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OspC in the pathogenesis of Borrelia burgdorferi

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OSPC IN THE PATHOGENESIS OF *BORRELIA BURGDORFERI*

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DEDICATION

"Worthy is the Lamb, who was slain, to receive power and wealth and wisdom and strength and honor and glory and praise."

Revelation 5:12

To my Father and Mother
Dr. Sarma V.K. Seemanapalli, Ph.D.
and Mrs. Santa K. Seemanapalli, M.P.A.

ACKNOWLEDGEMENTS

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ABSTRACT

Lyme disease is a multisystem disorder caused by the tick vector *Borrelia burgdorferi*. During its life cycle between the tick vector and the mammalian host, *Borrelia* up- and down-regulates the expression of its surface lipoproteins. Of its many surface lipoproteins, the Outer surface protein C (OspC) is crucial for initial mammalian infection. OspC has a common role shared with other lipoproteins of protection against host innate defences and a unique function in facilitating the dissemination of *B. burgdorferi* in the murine host.

The structure of OspC was solved in 2001 and the lipoprotein was found to be predominantly alpha-helical with 5 alpha helices and 2 beta sheets interconnected by 6 loops, and an N- and C-terminus. Not much is known as to how the OspC structure relates to the function of the lipoprotein and/or possible regulation. The up-and down-regulation of OspC is achieved by the *rpoS* alternative sigma factor and by an unidentified repressor, respectively.

Several truncated versions of OspC were tested in an OspC-deficient background to better understand the role of the amino acid sequences deleted. Structural deletions of 5 to 8-AA made within the OspC core, resulted in loss of infectivity. Longer deletions of 10- and 13 AA towards the N- and C-terminus of OspC, respectively, also resulted in loss of infectivity. Interestingly, a deletion of only 5-AA of the N-terminus of OspC did not affect the role of OspC in evasion but caused inefficient dissemination of *B. burgdorferi* in SCID mice.

Interestingly, whereas the deletion of N-terminus 5-AA of OspC resulted in upregulation, a deletion of 6- and 9-AA of the C-terminus of OspC resulted in downregulation of *ospC* mRNA in joints, indicating that OspC may be involved in self-regulation. This hypothesis was confirmed by the use of monoclonal antibody treatment. Deletion of 6-AA of the C-terminus of OspC resulted in lower spirochete burdens in heart and joint, whereas deletion of 9-AA resulted in higher spirochete burden in skin, indicating the tissue-dependent effects resulting from the

deletion of the C-terminal sequence. Taken together, these results indicate that OspC is involved in the pathogenesis of *B. burgdorferi*.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

<u>INTRODUCTION</u>

Borrelia burgdorferi, the agent of Lyme disease, differentially expresses several lipoproteins during its transmission cycle between a tick vector and a mammalian host (Burgdorfer *et al.*, 1982). Timely expression of the outer surface protein C (OspC) is crucial for the pathogenic strategy of *B. burgdorferi*. The pathogen expresses OspC abundantly during early infection of a mammal, when the antigen is required (Liang *et al.*, 2002a, Liang *et al.*, 2002b). However, OspC being a protective antigen, its continued presence poses a threat to the pathogen, once anti-OspC humoral response has developed. If the pathogen does not down-regulate OspC or undergo escape mutations on the *ospC* gene, infection would be cleared (Xu *et al.*, 2006).

When the crystal structure of OspC was resolved, OspC was shown to be predominantly alpha-helical in structure (Eicken *et al.*, 2001, Kumaran *et al.*, 2001). The molecule forms dimers in solution. Each subunit is made up of five alpha helices, two beta strands, six loops that connect the alpha helices and the beta strands and an amino (N-) and a carboxy (C-) terminus. Although the solution of the crystal structure did not shed much light to the function of OspC, through genetic and molecular methodologies, recent studies have shown that OspC has dual roles in early protection and in facilitation of dissemination of *B. burgdorferi* (Tilly *et al.*, 2007, Xu *et al.*, 2008).

In terms of regulation, the *ospC* gene is under the control of the sigma factor RpoS. Several virulent genes have an RpoS-dependent promoter (Yang *et al.*, 2000, Yang *et al.*, 2003a). In mice, RpoS appears to be constitutively expressed. The down-regulation of OspC when under immune pressure is thus dependent on an as-yet unidentified repressor that negatively regulates the gene via a specific interaction with the *ospC* operator (Xu *et al.*, 2007).

In this research project, we explored the crystal structure of OspC and made several types of strategic deletion mutants and analyzed the same for their effects on the functions of OspC and the regulation of the lipoprotein. The results show that deletion of the beta strands 1, 2 and alpha helix 4, 13-AA of the C-terminus and 10-AA of the N-terminus of OspC (βs_1, βs_2, αH_4, Ct13-AA and Nt10-AA) leads to non-infectious spirochete phenotypes, thus abrogating the role of OspC in early evasion of *B. burgdorferi* from host innate defences; that 5-AA sequence of the N-terminus of OspC is able to dissect the role of OspC in early immune evasion from that of dissemination, because the mutant is infectious but unable to disseminate efficiently in SCID mice; that the same 5-AA N-terminus sequence and along with the C-terminus of OspC are involved in self-regulation of *ospC*. Collectively, this study showed a correlation between the structure of OspC and its function in innate immune evasion, dissemination and self-regulation.

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LITERATURE REVIEW

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Discovery of Lyme Disease. Lyme disease was first recognized in 1975 as a distinct clinical disorder by Dr. Allen Steere. The disease derives its name from the small town of Lyme, Connecticut, USA (Steere *et al.*, 1977b). Awareness of the disease began when a cluster of children with an initial diagnosis of "juvenile rheumatoid arthritis" on one small street in Old Lyme, CT was reported by their parents to the State Health Department. Investigation of this unexplained arthritis in three contiguous Connecticut communities led to the description of Lyme arthritis. The geographical clustering of the patients in heavily wooded areas along with the peak occurrences in summer months suggested an infectious etiology and transmission of the infectious agent by an arthropod vector (Steere *et al.*, 1977a, Steere *et al.*, 1977b). However, the serendipitous discovery of the spirochetal etiology of Lyme disease was closely linked with studies on the natural history of spotted fever rickettsiae on Long Island, New York, where during 1971-1975, a total of 124 cases of spotted fever with eight deaths occurred. A scientist by

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the name Dr. Willy Burgdorfer along with another scientist, Dr. Jorge Benach from the New York State Health Department were primarily interested in obtaining a virulent strain of *Rickettsia rickettsii* from *Dermacentor variabilis* ticks collected near the homes of spotted fever patients on Long Island, New York. Instead, they found *R. montana*, a rickettsia non-pathogenic for humans. This then led them to speculate that possibly other tick species, such as the black-legged deer tick *Ixodes dammini* (now, *I. scapularis*), might play a role in the ecology of spotted fever group. In the fall of 1981, Dr. Benach supplied Dr. Burgdorfer with additional ticks from Shelter Island, NY. None of the ticks examined contained *Rickettsia* but Dr. Burgdorfer found several microfilarias in the hemolymph of two female ticks, and to determine if this nematode was present in other tissues, he dissected and prepared smears of all other tissues, including the midgut. Surprisingly, instead of additional microfilaria, he found poorly shaped, long, irregularly coiled spirochetes. Dark-field microscopy confirmed the nature of the organism. Thus, the previously unidentified spirochete was named *Borrelia burgdorferi*, after the scientist Willy Burgdorfer (Burgdorfer *et al.*, 1982, Glaser, 2006). The antigenic relationship between the *I. scapularis* spirochete and Lyme disease was established by indirect immunofluorescence and by Western Blot of sera from patients recovered from Lyme disease (Burgdorfer *et al.*, 1982). The discovery of this spirochete as the long-sought etiologic agent of Lyme disease was hailed as an important breakthrough that led to an exploration of research on other spirochetal diseases.

Clinical Manifestations of Lyme Disease. Lyme disease generally occurs in stages, with different clinical manifestations at each stage. Although the initial classification of Lyme disease was as simple as erythema migrans (stage 1), meningitis or Bell's palsy (stage 2) and arthritis (stage 3) (Steere *et al.*, 1983), due to the overlap of the clinical manifestations at the different stages, the disease was re-classified in a system similar to that used to classify Syphilis (Asbrink and Hovmark, 1988). Thus, Lyme disease was essentially divided into early and late

infection. Early infection consists of stage 1 (early localized), followed within days or weeks by stage 2 (early disseminated) and within weeks or months by intermittent symptoms. Late or persistent infection (stage 3) usually begins a year or more after the onset of the disease (Nadelman and Wormser, 1998, Steere, 1989, Yoshinari *et al.*, 1989).

The initial most common and clinical hallmark of the early localized stage of Lyme disease is a skin rash known as erythema chronicum migrans (Figure 1.1). The rash is recognized in about 90% of patients with objective evidence of *B. burgdorferi* infection (Gerber *et al.*, 1996). Erythema chronicum migrans typically develops at the site of the bite, within 7 to 10 days after the bite, as a small red annular macule or papule. As the redness around the center grows larger, the lesion tends to have red outer borders with partial central clearing that may expand to a diameter of up to 50 cm (Behera *et al.*, 2005, Steere *et al.*, 1978), for which reason the rash is also known as a "bull's eye rash". The lesion is usually asymptomatic but may be pruritic or painful and it may be accompanied by constitutional symptoms such as low-grade fever, fatigue, malaise, arthralgias, headaches, cough, stiff neck and regional lymphadenopathy (Steere *et al.*, 1983).

The most common manifestation of the early disseminated stage is multiple erythema migrans. These secondary skin lesions appear 3 to 5 weeks after the tick bite and consist of multiple annular erythematous lesions similar to, but usually smaller than the primary lesion. Other manifestations of this stage include cranial nerve palsy, especially facial nerve palsy (Bell's palsy), and meningitis. Unilateral or bilateral facial palsy is the most common cranial neuropathy, and it may be the only neurologic abnormality (Clark *et al.*, 1985). Rarely, carditis, manifested by various degrees of atrio-ventricular (A-V) block, may also occur at this stage (Steere *et al.*, 1980).

Figure 1.1. Erythema chronicum migrans. (Courtesy of Dori F Zaleznik, MD). The rash is often seen in the early stage of Lyme disease. It is not an allergic reaction to the tick bite but an actual skin infection by *Borrelia burgdorferi*. Because of its reddish outer borders and central clearing, it is commonly referred to as "Bull's eye rash".

In the United States, the most common manifestation of late Lyme disease is arthritis. Arthritis occurs weeks to months after the initial infection; it is usually monoarticular or oligoarticular (Steere *et al.*, 1977b) and affects the large joints, particularly the knee (Figure 1.2). Although the joint is swollen and tender, the intense pain associated with a septic arthritis is usually not present. At this stage, about 10% of adults and 5% of children are refractory to antimicrobial treatment.

Other manifestations highly indicative of Lyme disease are lymphocytoma, meningoradiculoneuritis (Garin-Bujadoux-Bannwarth syndrome), and acrodermatitis chronic atrophicans. Borrelial lymphocytoma, is a solitary bluish-red swelling with a diameter of up to few centimeters that appears most commonly on the ear lobe or nipple of the breast (Asbrink and Hovmark, 1988, Maraspin *et al.*, 2002, Strle *et al.*, 1992). Patients with meningoradiculoneuritis tend to have severe pain, usually in the thoracic or abdominal region, often belt-like and most pronounced during night (Kristoferitsch, 1991, Pachner and Steere, 1985, Steere, 1989). Acrodermatitis chronica atrophicans is a skin lesion that begins insidiously with bluish-red discoloration and swollen skin on an extremity. The lesion's inflammatory phase may persist for

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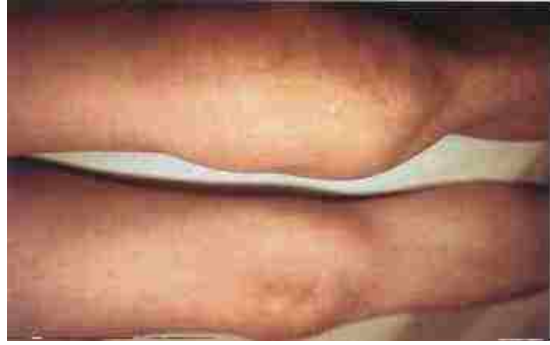
Figure 1.2. Lyme arthritis. (Rahn and Evans, 1998). Lyme arthritis occurs during the late stage of Lyme disease. It tends to be mono or oligoarticular, usually affecting the large joints, particularly the knee joint. Although the area is typically swollen, the intensity of joint pain is not comparable to that of a septic arthritis.

many years and it gradually leads to atrophy of the skin (Asbrink and Hovmark, 1988). It is most often located in the extensor sites of the hands and feet. It is frequent in patients older than 40 years and is more frequent in women than in men (Asbrink and Hovmark, 1988, Asbrink *et al.*, 1986, Steere, 1989).

Lyme disease is caused by *Borrelia burgdorferi sensu lato* strains. The human infection is caused primarily by three pathogenic genospecies, *Borrelia burgdorferi sensu strictu*, *B. garinii*, and *B. afzelii* (Johnson *et al.*, 1984). Whereas *B. burgdorferi* is the sole cause of infection in the United States, *B. garinii* and *B. afzelii* are the main causes of the disease in Europe and Asia (Baranton *et al.*, 1992, Canica *et al.*, 1993). The various clinical manifestations of chronic Lyme disease have been associated with different tissue tropisms of the three genospecies of *Borrelia*. Cutaneous and neurological manifestations such as acrodermatitis chronica atroficans and neuroborreliosis are often associated with *B.afzelii* and *B.garinii* infections, respectively, whereas Lyme arthritis is mainly a complication of *B. burgdorferi sensu strictu* infections (Balmelli and Piffaretti, 1995, van Dam *et al.*, 1993).

Diagnosis and Treatment of Lyme Disease. According to the Center for Disease Control and Prevention (CDC, 2009), the diagnosis of Lyme disease is based on symptoms, objective physical signs (such as the typical erythema migrans, facial palsy, or arthritis), and a

history of possible exposure to infected ticks. Validated laboratory tests can be very helpful, but are generally not recommended when a patient has erythema migrans. The most recommended laboratory tests are serodiagnostic.

CDC recommends a two-step process when testing blood for evidence of Lyme disease. The first step uses an enzyme immunoassay such as Enzyme-Linked Immunosorbant Assay (ELISA) or Immunofluoresence Assay (IFA) tests. These tests are designed for screening and are very sensitive. The ELISA provides a quantitative estimate of the concentration of antibodies against *B. burgdorferi*. If the ELISA or IFA is positive or indeterminate (equivocal), a second step should be performed to confirm the results. The second step uses a Western blot test, for its high specificity. The immunoblot provides antibodies against specific protein antigens of *Borrelia burgdorferi*. Most authorities require the presence of antibodies against at least 2 (for IgM) or 5 (for IgG) specific protein antigens of *B. burgdorferi* for the immunoblot to be considered positive (CDC, 1995). CDC does not recommend testing blood by Western blot without first testing it by ELISA or IFA. Dressler *et al*., (Dressler *et al.*, 1993) and Johnson, *et al*., (Johnson *et al.*, 1996) showed that the use of Western blot is important in determining whether "intermediate" results by ELISA are true positive.

