity shared between putative orthologs and B31 gene sequences will be performed using sequence algorithms, and iii) sequences will be examined for the presence of conserved regions which may indicate which nucleotides or amino acids are important for function and/or structure of each gene and its orthologs.

APPENDIX A

DISPHOS ANALYSIS FOR PUTATIVE PHOPHORYLATION SITES OF BBA66

The (Disorder-Enhanced Phosphorylation) DISPHOS Sites Predictor 3.1 (http://core.ist.temple.edu/pred/pred.html) was utilized to predict putative phosphorylation sites within the BBA66 amino acid sequence. The DISPHOS algorithm bases it's predictions upon sequence complexity, residue charge, disordered protein regions (considered an important prerequisite for phosphorylation to occur), and other additional characteristics. As shown in the figure below, 29 residues were predicted to be phosphorylated using this program. (A) A graphical representation depicts residues with a DISPHOS score above 0.5; scores between 0.5 and 1 are considered to be predictive of putative phosphorylation sights. Red triangles, serine (S); blue circles, threonine (T); green squares, tyrosine (Y). (B) The DISPHOS output for the BBA66 amino acid sequence showing all S, T, and Y using the color scheme noted in (A). Asterisks indicate S residues within the S-rich C-terminus that are putatively be phosphorylated. Coiled-coil regions and three residues predicted by RBDfinder to be putatively important in binding (Section 4.3.3) are indicated with yellow bars above the sequence or black bars below the sequence, respectively. (C) Tabular output of the colored residues shown in (B) with indication of residues predicted to be phosphorylated – denoted in the Yes/No column with "YES."

A.



B.

LKIKPLIQLKLLGLFLFSCTIDANLNEDYKNKVKGILNKAADDQETTSADTNSNAAKNIPIADNDKVAAELKKQSQAAKT
VAAAPNKGSQNQPQTTPNKGSQNQQAAPSPQLQSLSFSADLSNLPKTTAARAASLTKQRIPIQAVTTVPGNTRTFNSRNS
GLPTFALNYSFSQPTRQQTNSSSAVQTTTSSGSKLQTLKNELIRAISEEKNKTQNNFGFRETYDQFKMKDSAFELLDVIS
SAKVYDRSYAPQLNSNTPEAENERNKFYALMDFDQYKIEQFGSIMEALYNENQNHSLIRELMISGLGTQISFELALEEIN
KKIEIFNQDYLNAKINSFDFTMKLKELKSKLNQILDKRKEWSRQADGLIANASSNSSLSDSKSLAEYIKKRYLDNMQNAR
QSVLEAYISIM
* ** ** * * *

C.

Position	Residue	Score	Sequence	Yes/No
18	S	0.040	LFLFSCTID	
20	T	0.082	LFSCTIDAN	
29	Y	0.096	LNEDYKNKV	
46	T	0.249	DDQETTSAD	
47	T	0.443	DQETTSADT	
48	S	0.486	QETTSADTN	
51	T	0.422	TSADTNSNA	
53	S	0.303	ADTNSNAAK	
75	S	0.427	LKKQSQAAK	
80	T	0.412	QAAKTVAAA	
89	S	0.400	PNKGSQNQP	
95	T	0.139	NQPQTTPNK	
96	T	0.726	QPQTTPNKG	YES
101	S	0.419	PNKGSQNQQ	
109	S	0.699	QAAPSPQLQ	YES
114	S	0.490	PQLQSLSFS	
116	S	0.573	LQSLSFSAD	YES
118	S	0.524	SLSFSADLS	YES
122	S	0.365	SADLSNLPK	
127	T	0.259	NLPKTTAAR	
128	T	0.504	LPKTTAARA	YES
134	S	0.693	ARAASLTKQ	YES
136	T	0.277	AASLTKQRI	
146	T	0.133	IQAVTTVPG	
147	T	0.189	QAVTTVPGN	
152	T	0.358	VPGNTRTFN	
154	T	0.327	GNTRTFNSR	
157	S	0.128	RTFNSRNSG	
160	S	0.396	NSRNSGLPT	
164	T	0.444	SGLPTFALN	
169	Y	0.325	FALNYSFSQ	
170	S	0.195	ALNYSFSQP	
172	S	0.175	NYSFSQPTR	
175	T	0.546	FSQPTRQQT	YES
179	T	0.568	TRQQTNSSS	YES
181	S	0.464	QQTNSSSAV	
182	S	0.442	QTNSSSAVQ	
183	S	0.496	TNSSSAVQT	
187	T	0.508	SAVQTTTSS	YES
188	T	0.207	AVQTTTSSG	
189	T	0.281	VQTTTSSGS	
190	S	0.542	QTTTSSGSK	YES
191	S	0.699	TTTSSGSKL	YES
193	S	0.406	TSSGSKLQT	
197	T	0.556	SKLQTLKNE	YES
207	S	0.665	IRAISEEKN	YES
213	T	0.212	EKNKTQNNF	
222	T	0.782	GFRETYDQF	YES
223	Y	0.300	FRETYDQFK	
231	S	0.386	KMKDSAFEL	
240	S	0.393	LDVISSAKV	
241	S	0.332	DVISSAKVY	
245	Y	0.466	SAKVYDRSY	
248	S	0.526	VYDRSYAPQ	YES
249	Y	0.736	YDRSYAPQL	YES
255	S	0.227	PQLNSNTPE	
257	T	0.313	LNSNTPEAE	
268	Y	0.598	RNKFYALMD	YES
276	Y	0.340	DFDQYKIEQ	
283	S	0.263	EQFGSIMEA	
289	Y	0.393	MEALYNENQ	
296	S	0.223	NQNHSLIRE	
304	S	0.170	ELMISGLGT	
308	T	0.090	SGLGTQISF	
311	S	0.165	GTQISFELA	
330	Y	0.181	FNQDYLNAK	
337	S	0.184	AKINSFDFT	
341	T	0.128	SFDFTMKLK	
349	S	0.457	KELKSKLNQ	
362	S	0.509	RKEWSRQAD	YES
373	S	0.674	IANASSNSS	YES
374	S	0.705	ANASSNSSL	YES
376	S	0.778	ASSNSSLSD	YES
377	S	0.785	SSNSSLSDS	YES
379	S	0.749	NSSLSDSKS	YES
381	S	0.736	SLSDSKSLA	YES
383	S	0.750	SDSKSLAEY	YES
387	Y	0.176	SLAEYIKKR	
392	Y	0.095	IKKRYLDNM	
402	S	0.285	NARQSVLEA	
407	Y	0.135	VLEAYISIM	
409	S	0.199	EAYISIM**	

*An asterisk indicates prediction by similarity to the training sequences

BIBLIOGRAPHY

1. **Abbott, A.** 2006. Lyme disease: uphill struggle. Nature **439:**524-5.
2. **Afzelius, A.** 1921. Erythema chronicum migrans. Acta Derm Venereol **2:**120-125.
3. **Aguero-Rosenfeld, M. E., J. Nowakowski, S. Bittker, D. Cooper, R. B. Nadelman, and G. P. Wormser.** 1996. Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. J Clin Microbiol **34:**1-9.
4. **Ai, C. X., R. J. Hu, K. E. Hyland, Y. X. Wen, Y. G. Zhang, Q. C. Qiu, D. Y. Li, X. D. Liu, Z. X. Shi, J. H. Zhao, and et al.** 1990. Epidemiological and aetiological evidence for transmission of Lyme disease by adult *Ixodes persulcatus* in an endemic area in China. Int J Epidemiol **19:**1061-5.
5. **Ai, C. X., Y. X. Wen, Y. G. Zhang, S. S. Wang, Q. C. Qiu, Z. X. Shi, D. Y. Li, D. Q. Chen, X. D. Liu, and J. H. Zhao.** 1988. Clinical manifestations and epidemiological characteristics of Lyme disease in Hailin county, Heilongjiang Province, China. Ann N Y Acad Sci **539:**302-13.
6. **Alitalo, A., T. Meri, L. Ramo, T. S. Jokiranta, T. Heikkila, I. J. Seppala, J. Oksi, M. Viljanen, and S. Meri.** 2001. Complement evasion by *Borrelia burgdorferi*: serum-resistant strains promote C3b inactivation. Infect Immun **69:**3685-91.
7. **Almaviva, M., B. Hailu, G. Borgnolo, F. Chiabrera, G. Tolesse, and B. Gebre.** 1993. Louse-borne relapsing fever epidemic in Arssi Region, Ethiopia: a six months survey. Trans R Soc Trop Med Hyg **87:**153.
8. **Alverson, J., S. F. Bundle, C. D. Sohaskey, M. C. Lybecker, and D. S. Samuels.** 2003. Transcriptional regulation of the *ospAB* and *ospC* promoters from *Borrelia burgdorferi*. Mol Microbiol **48:**1665-1677.
9. **Anderson, J. F., L. A. Magnarelli, R. B. LeFebvre, T. G. Andreadis, J. B. McAninch, G. C. Perng, and R. C. Johnson.** 1989. Antigenically variable *Borrelia burgdorferi* isolated from cottontail rabbits and *Ixodes dentatus* in rural and urban areas. J Clin Microbiol **27:**13-20.
10. **Anguita, J., S. Samanta, B. Revilla, K. Suk, S. Das, S. W. Barthold, and E. Fikrig.** 2000. *Borrelia burgdorferi* gene expression in vivo and spirochete pathogenicity. Infect Immun **68:**1222-30.
11. **Anguita, J., V. Thomas, S. Samanta, R. Persinski, C. Hernanz, S. W. Barthold, and E. Fikrig.** 2001. *Borrelia burgdorferi*-induced inflammation facilitates spirochete adaptation and variable major protein-like sequence locus recombination. J Immunol **167:**3383-90.
12. **Antonara, S., R. M. Chafel, M. Lafrance, and J. Coburn.** 2007. *Borrelia burgdorferi* adhesins identified using in vivo phage display. Mol Microbiol **66:**262-76.

13. **Babb, K., N. El-Hage, J. C. Miller, J. A. Carroll, and B. Stevenson.** 2001. Distinct regulatory pathways control expression of *Borrelia burgdorferi* infection-associated OspC and Erp surface proteins. Infect Immun **69:**4146-53.
14. **Bachman, M. A., and M. S. Swanson.** 2001. RpoS co-operates with other factors to induce *Legionella pneumophila* virulence in the stationary phase. Mol Microbiol **40:**1201-14.
15. **Baig, S., T. Olsson, K. Hansen, and H. Link.** 1991. Anti-*Borrelia burgdorferi* antibody response over the course of Lyme neuroborreliosis. Infect Immun **59:**1050-6.
16. **Baranton, G., M. Assous, and D. Postic.** 1992. Three bacterial species associated with Lyme borreliosis. Clinical and diagnostic implications. Bull Acad Natl Med **176:**1075-85; discussion 1085-6.
17. **Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J. C. Piffaretti, M. Assous, and P. A. Grimont.** 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int J Syst Bacteriol **42:**378-83.
18. **Baranton, G., G. Seinost, G. Theodore, D. Postic, and D. Dykhuizen.** 2001. Distinct levels of genetic diversity of *Borrelia burgdorferi* are associated with different aspects of pathogenicity. Res Microbiol **152:**149-56.
19. **Barbour, A. G.** 2005. Relapsing Fever, p. 268-291. *In* J. L. Goodman, D. T. Dennis, and D. E. Sonenshine (ed.), Tick-Borne Diseases of Humans. ASM Press, Washington, DC.
20. **Barbour, A. G., Burgdorfer W., Hayes S. F., Peter O., Aeschlimann A.** 1983. Isolation of a cultivable spirochete from *Ixodes ricinus* ticks of Switzerland. Curr Microbiol **8:**123-126.
21. **Barbour, A. G., S. F. Hayes, R. A. Heiland, M. E. Schrumpf, and S. L. Tessier.** 1986. A *Borrelia*-specific monoclonal antibody binds to a flagellar epitope. Infect Immun **52:**549-54.
22. **Barbour, A. G., G. O. Maupin, G. J. Teltow, C. J. Carter, and J. Piesman.** 1996. Identification of an uncultivable *Borrelia* species in the hard tick *Amblyomma americanum*: possible agent of a Lyme disease-like illness. J Infect Dis **173:**403-9.
23. **Barbour, A. G., S. L. Tessier, and S. F. Hayes.** 1984. Variation in a major surface protein of Lyme disease spirochetes. Infect Immun **45:**94-100.
24. **Barbour, A. G., S. L. Tessier, and H. G. Stoenner.** 1982. Variable major proteins of *Borrellia hermsii*. J Exp Med **156:**1312-24.
25. **Barbour, A. G., S. L. Tessier, and W. J. Todd.** 1983. Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect Immun **41:**795-804.
26. **Barbour, A. G., W. J. Todd, and H. G. Stoenner.** 1982. Action of penicillin on *Borrelia hermsii*. Antimicrob Agents Chemother **21:**823-9.
27. **Barthold, S. W., D. S. Beck, G. M. Hansen, G. A. Terwilliger, and K. D. Moody.** 1990. Lyme borreliosis in selected strains and ages of laboratory mice. J Infect Dis **162:**133-8.
28. **Barthold, S. W., E. Fikrig, L. K. Bockenstedt, and D. H. Persing.** 1995. Circumvention of outer surface protein A immunity by host-adapted *Borrelia burgdorferi*. Infect Immun **63:**2255-61.

29. **Barthold, S. W., D. H. Persing, A. L. Armstrong, and R. A. Peeples.** 1991. Kinetics of *Borrelia burgdorferi* dissemination and evolution of disease after intradermal inoculation of mice. Am J Pathol **139:**263-73.
30. **Barthold, S. W., C. L. Sidman, and A. L. Smith.** 1992. Lyme borreliosis in genetically resistant and susceptible mice with severe combined immunodeficiency. Am J Trop Med Hyg **47:**605-13.
31. **Baughman, K. L.** 2005. Clinical presentations of myocarditis. Heart Fail Clin **1:**363-76.
32. **Bearson, S., B. Bearson, and J. W. Foster.** 1997. Acid stress responses in enterobacteria. FEMS Microbiol Lett **147:**173-80.
33. **Bearson, S. M., W. H. Benjamin, Jr., W. E. Swords, and J. W. Foster.** 1996. Acid shock induction of RpoS is mediated by the mouse virulence gene *mviA* of *Salmonella typhimurium*. J Bacteriol **178:**2572-9.
34. **Beermann, C., G. Lochnit, R. Geyer, P. Groscurth, and L. Filgueira.** 2000. The lipid component of lipoproteins from *Borrelia burgdorferi*: structural analysis, antigenicity, and presentation via human dendritic cells. Biochem Biophys Res Commun **267:**897-905.
35. **Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak.** 2004. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol **340:**783-95.
36. **Bensing, B. A., B. W. Gibson, and P. M. Sullam.** 2004. The *Streptococcus gordonii* platelet binding protein GspB undergoes glycosylation independently of export. J Bacteriol **186:**638-45.
37. **Berger, B. W.** 1989. Dermatologic manifestations of Lyme disease. Rev Infect Dis **11 Suppl 6:**S1475-81.
38. **Berger, B. W., R. C. Johnson, C. Kodner, and L. Coleman.** 1992. Cultivation of *Borrelia burgdorferi* from erythema migrans lesions and perilesional skin. J Clin Microbiol **30:**359-61.
39. **Berry, A. M., and J. C. Paton.** 1996. Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*. Infect Immun **64:**5255-62.
40. **Blevins, J. S., A. T. Revel, A. H. Smith, G. N. Bachlani, and M. V. Norgard.** 2007. Adaptation of a luciferase gene reporter and lac expression system to *Borrelia burgdorferi*. Appl Environ Microbiol **73:**1501-13.
41. **Bolz, D. D., and J. J. Weis.** 2004. Molecular mimicry to *Borrelia burgdorferi*: pathway to autoimmunity? Autoimmunity **37:**387-92.
42. **Bono, J. L., A. F. Elias, J. J. Kupko, 3rd, B. Stevenson, K. Tilly, and P. Rosa.** 2000. Efficient targeted mutagenesis in *Borrelia burgdorferi*. J Bacteriol **182:**2445-52.
43. **Bono, J. L., K. Tilly, B. Stevenson, D. Hogan, and P. Rosa.** 1998. Oligopeptide permease in *Borrelia burgdorferi*: putative peptide-binding components encoded by both chromosomal and plasmid loci. Microbiology **144:**1033-44.
44. **Bootsma, H. J., P. C. Aerts, G. Posthuma, T. Harmsen, J. Verhoef, H. van Dijk, and F. R. Mooi.** 1999. *Moraxella* (*Branhamella*) catarrhalis BRO beta-lactamase: a lipoprotein of gram-positive origin? J Bacteriol **181:**5090-3.
45. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem **256:**1604-7.
46. **Borgnolo, G., B. Denku, F. Chiabrera, and B. Hailu.** 1993. Louse-borne relapsing fever in Ethiopian children: a clinical study. Ann Trop Paediatr **13:**165-71.

