

Fall 1996

Characterization of P39, A *Borrelia burgdorferi* Specific Protein

Jill M. Troyer
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CHARACTERIZATION OF P39, A *Borrelia burgdorferi* SPECIFIC PROTEIN

by

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B.S. Biology, May 1990, Baylor University, Waco, Texas

A Dissertation Submitted to the Faculty of
Old Dominion University

and

Eastern Virginia Medical School

in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

October 1996

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ABSTRACT

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Director: Dr. Robert Ratzlaff

Borrelia burgdorferi, the causative agent of Lyme disease, expresses a species specific, 39 kiloDalton protein of unknown function called P39. This protein is highly immunogenic and the presence of anti-P39 antibodies in patient sera is used as an indicator of *B.burgdorferi* infection. P39 is also a candidate for vaccine development due to conserved expression of P39 among *B.burgdorferi* isolates. Since little is presently known about this important molecule, the present study was designed to characterize P39 and was carried out in six separate investigations. First, infectious and noninfectious isolates of *B.burgdorferi* were established in vitro, and the infectivity of each was assessed in a murine model. Second, the location of P39 within the spirochete was determined in infectious and noninfectious isolates by cell fractionation, and western blot analysis. A correlation was established between P39 localization and infectivity; infectious and noninfectious isolates localized P39 to both the outer envelope and inner membrane, but noninfectious isolates had increased levels of P39 in the inner membrane and cytosol as compared to infectious isolates. Third, flow cytometry was used to determine if P39 expression is uniform throughout a population

of spirochetes. Within a cloned population, organisms expressed similar levels of P39 on the outer surface. In contrast, uncloned populations displayed variable surface P39 expression, and uncloned noninfectious B31, WCH1, and JD1 all expressed lower levels of P39 as compared to the respective infectious organisms. Fourth, detergent extraction studies were employed to determine the solubility of the P39 molecule. P39 was shown to be hydrophobic in nature and the solubility of the molecule was not altered in infectious or noninfectious isolates nor was it altered in different cell fractions. Fifth, P39 was immunoprecipitated from infectious and noninfectious isolates in an attempt to characterize molecules which associate with P39. P39 coprecipitated with four molecules in all isolates, and six additional molecules common only to noninfectious isolates. Finally, in an attempt to determine if P39 elicits immunopathological responses in an infected host, site directed DNA mutagenesis was used to inactivate the gene encoding P39. No viable P39 deletion mutants were obtained, thereby precluding any studies regarding immunopathology caused by P39. The results of this study demonstrate P39 is a hydrophobic, membrane associated protein, and is expressed by both infectious and noninfectious isolates. Furthermore, infectious and noninfectious isolates exhibited differences in P39 localization, and coprecipitation.

ACKNOWLEDGMENTS

The author would like to thank the following individuals for the assistance given during the production of this work. Dr. Tom G. Schwan of Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, MT for generously donating the monoclonal antibody D1C9, and the plasmid pSPR33. Dr. Scott Samuels of University of Montana, Missoula, MT for his consultations and for sharing his expertise on electroporation of *Borrelia*. Dr. Timothy Bos of Eastern Virginia Medical School Norfolk, VA for donation of pCH110, assistance in designing the P39-LacZ construct and for taking the time to answer *all* of my questions. The author would like to thank the following individuals from the Department of Biological Sciences of Old Dominion University in Norfolk, VA: Dr. Andy Gordon for the use of his photographic equipment, and for consultation on silver staining techniques; Jessica Sweringen and Glenda Tillery of Biological Support Services for production of many reagents used in this study; special appreciation for the Dissertation Committee members for their time and guidance throughout the execution of this work; Dr. Daniel Sonenshine for showing me the ins and outs of acarology; Dr. Stephen Buescher for critical review of the overall experimental design and for introducing me to Triton X114; Dr. Chris Osgood for instruction on plasmid manipulation, and for allowing me to use his laboratory and equipment. And special acknowledgment to Dr. Robert Ratzlaff for his tireless instruction and unwavering interest in the education of a young scientist.

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I. INTRODUCTION

A. History of Lyme disease

Lyme disease is a vector-borne illness with symptoms ranging from mild arthritis to chronic cardiovascular and neurological abnormalities. It was first described as a clinical entity in 1977 by reason of a geographic clustering of children in Lyme, Connecticut thought to have juvenile rheumatoid arthritis (1, 2). Due to the rural location of Lyme and distinct rashes which were identified as a feature of the illness it was assumed that the disease was transmitted by an arthropod. It readily became apparent that Lyme disease was in fact a multisystem disorder that affected the nervous system and heart as well as the joints and skin. Epidemiological studies of patients suggested Lyme disease was caused by an infectious agent transmitted by ticks and identified the vector as belonging to the genus *Ixodes* (3). In 1982, almost five years after the search began, a previously unrecognized spirochete (thereafter titled *Borrelia burgdorferi*) was isolated from adult *Ixodes scapularis* ticks which were collected from an endemic focus of Lyme disease (4). Sixty one percent of *I. scapularis* collected in this area contained this newly discovered spirochete (4). Patient sera was demonstrated to contain antibodies recognizing this organism, and in laboratory studies rabbits developed the characteristic Lyme rash after exposure to this spirochete via tick bite (4). Subsequently, the spirochete was actually isolated from several Lyme patients and the spirochetal etiology of Lyme disease was demonstrated conclusively (5). In recent years, there has been an increasing number of reported Lyme disease cases. In 1994 alone there were greater than 13,000 reported cases of Lyme disease making it the most frequently diagnosed vector-borne disease in the United States (6), and consequently a disease of increasing importance.

B. Lyme Disease

1. Introduction

Humans who have been infected with *B.burgdorferi* may develop the pathology known as Lyme disease (7, 8, 9, 10). This disease is a multisystemic disorder, most notably affecting the skin, joints, heart, and nervous system. The clinical course of Lyme disease can be roughly divided into three stages. The first stage occurs in the first days or months following exposure and is characterized by a migrating rash (erythema migrans), fatigue, malaise, and flu-like symptoms. The second stage, occurring weeks to months after initial exposure, is characterized by swelling of joints (infiltration of monocytes into the joint space, synovial hyperplasia, intra- and peri-articular lesions), migratory musculoskeletal pain, as well as cardiovascular abnormalities (tachyarrhythmias, myopericarditis, atrioventricular conduction defect)(11), and neurologic complications (meningitis, encephalitis, optic neuritis, Bell's palsy) (12, 13). The third stage, occurring months to years post infection, is distinguished by severe arthritis, cartilage destruction, joint erosion, and chronic neurological disorders including numbness of limbs, facial palsies, heart palpitations, loss of vision, and memory impairment (12, 13, 14).

Borrelia burgdorferi has been divided into at least six genospecies including the following: *B.burgdorferi* sensu stricto, *B.afzelii*, and *B.garinii*. All three species can cause Lyme disease, and whether these species vary significantly in their physiology and ability to cause pathology is currently under scrutiny. European patients infected with *B.afzelii* or *B.garinii* have a preponderance of chronic skin manifestations as compared to U.S. patients infected with *B.burgdorferi* sensu stricto (15, 16, 17). In addition, arthritis is prevalent in *B.burgdorferi* sensu stricto infection, and neurological abnormalities are prevalent in both *B.burgdorferi* sensu stricto and *B.garinii* infections (17). These noted differences in pathology may be due to differences between the species of *B.burgdorferi* causing the disease. Alternatively the differences seen may be due to the genetic background and immune response of patients, or due to discernment of the doctor treating the patient. Further information

regarding the basic biology of the spirochete as well as the disease process itself must first be elucidated before such questions concerning strain variations and pathogenicity can be properly addressed.

2. Diagnosis

The symptoms of Lyme disease (*i.e.* myalgia, arthritis, myocarditis, and neurological abnormalities) mimic many other disorders (1, 18, 19, 20, 21). The progression of disease and the symptoms associated with it vary dramatically between individuals making a correct diagnosis difficult. Manifestations of Lyme disease often times are not recognized as being part of a single entity. Indeed, after identification of *B.burgdorferi* investigators began to implicate this spirochete as the causative agent of other previously recognized illnesses such as Bannwarth's syndrome (a neurological disorder), and acrodermatitis (a skin disorder). Obviously, the most direct course for diagnosis would be isolation of *B.burgdorferi* from the patient. However, tissue culturing is expensive and requires considerable time and experience; therefore, for many laboratories it is impractical (22). In one study, only 6 of 154 tissue samples from patients have yielded spirochetes (23). Currently, serodiagnosis is the method used to assess *B.burgdorferi* infection (22, 24, 25, 26). Indirect immunofluorescence assay (IFA) or enzyme linked immunosorbent assay (ELISA) are used as a primary screening method for serum samples. Samples which test positive for Lyme antibodies can be further characterized by a western blot analysis. The Center for Disease Control recommends a two test protocol for Lyme diagnosis; an EIA followed by a western blot for confirmation of positive results. A blot is considered positive if antibodies to two out of the three following bands are present: 39 kD (P39), 41 kD (flagellin), and 21-24 kD (OspC) (27). These assays have their limitations as well. Since flagellin is expressed by other organisms, crossreactivity to this antigen is common for patients with other spirochetal illnesses. OspC migration in polyacrylimide gels is variable, as well as its expression among *B.burgdorferi* isolates, making identification difficult. In addition, during the first few weeks of infection, the humoral immune response to the

organism may be weak resulting in a false negative in serologic assays. False positives can also result when patients are afflicted with other spirochetal illnesses. Another dilemma is a curtailed immune response due to the administration of antibiotic treatment early in the infection. All of these factors combine to make actual diagnosis of Lyme disease a challenge.

3. Treatment

Treatment of Lyme disease patients with high doses of oral antibiotics (tetracycline, amoxicillin, doxycycline, or in cases with neurological involvement, ceftriaxone) clears the infection and alleviates most symptoms of the disease if administered early in the course of infection (12, 14, 28, 29, 30). If treatment is given later than six months after initial exposure, the symptoms are usually not completely reversible. Persistent symptoms may be caused by a process which is no longer antibiotic-sensitive (not due to the spirochete). For example, *B. burgdorferi* infection may trigger fibromyalgia, a chronic pain syndrome which is not antibiotic responsive (31). Since no identifiable toxins are expressed by the spirochete (32) the pathology observed in affected tissues may result from specific and nonspecific immunological responses to *B. burgdorferi* (19, 33). Antibodies directed against myelin and myelin basic protein have been reported in patient sera (34, 35). Research into the dynamics of specific and nonspecific immune responses of affected individuals should yield clues to the cause of pathology.

4. Vaccine development

Due to difficulty in diagnosis and the occasional treatment failures, prevention of Lyme disease by vaccination has become an important alternative. The most studied vaccine candidate is a recombinant OspA molecule (rOspA). Active immunization with rOspA or passive transfer of rOspA specific antibodies are protective against *B. burgdorferi* infection in inbred strains of mice (36, 37, 38, 39). Immunized mice are

protected from needle inoculation of infectious spirochetes (37) as well as natural infection via infected ticks (40). However, this vaccine affords protection only at the beginning stages of tick feeding before the spirochetes ever enter the host (41). The tick imbibes blood containing rOspA specific antibodies, and the antibodies destroy the spirochetes while they are still in the tick midgut. Within three to five days of the feeding, spirochetes can be found in tissues other than the midgut, such as salivary glands, malphigian tubules, central ganglion, and ovary (42, 43, 44). Once they are out of the tick gut they are no longer susceptible to the rOspA antibodies as the spirochetes decrease surface expression of the OspA molecule (41). In addition, this protection was only effective against a few isolates of *B.burgdorferi* which share identical OspA sequences (45). This lack of cross protection occurs when using other outer surface proteins as immunogens: OspB, OspC, OspD, OspE, and OspF (46, 47, 48). Because of this lack of cross protection afforded by these molecules, research is currently underway for other vaccine candidates.

C. The Lyme cycle

In nature, the *B.burgdorferi* population is maintained by cycling between hematophagous tick vectors (*Ixodes* spp.) and various vertebrate hosts such as mice and rats (49). The primary tick vectors *Ixodes scapularis* (northeastern U.S.), *I.ricinus* (Europe), and *I. pacificus* (western U.S.) all have a four stage life cycle: egg, larva, nymph, and adult. Ticks feed once during each stage (with the exception of the egg stage). If a tick does not feed it is not able to molt and continue development into the next life stage. Ticks feed by using their elongated, serrated chelicerae to cut into the epidermis of the host, ripping open blood vessels beneath the surface. The host is ordinarily unaware of this event as ticks inject various pharmacological agents into the wound site, via the hypostome, to prevent pain and inflammation from occurring. These agents include prostaglandins and prostacyclins to prevent platelet aggregation, blood coagulation, and vasoconstriction; anti-histamines which decrease inflammation; and other unidentified substances which destroy bradykinin and block pain. Ticks also

secrete a substance produced in the salivary glands which acts as a cement to secure the mouthparts of the tick into the wound site. Over a period of two to five days, ticks slowly imbibe a blood meal from the wound site. After engorgement, the process of detachment is initiated by secretion of an uncharacterized substance which dissolves the cement and allows the tick to exit the wound without damage to the chelicerae.

Ticks may become infected with *B. burgdorferi* after ingesting a blood meal from a host carrying the spirochete. In the wild, the infection rate of ticks in endemic Lyme areas average 12-25% for nymphs, and 25-50% for adults (50, 51). The average spirochetal burden is 2,000 *B.b./tick* in endemic areas (52). After feeding, ticks retain the blood meal in the midgut which acts primarily as a storage organ. Digestion of the blood occurs intracellularly by pinocytosis of cells lining the gut allowing spirochetes and other bacteria to remain undisturbed by enzymatic digestion. Spirochetes may remain in the midgut (42) or migrate out of the mesothelium and into the surrounding tissue. The majority of ticks (>80%) have spirochetes localized primarily in the midgut until after the next blood meal (4, 42, 44). For example, a larval tick becomes infected with its first blood meal and the spirochetes remain in the midgut until feeding commences in the nymph stage. Notably, 80% of infected, fed ticks contain spirochetes in the salivary glands (44). This is a significant figure considering ticks may then infect a host by secreting the spirochetes from the salivary glands directly into the wound site during attachment to the host (43, 44, 53, 54). Additionally, some scientists postulate that ticks may also regurgitate midgut contents, including spirochetes, directly into the wound during feeding (42). Spirochetes may persist and multiply throughout the life of the tick. Therefore, if a tick is infected during the larval stage it has the potential to transmit *B. burgdorferi* to its subsequent hosts during both the nymph and adult stages.

After inoculation into the host via the bite of an infected tick, the spirochetes migrate out of the wound site and invade the surrounding tissue. Spirochetes are motile in viscous mediums (55), and their dissemination throughout the host is due to a combination of passive transport via the circulatory or lymphatic systems, and active migration through the extracellular milieu. *B. burgdorferi* are capable of adhering to a

wide range of cell types such as human umbilical vein endothelial cells (56), tick cells (57) and human epithelial cells (58). *B. burgdorferi* has been shown to adhere with high specificity (via 19 and 20 kD proteins) to the proteoglycan decorin (decorin is bound to collagen fibers), but not to collagens type I or II (59). *B. burgdorferi* has also been shown to penetrate endothelial cell monolayers (60). One study demonstrated that within 24 hours organisms were able to penetrate the blood-brain barrier of rats when injected intravenously (61). However, the spirochetal adhesion molecules and the cell surface receptors involved in these processes have not been described

D. Animal models for Lyme disease

Several animal models, including rabbit, hamster, rat, and mouse, have been developed to study experimental and natural infection with *B. burgdorferi*. After inoculation with *B. burgdorferi*, rabbits develop a skin lesion similar to that found in Lyme patients but do not develop any other specific pathology. Hamster, rats, and mice, however, have proven valuable for following the dissemination of the spirochete after infection as well as immune responses, and pathology. These animal models only partially mimic the human disease. No animal model has yet been described which duplicates the presentation of the human disease. The murine model is the most frequently used system due to the knowledge surrounding the murine genetic and immune systems, and it has the advantage of being a natural reservoir for the spirochete in nature (36). Upon infection mice develop multisystemic infections and remain infected for well over a year (9, 62). In addition, spirochetes can be transmitted to ticks feeding on the infected animals.

Xenodiagnosis is the term used for using ticks for detection of spirochetes in an infected animal. It is commonly used to assess the infectivity of newly isolated spirochetes. In general, transmission of spirochetes to feeding ticks is lower in inoculated mice than in naturally infected mice. Between 42-84% of ticks feeding on naturally infected hosts become infected, as compared to 6-35% for ticks feeding on experimentally inoculated hosts 14 to 100 days post inoculation (63, 64).

Tissue culture is another form of assessing the infectivity of *B. burgdorferi*. Spirochetes have been cultured from all of the following mouse tissues: bladder, spleen, kidney, liver, heart, eyes, brain, joint, skin and blood (7, 8, 9, 10, 36, 37, 62, 65, 66, 67, 68, 69, 70, 71, 72). The bladder has been demonstrated as being the most consistent source for isolation of *B. burgdorferi* in murine tissue culture (7, 24, 37, 65, 66, 67, 68, 69, 73, 74). Recovery of spirochetes from 94% of bladders of infected mice as compared to 61% from spleen tissue, and even lower percentages for blood and kidney (67, 69.). It is unclear whether this high recovery rate from bladder tissue is due to a possible spirochetal predilection for this organ, or due to an artifact of tissue culturing since the spirochetes may be sensitive to components present in the immascerated tissue of certain organs. Interestingly, *B. burgdorferi* gradually lose their infectivity during prolonged in vitro cultivation (7, 9, 24, 65, 66, 67, 69, 73, 75, 76, 77). This loss of infectivity is monitored by tissue culture, and xenodiagnosis.

Histopathology of Lyme disease in mice is wide spread; carditis and arthritis are common, and joints may become inflamed and swollen. Affected joints contain infiltrating polymorphonuclear leucocytes, immune complexes, and occasionally spirochetes. Though inflammation in various tissues is common, spirochetes are not always detected in or around areas of inflammation (78). This fact once again raises the issue of whether the spirochete or the immune response to the spirochete is responsible for tissue pathology.

Borrelia burgdorferi may persist in a host for years after the initial infection (62, 68, 71, 79). The mechanism by which *B. burgdorferi* is capable of surviving the vigorous B and T cell responses generated in an immunocompetent host is currently unknown, and is under intensive scrutiny. Animals inoculated with spirochetes develop an early humoral response (<14 days) to OspA, OspB, P39 and flagellin. Naturally infected animals, including humans, respond with antibodies against the 20 kD, 22 kD (OspC), 35 kD, P39, and 58 kD proteins of *B. burgdorferi*, whereas few recognize flagellin, OspA, OspB until very late into the infection, and sometimes not at all (19, 70, 80, 81). The spirochetes are susceptible to phagocytosis and complement mediated cytolysis (19, 33, 82). Additionally, monoclonal antibodies specific for P39,

OspA, and OspB are bactericidal for *B.burgdorferi* in vitro even in the absence of complement (83). There is no evidence to indicate *B.burgdorferi* can vary surface antigens to escape immune response (84, 85). However, nonspecific interactions between extracellular *B.burgdorferi* proteins and IgM antibodies have been reported (86), raising the possibility that nonspecific immune complexes may contribute to immune evasion by camouflaging the cell surface of the spirochete (86). Much remains to be learned regarding the spirochetes ability to survive an immune response. Indeed, specific factors required by the spirochete to maintain an infection in a mammalian host are currently unknown.

E. Biology of *B.burgdorferi*

The causative agent of Lyme disease is the gram-negative spirochete, *Borrelia burgdorferi* (4). It was described as a new species of the genus *Borrelia* on the basis of DNA-DNA homology and G + C content similarities (87). It was originally believed only a single species of *Borrelia* caused Lyme disease. However, at least six different genospecies of *B.burgdorferi sensu lato* have now been recognized which are classified according to sequence differences in 16s rRNA genes and restriction fragment length polymorphism. Three of these are *B.burgdorferi sensu stricto* (88, 89), *B.afzelii* (90, 91), and *B.garinii* (89, 92). The DNA homology among the different strains is 76 to 100%. All three groups are found in Europe and Asia, but only *B.burgdorferi sensu stricto* has been isolated in the U.S (89). All three can cause Lyme disease but whether these strains vary significantly in their physiology or ability to cause pathology is currently under scrutiny.