Treatment of Lyme disease involves primarily antibiotic therapy. The recommended treatment for early localized or disseminated Lyme disease, in the absence of specific neurologic manifestations, involves oral administration of doxycycline, amoxicillin, or cefuroxime axetil for 14 days. The intravenous use of ceftriaxone for 14 days in early Lyme disease is recommended for adult patients with acute neurologic disease manifested by meningitis or radiculopathy (Wormser *et al.*, 2006).

Lyme arthritis can usually be treated successfully with antimicrobials administered orally. Doxycycline, amoxicillin, or cefuroxime axetil for 28 days is recommended for adult

patients without clinical evidence of neurologic disease. Adult patients with arthritis and objective evidence of neurologic disease should receive ceftriaxone IV (intravenous) (Wormser *et al.*, 2006). For persons who have persistent or recurrent joint swelling after a recommended course of oral antibiotic therapy, the panel formed by Wormser, GP and colleagues recommend re-treatment with another 4-week course of oral antibiotics or with 2-4- week course of ceftriaxone IV (Wormser *et al.*, 2006).

The severity and the duration of Lyme arthritis are increased in patients with IgG antibodies against OspA (Kalish *et al.*, 1993, Steere *et al.*, 1994). Particular class II major histocompatibility molecule (MHC) genes can determine a host immune response to *B. burgdorferi* that results in chronic arthritis and lack of response to antibiotic therapy (Steere *et al.*, 1990, Steere *et al.*, 1994). Due to the increased frequency of certain MHC molecules such as HLA-DR3 and HLA-DR4 alleles (Nocton, 1994, Steere *et al.*, 1988, Steere *et al.*, 2006) and the identification of human leukocyte function-associated antigen-1 (hLFA-1) as a candidate autoantigen (Gross *et al.*, 1998) in these patients, an autoimmune mechanism has been proposed. However, LFA-1 is unlikely a relevant autoantigen, instead, an OspA epitope may be critical in triggering an antibiotic treatment-resistant Lyme arthritis (Steere *et al.*, 2003). Although the pathogenesis of this refractory arthritis is not well understood, future technologies may allow the identification of spirochetal components or a relevant autoantigen, or both in the joint synovia of these patients. Since most patients with treatment-resistant Lyme arthritis yield consistently negative PCR results after antibiotic treatment, it is possible that chronic synovitis is maintained in the absence of *B. burgdorferi*.

Vaccine Development against Lyme Disease. Considerable morbidity is associated with Lyme disease and there are areas in the United States and Europe where up to 3% of the population is infected annually (Fahrer *et al.*, 1991). Based on pharmaco-economic studies, there

is a clear need for a Lyme disease vaccine, particularly in populations where the annual risk exceeds 1% (Meltzer *et al.*, 1999, Shadick *et al.*, 2001). However, at the present time a human vaccine is not commercially available. The first human Lyme disease vaccine was an Outer surface protein A or OspA-based formulation named as LYMErix (GlaxoSmithKline); however, its tenure was short, and citing a drop in sales, it was voluntarily pulled from the market in 2002. The decline in sales could be traced to concerns, real or perceived, of possible adverse effects including a chronic inflammatory arthritis that could theoretically develop in HLA-DR4 positive patients suggesting a vaccine associated auto-immune arthritis (Kalish *et al.*, 1993). While new OspA based vaccinogens are being developed to mitigate this potential complication (Koide *et al.*, 2005, Willett *et al.*, 2004), questions remain about the viability of an OspA-based vaccine. One concern is the frequency of boosts required to maintain long term protection. OspA is expressed in the tick midgut, is rapidly down-regulated upon tick feeding, and is not expressed in mammals. The inherent problems associated with OspA-based vaccines can be avoided by use of antigens that are expressed at high levels during early mammalian infection and that elicit bactericidal antibody, such as the outer surface protein C or OspC.

OspC has received considerable attention in Lyme disease vaccine development. Its expression is induced during tick feeding and maintained during early mammalian infection (Schwan, 2003). Anti-OspC antibodies have been demonstrated to protect against infection, but only against strains that are closely related in sequence to the vaccinogen (Bockenstedt *et al.*, 1997, Gilmore *et al.*, 1996, Scheiblhofer *et al.*, 2003). Analysis of OspC sequences has delineated 21 phyletic *ospC* clusters or types that are differentiated by letter designation (A-U) (Seinost *et al.*, 1999, Wang *et al.*, 1999). Although OspC exhibits diversity, it is genetically stable during infection (Hodzic *et al.*, 2000, Stevenson *et al.*, 1994). Identification of protective epitopes of each OspC type will allow for construction of, recombinant, polyvalent, chimeric

vaccines that will elicit broad protection. Thus, a tetravalent (Earnhart *et al.*, 2007) and an octavalent (Earnhart and Marconi, 2007) vaccine based on OspC types have been developed and resulted in induction of complement-dependent bactericidal activity. Interestingly, a DNA-vaccine encoding *ospC* gene was also shown to be suitable for inducing protection against Lyme borreliosis (Scheiblhofer *et al.*, 2003).

EPIDEMIOLOGY AND TICK VECTOR LIFE CYCLE

According to the Center for Disease Control and Prevention, since 1985, Lyme disease has been the most prevalent vector-borne illness in the United States (CDC, 2000). In 2007, more than 27,000 new cases of Lyme disease were reported in the United States, yielding a national average of 9.1 cases per 100,000 persons (CDC, 2007). In 2008, State health departments reported 28,921 confirmed cases and 6,277 probable cases of Lyme disease to CDC (Figure 1.3).

The 2008 Case Definition for Lyme disease, for the purpose of surveillance, states a confirmed case as:

a) a case of EM with a known exposure. Exposure is defined as having been (less than or equal to 30 days before onset of EM) in wooded, brushy, or grassy areas (i.e., potential tick habitats) in a county in which Lyme disease is endemic. A history of tick bite is not required;

b) a case of EM with laboratory evidence of infection and without a known exposure; or

c) a case with at least one late manifestation that has laboratory evidence of infection; and a probable case as: any other case of physician-diagnosed Lyme disease that has laboratory evidence of infection. For the purposes of surveillance, the definition of a qualified laboratory assay is

(1) a positive culture for *B. burgdorferi*,

(2) two-tier testing interpreted using established criteria (CDC, 1995), or
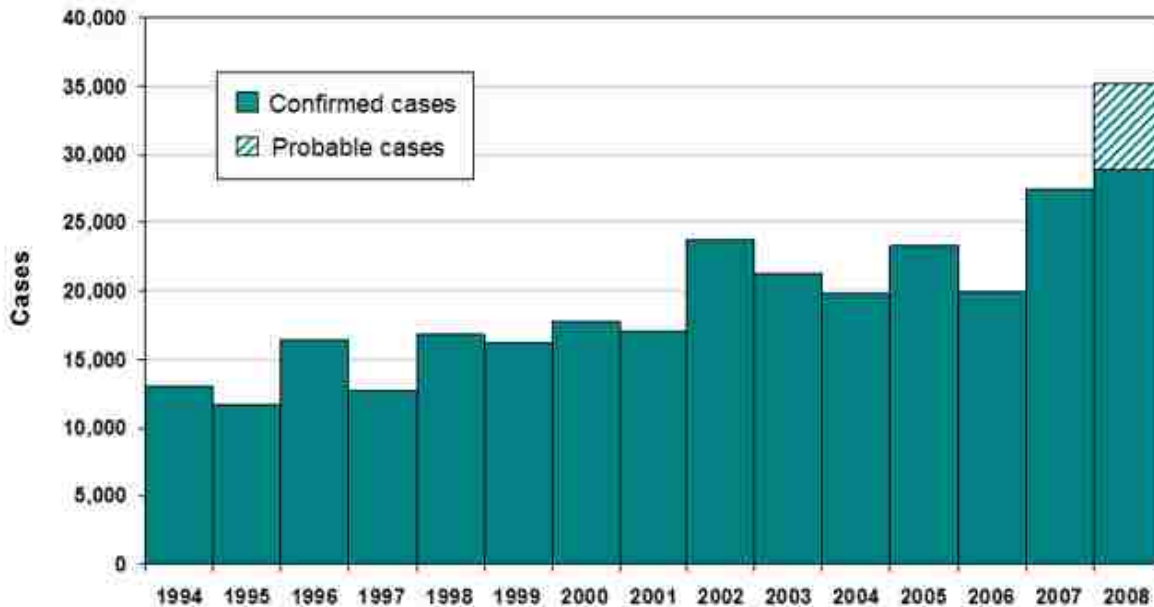
Figure 1.3. Reported cases of Lyme disease by year, United States, 1994-2008. (CDC-Division of Vector-borne Infectious Diseases-last reviewed August 2009). http://www.cdc.gov/ncidod/dvbid/lyme/ld_UpClimbLymeDis.htm. State health departments reported 28,921 confirmed cases and 6,277 probable cases of Lyme disease to CDC in 2008. This represents a 5% increase in confirmed cases compared to 2007. The definition and reporting of probable cases was initiated in 2008 based on revisions to the national surveillance case definition.

(3) single-tier IgG immunoblot seropositivity interpreted using established criteria (CDC, 2005, CDC, 1995, Dressler *et al.*, 1993, Engstrom *et al.*, 1995).

Lyme disease is endemic in several regions of the United States, particularly areas of the Northeast, upper Midwest, and northern California (CDC, 2004, CDC, 2000) (Figure 1.4). The ten states of the United States with the highest incidence rates are Connecticut, Delaware, New Hampshire, Massachusetts, Maryland, Maine, New Jersey, Pennsylvania, Minnesota, and Wisconsin.

Lyme disease is considered a zoonosis because *Borrelia burgdorferi*, the causative agent is maintained in a natural infectious cycle that does not include humans, who only inadvertently

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become infected. Transmission of *B. burgdorferi* to humans occurs by the bite of an infected tick

vector of the genus *Ixodes*, which is a hard-tick (Steere *et al.*, 1978, Wallis *et al.*, 1978). The tick

vectors vary depending on the region of the United States. The main species involved are *I.*

scapularis in northeastern and upper midwestern United States, *I. pacificus* in the far-western
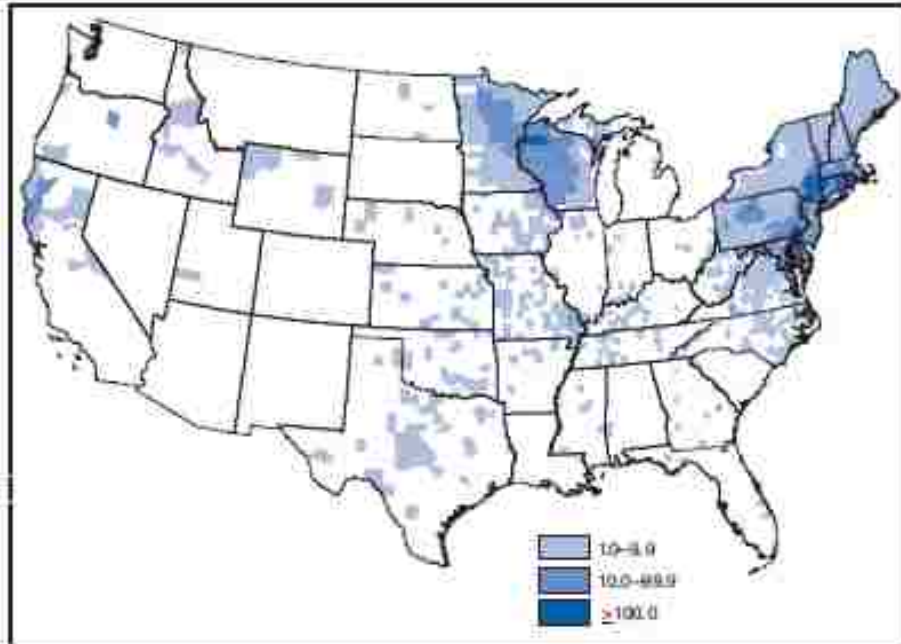
* Per 100,000 population.
† County of residence was available for 98.1% of cases reported during 1992–2006.
§ During 2003, Pennsylvania reported 4,722 confirmed cases and 1,008 suspected cases.

Figure 1.4. Average rate* of Lyme disease by County of Residence† –United States, 1992-2006. (CDC 2008). During the 15-year study period, the number of cases reported increased 101%. The highest incidence rates were found in Northeastern and North-Central States.

United States, *I. ricinus* and *I. persulcatus* in Eurasia (Lane *et al.*, 1991). In the southeastern and

western United States, immature stages of the vector ticks feed primarily on lizards (Apperson *et*

al., 1993, Durden *et al.*, 2002, Wright *et al.*, 1998). Lizards, however, rarely serve as competent

reservoir hosts for the pathogenic Lyme disease spirochetes in the USA, probably because

proteins comprising the alternative complement pathway are responsible for the borreliacidal activity observed in the blood of certain species of lizards (Kuo *et al.*, 2000).

Ixodes scapularis, also known as the blacklegged tick, was first described by Spielman *et al.,* (Spielman *et al.*, 1979) as a vector of *Babesia microti* infecting man in New England. This tick vector can also transmit *Anaplasma* the agent of human granulocytic anaplasmosis (HGA) (Dumler *et al.*, 2001). *Ixodes scapularis* Say is the same species as *I. dammini* (Cooley and Kohls, 1945) and thus has retained the older name (Oliver *et al.*, 1993). *I. scapularis*, particularly the immature stages, has a broad host range (mainly small mammals and birds) (Anderson, 1989a, Anderson, 1989b, Anderson, 1988) and the adult ticks feed on large mammals. However, two hosts stand-out as central to the survival of the tick *I. scapularis* and of the spirochete *B. burgdorferi*: the white-footed mouse, *P. leucopus* (Anderson and Magnarelli, 1980), which serves as the host for larval and nymphal ticks, and the white-tailed deer, *Odocoileus virginianus* (Bosler *et al.*, 1984, Carey *et al.*, 1980, Piesman *et al.*, 1979, Wilson *et al.*, 1990)*,* which serves as the host for adult ticks.