47. **Borgnolo, G., B. Hailu, A. Ciancarelli, M. Almaviva, and T. Woldemariam.** 1993. Louse-borne relapsing fever. A clinical and an epidemiological study of 389 patients in Asella Hospital, Ethiopia. Trop Geogr Med **45:**66-9.
48. **Bowman, A. S., L. B. Coons, G. R. Needham, and J. R. Sauer.** 1997. Tick saliva: recent advances and implications for vector competence. Med Vet Entomol **11:**277-85.
49. **Brandt, M. E., B. S. Riley, J. D. Radolf, and M. V. Norgard.** 1990. Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. Infect Immun **58:**983-91.
50. **Breitner-Ruddock, S., R. Wurzner, J. Schulze, and V. Brade.** 1997. Heterogeneity in the complement-dependent bacteriolysis within the species of *Borrelia burgdorferi*. Med Microbiol Immunol (Berl) **185:**253-60.
51. **Brisson, D., D. E. Dykhuizen, and R. S. Ostfeld.** 2008. Conspicuous impacts of inconspicuous hosts on the Lyme disease epidemic. Proc Biol Sci **275:**227-35.
52. **Brooks, C. S., P. S. Hefty, S. E. Jolliff, and D. R. Akins.** 2003. Global analysis of *Borrelia burgdorferi* genes regulated by mammalian host-specific signals. Infect Immun **71:**3371-83.
53. **Brooks, C. S., S. R. Vuppala, A. M. Jett, and D. R. Akins.** 2006. Identification of *Borrelia burgdorferi* outer surface proteins. Infect Immun **74:**296-304.
54. **Brouqui, P., F. Bacellar, G. Baranton, R. J. Birtles, A. Bjoersdorff, J. R. Blanco, G. Caruso, M. Cinco, P. E. Fournier, E. Francavilla, M. Jensenius, J. Kazar, H. Laferl, A. Lakos, S. Lotric Furlan, M. Maurin, J. A. Oteo, P. Parola, C. Perez-Eid, O. Peter, D. Postic, D. Raoult, A. Tellez, Y. Tselentis, and B. Wilske.** 2004. Guidelines for the diagnosis of tick-borne bacterial diseases in Europe. Clin Microbiol Infect **10:**1108-32.
55. **Brouqui, P., A. Stein, H. T. Dupont, P. Gallian, S. Badiaga, J. M. Rolain, J. L. Mege, B. La Scola, P. Berbis, and D. Raoult.** 2005. Ectoparasitism and Vector-Borne Diseases in 930 Homeless People From Marseilles. Medicine (Baltimore) **84:**61-68.
56. **Brown, E. L., B. P. Guo, P. O'Neal, and M. Hook.** 1999. Adherence of *Borrelia burgdorferi*. Identification Of critical lysine residues in *dbpA* required for decorin binding. J Biol Chem **274:**26272-8.
57. **Brown, N. L., J. V. Stoyanov, S. P. Kidd, and J. L. Hobman.** 2003. The MerR family of transcriptional regulators. FEMS Microbiol Rev **27:**145-63.
58. **Brown, V., B. Larouze, G. Desve, J. J. Rousset, M. Thibon, A. Fourrier, and V. Schwoebel.** 1988. Clinical presentation of louse-borne relapsing fever among Ethiopian refugees in northern Somalia. Ann Trop Med Parasitol **82:**499-502.
59. **Bryceson, A. D., E. H. Parry, P. L. Perine, D. A. Warrell, D. Vukotich, and C. S. Leithead.** 1970. Louse-borne relapsing fever. Q J Med **39:**129-70.
60. **Bunikis, J., and A. G. Barbour.** 1999. Access of antibody or trypsin to an integral outer membrane protein (P66) of *Borrelia burgdorferi* is hindered by Osp lipoproteins. Infect Immun **67:**2874-83.
61. **Bunikis, J., L. Noppa, and S. Bergstrom.** 1995. Molecular analysis of a 66-kDa protein associated with the outer membrane of Lyme disease *Borrelia*. FEMS Microbiol Lett **131:**139-45.
62. **Burgdorfer, W.** 1991. Lyme borreliosis: ten years after discovery of the etiologic agent, *Borrelia burgdorferi*. Infection **19:**257-62.
63. **Burgdorfer, W.** 1989. Vector/host relationships of the Lyme disease spirochete, *Borrelia burgdorferi*. Rheum Dis Clin North Am **15:**775-87.

64. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease-A tick borne spirochetosis? Science **216:**1317-1319.
65. **Burgdorfer, W., and M. G. Varma.** 1967. Trans-stadial and transovarial development of disease agents in arthropods. Annu Rev Entomol **12:**347-76.
66. **Burtnick, M. N., J. S. Downey, P. J. Brett, J. A. Boylan, J. G. Frye, T. R. Hoover, and F. C. Gherardini.** 2007. Insights into the complex regulation of *rpoS* in *Borrelia burgdorferi*. Mol Microbiol **65:**277-93.
67. **Bykowski, T., K. Babb, K. von Lackum, S. P. Riley, S. J. Norris, and B. Stevenson.** 2006. Transcriptional regulation of the *Borrelia burgdorferi* antigenically variable VlsE surface protein. J Bacteriol **188:**4879-89.
68. **C.D.C.** 2007. Acute respiratory distress syndrome in persons with tickborne relapsing fever--three states, 2004-2005. MMWR Morb Mortal Wkly Rep **56:**1073-6.
69. **C.D.C.** 2001. Lyme Disease. http://www.cdc.gov/ncidod/dvbid/lyme/who_cc/.
70. **C.D.C.** 2007. Lyme disease--United States, 2003-2005. MMWR Morb Mortal Wkly Rep **56:**573-6.
71. **C.D.C.** 1995. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. MMWR Morb Mortal Wkly Rep **44:**590-1.
72. **Caimano, M. J., C. H. Eggers, C. A. Gonzalez, and J. D. Radolf.** 2005. Alternate Sigma Factor RpoS Is Required for the In Vivo-Specific Repression of *Borrelia burgdorferi* Plasmid lp54-Borne *ospA* and *lp6.6* Genes. J Bacteriol **187:**7845-52.
73. **Caimano, M. J., C. H. Eggers, K. R. Hazlett, and J. D. Radolf.** 2004. RpoS is not central to the general stress response in *Borrelia burgdorferi* but does control expression of one or more essential virulence determinants. Infect Immun **72:**6433-45.
74. **Caimano, M. J., R. Iyer, C. H. Eggers, C. Gonzalez, E. A. Morton, M. A. Gilbert, I. Schwartz, and J. D. Radolf.** 2007. Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals provides insight into RpoS function during the enzootic cycle. Mol Microbiol **65:**1193-1217.
75. **Callister, S. M., K. L. Case, W. A. Agger, R. F. Schell, R. C. Johnson, and J. L. Ellingson.** 1990. Effects of bovine serum albumin on the ability of Barbour-Stoenner-Kelly medium to detect *Borrelia burgdorferi*. J Clin Microbiol **28:**363-5.
76. **Canica, M. M., F. Nato, L. du Merle, J. C. Mazie, G. Baranton, and D. Postic.** 1993. Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. Scand J Infect Dis **25:**441-8.
77. **Carroll, J. A., R. M. Cordova, and C. F. Garon.** 2000. Identification of 11 pH-regulated genes in *Borrelia burgdorferi* localizing to linear plasmids. Infect Immun **68:**6677-84.
78. **Carroll, J. A., C. F. Garon, and T. G. Schwan.** 1999. Effects of environmental pH on membrane proteins in *Borrelia burgdorferi*. Infect Immun **67:**3181-7.
79. **Carroll, J. A., and F. C. Gherardini.** 1996. Membrane protein variations associated with in vitro passage of *Borrelia burgdorferi*. Infect Immun **64:**392-8.
80. **Carroll, J. A., P. E. Stewart, P. Rosa, A. F. Elias, and C. F. Garon.** 2003. An enhanced GFP reporter system to monitor gene expression in *Borrelia burgdorferi*. Microbiology **149:**1819-1828.

81. **Cascales, E., A. Bernadac, M. Gavioli, J. C. Lazzaroni, and R. Lloubes.** 2002. Pal lipoprotein of *Escherichia coli* plays a major role in outer membrane integrity. J Bacteriol **184:**754-9.
82. **Casjens, S., N. Palmer, R. van Vugt, W. M. Huang, B. Stevenson, P. Rosa, R. Lathigra, G. Sutton, J. Peterson, R. J. Dodson, D. Haft, E. Hickey, M. Gwinn, O. White, and C. M. Fraser.** 2000. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. Mol Microbiol **35:**490-516.
83. **Cedervall, T., M. U. Johansson, and B. Akerstrom.** 1997. Coiled-coil structure of group A streptococcal M proteins. Different temperature stability of class A and C proteins by hydrophobic-nonhydrophobic amino acid substitutions at heptad positions a and d. Biochemistry **36:**4987-94.
84. **CentersforDiseaseControlandPrevention.** 2002. Lyme Disease — United States, 2000. MMWR Morb Mortal Wkly Rep **51:**29-31.
85. **CentersforDiseaseControlandPrevention.** 2001. Lyme disease--United States, 1999. MMWR Morb Mortal Wkly Rep **50:**181-5.
86. **CentersforDiseaseControlandPrevention.** 2004. Lyme disease--United States, 2001-2002. MMWR Morb Mortal Wkly Rep **53:**365-9.
87. **Christen, H. J., H. Eiffert, A. Ohlenbusch, and F. Hanefeld.** 1995. Evaluation of the polymerase chain reaction for the detection of *Borrelia burgdorferi* in cerebrospinal fluid of children with acute peripheral facial palsy. Eur J Pediatr **154:**374-7.
88. **Clackson, T., and J. A. Wells.** 1995. A hot spot of binding energy in a hormone-receptor interface. Science **267:**383-6.
89. **Clifton, D. R., C. L. Nolder, J. L. Hughes, A. J. Nowalk, and J. A. Carroll.** 2006. Regulation and expression of *bba66* encoding an immunogenic infection-associated lipoprotein in *Borrelia burgdorferi*. Mol Microbiol **61:**243-58.
90. **Coburn, J., W. Chege, L. Magoun, S. C. Bodary, and J. M. Leong.** 1999. Characterization of a candidate *Borrelia burgdorferi* beta3-chain integrin ligand identified using a phage display library. Mol Microbiol **34:**926-940.
91. **Coburn, J., and C. Cugini.** 2003. Targeted mutation of the outer membrane protein P66 disrupts attachment of the Lyme disease agent, *Borrelia burgdorferi*, to integrin alphavbeta3. Proc Natl Acad Sci U S A **100:**7301-6.
92. **Cornelissen, C. N.** 2003. Transferrin-iron uptake by Gram-negative bacteria. Front Biosci **8:**d836-47.
93. **Cox, D. L., D. R. Akins, K. W. Bourell, P. Lahdenne, M. V. Norgard, and J. D. Radolf.** 1996. Limited surface exposure of *Borrelia burgdorferi* outer surface lipoproteins. Proc Natl Acad Sci U S A **93:**7973-8.
94. **Coyle, B. S., G. T. Strickland, Y. Y. Liang, C. Pena, R. McCarter, and E. Israel.** 1996. The public health impact of Lyme disease in Maryland. J Infect Dis **173:**1260-2.
95. **Cozzone, A. J.** 2005. Role of protein phosphorylation on serine/threonine and tyrosine in the virulence of bacterial pathogens. J Mol Microbiol Biotechnol **9:**198-213.
96. **Cozzone, A. J., C. Grangeasse, P. Doublet, and B. Duclos.** 2004. Protein phosphorylation on tyrosine in bacteria. Arch Microbiol **181:**171-81.
97. **Crother, T. R., C. I. Champion, J. P. Whitelegge, R. Aguilera, X. Y. Wu, D. R. Blanco, J. N. Miller, and M. A. Lovett.** 2004. Temporal analysis of the antigenic

composition of *Borrelia burgdorferi* during infection in rabbit skin. Infect Immun **72:**5063-72.

98. **Crother, T. R., C. I. Champion, X. Y. Wu, D. R. Blanco, J. N. Miller, and M. A. Lovett.** 2003. Antigenic composition of *Borrelia burgdorferi* during infection of SCID mice. Infect Immun **71:**3419-28.
99. **Cserzo, M., E. Wallin, I. Simon, G. von Heijne, and A. Elofsson.** 1997. Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. Protein Eng **10:**673-6.
100. **Cunningham, T. M., E. M. Walker, J. N. Miller, and M. A. Lovett.** 1988. Selective release of the *Treponema pallidum* outer membrane and associated polypeptides with Triton X-114. J Bacteriol **170:**5789-96.
101. **Cutler, S. J.** 2006. Possibilities for relapsing fever reemergence. Emerg Infect Dis **12:**369-74.
102. **Cutler, S. J., D. Fekade, K. Hussein, K. A. Knox, A. Melka, K. Cann, A. R. Emilianus, D. A. Warrell, and D. J. Wright.** 1994. Successful in-vitro cultivation of *Borrelia recurrentis*. Lancet **343:**242.
103. **Cutler, S. J., J. Moss, M. Fukunaga, D. J. Wright, D. Fekade, and D. Warrell.** 1997. *Borrelia recurrentis* characterization and comparison with relapsing-fever, Lyme-associated, and other *Borrelia* spp. Int J Syst Bacteriol **47:**958-68.
104. **Daniel, E., H. Beyene, and T. Tessema.** 1992. Relapsing fever in children--demographic, social and clinical features. Ethiop Med J **30:**207-14.
105. **de Mik, E. L., W. van Pelt, B. D. Docters-van Leeuwen, A. van der Veen, J. F. Schellekens, and M. W. Borgdorff.** 1997. The geographical distribution of tick bites and erythema migrans in general practice in The Netherlands. Int J Epidemiol **26:**451-7.
106. **de Silva, A. M., S. R. Telford, 3rd, L. R. Brunet, S. W. Barthold, and E. Fikrig.** 1996. *Borrelia burgdorferi* OspA is an arthropod-specific transmission- blocking Lyme disease vaccine. J Exp Med **183:**271-5.
107. **De Silva, R. S., G. Kovacikova, W. Lin, R. K. Taylor, K. Skorupski, and F. J. Kull.** 2005. Crystal structure of the virulence gene activator AphA from *Vibrio cholerae* reveals it is a novel member of the winged helix transcription factor superfamily. J Biol Chem **280:**13779-83.
108. **Debue, M., P. Gautier, C. Hackel, A. Van Elsen, A. Herzog, G. Bigaignon, and A. Bollen.** 1991. Detection of *Borrelia burgdorferi* in biological samples using the polymerase chain reaction assay. Res Microbiol **142:**565-72.
109. **Dec, G. W., Jr., I. F. Palacios, J. T. Fallon, H. T. Aretz, J. Mills, D. C. Lee, and R. A. Johnson.** 1985. Active myocarditis in the spectrum of acute dilated cardiomyopathies. Clinical features, histologic correlates, and clinical outcome. N Engl J Med **312:**885-90.
110. **Dec, G. W., Jr., H. Waldman, J. Southern, J. T. Fallon, A. M. Hutter, Jr., and I. Palacios.** 1992. Viral myocarditis mimicking acute myocardial infarction. J Am Coll Cardiol **20:**85-9.
111. **Decatur, A. L., and D. A. Portnoy.** 2000. A PEST-like sequence in listeriolysin O essential for Listeria monocytogenes pathogenicity. Science **290:**992-5.
112. **DePietropaolo, D. L., J. H. Powers, J. M. Gill, and A. J. Foy.** 2005. Diagnosis of lyme disease. Am Fam Physician **72:**297-304.