Borrelia are the longest and thinnest of the spirochetes ranging in size from 0.2 to 0.5 μm wide and 8 to 30 μm long (93). The cell is comprised of five basic structures: outer membrane, periplasmic space, flagella, inner cell membrane, and cytoplasmic compartment. The membrane composition of *B. burgdorferi* differs from that of other gram negative spirochetes in that it does not contain lipopolysaccharide (LPS) or phosphatidylethanolamine (94, 95). In addition, *B.burgdorferi* have a substantial number of lipidated membrane proteins yet few transmembrane proteins in the outer

envelope (96, 97, 98). *B.burgdorferi* expresses over 30 major proteins, but few have been well characterized. Several techniques have been used to characterize outer membrane proteins, including surface radioiodination or biotinylation of surface exposed proteins. From such experiments thirteen proteins have been identified: 22, 24, 29, 31, 34, 37, 39, 41, 52, 66, 70, 73, and a 93 kD molecule (99). Unfortunately, the further identification of these surface molecules using monoclonals was not performed. Six lipoproteins (designated outer surface proteins or Osp) of unknown function have been identified using monoclonal antibodies: OspA, OspB, OspC, OspD, OspE, and OspF (46, 75, 100, 101). However, the Osps are not exclusively located in the outer envelope as their name might suggest, lipidated Osps are also located in the inner membrane (102). The molecular weight of these molecules has been shown to vary between different isolates of *B.burgdorferi* (103, 104) making the use of monoclonal antibodies essential in the identification of these molecules. Expression of the Osps are affected by changes in temperature (51, 105, 106). When a tick imbibes a blood meal the temperature in the gut increases. This increase in temperature results in a decreased surface expression of OspA and an increased surface expression of OspC, OspE, and OspF. This change in expression of these molecules is not rapid but occurs over several cycles of cell division (105). Early studies indicated that OspA and OspB may be important for adherence or invasion of host cells (107, 108), and OspC for establishment of infection in a host. However, a mutant isolate which does not express OspA, OspB, OspC or OspD was shown to have decreased adhesion to human umbilical vein endothelial cells in vitro yet the infectivity of the organism was unaffected (109). Even though OspC is not critical for infectivity, it is theorized that OspC may be important in the migration of the spirochete from the midgut to the salivary gland (105).

Other *Borrelia* proteins include flagellin (41 kD), P39 (39 kD), two heat shock protein homologs (60 and 73 kD), a 93 kD and a 13 kD protein. Two proteins which appear conserved among all strains of *B.burgdorferi* are P39 and flagellin. P39 is an immunogenic protein of unknown function, and a member of a small family of proteins known as *Borrelia* membrane proteins (bmp). There are four members in this group:

bmpA(P39), bmpB, bmpC, and bmpD. The function of these molecules is unknown but they are highly conserved among all three *B. burgdorferi* genospecies (110). The four genes are located on the chromosome and are arranged in the following order: bmpD, bmpC, P39, bmpB. The bmpD has been shown to be monocistronic transcript (110). BmpC is also believed to be monocistronic (111), but P39 and bmpB are believed to constitute a single operon since P39 has a promoter region whereas bmpB does not (112). All contain basic charged residues followed by a hydrophobic region in the very N-terminus of the protein which is a typical signal sequence of membrane proteins (110, 111, 112). In addition, they all contain putative signal II peptidase site (LFIVAC) which suggests that they may also be lipoproteins yet no evidence of lipid modification has been shown (110, 112). P39, in contrast to the Osps, is not differentially expressed in relation to temperature (105), and no P39-less mutants have ever been described in the literature.

Flagellin is the core protein unit of the spirochetes flagella. *Borrelia* have between 7 and 11 flagella that are sheathed and located between the inner and outer cell membranes, which is characteristic of most spirochetes (93). Only a single flagellar protein constitutes the core structure of the *B. burgdorferi* flagella, unlike other organisms which have a complex of several protein subunits constituting the flagellar filament (113). Each flagellum is anchored to the inner membrane at one end of the spirochete and runs the length of the organism (93). In the middle of the spirochete the flagella from one end overlaps with flagella from the other end and forms a distinct bundle (114). The arrangement of the flagella dictates the helical shape of the cell and allows the spirochete to move in a spiraling motion. It has been shown that the spirochetes need a viscous medium, similar to the intercellular matrix of skin, in order to be motile (55). Spirochetes lose their motility in less viscous fluids such as blood, and appear to simply spin in place instead of progressing in any direction (55).

Growth of *B. burgdorferi* in vitro is slow and requires microaerophilic conditions, temperatures between 30 and 35°C, and a complex liquid medium known as Barbour-Stoenner-Kelly medium (BSK). The generation time is 12-24 hours (32)

and *Borrelia* divide by forming a septum as opposed to division by constriction (93). As previously mentioned, the organisms gradually lose their infectivity during prolonged in vitro cultivation (7, 9, 24, 65, 66, 67, 73, 75- 77). The loss is irreversible and occurs as early as the fifth in vitro passage or as late as the 10th passage. The direct reason for this loss is unclear, but investigators have documented a variety of changes that occur during culture including loss of DNA plasmids, changes in morphology, protein expression, and antigenicity (7, 65, 75-77, 111, 115-117). Loss of extrachromosomal elements and subtle changes in antigenicity do occur in the zoonotic cycle, but any variation is limited compared with changes seen during in vitro cultivation (111). Investigators have tried to identify factors responsible for the infectivity of the organism by comparing whole cell sonicates of infectious and noninfectious organisms and have documented specific DNA plasmids and proteins which are no longer present in some noninfectious isolates (65, 68, 75, 76, 115-117). However, the loss of plasmids or protein expression is not consistent among isolates which have lost infectivity. This raises the possibility that infectivity is dependent upon a number of factors; the loss of any one factor results in loss of infectivity. To date, no factors critical to the infectivity of the organism have been identified.

The genetic makeup of *B. burgdorferi* consists of a 1000 kilobase chromosome and has a cytosine/guanine ratio of 27 to 30%. In addition to the chromosome, four to nine circular and linear plasmids (linear plasmids are unique to the genus *Borrelia*) ranging in size between 16- to 175-kilo bases have been identified in isolates of *B. burgdorferi* (49, 65, 118, 119). The spirochetes are clonal and show no evidence of lateral gene transfer between organisms (91). Therefore, all genes within a strain will share a common evolutionary history in the absence of recombination. In addition, no antigenic variation involving altered expression of proteins by genetic modification, conferring a selective advantage to the organism has been documented for *B. burgdorferi*. Fifteen other *Borrelia* species, such as *Borrelia hermsii* the causative agent of relapsing fever, exhibit antigenic variation by changing the expression of immunodominant, surface exposed, lipoproteins known as Variable Major Proteins or VMP (77). The genetic switch occurs between two linear plasmids, one carrying the

silent VMP gene and the other carrying the expressed VMP gene proceeded by a promoter. The silent gene physically replaces the active gene during genetic recombination, and the formerly active gene is lost. *Borrelia burgdorferi*'s close relation to these organisms, and possession of linear plasmids, led researchers to investigate antigenic variation in the Lyme disease spirochete. Initially data regarding the discrepancy between protein profiles and antigenicity of spirochetes recovered from inoculated hosts versus the profiles of the original inoculum (usually an uncloned population) was misinterpreted as possible antigenic variation (77, 104). However, subsequent analysis using cloned organisms revealed the inaccuracy of the conclusion.

F. Discovery of P39

P39 was initially described in an attempt to identify *B. burgdorferi* specific antigens that could enhance the specificity of diagnostic assays. P39 was shown to be consistently immunogenic in experimentally and naturally infected mice (24, 74) and one hundred percent (n=94) of confirmed Lyme patients tested produced antibodies specific for P39 (74). More importantly, no anti-P39 antibodies were found in control sera collected from 25 patients with other spirochetal illnesses such as syphilis, and relapsing fever, or amyotrophic lateral sclerosis (an auto immune disorder) or normal individuals (74). It was also demonstrated that P39 expression is conserved among North American and European isolates (24), a trait not shared by several other *B. burgdorferi* proteins such as OspA, OspB, and OspC (103, 104). As a result, the immunogenicity, species specificity, and conserved expression of P39 have made it an indispensable tool for the diagnosis Lyme disease. Currently microtiter plates coated with *E. coli* expressing recombinant P39 are used in screening of sera for Lyme disease (General Biometrics, San Diego, CA). Purification of P39 does not enhance the effectiveness of this assay, therefore the *E. coli* expressing P39 is used.

Two different monoclonals were developed for further study of P39. The first monoclonal was developed using recombinant P39 antigen. The gene encoding P39 was cloned in a pBluescript vector designated pSPR33. This recombinant P39 antigen was expressed in *E. coli* and was injected into Balb/c mice. The monoclonal antibody

D1C9 was obtained from this experiment and subsequently shown to be specific for *B.burgdorferi* P39 antigen by western blot analysis (24, 74). The epitope for D1C9 has not yet been mapped, but the monoclonal has a high titer and is commonly used in our laboratory at a 1/250 or greater dilution for IFA or ELISA. Another monoclonal to P39, Hkmy, was developed by injecting *B.burgdorferi* into mice and screening hybridomas for reactivity to P39. However this monoclonal is of very low titer and must be used undiluted. This monoclonal was not used in the present study due to the weak reactivity to P39. Accurate detection and quantitation of P39 was necessary for this study and the weak reactivity of this antibody would not allow such analysis.

The location and function of P39 within the organism is unknown. However, a sequence analysis of the cloned P39 gene revealed the coding sequence to be 1020 nucleotides in length (112) and the deduced amino acid sequence has the highest homology (27%) with TmpC, a putative membrane protein of the spirochete *Treponema pallidum* (112) also of an unknown function. The amino terminus of P39 contains charged residues followed by hydrophobic residues consistent with a signal peptide sequence, and the amino acids from residues 14-18 are consistent with a signal peptidase II site (112). These observations suggest that P39 may be associated with either the outer membrane or the inner membrane of *B.burgdorferi* (112). A study attempting to locate P39 in whole cell *Borrelia* using immuno-electron microscopy (EM) with monoclonal antibody Hkmy gave some evidence for membrane as well as cytoplasmic localization (120). However, most EM studies of P39 have been unsuccessful (this lab, Schwan unpublished data) making confirmation of this finding difficult.

G. Focus of the present study

1. Rational

P39 is an important molecule in respect to the diagnosis of Lyme disease, yet little is currently known about the molecule. For instance where it resides, what it associates with in the cell, and if it is related to the infectivity of the organism, or a

cause in the pathology of Lyme disease are all unknown. The objective of the present study is to more fully understand the physical and biochemical nature of the P39 molecule expressed by *B.burgdorferi*. If the molecules responsible for *B.burgdorferi* infectivity or pathology in Lyme disease are elucidated, more effective treatments and therapies for Lyme disease patients would be forthcoming.

2. Specific Aims

1. The first objective was to determine where P39 resides within infectious and noninfectious spirochetes and determine if there is a correlation between P39 localization and infectivity. Whole cell spirochetes were separated into three subcellular fractions: outer envelope, innermembrane, and cytosolic fractions. Cell fractions were analyzed by SDS-PAGE, silver stain and western blot to determine the protein composition of each fraction. This procedure was performed on both infectious and noninfectious isolates, allowing for correlation between P39 localization and infectivity of the organism.

2. The next aim was to determine if P39 surface expression was uniform throughout a population of spirochetes. Since several of the isolates used in the study were uncloned populations, it was important to determine the variability in P39 surface expression within an isolate. Both cloned and uncloned populations of *B.burgdorferi* were analyzed by flow cytometry after staining with a monoclonal specific for P39.

3. The next aim was to determine the solubility of P39 and determine if the solubility of P39 differed in infectious and noninfectious isolates. Detergent extraction techniques were employed to separate hydrophobic and hydrophilic components of cell fractions from infectious and noninfectious isolates, and they were analyzed by SDS-PAGE and western blot.

4. The next aim was to determine what molecules associate with P39, and if those molecules are different in infectious and noninfectious isolates. Whole cell sonicates were subjected to immunoprecipitation using a monoclonal specific for P39. The immunoprecipitants from infectious and noninfectious isolates were then analyzed by SDS-PAGE and silver staining.

5. The final aim was to assess the role of P39 in the infectivity of the spirochete and in the pathology of Lyme disease. The cloned P39 gene was mutated by deletion of a 300 bp fragment from the front of the reading frame. The resulting mutant P39 gene was used to transform low passage infectious organisms in order to create *B.burgdorferi* P39-less mutants. Using this approach, one can specifically determine if expression of P39 is required for *B.burgdorferi* viability, infectivity, or if P39 is involved in the pathology of Lyme disease.

II. MATERIALS AND METHODS

A. Materials

1. *Borrelia* isolates

Characteristics of P39 (localization, etc.) were investigated by comparing various infectious and noninfectious isolates of *B.burgdorferi*. All isolates used in this study are members of the group *B.burgdorferi sensu stricto* originally isolated from *Ixodes scapularis* ticks (4). Isolate CN40 is a clonal population derived from isolate N40 after the fourth in vitro passage (62). N40 was originally isolated in 1988 from a tick in Westchester County, New York (121). CN40 is the only population of *B.burgdorferi* known to retain infectivity during in vitro culture. This isolate allows observation of changes in *B.burgdorferi* which are due to in vitro culture and not related to loss of infectivity. CN40 was kindly provided by Dr. Stephen Barthold (Yale University, New Haven, CT).

All remaining isolates are uncloned populations, and serve as model isolates which lose infectivity after in vitro culture. These isolates include B31, WCH1, and JD1. Isolate B31 (purchased from American Type Tissue Culture Collection ATCC 35210) is the prototype *B.burgdorferi* isolate, and was recovered from a pool of adult *Ixodes scapularis* from Shelter Island, NY by Willy Burgdorfer in 1982 (4). Isolate WCH1 was isolated from adult *Ixodes scapularis* ticks from Westchester County, N Y in 1986. Isolate JD1 was isolated from nymphal *Ixodes scapularis* ticks collected in Ipswich, MA in 1987 (53). Both JD1 and WCH1 were a gift from Dr. Joseph Piesman (Center for Disease Control, Fort Collins, CO) for use in this study.

Nine additional clones were generated from WCH1 by the author by limiting dilution, and analyzed by flow cytometry (see below).

2. Ticks

Laboratory raised, spirochete-free *Ixodes scapularis* nymphal ticks were a gift from Dr. Joseph Piesman (Center for Disease Control, Fort Collins, CO), and were kept in mesh covered glass vials and placed in a climate controlled incubator ($26^{\circ}\text{C} \pm 1^{\circ}$) with $92 \pm 2\%$ relative humidity and 14:10 light dark cycle.

3. Mice

Six to 12 week old, female B6CBAF1/J mice (Jackson Laboratory, Bar Harbor, ME) were used for the infectivity assay. Prior to the assay, daily care for the animals were provided by the Animal Facility at Old Dominion University. All treatment of the animals was approved by the University IACUC and was in accordance with the Federal Guide for the Care and Use of Laboratory Animals.

4. Medium

Borrelia burgdorferi isolates were cultured in liquid BSK II or BSKH medium supplemented with 6% rabbit serum (Pel Freeze Biologicals, Rogers, Arkansas) and antibiotics (phosphomycin 100 mg/ml and rifampicin 50 mg/ml, SIGMA chemical company, St. Louis, MO). For infectivity studies cultures were grown in 15 ml screw cap vials, and for fractionation studies isolates were grown in three 30 ml culture flasks.

5. Monoclonal antibodies

Monoclonal antibodies (Mab) used in this study were as follows: Mab D1C9, specific for P39 was provided by Dr. Tom Schwan (Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, MT). Mab H5332 specific for OspA, Mab H6831 specific for OspB, Mab H9724 specific for flagellin, and H4825 specific for *B. hermsii* were provided by Allen G. Barbour (Health Science Center, Univ. of Texas, San Antonio, TX). Goat anti-mouse IgG fluorescein-isothiocyanate conjugate (FITC) was purchased from Kirkegaard Perry Labs, Gaithersburg, MD, as well as peroxidase labeled goat anti-mouse IgG.

6. Plasmids

a. pSPR33

The pBluescript plasmid pSPR33 contains an 6.3 kb insert encoding P39 and a second open reading frame (ORF2) (24, 74). The plasmid was given to this lab in a glycerol stock by Dr. Tom Schwan (Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, MT).

b. pCH110

The plasmid pCH110 contains an insert (3.6 kb) encoding the entire Beta galactosidase gene (LacZ). LacZ metabolizes B-galactose into glucose and lactose molecules. The plasmid was given to this lab by Dr. Timothy Bos (Eastern Virginia Medical School, Norfolk, VA).

7. PCR Primers

The primer sequences were derived from a randomly cloned, chromosomal DNA of isolate B31. The sequence is specific for *B.burgdorferi* and is not present in other *Borrelia* species (122, 123, 124, 125). Initially described by Rosa and Schwan (123, 125), this genomic sequence has been analyzed by several databases and no homology has been found between the deduced amino acid sequence and any known proteins. Regardless, the value of this sequence lies in its conservation among *B.burgdorferi* and its absence in other *Borrelia* species. In addition, it is a chromosomally located gene sequence as opposed to a plasmid encoded gene; this is an important feature since plasmids are heterogenous among isolates and can be lost during in vitro culture.

Forward primer sequence: CGAAGATACTAAATCTGT

Reverse primer sequence: GATCAAATATTTTCAGCTT

The primers were synthesized at the Core Molecular Biology facility at Eastern Virginia Medical School.

8. Buffers

Sodium bicarbonate buffer: 14 mM Na_2CO_3 , 34 mM NaHCO_3 , pH=9.6

Phosphate buffered saline (PBS): 10 mM NaCl , 8 mM Na_2HPO_4 , 8 mM NaH_2PO_4 , pH=7.4

PBS-Tween: PBS supplemented with 0.05% Tween 20 detergent (Sigma).

PBS-Tween/BSA: PBS-Tween supplemented with 1 mg/ml Bovine Serum Albumin (BSA, Sigma).

PBS/ MgCl_2 : PBS supplemented with 5 mM MgCl_2 pH=7.8

Ketamine/xylazine solution: xylazine 20 mg/ml (Butler, Columbus, OH) and ketamine 62.5 mg/ml (Aveco Co., Fort Dodge, IA)

Lysis buffer: PBS, 1% SDS, and 0.1 mM proteinase K

2x sample buffer: 1.25 mM Tris[hydroxymethyl]amino-methane (Tris), 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, H_2O , pH=6.8

Coomassie fixing solution: 40% methanol, 10% acetic acid, 0.5% coomassie brilliant blue

Destaining solution: 40% methanol, 10% acetic acid solution

Silver equilibration solution: 0.5% silver nitrate in water

Development solution: 10% sodium carbonate, 0.05% formaldehyde in water

Stop solution: 1% glacial acetic acid in water

Reducer solution : 0.6% Potassium ferricyanide, 1.3% Sodium thiosulfate, 0.2% Sodium carbonate

Electroporation solution (EPS): 272 mM sucrose and 15% glycerol.

TE buffer: 50 mM Tris, 50 mM EDTA, pH=8.0

10x blunt end buffer: 100 mM Tris pH=7.5, 0.5 mM EDTA, 50 mM MgCl_2

NET buffer: 150 mM NaCl , 5 mM EDTA, 50 mM Tris pH=7.5, 0.05% NP-40

Resuspension Buffer P1: 100 $\mu\text{g/ml}$ RNase A, 50 mM Tris/HCL, 10 mM EDTA, pH=8.0

Lysis Buffer P2: 200 mM NaOH, 1% SDS

Neutralization Buffer P3: 3.0 M potassium acetate KOAc, pH=5.5

Equilibration Buffer QBT: 750 mM NaCl, 50 mM MOPS, 15% ethanol, pH=7.0, 0.15% Triton X-100

Wash Buffer QC: 1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH=7.0

Elution Buffer QF: 1.25 M NaCl, 50 mM Tris/HCL, 15% ethanol, pH=8.5

B. Methods

1. Establishment of infectious and noninfectious populations of *Borrelia burgdorferi*

Noninfectious isolates were generated from infectious WCH1 passage 5 (p5), JD1 passage 3 (p3), and B31 passage 6 (p6) by culturing each in vitro for approximately 3 months. The *Borrelia* were grown in screw cap vials with 15 ml of BSK II incubated at 33°C, and were passed once a week by transferring 5% of spent culture to a new tube of fresh media (equivalent to one passage). Isolates were routinely analyzed by dark field microscopy and motility, activity, and general appearance were noted. An isolate was designated high passage after passage 15. Passage fifteen was considered a sufficient end point for high passage since most isolates lose infectivity by the tenth passage. In this study, isolate CN40 was cultured as described above and harvested at p9 and p22. Isolate CN40 is unique in its ability to retain infectivity during in vitro culture and was used in this study as a control for changes induced by in vitro culture which have no effect on infectivity. All isolates were counted using Petroff-Hauser bacterial counting chamber (Baxter Scientific, McGaw Park, Ill.). Each isolate was counted three times and the values averaged. The averaged value was recorded as the concentration of spirochetes in culture.