The tick has a 2-year life cycle and has four life stages: the egg, and 3 developmental stages: larva, nymph and adult stages (Figure 1.5). *Ixodes* ticks feed once at each of the three developmental stages. The cycle begins with eggs being laid during the spring. The larvae hatch in the summer and are usually uninfected with *B. burgdorferi*. The uninfected larval tick feeds on an infectious vertebrate (during August, September), primarily the white footed mouse, *Peromyscus leucopus* which is the natural reservoir for *B. burgdorferi*. After feeding slowly for 3 to 5 days, engorged larvae drop from the host to the ground where they overwinter. The following spring, the larvae molt into nymphs. In a similar manner, nymphs also feed for 3-5 days on mammals (during May, June), detach and drop to the forest floor, where they molt into the adult stage during the fall. After mating, the females lay eggs on the ground the following

spring before they die and the 2-year life cycle begins again. (Burgdorfer *et al.*, 1988b, Lane and Loye, 1991, Magnarelli and Anderson, 1988).

Transovarial transmission of *B. burgdorferi* does occur in ticks (Burgdorfer *et al.*, 1983, Magnarelli *et al.*, 1986, Piesman *et al.*, 1986). However, it is rare and inadequate to maintain infected populations of either the tick vector or vertebrate hosts (Schulze *et al.*, 1985). Adult ticks are infected through transtadial transmission of the spirochetes from immature stage ticks which had fed earlier in their life history on infected animals (Bosler *et al.*, 1984). It is critical that the tick feeds on the same host species (particularly rodents and chipmunks) in both of its immature stages (larval and nymphal), because the life cycle of the spirochete depends on horizontal transmission (Steere *et al.*, 2004). Studies using animals have shown that the infected nymphal ticks must remain attached for 36 to 48 hours or longer and infected adult ticks must remain attached for 48 to 72 hours or longer before the risk of transmission of *B. burgdorferi* becomes substantial (Falco *et al.*, 1996, Piesman, 1993, Piesman *et al.*, 1987, Piesman *et al.*, 1991).

Although all stages have been observed to feed on humans (Main *et al.*, 1981), the nymphal stage tick is the most likely to transmit the infection to humans. Presumably, because (1) the nymphs are smaller than the adult ticks, and thus are difficult to be identified and removed soon; (2) they become engorged quicker than the adults and engorgement is necessary before the bacteria can be transmitted; and (3) they are prevalent during spring and summer, when humans enter the habitats where ticks thrive. Interestingly, the seasonal questing (host-seeking pattern) of nymphal *I. scapularis* precedes the peak of human disease cases (Lane *et al.*, 1991).

Thus, uninfected larval ticks generally acquire *B. burgdorferi* by feeding on infectious mammals and uninfected mammals acquire *B. burgdorferi* by feeding on infected nymphal ticks.
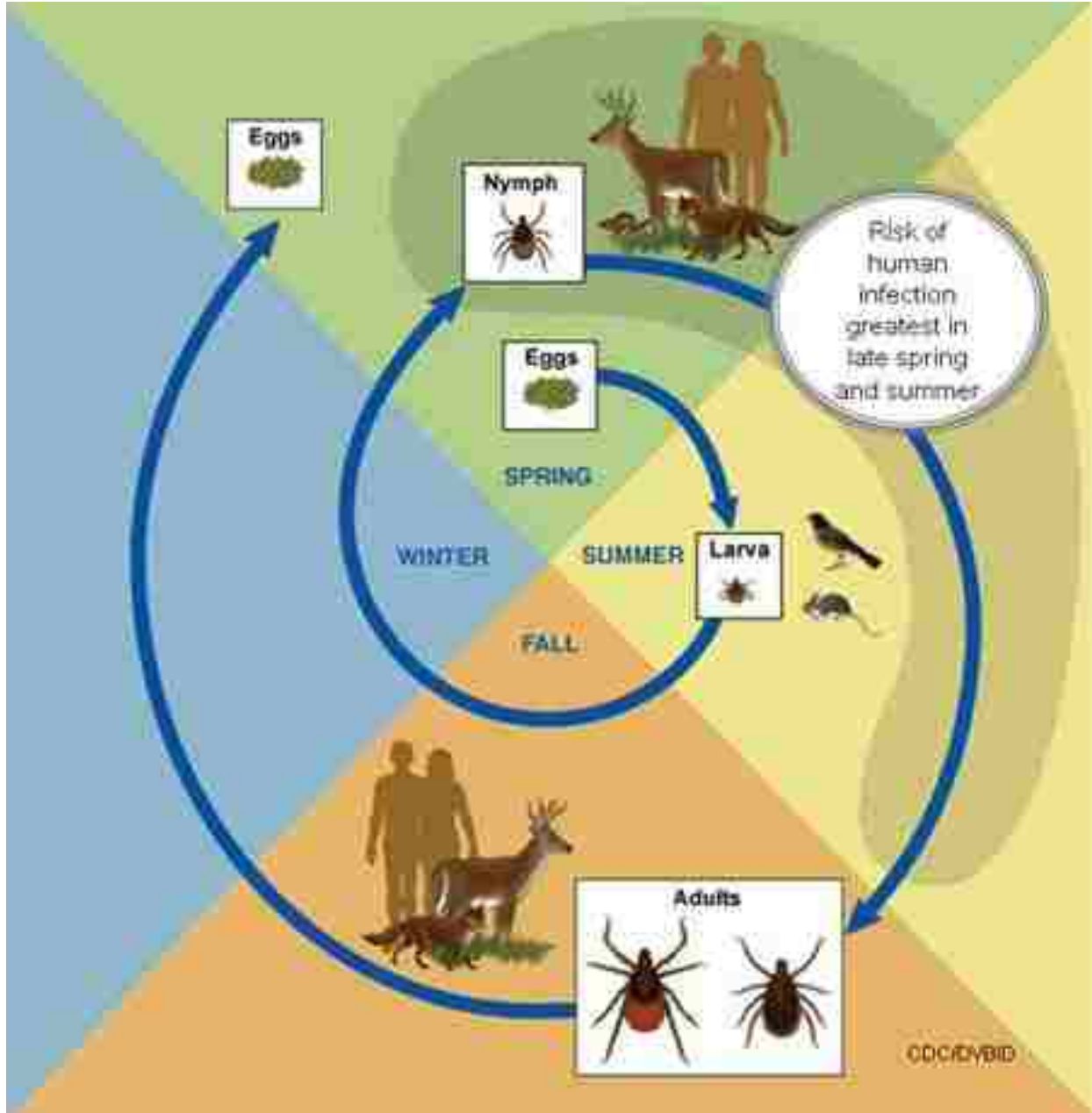
Figure 1.5. The 2 year enzootic life cycle of the Lyme disease vector. (CDC-Division of Vector-borne Infectious Diseases-last modified November 2007). http://www.cdc.gov/ncidod/dvbid/lyme/ld_transmission.htm. *Ixodes scapularis* ticks feed once at each of the three stages of their usual life cycle. Eggs are laid on the ground during the spring, larvae hatch from the eggs in the summer and take one blood meal on a small mammal or bird, molt to nymphs the following spring and feed once during late spring or early summer when the risk of human infection is greatest, and molt into adult stage during fall. After mating, the adult females feed once, usually on large mammals, and lay their eggs the following spring before they die.

Larval feeding follows nymphal feeding and thus it increases the efficiency of the enzootic cycle of *B. burgdorferi* because it ensures that juvenile rodents become infected with spirochetes (May, June) before serving as hosts to larvae (August, September), thus perpetuating the infection from year to year (Spielman *et al.*, 1985, Wilson and Spielman, 1985).

MOUSE MODEL OF LYME DISEASE

In 1988, Schaible, *et al.*, found that experimental inoculation of several inbred mouse strains with high-passage isolate of *B. burgdorferi* led to pathomorphological changes in several organs, comparable with those found in patients with Lyme disease (Schaible *et al.*, 1988). The following year, Schaible, *et al.* reported that severe combined immunodeficiency (SCID) mice with severely impaired T and B cell functions develop multisystemic disease with a preponderance for polyarthritis and carditis after inoculation with tick isolate of *B. burgdorferi* (Schaible *et al.*, 1989). Although natural rodent hosts and most laboratory mice can have persistent *B. burgdorferi* infections without signs of disease, some inbred mice develop joint and heart pathologies that resemble human infection of Lyme disease (Barthold *et al.*, 1990). Thus, the mouse serves as a useful model for Lyme disease.

In a mouse model of infection, *B. burgdorferi* initially establishes a localized infection at the site of the tick bite, transiently disseminates via the bloodstream to various tissues, including skin, joints, heart and bladder (Barthold *et al.*, 1991). In addition to mice, other experimentally infected animals include rats, hamsters, gerbils, rabbits, dogs, and monkeys (Kornblatt *et al.*, 1984, Kornblatt *et al.*, 1985, Mursic *et al.*, 1990, Piesman *et al.*, 1987). Significantly, infected primates can develop infection of the central nervous system (CNS) and neurological manifestations that are not common in infected rodents (Philipp and Johnson, 1994). In 2000, a study showed for the first time, evidence of induced infection in ponies by exposure to adult ticks infected with *B. burgdorferi* (Chang *et al.*, 2000).

BORRELIA BURGDORFERI

Taxonomy. Spirochetes are a phylogenetically ancient group of eubacteria (Paster *et al.*, 1984). The phylum Spirochetes is composed of a single class (Spirochetes) and order (Spirochetales). The order is divided into three families: Brachyspiraceae, Leptospiraceae, Spirochaetaceae (Paster and Dewhirst, 2000). Within the family Spirochaetaceae is the genus *Borrelia*. Of the 37 known *Borrelia* species, 12 can cause Lyme disease (LD) transmitted by ticks (Hengge *et al.*, 2003) (Figure 1.6).

Spirochaetes (phylum)
Spirochaetes (class)
Spirochaetales (order)
Brachyspiraceae (family)
Brachyspira (genus)
Leptospiraceae
Leptonema
Leptospira
Spirochaetaceae
Borrelia
Brevinema
Cristispira
Spirochaeta
Spironema
Treponema
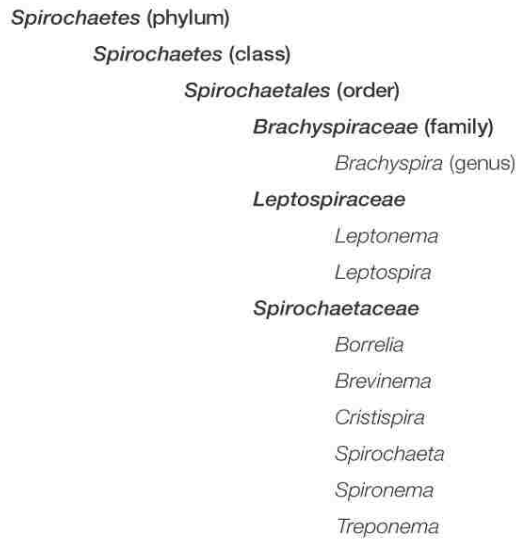
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Figure 1.6. Phylogeny of Spirochetes. (Rosa *et al.*, 2005). Members of the phylum Spirochaetes are easily identified by their unique spiral morphology and periplasmic flagella. The phylum is composed of a single class and order, divided into three families. Spirochetes include the genera Leptospira, Treponema and Borrelia, which all contain species pathogenic for the human host. *Borrelia* is the only known member of the phylum that contains linear DNA molecules.

Since *Borrelia burgdorferi* was first isolated in 1982 and identified as a new species of the genus *Borrelia* in 1984, hundreds of *B. burgdorferi* isolates have been cultured worldwide. Molecular analysis has indicated that these isolates are genetically and phenotypically divergent. A closely related cluster containing at least 10 tick-borne *Borrelia* species associated with Lyme Borreliosis has been defined (Baranton *et al.*, 1992, Canica *et al.*, 1993, Fukunaga *et al.*, 1996,

Kawabata *et al.*, 1993, Marconi *et al.*, 1995, Postic *et al.*, 1998, Wang *et al.*, 1997). The term "*Borrelia burgdorferi sensu lato*" is now collectively used for all *Borrelia* isolates within this cluster and to distinguish it from the species "*Borrelia burgdorferi sensu strictu*" (strict sense of *Borrelia burgdorferi*) (Baranton *et al.*, 1992). Hence, the *Borrelia burgdorferi sensu lato* complex includes species such as *B. burgdorferi sensu strictu*, *B. afzelii, B. garinii,* and *B. japonica*.

Biology of *B. burgdorferi.* Borreliae belong to the spirochete Family and as such have in common with other spirochetes the following structural characteristics: 1) helical shape and motile; 2) a double-membrane: an outer membrane, which surrounds the protoplasmic cylinder complex, consisting of the cytoplasm, the peptidoglycan complex, and the inner membrane; 3) flagella, located in the periplasmic space between the outer membrane and the protoplasmic cylinder (Barbour and Hayes, 1986, Holt, 1978, Johnson, 1977) (Figure 1.7).

In terms of size, borreliae are 10-30 μm long and 0.2-.3μm wide; the 7 to 11 periplasmic flagella give the bacteria its spiral shape (Burgdorfer *et al.*, 1982, Hovind-Hougen *et al.*, 1986). Borreliae are extracellular, fastidious bacteria requiring a rich growth medium. Dr. Alan Barbour first grew *B. burgdorferi* (strain B31) in BSK (Barbour-Stoenner-Kelly) medium (Burgdorfer *et al.*, 1982). The spirochete is grown at temperatures between 30 and 37 °C in the laboratory in microaerophilic conditions (Kelly, 1971), hence, it is usually grown in capped tubes with limited air space. The generation time for *Borrelia* is 8 to 24 h and culture adapted strains achieve cell densities of about 10^8/ml (Barbour, 1984).

Although *B. burgdorferi* contains a double-membrane similar to enteric Gram-negative bacteria, it has several distinct features (Figure 1.8).These features include: an extraordinary abundance of membrane proteins covalently linked with lipids also known as lipoproteins (Bergstrom *et al.*, 1989, Brandt *et al.*, 1990); the absence of lipopolysaccharide (LPS)

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Figure 1.7. Schematic representation of a spirochete. (Canale-Parola, 1977). The broken line indicates the outer membrane. The area delimited by the thick solid line represents the protoplasmic cylinder. The circles near the ends of the protoplasmic cylinder indicate the insertion points of the periplasmic flagella. The thin solid lines denote periplasmic flagella.

(Takayama *et al.*, 1987) and phosphatidylethanolamine (Belisle *et al.*, 1994); the presence of glycolipids other than LPS (Belisle *et al.*, 1994, Eiffert *et al.*, 1991) and an outer membrane which contains a relatively low density of transmembrane proteins (Radolf *et al.*, 1994).