113. **des Vignes, F., J. Piesman, R. Heffernan, T. L. Schulze, K. C. Stafford, 3rd, and D. Fish.** 2001. Effect of tick removal on transmission of *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* nymphs. J Infect Dis **183:**773-8.
114. **Donahue, J. G., J. Piesman, and A. Spielman.** 1987. Reservoir competence of white-footed mice for Lyme disease spirochetes. Am J Trop Med Hyg **36:**92-6.
115. **Dressler, F., J. A. Whalen, B. N. Reinhardt, and A. C. Steere.** 1993. Western blotting in the serodiagnosis of Lyme disease. J Infect Dis **167:**392-400.
116. **Dunn, J. J., B. N. Lade, and A. G. Barbour.** 1990. Outer surface protein A (OspA) from the Lyme disease spirochete, *Borrelia burgdorferi*: high level expression and purification of a soluble recombinant form of OspA. Protein Expr Purif **1:**159-68.
117. **Dupont, H. T., B. La Scola, R. Williams, and D. Raoult.** 1997. A focus of tick-borne relapsing fever in southern Zaire. Clin Infect Dis **25:**139-44.
118. **Dworkin, M. S., P. C. Shoemaker, C. L. Fritz, M. E. Dowell, and D. E. Anderson, Jr.** 2002. The epidemiology of tick-borne relapsing fever in the United States. Am J Trop Med Hyg **66:**753-8.
119. **Edlow, J. A.** 2002. Erythema migrans. Med Clin North Am **86:**239-60.
120. **Eggers, C. H., M. J. Caimano, and J. D. Radolf.** 2004. Analysis of promoter elements involved in the transcriptional initiation of RpoS-dependent *Borrelia burgdorferi* genes. J Bacteriol **186:**7390-402.
121. **El-Hage, N., K. Babb, J. A. Carroll, N. Lindstrom, E. R. Fischer, J. C. Miller, R. D. Gilmore, Jr., M. L. Mbow, and B. Stevenson.** 2001. Surface exposure and protease insensitivity of *Borrelia burgdorferi* Erp (OspEF-related) lipoproteins. Microbiology **147:**821-30.
122. **Elias, A. F., J. L. Bono, J. J. Kupko, 3rd, P. E. Stewart, J. G. Krum, and P. A. Rosa.** 2003. New antibiotic resistance cassettes suitable for genetic studies in *Borrelia burgdorferi*. J Mol Microbiol Biotechnol **6:**29-40.
123. **Elias, A. F., P. E. Stewart, D. Grimm, M. J. Caimano, C. H. Eggers, K. Tilly, J. L. Bono, D. R. Akins, J. D. Radolf, T. G. Schwan, and P. Rosa.** 2002. Clonal polymorphism of *Borrelia burgdorferi*B31 MI: implications for mutagenesis in an infectious strain background. Infect Immun **70:**2139-50.
124. **Ellis, C. R., and T. Di Salvo.** 2007. Myocarditis: basic and clinical aspects. Cardiol Rev **15:**170-7.
125. **Engstrom, S. M., E. Shoop, and R. C. Johnson.** 1995. Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. J Clin Microbiol **33:**419-27.
126. **Estrada-Pena, A., and F. Jongejan.** 1999. Ticks feeding on humans: a review of records on human-biting Ixodoidea with special reference to pathogen transmission. Exp Appl Acarol **23:**685-715.
127. **F.D.A.** 2001. LYMErix safety data reported to the vaccine adverse event reporting system (VAERS) from December 21, 1998 through October 31, 2000. http://www.fda.gov/ohrms/dockets/ac/01/briefing/3680b2_06.doc.
128. **Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and D. G. Guiney.** 1992. The alternative sigma factor *katF* (*rpoS*) regulates *Salmonella* virulence. Proc Natl Acad Sci U S A **89:**11978-82.
129. **Fearon, D. T., and K. F. Austen.** 1977. Activation of the alternative complement pathway due to resistance of zymosan-bound. Proc Natl Acad Sci U S A **74:**1683-7.

130. **Feng, J., H. Wehbi, and M. F. Roberts.** 2002. Role of tryptophan residues in interfacial binding of phosphatidylinositol-specific phospholipase C. J Biol Chem **277:**19867-75.
131. **Feng, S., E. Hodzic, and S. W. Barthold.** 2000. Lyme arthritis resolution with antiserum to a 37-kilodalton *Borrelia burgdorferi* protein. Infect Immun **68:**4169-73.
132. **Ferdows, M. S., P. Serwer, G. A. Griess, S. J. Norris, and A. G. Barbour.** 1996. Conversion of a linear to a circular plasmid in the relapsing fever agent *Borrelia hermsii*. J Bacteriol **178:**793-800.
133. **Fernandez-Prada, C. M., E. B. Zelazowska, M. Nikolich, T. L. Hadfield, R. M. Roop, 2nd, G. L. Robertson, and D. L. Hoover.** 2003. Interactions between *Brucella melitensis* and human phagocytes: bacterial surface O-Polysaccharide inhibits phagocytosis, bacterial killing, and subsequent host cell apoptosis. Infect Immun **71:**2110-9.
134. **Fingerle, V., U. Hauser, G. Liegl, B. Petko, V. Preac-Mursic, and B. Wilske.** 1995. Expression of outer surface proteins A and C of *Borrelia burgdorferi* in *Ixodes ricinus*. J Clin Microbiol **33:**1867-9.
135. **Fingeroth, J. D., J. J. Weis, T. F. Tedder, J. L. Strominger, P. A. Biro, and D. T. Fearon.** 1984. Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. Proc Natl Acad Sci U S A **81:**4510-4.
136. **Fischer, J. R., K. T. LeBlanc, and J. M. Leong.** 2006. Fibronectin binding protein BBK32 of the Lyme disease spirochete promotes bacterial attachment to glycosaminoglycans. Infect Immun **74:**435-41.
137. **Fischer, J. R., N. Parveen, L. Magoun, and J. M. Leong.** 2003. Decorin-binding proteins A and B confer distinct mammalian cell type-specific attachment by *Borrelia burgdorferi*, the Lyme disease spirochete. Proc Natl Acad Sci U S A **100:**7307-12.
138. **Fisher, M. A., D. Grimm, A. K. Henion, A. F. Elias, P. E. Stewart, P. A. Rosa, and F. C. Gherardini.** 2005. *Borrelia burgdorferi* sigma54 is required for mammalian infection and vector transmission but not for tick colonization. Proc Natl Acad Sci U S A **102:**5162-7.
139. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, and et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496-512.
140. **Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, J. C. Venter, and et al.** 1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature **390:**580-6.
141. **Fraser, C. M., J. D. Gocayne, O. White, M. D. Adams, R. A. Clayton, R. D. Fleischmann, C. J. Bult, A. R. Kerlavage, G. Sutton, J. M. Kelley, R. D. Fritchman, J. F. Weidman, K. V. Small, M. Sandusky, J. Fuhrmann, D. Nguyen, T. R. Utterback, D. M. Saudek, C. A. Phillips, J. M. Merrick, J. F. Tomb, B. A. Dougherty, K. F. Bott, P. C. Hu, T. S. Lucier, S. N. Peterson, H. O. Smith, C. A. Hutchison, 3rd, and J. C. Venter.** 1995. The minimal gene complement of *Mycoplasma genitalium*. Science **270:**397-403.

142. **Fraser, C. M., S. J. Norris, G. M. Weinstock, O. White, G. G. Sutton, R. Dodson, M. Gwinn, E. K. Hickey, R. Clayton, K. A. Ketchum, E. Sodergren, J. M. Hardham, M. P. McLeod, S. Salzberg, J. Peterson, H. Khalak, D. Richardson, J. K. Howell, M. Chidambaram, T. Utterback, L. McDonald, P. Artiach, C. Bowman, M. D. Cotton, J. C. Venter, and et al.** 1998. Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. Science **281:**375-88.
143. **Fuchs, P. C., and A. A. Oyama.** 1969. Neonatal relapsing fever due to transplacental transmission of *Borrelia*. Jama **208:**690-2.
144. **Fukunaga, M., A. Hamase, K. Okada, and M. Nakao.** 1996. *Borrelia tanukii* sp. nov. and *Borrelia turdae* sp. nov. found from ixodid ticks in Japan: rapid species identification by 16S rRNA gene- targeted PCR analysis. Microbiol Immunol **40:**877-81.
145. **Fukunaga, M., K. Okada, M. Nakao, T. Konishi, and Y. Sato.** 1996. Phylogenetic analysis of *Borrelia* species based on flagellin gene sequences and its application for molecular typing of Lyme disease borreliae. Int J Syst Bacteriol **46:**898-905.
146. **Fukunaga, M., Y. Takahashi, Y. Tsuruta, O. Matsushita, D. Ralph, M. McClelland, and M. Nakao.** 1995. Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan. Int J Syst Bacteriol **45:**804-10.
147. **Fung, B. P., G. L. McHugh, J. M. Leong, and A. C. Steere.** 1994. Humoral immune response to outer surface protein C of *Borrelia burgdorferi* in Lyme disease: role of the immunoglobulin M response in the serodiagnosis of early infection. Infect Immun **62:**3213-21.
148. **Gallet, X., B. Charloteaux, A. Thomas, and R. Brasseur.** 2000. A fast method to predict protein interaction sites from sequences. J Mol Biol **302:**917-26.
149. **Gautam, A., M. Hathaway, N. McClain, G. Ramesh, and R. Ramamoorthy.** 2008. Analysis of the determinants of *bba64* (P35) gene expression in *Borrelia burgdorferi* using a gfp reporter. Microbiology **154:**275-85.
150. **Gawande, P. V., and M. W. Griffiths.** 2005. Effects of environmental stresses on the activities of the *uspA*, *grpE* and *rpoS* promoters of *Escherichia coli* O157:H7. Int J Food Microbiol **99:**91-8.
151. **Gebrehiwot, T., and A. Fiseha.** 1992. Tetracycline versus penicillin in the treatment of louse-borne relapsing fever. Ethiop Med J **30:**175-81.
152. **Gilbert, M. A., E. A. Morton, S. F. Bundle, and D. S. Samuels.** 2007. Artificial regulation of *ospC* expression in *Borrelia burgdorferi*. Mol Microbiol **63:**1259-73.
153. **Gilmore, R. D., Jr., R. R. Howison, V. L. Schmit, A. J. Nowalk, D. R. Clifton, C. Nolder, J. L. Hughes, and J. A. Carroll.** 2007. Temporal expression analysis of the *Borrelia burgdorferi* paralogous gene family 54 genes *bba64*, *bba65*, and *bba66* during persistent infection in mice. Infect Immun **75:**2753-2764.
154. **Gilmore, R. D., Jr., K. J. Kappel, and B. J. Johnson.** 1997. Molecular characterization of a 35-kilodalton protein of *Borrelia burgdorferi*, an antigen of diagnostic importance in early Lyme disease. J Clin Microbiol **35:**86-91.
155. **Ginsberg, H. S., and C. P. Ewing.** 1989. Habitat distribution of *Ixodes dammini* (Acari: Ixodidae) an Lyme disease spirochetes on Fire Island, New York. J Med Entomol **26:**183-9.
156. **Glatz, M., M. Golestani, H. Kerl, and R. R. Mullegger.** 2006. Clinical relevance of different IgG and IgM serum antibody responses to *Borrelia burgdorferi* after antibiotic

therapy for erythema migrans: long-term follow-up study of 113 patients. Arch Dermatol **142:**862-8.
157. **Glockner, G., R. Lehmann, A. Romualdi, S. Pradella, U. Schulte-Spechtel, M. Schilhabel, B. Wilske, J. Suhnel, and M. Platzer.** 2004. Comparative analysis of the *Borrelia garinii* genome. Nucleic Acids Res **32:**6038-46.
158. **Glockner, G., U. Schulte-Spechtel, M. Schilhabel, M. Felder, J. Suhnel, B. Wilske, and M. Platzer.** 2006. Comparative genome analysis: selection pressure on the *Borrelia vls* cassettes is essential for infectivity. BMC Genomics **7:**211.
159. **Golde, W. T., and M. C. Dolan.** 1995. Variation in antigenicity and infectivity of derivatives of *Borrelia burgdorferi*, strain B31, maintained in the natural, zoonotic cycle compared with maintenance in culture. Infect Immun **63:**4795-801.
160. **Goldoft, M. J., T. L. Schulze, W. E. Parkin, and R. A. Gunn.** 1990. Lyme disease in New Jersey. N J Med **87:**579-84.
161. **Goodman, J. L., J. F. Bradley, A. E. Ross, P. Goellner, A. Lagus, B. Vitale, B. W. Berger, S. Luger, and R. C. Johnson.** 1995. Bloodstream invasion in early Lyme disease: results from a prospective, controlled, blinded study using the polymerase chain reaction [published erratum appears in Am J Med 1996 Aug;101(2):239]. Am J Med **99:**6-12.
162. **Goubau, P. F.** 1984. Relapsing fevers. A review. Ann Soc Belg Med Trop **64:**335-64.
163. **Grab, D. J., C. Givens, and R. Kennedy.** 1998. Fibronectin-binding activity in Borrelia burgdorferi1. Biochim Biophys Acta **1407:**135-45.
164. **Gray, J.** 1999. Risk assessment in Lyme borreliosis. Wien Klin Wochenschr **111:**990-3.
165. **Gray, J. S., O. Kahl, C. Janetzki, and J. Stein.** 1992. Studies on the ecology of Lyme disease in a deer forest in County Galway, Ireland. J Med Entomol **29:**915-20.
166. **Gray, J. S., O. Kahl, J. N. Robertson, M. Daniel, A. Estrada-Pena, G. Gettinby, T. G. Jaenson, P. Jensen, F. Jongejan, E. Korenberg, K. Kurtenbach, and P. Zeman.** 1998. Lyme borreliosis habitat assessment. Zentralbl Bakteriol **287:**211-28.
167. **Grimm, D., C. H. Eggers, M. J. Caimano, K. Tilly, P. E. Stewart, A. F. Elias, J. D. Radolf, and P. A. Rosa.** 2004. Experimental assessment of the roles of linear plasmids lp25 and lp28-1 of *Borrelia burgdorferi* throughout the infectious cycle. Infect Immun **72:**5938-46.
168. **Grimm, D., A. F. Elias, K. Tilly, and P. A. Rosa.** 2003. Plasmid stability during in vitro propagation of *Borrelia burgdorferi* assessed at a clonal level. Infect Immun **71:**3138-45.
169. **Grimm, D., K. Tilly, R. Byram, P. E. Stewart, J. G. Krum, D. M. Bueschel, T. G. Schwan, P. F. Policastro, A. F. Elias, and P. A. Rosa.** 2004. Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. Proc Natl Acad Sci U S A **101:**3142-7.
170. **Gross, D. M., T. Forsthuber, M. Tary-Lehmann, C. Etling, K. Ito, Z. A. Nagy, J. A. Field, A. C. Steere, and B. T. Huber.** 1998. Identification of LFA-1 as a candidate autoantigen in treatment- resistant Lyme arthritis. Science **281:**703-6.
171. **Guerau-de-Arellano, M., and B. T. Huber.** 2002. Development of autoimmunity in Lyme arthritis. Curr Opin Rheumatol **14:**388-93.
172. **Guilherme, L., J. Kalil, and M. Cunningham.** 2006. Molecular mimicry in the autoimmune pathogenesis of rheumatic heart disease. Autoimmunity **39:**31-9.
173. **Guo, B. P., E. L. Brown, D. W. Dorward, L. C. Rosenberg, and M. Hook.** 1998. Decorin-binding adhesins from *Borrelia burgdorferi*. Mol Microbiol **30:**711-23.