The method used to assess infectivity of *B. burgdorferi* has been previously described (7, 67, 68). Briefly, low passage ($p < 10$) and high passage ($p \geq 15$) isolates were harvested by centrifugation at 10,000 x g for 10 min at 4°C. Spent media was decanted and cells were washed to remove all traces of media by resuspending the cell

pellet in 10 ml of PBS/MgCl₂ and repeating the centrifugation step. This washing step was repeated once more and the final cell pellet was resuspended in 5 ml of PBS/MgCl₂. The *Borrelia* were counted on a Petroff-Hauser chamber and adjusted to 10⁶ organisms per ml. Viability of washed organisms was confirmed by re-inoculation of 20 µl into 2 ml of BSK II media.

For each isolate, two B6CBAF1/J mice received an intraperitoneal (i.p.) injection of 100,000 spirochetes. After 15 days, mice were sedated with a 50 µl injection of ketamine/xylazine solution, and 25 *I. scapularis* nymphs were allowed to feed on the mice for xenodiagnosis. Mice were then placed in individual chambers with wire mesh flooring. The chambers were elevated over collecting trays lined with double sided tape which served to retain the ticks once they detached from the mice after feeding. The mice were kept in these cages for five days. The engorged nymphs were collected and kept in glass vials for 6 days at room temperature to allow *Borrelia* to multiply. Using a sterile razor blade, ticks were dissected and gut contents were smeared onto a glass slide and processed by immunofluorescent antibody assay (IFA) for detection of *Borrelia* as described below.

Mice were sacrificed by cervical dislocation 21 days post-inoculation and the bladders were surgically removed and divided into two equal halves. One half was cultured in BSK-II media (tissue culture) and the other half was frozen in BSK II media at -80°C until needed for further analysis by PCR as described below. An isolate was considered infectious if spirochetes were recovered from mouse tissue, detected in tick smears, or detected by PCR in tissue samples. All negative results were confirmed by repeating the experiment.

For tissue culture, one half of a mouse bladder was placed in an eppendorf tube containing 500 µl BSK II media. The tissue was mascerated for one minute using a sterile pestle. The mascerated tissue and media was divided into two aliquots and each aliquot was added to a 10 ml tube of BSK II. Cultures were incubated at 33°C, and were viewed by dark field microscopy every 3-4 days for detection of *B. burgdorferi*. The presence of the spirochete was confirmed by immunofluorescence assay as described below.

a. Immunofluorescence assay (IFA)

Culture supernatants (20 µl) or tick guts (each tick analyzed individually) were smeared onto glass slides, air dried for 10 minutes, then fixed with acetone (Mallinckrodt Inc., Paris, KY) for 20 minutes at room temperature. The slides were rinsed 3 times in PBS and 10 µl of undiluted monoclonal antibody (Mab) H5332 specific for *B.burgdorferi* outer surface protein A (OspA) was added to each slide and incubated for 1 hour in a humid, dark chamber at 37°C. The slides were rinsed 3 times in PBS and 10 µl of goat anti-mouse IgG fluorescein-isothiocyanate conjugate (1/40 in PBS) was added to each slide and incubated for 1 hour at 37°C in a humid, dark chamber. The slides were rinsed 3 times in PBS and frozen at -20°C until viewed by fluorescence microscopy using an Olympus AH-2, Vanox model T microscope.

OspA was selected as the target antigen due to the high surface expression of this antigen in all the isolates used in this study (53, 126). IFA staining of OspA gives very high fluorescence and is readily detectable. Other antigens, such as P39, are not readily detectable by IFA presumably because the epitope is fixative sensitive. Fluorescent intensity was monitored and given a rating: - (no fluorescence) or + (fluorescence equivalent to a positive control slide containing B31 spirochetes). Tissue cultures were designated positive if one or more spirochetes were detected by IFA, and negative if no spirochetes were detected by IFA.

b. Polymerase chain reaction (PCR)

PCR was used to detect *B.burgdorferi* DNA in bladder tissue samples taken from mice using methods previously described (122, 123, 124, 125). Frozen tissues (one half of the total organ) were thawed at room temperature then rinsed in sterile PBS and minced with a sterile scalpel. The minced tissues were suspended in 1 ml of lysis buffer and incubated at 37°C for 24 hr. Samples were then extracted twice with 1:1 volume of phenol/chloroform followed by a final 1:1 chloroform extraction. The DNA was precipitated by the addition of 1 ml of ice cold 100% ethanol and 10 µl of 3M

sodium acetate. The samples were incubated at -20°C overnight and DNA was pelleted by centrifugation at 14,000 rpm for 15 min at 4°C. The pellets were air dried and resuspended in 50 µl sterile water. Samples were heated to 100°C for 5 min to inactivate proteinase K. The DNA concentration was determined by OD 260. Each PCR reaction contained 500 ng of template DNA, 5 µl of each primer (10 µM), 10 µl of dNTP (1.25 mM), 1 µl of Taq polymerase, 10 µl of 10x PCR buffer, and a final magnesium concentration of 1.5 mM in a 100 µl volume. Controls included the following: a water control for PCR contamination, 500 ng of DNA extracted from uninfected mouse tissue spiked with 1 µg, 10 ng, or 10 pg of *B.burgdorferi* template DNA. After an initial 5 min denaturing step at 94°C, 30 cycles were executed as follows: denature 1 min at 94°C, anneal 30 sec at 37°C, and extension 2 min at 72°. PCR was performed on an automated thermal cycler (Perkin Elmer). Ten microliters of each PCR reaction was electrophoresed on a 0.8% agarose gel followed by staining with ethidium bromide to detect DNA bands.

2. Protein localization in *B.burgdorferi*

a. Cell fractionation of *Borrelia burgdorferi*

Spirochetes were collected from culture by centrifugation at 10,000 x g at 4°C for 10 min and adjusted to 35×10^6 organisms/ml in PBS. Spirochetes were not washed further due to the fragile nature of the outer envelope (127). The outer envelope was removed by adding sodium dodecyl sulfate (SDS) to a final concentration of 0.03% in a 10 ml volume of spirochetes, followed by incubation at room for 15 min. During this step the outer envelope is solubilized and remains in the aqueous phase. The solution was centrifuged at 15,000 x g for 90 min to pellet the intact protoplasmic cylinders (intact cell without outer envelope). The supernatant containing the outer envelope fraction (OE) was removed and the protoplasmic cylinders were resuspended to the original volume in PBS thereby retaining equivalent concentration of spirochetes. The protoplasmic cylinders were then sonicated on ice for 3 minutes in 20 second pulses to break apart the cell. The sonicate was centrifuged

for two hours at 138,000 x g at 4°C to separate the inner membrane fraction and the cytosolic fraction (pellet and supernatant respectively). The cytosolic fraction (CY) was removed and the inner membrane fraction (IM) was resuspended in PBS to original volume to maintain equivalence. All fractions were stored at -20°C. A media sample was run through the fractionation procedure and 8 ul were included on gels developed by silver staining (media control).

b. Protein assay of cell fractions

The protein concentration of each cell fraction was determined using Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Standards were tested in duplicate at the following concentrations: 0.5, 1, 2, 4, 8, 12, 16, and 20 µg/ml. Each sample was tested in triplicate at the following volumes: 5 ul of outer envelope, 10 ul of inner membrane, and 20 ul of cytosolic fraction. Due to the low protein content of the inner membrane and cytosolic fractions, a larger sample size was needed to assess the protein concentration within the range of the assay (0.5 ug/ml).

c. SDS-PAGE

All reagents were prepared according to the method of Laemmli (128). The cell fractions collected from isolates were subjected to SDS-PAGE. Equal volumes (8 microliters) of each cell fraction (OE, IM, CY), representing material derived from 10⁷ organisms, were added to 20 microliters of 2x sample buffer. The samples were boiled for 1 min then loaded onto a SDS-PAGE 12-15% gradient gel and electrophoresed at 250 volts for approximately 3.5 hours. Gels were then processed by silver staining or western blot as described below.

d. Western Blot

The electrophoresed proteins were transferred to 0.22 micron nitrocellulose paper (Micron Separations Inc., Westboro, MA) via western blot at 14 volts overnight. The blot was soaked for 1 hour in 1/20 milk diluent (KPL) in PBS-Tween 20. Spirochetal antigens (P39, Osp B, Osp A, and flagellin) were detected using the

following monoclonal antibodies: D1C9 (anti-P39, 1/250), H6831 (anti-OspB, 1/1500), H5332 (anti-OspA, 1/2000), H9724 (anti-Fla, 1/100). Blots were incubated with the monoclonal antibodies for 1 hour on a rotating plate. The blots were rinsed 4 times with PBS/Tween 20, then incubated with a 1/2000 dilution of goat anti-mouse IgG peroxidase (KPL) conjugate for 1 hour on a rotating plate, followed by four successive washes with PBS/Tween 20. Blots were developed with TMB peroxidase substrate (KPL) for 5-10 min followed by one thorough wash with distilled water to stop the reaction. Blots were scanned by a LKB 2222-20 Ultrosan laser densitometer (Pharmacia, Bromma, Sweden) for quantitation of reactive proteins in each fraction. Data was analyzed by regression analysis.

e. Silver Stain

Gels were silver stained using Sigma's Silver Stain Kit AG-25 as directed by the manufacturer. Gels were placed in 300 ml of silver equilibration solution for 30 min with constant agitation. The silver solution was discarded and the gels quickly rinsed in water for 10-20 seconds. Developer solution was then added to the gels in two successive washes of 150 ml each. The development of bands was aborted by washing the gels in Stop solution for 5 min, followed by three washes in water for 10 min each. Gels were then placed for 20 seconds in 300 ml of Reducer solution in order to reduce background staining. Gels were subsequently washed three times in water for 10 min each rinse. Gels were then photographed for preservation of the data. According to the manufacturer, silver staining can detect as little as 50 ng of protein, but the intensity of staining depends upon the characteristics of each individual protein (129, 130).

3. Flow Cytometry

a. Generation of clonal populations of WCH1

Clones of isolate WCH1 p5 were generated by limiting dilution (131). Cells were counted using a Petrof-Hauser chamber, and one ml of culture was serially diluted

10-fold in BSK II media until a concentration of approximately one *B.burgdorferi*/ml was obtained. Fifty cultures (one ml each) were grown as described above and checked every week using dark field microscopy for detection of *B.burgdorferi*. A total of nine clones were obtained from the 50 cultures. Clones were designated WCH1 clone 1, clone 2, etc. through clone 9 and were harvested at p6 and p17. Clone 4, 7, and 8 were discarded because of fungal contamination.

The remaining clones were analyzed by ELISA for differences in P39 expression using the following procedure: Whole cell antigen was prepared by harvesting organisms from a 30 ml culture (passage 6) by centrifugation at 10,000 x g for 10 min and adjusting in PBS to a cell density of 0.25 OD at 650 nm (approximately 10^6 org./ml). Sonicated antigen of these six clones was prepared by sonicating on ice one ml of whole cell suspension four times for 30 seconds at 30 W (Sonifier cell disrupter, Branson Sonic Cc., Danbury, CN). Whole cell and sonicate preparations of WCH1 p.5, and WCH1 clones at p.6 were plated in duplicate on 96 well microtiter plates (Falcon 3911 assay plates, Becton, Dickinson Labware, Oxnard, CA) using sodium bicarbonate buffer. The final volume in each well was 100 ul. Plates were covered with saran wrap and stored at 4°C overnight. Plates were washed three times in PBS-Tween. Each well was blocked using PBS-Tween/BSA at a final volume of 100 ul/well. Plates were incubated with constant agitation 0.5 hour. Plates were washed three times in PBS-Tween. Antigen was detected using Mab D1C9 (specific for P39) at 1/1000 in PBS-Tween/BSA. Plates were incubated with constant agitation for 0.5 hour, then washed three times in PBS-Tween. Bound antibody was detected using peroxidase conjugated Goat anti-Mouse IgG at 1/800 in PBS-Tween. Plates were incubated with constant agitation for 0.5 hour. Plates were washed three times in PBS-Tween. TMB Peroxidase substrate (KPL) was added at a final volume of 100 ul/well. After five minutes, plates were read on a spectrophotometer at 650 nm. Two wells containing no antigen were included on each plate. The readings obtained in these two "blank" wells were averaged and this value, designated nonspecific reactivity, was subtracted from the values obtained for whole cell and sonicate antigen.

The adjusted values of each duplicate sample was then averaged and used as the final value of absorbance at 650 nm. Interassay sample variation was less than 0.2 units of absorbance.

b. Flow cytometry analysis of cloned and uncloned *B.burgdorferi*

Whole cells were assayed by flow cytometry to determine if outer surface expression of P39, OspB, and OspA were uniform among cloned, uncloned, infectious, and noninfectious populations of *B.burgdorferi*. Two hundred microliters of uncloned, whole cell WCH1, JD1, and B31, as well as the cloned populations of CN40, and WCH1 clone 1 were incubated with 20 ul of undiluted anti-OspA, anti-OspB, anti-Fla, or anti-P39 for 30 min at room temp. Cells were pelleted by centrifugation at 14,000 rpm for 1 min, the supernatant was aspirated, and the pellet resuspended in 200 ul of PBS. Five microliters of goat anti-mouse IgG fluorescein-isothiocyanate conjugate (1/40 in PBS) was added to each sample. All samples were covered with foil to protect from the light. Samples were incubated for 30 min at room temp, washed twice in PBS, then resuspended to 500 ul. PBS. Samples were filtered through a 0.44 micron filter to remove any particulate debris, then read on a FACScan single laser flow cytometer (Becton Dickinson, Mountain View, CA). Settings were as follows: 0.250mW at 430 volts, 5000 organisms read for each sample. The control for nonspecific staining was B31 incubated without primary antibody, but with the secondary antibody.

4. Triton X-114 phase partitioning of hydrophobic proteins

Triton X-114 phase partitioning of *Borrelia* proteins (132, 133, 134, 135) was performed in order to determine the soluble character of P39. Two hundred microliters of whole cell sonicate or cell fractions from CN40 or B31 were mixed with 600 ml of TE buffer and Triton X-114 (SIGMA) was added to a final concentration of 1%. The samples were placed on ice for 15 minutes and inverted occasionally. The samples were then warmed to 36°C for 5 min. When the solutions became cloudy, the samples were centrifuged at 2,500 rpm for 3 min in order to separate the phases. The aqueous

phase was transferred to a new tube. All detergent soluble phases were washed three times by resuspension in TE and Triton X-114 (final concentration of 1%), followed by incubation on ice for 15 min, incubation at 36°C for 5 min, and centrifugation.

Aqueous phases were washed 3 times by adding Triton X-114 to a final concentration of 2% (to ensure complete removal of hydrophobic molecules), followed by incubation on ice for 15 min, incubation at 36°C for 5 min, and centrifugation. The final aqueous and detergent soluble fractions were then acetone precipitated with 2 volumes of cold acetone and incubated overnight at 4°C. The samples were centrifuged at 14,000 rpm for 10 min at 4°C. The pellets were air dried for 20 min and resuspended in 100 µl of 1x Laemmli sample buffer. Prior to loading onto a SDS-PAGE, the samples were boiled for 5 min then centrifuged for 1 min at 10,000 rpm to pellet any insoluble material. Thirty microliters were loaded onto a 12.5% gel. Electrophoresis was conducted for 3 hours at 250 volts. The gels were subjected to western blot and developed using antibody D1C9 specific for P39 as described previously.

5. Immunoprecipitation

Immunoprecipitation of P39 from whole cell sonicates of infectious and noninfectious spirochetes was performed. Two hundred microliters (approximately 7×10^6 organisms) of whole cell sonicate were precleared by adding 450 µl of NET buffer, 1 mg/ml BSA, and 50 µl of prewashed Pansorbin. The mixture was incubated for 1 hr at 22°C, followed by centrifugation at 10,000 x g for 1 min. The supernatant was removed and placed in a clean eppendorf tube. Ten microliters of monoclonal antibody D1C9 (specific for P39), or an isotype matched control, Mab H4825 specific for a *Borrelia hermsii* antigen (104), was added to each tube and incubated at 22°C for 1 hr. Seventy five microliters of prewashed Pansorbin was added and the mixture was incubated for 10 min at 22°C. The Pansorbin was collected by centrifugation for 1 min at 10,000 x g. The samples were washed three times in 750 µl of NET buffer. The final pellet was resuspended in 50 µl of sample buffer and boiled for 5 min. Samples were centrifuged at 3,000 x g to pellet insoluble material, and the supernatant was loaded onto 12.5% SDS-PAGE gel. Gels were electrophoresed for 3.5 hr at 250 volts

and subsequently silver stained. Immunoprecipitation was also attempted with ZYMED (recombinant protein A-sepharose 4B conjugate, ZYMED Laboratories, Inc., San Francisco, CA), but high background and low recovery of P39 were found to occur with this product and therefore was not used in this study.

6. Genetic manipulation of the cloned P39 gene

To determine if P39 expression in *B.burgdorferi* is required for the infectivity of the organism, or involved in the pathology in Lyme disease an attempt was made to inactivate the P39 gene in the spirochete. Two strategies were employed to inactivate the endogenous P39 gene: first, a P39-LacZ gene fusion, and second, a P39 deletion mutant construct.

a. Attempts to construct a P39-LacZ fusion gene

A unique restriction site Bsu36I was detected 90 nucleotides 3' of the start codon of P39 (Appendix 1). This site was selected as the insertion point for the LacZ gene. The proximity to the start site ensures no functional P39 activity will exist in the P39 lacZ fusion. The lacZ gene already contains a Bsu36I site at the 5' end of the gene but does not contain a Bsu36I site at the 3' end of the gene (Appendix 2). Two different strategies were employed for creating a 3' sticky end compatible with the P39 Bsu36I site. The first approach required using oligo linkers to insert a new Bsu36I site within the BamHI site 3' of LacZ gene. The second strategy utilized a MaeI site (sequence compatible sticky end) 3' of the LacZ gene instead of creating a new Bsu36I site.

(1). Plasmid pSPR33

The plasmid pSPR33, encoding P39, was given to this lab in a glycerol stock of JM109 *E.coli* cells. A sample from the glycerol stock was streaked onto a LB agar plate containing 200 mg/ml ampicillin (LB/Amp). Plates were incubated overnight at 37°C. One colony was selected from the plate, inoculated into 5 ml of LB/Amp media, and incubated overnight at 37°C with constant rotation. For large scale production of the plasmid, 150 ml of LB/Amp was inoculated with 1.5 ml of overnight culture, and

incubated overnight at 37°C with constant rotation. The culture was harvested the next day by centrifugation at 14,000 rpm x 2 min x 4°C. Purified plasmid was collected from the culture using QIAGEN midi kit as directed by the manufacturer (QIAGEN Inc., Chatsworth, CA). The directions for the midi prep are shown below. The cell pellet was resuspended in 4 ml of Resuspension Buffer P1. Four ml of Lysis Buffer P2 are added, mixed gently by inversion of tube, and incubated at room temp for 5 min. Four ml of Neutralization Buffer P3 are added and mixed immediately by inversion of the tube, and incubated on ice for 15 min. The samples are then centrifuged for 30 min. at 30,000 x g at 4°C. The supernatant is removed promptly and is passed over an equilibrated QIAGEN column (previously washed with Equilibration Buffer QBT) which acts to retain the plasmids. The column is then washed twice with 10 ml of Wash Buffer QC. This removes all nonspecifically bound molecules from the column. The plasmid prep is then eluted off of the column with 5 ml of Elution Buffer QF. The eluate, containing the plasmid prep, is collected in a clean tube. The plasmid DNA is then precipitated with 0.7 volumes of room temperature isopropanol, and centrifuged immediately at 15,000 x g at 4°C for 30 min. The supernatant is carefully removed. The pellet, containing plasmid DNA, is washed with 5 ml of cold 70% EtOH. The pellet is then air dried, and resuspended in 100 µl of sterile distilled H₂O (dH₂O). The DNA concentration is determined by absorbance at 260nm on a UV spectrophotometer.