Genome. The genome of *B. burgdorferi* sensu strictu strain B31 was completed in 1997 (Fraser *et al.*, 1997). The genome size is relatively small, about 1.5 MB. One of the most striking features of *B. burgdorferi* is its unusual genome, which includes a linear chromosome of approximately 910 kilobases (kb) (Baril *et al.*, 1989, Davidson *et al.*, 1992), and 12 linear and 9 circular plasmids that total 610kb (Figure 1.9). The linear plasmids (lp) and circular plasmids (cp) are numbered according to their sizes in kilo base pairs. The isolate B31 contains the following linear plasmids: lp5, lp17, lp21, lp25, four different homologous plasmids of 28 kbp (lp28-1, lp28-2, lp28-3, and lp28-4), lp36, lp38, lp54, and lp56; and the following circular plasmids: cp9, cp26 and six or seven homologous plasmids of 32 kbp (cp32-1, cp32-3, cp32-4, cp32-6, cp32-7, cp32-8 and cp32-9) (Fraser *et al.*, 1997). Comparisons with other B31 cultures suggest that this isolate may have lost one linear plasmid (lp21) and one or two 32 kbp circular plasmids during growth in culture since its original isolation (Barbour, 1988, Barbour and Garon, 1987, Norris *et al.*, 1995). Eight plasmids: lp5, lp21, lp28-1, lp28-2, lp28-4, lp56, cp9 and cp32-
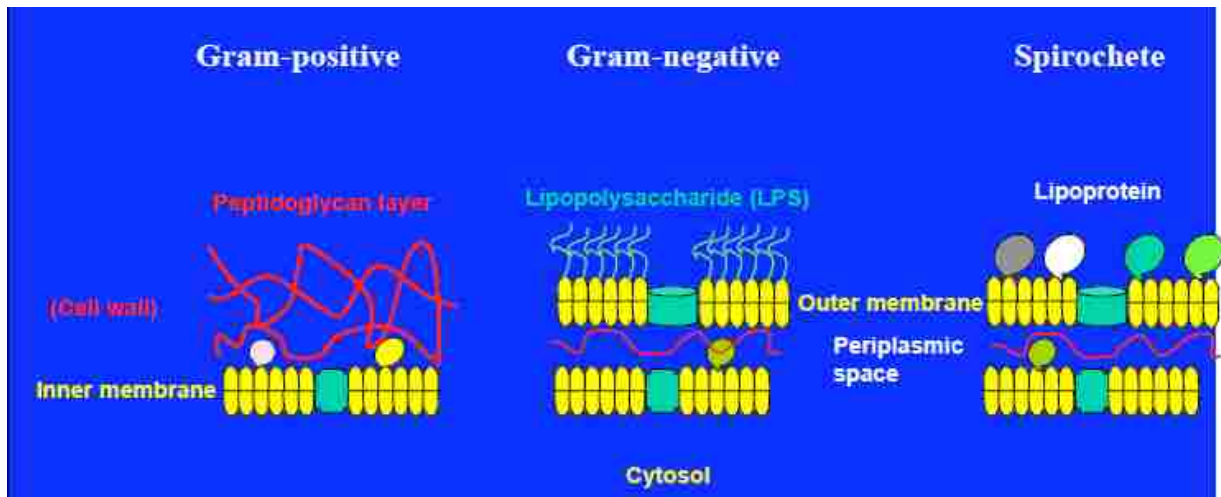
Figure 1.8. Membrane structure of spirochetes compared to that of Gram-negative and Gram-positive bacteria. (Courtesy of Dr. Fang-ting Liang). All three types of bacteria contain an inner membrane. Whereas Gram-positive bacteria do not contain an outer membrane and instead contain an abundance of peptidoglycans, both Gram-negative bacteria and spirochetes contain an inner and an outer membrane. The difference between the last two is the presence of lipopolysaccharides on the outer surface of Gram-negative bacteria and the absence thereof in spirochetes. Instead, spirochetes have an abundance of lipoproteins.

3, tend to be spontaneously lost during in vitro propagation (Purser and Norris, 2000). The overall G+C contents of the B31 plasmids vary from 20.7% to 31.6% (Casjens, 2000).

Some of the most unusual aspects of the *Borrelia* genome are the presence of (1) linear replicons; (2) more than 20 replicons in a single bacterium; and (3) large tracts of directly repeated short DNA sequences. Approximately 5% of *B. burgdorferi* chromosomal genes and 5% of plasmid genes are de voted to encoding more than 130 lipoproteins (Casjens, 2000). These percentages are larger than any for lipoproteins encoded in a sequenced bacterial genome (Templeton, 2004).

Lipoproteins. Most of the outer surface proteins (osps) that have been characterized in *Borrelia* are lipoproteins (Rosa, 1997). Orthologues of the genes encoding the essential components of the bacterial secretory machinery and homologues of the three enzymes required
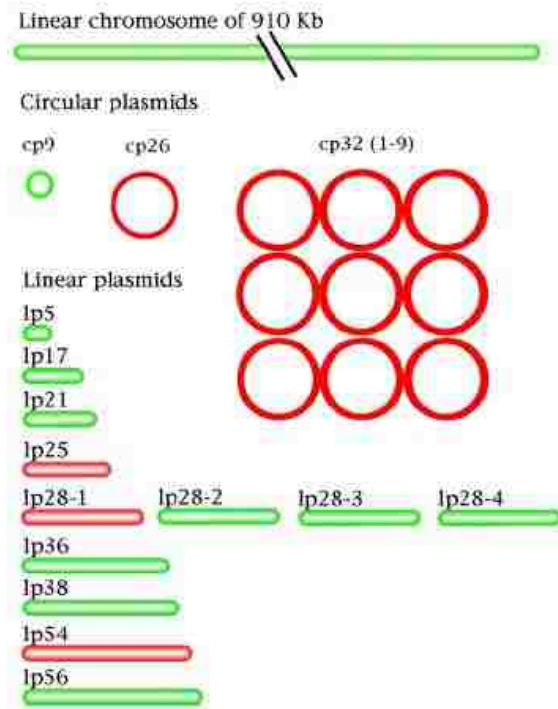
Figure 1.9. The segmented genome of *B.burgdorferi*. *Borrelia* is a unique bacterium that contains one linear chromosome and up to 21 extrachromosomal elements. (Stewart *et al.*, 2005). Linear plasmids are abbreviated lp and circular plasmids cp, the number represents the approximate size of the plasmid in kilobases. Evidence supports that plasmids shown in red are required for infectivity or persistence in the tick or vertebrate host.

for biosynthesis of lipoproteins were found when sequencing the *B. burgdorferi* genome (Fraser *et al.*, 1997, Haake, 2000).

Lipoproteins are synthesized as precursors in the cytoplasm and then translocated across the inner membrane by a preprotein translocase complex, in an energy-dependent cycle. Subsequent modifications take place sequentially leading to the formation of mature lipoproteins having a lipid-modified cystein at the N-terminus (Hayashi and Wu, 1990, Pugsley, 1993). Thus, the characteristic feature of all lipoproteins is a signal sequence in the N-terminal end, followed by a cystein (Hayashi and Wu, 1990). Lipoprotein processing is similar to that in *E.coli*. The first of the three enzymes in the pathway is phosphadidyl-glycerol: prolipoprotein diacylglyceryl transferase (Lgt). This enzyme transfers a diacylglyceryl group (containing two fatty acids) from

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phosphatidylglycerol to the sulfur atom of cystein. The second enzyme in the pathway is

prolipoprotein signal peptidase (Lsp), which proteolytically removes the signal peptide, making

cystein the new N-terminal amino acid. The third enzyme is phospholipid:apolipoprotein

transacylase (Lnt) which transfers a third fatty acid from a membrane phospholipid to the

nitrogen atom of cystein. After processing, lipoproteins will have three fatty acids attached to the

cystein residue (Wu, 1996). Figure 1.10 summarizes the biosynthesis of lipoproteins.

Among the various lipoproteins of *B. burgdorferi* described are the outer surface proteins

OspA, which was the first Osp identified in culture (Barbour *et al.*, 1983, Bergstrom *et al.*,

1989), OspB (Bergstrom *et al.*, 1989, Howe *et al.*, 1985), OspC (Fuchs *et al.*, 1992, Wilske *et al.*,

1993), OspD (Norris *et al.*, 1992), OspE (Lam *et al.*, 1994), and OspF (Lam *et al.*, 1994).

Coordinated Expression of OspA and OspC. The spirochetes are transmitted to small

mammals, mainly rodents, through the bite of *Ixodes* ticks (Burgdorfer *et al.*, 1982). In unfed

ticks, the spirochetes remain in the tick midgut. During tick feeding, which lasts several days,

bacteria migrate from the tick midgut to the salivary glands, from which they are transmitted

through the saliva to the dermis of the mammalian host (Ribeiro, 1987). Studies have shown that

spirochetes in unfed ticks have OspA, but no OspC; yet, immediately after a blood meal, a

significant number of spirochetes are stained positive for OspC and negative for OspA (Schwan

et al., 1995). This switch from OspA to OspC accompanies a change in location of the

spirochetes within the tick, from the tick midgut to the salivary glands (de Silva *et al.*, 1997,

Schwan *et al.*, 1995). Figure 1.11 summarizes the changes in the expression of the surface

lipoproteins, especially OspA and OspC as *Borrelia* transits between the tick vector and the

mammalian host. Aspects of the midgut environment that change during tick feeding, such as

temperature, pH, and nutrients, influence the expression of many genes, including *ospA* and

ospC (Carroll *et al.*, 1999, Ojaimi *et al.*, 2003, Revel *et al.*, 2002, Schwan *et al.*, 1995).
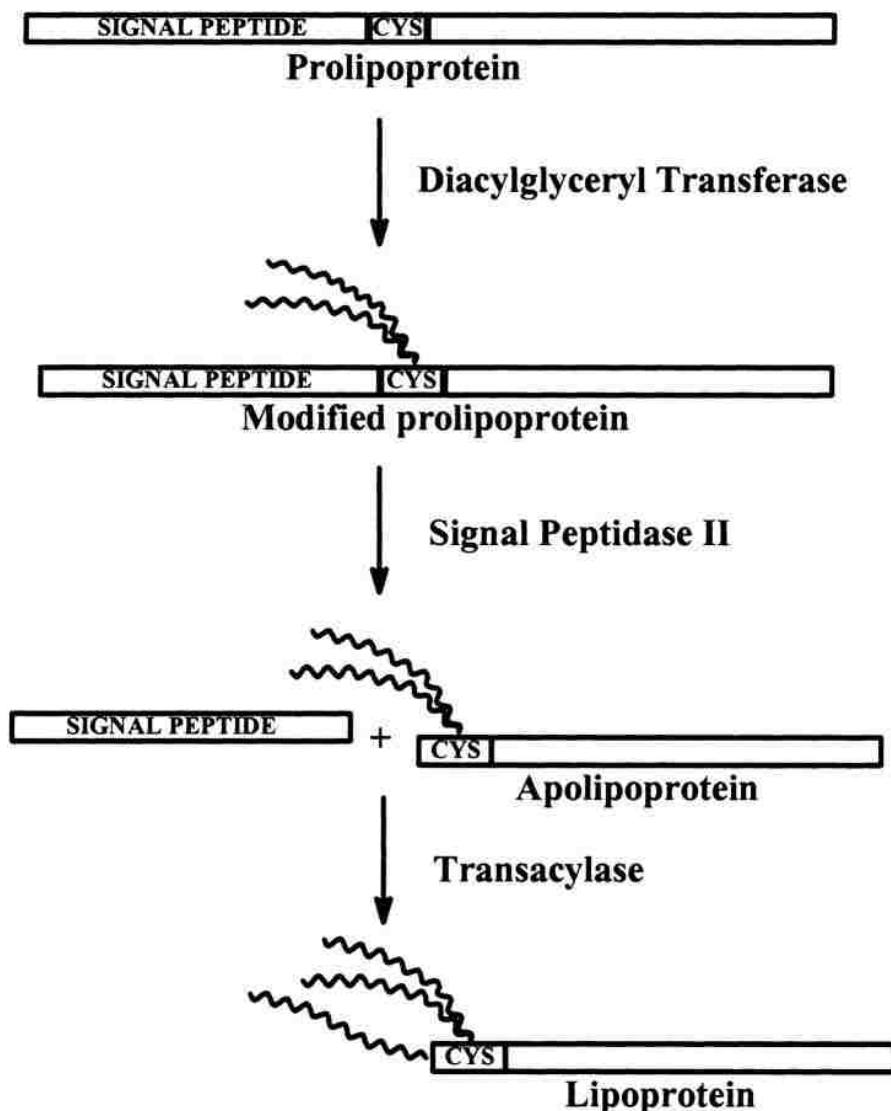
Figure 1.10. Biosynthesis of lipoproteins. (Juncker *et al.*, 2003). Shown here are the transfer of a diacylglyceride to the cystein sulphydryl group of the unmodified prolipoprotein; cleavage of the signal peptide by signal peptidase II, forming an apolipoprotein; and finally, acylation of the α-amino group of the N-terminal cystein of the apolipoprotein. Lipids are attached to cystein. Peptides are shown to the left and to the right of the cystein residue. Catalytic enzymes are written beside reaction arrows.

According to some findings, a tick midgut receptor for OspA which has been identified and has

an acronym as TROSPA was shown to be required for spirochetal colonization of *I. scapularis*

(Pal *et al.*, 2004a). On the other hand, Salp 15 a tick salivary gland ligand was shown to be a

ligand for both OspC and the CD4 molecule of CD4+ T cells (Garg *et al.*, 2006, Ramamoorthi *et al.*, 2005).

OspC Groups and Invasiveness. The *ospC* gene has a high degree of sequence variability (Jauris-Heipke *et al.*, 1995, Theisen *et al.*, 1993, Wilske *et al.*, 1995). On the basis of *ospC* sequence analysis of *ospC* alleles collected from a single site on Shelter Island, NY, Wang *et al.*, (Wang *et al.*, 1999) defined 19 major *ospC* groups or types (from A to S, with divergence of < 2% within a group and > 8% between groups) for *B. burgdorferi sensu strictu* strain. Two new groups (groups T and U) were described by Seinost, *et al.*, 1999 (Seinost *et al.*, 1999). Of the 21 major groups, only four (A, B, I and K) contain invasive clones and therefore cause infections of the skin (or primary site) and extra-cutaneous sites, also known as secondary sites (such as heart, joint, and nervous system), while the others are non-human pathogens, or infect the skin only (Seinost *et al.*, 1999). The *ospC* gene could, therefore, be one of the determinants involved in the invasiveness of *Borrelia* strains leading to disseminated forms of the disease. The *B. burgdorferi sensu strictu* strain B31 belongs to the *ospC* invasive group A.