174. **Guo, B. P., S. J. Norris, L. C. Rosenberg, and M. Hook.** 1995. Adherence of *Borrelia burgdorferi* to the proteoglycan decorin. Infect Immun **63:**3467-72.
175. **Guy, E. C., and G. Stanek.** 1991. Detection of *Borrelia burgdorferi* in patients with Lyme disease by the polymerase chain reaction. J Clin Pathol **44:**610-1.
176. **Gygi, S. P., Y. Rochon, B. R. Franza, and R. Aebersold.** 1999. Correlation between protein and mRNA abundance in yeast. Mol Cell Biol **19:**1720-30.
177. **Haddad, F., G. Berry, R. L. Doyle, P. Martineau, T. K. Leung, and N. Racine.** 2007. Active bacterial myocarditis: a case report and review of the literature. J Heart Lung Transplant **26:**745-9.
178. **Hagman, K. E., P. Lahdenne, T. G. Popova, S. F. Porcella, D. R. Akins, J. D. Radolf, and M. V. Norgard.** 1998. Decorin-binding protein of *Borrelia burgdorferi* is encoded within a two- gene operon and is protective in the murine model of Lyme borreliosis. Infect Immun **66:**2674-83.
179. **Halperin, J. J., L. B. Krupp, M. G. Golightly, and D. J. Volkman.** 1990. Lyme borreliosis-associated encephalopathy. Neurology **40:**1340-3.
180. **Halperin, J. J., B. J. Luft, A. K. Anand, C. T. Roque, O. Alvarez, D. J. Volkman, and R. J. Dattwyler.** 1989. Lyme neuroborreliosis: central nervous system manifestations. Neurology **39:**753-9.
181. **Hammers-Berggren, S., A. M. Lebech, M. Karlsson, U. Andersson, K. Hansen, and G. Stiernstedt.** 1994. Serological follow-up after treatment of *Borrelia arthritis* and acrodermatitis chronica atrophicans. Scand J Infect Dis **26:**339-47.
182. **Hammers-Berggren, S., A. M. Lebech, M. Karlsson, B. Svenungsson, K. Hansen, and G. Stiernstedt.** 1994. Serological follow-up after treatment of patients with erythema migrans and neuroborreliosis. J Clin Microbiol **32:**1519-25.
183. **He, M., B. K. Boardman, D. Yan, and X. F. Yang.** 2007. Regulation of expression of the fibronectin-binding protein BBK32 in *Borrelia burgdorferi*. J Bacteriol **189:**8377-80.
184. **Heiskanen, P., S. Taira, and M. Rhen.** 1994. Role of *rpoS* in the regulation of *Salmonella* plasmid virulence (*spv*) genes. FEMS Microbiol Lett **123:**125-30.
185. **Hellman, J., J. D. Roberts, Jr., M. M. Tehan, J. E. Allaire, and H. S. Warren.** 2002. Bacterial peptidoglycan-associated lipoprotein is released into the bloodstream in gram-negative sepsis and causes inflammation and death in mice. J Biol Chem **277:**14274-80.
186. **Hellwage, J., T. Meri, T. Heikkila, A. Alitalo, J. Panelius, P. Lahdenne, I. J. Seppala, and S. Meri.** 2001. The complement regulator factor H binds to the surface protein OspE of *Borrelia burgdorferi*. J Biol Chem **276:**8427-35.
187. **Hemmer, B., B. Gran, Y. Zhao, A. Marques, J. Pascal, A. Tzou, T. Kondo, I. Cortese, B. Bielekova, S. E. Straus, H. F. McFarland, R. Houghten, R. Simon, C. Pinilla, and R. Martin.** 1999. Identification of candidate T-cell epitopes and molecular mimics in chronic Lyme disease. Nat Med **5:**1375-1382.
188. **Hengge-Aronis, R.** 1996. Back to log phase: sigma S as a global regulator in the osmotic control of gene expression in *Escherichia coli*. Mol Microbiol **21:**887-93.
189. **Hengge-Aronis, R.** 1993. Survival of hunger and stress: the role of rpoS in early stationary phase gene regulation in *E. coli*. Cell **72:**165-8.
190. **Hengge-Aronis, R., and D. Fischer.** 1992. Identification and molecular analysis of *glgS*, a novel growth-phase-regulated and *rpoS*-dependent gene involved in glycogen synthesis in *Escherichia coli*. Mol Microbiol **6:**1877-86.

191. **Hinnebusch, B. J., A. G. Barbour, B. I. Restrepo, and T. G. Schwan.** 1998. Population structure of the relapsing fever spirochete *Borrelia hermsii* as indicated by polymorphism of two multigene families that encode immunogenic outer surface lipoproteins. Infect Immun **66:**432-40.
192. **Hodzic, E., S. Feng, K. J. Freet, D. L. Borjesson, and S. W. Barthold.** 2002. *Borrelia burgdorferi* population kinetics and selected gene expression at the host-vector interface. Infect Immun **70:**3382-8.
193. **Horstmann, R. D., H. J. Sievertsen, J. Knobloch, and V. A. Fischetti.** 1988. Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. Proc Natl Acad Sci U S A **85:**1657-61.
194. **Houhamdi, L., and D. Raoult.** 2005. Excretion of living *Borrelia recurrentis* in feces of infected human body lice. J Infect Dis **191:**1898-906.
195. **Hovind-Hougen, K.** 1984. Ultrastructure of spirochetes isolated from *Ixodes ricinus* and *Ixodes dammini*. Yale J Biol Med **57:**543-8.
196. **Hovis, K. M., J. V. McDowell, L. Griffin, and R. T. Marconi.** 2004. Identification and characterization of a linear-plasmid-encoded factor H-binding protein (FhbA) of the relapsing fever spirochete *Borrelia hermsii*. J Bacteriol **186:**2612-8.
197. **Hovis, K. M., E. Tran, C. M. Sundy, E. Buckles, J. V. McDowell, and R. T. Marconi.** 2006. Selective binding of *Borrelia burgdorferi* OspE paralogs to factor H and serum proteins from diverse animals: possible expansion of the role of OspE in Lyme disease pathogenesis. Infect Immun **74:**1967-72.
198. **Hovius, J. W., A. P. van Dam, and E. Fikrig.** 2007. Tick-host-pathogen interactions in Lyme borreliosis. Trends Parasitol **23:**434-8.
199. **Hu, L. T., S. D. Pratt, G. Perides, L. Katz, R. A. Rogers, and M. S. Klempner.** 1997. Isolation, cloning, and expression of a 70-kilodalton plasminogen binding protein of *Borrelia burgdorferi*. Infect Immun **65:**4989-95.
200. **Hubner, A., X. Yang, D. M. Nolen, T. G. Popova, F. C. Cabello, and M. V. Norgard.** 2001. Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN-RpoS regulatory pathway. Proc Natl Acad Sci U S A **98:**12724-9.
201. **Hughes, C. A., and R. C. Johnson.** 1990. Methylated DNA in *Borrelia* species. J Bacteriol **172:**6602-4.
202. **Hurme, R., K. D. Berndt, E. Namork, and M. Rhen.** 1996. DNA binding exerted by a bacterial gene regulator with an extensive coiled-coil domain. J Biol Chem **271:**12626-31.
203. **Hyde, F. W., and R. C. Johnson.** 1984. Genetic relationship of lyme disease spirochetes to *Borrelia*, *Treponema*, and *Leptospira* spp. J Clin Microbiol **20:**151-4.
204. **Hyde, J. A., J. P. Trzeciakowski, and J. T. Skare.** 2007. *Borrelia burgdorferi* alters its gene expression and antigenic profile in response to CO2 levels. J Bacteriol **189:**437-45.
205. **Indest, K. J., R. Ramamoorthy, M. Sole, R. D. Gilmore, B. J. Johnson, and M. T. Philipp.** 1997. Cell-density-dependent expression of *Borrelia burgdorferi* lipoproteins in vitro. Infect Immun **65:**1165-71.
206. **Iriarte, M., I. Stainier, and G. R. Cornelis.** 1995. The *rpoS* gene from *Yersinia enterocolitica* and its influence on expression of virulence factors. Infect Immun **63:**1840-7.

207. **Jacks-Weis, J., Y. Kim, and P. P. Cleary.** 1982. Restricted deposition of C3 on M+ group A streptococci: correlation with resistance to phagocytosis. J Immunol **128:**1897-902.
208. **Jacobs, M. B., S. J. Norris, K. M. Phillippi-Falkenstein, and M. T. Philipp.** 2006. Infectivity of the Highly Transformable BBE02- lp56- Mutant of *Borrelia burgdorferi*, the Lyme Disease Spirochete, via Ticks. Infect Immun **74:**3678-81.
209. **Johnson, R. C., F. W. Hyde, and C. M. Rumpel.** 1984. Taxonomy of the Lyme disease spirochetes. Yale J Biol Med **57:**529-37.
210. **Johnson, R. C., G. P. Schmid, F. W. Hyde, A. G. Steigerwalt, and D. J. Brenner.** 1984. *Borrelia burgdorferi* sp. nov.: Etiologic agent of Lyme disease. Int J Syst Bacteriol **34:**496-497.
211. **Juncker, A. S., H. Willenbrock, G. Von Heijne, S. Brunak, H. Nielsen, and A. Krogh.** 2003. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci **12:**1652-62.
212. **Kalish, R. A., R. F. Kaplan, E. Taylor, L. Jones-Woodward, K. Workman, and A. C. Steere.** 2001. Evaluation of study patients with Lyme disease, 10-20-year follow-up. J Infect Dis **183:**453-60.
213. **Kalish, R. A., G. McHugh, J. Granquist, B. Shea, R. Ruthazer, and A. C. Steere.** 2001. Persistence of Immunoglobulin M or Immunoglobulin G Antibody Responses to *Borrelia burgdorferi* 10-20 Years after Active Lyme Disease. Clin Infect Dis **33:**780-5.
214. **Kaplan, R. F., L. Jones-Woodward, K. Workman, A. C. Steere, E. L. Logigian, and M. E. Meadows.** 1999. Neuropsychological deficits in Lyme disease patients with and without other evidence of central nervous system pathology. Appl Neuropsychol **6:**3-11.
215. **Karlsson, M., K. Hovind-Hougen, B. Svenungsson, and G. Stiernstedt.** 1990. Cultivation and characterization of spirochetes from cerebrospinal fluid of patients with Lyme borreliosis. J Clin Microbiol **28:**473-9.
216. **Kawabata, H., T. Masuzawa, and Y. Yanagihara.** 1993. Genomic analysis of *Borrelia japonica* sp. nov. isolated from *Ixodes ovatus* in Japan. Microbiol Immunol **37:**843-8.
217. **Kawabata, H., S. J. Norris, and H. Watanabe.** 2004. BBE02 disruption mutants of *Borrelia burgdorferi* B31 have a highly transformable, infectious phenotype. Infect Immun **72:**7147-54.
218. **Kazmierczak, M. J., M. Wiedmann, and K. J. Boor.** 2005. Alternative sigma factors and their roles in bacterial virulence. Microbiol Mol Biol Rev **69:**527-43.
219. **Keller, T. L., J. J. Halperin, and M. Whitman.** 1992. PCR detection of *Borrelia burgdorferi* DNA in cerebrospinal fluid of Lyme neuroborreliosis patients. Neurology **42:**32-42.
220. **Kelly, R.** 1971. Cultivation of *Borrelia hermsi*. Science **173:**443-4.
221. **Kimball, S. A., P. A. Janson, and P. J. LaRaia.** 1989. Complete heart block as the sole presentation of Lyme disease. Arch Intern Med **149:**1897-8.
222. **Kindstrand, E., B. Y. Nilsson, A. Hovmark, R. Pirskanen, and E. Asbrink.** 1997. Peripheral neuropathy in acrodermatitis chronica atrophicans - a late *Borrelia* manifestation. Acta Neurol Scand **95:**338-45.
223. **Kisinza, W. N., P. J. McCall, H. Mitani, A. Talbert, and M. Fukunaga.** 2003. A newly identified tick-borne *Borrelia* species and relapsing fever in Tanzania. Lancet **362:**1283-4.

224. **Klempner, M. S., and B. T. Huber.** 1999. Is it thee or me?--autoimmunity in Lyme disease. Nat Med **5:**1346-7.
225. **Klose, K. E., and J. J. Mekalanos.** 1998. Distinct roles of an alternative sigma factor during both free-swimming and colonizing phases of the *Vibrio cholerae* pathogenic cycle. Mol Microbiol **28:**501-20.
226. **Konik, P., V. Slavikova, J. Salat, J. Reznickova, E. Dvoroznakova, and J. Kopecky.** 2006. Anti-tumour necrosis factor-alpha activity in *Ixodes ricinus* saliva. Parasite Immunol **28:**649-56.
227. **Kopecky, J., M. Kuthejlova, and J. Pechova.** 1999. Salivary gland extract from *Ixodes ricinus* ticks inhibits production of interferon-gamma by the upregulation of interleukin-10. Parasite Immunol **21:**351-6.
228. **Kotsyfakis, M., A. Sa-Nunes, I. M. Francischetti, T. N. Mather, J. F. Andersen, and J. M. Ribeiro.** 2006. Antiinflammatory and immunosuppressive activity of sialostatin L, a salivary cystatin from the tick *Ixodes scapularis*. J Biol Chem **281:**26298-307.
229. **Kowarz, L., C. Coynault, V. Robbe-Saule, and F. Norel.** 1994. The *Salmonella typhimurium katF* (*rpoS*) gene: cloning, nucleotide sequence, and regulation of *spvR* and *spvABCD* virulence plasmid genes. J Bacteriol **176:**6852-60.
230. **Kraiczy, P., J. Hellwage, C. Skerka, H. Becker, M. Kirschfink, M. M. Simon, V. Brade, P. F. Zipfel, and R. Wallich.** 2004. Complement resistance of *Borrelia burgdorferi* correlates with the expression of BbCRASP-1, a novel linear plasmid-encoded surface protein that interacts with human factor H and FHL-1 and is unrelated to Erp proteins. J Biol Chem **279:**2421-9.
231. **Kraiczy, P., J. Hellwage, C. Skerka, M. Kirschfink, V. Brade, P. F. Zipfel, and R. Wallich.** 2003. Immune evasion of Borrelia burgdorferi: mapping of a complement-inhibitor factor H-binding site of BbCRASP-3, a novel member of the Erp protein family. Eur J Immunol **33:**697-707.
232. **Kraiczy, P., E. Rossmann, V. Brade, M. M. Simon, C. Skerka, P. F. Zipfel, and R. Wallich.** 2006. Binding of human complement regulators FHL-1 and factor H to CRASP-1 orthologs of *Borrelia burgdorferi*. Wien Klin Wochenschr **118:**669-76.
233. **Kraiczy, P., C. Skerka, V. Brade, and P. F. Zipfel.** 2001. Further characterization of complement regulator-acquiring surface proteins of *Borrelia burgdorferi*. Infect Immun **69:**7800-9.
234. **Kraiczy, P., C. Skerka, M. Kirschfink, V. Brade, and P. F. Zipfel.** 2001. Immune evasion of *Borrelia burgdorferi* by acquisition of human complement regulators FHL-1/reconectin and Factor H. Eur J Immunol **31:**1674-84.
235. **Kraiczy, P., C. Skerka, M. Kirschfink, P. F. Zipfel, and V. Brade.** 2001. Mechanism of complement resistance of pathogenic *Borrelia burgdorferi* isolates. Int Immunopharmacol **1:**393-401.
236. **Kristoferitsch, W., E. Sluga, M. Graf, H. Partsch, R. Neumann, G. Stanek, and H. Budka.** 1988. Neuropathy associated with acrodermatitis chronica atrophicans. Clinical and morphological features. Ann N Y Acad Sci **539:**35-45.
237. **Kurtenbach, K., A. Dizij, H. M. Seitz, G. Margos, S. E. Moter, M. D. Kramer, R. Wallich, U. E. Schaible, and M. M. Simon.** 1994. Differential immune responses to *Borrelia burgdorferi* in European wild rodent species influence spirochete transmission to *Ixodes ricinus* L. (Acari: Ixodidae). Infect Immun **62:**5344-52.