(2). plasmid pCH110

The plasmid pCH110 (encoding LacZ) was given to this lab as a glycerol stock. A sample from the glycerol stock was streaked onto a Luria broth (LB) agar plate containing 200 µg/ml ampicillin (LB/Amp). Plates were incubated overnight at 37°C. One positive colony was selected and a plasmid prep was prepared as described above.

(3). Creating new Bsu36I site 3' of LacZ gene

Ten micrograms of pCH110 was digested with BamHI, and the digested DNA was purified on a 5% acrylamide gel, electroeluted and ethanol precipitated as

previously described. The ends of the linearized pCH110 was dephosphorylated using calf intestinal alkaline phosphatase treatment (CIAP, Promega). The purpose of CIAP is to remove the phosphate group from the ends of the digested plasmid, thereby preventing religation of the plasmid in the absence of an insert. The DNA was then purified using phenol chloroform extraction and ethanol precipitation. The oligonucleotides (synthesized at the Core Molecular Biology Laboratory, EVMS) encoding a new Bsu36I site were first annealed to each other prior to use in the ligation with pCH110. The ligation was performed at three different ratios of vector and insert DNA; 1:1, 1:3, and 3:1. Ligation reactions were screened in JM109 *E.coli* Amp^r.

(4). Using sequence compatible ends for insertion of LacZ into P39

Ten micrograms of unmodified pCH110 (Appendix 2) was digested with Bsu36I and MaeI. The digested DNA (pCH110*B*M) was electrophoresed on an agarose gel. The insert was excised from the gel and electroeluted. The insert DNA was then concentrated by ethanol precipitation. Ten micrograms of P39 was digested with Bsu36I followed by CIAP. The digested DNA was purified as described above. Ligation of vector and insert DNA was performed at three different ratios 1:1, 1:3, and 3:1. Ligation reactions were screened in JM109 *E.coli* Amp^r.

b. Attempts to construct a P39 deletion mutant

A 10 µg aliquot of p39 was digested with restriction enzymes Bsu36 I and Bal I (New England BioLabs). The digestion results in liberation of a ~300 base pair fragment from the P39 open reading frame (approximately 300 bases 3' of the start site). To this reaction was added 1.5ml of Klenow fragment (3 units Boehringer Mannheim digitonin label kit), 2 ml of 0.5 mM dATP and 2 ml of 0.5 mM dTTP. This mixture was incubated at 30°C for 15 min. The mixture was then heated to 75°C for 10 min to heat inactivate the enzymes. The DNA was purified by phenol extraction, followed by ethanol precipitation. The DNA pellet was resuspended in 8 ml of water and used in a ligation reaction with 1 ml of T4 DNA ligase, and 1 ml of 10x blunt end buffer. The ligation reaction was incubated at 17°C for 5 hours. The ligation was

screened in JM109 *E.coli* cells. *E.coli* cells were thawed on ice for 10 min and 40 ml of cells are placed in a prechilled 0.2cm electroporation cuvette. Eight microliters of ligation mix were added to the cuvette, and the cells were electroporated at 2.5 volts, 200 Ohms, 25mF. One ml of S.O.C. medium was immediately added to the cuvette, then transferred to a culture tube. The tube was incubated for 1 hour at 37°C with constant rotation. Cells (200 µl) were streaked on LB/amp plates, and incubated at 37°C, overnight. Several colonies were selected, grown overnight in 5 ml of LB/amp broth, and were screened by Pst I digestion of miniplasmid preps. Colonies containing the P39 deletion construct had digestion products migrating at 1.4kb, 3.5kb, and 4.5kb, as compared to the wild type plasmid which had digestion products migrating at 1.7kb, 3.5kb, and 4.5kb. One positive colony (designated P39dm) was grown overnight in 150 ml of LB/amp, and the pSPR33 deletion-mutant plasmid collected with using a QIAGEN prep kit as described above.

(1). Lack of P39 expression by P39 deletion mutant construct in *E.coli*

An aliquot of P39dm was harvested and washed three times in PBS to remove all traces of media. The cells were adjusted to 0.20 OD at 650 nm and subsequently sonicated for one min on ice to break apart the cells. Two fold dilutions of P39dm were plated on a microtiter plate in sodium bicarbonate buffer. Controls included untransformed JM109 and JM109 expressing pSPR33 at the same concentration. All samples were tested in triplicate. Plates were incubated overnight at 4°C and screened by ELISA for P39 expression as described above.

c. Transformation of *B.burgdorferi*

The P39 deletion mutant construct was excised from the plasmid using Pst I digestion. The digested plasmid was ethanol precipitated overnight. The digested DNA was used to transform low passage infectious CN40. Spirochetes were collected from mid-log phase culture, washed twice with cold PBS, and then washed three times in cold EPS. The spirochetes were resuspended to a final concentration of 2×10^5 cells/ml. Fifty microliters were transferred to a prechilled 0.2 cm electroporation

cuvette along with 2 µg P39 deletion mutant DNA. One pulse was administered at 2.5kV, 25mF, 200 Ohms (Gene Pulser II, Bio Rad, Melville, NY) producing a time constant of 4-5 millise. One ml of BSK II medium with no antibiotics was added to the cuvette and transferred to 5 ml of BSK II medium and incubated for 24 hours at 33°C.

The electroporated spirochetes were then screened by ELISA for expression of flagellin (to confirm presence of spirochete) and loss of P39 expression (to confirm transformation of spirochete) using the following protocol. Twenty four hours after electroporation, the spirochetes were counted on a Petroff-Hauser chamber. The organisms were diluted in BSK II medium and plated out on 96 well tissue culture plates at approximately one spirochete per well. The plates were incubated at 33°C for 10 days. A 50 µl aliquot was removed and saved from each well, then the original plates were centrifuged at 3,000 rpm for 5 min to pellet the cells. Media was removed from each well by aspiration. The cells were resuspended in 200 µl of sodium bicarbonate buffer. The solution was divided equally between two microtiter plates (one hundred microliters per well) and both plates were incubated at 4°C overnight. The plates were rinsed 3 times with PBS, and a 1/200 dilution of Mab D1C9 (specific for P39) was added to one plate and a 1/100 dilution of Mab H9724 (specific for flagellin). The plates were incubated at room temperature for 30 min with constant rotation. The plates were washed 3 times with PBS, and bound antibody was detected with a peroxidase conjugate (goat anti-mouse IgG) at 1/2000 dilution. The plates were incubated at room temperature for 30 min with constant rotation. Plates were washed 3 times with PBS, and 100 µl of peroxidase substrate (KPL) was added to each well. Reactivity was detected on a spectrophotometer at 650 nm.

Flagellin reactivity was used to detect the wells containing spirochetes (as opposed to being an empty well). P39 reactivity of each well was compared to that of control wells containing unelectroporated *B.burgdorferi*. Readings which fell below (> 0.1 OD below control well) those obtained from the untransformed spirochetes was considered a P39-low reading. This cut off level was selected in order to ensure all

possible transformants would be detected. Samples that exhibited flagellin reactivity, but were scored as low for P39 reactivity, were grown in culture for 10 days. These cultures were subsequently examined by western blot to assess P39 expression in these organisms.

III. RESULTS

A. Establishment of infectious and noninfectious isolates of *Borrelia burgdorferi*

The infectivity of the low and high passage isolates, as defined by the ability to survive in a host, was assessed in mice by needle inoculation. It was important to confirm the infectivity status of these isolates since the remainder of the study involves comparison of the infectious and noninfectious isolates. Accordingly, three separate methods were used for verifying isolate infectivity: culture of mouse tissue, xenodiagnosis using ticks, and finally PCR of mouse tissue. Low passage CN40 p9, B31 p6, WCH1 p6 and JD1 p3 and high passage CN40 p22 were all infectious based on recovery of spirochetes from mouse bladder tissue (Table 1). In contrast, the high passage isolates B31 p.32, WCH1 p.15, and JD1 p.15 were not recovered from mouse tissue.

In addition, *Borrelia* were detected in ticks which fed on mice infected with low passage WCH1 and JD1 respectively (Table 1). No *Borrelia* were detected in ticks which fed on mice inoculated with high passage WCH1 and JD1.

The tissues from infected mice (inoculated with low passage B31, WCH1, JD1, low and high passage CN40) were also positive for *B.burgdorferi* infection when assayed by PCR using primers specific for *B.burgdorferi* chromosomal DNA (Fig. 1). Tissues from noninfected mice (inoculated with high passage B31, WCH1, and JD1) were not positive by PCR. *Borrelia burgdorferi* DNA was detected at a sensitivity of 10 pg of spirochetal DNA (control lanes panel A, lane 9-12).

Table 1. Infectivity of low and high passage *Borrelia burgdorferi* isolates.

Isolate	Passage	Tissue^a	Tick^b	PCR^c	Infectivity^d
CN40	9	2/2	nd	2/2	I
CN40	22	2/2	nd	nd	I
B31	6	2/2	nd	2/2	I
B31	32	0/4	nd	0/4	N
WCH1	6	2/2	1/14	2/2	I
WCH1	15	0/4	0/12	0/4	N
JD1	3	2/2	3/20	2/2	I
JD1	15	0/4	0/14	0/4	N

^a Recovery of *B.burgdorferi* from cultured bladder tissues. Total number positive/total number tested. All negative cultures were confirmed by repeating the experiment.

^b IFA detection of *B.burgdorferi* in ticks which had fed on inoculated mice. Total number of positive ticks/total number of ticks tested. nd=experiment not done.

^c Detection of *B.burgdorferi* DNA in mouse bladder tissue by PCR.

^d Infectivity of *B.burgdorferi* isolate I=infectious, N=noninfectious.

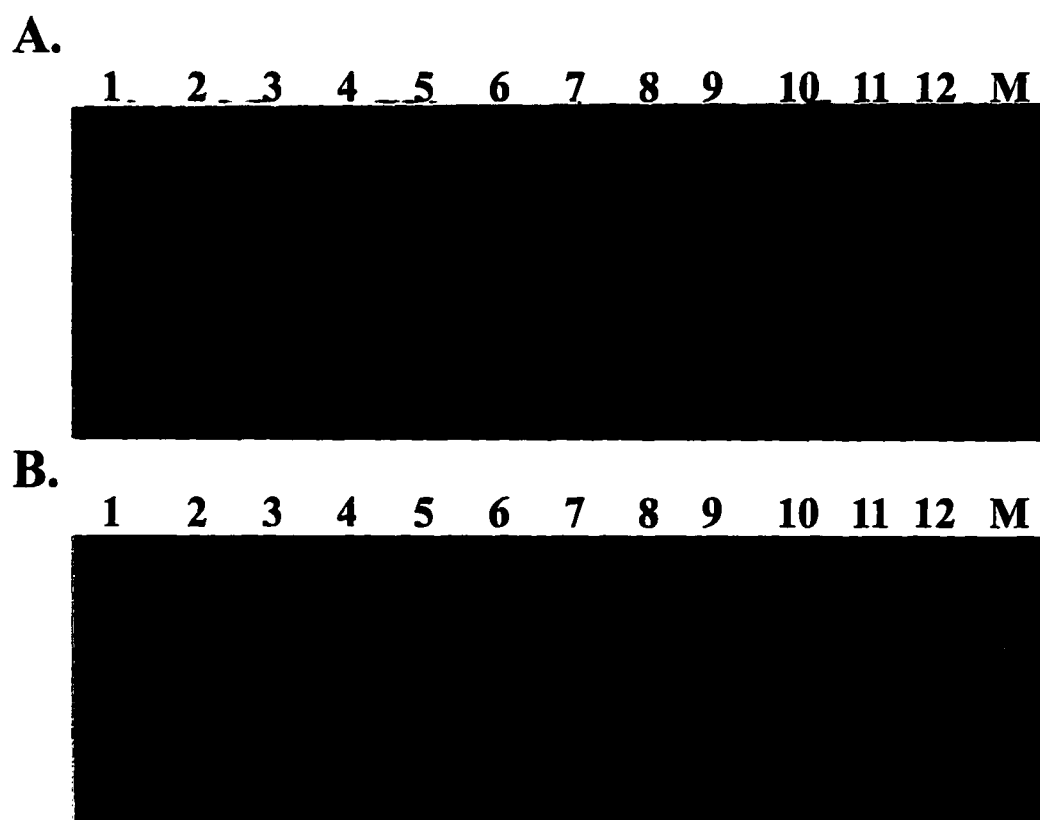


Figure 1. Detection of *Borrelia burgdorferi* DNA in mouse bladder tissue using PCR. Template DNA was collected from mice inoculated with the following low or high passage isolates: CN40 p.9 (panel A, lane 1 and 2), B31 p.6 (panel A, lane 3 and 4), B31 p.32 (panel A, lanes 5-8), WCH1 p.6 (panel B, lane 1 and 2), WCH1 p.15 (panel B, lanes 3-6), JD1 p.3 (panel B, lane 7 and 8), JD1 p.15 (panel B, lanes 9-12). DNA size markers are shown in lane 13 of panel A and B. Control for PCR sensitivity shown in panel A: 500ng of DNA extracted from uninfected mouse tissue was mixed with 1 μ g (lane 9), 10ng (lane 10), or 10 pg (lane 11) of template *B.burgdorferi* DNA to determine interference with PCR. A negative control is shown in A, lane 12.

Based on these three separate criteria (tissue culture, xenodiagnosis, and PCR) low passage CN40, B31, WCH1, JD1, and high passage CN40 were judged to be infectious and high passage B31, WCH1, and JD1 were noninfectious. These infectious and noninfectious organisms were next analyzed for P39, OspB, OspA, and Fla localization within the spirochetal cell to determine if a correlation exists between P39 localization and infectivity.

B. Subcellular localization and quantitation of P39, OspB, OspA, and Fla in infectious and noninfectious isolates

1. Western blot of cell fractions

Infectious and noninfectious *B. burgdorferi* were separated into outer envelope, inner membrane, and cytosolic fractions and analyzed by western blot /densitometry to quantitate the antibody reactivity for each epitope (hereafter referred to as levels). The P39, OspB, and OspA levels for infectious and noninfectious isolates were analyzed by regression analysis to determine the statistical significance.

a. P39 levels

For all isolates, a decrease in P39 levels in the outer envelope correlated with loss of infectivity ($P = 0.019$) (Table 2). However, no significant interaction exists between the amount of P39 in the inner membrane ($P = 0.24$) or cytosolic fraction and infectivity ($P = 0.32$).

There was considerable variability in P39 localization among isolates. Most notably the P39 levels in the cloned, infectious isolate CN40 was located exclusively in the outer envelope fraction. No P39 was detected in the inner membrane or cytosolic fractions (Table 2, Fig. 2). Both outer envelope fractions of CN40 had comparable P39 levels, therefore P39 expression did not increase or decrease during in vitro culture.

In contrast, the P39 levels in the remaining uncloned isolates did change. For B31, P39 was detected in all cell fractions, except infectious cytosol (Table 2, Fig. 3).

Table 2. P39 levels in *Borrelia burgdorferi* cell fractions measured by westernblot densitometry.^a

Isolate	Infectivity	OE	IM	CY	Cumulative
CN40 (p5)	I	0.639 ± 0.10	0	0	0.639
CN40 (p23)	I	0.727 ± 0.11	0	0	0.727
B31	I	0.187 ± 0.11	0.124 ± 0.10	0	0.311
B31	N	0.467 ± 0.14	0.171 ± 0.07	1.09 ± 0.17	1.728
WCH1	I	1.534 ± 0.28	1.076 ± 0.22	0.177 ± 0.11	2.787
WCH1	N	0.883 ± 0.15	1.254 ± 0.25	0.235 ± 0.15	2.372
JD1	I	1.154 ± 0.14	1.213 ± 0.18	0.459 ± 0.12	2.826
JD1	N	0.713 ± 0.13	1.406 ± 0.22	0.654 ± 0.08	2.773
Mean	I	0.847	0.603	0.159	1.573
	N	0.687	0.708	0.495	1.901

^aData shown represent the mean of four independent experiments ± SEM.

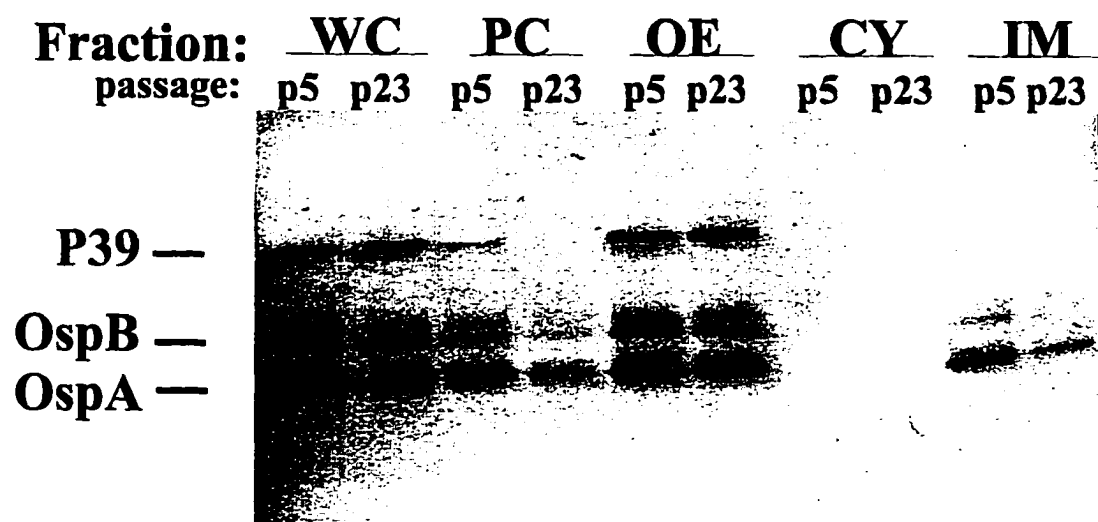


Figure 2. Detection of P39, OspB, and OspA in infectious CN40 cell fractions at p5 and p23 by westernblot. Eight microliters of whole cell sonicate (WC), protoplasmic cylinder (PC), outer envelope (OE), cytosolic (CY), or inner membrane (IM) fraction were analyzed.

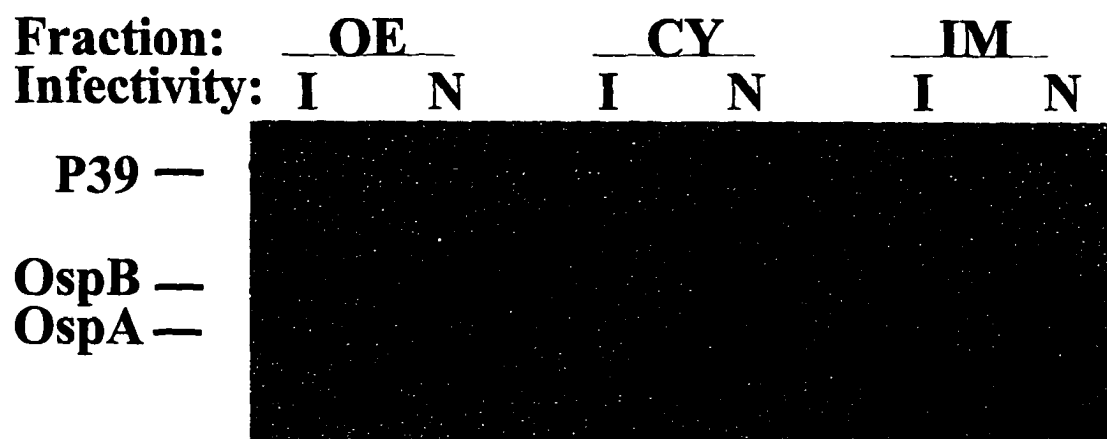


Figure 3. Detection of P39, OspB, and OspA in low and high passage B31 cell fractions by western blot. A total of eight microliters of outer envelope (OE), cytosolic (CY), or inner membrane (IM) fractions were analyzed.