Regulation of *ospC* Gene Expression. *Borrelia burgdorferi* has an enzootic life cycle alternating between *Ixodes* ticks and mammalian hosts (mice or deer) (Lane *et al.*, 1991). Therefore, the spirochete must transition between, adapt to and survive in the tick and mammalian host, which are two vastly different environments. Similar to many other pathogenic bacteria, *B. burgdorferi* senses and responds to environmental cues by regulating the synthesis of various proteins. These cues include changes in temperature, pH, cell density, oxygen and/or exposure to host factors (Carroll *et al.*, 1999, Indest and Philipp, 2000, Indest *et al.*, 1997, Ojaimi *et al.*, 2002, Ramamoorthy and Philipp, 1998, Schwan and Piesman, 2000, Schwan *et al.*, 1995, Seshu *et al.*, 2004, Stevenson *et al.*, 1995).
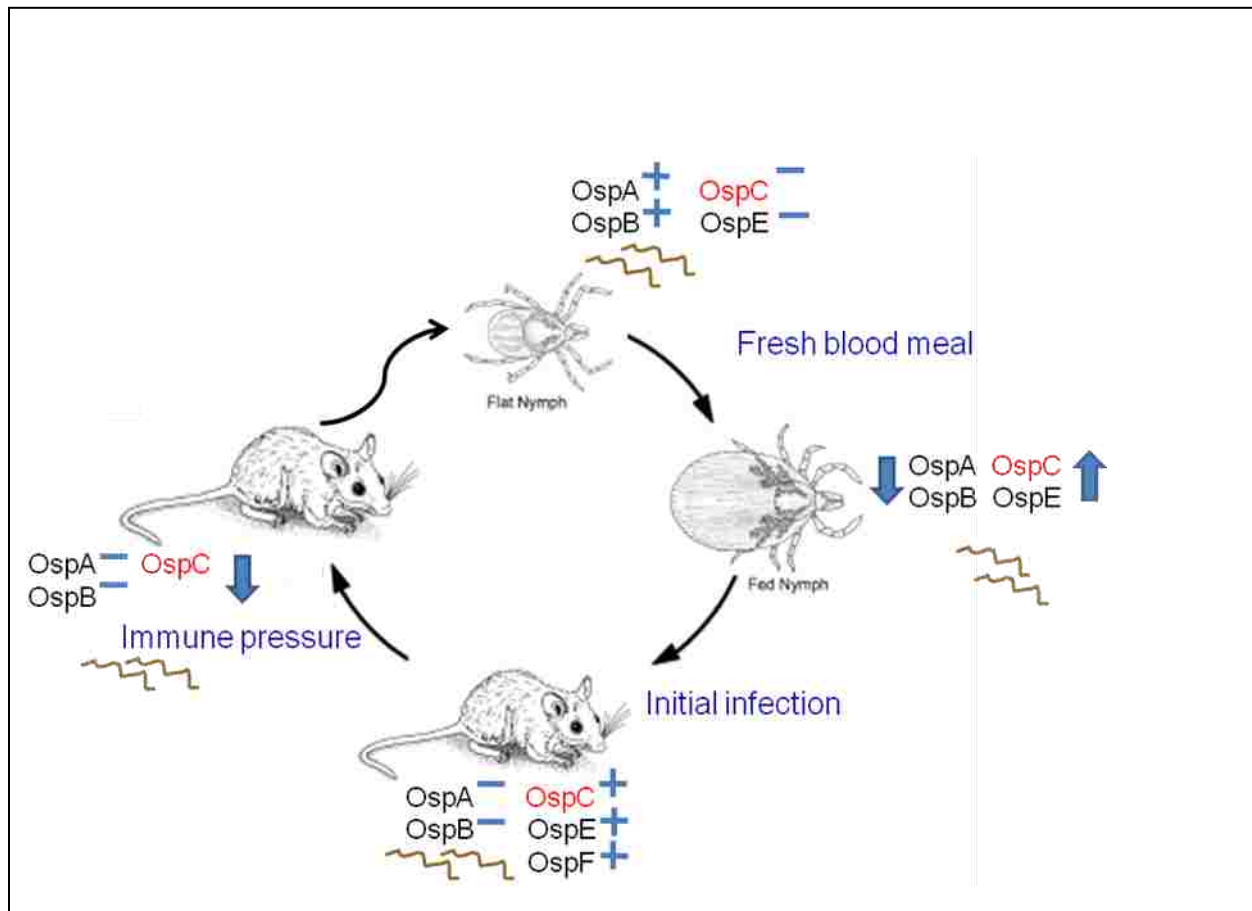
Figure 1.11. Coordinated expression of OspA and OspC as *Borrelia* cycles between the tick vector and the mammalian host. (Mulay *et al.*, 2007). In the flat tick, *Borrelia* expresses high levels of OspA and does not express OspC (shown in red). A fresh blood meal changes the microenvironment in the tick midgut inducing the downregulation of OspA and the upregulation of OspC, preparing the bacteria to infect a mammalian host. This switch of OspA to OspC coincides with the migration of the bacteria from the tick midgut to the salivary glands. Within the host, *Borrelia* abundantly produces OspC. However, in order to avoid clearance by specific antibody response, *Borrelia* downregulates OspC. Once *Borrelia* enters a naïve tick, it upregulates OspA which remains upregulated in the flat tick. Figure was modified from its original version.

For example, *B. burgdorferi* alters its Osps with changes in temperature. During *in vitro* growth, a temperature shift from 24 °C to 37 °C stimulates the spirochetes to synthesize OspC, whereas decreasing the temperature back to 24 °C caused OspC to be decreased to below detectable levels (Schwan *et al.*, 1995). Also, high spirochete density at least *in vitro* influences the upregulation of OspC (Indest *et al.*, 1997, Ramamoorthy and Philipp, 1998). *In vitro*, the

expression of OspC is thus phase-dependent. Finally, although *B. burgdorferi* produces OspC at pH 6.0 and 7.0, it does not at pH 8.0 (Carroll *et al.*, 1999).

Recent advances in genetic manipulation of *B. burgdorferi* have culminated in the discovery of the first genetic regulatory network, the RpoN-RpoS pathway (Caimano *et al.*, 2004, Yang *et al.*, 2003b). In this pathway, the response regulator, Rrp2, functions as an enhancer binding protein (EBP), along with the alternative sigma factor RpoN (σ^N), to control the expression of another alternative sigma factor, RpoS (σ^S). RpoS, in turn regulates the expression of OspC, and other lipoproteins, such as DbpA and Mlp family of lipoproteins (Yang *et al.*, 2000, Yang *et al.*, 2003b). In *B. burgdorferi*, interestingly, the regulation of the *ospC* gene expression involves not only the RpoN-RpoS signaling pathway but also DNA supercoiling (Alverson *et al.*, 2003). The discovery of the RpoN-RpoS regulatory network prompts an important question concerning how σ^S induces the expression of o*spC*.

Mechanisms governing *in vitro OspC* regulation may be controlled solely by an RpoS-dependent promoter (Eggers *et al.*, 2004, Yang *et al.*, 2005). In the mammalian host, it would be unfeasible for *B. burgdorferi* to turn off *rpoS* to achieve selective OspC downregulation. Two sets of conserved inverted repeats (IRs) of 20 bp have been proposed to be candidate binding sites for a potential transactivator (Fraser *et al.*, 1997). Xu *et al*., (Xu *et al.*, 2007) have identified the operator region of OspC immediately upstream of the promoter region. To negatively regulate its controlled gene, the operator must interact with an as yet unidentified regulatory protein(s) called a repressor.

Characteristics of OspC. The *ospC* gene (named as BBB19 on the genome) is located on the circular plasmid cp26 which is ubiquitously present in *B. burgdorferi* isolates (Casjens *et al.*, 2000, Tilly *et al.*, 1997). *B. burgdorferi* exhibits tissue-specific *ospC* gene expression during mammalian infection (Narasimhan *et al.*, 2003). The *ospC* gene is more abundantly expressed in

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both heart and skin than in the joints in the absence of immune pressure (Liang *et al.*, 2004b). The expression of *ospC* gene is also dependent on the present or absence of adaptive immune pressure. The spirochete persistently expresses OspC during infection of severe combined immunodeficient (SCID) mice (Liang *et al.*, 2002a, Liang *et al.*, 2002b). The development of OspC antibody, however, preferentially selects for spirochetes that do not abundantly express OspC in wild type mice. It is likely that antibodies against OspC antigens exert an immune pressure on spirochetes that express OspC (Liang *et al.*, 2004a).

Of all the Outer surface proteins, OspC is one of the well-characterized lipoproteins in *B. burgdorferi*. OspC is a basic protein (with an isoelectric point of ~ 9.0) and has a subunit molecular weight of 22 kDa (Fuchs *et al.*, 1992). The full length lipoprotein is 220 amino acids long. The signal peptide is 18-AA long, the N-terminal linker is 23-AA long, and the C terminal stretch (CTS) is 13-AA long. Compared to the N-terminal linker, the C-terminal stretch is conserved among borrelial isolates and highly immunogenic (Mathiesen *et al.*, 1998). The lipid moiety of OspC is highly immunogenic; the lipoprotein is a strong innate agonist which interacts with toll-like receptor TLR2 (Aliprantis *et al.*, 1999, Brightbill *et al.*, 1999, Hirschfeld *et al.*, 1999).

Crystal Structure of OspC. The crystal structure of OspC was resolved in 2001 by two different scientific groups (Eicken *et al.*, 2001, Kumaran *et al.*, 2001) (Figure 1.12). Whereas Kumaran *et al*., used the *B. burgdorferi* strains HB19 (*ospC* group I) and B31 (*ospC* group A), Eicken *et al*., used strain N40 (*ospC* group E) to study OspC structure (Seinost *et al.*, 1999). The OspC monomer is predominantly an up and down alpha helical bundle. It is composed of five parallel α-helices and two short β-strands. The α-helices consist of α1 (residues 43-74), α2 (residues 94-112), α3 (residues 119-144), α4 (residues 151-154), and α5 (residues 169-196). The two β-strands, β1 (residues 78-82) and β2 (residues 85-89) connect helices α1 and α2. The long

helices α1 and α5 are closely packed together such that the N and C termini, which define the membrane proximal end of both subunits, are in close proximity to each other. It is suggested that the biologically functional OspC molecule may be a dimer with a characteristic central four-helical bundle formed by the association of the two longest helices of each subunit, namely α helix 1 and α helix 5 (Eicken *et al.*, 2001, Kumaran *et al.*, 2001). Both the N- and the C-terminal regions of the protein are quite flexible and unable to produce crystals. Neither the N terminal linker nor the C terminal stretch contributes to the core structure of OspC. The N- and C-termini are on the same side of the molecule and are close to each other, suggesting that the C-terminus is also close to the membrane surface on which the lipidated N-terminus is anchored (Kumaran *et al.*, 2001). The topology diagram of the OspC monomer is shown in Figure 1.13.

All helices, except for α4 and α5, are anti-parallel. The dimeric interaction is mostly between helices α1 and α1' (of each monomer), which are parallel. The interaction in the dimeric interface is almost completely hydrophobic (Kumaran *et al.*, 2001). The surface that projects away from the membrane has a strong negative electrostatic potential. It also has two 50 Å cavities ~8 Å below the exposed surface. Thus the pocket formed by these cavities may bind an as yet unknown ligand of OspC. A Dali server (network service for comparing protein structures in 3D) search (Holm and Sander, 1996) indicates that OspC has similarity to the periplasmic domain of the *Salmonella* aspartate receptor (AR), which is also a dimer (Yeh *et al.*, 1996).

Functions of OspC. The structural similarity between the aspartate receptor (AR) of *Salmonella* and OspC HB19 suggests that OspC may possibly be a binding protein. If OspC is a binding-protein, the region that projects away from the membrane may be involved in the binding (Kumaran *et al.*, 2001). As an extracellular bacterium, *Borrelia* has a predilection for extracellular matrix (ECM) and connective tissue. Although some lipoproteins are known to bind ECM components such as the Decorin binding protein (DbpA) which binds to the proteoglycan

decorin (Guo *et al.*, 1995), no such role has been attributed to OspC yet. A better understanding of the function of OspC began with a better understanding of the requirements for OspC by *Borrelia*.

In 2003, Schwan T.G., stated that *B. burgdorferi* probably requires OspC for initial infection of mammals but that no definitive proof of this hypothesis existed (Schwan, 2003). The analysis of OspC knock-out mutants has demonstrated that the OspC protein is required for the spirochete to establish infection in a mammal, following either a tick bite or needle inoculation (Grimm *et al.*, 2004, Stewart *et al.*, 2006, Tilly *et al.*, 2007, Tilly *et al.*, 2006).

Concerning the role of OspC in tick colonization, while Grimm *et al*., March 2004 (PNAS) of Patricia Rosa group, concluded that OspC is not required for migration in the tick (from the *I. scapularis* tick midgut to the salivary glands), Utpal Pal *et al*., January 2004 (JCI) of Erol Fikrig group, concluded that OspC is required for migration of *B. burgdorferi* in the tick (Pal *et al.*, 2004b). The discrepancy between the two studies may be due to the fact that the two groups used different types of OspC knock-out mutants. As proof of principle, the Patricia Rosa group (Tilly *et al.*, 2006) constructed a complete deletion of OspC, and was able to confirm that OspC is not required for migration of spirochetes from tick midguts to salivary glands. It was however, shown to be required for mammalian infection. Furthermore, the study concluded that OspC is required only during an early period (first 28 days post-inoculation) in mammalian infection.