238. **Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss.** 1989. Expression of sigma 54 (ntrA)-dependent genes is probably united by a common mechanism. Microbiol Rev **53:**367-76.
239. **Kwon, O., D. Georgellis, and E. C. Lin.** 2003. Rotational on-off switching of a hybrid membrane sensor kinase Tar-ArcB in *Escherichia coli*. J Biol Chem **278:**13192-5.
240. **Labandeira-Rey, M., J. Seshu, and J. T. Skare.** 2003. The absence of linear plasmid 25 or 28-1 of *Borrelia burgdorferi* dramatically alters the kinetics of experimental infection via distinct mechanisms. Infect Immun **71:**4608-13.
241. **Labandeira-Rey, M., and J. T. Skare.** 2001. Decreased infectivity in *Borrelia burgdorferi* strain B31 is associated with loss of linear plasmid 25 or 28-1. Infect Immun **69:**446-55.
242. **Lam, T. T., T. P. Nguyen, R. R. Montgomery, F. S. Kantor, E. Fikrig, and R. A. Flavell.** 1994. Outer surface proteins E and F of *Borrelia burgdorferi*, the agent of Lyme disease. Infect Immun **62:**290-8.
243. **Landbo, A. S., and P. T. Flong.** 1992. *Borrelia burgdorferi* infection in *Ixodes ricinus* from habitats in Denmark. Med Vet Entomol **6:**165-7.
244. **Lane, R. S., and J. E. Loye.** 1989. Lyme disease in California: interrelationship of *Ixodes pacificus* (Acari: Ixodidae), the western fence lizard (*Sceloporus occidentalis*), and *Borrelia burgdorferi*. J Med Entomol **26:**272-8.
245. **Lane, R. S., and J. A. Pascocello.** 1989. Antigenic characteristics of *Borrelia burgdorferi* isolates from ixodid ticks in California. J Clin Microbiol **27:**2344-9.
246. **Lane, R. S., J. Piesman, and W. Burgdorfer.** 1991. Lyme borreliosis: relation of its causative agent to its vectors and hosts in North America and Europe. Annu Rev Entomol **36:**587-609.
247. **Lane, R. S., and H. A. Stubbs.** 1990. Host-seeking behavior of adult *Ixodes pacificus* (Acari: Ixodidae) as determined by flagging vegetation. J Med Entomol **27:**282-7.
248. **Lange, R., and R. Hengge-Aronis.** 1994. The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. Genes Dev **8:**1600-12.
249. **Lawrenz, M. B., R. M. Wooten, and S. J. Norris.** 2004. Effects of *vlsE* complementation on the infectivity of *Borrelia burgdorferi* lacking the linear plasmid lp28-1. Infect Immun **72:**6577-85.
250. **Le Fleche, A., D. Postic, K. Girardet, O. Peter, and G. Baranton.** 1997. Characterization of *Borrelia lusitaniae* sp. nov. by 16S ribosomal DNA sequence analysis. Int J Syst Bacteriol **47:**921-5.
251. **Lebech, A. M., and K. Hansen.** 1992. Detection of *Borrelia burgdorferi* DNA in urine samples and cerebrospinal fluid samples from patients with early and late Lyme neuroborreliosis by polymerase chain reaction. J Clin Microbiol **30:**1646-53.
252. **Leboulle, G., M. Crippa, Y. Decrem, N. Mejri, M. Brossard, A. Bollen, and E. Godfroid.** 2002. Characterization of a novel salivary immunosuppressive protein from *Ixodes ricinus* ticks. J Biol Chem **277:**10083-9.
253. **Lee, I. S., J. Lin, H. K. Hall, B. Bearson, and J. W. Foster.** 1995. The stationary-phase sigma factor sigma S (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. Mol Microbiol **17:**155-67.
254. **Levine, J. F., M. L. Wilson, and A. Spielman.** 1985. Mice as reservoirs of the Lyme disease spirochete. Am J Trop Med Hyg **34:**355-60.

255. **Li, X., X. Liu, D. S. Beck, F. S. Kantor, and E. Fikrig.** 2006. *Borrelia burgdorferi* Lacking BBK32, a Fibronectin-Binding Protein, Retains Full Pathogenicity. Infect Immun **74:**3305-13.
256. **Liang, F. T., E. L. Brown, T. Wang, R. V. Iozzo, and E. Fikrig.** 2004. Protective niche for *Borrelia burgdorferi* to evade humoral immunity. Am J Pathol **165:**977-85.
257. **Liang, F. T., F. K. Nelson, and E. Fikrig.** 2002. DNA microarray assessment of putative *Borrelia burgdorferi* lipoprotein genes. Infect Immun **70:**3300-3.
258. **Liang, F. T., F. K. Nelson, and E. Fikrig.** 2002. Molecular adaptation of *Borrelia burgdorferi* in the murine host. J Exp Med **196:**275-80.
259. **Liebling, M. R., M. J. Nishio, A. Rodriguez, L. H. Sigal, T. Jin, and J. S. Louie.** 1993. The polymerase chain reaction for the detection of *Borrelia burgdorferi* in human body fluids. Arthritis Rheum **36:**665-75.
260. **Lin, B., S. A. Short, M. Eskildsen, M. S. Klempner, and L. T. Hu.** 2001. Functional testing of putative oligopeptide permease (Opp) proteins of *Borrelia burgdorferi*: a complementation model in *opp*(-) *Escherichia coli*. Biochim Biophys Acta **1499:**222-31.
261. **Lindberg, A. A., A. Karnell, and A. Weintraub.** 1991. The lipopolysaccharide of *Shigella* bacteria as a virulence factor. Rev Infect Dis **13 Suppl 4:**S279-84.
262. **Livengood, J. A., V. L. Schmit, and R. D. Gilmore, Jr.** 2008. Global transcriptome analysis of *Borrelia burgdorferi* during association with human neuroglial cells. Infect Immun **76:**298-307.
263. **Liveris, D., S. Varde, R. Iyer, S. Koenig, S. Bittker, D. Cooper, D. McKenna, J. Nowakowski, R. B. Nadelman, G. P. Wormser, and I. Schwartz.** 1999. Genetic diversity of *Borrelia burgdorferi* in lyme disease patients as determined by culture versus direct PCR with clinical specimens. J Clin Microbiol **37:**565-9.
264. **Liveris, D., G. Wang, G. Girao, D. W. Byrne, J. Nowakowski, D. McKenna, R. Nadelman, G. P. Wormser, and I. Schwartz.** 2002. Quantitative detection of *Borrelia burgdorferi* in 2-millimeter skin samples of erythema migrans lesions: correlation of results with clinical and laboratory findings. J Clin Microbiol **40:**1249-53.
265. **Logigian, E. L., R. F. Kaplan, and A. C. Steere.** 1990. Chronic neurologic manifestations of Lyme disease. N Engl J Med **323:**1438-44.
266. **Ma, B., T. Elkayam, H. Wolfson, and R. Nussinov.** 2003. Protein-protein interactions: structurally conserved residues distinguish between binding sites and exposed protein surfaces. Proc Natl Acad Sci U S A **100:**5772-7.
267. **Ma, Y., K. P. Seiler, E. J. Eichwald, J. H. Weis, C. Teuscher, and J. J. Weis.** 1998. Distinct characteristics of resistance to *Borrelia burgdorferi*-induced arthritis in C57BL/6N mice. Infect Immun **66:**161-8.
268. **Marconi, R. T., D. S. Samuels, and C. F. Garon.** 1993. Transcriptional analyses and mapping of the *ospC* gene in Lyme disease spirochetes. J Bacteriol **175:**926-32.
269. **Marconi, R. T., D. S. Samuels, T. G. Schwan, and C. F. Garon.** 1993. Identification of a protein in several *Borrelia* species which is related to OspC of the Lyme disease spirochetes. J Clin Microbiol **31:**2577-83.
270. **Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem **87:**206-10.
271. **Maruo, S., L. Yang, and K. Takada.** 2001. Roles of Epstein-Barr virus glycoproteins gp350 and gp25 in the infection of human epithelial cells. J Gen Virol **82:**2373-83.

272. **Maruskova, M., M. D. Esteve-Gassent, V. L. Sexton, and J. Seshu.** 2007. The role of *bba64* locus of *Borrelia burgdorferi* in early stages of infectivity in the murine model of Lyme disease. Infect Immun **76:**391-402.
273. **Masuzawa, T.** 2004. Terrestrial Distribution of the Lyme Borreliosis Agent *Borrelia burgdorferi* Sensu Lato in East Asia. Jpn J Infect Dis **57:**229-35.
274. **Masuzawa, T., T. Kurita, H. Kawabata, and Y. Yanagihara.** 1994. Relationship between infectivity and OspC expression in Lyme disease *Borrelia*. FEMS Microbiol Lett **123:**319-24.
275. **Masuzawa, T., Y. Okada, Y. Yanagihara, and N. Sato.** 1991. Antigenic properties of *Borrelia burgdorferi* isolated from *Ixodes ovatus* and *Ixodes persulcatus* in Hokkaido, Japan [published erratum appears in J Clin Microbiol 1992 Mar;30(3):755]. J Clin Microbiol **29:**1568-73.
276. **Masuzawa, T., N. Takada, M. Kudeken, T. Fukui, Y. Yano, F. Ishiguro, Y. Kawamura, Y. Imai, and T. Ezaki.** 2001. *Borrelia sinica* sp. nov., a lyme disease-related *Borrelia* species isolated in China. Int J Syst Evol Microbiol **51:**1817-24.
277. **Mathiesen, D. A., J. H. Oliver, Jr., C. P. Kolbert, E. D. Tullson, B. J. Johnson, G. L. Campbell, P. D. Mitchell, K. D. Reed, S. R. Telford, 3rd, J. F. Anderson, R. S. Lane, and D. H. Persing.** 1997. Genetic heterogeneity of *Borrelia burgdorferi* in the United States. J Infect Dis **175:**98-107.
278. **Matuschka, F. R., P. Fischer, M. Heiler, S. Blumcke, and A. Spielman.** 1992. Stage-associated risk of transmission of the Lyme disease spirochete by European *Ixodes* ticks. Parasitol Res **78:**695-8.
279. **Maupin, G. O., D. Fish, J. Zultowsky, E. G. Campos, and J. Piesman.** 1991. Landscape ecology of Lyme disease in a residential area of Westchester County, New York. Am J Epidemiol **133:**1105-13.
280. **Maxwell, S. S., T. A. Stoklasek, Y. Dash, K. R. Macaluso, and S. K. Wikel.** 2005. Tick modulation of the in-vitro expression of adhesion molecules by skin-derived endothelial cells. Ann Trop Med Parasitol **99:**661-72.
281. **Mayer, W., F. X. Kleber, B. Wilske, V. Preac-Mursic, W. Maciejewski, H. Sigl, E. Holzer, and W. Doering.** 1990. Persistent atrioventricular block in Lyme borreliosis. Klin Wochenschr **68:**431-5.
282. **McAlister, H. F., P. T. Klementowicz, C. Andrews, J. D. Fisher, M. Feld, and S. Furman.** 1989. Lyme carditis: an important cause of reversible heart block. Ann Intern Med **110:**339-45.
283. **McCarthy, R. E., 3rd, J. P. Boehmer, R. H. Hruban, G. M. Hutchins, E. K. Kasper, J. M. Hare, and K. L. Baughman.** 2000. Long-term outcome of fulminant myocarditis as compared with acute (nonfulminant) myocarditis. N Engl J Med **342:**690-5.
284. **McConnell, J.** 2003. Tick-borne relapsing fever under-reported. Lancet Infect Dis **3:**604.
285. **McDowell, J. V., M. E. Harlin, E. A. Rogers, and R. T. Marconi.** 2005. Putative coiled-coil structural elements of the BBA68 protein of Lyme disease spirochetes are required for formation of its factor H binding site. J Bacteriol **187:**1317-23.
286. **McDowell, J. V., J. Wolfgang, L. Senty, C. M. Sundy, M. J. Noto, and R. T. Marconi.** 2004. Demonstration of the involvement of outer surface protein e coiled coil structural domains and higher order structural elements in the binding of infection-induced antibody and the complement-regulatory protein, factor H1. J Immunol **173:**7471-80.

287. **McSweegan, E.** 2006. Lyme vaccine demonized by advocacy groups. Nature **440:**278.
288. **Meek, J. I., C. L. Roberts, E. V. Smith, Jr., and M. L. Cartter.** 1996. Underreporting of Lyme disease by Connecticut physicians, 1992. J Public Health Manag Pract **2:**61-5.
289. **Meier, J. T., M. I. Simon, and A. G. Barbour.** 1985. Antigenic variation is associated with DNA rearrangements in a relapsing fever *Borrelia*. Cell **41:**403-9.
290. **Mejlon, H. A., and T. G. Jaenson.** 1993. Seasonal prevalence of *Borrelia burgdorferi* in *Ixodes ricinus* in different vegetation types in Sweden. Scand J Infect Dis **25:**449-56.
291. **Mekasha, A., and S. Meharie.** 1996. Outbreak of louse-borne relapsing fever in Jimma, south western Ethiopia. East Afr Med J **73:**54-8.
292. **Melski, J. W., K. D. Reed, P. D. Mitchell, and G. D. Barth.** 1993. Primary and secondary erythema migrans in central Wisconsin. Arch Dermatol **129:**709-16.
293. **Millner, M. M., R. R. Mullegger, K. D. Spork, and G. Stanek.** 1991. Lyme borreliosis of central nervous system (CNS) in children: a diagnostic challenge. Infection **19:**273-8.
294. **Montgomery, R. R., D. Lusitani, A. De Boisfleury Chevance, and S. E. Malawista.** 2004. Tick saliva reduces adherence and area of human neutrophils. Infect Immun **72:**2989-94.
295. **Montgomery, R. R., S. E. Malawista, K. J. Feen, and L. K. Bockenstedt.** 1996. Direct demonstration of antigenic substitution of *Borrelia burgdorferi* ex vivo: exploration of the paradox of the early immune response to outer surface proteins A and C in Lyme disease. J Exp Med **183:**261-9.
296. **Motaleb, M. A., L. Corum, J. L. Bono, A. F. Elias, P. Rosa, D. S. Samuels, and N. W. Charon.** 2000. *Borrelia burgdorferi* periplasmic flagella have both skeletal and motility functions. Proc Natl Acad Sci U S A **97:**10899-10904.
297. **Muffler, A., D. D. Traulsen, R. Lange, and R. Hengge-Aronis.** 1996. Posttranscriptional osmotic regulation of the sigma(s) subunit of RNA polymerase in *Escherichia coli*. J Bacteriol **178:**1607-13.
298. **Mullegger, R. R.** 2004. Dermatological manifestations of Lyme borreliosis. Eur J Dermatol **14:**296-309.
299. **Munk, P. S., S. Orn, and A. I. Larsen.** 2007. Lyme carditis: persistent local delayed enhancement by cardiac magnetic resonance imaging. Int J Cardiol **115:**e108-10.
300. **Nadelman, R. B., J. Nowakowski, G. Forseter, N. S. Goldberg, S. Bittker, D. Cooper, M. Aguero-Rosenfeld, and G. P. Wormser.** 1996. The clinical spectrum of early Lyme borreliosis in patients with culture-confirmed erythema migrans. Am J Med **100:**502-8.
301. **Neufert, C., R. K. Pai, E. H. Noss, M. Berger, W. H. Boom, and C. V. Harding.** 2001. Mycobacterium tuberculosis 19-kDa lipoprotein promotes neutrophil activation. J Immunol **167:**1542-9.
302. **Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng **10:**1-6.
303. **Nigrovic, L. E., and K. M. Thompson.** 2007. The Lyme vaccine: a cautionary tale. Epidemiol Infect **135:**1-8.
304. **Nocton, J. J., F. Dressler, B. J. Rutledge, P. N. Rys, D. H. Persing, and A. C. Steere.** 1994. Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. N Engl J Med **330:**229-34.