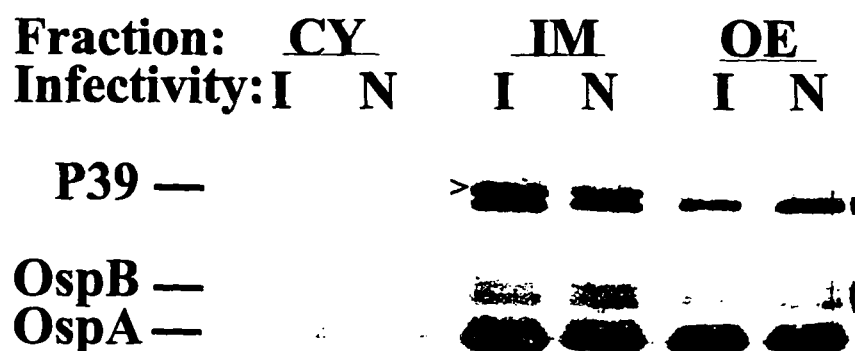


Figure 4. Detection of P39, OspB, and OspA in low and high passage WCH1 cell fractions by western blot. Eight microliters of cytosolic (CY), inner membrane (IM), and outer envelope (OE) fractions were analyzed. Flagellin control is marked (>) in IM fraction.

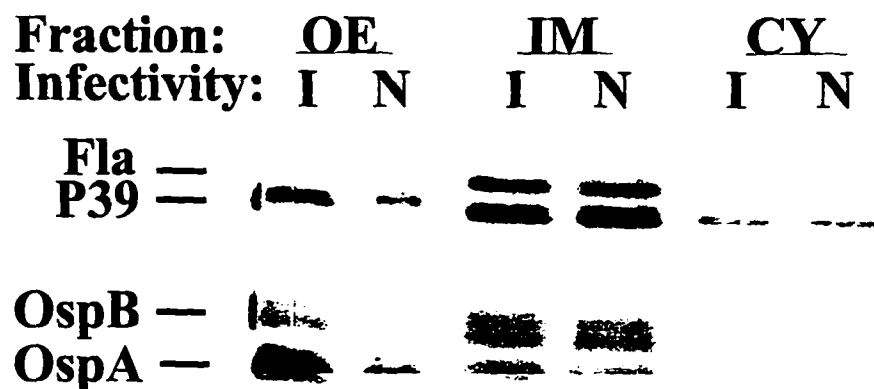


Figure 5. Detection of Fla, P39, OspB, and OspA in low and high passage JD1 cell fractions by western blot. Eight microliters of outer envelope (OE), cytosolic (CY), or inner membrane (IM) fractions were analyzed.

Table 3. OspB levels in *Borrelia burgdorferi* cell fractions measured by western blot densitometry.^a

Isolate	Infectivity	OE	IM	CY	Cumulative
CN40 (p5)	I	0.863 ± 0.20	0.181 ± 0.11	0	1.044
CN40 (p23)	I	0.988 ± 0.30	0.098 ± 0.15	0	1.086
B31	I	0.426 ± 0.20	0.850 ± 0.20	0.286 ± 0.14	1.562
B31	N	1.16 ± 0.22	1.99 ± 0.31	1.8 ± 0.45	4.950
WCH1	I	1.793 ± 0.32	1.796 ± 0.42	0.298 ± 0.10	3.887
WCH1	N	1.246 ± 0.27	1.834 ± 0.48	0.248 ± 0.04	3.328
JD1	I	0.954 ± 0.34	1.350 ± 0.41	0.426 ± 0.17	3.684
JD1	N	0.481 ± 0.20	1.520 ± 0.49	0.323 ± 0.15	2.324
Mean	I	1.009	1.044	0.252	2.544
Mean	N	0.968	1.360	0.592	2.922

^aData shown represent the mean of four independent experiments ± SEM.

Table 4. OspA levels in *Borrelia burgdorferi* cell fractions measured by western blot densitometry.^a

Isolate	Infectivity	OE	IM	CY	Cumulative
CN40 (p5)	I	0.922 ± 0.20	0.732 ± 0.30	0	1.654
CN40 (p23)	I	0.994 ± 0.20	0.435 ± 0.40	0	1.429
B31	I	0.555 ± 0.20	1.138 ± 0.22	0.413 ± 0.21	2.106
B31	N	1.05 ± 0.70	1.93 ± 0.40	1.25 ± 0.32	4.230
WCH1	I	1.781 ± 0.18	2.079 ± 0.17	0.742 ± 0.18	4.602
WCH1	N	1.825 ± 0.12	2.008 ± 0.25	0.675 ± 0.18	4.508
JD1	I	1.238 ± 0.50	2.590 ± 0.61	1.120 ± 0.37	4.948
JD1	N	1.150 ± 0.40	2.240 ± 0.43	1.090 ± 0.38	4.480
Mean	I	1.124	1.634	0.568	3.327
Mean	N	1.254	1.653	0.753	3.661

^aData shown represent the mean of four independent experiments.

Table 5. Flagellin levels in *Borrelia burgdorferi* cell fractions measured by westernblot densitometry.^a

Isolate	Infectivity	OE	IM	CY	Cumulative
CN40 (p5)	I	0	nd ^b	nd	nd
CN40 (p23)	I	0	nd	nd	nd
B31	I	0	nd	nd	nd
B31	N	0	nd	nd	nd
WCH1	I	0	0.426	0.030	0.456
WCH1	N	0	0.611	0.050	0.661
JD1	I	0	0.734 ^c	0.155	0.889
JD1	N	0	0.909 ^d	0.045	0.954
Mean	I	0	0.58	0.092	0.672
Mean	N	0	0.76	0.047	0.807

^aData shown represent the mean of four independent experiments. SEM < 0.05 unless otherwise indicated.

^bnd = not determined.

^cSEM = 0.2

^dSEM = 0.1

In infectious B31 cell fractions, the P39 was located in the membrane fractions only. However, noninfectious fractions had considerably more P39 and the majority was in the cytosolic fraction. For the other uncloned isolates WCH1 and JD1, P39 was detected in all fractions (Fig. 4 and 5). However, decrease in P39 was detected in the noninfectious outer envelope fraction of WCH1 and JD1 (Table 2). This decrease corresponds with a slight increase in noninfectious inner membrane fractions, and cytosolic fraction for both isolates.

b. OspB levels

No significant interaction exists between infectivity and OspB localization in any cell fraction: outer envelope ($P = 0.65$) inner membrane ($P = 0.20$), cytosol ($P = 0.65$). For the infectious CN40, OspB levels were observed in both the outer envelope and inner membrane fractions but were not seen in the cytosolic fractions (Table 3, Fig. 2). Notably, the OspB levels for CN40 did not change from low to high passage. In contrast, OspB (Table 3, Fig.3) was detected in all fractions of B31, but noninfectious cell fractions contained twice the amount of OspB. OspB was also detected in all fractions of WCH1 and JD1 (Table 3). The amount of OspB in the outer envelope fraction decreased in both noninfectious WCH1 and JD1, but inner membrane and cytosolic levels remained the same.

c. OspA levels

No significant interaction exists between infectivity and OspA localization in any cell fraction: outer envelope ($P = 0.15$), inner membrane ($P = 0.27$), cytosol ($P = 0.19$). For CN40, OspA was detected in both the outer envelope and inner membrane fractions but were not seen in the cytosolic fractions (Fig. 2, Table 4). CN40 OspA levels did not change with in vitro culture, this is consistent with CN40 P39 and OspB levels. In contrast, OspA was detected in all fractions of B31, but noninfectious cell fractions contained twice the amount of OspA (Fig.3, Table 4). OspA was also detected in all cell fractions of WCH1 and JD1 (Table 4). OspA was equally distributed between the membrane fractions of WCH1, and did not change with

infectivity. For JD1, the majority of OspA was located in the inner membrane fraction, and this did not change with infectivity. For all isolates there was no significant correlation between OspA localization and infectivity .

d. Flagellin levels

Flagellin levels were monitored simply as a quality control measure for the cell fractionation procedure. No flagellin was detected in any outer envelope fraction, indicating proper fractionation of the isolates (Table 5). Flagellin was detected in the inner membrane fraction and trace amounts in the cytosol. Noninfectious and infectious isolates had similar flagellin levels in the inner membrane fraction.

2. Quality control measures for fractionation technique and densitometry analysis

a. Interassay variation of cell fractionation

The reproducibility of the fractionation procedure was monitored by protein assay. The protein distribution within spirochete cell fractions was as follows: 90-93% in the outer envelope, 4-8% in the inner membrane, and 0.6-1.8% in the cytosolic fraction. The average amount of protein from 1×10^6 infectious spirochetes was 2.0 mg (93% of total) in the outer envelope, 0.1 mg (4.6% of total) in the inner membrane, and 0.04 mg (1.8% of total) in the cytosolic fraction. When the protein concentrations of the cell fractions were added together in all cases the total was equal to (100%) or slightly higher than (101-107%) the protein concentration for the whole cell sonicate preparation. For noninfectious organisms, there was nearly twice as much protein from the same number of spirochetes: 4.0 mg in the outer envelope, 0.2 mg in the inner membrane and 0.07 mg in the cytosolic fraction. The protein concentration of cell fractions generated from duplicate samples of CN40 (two populations of CN40 subjected to cell fractionation on different occasions) were analyzed by students T test. No significant differences exist between protein concentrations of cell fractions from duplicate samples ($P = 0.3736$).

b. Localization of cell fraction markers

The flagellin protein is the major component of the spirochete flagella. Physiologically it is anchored to the inner membrane. When the cell is fractioned, the flagellin should partition with the inner membrane fraction. Any detection of flagellin in the outer envelope fraction would be evidence of improper separation of the fractions. There was no control for outer membrane contamination of the inner membrane or cytosolic fractions as no outer envelope specific marker had been identified for *B.burgdorferi*. For all isolates analyzed, no flagellin was detected in the outer envelope fraction indicating proper fractionation of the cell (Table 5). Flagellin was detected primarily in the inner membrane fraction, and trace amounts (0-2%) were detected in the cytoplasmic fraction of WCH1 and JD1.

c. Western blot/densitometry assessment

To ensure proper detection of bands by the densitometer, the densitometer sensitivity was assessed. Duplicate serial dilutions of whole cell *B.burgdorferi* antigen (0ug-30ug) were subjected to SDS-PAGE and western blot (purified P39, OspB, or OspA were not used for assessment of the densitometer since cell fractions, not purified proteins, are the subject of this study). The blot was treated with monoclonal antibodies specific for P39, OspA, and OspB and developed as described above. A linear relationship was demonstrated between densitometer absorbance readings and serial dilutions of whole cell *B.burgdorferi* antigen less than 30 ug ($P39\ Y = 0.04X + 0.628$; $OspB\ y = 0.037X + 0.167$; $OspA\ Y = 0.0368X + 0.3568$) (Appendix 3). Therefore, the densitometer was able to quantitate P39, OspA, and OspB in antigen samples less than 30ug. The protein concentration in 8ul of each cell fraction (2.8×10^5 spirochetes, the amount used in the SDS-PAGE in this study) is less than 15 ug. All fractions from one isolate (both low and high passage) were analyzed on the same blot, thereby minimizing differences in development. A minimum of three experiments were performed with each isolate, and densitometry readings were analyzed by linear regression analysis to determine the statistical significance.

3. Silver stain analysis of cell fractions

Cell fractions of the infectious and noninfectious spirochetes were also analyzed by SDS-PAGE and silver stain to detect any other changes in protein expression which might correlate with loss of infectivity. All isolates (infectious and noninfectious) had bands migrating at 14, 20, 22 (corresponding to OspC), 29, 32 (corresponding to OspA), 34 (corresponding to OspB), and a large band at 50-66 kD in the whole cell sonicate (Figs.6-9).

a. Outer envelope fraction

For all isolates, the majority of detectable proteins were in the outer envelope fraction. Most bands detected in the whole cell sonicate were also detected in the outer envelope. For the infectious isolate CN40, all corresponding bands in low and high passage fractions appeared to stain with equal intensity (Fig. 6). For isolate B31, infectious and noninfectious outer envelope fractions displayed different staining intensities for many bands; most notably bands of 24, 22 and 20 kD were all detectable in the infectious outer envelope fraction yet absent from the noninfectious fraction (Fig. 7). WCH1 also exhibited several differences between infectious and noninfectious outer envelope fractions (Fig. 8). Infectious outer envelope had distinct bands migrating at 36 and 39 kD. In the noninfectious fraction the 36 kD band was greatly diminished and the 39 kD band was completely absent. All other corresponding bands in WCH1 outer envelope fractions appeared to have equal intensity. JD1 also exhibited changes in band intensities between infectious and noninfectious outer envelope fractions (Fig. 9). The noninfectious outer envelope fraction contained darker bands migrating at 47, 45, 34, 32, and 14 kD, but a lighter band at 29 kD as compared to the corresponding infectious fraction.

b. Innermembrane fraction

For all isolates very few bands were detected in the inner membrane fraction. All isolates (except JD1) had a 66 kD band. CN40 also had a band at 20 kD in the high passage fraction. For B31, faint bands at 41, 33, and 21 kD could be seen in the

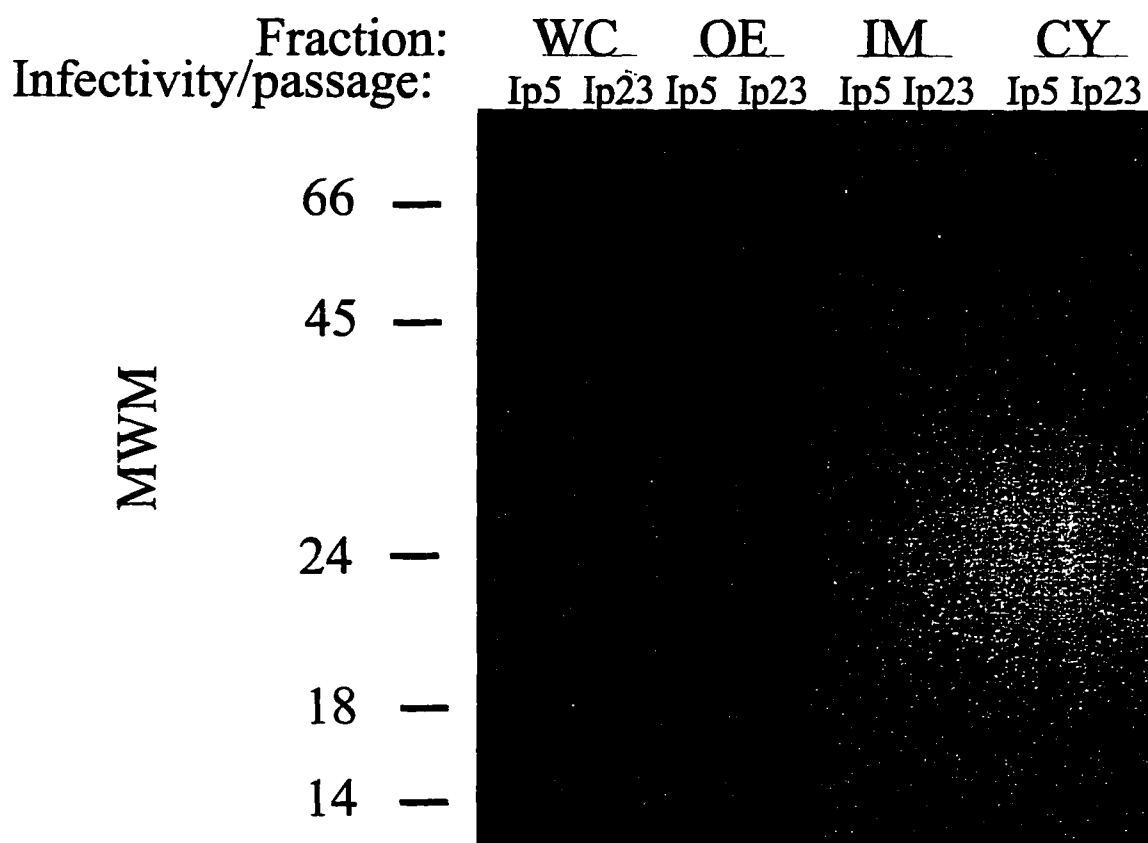


Figure 6. Silver stain analysis of infectious isolate CN40 cell fractions. Eight microliters of the cell fractions are represented in each lane. Whole cell sonicate (WC), outer envelope (OE), inner membrane (IM) and cytosolic (CY) fractions are shown. Molecular weight markers are indicated to the left of the gel.

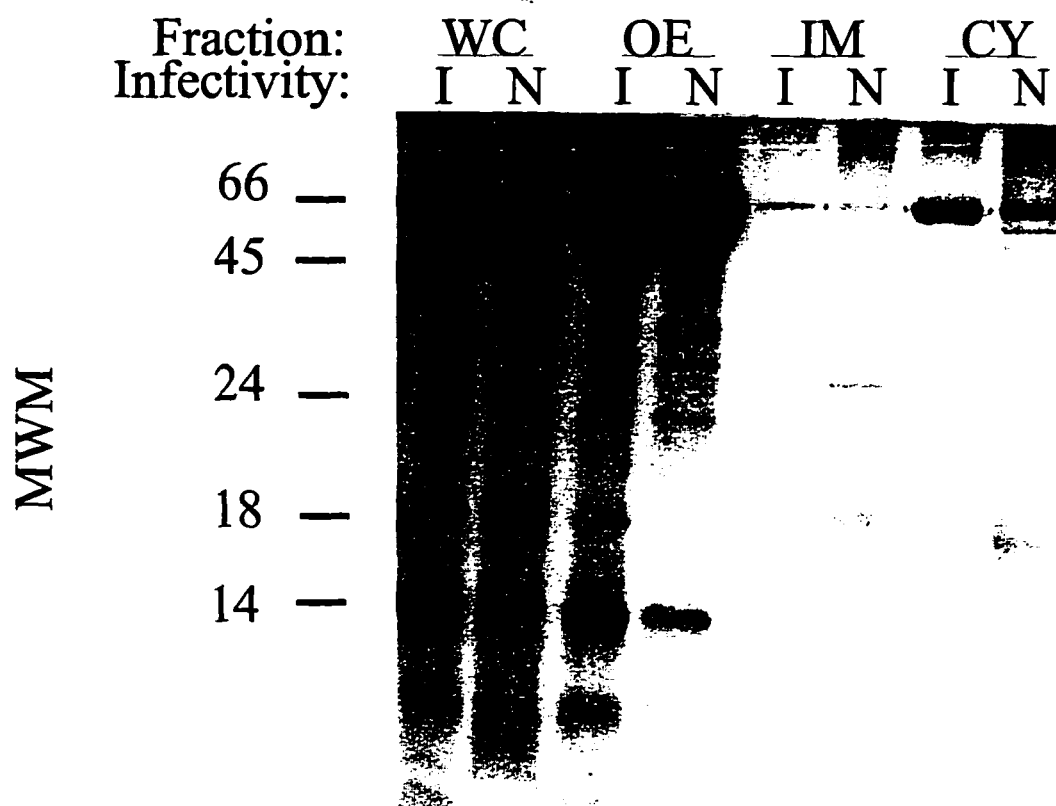


Figure 7. Silver stain analysis of B31 cell fractions. Eight microliters of cell fractions were electrophoresed on a 12.5% gel and silver stained to detect proteins. Whole cell sonicate (WC), outer envelope (OE), inner membrane (IM) and cytosolic (CY) fractions from infectious (I) and noninfectious (N) B31 are shown. Molecular weight markers are indicated to the left of the gel.