OspC is the only surface lipoprotein to be essential for mammalian infection (Grimm *et al.*, 2004, Stewart *et al.*, 2006, Tilly *et al.*, 2007, Tilly *et al.*, 2006). Rosa and colleagues had hypothesized that OspC is required for evasion of innate immunity during initial mammalian infection. One pertinent study by Tilly, *et al.*, showed that OspC-knockout mutants were cleared
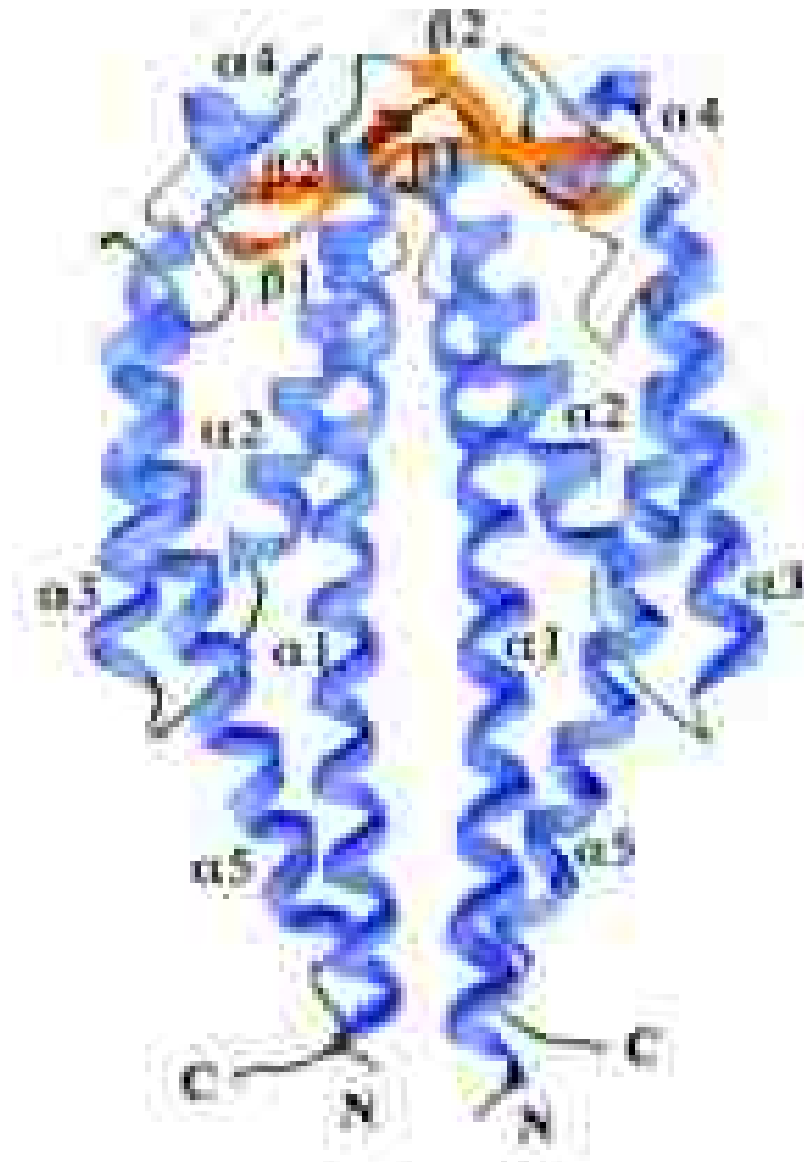
Figure 1.12. Crystal Structure of OspC. (Kumaran *et al.*, 2001). A ribbon representation of the OspC dimer is shown. The core structure of OspC is predominantly α-helical. It contains five parallel α helices and two short β sheets. The α helices pack closely together such that the N and C termini are in close proximity to each other. These termini are quite flexible and unable to produce crystals. The membrane distal end is towards the β sheets whereas the membrane proximal end is defined by the N and C termini. The lipoprotein is anchored to the outer surface of the outer membrane of *B. burgdorferi* by the N-terminus.
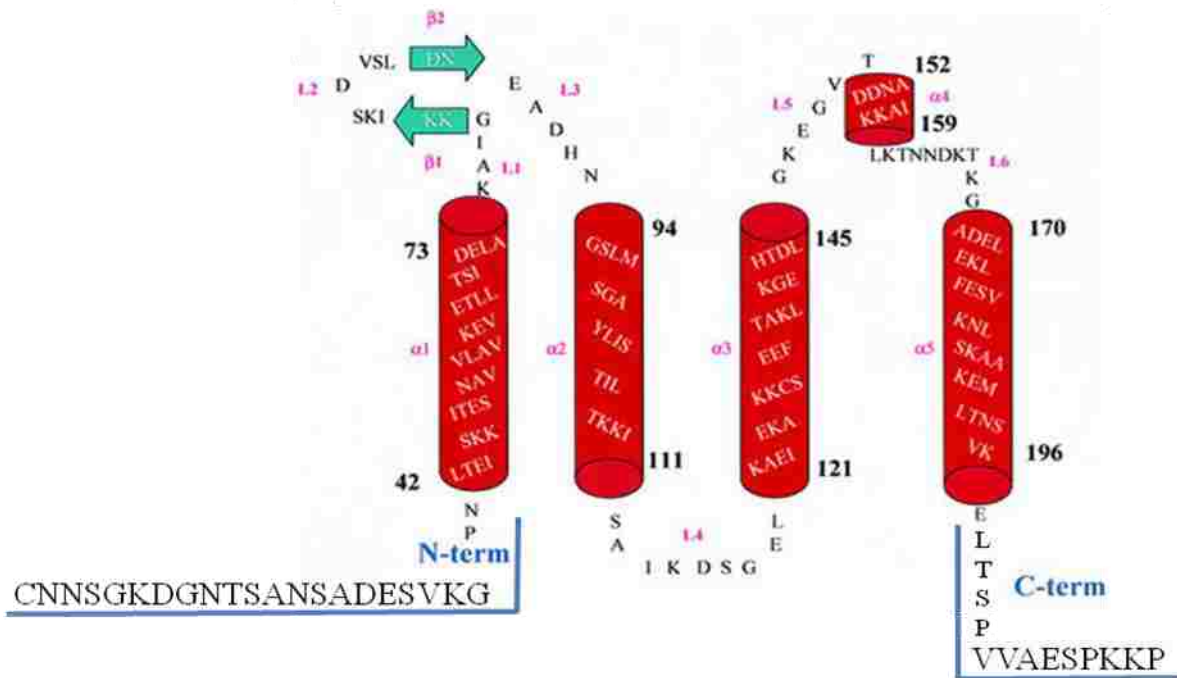
Figure 1.13. Topology diagram of OspC-HB19 monomer. (Kumaran *et al.*, 2001). Red cylinders represent α-helices and green arrows β-sheets. The N terminus amino acids were added to represent the peptide sequence from the lipidated cystein. The C-terminus aminoacids were added to represent the complete peptide sequence (Figure was modified from its original version).

within 48h from C3H wild type mice (Tilly *et al.*, 2007). However, Xu., *et al*, 2008 (Xu *et al.*, 2008) using OspC-deficient *B. burgdorferi,* which were modified to increase expression of other well-defined surface lipoproteins (such as OspA, OspE, VlsE and DbpA), were able to show that the essential protective role of OspC against early elimination can be overridden by increasing the expression of any of the four lipoproteins. Thus, although during natural infection OspC is exclusively required for initial infection of a mammalian host, on a laboratory experimental basis, it is not so. Heterologous lipoproteins can substitute for the function of OspC when overexpressed, suggesting that an abundance of lipoprotein(s) is needed to stabilize the borrelial outer membrane against innate defences. Furthermore, the study showed that OspC is required for efficient dissemination and this function can be substituted to varying extents by other outer

surface lipoproteins; OspC was also shown not to be required for the efficient colonization of the joint or skin, but for the heart tissues of SCID mice. Hence, the following functions for OspC have been identified so far: evasion of innate immunity and facilitating dissemination in the vertebrate host.

Recently, a study by the P. Rosa group (Tilly *et al.*, 2009), has shown that mammalian host-adapted spirochetes can infect and disseminate in mice in the absence of OspC. They proposed a model in which OspC is one of a succession of functionally equivalent, essential proteins that are synthesized at different stages of mammalian infection.

STATEMENT OF THE PROBLEM

More than three decades have elapsed since the discovery of the tick-borne illness named Lyme disease in 1975. Since then, much has been learnt about the life cycle of the tick vector, *I. scapularis*, and the molecular changes that *Borrelia,* the agent of Lyme disease, undergoes in order to survive in two very disparate environments, the tick vector and the mammalian host (Burgdorfer *et al.*, 1988a, de Silva and Fikrig, 1997, Lane and Loye, 1991, Schwan *et al.*, 1995). Of the molecular changes that *Borrelia* undergoes, the up- and down-regulation of its many lipoproteins, both during its transmission between the tick vector and the mammalian host and within the mammalian host itself, are obviously crucial for its survival and maximal virulence (de Silva *et al.*, 1997, Schwan *et al.*, 1995). OspC is a known virulence factor of *B. burgdorferi.* Since the upregulation of OspC within the tick vector is required for initial mammalian infection (Schwan *et al.*, 1995, Tilly *et al.*, 2006, Yang *et al.*, 2000) whereas its downregulation is required for avoidance of spirochetal clearance (Liang *et al.*, 2002a, Xu *et al.*, 2007, Xu *et al.*, 2006), many researchers are focused on the function and regulation of OspC in order to better understand the role of OspC in the pathogenesis of *B. burgdorferi*.

Recent studies have shown that a common role of all surface lipoproteins, including OspC, is that of evasion from host innate immune defences (Tilly *et al.*, 2007, Xu *et al.*, 2008) and that the unique role of OspC is that of a dissemination-facilitating factor of *B. burgdorferi* (Xu *et al.*, 2008). Since the solution of the three-dimensional structure of OspC (Eicken *et al.*, 2001, Kumaran *et al.*, 2001), not much is known as to how the various secondary structures that make up OspC as a whole lipoprotein relate to its function(s) and/or possible *ospC* mRNA regulation.

GOAL, SPECIFIC OBJECTIVES AND HYPOTHESES

The overall goal of this dissertation research was to make strategic structural deletions of OspC and study their phenotypic effects on infectivity, dissemination, pathogenicity and colonization of *B. burgdorferi* and on the regulation of *ospC* mRNA expression in the murine model. The specific objectives were:

1. To create beta sheets 1 and 2 (βs_1 and βs_2) and alpha helix 4 (αH_4) deletions of OspC and study the effects of such deletions on the infectivity of *B. burgdorferi*.

2. To create an N-terminus 5-AA and 10-AA deletion of OspC in order to dissect the role of OspC in evasion from innate immunity and in dissemination of *B. burgdorferi* to distal tissues during mammalian infection.

3. To study the role of the N-terminus 5-AA sequence in regulation of *ospC* mRNA expression.

4. To create C-terminus 6-,9-, and 13-AA deletions of OspC and study their contributions to the overall function(s) of OspC and to the regulation of *ospC* mRNA expression.

The hypotheses of the proposed work for the above objectives were the following: 1) that there is a correlation between the OspC secondary structure and function of the lipoprotein that can be well delineated; and 2) that OspC is involved in self-regulation.

Chapter 2 describes the selective, relatively small, deletions that were made within the core of OspC structure of beta sheets 1and 2 and alpha helix 4 (βs_1 and βs_2 and αH_4). Included in this work were longer deletions of the N- and C-terminus of OspC (Nt10 and Ct13). All these deletions resulted in complete abolishment of the immune evasion role of OspC, indicating that these regions of OspC are critical for the survival of the spirochetes during the initial infection of the mammalian host.

Chapter 3 describes a deletion of 5-AA of the N-terminus of OspC. This deletion did not affect the innate immune evasive role of OspC as measured by a 50% infectious dose (ID_{50}) study. The tissue spirochetal load measured by qPCR was used as another criterion of the evasion of the spirochete from elimination. There was no significant difference in the tissue spirochetal burden in the joint and skin; however, there was a significant decrease in the heart tissues as compared to controls. Deletion of N-terminus 5-AA of OspC resulted in inefficient dissemination of *B. burgdorferi* to remote tissues in the SCID mice. This dissemination defect was even more pronounced in wild type mice. This particular deletion, therefore, led to a dissection of the role of OspC in evasion from that of facilitating dissemination of *B. burgdorferi*.

Chapter 4 describes the influence of the 5-AA N-terminus deletion of OspC on *ospC* mRNA levels as measured by RT-qPCR in the heart, joints and skin tissues of SCID mice. The results show an upregulation in *ospC* expression in the joint of mutant spirochetes as compared to control spirochetes containing a full length OspC copy, indicating that OspC is involved in self-regulation. There was no significant difference in *ospC* mRNA levels the heart and skin tissues between the mutant and control spirochetes. The role of OspC in self-regulation was further confirmed by OspC monoclonal antibody treatment of SCID mice inoculated with *Borrelia* expressing the truncated OspC. The treatment resulted in clearance of spirochetes from

all sites tested, except the joints, indicating that the joint tissue may serve as an immune privileged site.

Chapter 5 describes the phenotypic effects of sequential deletion of 6-, 9-, and 13-AA of the C-terminus of OspC on infectivity and pathogenicity of *B. burgdorferi* and on *ospC* expression. While spirochetes containing a deletion of 13-AA resulted in a non-infectious phenotype, as described earlier, spirochetes containing deletions of 6- and 9-AA retained their innate immune evasive roles as measured by ID_{50} values in both SCID and wild type mice, which were similar to those of control spirochetes. However, interestingly, whereas spirochetes with 6-AA deletion had lost their arthritis virulence, spirochetes with 9-AA C-terminus deletion retained their arthritis virulence. In terms of tissue spirochete burden, the study highlighted the tissue-dependent effects of the C-terminus deletion. Finally, deletion of C-terminus 9-AA sequence of OspC led to significant changes in *ospC* mRNA accumulation in all tissues, whereas deletion of C-terminus 6-AA sequence of OspC led to significant changes in *ospC* expression in the joint tissue only. Interestingly, in the joints, the deletion of 6-AA and 9-AA sequence of the C-terminus of OspC resulted in a down-regulation of *ospC* transcripts, indicating that OspC is involved in self-regulation.

Chapter 6, the last chapter, describes the general conclusions of this study. It summarizes the major findings of this dissertation research in a table format. It also provides an overview of our current accumulated knowledge in regards to the function(s) of OspC and how this dissertation research has contributed to a better understanding of those functions and of the regulation of OspC. Ultimately, this work contributes to an appreciation of the role that OspC plays in the pathogenesis of *B. burgdorferi*.

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CHAPTER 2

SELECTIVE STRUCTURAL DELETIONS OF OSPC ABOLISH THE ROLE OF OSPC IN EVASION OF *BORRELIA BURGDORFERI* FROM HOST INNATE DEFENCES

<u>INTRODUCTION</u>

Borrelia burgdorferi, the agent of Lyme disease, requires OspC upon initial infection of a mammalian host. OspC-knock out mutants were shown to be cleared from skin after intradermal/subcutaneous inoculation of *Borrelia burgdorferi* (Tilly *et al*., 2007). The requirement for OspC may be exclusive in nature; however, under laboratory setting, this role of OspC in evasion from host innate defences can be overridden by other heterologous lipoproteins (Xu *et al.*, 2008). OspC has been shown to have dual roles: in protection against (or evasion from) host innate immune response and in dissemination during infection of murine hosts (Xu *et al.*, 2008). To understand the function of a lipoprotein at a molecular level, it is often necessary to determine the three-dimensional structure of that lipoprotein. Based on this premise, the crystal structure of OspC was explored for its possible role in infectivity of *B. burgdorferi*. OspC is a predominantly α-helical in structure. A single unit of OspC is composed of five parallel α-helices and two short β-strands inter-connected by six loops, apart from an amino (N) - and carboxy (C)-terminus. The N-terminus linker is 23 AA long, whereas the C-terminal stretch is 13-AA long (Eicken *et al.*, 2001, Kumaran *et al.*, 2001). Of these, the nucleotides encoding for the two β strands (βs$_1$ and βs$_2$), alpha helix 4 (αH$_4$), C-terminus 13-AA (Ct13) and N-terminus 10-AA (Nt10) sequence of OspC were deleted, and the resulting truncated OspC for each clone was tested for infectivity potential in the SCID mouse. All five (βs$_1$, βs$_2$, αH$_4$, Ct13 and Nt10) OspC mutations produced non-infectious phenotypes. There was, however, recovery of OspC-deficient spirochetes containing a mutation in DbpA lipoprotein. The truncated OspC mutants

revealed that these corresponding regions of the *ospC* gene are required for the function of the lipoprotein in protection against host innate defences.