305. **Norris, S. J., C. J. Carter, J. K. Howell, and A. G. Barbour.** 1992. Low-passage-associated proteins of *Borrelia burgdorferi* B31: characterization and molecular cloning of OspD, a surface-exposed, plasmid-encoded lipoprotein. Infect Immun **60:**4662-72.
306. **Norris, S. J., J. K. Howell, S. A. Garza, M. S. Ferdows, and A. G. Barbour.** 1995. High- and low-infectivity phenotypes of clonal populations of in vitro- cultured *Borrelia burgdorferi*. Infect Immun **63:**2206-12.
307. **Nowakowski, J., I. Schwartz, D. Liveris, G. Wang, M. E. Aguero-Rosenfeld, G. Girao, D. McKenna, R. B. Nadelman, L. F. Cavaliere, and G. P. Wormser.** 2001. Laboratory diagnostic techniques for patients with early Lyme disease associated with erythema migrans: a comparison of different techniques. Clin Infect Dis **33:**2023-7.
308. **Nowalk, A. J., R. D. Gilmore, Jr., and J. A. Carroll.** 2006. Serologic proteome analysis of *Borrelia burgdorferi* membrane-associated proteins. Infect Immun **74:**3864-73.
309. **Nowalk, A. J., C. Nolder, D. R. Clifton, and J. A. Carroll.** 2006. Comparative proteome analysis of subcellular fractions from *Borrelia burgdorferi* by NEPHGE and IPG. Proteomics **6:**2121-2134.
310. **Obert, C., J. Sublett, D. Kaushal, E. Hinojosa, T. Barton, E. I. Tuomanen, and C. J. Orihuela.** 2006. Identification of a Candidate *Streptococcus pneumoniae* core genome and regions of diversity correlated with invasive pneumococcal disease. Infect Immun **74:**4766-77.
311. **Obonyo, M., U. G. Munderloh, V. Fingerle, B. Wilske, and T. J. Kurtti.** 1999. *Borrelia burgdorferi* in tick cell culture modulates expression of outer surface proteins A and C in response to temperature. J Clin Microbiol **37:**2137-41.
312. **Ohnishi, J., J. Piesman, and A. M. de Silva.** 2001. Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks. Proc Natl Acad Sci U S A **98:**670-5.
313. **Ohnishi, J., B. Schneider, W. B. Messer, J. Piesman, and A. M. de Silva.** 2003. Genetic variation at the *vlsE* locus of *Borrelia burgdorferi* within ticks and mice over the course of a single transmission cycle. J Bacteriol **185:**4432-41.
314. **Ojaimi, C., C. Brooks, S. Casjens, P. Rosa, A. Elias, A. Barbour, A. Jasinskas, J. Benach, L. Katona, J. Radolf, M. Caimano, J. Skare, K. Swingle, D. Akins, and I. Schwartz.** 2003. Profiling of temperature-induced changes in *Borrelia burgdorferi* gene expression by using whole genome arrays. Infect Immun **71:**1689-705.
315. **O'Neal, C. R., W. M. Gabriel, A. K. Turk, S. J. Libby, F. C. Fang, and M. P. Spector.** 1994. RpoS is necessary for both the positive and negative regulation of starvation survival genes during phosphate, carbon, and nitrogen starvation in *Salmonella typhimurium*. J Bacteriol **176:**4610-6.
316. **Orloski, K. A., E. B. Hayes, G. L. Campbell, and D. T. Dennis.** 2000. Surveillance for Lyme disease--United States, 1992-1998. MMWR CDC Surveill Summ **49:**1-11.
317. **Pachner, A. R., and A. C. Steere.** 1985. The triad of neurologic manifestations of Lyme disease: meningitis, cranial neuritis, and radiculoneuritis. Neurology **35:**47-53.
318. **Padula, S. J., F. Dias, A. Sampieri, R. B. Craven, and R. W. Ryan.** 1994. Use of recombinant OspC from *Borrelia burgdorferi* for serodiagnosis of early Lyme disease. J Clin Microbiol **32:**1733-8.

319. **Padula, S. J., A. Sampieri, F. Dias, A. Szczepanski, and R. W. Ryan.** 1993. Molecular characterization and expression of p23 (OspC) from a North American strain of *Borrelia burgdorferi*. Infect Immun **61:**5097-105.
320. **Paget, M. S., and J. D. Helmann.** 2003. The sigma70 family of sigma factors. Genome Biol **4:**203.
321. **Pal, U., P. Wang, F. Bao, X. Yang, S. Samanta, R. Schoen, G. P. Wormser, I. Schwartz, and E. Fikrig.** 2008. *Borrelia burgdorferi* basic membrane proteins A and B participate in the genesis of Lyme arthritis. J Exp Med **205:**133-41.
322. **Pal, U., X. Yang, M. Chen, L. K. Bockenstedt, J. F. Anderson, R. A. Flavell, M. V. Norgard, and E. Fikrig.** 2004. OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands. J Clin Invest **113:**220-30.
323. **Park, K. J., M. J. Kang, S. H. Kim, H. J. Lee, J. K. Lim, S. H. Choi, S. J. Park, and K. H. Lee.** 2004. Isolation and characterization of *rpoS* from a pathogenic bacterium, *Vibrio vulnificus*: role of sigmaS in survival of exponential-phase cells under oxidative stress. J Bacteriol **186:**3304-12.
324. **Parola, P., and D. Raoult.** 2001. Tick-borne bacterial diseases emerging in Europe. Clin Microbiol Infect **7:**80-3.
325. **Parola, P., and D. Raoult.** 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin Infect Dis **32:**897-928.
326. **Parveen, N., and J. M. Leong.** 2000. Identification of a candidate glycosaminoglycan-binding adhesin of the Lyme disease spirochete *Borrelia burgdorferi*. Mol Microbiol **35:**1220-34.
327. **Persing, D. H., D. Mathiesen, D. Podzorski, and S. W. Barthold.** 1994. Genetic stability of *Borrelia burgdorferi* recovered from chronically infected immunocompetent mice. Infect Immun **62:**3521-7.
328. **Phillips, S. E., J. J. Burrascano, N. S. Harris, L. Johnson, P. V. Smith, and R. B. Stricker.** 2005. Chronic infection in 'post-Lyme borreliosis syndrome'. Int J Epidemiol **34:**1439-40; author reply 1440-3.
329. **Piesman, J., T. N. Mather, R. J. Sinsky, and A. Spielman.** 1987. Duration of tick attachment and *Borrelia burgdorferi* transmission. J Clin Microbiol **25:**557-8.
330. **Piesman, J., N. S. Zeidner, and B. S. Schneider.** 2003. Dynamic changes in *Borrelia burgdorferi* populations in Ixodes scapularis (Acari: Ixodidae) during transmission: studies at the mRNA level. Vector Borne Zoonotic Dis **3:**125-32.
331. **Pinne, M., K. Denker, E. Nilsson, R. Benz, and S. Bergstrom.** 2006. The BBA01 protein, a member of paralog family 48 from *Borrelia burgdorferi*, is potentially interchangeable with the channel-forming protein P13. J Bacteriol **188:**4207-17.
332. **Pinto, D. S.** 2002. Cardiac manifestations of Lyme disease. Med Clin North Am **86:**285-96.
333. **Plasterk, R. H., M. I. Simon, and A. G. Barbour.** 1985. Transposition of structural genes to an expression sequence on a linear plasmid causes antigenic variation in the bacterium *Borrelia hermsii*. Nature **318:**257-63.
334. **Plesa, M., J. P. Hernalsteens, G. Vandenbussche, J. M. Ruysschaert, and P. Cornelis.** 2006. The SlyB outer membrane lipoprotein of *Burkholderia multivorans* contributes to membrane integrity. Res Microbiol **157:**582-92.

335. **Plummer, C., H. Wu, S. W. Kerrigan, G. Meade, D. Cox, and C. W. Ian Douglas.** 2005. A serine-rich glycoprotein of *Streptococcus sanguis* mediates adhesion to platelets via GPIb. Br J Haematol **129:**101-9.
336. **Poole, D. M., G. P. Hazlewood, N. S. Huskisson, R. Virden, and H. J. Gilbert.** 1993. The role of conserved tryptophan residues in the interaction of a bacterial cellulose binding domain with its ligand. FEMS Microbiol Lett **106:**77-83.
337. **Postic, D., M. Garnier, and G. Baranton.** 2007. Multilocus sequence analysis of atypical *Borrelia burgdorferi* sensu lato isolates - Description of *Borrelia californiensis* sp. nov., and genomospecies 1 and 2. Int J Med Microbiol **297:**263-271.
338. **Postic, D., N. M. Ras, R. S. Lane, M. Hendson, and G. Baranton.** 1998. Expanded diversity among Californian borrelia isolates and description of *Borrelia bissettii* sp. nov. (formerly Borrelia group DN127). J Clin Microbiol **36:**3497-504.
339. **Power, C., J. H. Wang, S. Sookhai, J. T. Street, and H. P. Redmond.** 2001. Bacterial wall products induce downregulation of vascular endothelial growth factor receptors on endothelial cells via a CD14-dependent mechanism: implications for surgical wound healing. J Surg Res **101:**138-45.
340. **Probert, W. S., and B. J. Johnson.** 1998. Identification of a 47 kDa fibronectin-binding protein expressed by *Borrelia burgdorferi* isolate B31. Mol Microbiol **30:**1003-15.
341. **Probert, W. S., J. H. Kim, M. Hook, and B. J. Johnson.** 2001. Mapping the ligand-binding region of *Borrelia burgdorferi* fibronectin- binding protein BBK32. Infect Immun **69:**4129-33.
342. **Purser, J. E., M. B. Lawrenz, M. J. Caimano, J. K. Howell, J. D. Radolf, and S. J. Norris.** 2003. A plasmid-encoded nicotinamidase (PncA) is essential for infectivity of *Borrelia burgdorferi* in a mammalian host. Mol Microbiol **48:**753-64.
343. **Purser, J. E., and S. J. Norris.** 2000. Correlation between plasmid content and infectivity in *Borrelia burgdorferi*. Proc Natl Acad Sci U S A **97:**13865-70.
344. **Rahn, D. W.** 2001. Lyme vaccine: issues and controversies. Infect Dis Clin North Am **15:**171-87.
345. **Ramamoorthy, R., and M. T. Philipp.** 1998. Differential expression of *Borrelia burgdorferi* proteins during growth In vitro. Infect Immun **66:**5119-24.
346. **Ramamoorthy, R., and D. Scholl-Meeker.** 2001. *Borrelia burgdorferi* proteins whose expression is similarly affected by culture temperature and pH. Infect Immun **69:**2739-42.
347. **Ramos, J. M., E. Malmierca, F. Reyes, W. Wolde, A. Galata, A. Tesfamariam, and M. Gorgolas.** 2004. Characteristics of louse-borne relapsing fever in Ethiopian children and adults. Ann Trop Med Parasitol **98:**191-6.
348. **Raoult, D., and V. Roux.** 1999. The body louse as a vector of reemerging human diseases. Clin Infect Dis **29:**888-911.
349. **Ras, N. M., B. Lascola, D. Postic, S. J. Cutler, F. Rodhain, G. Baranton, and D. Raoult.** 1996. Phylogenesis of relapsing fever *Borrelia* spp. Int J Syst Bacteriol **46:**859-65.
350. **Rathinavelu, S., and A. M. de Silva.** 2001. Purification and characterization of *Borrelia burgdorferi* from feeding nymphal ticks (Ixodes scapularis). Infect Immun **69:**3536-41.
351. **Rautemaa, R., and S. Meri.** 1999. Complement-resistance mechanisms of bacteria. Microbes Infect **1:**785-94.

352. **Raveche, E. S., S. E. Schutzer, H. Fernandes, H. Bateman, B. A. McCarthy, S. P. Nickell, and M. W. Cunningham.** 2005. Evidence of *Borrelia* autoimmunity-induced component of Lyme carditis and arthritis. J Clin Microbiol **43:**850-6.
353. **Rebaudet, S., and P. Parola.** 2006. Epidemiology of relapsing fever borreliosis in Europe. FEMS Immunol Med Microbiol **48:**11-5.
354. **Rechsteiner, M., and S. W. Rogers.** 1996. PEST sequences and regulation by proteolysis. Trends Biochem Sci **21:**267-71.
355. **Reece, S., C. P. Simmons, R. J. Fitzhenry, M. Batchelor, C. Hale, S. Matthews, A. D. Phillips, G. Dougan, and G. Frankel.** 2002. Mutagenesis of conserved tryptophan residues within the receptor-binding domain of intimin: influence on binding activity and virulence. Microbiology **148:**657-65.
356. **Reeves, P.** 1995. Role of O-antigen variation in the immune response. Trends Microbiol **3:**381-6.
357. **Reglier-Poupet, H., C. Frehel, I. Dubail, J. L. Beretti, P. Berche, A. Charbit, and C. Raynaud.** 2003. Maturation of lipoproteins by type II signal peptidase is required for phagosomal escape of *Listeria monocytogenes*. J Biol Chem **278:**49469-77.
358. **Reik, L., A. C. Steere, N. H. Bartenhagen, R. E. Shope, and S. E. Malawista.** 1979. Neurologic abnormalities of Lyme disease. Medicine (Baltimore) **58:**281-94.
359. **Reitzer, L.** 2003. Nitrogen assimilation and global regulation in *Escherichia coli*. Annu Rev Microbiol **57:**155-76.
360. **Revel, A. T., J. S. Blevins, C. Almazan, L. Neil, K. M. Kocan, J. de la Fuente, K. E. Hagman, and M. V. Norgard.** 2005. *bptA* (*bbe16*) is essential for the persistence of the Lyme disease spirochete, *Borrelia burgdorferi*, in its natural tick vector. Proc Natl Acad Sci U S A **102:**6972-7.
361. **Revel, A. T., A. M. Talaat, and M. V. Norgard.** 2002. DNA microarray analysis of differential gene expression in *Borrelia burgdorferi*, the Lyme disease spirochete. Proc Natl Acad Sci U S A **99:**1562-7.
362. **Reznick, J. W., D. B. Braunstein, R. L. Walsh, C. R. Smith, P. M. Wolfson, L. W. Gierke, L. Gorelkin, and F. W. Chandler.** 1986. Lyme carditis. Electrophysiologic and histopathologic study. Am J Med **81:**923-7.
363. **Ribeiro, J. M., G. T. Makoul, J. Levine, D. R. Robinson, and A. Spielman.** 1985. Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, Ixodes dammini. J Exp Med **161:**332-44.
364. **Richter, D., D. B. Schlee, R. Allgower, and F. R. Matuschka.** 2004. Relationships of a novel Lyme disease spirochete, *Borrelia spielmani* sp. nov., with its hosts in Central Europe. Appl Environ Microbiol **70:**6414-9.
365. **Roehrig, J. T., J. Piesman, A. R. Hunt, M. G. Keen, C. M. Happ, and B. J. Johnson.** 1992. The hamster immune response to tick-transmitted *Borrelia burgdorferi* differs from the response to needle-inoculated, cultured organisms. J Immunol **149:**3648-53.
366. **Rogers, E. A., and R. T. Marconi.** 2007. Delineation of species-specific binding properties of the CspZ protein (BBH06) of Lyme disease spirochetes: evidence for new contributions to the pathogenesis of *Borrelia* spp. Infect Immun **75:**5272-81.
367. **Rogers, S., R. Wells, and M. Rechsteiner.** 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science **234:**364-8.
368. **Rosa, P., J. Bono, A. Elias, J. Errett, J. Kupko, B. Stevenson, G. Taylor, and K. Tilly.** 1998. Genetic studies in *Borrelia burgdorferi*. Wien Klin Wochenschr **110:**859-62.