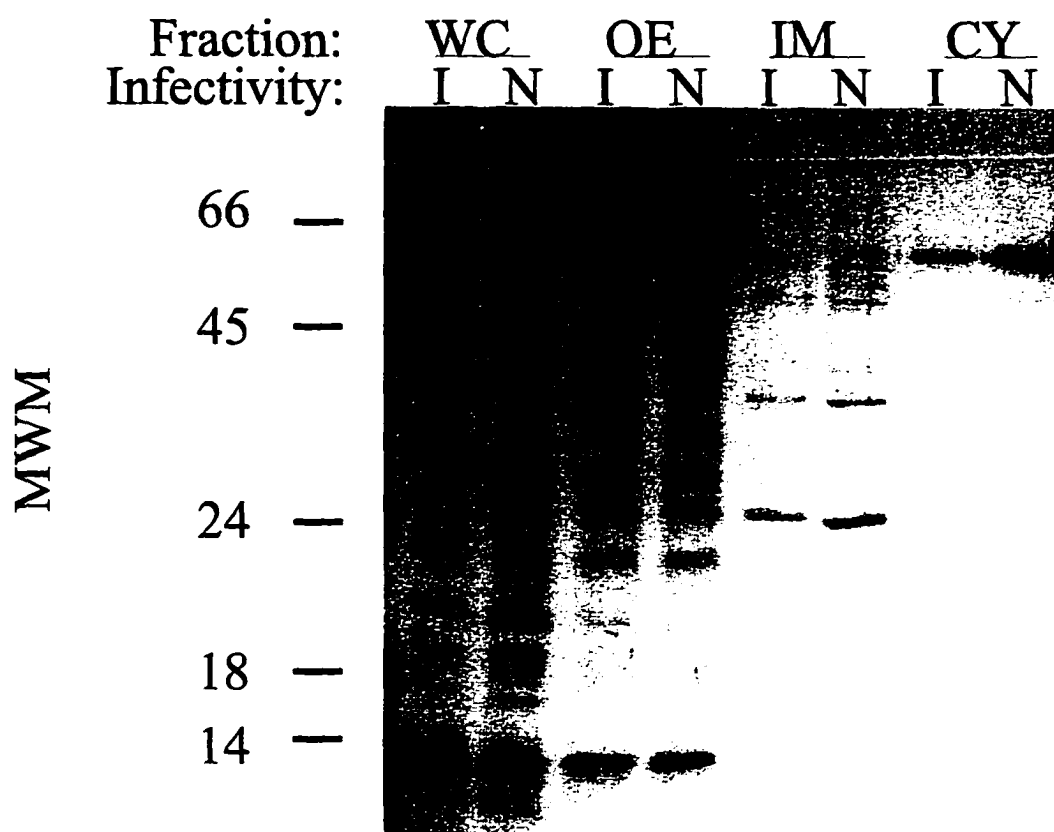


Figure 8. Silver stain analysis of WCH1 cell fractions. Eight microliters of cell fractions were electrophoresed on a 12.5% gel and silver stained to detect proteins. Whole cell sonicate (WC), outer envelope (OE), inner membrane (IM) and cytosolic (CY) fractions from infectious (I) and noninfectious (N) WCH1 are shown. Molecular weight markers are indicated to the left of the gel.

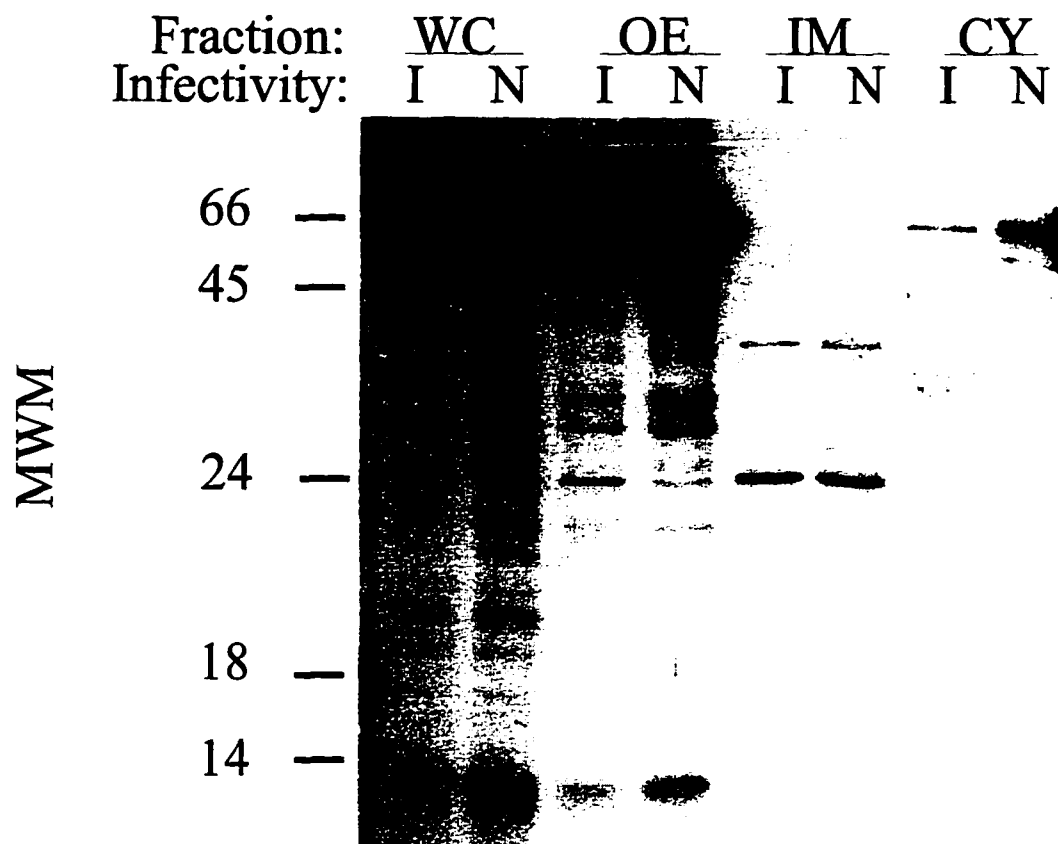


Figure 9. Silver stain analysis of JD1 cell fractions. Eight microliters of cell fractions were electrophoresed on a 12.5% gel and silver stained to detect proteins. Whole cell sonicate (WC), outer envelope (OE), inner membrane (IM) and cytosolic (CY) fractions of infectious (I) and noninfectious (N) JD1 are shown. Molecular weight markers are indicated to the left of the gel.

noninfectious inner membrane fraction, but were not seen in the infectious inner membrane fraction. For WCH1, bands migrating at 29, 41, and 60 kD were detected in both inner membrane fractions but appeared to be darker in the noninfectious fraction. Additional bands at 52, 54, and 60 kD were also evident in the noninfectious inner membrane fraction. For JD1 both inner membrane fractions had bands of equal intensity migrating at 29, 31, and 41 kD.

c. Cytosolic fraction

All isolates displayed a single large band at 66 kD for both cytosolic fractions. For CN40 this was the only band detected. A distinct band in the cytosolic fraction of infectious B31 migrated at 66 kD, while the noninfectious cytosolic fraction contained several additional faint bands at 60, 40, 34, 33, 22, and 18 kD. A single band of 66 kD was detected in both cytosolic fractions of WCH1, although it appeared to be more intense in the noninfectious passage. Additionally, both cytosolic fractions from JD1 contained a single band migrating at 66 kD, but the band in the noninfectious fraction was considerably darker. Proteins are present in the inner membrane and cytosolic fractions as evidenced by BCA protein assay; however, not all proteins are detected by silver staining, and the intensity of staining depends upon the individual characteristics of each protein.

These data demonstrate several differences in protein expression between infectious organisms and noninfectious organisms. CN40 alone remains unchanged in low and high passage. B31 had two bands in the outer envelope fractions with different intensities (29 kD and 20 kD), and the noninfectious inner membrane and cytosolic fractions both contained bands which were not found in the corresponding infectious fractions. WCH1 noninfectious outer envelope fraction lacked two bands (36 kD and 39 kD) found in infectious fraction, and overall noninfectious fraction bands were darker in both inner membrane and cytosolic fractions. JD1 had disparity in band intensities between infectious and noninfectious cell fractions, but the loss or gain of bands was not noted. The fact that B31, WCH1, and JD1 are uncloned

populations of spirochetes may account for the differences seen between low and high passage organisms.

C. Surface expression of P39, OspB, and OspA as detected by flow cytometry

The uniformity of P39, OspB, or OspA surface expression in a population of spirochetes was assessed by flow cytometry. Distinct peaks show uniformity of expression in a population. Flat or broad peaks are a sign of variability in surface expression among the population of spirochetes.

1. Differences in levels of P39 expression in clones derived from parental isolate WCH1

Clones of isolate WCH1 were generated for this study in order to compare P39 surface expression of clones to the original uncloned population of spirochetes. Parental, uncloned WCH1 and WCH1 clones were first analyzed by ELISA for P39 expression. A single clone, Clone 1, showed marked differences in P39 reactivity as compared to uncloned WCH1. Clone 1 had higher P39 reactivity in whole cell antigen preparations than uncloned WCH1 (O.D. values at 260 nm were 0.62 versus 0.38 respectively). All other clones had P39 reactivity similar to uncloned WCH1. Due to the differences in P39 reactivity, Clone 1 was selected for further study by flow cytometry.

2. P39 surface expression

In order to determine the variability of P39 surface expression in cloned and uncloned isolates, low and high passage whole cell B31, WCH1, JD1, WCH1 clone 1, and high passage CN40 were analyzed by flow cytometry. The infectious, cloned isolate CN40 had a single, very low intensity peak for P39 indicating the cloned spirochetes' uniform reactivity to Mab D1C9 (mean fluorescence = 72.9, Table 6, Fig.10). A single peak was also observed in both low and high passage WCH1 clone 1, and had a mean fluorescence of 111.5 for p7 and a mean of 104.0 for p17. The P39 reactivity was not uniform in the uncloned isolates B31, WCH1, and JD1. Infectious

Table 6. Surface expression of P39, OspB, and OspA as detected by flow cytometry.

Isolate	Infectivity	P39	OspB	OspA
CN40 p22	I	72.9 ± 31.4	165.6 ± 35.7	120.7 ± 45.3
clone 1 p7	nd^b	111.5 ± 29.1	100.7 ± 30.4	59.2 ± 26.8
clone 1 p17	nd	104.0 ± 35.4	136.0 ± 36.1	50.4 ± 23.8
B31	I	140.8 ± 44.0	nd	nd
B31	N	84.7 ± 34.1	103.4 ± 26.5	41.6 ± 22.8
WCH1	I	130.8 ± 51.3	135.4 ± 33.4	54.7 ± 25.8
WCH1	N	107.4 ± 38.3	143.4 ± 32.7	73.2 ± 30.0
JD1	I	136.6 ± 51.3	126.9 ± 33.5	51.2 ± 24.3
JD1	N	88.3 ± 33.8	116.2 ± 32.0	66.8 ± 26.2

^a Mean fluorescence ± standard error are shown.

^b nd= not determined.

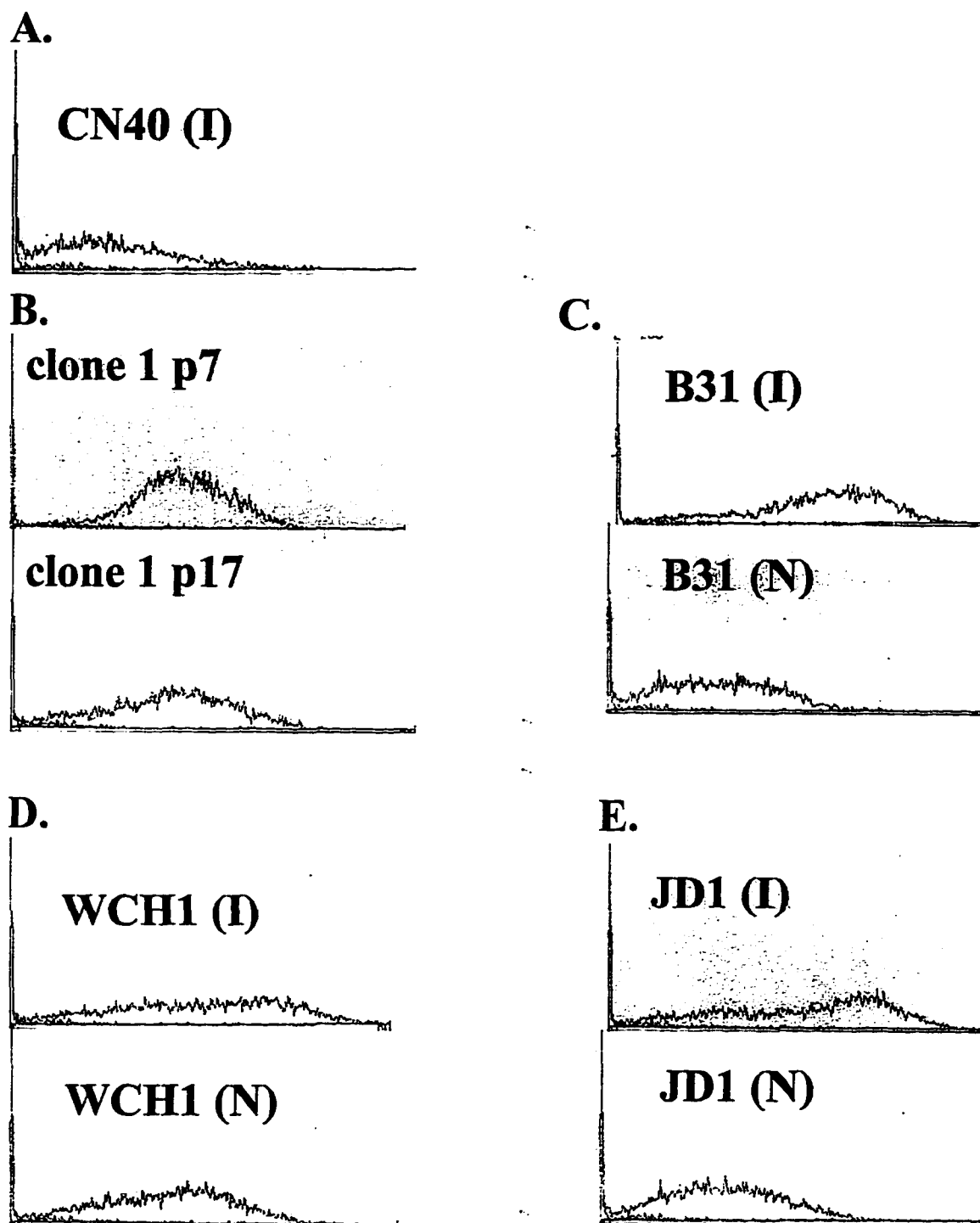


Figure 10. P39 surface expression as detected by flow cytometry. Infectious (I) and noninfectious (N) spirochetes were incubated with monoclonal D1C9, followed by a fluorescein labeled secondary antibody, and assessed by flow cytometry. Panel A isolate CN40, panel B WCH1 clone 1 p7 and p17 (infectivity unknown), panel C isolate B31, panel D isolate WCH1, and panel E isolate JD1.

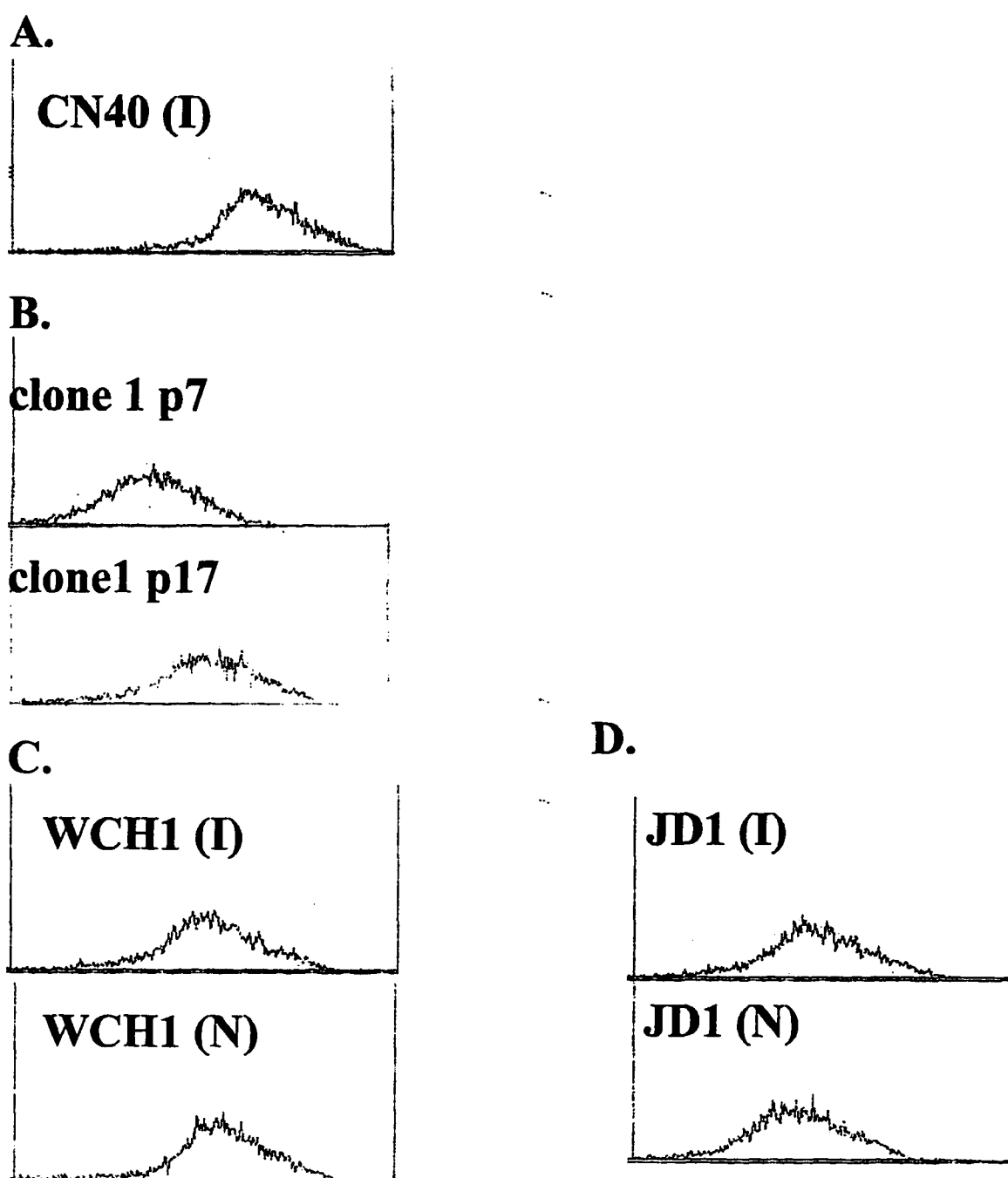


Figure 11. OspB surface expression as detected by flow cytometry. Infectious (I) and noninfectious (N) spirochetes were incubated with monoclonal H6831, followed by a fluorescein labeled secondary antibody and assessed by flow cytometry. Panel A isolate CN40, panel B WCH1 clone 1 p7 and p17 (infectivity unknown), panel C isolate WCH1 and panel Disolate JD1.