MATERIALS AND METHODS

Strains Generated Previously and Used in This Study. The *B. burgdorferi* strain B31 clone 13A was generated previously (Xu *et al.*, 2007). Serially diluting the *B. burgdorferi* B31 5A13 culture led to the identification of a single clone designated 13A, which lost lp25 and lp56 but contained the remaining 19 plasmids. This clone is highly transformable because lp25 and lp56 may carry restriction enzymes to negatively affect transformation efficiency of *B. burgdorferi* (Kawabata *et al.*, 2004, Lawrenz *et al.*, 2002). The *ospC* mutant clone was also generated previously by insertion of the *aacC1* cassette within the *ospC* locus and was confirmed by PCR (Xu *et al.*, 2007). This clone contained lp28-1 but lost cp9, lp5 and lp21, in addition to lp25 and lp56.

Construction of Plasmids pBBE22-o*spCβs₁*, pBBE22-*ospCβs₂*, pBBE22-*ospCαH₄* and pBBE22-*ospCNt10*. As illustrated in Fig. 2.1 through Fig. 2.4, to efficiently generate constructs encoding the two beta strands, alpha helix 4 and N-terminus 10 amino acid deletions, a 1057-bp fragment covering the *ospC*-coding region and up- and down-stream sequences was amplified by PCR using primers P1F and P1R (Table 2.1) and cloned into TA cloning vector pNCO1T (Downie *et al.*, 2004), creating an intermediate vector designated pNCO1T-*ospC*. Four large amplicons, βs₁, βs₂, αH₄ and Nt10 were generated by PCR using pNCO1T-*ospC* as a template and each of four forward primers P2F, P3F, P4F, P5F and each of four reverse primers, P2R, P3R, P4R and P5R, respectively (Table 2.1).

Primers P4F and P4R for αH₄ were designed for blunt end ligation; these primers were phosphorylated using T4 polynucleotide kinase, ATP and appropriate buffer (New England
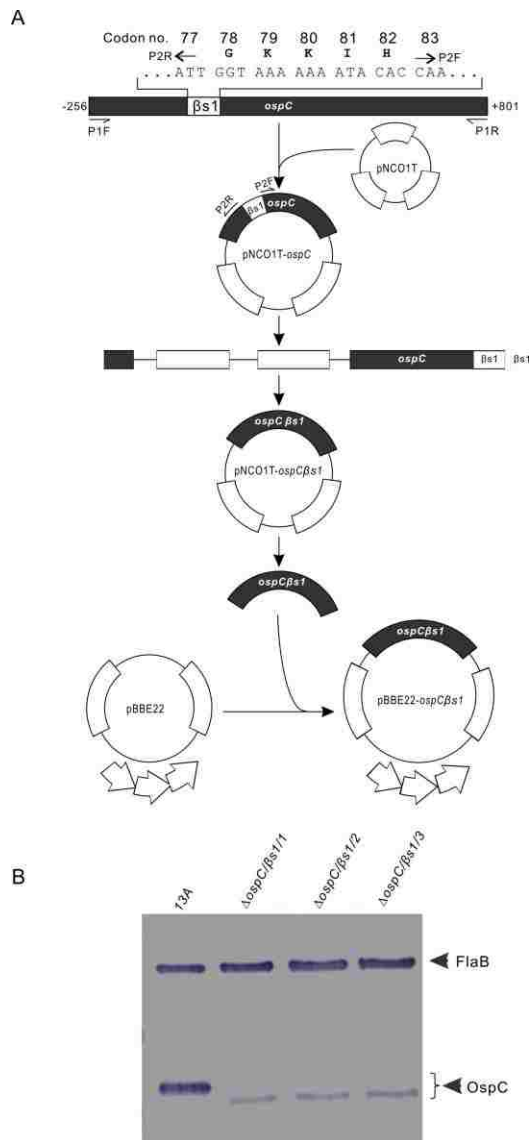
Figure 2.1. Generation of *B. burgdorferi* producing OspC with βs₁ deletion. A. Construction of pBBE22-*ospCβs1* coding for OspC with beta sheet 1 deletion. The five codons coding for the five amino acid residues (residues 78—82) and their adjacent codons are presented. The amplification starting sites and directions of four primers, P1F, P1R, P2F and P2R, used for plasmid construction are also marked. The long bar represents the 1057-bp fragment, covering the entire *ospC*-coding region and down- (extending to +801 from the transcriptional start site) and up-stream sequences (extending to -256). The detailed construction of the vector was described in Materials and Methods section. **B. Restoration of OspC production.** The parental clone *13A* and the clones *ΔospC/βs₁*/1 through *ΔospC/βs₁*/3 (of the total of 7 transformants) were verified for OspC expression by immunoblot probed with a mixture of FlaB and OspC anti-sera.
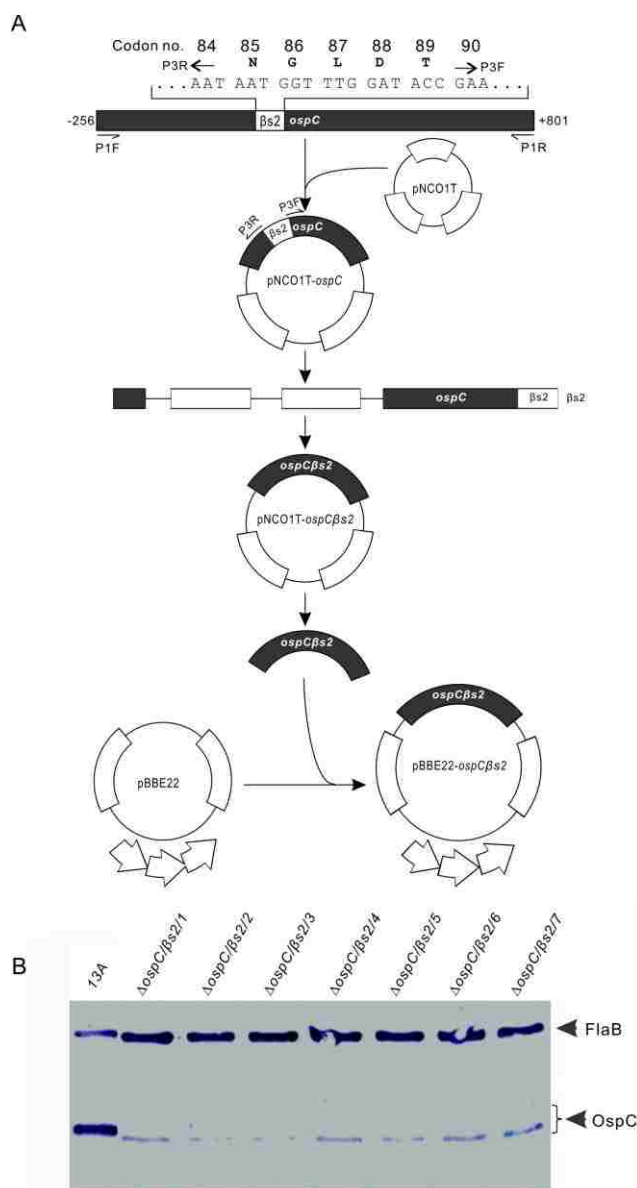
Figure 2.2. Generation of *B. burgdorferi* producing OspC with βs$_2$ deletion. A. Construction of pBBE22-*ospCβs2* coding for OspC with beta sheet 2 deletion. The five codons coding for the five amino acid residues (residues 85—89) and their adjacent codons are presented. The amplification starting sites and directions of four primers, P1F, P1R, P3F and P3R, used for plasmid construction are also marked. The long bar represents the 1057-bp fragment, covering the entire *ospC*-coding region and down- (extending to +801 from the transcriptional start site) and up-stream sequences (extending to -256). The detailed construction of the vector was described in Materials and Methods section. **B. Restoration of OspC production.** The parental clone *13A* and the clones *ΔospC*/*βs$_2$*/1 through *ΔospC*/*βs$_2$*/7 were verified for OspC expression by immunoblot probed with a mixture of FlaB and OspC anti-sera.

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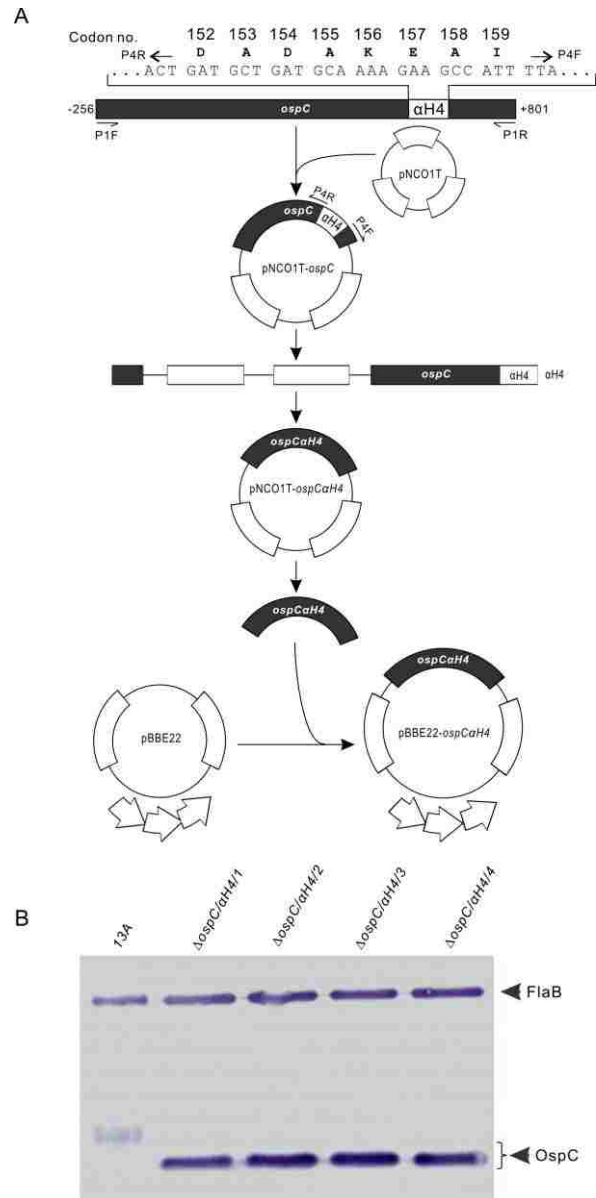
Figure 2.3 Generation of *B. burgdorferi* producing OspC with αH₄ deletion. A. Construction of pBBE22-*ospCαH₄* coding for OspC with alpha helix 4 deletion. The eight codons coding for the eight amino acid residues (residues 152—159) and their adjacent codons are presented. The amplification starting sites and directions of four primers, P1F, P1R, P4F and P4R, used for plasmid construction are also marked. The long bar represents the 1057-bp fragment, covering the entire *ospC*-coding region and down- (extending to +801 from the transcriptional start site) and up-stream sequences (extending to -256). The detailed construction of the vector was described in Materials and Methods section**. B. Restoration of OspC** production. The parental clone *13A* and the clones *ΔospC/αH₄*/1 through *ΔospC/αH₄*/4 were verified for OspC expression by immunoblot probed with a mixture of FlaB and OspC anti-sera.

A

Codon no.   24   25   26   27   28   29   30   31   32   33   34   35   36   37   38
            K   D   G   N   T   S   A   N   S   A   D   E   S*   V   K
           AAA GAT GGG AAT ACA TCT GCA AAT TCT GCT GAT GAG TCT GTT AAA

            P5R   P5F

     -256   Nt10   ospC   +801
           P1F   P1R

                                              pNCO1T

                    P5R Nt10 ospC
                         pNCO1T-ospC

                                        ospC   Nt10 Nt10

                         ospCNt10
                         pNCO1T
                         -ospCNt10

                         ospCNt10

                                              ospCNt10
         pBBE22   pBBE22
                                              -ospCNt10

B

13A   ΔospC/Nt10/1   ΔospC/Nt10/2   ΔospC/Nt10/3   ΔospC/Nt10/4   ΔospC/Nt10/5   ΔospC/Nt10/6

◄ FlaB
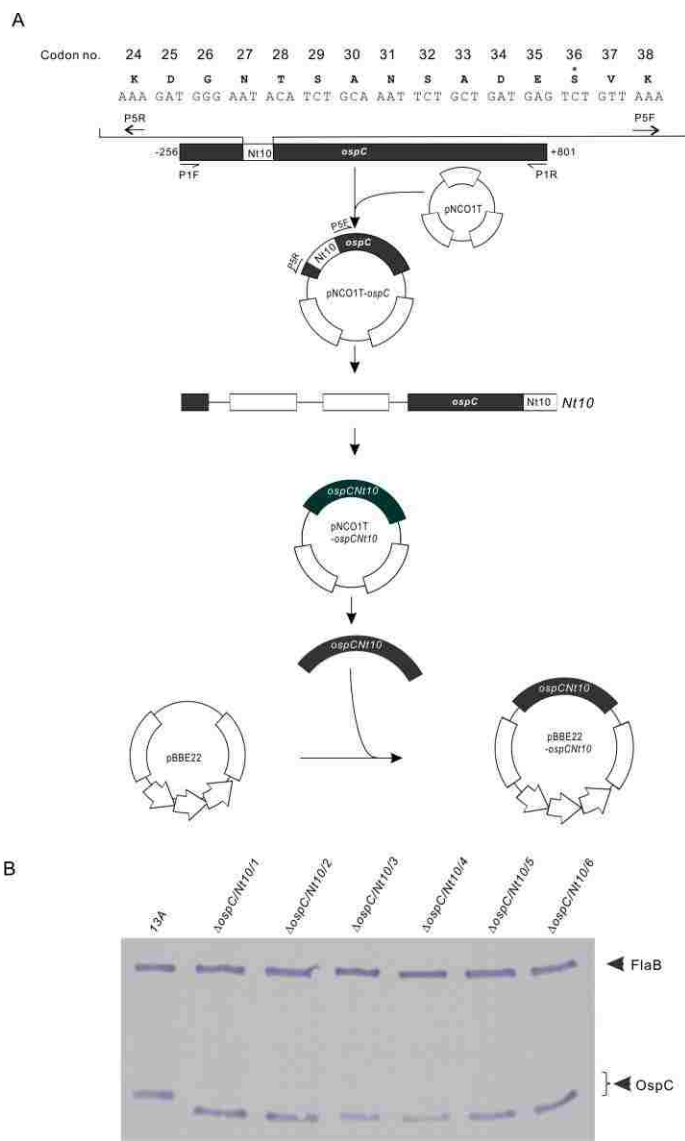
◄ OspC

Figure 2.4. Generation of *B. burgdorferi* producing OspC with Nt10 deletion. A. Construction of pBBE22-*ospCNt10* coding for OspC with N-terminus 10 amino acid deletion. The ten codons coding for the ten amino acid residues (residues 26—35) and their adjacent codons are presented. The amplification starting sites and directions of four primers, P1F, P1R, P5F and P5R, used for plasmid construction are also marked. Downstream of the deletion, the S was changed to a D as marked by an asterisk. The long bar represents the 1057-bp fragment, covering the entire *ospC*-coding region and down- (extending to +801 from the transcriptional start site) and up-stream sequences (extending to -256). The detailed construction of the vector was described in Materials and Methods section. **B. Restoration of OspC production.** The parental clone *13A* and the clones *ΔospC*/*Nt10*/1 through *ΔospC*/*Nt10*/6 were verified for OspC expression by immunoblot probed with a mixture of FlaB and OspC MAbs.