369. **Rosa, P., D. S. Samuels, D. Hogan, B. Stevenson, S. Casjens, and K. Tilly.** 1996. Directed insertion of a selectable marker into a circular plasmid of *Borrelia burgdorferi*. J Bacteriol **178:**5946-53.
370. **Rosa, P. A., K. Tilly, and P. E. Stewart.** 2005. The burgeoning molecular genetics of the Lyme disease spirochaete. Nat Rev Microbiol **3:**129-43.
371. **Rose, C. D., P. T. Fawcett, and K. M. Gibney.** 2001. Arthritis following recombinant outer surface protein A vaccination for Lyme disease. J Rheumatol **28:**2555-7.
372. **Rosengarten, R., and K. S. Wise.** 1990. Phenotypic switching in mycoplasmas: phase variation of diverse surface lipoproteins. Science **247:**315-8.
373. **Roux, F., E. Boyer, B. Jaulhac, E. Dernis, F. Closs-Prophette, and X. Puechal.** 2007. Lyme meningoradiculitis: prospective evaluation of biological diagnosis methods. Eur J Clin Microbiol Infect Dis **26:**685-93.
374. **Ryffel, K., P. t. O, B. Rutti, A. Suard, and E. Dayer.** 1999. Scored Antibody Reactivity Determined by Immunoblotting Shows an Association between Clinical Manifestations and Presence of *Borrelia burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. Valaisiana* in Humans. J Clin Microbiol **37:**4086-4092.
375. **Saito, K., T. Ito, N. Asashima, M. Ohno, R. Nagai, H. Fujita, N. Koizumi, A. Takano, H. Watanabe, and H. Kawabata.** 2007. Case report: *Borrelia valaisiana* infection in a Japanese man associated with traveling to foreign countries. Am J Trop Med Hyg **77:**1124-7.
376. **Salih, S. Y., D. Mustafa, S. M. Abdel Wahab, M. A. Ahmed, and A. Omer.** 1977. Louse-borne relapsing fever: I. A clinical and laboratory study of 363 cases in the Sudan. Trans R Soc Trop Med Hyg **71:**43-8.
377. **Samanta, U., and P. Chakrabarti.** 2001. Assessing the role of tryptophan residues in the binding site. Protein Eng **14:**7-15.
378. **Sambri, V., F. Massaria, M. Ardizzoni, C. Stefanelli, and R. Cevenini.** 1993. Glycoprotein patterns in *Borrelia* spp. Int J Med Microbiol Virol Parasitol Infect Dis **279:**330-5.
379. **Sambri, V., C. Stefanelli, and R. Cevenini.** 1992. Detection of glycoproteins in *Borrelia burgdorferi*. Arch Microbiol **157:**205-8.
380. **Sa-Nunes, A., A. Bafica, D. A. Lucas, T. P. Conrads, T. D. Veenstra, J. F. Andersen, T. N. Mather, J. M. Ribeiro, and I. M. Francischetti.** 2007. Prostaglandin E2 is a major inhibitor of dendritic cell maturation and function in *Ixodes scapularis* saliva. J Immunol **179:**1497-505.
381. **Sartakova, M., E. Dobrikova, and F. C. Cabello.** 2000. Development of an extrachromosomal cloning vector system for use in *Borrelia burgdorferi*. Proc Natl Acad Sci U S A **97:**4850-4855.
382. **Schaarschmidt, D., R. Oehme, P. Kimmig, R. D. Hesch, and S. Englisch.** 2001. Detection and molecular typing of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks and in different patient samples from southwest Germany. Eur J Epidemiol **17:**1067-74.
383. **Schmid, G. P.** 1984. The global distribution of Lyme disease. Yale J Biol Med **57:**617-8.
384. **Schmid, G. P., A. G. Steigerwalt, S. E. Johnson, A. G. Barbour, A. C. Steere, I. M. Robinson, and D. J. Brenner.** 1984. DNA characterization of the spirochete that causes Lyme disease. J Clin Microbiol **20:**155-8.
385. **Schoen, R. T., T. Deshefy-Longhi, C. Van-Hoecke, C. Buscarino, and E. Fikrig.** 2003. An open-label, nonrandomized, single-center, prospective extension, clinical trial

of booster dose schedules to assess the safety profile and immunogenicity of recombinant outer-surface protein A (OspA) Lyme disease vaccine. Clin Ther **25:**210-24.
386. **Schuch, R., and A. T. Maurelli.** 1999. The mxi-Spa type III secretory pathway of *Shigella flexneri* requires an outer membrane lipoprotein, MxiM, for invasin translocation. Infect Immun **67:**1982-91.
387. **Schulze, R. J., and W. R. Zuckert.** 2006. *Borrelia burgdorferi* lipoproteins are secreted to the outer surface by default. Mol Microbiol **59:**1473-84.
388. **Schwan, T. G., W. Burgdorfer, and C. F. Garon.** 1988. Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of in vitro cultivation. Infect Immun **56:**1831-6.
389. **Schwan, T. G., and J. Piesman.** 2000. Temporal Changes in Outer Surface Proteins A and C of the Lyme Disease- Associated Spirochete, *Borrelia burgdorferi*, during the Chain of Infection in Ticks and Mice. J Clin Microbiol **38:**382-388.
390. **Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa.** 1995. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. Proc Natl Acad Sci U S A **92:**2909-13.
391. **Schwan, T. G., P. F. Policastro, Z. Miller, R. L. Thompson, T. Damrow, and J. E. Keirans.** 2003. Tick-borne relapsing fever caused by *Borrelia hermsii*, Montana. Emerg Infect Dis **9:**1151-4.
392. **Schwan, T. G., and W. J. Simpson.** 1991. Factors influencing the antigenic reactivity of *Borrelia burgdorferi*, the Lyme disease spirochete. Scand J Infect Dis Suppl **77:**94-101.
393. **Schwan, T. G., W. J. Simpson, M. E. Schrumpf, and R. H. Karstens.** 1989. Identification of *Borrelia burgdorferi* and *B. hermsii* using DNA hybridization probes. J Clin Microbiol **27:**1734-8.
394. **Schwartz, I., G. P. Wormser, J. J. Schwartz, D. Cooper, P. Weissensee, A. Gazumyan, E. Zimmermann, N. S. Goldberg, S. Bittker, G. L. Campbell, and et al.** 1992. Diagnosis of early Lyme disease by polymerase chain reaction amplification and culture of skin biopsies from erythema migrans lesions. J Clin Microbiol **30:**3082-8.
395. **Scrimenti, R. J.** 1970. Erythema chronicum migrans. Arch Dermatol **102:**104-5.
396. **Seboxa, T., and S. I. Rahlenbeck.** 1995. Treatment of louse-borne relapsing fever with low dose penicillin or tetracycline: a clinical trial. Scand J Infect Dis **27:**29-31.
397. **Seifert, K. N., E. E. Adderson, A. A. Whiting, J. F. Bohnsack, P. J. Crowley, and L. J. Brady.** 2006. A unique serine-rich repeat protein (Srr-2) and novel surface antigen (epsilon) associated with a virulent lineage of serotype III *Streptococcus agalactiae*. Microbiology **152:**1029-40.
398. **Seiler, K. P., Z. Vavrin, E. Eichwald, J. B. Hibbs, Jr., and J. J. Weis.** 1995. Nitric oxide production during murine Lyme disease: lack of involvement in host resistance or pathology. Infect Immun **63:**3886-95.
399. **Seshu, J., J. A. Boylan, F. C. Gherardini, and J. T. Skare.** 2004. Dissolved oxygen levels alter gene expression and antigen profiles in *Borrelia burgdorferi*. Infect Immun **72:**1580-6.
400. **Seshu, J., M. D. Esteve-Gassent, M. Labandeira-Rey, J. H. Kim, J. P. Trzeciakowski, M. Hook, and J. T. Skare.** 2006. Inactivation of the fibronectin-binding adhesin gene *bbk32* significantly attenuates the infectivity potential of *Borrelia burgdorferi*. Mol Microbiol **59:**1591-601.

401. **Setubal, J. C., M. Reis, J. Matsunaga, and D. A. Haake.** 2006. Lipoprotein computational prediction in spirochaetal genomes. Microbiology **152:**113-21.
402. **Severinova, J., J. Salat, Z. Krocova, J. Reznickova, H. Demova, H. Horka, and J. Kopecky.** 2005. Co-inoculation of *Borrelia afzelii* with tick salivary gland extract influences distribution of immunocompetent cells in the skin and lymph nodes of mice. Folia Microbiol (Praha) **50:**457-63.
403. **Sha, J., A. A. Fadl, G. R. Klimpel, D. W. Niesel, V. L. Popov, and A. K. Chopra.** 2004. The two murein lipoproteins of *Salmonella enterica* serovar Typhimurium contribute to the virulence of the organism. Infect Immun **72:**3987-4003.
404. **Shi, Y., Q. Xu, K. McShan, and F. T. Liang.** 2008. Both decorin-binding proteins A and B are critical for the overall virulence of *Borrelia burgdorferi*. Infect Immun **76:**1239-46.
405. **Siboo, I. R., H. F. Chambers, and P. M. Sullam.** 2005. Role of SraP, a Serine-Rich Surface Protein of *Staphylococcus aureus*, in binding to human platelets. Infect Immun **73:**2273-80.
406. **Sidi, G., N. Davidovitch, R. D. Balicer, E. Anis, I. Grotto, and E. Schwartz.** 2005. Tickborne relapsing fever in Israel. Emerg Infect Dis **11:**1784-6.
407. **Silver, H. M.** 1997. Lyme disease during pregnancy. Infect Dis Clin North Am **11:**93-7.
408. **Skare, J. T., E. S. Shang, D. M. Foley, D. R. Blanco, C. I. Champion, T. Mirzabekov, Y. Sokolov, B. L. Kagan, J. N. Miller, and M. A. Lovett.** 1995. Virulent strain associated outer membrane proteins of *Borrelia burgdorferi*. J Clin Invest **96:**2380-92.
409. **Small, P., D. Blankenhorn, D. Welty, E. Zinser, and J. L. Slonczewski.** 1994. Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of *rpoS* and growth pH. J Bacteriol **176:**1729-37.
410. **Smith, R., S. O'Connell, and S. Palmer.** 2000. Lyme disease surveillance in England and Wales, 1986 1998. Emerg Infect Dis **6:**404-7.
411. **Southern P., and S. J.** 1969. Relapsing fever. A clinical and microbiological review. Medicine **48:**129-149.
412. **Spach, D. H., W. C. Liles, G. L. Campbell, R. E. Quick, D. E. Anderson, Jr., and T. R. Fritsche.** 1993. Tick-borne diseases in the United States. N Engl J Med **329:**936-47.
413. **Stanek, G.** 1995. Borreliosis and Travel Medicine. J Travel Med **2:**244-251.
414. **Steere, A. C.** 1989. Lyme disease. N. Engl. J. Med. **321:**586-596.
415. **Steere, A. C., W. P. Batsford, M. Weinberg, J. Alexander, H. J. Berger, S. Wolfson, and S. E. Malawista.** 1980. Lyme carditis: cardiac abnormalities of Lyme disease. Ann Intern Med **93:**8-16.
416. **Steere, A. C., T. F. Broderick, and S. E. Malawista.** 1978. Erythema chronicum migrans and Lyme arthritis: epidemiologic evidence for a tick vector. Am J Epidemiol **108:**312-21.
417. **Steere, A. C., J. Coburn, and L. Glickstein.** 2004. The emergence of Lyme disease. J Clin Invest **113:**1093-101.
418. **Steere, A. C., J. E. Craft, G. J. Hutchinson, J. H. Newman, D. W. Rhan, L. H. Sigal, P. N. Spieler, K. S. Stenn, and S. E. Malawista.** 1983. The early clinical manifestations of Lyme disease. Ann. Intern. Med **99:**76-82.
419. **Steere, A. C., R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson, and S. E. Malawista.** 1983. The spirochetal etiology of Lyme disease. N Engl J Med **308:**733-40.

420. **Steere, A. C., J. A. Hardin, and S. E. Malawista.** 1978. Lyme arthritis: a new clinical entity. Hosp Pract **13:**143-58.
421. **Steere, A. C., S. E. Malawista, J. A. Hardin, S. Ruddy, W. Askenase, and W. A. Andiman.** 1977. Erythema chronicum migrans and Lyme arthritis. The enlarging clinical spectrum. Ann Intern Med **86:**685-98.
422. **Steere, A. C., S. E. Malawista, D. R. Snydman, R. E. Shope, W. A. Andiman, M. R. Ross, and F. M. Steele.** 1977. Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities. Arthritis Rheum **20:**7-17.
423. **Steere, A. C., and V. K. Sikand.** 2003. The presenting manifestations of Lyme disease and the outcomes of treatment. N Engl J Med **348:**2472-4.
424. **Steere, A. C., V. K. Sikand, F. Meurice, D. L. Parenti, E. Fikrig, R. T. Schoen, J. Nowakowski, C. H. Schmid, S. Laukamp, C. Buscarino, and D. S. Krause.** 1998. Vaccination against Lyme disease with recombinant *Borrelia burgdorferi* outer-surface lipoprotein A with adjuvant. Lyme Disease Vaccine Study Group. N Engl J Med **339:**209-15.
425. **Steere, A. C., E. Taylor, G. L. McHugh, and E. L. Logigian.** 1993. The overdiagnosis of Lyme disease. Jama **269:**1812-6.
426. **Stevenson, B., J. L. Bono, T. G. Schwan, and P. Rosa.** 1998. *Borrelia burgdorferi erp* proteins are immunogenic in mammals infected by tick bite, and their synthesis is inducible in cultured bacteria. Infect Immun **66:**2648-54.
427. **Stevenson, B., N. El-Hage, M. A. Hines, J. C. Miller, and K. Babb.** 2002. Differential binding of host complement inhibitor factor H by *Borrelia burgdorferi* Erp surface proteins: a possible mechanism underlying the expansive host range of Lyme disease spirochetes. Infect Immun **70:**491-7.
428. **Stevenson, B., S. F. Porcella, K. L. Oie, C. A. Fitzpatrick, S. J. Raffel, L. Lubke, M. E. Schrumpf, and T. G. Schwan.** 2000. The relapsing fever spirochete *Borrelia hermsii* contains multiple, antigen-encoding circular plasmids that are homologous to the cp32 plasmids of Lyme disease spirochetes. Infect Immun **68:**3900-8.
429. **Stevenson, B., T. G. Schwan, and P. A. Rosa.** 1995. Temperature-related differential expression of antigens in the Lyme disease spirochete, *Borrelia burgdorferi*. Infect Immun **63:**4535-9.
430. **Stewart, P. E., R. Thalken, J. L. Bono, and P. Rosa.** 2001. Isolation of a circular plasmid region sufficient for autonomous replication and transformation of infectious *Borrelia burgdorferi*. Mol Microbiol **39:**714-21.
431. **Stewart, P. E., X. Wang, D. M. Bueschel, D. R. Clifton, D. Grimm, K. Tilly, J. A. Carroll, J. J. Weis, and P. A. Rosa.** 2006. Delineating the Requirement for the *Borrelia burgdorferi* Virulence Factor OspC in the Mammalian Host. Infect Immun **74:**3547-53.
432. **Stoenner, H. G., T. Dodd, and C. Larsen.** 1982. Antigenic variation of *Borrelia hermsii*. J Exp Med **156:**1297-311.
433. **Strle, F., R. B. Nadelman, J. Cimperman, J. Nowakowski, R. N. Picken, I. Schwartz, V. Maraspin, M. E. Aguero-Rosenfeld, S. Varde, S. Lotric-Furlan, and G. P. Wormser.** 1999. Comparison of culture-confirmed erythema migrans caused by *Borrelia burgdorferi* sensu stricto in New York State and by Borrelia afzelii in Slovenia. Ann Intern Med **130:**32-6.