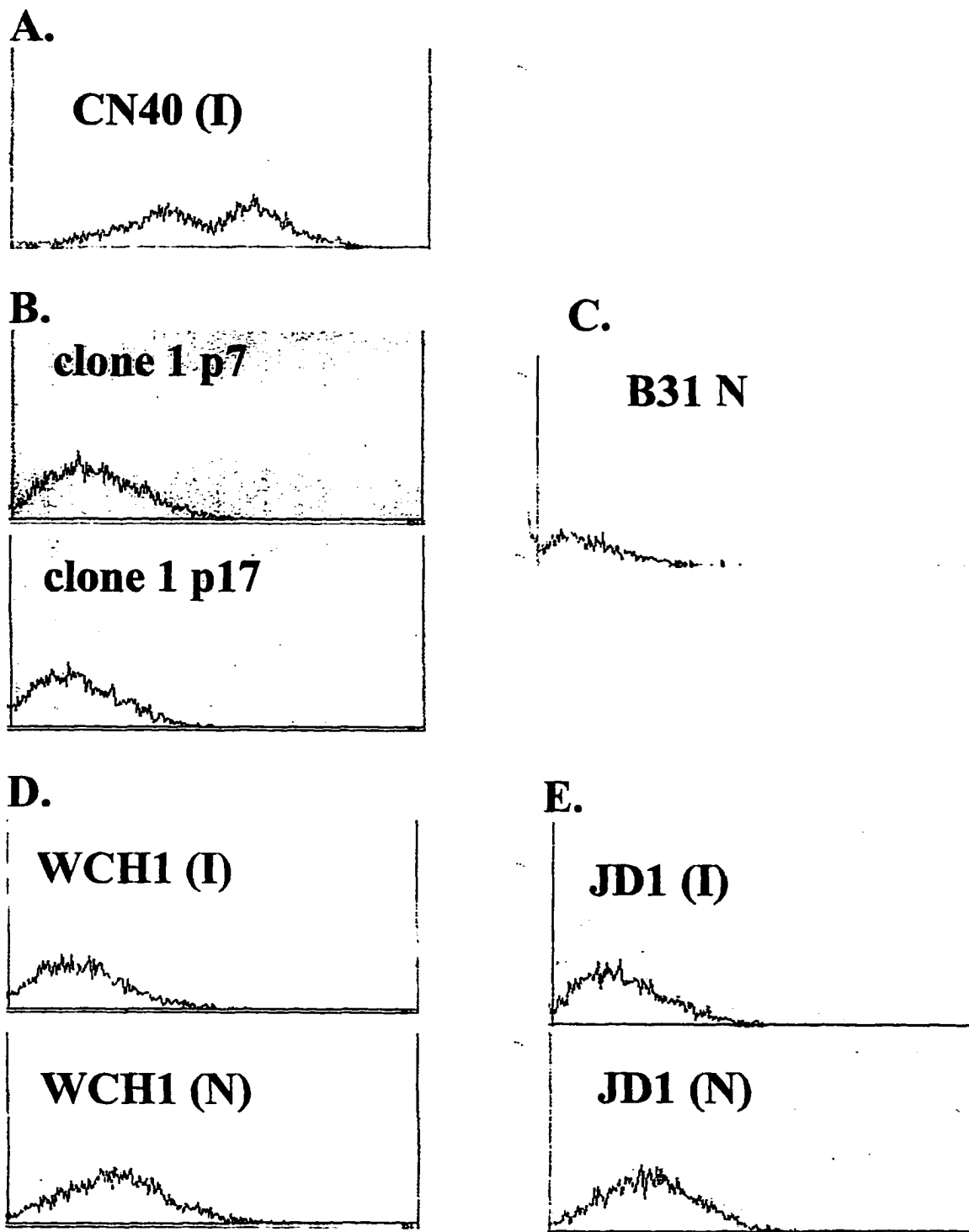


Figure 12. OspA surface expression as detected by flow cytometry. Infectious (I) and noninfectious (N) spirochetes were incubated with monoclonal antibody H5332, followed by a fluoroscein labeled secondary antibody and assessed by flow cytometry. Panel A isolate CN40, panel B WCH1 clone 1 p7 and p17 (infectivity unknown), panel C isolate B31, panel D isolate WCH1, and panel E isolate JD1.

B31, JD1, and WCH1 all displayed skewed peaks. In addition, all uncloned isolates exhibited higher P39 surface expression in the infectious population than the respective noninfectious population. The noninfectious samples' peaks remained skewed but there was a noticeable overall decrease in P39 reactivity. These data demonstrate the shift in outer surface P39 expression in uncloned organisms upon in vitro cultivation. This change may be due to a selection during in vitro culture of a particular subpopulation of spirochetes, or a change in the expression of the protein within the whole population of spirochetes.

3. OspB surface expression

All isolates had very distinct peaks for OspB (Fig.11). Clone 1 increased from a mean fluorescence of 100 to a mean of 136 from p7 to p17, but all other isolates remained relatively unchanged (Table 6). Overall OspB expression appeared uniform throughout cloned and uncloned populations, and did not change in intensity from low to high passage.

4. OspA surface expression

CN40 displayed two distinct peaks for OspA reactivity demonstrating two subpopulations varying in OspA expression (Fig.12). Either there are two subpopulations within this cloned population of spirochetes, or the spirochetes are differentially regulating the OspA expression. All other isolates, cloned and uncloned, had single distinct peaks for OspA expression indicating uniform surface expression of this molecule.

D. Characterization of P39

1. Solubility of P39 as determined by detergent extraction

In the cell fractionation experiment, P39 was detected in membrane fractions as well as cytosolic fractions. Therefore the question of the solubility of P39 was raised. Is P39 localizing to the cytosolic fractions because of a change in the solubility of the molecule? B31 was the isolate with the most dramatic shift in P39 localization

in infectious and noninfectious populations (from membrane to cytosol, Table 2) and was therefore selected for further study. For comparison CN40 was selected because it retains infectivity and does not exhibit a change in P39 localization. Based on these criteria, the localization of P39 within the isolates B31 and CN40 was determined utilizing detergent solubilization of whole cell sonicates, outer envelope, inner membrane, and cytosolic cell fractions.

P39 consistently localized to the Triton X114 soluble fraction indicating the hydrophobic nature of the molecule. For CN40, P39 was detected in the Triton X114 soluble phase of the outer envelope fraction (Fig. 13). For B31, P39 was detected in the Triton X114 soluble phase of whole cell sonicate and all three subcellular fractions (Fig. 14). This pattern of P39 localization in CN40 and B31 was also seen in the western blot analysis (Fig. 2 and 3). The consistent partitioning to the Triton X114 fraction indicates P39 is hydrophobic in nature and does not exhibit any change in solubility in infectious or noninfectious spirochetes or in different cell fractions. Therefore, the reason for the change of P39 localization in infectious and noninfectious B31 is not due to a change in the solubility of the P39 molecule.

2. Proteins associated with P39

If the change in P39 localization in infectious and noninfectious isolates is not due to a change in the solubility of the P39 molecule, perhaps it is due to the molecules with which P39 associates. This was investigated by immunoprecipitating P39 (using Mab D1C9) from whole cell sonicates of CN40, B31, WCH1, and JD1 and identifying any coprecipitating molecules. Using Pansorbin for the affinity purification of these complexes, a band corresponding to P39 was detected in all isolates. Figure 15 shows the results of immunoprecipitation from whole cell sonicates. Controls include the following: whole cell antigen immunoprecipitated in the absence of D1C9 (Ag); D1C9 immunoprecipitated in the absence of antigen (Mab); and Pansorbin with no antigen or antibody (Pan). Bands seen in the control lanes (designated nonspecific bands) include a 65, 54, 52, 41, 36, 26, and 16 kD bands. These bands appeared in the immunoprecipitations from the isolates and will not be discussed further. P39 was

<u>PCp5</u>		<u>OEp5</u>		<u>PCp23</u>		<u>OEp23</u>	
dt	aq	dt	aq	dt	aq	dt	aq

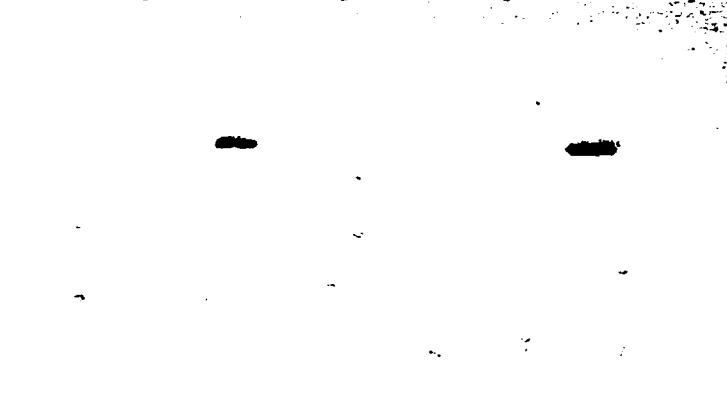


Fig. 13 Western blot detection of P39 in detergent extracted cell fractions of infectious CN40. Triton X114 was used to separate hydrophobic and hydrophilic molecules in cell fractions of isolate CN40. Detergent soluble (dt) and aqueous (aq) phases of CN40 protoplasmic cylander (PC), and outer envelope (OE) fractions from p5 and p23 are shown.

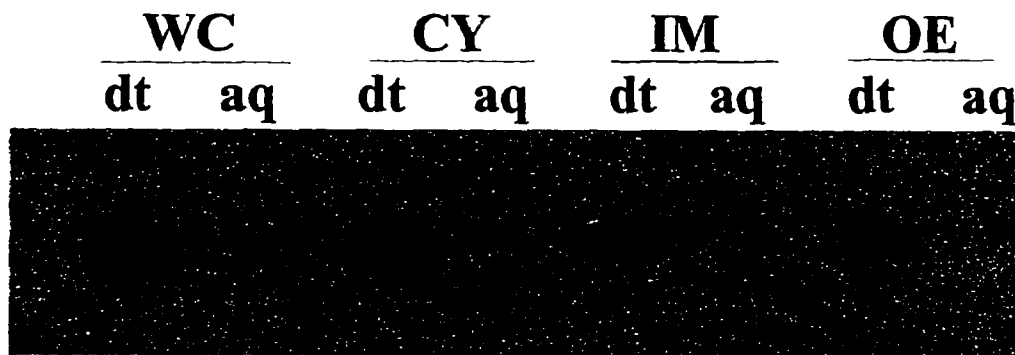


Figure 14. Western blot detection of P39 in detergent extracted cell fractions of noninfectious B31. Triton X114 was used to separate hydrophobic and hydrophilic molecules in cell fractions of isolate B31. Detergent soluble (dt) and aqueous (aq) phases of high passage B31 whole cell (WC), cytosolic (CY), inner membrane (IM), and outer envelope (OE) fractions are shown.

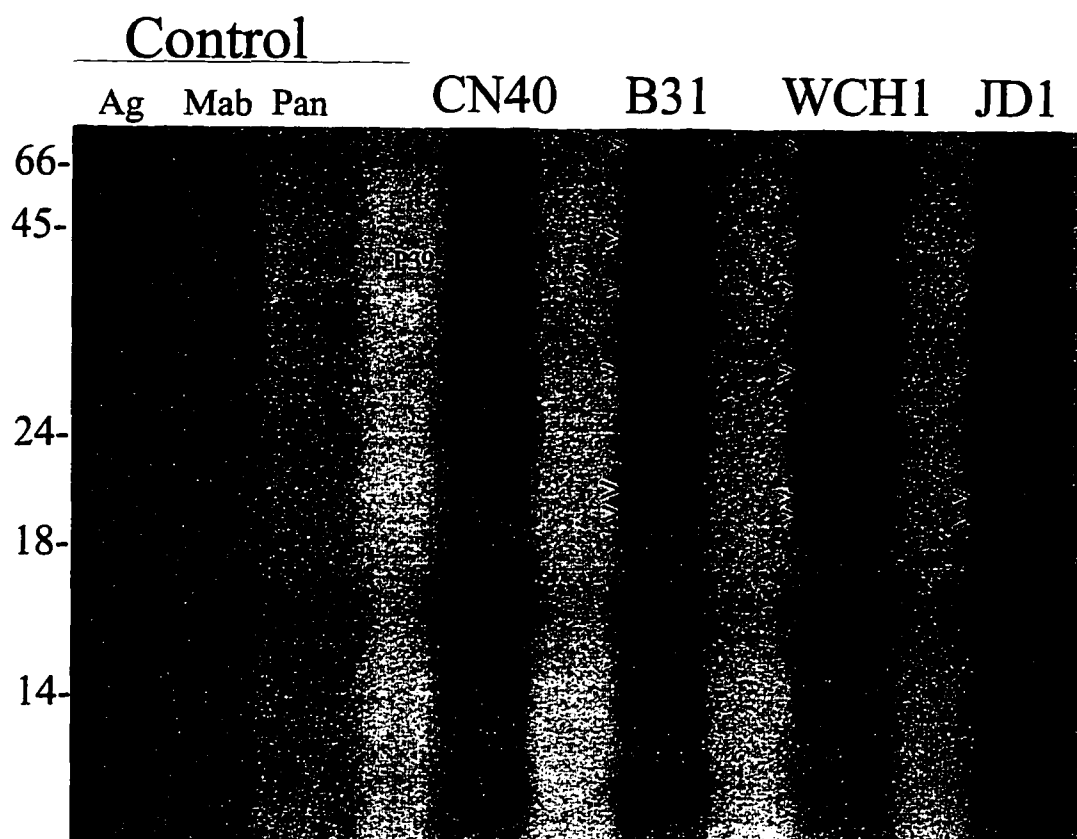


Figure 15. Immunoprecipitation of P39 from high passage whole cell sonicate of infectious isolate CN40 and noninfectious isolates B31, WCH1, and JD1. Controls include B31 sonicate (Ag), Monoclonal D1C9 (Mab), and Pansorbin cells (Pan). P39 was detected in all isolates (P39). Coprecipitating molecules found only in B31, WCH1 and JD1 are indicated with an arrow (>). Molecular weight markers, in kilodaltons, are indicated at left.

detected in all isolates. Bands common to all four isolates include 50, 39, 15, and 13 kD. Six additional bands found in all three noninfectious isolates, but not in the infectious isolate CN40 include 60, 46, 38, 32, 22, and 21 kD. In addition, there were a 29 kD and 9 kD band detected only in B31.

E. Attempts to genetically mutate the cloned P39 gene

1. P39-LacZ fusion

The first attempt to knock out P39 in *B.burgdorferi* was based on creating a P39-LacZ fusion gene. However, no successful ligations of P39 and LacZ DNA were detected in *E.coli*. Twenty five different reactions were attempted, utilizing enzymes and buffers from different manufacturers in an attempt to optimize the reaction. No successful P39-LacZ fusion gene was obtained. Therefore, instead of mutating the gene by insertion of a selectable marker, the P39 gene was mutated by deletion.

2. P39 deletion-mutant

Deletion of a 300 bp fragment from the cloned P39 gene and religation to yield a product which was out of frame and no longer expressed P39 was successful on the first attempt. The P39 deletion mutant construct did not express a protein recognized by the Mab D1C9 as demonstrated by ELISA. The absorbance for the wild type P39 (*E.coli* expressing cloned P39 gene) at 260 nm was 2.30 O.D., whereas the absorbance for the P39 deletion mutant (*E.coli* expressing mutant construct) was 1.40 O.D, and the negative control (*E.coli* with control vector) was 1.47 O.D.. In addition to providing a deletion-mutant construct for use in transforming *B.burgdorferi*, this experiment confirmed that the epitope for D1C9 is not contained within the first 100 amino acids (300 nucleotides) of P39.

3. Screening for transformed *B.burgdorferi*

CN40 was the isolate selected for use in this experiment because it does not lose infectivity during in vitro culture. CN40 was electroporated with the linearized P39 deletion mutant gene. Electroporated spirochetes were assayed by ELISA for

expression of flagellin and P39. Out of 2430 organisms 18 were concurrently positive for flagellin expression and low for P39 expression (as compared to untransformed organisms.) The qualification for a spirochete being P39 “low” was 0.1 O.D. units below the untransformed control spirochetes. This generous cut off point will invariably include P39 expressing spirochetes since this value is within the standard error of the mean (0.2). The purpose was to ensure no transformants were excluded, even if this meant including several nontransformed spirochetes. The 18 putative transformants were grown in culture for two weeks to increase the number of organisms. The spirochetes were subsequently harvested from culture and assayed by western blot to assess the P39 expression. However, all spirochetes expressed P39 as detected by western blot. Therefore, no *B.burgdorferi* P39 deletion mutants were obtained, and the question of P39’s role in infectivity of *B.burgdorferi*, and its role in the pathology of Lyme disease could not be directly addressed.

IV. CONCLUSIONS

The purpose of this study was to biochemically characterize the P39 molecule and determine if a correlation exists between P39 expression and infectivity of *Borrelia burgdorferi*. The first aim was to determine where P39 is located within the spirochete, and determine if localization of P39 correlates with infectivity. This comparative analysis necessitated the establishment of infectious and noninfectious isolates of *B. burgdorferi*.

Four different isolates were used in this study, and three separate methods were used for verifying isolate infectivity: culture of mouse tissue, xenodiagnosis using ticks, and PCR of mouse tissue. The first isolate CN40, which is the only isolate known to retain infectivity during prolonged in vitro culture, was shown to be infectious at passage 9 and passage 22 by tissue culture. The remaining isolates B31, WCH1, and JD1 (all uncloned organisms) were infectious at or below passage 6. Each isolate was recovered from the tissues of inoculated mice demonstrating persistence of spirochetes in tissues of the host. Transmission of spirochetes from infected mice to ticks, an important part of maintaining the *B. burgdorferi* population in the wild, was also demonstrated for isolates WCH1 and JD1. Other investigators report the transmission rate of WCH1 or JD1 from experimentally infected mice to feeding ticks is 1-10% (personal communication). Thus the transmission rate in this experiment, 4.3% for WCH1 and 15% for JD1, is consistent with similar findings made elsewhere.

When B31, WCH1, and JD1 were passed 15 or more times they were not recovered from the tissues of inoculated mice and were not transmitted to ticks. To ensure that the high passage isolates were not simply more difficult to isolate from tissue, PCR was performed on mouse tissues. No *B. burgdorferi* DNA was detected at a sensitivity of 10 pg of spirochete DNA/500 µg mouse DNA, confirming the lack of infectivity of these high passage isolates. These infectious and noninfectious

spirochetes were next analyzed for P39 localization to determine if a correlation exists between P39 localization and infectivity.

As shown in the western blot/densitometry analysis of cell fractions, there was a statistically significant interaction between P39 localization (outer envelope fraction only) and the infectivity of *B.burgdorferi*. High levels of P39 in the outer envelope correlated with infectivity of the spirochete. A decrease in P39 levels in the outer envelope correlated with a loss of infectivity. No significant interaction with infectivity was detected for P39 in the remaining cell fractions, nor for OspB or OspA in any cell fraction. This supports the hypothesis that P39 localization is related to the infectivity of *B.burgdorferi*.

Interestingly, the cloned infectious isolate CN40 localized P39 exclusively to the outer envelope. Continuous culture of CN40 did not affect the localization of P39 nor the quantity produced. In contrast, P39 was localized to all three cell fractions of uncloned isolates WCH1, JD1, and B31 and continuous culture did affect the localization of P39 in these three isolates. Noninfectious WCH1 and JD1 had far less P39 in the outer envelope and more P39 in the inner membrane and cytosol, whereas noninfectious B31 had a dramatic increase of P39 in the cytosol. This shift in P39 localization was also demonstrated in the flow cytometry analysis; noninfectious B31, WCH1, and JD1 had uniformly decreased surface expression of P39 as compared to the corresponding infectious isolates. The changes noted in P39 localization in noninfectious B31, WCH1, and JD1 may have been due to clonal expansion of a particular subgroup of spirochetes in the uncloned population, or a widespread phenotypic change by the whole population of spirochetes. The cell fractionation/western blot procedure does not allow for differentiation between the two possibilities.

Other changes were detected in these three uncloned isolates as shown by silver staining. In comparison of infectious and noninfectious outer envelope, each uncloned isolate had at least two bands which differed in intensity: B31 had bands at 24, 22 and 20 kD; WCH1 had bands at 39 and 36 kD; JD1 had bands at 47, 45, 34, 32, 29, and 14 kD. The cloned, infectious isolate CN40 exhibited no detectable changes in banding patterns in low and high passage cell fractions. For all isolates the majority of proteins

detected by silver stain were located in the outer envelope fraction. Few proteins were detected in the inner membrane and cytosolic fractions even though proteins were present in those fractions as evidenced by western blot.

Previous studies have compared infectious and noninfectious isolates by SDS-PAGE silver stain and western blot (65, 68, 73, 75, 103). All of these studies have used whole cell organisms and have not analyzed possible differences in protein localization within the spirochetal cell. Subtle changes in protein localization, such as those described in this study, would be easily overlooked by simple total protein analysis. Some previously documented changes of B31 as a result of in vitro culture are as follows; decrease in 24 and 20 kD molecules (also documented in the present study) and loss of 35 and 28 kD molecules (75), and in another study using B31 documented the loss of 28, 26, 18 and 16 kD proteins (117).

Other investigators have selectively studied the outer envelope of the *B. burgdorferi* in hopes of identifying virulence factors, and have separated the spirochetal cell using isopicnic centrifugation after disruption of the cell in a French pressure cell (133). However this allows mixing of the different cell fractions prior to separation of the fractions. This process may artificially allow proteins to separate into fractions in which they were not originally located. In order to avoid this dilemma in the present study the outer envelope was first removed from the protoplasmic cylinder using a mild detergent solution. This outer envelope fraction was then separated from the protoplasmic cylinder by centrifugation. The protoplasmic cylinder was then sonicated to break apart the cell, and inner membrane and cytosolic constituents were separated by centrifugation. This modified procedure reduces the possibility of artifactual redistribution of antigens among the cell fractions.

Perhaps what P39 is "doing" in the outer envelope is important. What is P39 bound to or complexed with in the outer envelope? As shown by immunoprecipitation, molecules associating with P39 in all four isolates include 50, 39, 15 and 13 kD. All noninfectious isolates had the following additional molecules: 60, 46, 38, 22, and 21 kD. In addition, isolate B31 had bands at 29 and 9 kD. Therefore there is a distinct difference in the molecules associating with P39 in infectious and noninfectious

isolates. One must note that P39's association with other molecules may result in the masking of the P39 epitope recognized by D1C9, therefore it is possible not all molecules interacting with P39 will be identified.

It also should be noted that the amount of a molecule contained within the outer envelope fraction is not necessarily indicative of how much of that molecule is actually surface exposed. For example, in the western blot/densitometry analysis infectious B31 had the least amount of P39 in the outer envelope of all the isolates, yet it had the highest flow cytometry reading for surface exposed P39. From these data it appears that all or most of the P39 in the outer envelope of infectious B31 is surface exposed. In contrast, the infectious isolate CN40 flow cytometry analysis revealed that very little P39 is actually surface exposed. Indeed, the surface exposed P39 in high passage infectious CN40 was less than any other isolate either infectious or noninfectious. Either the P39 in CN40 outer envelope is located on the inner leaflet of the outer envelope thereby limiting surface exposure, or the P39 epitope is masked by another molecule at the cell surface.

Flow cytometry analysis also revealed that uncloned infectious isolates did have more P39 surface exposed than the corresponding noninfectious isolates as was seen in the western blot/densitometry analysis. Furthermore, infectious B31, JD1, and WCH1 had variable surface expression of P39. All exhibited broad, skewed peaks illustrating a lack of uniformity in P39 surface expression within the population of spirochetes. These same isolates did have more uniform reactivity in the noninfectious populations. Notably, all three uncloned populations exhibited the same shift from high P39 surface expression to low P39 surface expression. This may be due to a selection of the lower expressing population over a period of time, or it may be due to a change in the expression of the P39 protein by all spirochetes within the population.

Another point to note is that surface protein expression can vary even within a cloned population as demonstrated by CN40 expression of OspA. Two distinct populations were detected by flow cytometry. In past research OspA expression has been demonstrated as being differentially regulated by temperature, but all cultures in this study were maintained at the same temperature. Therefore another regulatory

mechanism must be involved. Another point is that uncloned isolates such as B31, WCH1 and JD1 can vary in surface expression of one molecule (P39) and yet have uniform expression in other surface exposed molecules (OspB and OspA). This also occurs for cloned isolate CN40, OspA expression is varied and yet P39 and OspB expression is uniform throughout the population. This varying of surface exposed molecules may be advantageous to the spirochete in evasion of immune responses. A recent study documenting Osp localization to both outer envelope and inner membrane fractions concluded that *B. burgdorferi* may uncouple lipoprotein expression from surface localization as part of their parasitic strategy for evasion of humoral immune response (102). This theory is supported in part by the findings in the present study; Osps located in both membrane fractions of the spirochete, and a shift of location of surface exposed molecules by cloned and uncloned organisms. No immune pressure was placed on these organisms since they were grown in vitro, therefore the primary purpose for this shift may be entirely unrelated to immune evasion.

In the present study P39 was detected primarily in membrane fractions, therefore it would seem logical that P39 be hydrophobic. Since P39 was also detected in the cytosol of the uncloned isolates (most prominently in noninfectious B31), it raised the possibility that P39 may be modified in a way which would affect the solubility of the molecule. The solubility of P39 has not previously been investigated. Other researchers utilizing Triton X114 have reported a detergent soluble protein of 39 kD obtained from isolate B31 (132), but did not confirm the identification of the molecule with a monoclonal antibody. The present study demonstrated, using western blot as confirmation, that P39 consistently localized to the detergent soluble fraction of B31 in all cell fractions tested. This was also the case for isolate CN40. This indicates P39 is hydrophobic in nature and does not exhibit any change in solubility from low to high passage or in different cell fractions. Therefore, the reason for the change of P39 localization in infectious and noninfectious B31 is not due to a change in the solubility of the P39 molecule. P39 may be associating with additional molecules in B31, such as the 29 and 9 kD molecules seen in the immunoprecipitation experiment, and this

may be responsible for the dramatic increase of P39 in the cytosol of noninfectious B31.

There is a statistically significant relationship between P39 levels in the outer envelope of a spirochete and the infectivity of that spirochete. However, statistical significance does not necessarily translate into a cause and effect relationship in regards to infectivity. The only way to prove that P39 expression is required for the infectivity of *B.burgdorferi* is to create a P39-less mutant and assess the infectivity of that mutant. Even if a P39-less mutant remained infectious, it could be used to assess the role of P39 in the pathology of Lyme disease. For this study an attempt was made to create a P39-less mutant *B.burgdorferi*. The use of electroporation and homologous recombination has previously been demonstrated as an effective route of genetic manipulation in *B.burgdorferi* (136). The first attempt consisted of creating a P39-LacZ fusion for use in electroporation. Despite much effort, no functional construct was obtained. The second attempt utilized a P39 deletion mutant construct; instead of inserting DNA as a mode of gene disruption, DNA was deleted. Screening by loss of P39 signal was not ideal but still an option. The transformation efficiency in *B.burgdorferi* using linear DNA is $10^3/\mu\text{g}$ of DNA, therefore at least 2,000 of the 10,000 electroporated organisms were expected to be successfully transformed under the conditions used. The putative transformants were screened both by ELISA and by western blot. No spirochetes could be detected which lacked expression of P39. It is possible that P39 is an essential protein for routine function of the bacterial cell and therefore any transformants carried a lethal mutation.

The changes in P39 localization of infectious and noninfectious organisms seen in this study may be indicative of a novel protein localization regulatory mechanism operating in vivo. This regulation differs from the temperature regulation of OspA, and OspC; changes in temperature have no effect on the expression of P39 (105). The change in P39 localization seen in this study also differs from the antigenic variation seen in *Borrelia hermsii*, the causative agent of relapsing fever, which alters the expression of surface exposed lipoproteins by actual rearrangement of the DNA. This

novel change in P39 localization may explain *B.burgdorferi*'s successful immune evasion in vivo. Clinical evidence shows the strong anti-P39 humoral response seen in an infected host fails to clear the *B.burgdorferi* infection. Yet P39 specific antibodies have been shown to be highly bactericidal in vitro (83). Perhaps P39 surface expression is required only during the initial establishment of infection, and the surface exposure of P39 facilitates development of the early P39 specific humoral response. It is possible that after the initial infection all or some of the spirochetes decrease the amount of P39 that is surface exposed thereby evading the anti-P39 antibodies. This type of regulation of surface exposed molecules may be the key to infectivity and to how *B.burgdorferi* successfully evade the hosts immune response.

In summary, this study provides groundwork information which is necessary for a more complete understanding of P39 and *B.burgdorferi* infectivity. The results of this study show that P39 is localized primarily to the outer envelope and inner membrane of *B.burgdorferi*. Infectious isolates have a higher level of P39 in the outer envelope as compared to noninfectious isolates. P39 was shown to be hydrophobic in nature and this remained unchanged in all cell fractions of infectious and noninfectious organisms. Other differences were noted between infectious and noninfectious isolates by silver stain, and immunoprecipitation. P39 associated with three molecules common to all isolates, and at least six additional molecules in noninfectious isolates only. And finally, P39 surface expression was found to change in all uncloned isolates during in vitro culture; uncloned isolates had a uniform decrease in surface expression of P39 which corresponded with their loss of infectivity.

ANIMAL STUDIES

Approximately 32 female, B6CBA strain, *Mus musculus*, ages six to 12 weeks, were used in the following infectivity experiment. Mice were anesthetized with ether, and blood samples were collected from each mouse using the retro-orbital technique. Ether serves to minimize any pain the animals feel while blood is collected. Blood samples were required to monitor any serological changes that occur during the attempted infection process. Mice were inoculated with 0.1 ml of a *Borrelia burgdorferi* suspension (100,000 organisms). Two to eight mice were used per isolate (low or high passage). Fifteen days later mice received a 0.05 ml intraperitoneal injection of ketamine/xylazine solution. This causes the mice to sleep for approximately twenty minutes. At this time 10-15 *Ixodes scapularis* nymphs were allowed to feed on each mouse. Anesthetizing the mice allows the ticks a better opportunity to attach and feed on the mice. Unanesthetized mice will readily eat the ticks before they can attach. The ticks were used to determine if spirochetes can be transmitted from infected mice to ticks as occurs in the wild. Four days after feeding commences, the ticks were collected and held for processing. The mice were anesthetized with ether and bled by the retro-orbital technique for reasons already stated. The mice were euthanized by cervical dislocation (placing forceps at the base of the mouse's skull and giving a quick and strong jerk on the tail). This method results in complete ablation of central nervous system function and is approved by the Federal Guide for the Care and Use of Laboratory Animals. The tissues were collected and cultured in BSK-H media for detection of *B.burgdorferi*.

Daily care for the animals was provided by the Animal Facility located in room 406, Mills Godwin building. The use and treatment of the animals were approved by the Animal Care and Use Committee of Old Dominion University. A mammalian host was needed to determine the infectivity of *B.burgdorferi*. Mice are reservoir hosts for *B.burgdorferi* in the wild, and are commonly used in laboratory studies

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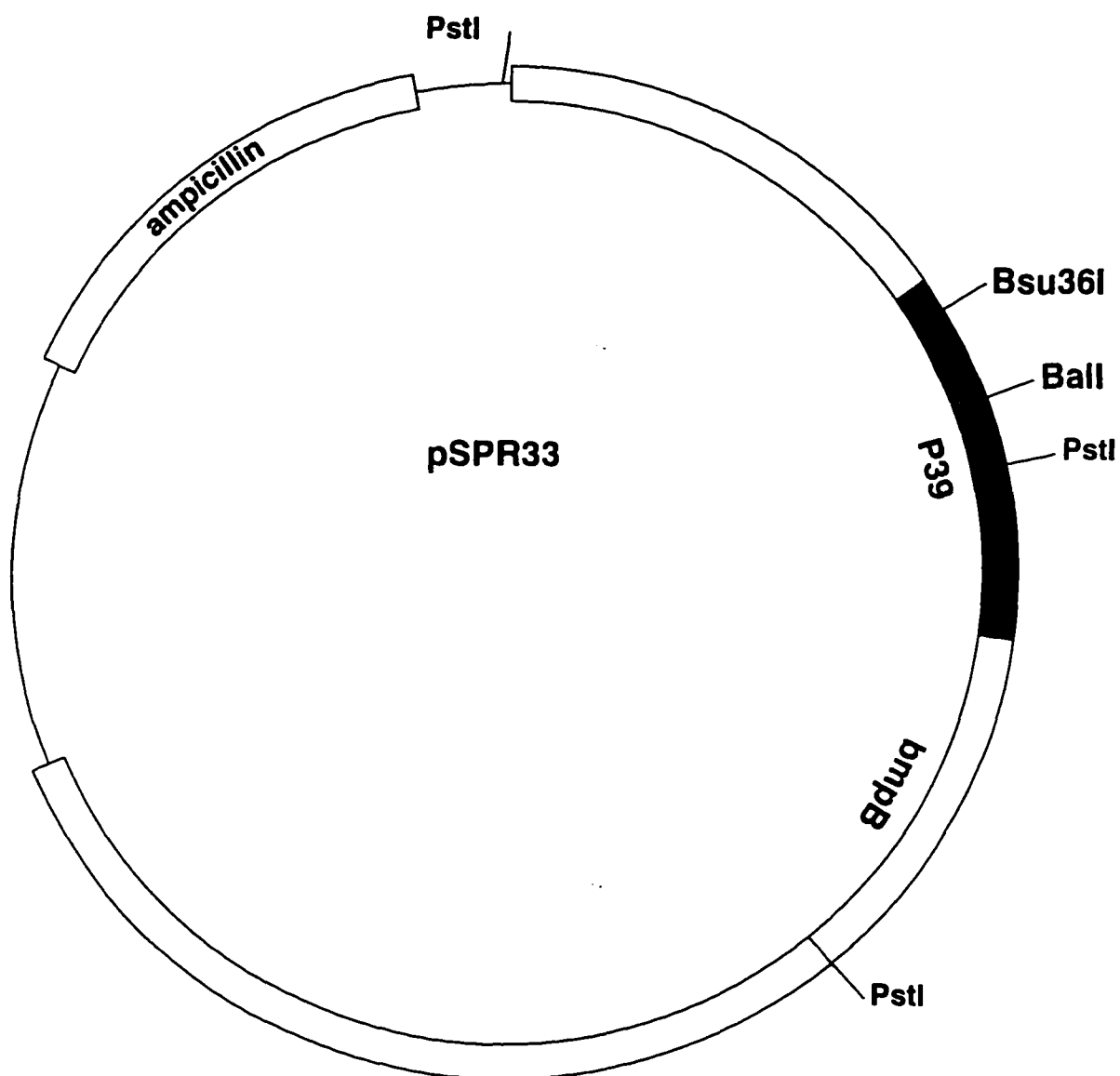
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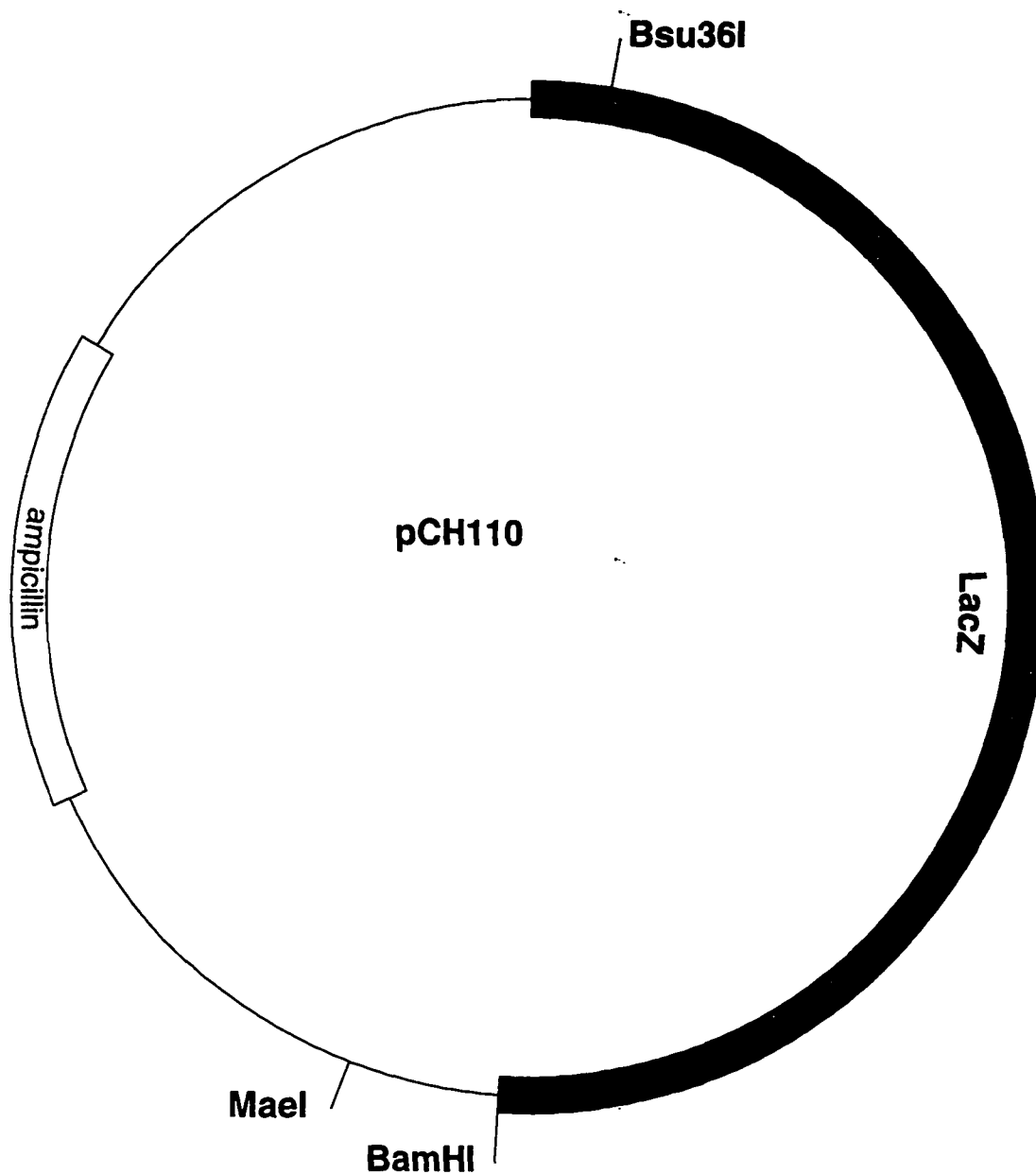
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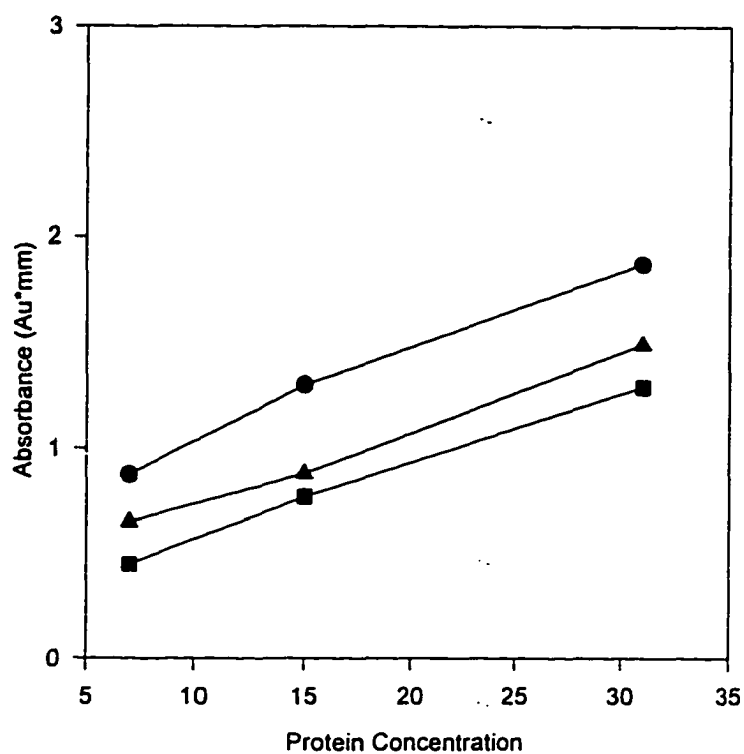
APPENDIX



Appendix 1. Plasmid map of pSPR33. P39 gene, bmpB gene, ampicillin resistance gene, and restriction sites are shown.



Appendix 2. Plasmid map of pCH110. LacZ gene, ampicillin resistance gene, and restriction sites are shown.



Appendix 3. Western blot densitometry assessment. Densitometry absorbance readings from a western blot of serial dilutions of *B. burgdorferi* (protein concentration given in μg) developed with monoclonals specific for P39 (●), Osp A (◆) and Osp B (■).

VITA

Jill M. Troyer earned her Bachelor of Science degree in Biology at Baylor University in Waco, Texas in 1990, and in 1996 she completed her Ph.D. in Biomedical Sciences at Old Dominion University in Norfolk, VA. She is a member of both Sigma Xi, American Society for Microbiology, and the American Society for Rickettsiology and Rickettsial Diseases. She currently resides in Baltimore, Maryland and holds a Postdoctoral Fellowship with Dr. Abdu Azad in the Microbiology and Immunology Department of University of Maryland School of Medicine. Below is a abbreviated list of her publications.

Published Articles

Daniel E. Sonenshine, Robert E. Ratzlaff, Jill Troyer, Shawn Demerlee, Elizabeth R. Demerlee, Warren E. Austin, Sigmund Tan, Barry A. Annis, Suzanne Jenkins. Lyme borreliosis in eastern Virginia: comparison between a coastal and inland locality. *Am. J. Trop. Med. Hyg.* 53(2):123-133.

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