434. **Strobino, B. A., C. L. Williams, S. Abid, R. Chalson, and P. Spierling.** 1993. Lyme disease and pregnancy outcome: a prospective study of two thousand prenatal patients. Am J Obstet Gynecol **169:**367-74.
435. **Suh, S. J., L. Silo-Suh, D. E. Woods, D. J. Hassett, S. E. West, and D. E. Ohman.** 1999. Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. J Bacteriol **181:**3890-7.
436. **Sukupolvi, S., and C. D. O'Connor.** 1990. TraT lipoprotein, a plasmid-specified mediator of interactions between gram-negative bacteria and their environment. Microbiol Rev **54:**331-41.
437. **Sundnes, K. O., and A. T. Haimanot.** 1993. Epidemic of louse-borne relapsing fever in Ethiopia. Lancet **342:**1213-5.
438. **Surette, M. G., and J. B. Stock.** 1996. Role of alpha-helical coiled-coil interactions in receptor dimerization, signaling, and adaptation during bacterial chemotaxis. J Biol Chem **271:**17966-73.
439. **Takahashi, Y., K. Konishi, J. O. Cisar, and M. Yoshikawa.** 2002. Identification and characterization of *hsa*, the gene encoding the sialic acid-binding adhesin of *Streptococcus gordonii* DL1. Infect Immun **70:**1209-18.
440. **Telford, S. R. d., T. N. Mather, S. I. Moore, M. L. Wilson, and A. Spielman.** 1988. Incompetence of deer as reservoirs of the Lyme disease spirochete. Am J Trop Med Hyg **39:**105-9.
441. **Thomas, V., J. Anguita, S. Samanta, P. A. Rosa, P. Stewart, S. W. Barthold, and E. Fikrig.** 2001. Dissociation of infectivity and pathogenicity in *Borrelia burgdorferi*. Infect Immun **69:**3507-9.
442. **Thoma-Uszynski, S., S. M. Kiertscher, M. T. Ochoa, D. A. Bouis, M. V. Norgard, K. Miyake, P. J. Godowski, M. D. Roth, and R. L. Modlin.** 2000. Activation of toll-like receptor 2 on human dendritic cells triggers induction of IL-12, but not IL-10. J Immunol **165:**3804-10.
443. **Tilly, K., A. Bestor, M. W. Jewett, and P. Rosa.** 2006. Rapid clearance of Lyme disease spirochetes lacking OspC from skin. Infect Immun **75:**1517-1519.
444. **Tilly, K., A. F. Elias, J. Errett, E. Fischer, R. Iyer, I. Schwartz, J. L. Bono, and P. Rosa.** 2001. Genetics and Regulation of Chitobiose Utilization in *Borrelia burgdorferi*. J Bacteriol **183:**5544-53.
445. **Tilly, K., J. G. Krum, A. Bestor, M. W. Jewett, D. Grimm, D. Bueschel, R. Byram, D. Dorward, M. J. Vanraden, P. Stewart, and P. Rosa.** 2006. *Borrelia burgdorferi* OspC Protein Required Exclusively in a Crucial Early Stage of Mammalian Infection. Infect Immun **74:**3554-64.
446. **Tokarz, R., J. M. Anderton, L. I. Katona, and J. L. Benach.** 2004. Combined effects of blood and temperature shift on *Borrelia burgdorferi* gene expression as determined by whole genome DNA array. Infect Immun **72:**5419-32.
447. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A **76:**4350-4.
448. **Trape, J. F., J. M. Duplantier, H. Bouganali, B. Godeluck, F. Legros, J. P. Cornet, and J. L. Camicas.** 1991. Tick-borne borreliosis in west Africa. Lancet **337:**473-5.

449. **Trollmo, C., A. L. Meyer, A. C. Steere, D. A. Hafler, and B. T. Huber.** 2001. Molecular mimicry in Lyme arthritis demonstrated at the single cell level: LFA-1 alpha L is a partial agonist for outer surface protein A- reactive T cells. J Immunol **166:**5286-91.
450. **Valsangiacomo, C., T. Balmelli, and J. C. Piffaretti.** 1997. A phylogenetic analysis of *Borrelia burgdorferi* sensu lato based on sequence information from the hbb gene, coding for a histone-like protein. Int J Syst Bacteriol **47:**1-10.
451. **van Dam, A. P., H. Kuiper, K. Vos, A. Widjojokusumo, B. M. de Jongh, L. Spanjaard, A. C. Ramselaar, M. D. Kramer, and J. Dankert.** 1993. Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. Clin Infect Dis **17:**708-17.
452. **van der Linde, M. R.** 1991. Lyme carditis: clinical characteristics of 105 cases. Scand J Infect Dis Suppl **77:**81-4.
453. **Vasseur, P., C. Soscia, R. Voulhoux, and A. Filloux.** 2007. PelC is a *Pseudomonas aeruginosa* outer membrane lipoprotein of the OMA family of proteins involved in exopolysaccharide transport. Biochimie **89:**903-15.
454. **Vial, L., G. Diatta, A. Tall, H. Ba el, H. Bouganali, P. Durand, C. Sokhna, C. Rogier, F. Renaud, and J. F. Trape.** 2006. Incidence of tick-borne relapsing fever in west Africa: longitudinal study. Lancet **368:**37-43.
455. **Vidal, V., S. Cutler, I. G. Scragg, D. J. Wright, and D. Kwiatkowski.** 2002. Characterisation of silent and active genes for a variable large protein of *Borrelia recurrentis*. BMC Infect Dis **2:**25.
456. **von Stedingk, L. V., I. Olsson, H. S. Hanson, E. Asbrink, and A. Hovmark.** 1995. Polymerase chain reaction for detection of *Borrelia burgdorferi* DNA in skin lesions of early and late Lyme borreliosis. Eur J Clin Microbiol Infect Dis **14:**1-5.
457. **Vorou, R. M., V. G. Papavassiliou, and S. Tsiodras.** 2007. Emerging zoonoses and vector-borne infections affecting humans in Europe. Epidemiol Infect **135:**1231-47.
458. **Wallich, R., O. Jahraus, T. Stehle, T. T. Tran, C. Brenner, H. Hofmann, L. Gern, and M. M. Simon.** 2003. Artificial-infection protocols allow immunodetection of novel *Borrelia burgdorferi* antigens suitable as vaccine candidates against Lyme disease. Eur J Immunol **33:**708-19.
459. **Walter, S., and H. Schrempf.** 2003. Oligomerization, membrane anchoring, and cellulose-binding characteristics of AbpS, a receptor-like *Streptomyces* protein. J Biol Chem **278:**26639-47.
460. **Wang, G., R. Iyer, S. Bittker, D. Cooper, J. Small, G. P. Wormser, and I. Schwartz.** 2004. Variations in Barbour-Stoenner-Kelly culture medium modulate infectivity and pathogenicity of *Borrelia burgdorferi* clinical isolates. Infect Immun **72:**6702-6.
461. **Wang, G., A. P. van Dam, A. Le Fleche, D. Postic, O. Peter, G. Baranton, R. de Boer, L. Spanjaard, and J. Dankert.** 1997. Genetic and phenotypic analysis of *Borrelia valaisiana* sp. nov. (Borrelia genomic groups VS116 and M19). Int J Syst Bacteriol **47:**926-32.
462. **Wang, G., A. P. van Dam, I. Schwartz, and J. Dankert.** 1999. Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. Clin Microbiol Rev **12:**633-53.
463. **Wang, X. G., B. Lin, J. M. Kidder, S. Telford, and L. T. Hu.** 2002. Effects of environmental changes on expression of the oligopeptide permease (*opp*) genes of *Borrelia burgdorferi*. J Bacteriol **184:**6198-206.

464. **Wang, Y., R. Gao, and D. G. Lynn.** 2002. Ratcheting up vir gene expression in *Agrobacterium tumefaciens*: coiled coils in histidine kinase signal transduction. Chembiochem **3:**311-7.
465. **Ward, H. B.** 1908. The spirochetes and their relationship to other organisms. The American Naturalist **42:**374-387.
466. **Washburn, L. R., E. J. Miller, and K. E. Weaver.** 2000. Molecular characterization of *Mycoplasma arthritidis* membrane lipoprotein MAA1. Infect Immun **68:**437-42.
467. **Weiner, L., J. L. Brissette, and P. Model.** 1991. Stress-induced expression of the *Escherichia coli* phage shock protein operon is dependent on sigma 54 and modulated by positive and negative feedback mechanisms. Genes Dev **5:**1912-23.
468. **Weinstein, A., and M. Britchkov.** 2002. Lyme arthritis and post-Lyme disease syndrome. Curr Opin Rheumatol **14:**383-7.
469. **Weis, J. J., B. A. McCracken, Y. Ma, D. Fairbairn, R. J. Roper, T. B. Morrison, J. H. Weis, J. F. Zachary, R. W. Doerge, and C. Teuscher.** 1999. Identification of quantitative trait loci governing arthritis severity and humoral responses in the murine model of Lyme disease. J Immunol **162:**948-56.
470. **Wikel, S. K.** 1999. Tick modulation of host immunity: an important factor in pathogen transmission. Int J Parasitol **29:**851-9.
471. **Williams, C. L., B. Strobino, A. Weinstein, P. Spierling, and F. Medici.** 1995. Maternal Lyme disease and congenital malformations: a cord blood serosurvey in endemic and control areas. Paediatr Perinat Epidemiol **9:**320-30.
472. **Wilske, B., V. Preac-Mursic, G. Schierz, and K. V. Busch.** 1986. Immunochemical and immunological analysis of European *Borrelia burgdorferi* strains. Zentralbl Bakteriol Mikrobiol Hyg [A] **263:**92-102.
473. **Wormser, G. P.** 2006. Hematogenous dissemination in early Lyme disease. Wien Klin Wochenschr **118:**634-7.
474. **Wormser, G. P., S. Bittker, D. Cooper, J. Nowakowski, R. B. Nadelman, and C. Pavia.** 2000. Comparison of the yields of blood cultures using serum or plasma from patients with early lyme disease. J Clin Microbiol **38:**1648-50.
475. **Wormser, G. P., S. Bittker, D. Cooper, J. Nowakowski, R. B. Nadelman, and C. Pavia.** 2001. Yield of large-volume blood cultures in patients with early lyme disease. J Infect Dis **184:**1070-2.
476. **Wormser, G. P., R. J. Dattwyler, E. D. Shapiro, J. J. Halperin, A. C. Steere, M. S. Klempner, P. J. Krause, J. S. Bakken, F. Strle, G. Stanek, L. Bockenstedt, D. Fish, J. S. Dumler, and R. B. Nadelman.** 2006. The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis **43:**1089-134.
477. **Wormser, G. P., D. McKenna, J. Carlin, R. B. Nadelman, L. F. Cavaliere, D. Holmgren, D. W. Byrne, and J. Nowakowski.** 2005. Brief communication: hematogenous dissemination in early Lyme disease. Ann Intern Med **142:**751-5.
478. **Wormser, G. P., R. B. Nadelman, R. J. Dattwyler, D. T. Dennis, E. D. Shapiro, A. C. Steere, T. J. Rush, D. W. Rahn, P. K. Coyle, D. H. Persing, D. Fish, and B. J. Luft.** 2000. Practice guidelines for the treatment of Lyme disease. The Infectious Diseases Society of America. Clin Infect Dis **31 Suppl 1:**1-14.

479. **Wormser, G. P., J. Nowakowski, R. B. Nadelman, S. Bittker, D. Cooper, and C. Pavia.** 1998. Improving the yield of blood cultures for patients with early Lyme disease. J Clin Microbiol **36:**296-8.
480. **Wu, H., K. P. Mintz, M. Ladha, and P. M. Fives-Taylor.** 1998. Isolation and characterization of Fap1, a fimbriae-associated adhesin of *Streptococcus parasanguis* FW213. Mol Microbiol **28:**487-500.
481. **Xu, Q., S. V. Seemanapalli, L. Lomax, K. McShan, X. Li, E. Fikrig, and F. T. Liang.** 2005. Association of linear plasmid 28-1 with an arthritic phenotype of *Borrelia burgdorferi*. Infect Immun **73:**7208-15.
482. **Xu, Q., S. V. Seemanaplli, K. McShan, and F. T. Liang.** 2007. Increasing the interaction of *Borrelia burgdorferi* with decorin significantly reduces the 50 percent infectious dose and severely impairs dissemination. Infect Immun **75:**4272-81.
483. **Yang, L., J. H. Weis, E. Eichwald, C. P. Kolbert, D. H. Persing, and J. J. Weis.** 1994. Heritable susceptibility to severe *Borrelia burgdorferi*-induced arthritis is dominant and is associated with persistence of large numbers of spirochetes in tissues. Infect Immun **62:**492-500.
484. **Yang, X., M. S. Goldberg, T. G. Popova, G. B. Schoeler, S. K. Wikel, K. E. Hagman, and M. V. Norgard.** 2000. Interdependence of environmental factors influencing reciprocal patterns of gene expression in virulent *Borrelia burgdorferi*. Mol Microbiol **37:**1470-1479.
485. **Yang, X. F., S. M. Alani, and M. V. Norgard.** 2003. The response regulator Rrp2 is essential for the expression of major membrane lipoproteins in *Borrelia burgdorferi*. Proc Natl Acad Sci U S A **100:**11001-6.
486. **Yang, X. F., A. Hubner, T. G. Popova, K. E. Hagman, and M. V. Norgard.** 2003. Regulation of Expression of the Paralogous Mlp Family in *Borrelia burgdorferi*. Infect Immun **71:**5012-20.
487. **Yang, X. F., M. C. Lybecker, U. Pal, S. M. Alani, J. Blevins, A. T. Revel, D. S. Samuels, and M. V. Norgard.** 2005. Analysis of the *ospC* regulatory element controlled by the RpoN-RpoS regulatory pathway in *Borrelia burgdorferi*. J Bacteriol **187:**4822-9.
488. **Yang, X. F., U. Pal, S. M. Alani, E. Fikrig, and M. V. Norgard.** 2004. Essential role for OspA/B in the life cycle of the Lyme disease spirochete. J Exp Med **199:**641-8.
489. **Zgurskaya, H. I., and H. Nikaido.** 2000. Multidrug resistance mechanisms: drug efflux across two membranes. Mol Microbiol **37:**219-25.
490. **Zhang, H., D. W. Niesel, J. W. Peterson, and G. R. Klimpel.** 1998. Lipoprotein release by bacteria: potential factor in bacterial pathogenesis. Infect Immun **66:**5196-201.
491. **Zhang, J. R., J. M. Hardham, A. G. Barbour, and S. J. Norris.** 1997. Antigenic variation in Lyme disease borreliae by promiscuous recombination of VMP-like sequence cassettes. Cell **89:**275-85.
492. **Zhang, J. R., and S. J. Norris.** 1998. Genetic variation of the *Borrelia burgdorferi* gene *vlsE* involves cassette-specific, segmental gene conversion. Infect Immun **66:**3698-704.
493. **Zhang, J. R., and S. J. Norris.** 1998. Kinetics and in vivo induction of genetic variation of *vlsE* in *Borrelia burgdorferi*. Infect Immun **66:**3689-97.
494. **Zhong, W., L. Gern, M. Kramer, R. Wallich, and M. M. Simon.** 1997. T helper cell priming of mice to *Borrelia burgdorferi* OspA leads to induction of protective antibodies following experimental but not tick- borne infection. Eur J Immunol **27:**2942-7.