Table 2.1. Primers used to make the pBBE22-*ospCβs₁*, pBBE22-*ospCβs₂*, pBBE22-*ospCαH₄* and pBBE22-*ospCNt10*.

Construct	Primer name	Primer sequence (5'-3')[a]
FL	P1F	5'-TAGTTGGCTATATTG<u>GGATCC</u>AA-3'
	P1R	5'-TTCC<u>TCTAGA</u>GAAGAGCTTAAAGTTAA-3'
βs₁	P2F	5'-AC<u>GCTCTTC</u>TTCAAAATAATGGTTTGGATACC-3'
	P2R	5'-AT<u>GCTCTTC</u>TTGAATAGCTTTAGCAGCAATTTC-3'
βs₂	P3F	5'-AC<u>GCTCTTC</u>TTGAAAATAATCACAATGGATCATTG-3'
	P3R	5'-AC<u>GCTCTTC</u>TTCATTTTGGTGTATTTTTTACC-3'
αH₄	P4F	5'-AGTAACACCTTCTTTACCAAG-3'
	P4R	5'-TTAAAAACAAATGGTACTAAAAC-3'
Nt10	P5F	5'-AT<u>GACGTC</u>AAAGGGCCTA-3'
	P5R	5'-CT<u>GACGTC</u>TTCCCCTGAATT-3'

[a] The underlined sequences are restriction enzyme sites. P1F contains a *Bam*HI site; P1R has an *Xba*I site; P2F, P2R, P3F and P3R have *Sap*I sites; P4F and P4R are blunt end primers, with no restriction sites; P5F and P5R contain *Aat*II sites.

Biolabs, Ipswich, MA). After PCR, the termini of the PCR product was polished using T4 DNA polymerase (New England Biolabs, Ipswich, MA); digested with *Dpn*I (to cut methylated Adenine), purified, and underwent T4 DNA ligation with the vector pNCO1T.

To construct the N terminus 10-AA deletion, an *Aat*II restriction enzyme site was chosen as the appropriate site to be added to primers P5F and P5R. In order to introduce the site, the S (serine) residue was changed to a D (aspartic acid) residue at position 36 from start codon.

After digestion with *Sap*I for βs₁ and βs₂ and digestion with *Aat*II for Nt10 and subsequent purification, the four amplicons were circularized via ligation (blunt-end ligation in the case of αH4) and then digested with *Bam*HI and *Xba*I to release, βs₁, βs₂, αH₄ and Nt10. These four fragments, encoding an OspC variant with βs₁, βs₂ αH₄, and Nt10 deletions, were

cloned into the recombinant plasmid pBBE22 (a gift from S. Norris) after the vector was digested with *Bam*H1 and *Xba*I. Resulting constructs were designated, pBBE22-*ospCβs₁*, pBBE22-*ospCβs₂*, pBBE22-*ospCαH₄* and pBBE22-*ospCNt10* respectively. The inserts and their flanking regions within pBBE22 were sequenced to ensure the constructs were as designed.

Additional Plasmids Generated Previously and Used in This Study. Construct pBBE22-*ospCct13* was generated and described further in Chapter 5. *OspCct13* is a truncated *ospC* in which the nucleotides corresponding to 13-AA of the carboxy-terminus of OspC were deleted. Mouse infectivity studies (included in Chapter 5) had shown that clone Δ*ospC/ospCct13* was non-infectious at one month post-inoculation in SCID mice. Therefore, to test if infectivity was lost at an earlier time point, clone Δ*ospC/ospCct13* now named as Δ*ospC/Δct13* was included in this study. Construct pBBE22-*dbpAmt₈₂* was also generated in a previous study (unpublished data). The *dbpAmt₈₂* has a point mutation at the nucleotide coding for one of the three critical lysine residues for the binding of decorin-binding protein A (DbpA) to decorin. This was the lysine at amino acid position 82 from the methionine start codon (Brown *et al.*, 1999, Pikas *et al.*, 2003). The construct pBBE22-*ospC'* which has a flaB-promoter to constitutively express a full length copy of OspC was used as a positive control (Xu *et al.*, 2006). All three plasmids were electroporated into an OspC mutant. The resulting clones were designated Δ*ospC/ospCct13*, Δ*ospC/dbpAmt₈₂* and Δ*ospC/ospC'* and were used in the SCID mice infection study.

Generation of Δ*ospC/Δβs₁*, Δ*ospC/Δβs₂,* Δ*ospC/ΔαH₄*, Δ*ospC/ΔNt10* and Δ*ospC/Δct13* Transformants. Complementation plasmids were electroporated into an *ospC* mutant, namely, Δ*ospC*, which was generated in our previous study (Xu *et al.*, 2007). Briefly, Δ*ospC* spirochetes were grown in 10 ml of BSK-H medium at densities of 5×10^7 to 1×10^8 cells/ml (mid to late exponential phase), harvested, washed, and transformed with 1.5 µg of plasmid pBBE22-

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ospCαH₄, pBBE22-*ospCβs* , pBBE22-*ospCβs₂*, and pBBE22-*ospCNt10* under standard

electroporation conditions (Lawrenz *et al.*, 2002, Stewart *et al.*, 2001). The plasmid pBBE22 was

derived from shuttle vector pBSV2 by inserting the gene BBE22 (Purser *et al.*, 2003, Stewart *et al.*, 2001). The cells were allowed to recover in 20 ml of BSK-H medium at 33 ℃ for 18h. After

adding kanamycin at a concentration of 200 µg/ml and gentamycin at 50 µg/ml, the suspension

was transferred into 96 PCR tubes (200 µl/tube). The limited dilution assay has been widely used

in the screening of transformants (Blevins *et al.*, 2004, Caimano *et al.*, 2004, Revel *et al.*, 2005,

Yang *et al.*, 2004). Aliquots were incubated at 33 ℃ for 10 days and live spirochetes were

examined under a dark-field microscope. Viable spirochetes were found in 7, 7, 4, 6 and 5 of the

96 tubes for Δ*ospC*/Δ*βs₁*, Δ*ospC*/Δ*βs₂,* Δ*ospC*/Δ*αH₄*, Δ*ospC*/Δ*Nt10* and Δ*ospC*/Δ*ct13* clones,

respectively. Approximately 30 µl of the kanamycin/gentamycin resistant culture was transferred

to 1.3 ml of BSK-H medium in a 1.5 ml microcentrifuge tube and grown to stationary phase at

33 ℃. Spirochetes were harvested from 200 µl of culture by centrifugation at 11,000xg for 5 min

at room temperature and washed twice with excess volumes of phosphate-buffered saline (pH

7.3) to remove residual plasmid DNA and resuspended in 200 µl and resuspended in 200 µl of

deionized H_2O. One microliter of suspension was used as a DNA template source for

examination of recombinant plasmid pBBE22 by examining the kanamycin-resistant cassette by

PCR. The specific kanamycin primers (forward: 5'-ATGAGCCATATTCAACGGGAAACGT-

3'; reverse: 5'-TCAGCGTAATGCTCTGCCAGTGT-3') were used. The PCR conditions

included: 94 ℃ for 1 min; 94 ℃ for 30 seconds, 54 ℃ for 30 seconds, 72 ℃ for 1 min, 35

cycles; 72 ℃ for 10 min. PCR products were separated on an ethidium bromide-agarose gel.

Immunoblot Analysis. Transformants were grown in BSK-H complete medium to late

log phase at 33 ℃ and harvested by centrifugation. Spirochetes were dissolved in sodium

dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, separated by electrophoresis (Biorad, Hercules, CA), and electrotransferred onto nitrocellulose membranes (Thermo Scientific, Rockford, IL). Restoration of OspC expression was verified using immunoblots probed with a mixture of FlaB and OspC monoclonal antibodies (MAbs) for clone $\Delta ospC/\Delta Nt10$, or with the FlaB MAb and mouse anti-OspC sera raised against a recombinant OspC antigen for clones $\Delta ospC/\Delta \beta s_1$, $\Delta ospC/\Delta \beta s_2$, $\Delta ospC/\Delta \alpha H_4$ and $\Delta ospC/\Delta ct13$. The flaB and OspC MAbs were developed by Barbour *et al*., (Barbour *et al*., 1986) and Mbow *et al*., (Mbow *et al*., 1999) and used at 1:100 and 1:10,000 final dilutions, respectively. The secondary antibody used was goat anti-mouse IgG conjugated with horseradish peroxidase (hrp) at a 1:5,000 final dilution (Thermo Scientific, Waltham, MA). To develop the nitrocellulose membrane, a colorimetric blotting substrate solution (4CN) was used (Kirkegaard and Perry Laboratories, Gaithesburg, MD). The clone $\Delta ospC/FL/1$, which was generated in our previous study (Xu *et al*., 2007) and expressed wild-type OspC, was included as a control.

SCID Mice Infection Study. BALB/c SCID mice (ages 4-8 weeks, provided by the Division of LSU Laboratory Animal Medicine) were given two intradermal/subcutaneous injections of 10^5 spirochetes. The two inoculation sites were at least 2 cm apart. Animals were sacrificed at 24 and 48h later; inoculation skin site tissues were harvested for spirochete isolation as previously described (Xu *et al*., 2005).

Examination of Plasmid Content by PCR. The presence of lp28-1, lp28-2, lp28-4 and cp32-3, that tend to be spontaneously lost, was surveyed by PCR with primer pairs specific for each of the four plasmids (Table 2.2). Approximately 1 µl of spirochete culture was used as a DNA template source in a PCR volume of 25 µl. *Taq* polymerase was purchased from Takara Mirus Bio Inc (Madison, WI). The PCR conditions included: 94 °C for 1 min; 94 °C for 30

seconds, 54 °C for 30 seconds, 72 °C for 1 min, 35 cycles; 72 °C for 10 min. PCR products were separated on an ethidium bromide-agarose gel.

Table 2.2. PCR primers for examination of plasmid content in *B. burgdorferi*

Plasmid	Sequence	
	Forward	Reverse
lp28-1	5'-TTTCATTATAAGGAGACGATGA-3'	5'-CGTCGTACTACTTATATCGC-3'
lp28-2	5'-TTCTAGTATCTACTAGACCG-3'	5'-AGCCAAGTATTTCTTCTGCA-3'
lp28-4	5'-GTGTCGCTTAATTTTAGAGTTA-3'	5'-ATAGAGATCCTCTATTAGAGA-3'
cp32-3	5'-CTGTTTAATCCACCACCAGTA-3'	5'-ACTTTCTCAGTACTTTCTCC-3'

RESULTS

The Plasmid Contents of The Various Transformants Generated. All of the identified transformants had the kanamycin-resistant cassette as tested by PCR, indicating the presence of pBBE22. Then, the transformants were surveyed for the presence of lp28-1, a plasmid essential for infection of immunocompetent hosts (Labandeira-Rey *et al.*, 2003, Purser and Norris, 2000), but not required for infectivity in SCID mice (Labandeira-Rey *et al.*, 2003). Only clones containing lp28-1 were further analyzed for the content of those plasmids that tend to be spontaneously lost during in vitro propagation, described in the Materials and Methods section, with the exception of αH$_4$ construct for which only one of the four transformants contained lp28-1. For the majority of clones, two transformants from each clone that had identical plasmid contents were chosen for inoculation into SCID mice, except for Δ*ospC*/Δ*Nt10* and Δ*ospC*/Δα*H$_4$*. For Δ*ospC*/Δ*Nt10*, the plasmid content was identical for lp28-2 but not lp28-4. For Δ*ospC*/Δα*H$_4$*, the two transformants had lp28-2 and lp28-4, although one clone had lp28-1 and the other did not.

Various Truncated OspC Mutants Are Unable to Aid *Borrelia* Evade Quick

Clearance. Groups of four SCID mice each received two intradermal/subcutaneous inoculations of the clones Δ*ospC/ospC'/1*, Δ*ospC/ospC'/2*, Δ*ospC/Δβs₁/1*, Δ*ospC/Δβs₁/2,* Δ*ospC/Δβs₂/1,* Δ*ospC/Δβs₂/2*, Δ*ospC/ΔαH₄/1,* Δ*ospC/ΔαH₄/2*, Δ*ospC/ΔNt10/1*, Δ*ospC/ΔNt10/2*, Δ*ospC/ΔCt13/1*, Δ*ospC/ΔCt13/2*, Δ*ospC/dbpAmt₈₂/1*, or Δ*ospC/dbpAmt₈₂/2*. The control spirochetes Δ*ospC/ospC'/1* and Δ*ospC/ospC'/2* were consistently grown from each of the 16 inoculation sites from all the eight inoculated mice (Table 2.3). In contrast, none of the Δ*ospC/Δβs₁/1*, Δ*ospC/Δβs₁/2,* Δ*ospC/Δβs₂/1,* Δ*ospC/Δβs₂/2*, Δ*ospC/ΔαH₄/1,* Δ*ospC/ΔαH₄/2*, Δ*ospC/ΔNt10/1*, Δ*ospC/ΔNt10/2*, Δ*ospC/ΔCt13/1*, or Δ*ospC/ΔCt13/2* were grown from either the 8 inoculation sites at 24h time point or the 48h time point. Like the positive control, the clone Δ*ospC/dbpAmt₈₂/1* and Δ*ospC/dbpAmt₈₂/2* were also recovered from all inoculation sites at both time points. These results indicate that strategic deletions of OspC of small number of amino acids within the core region or of large number of amino acids in the termini of OspC abolish the ability of the lipoprotein to aid *B. burgdorferi* evade from quick clearance. In contrast, the DbpA mutant is able to compensate for the role of OspC in evasion of host innate defenses, although one of its decorin binding sites was mutated.

DISCUSSION

B. burgdorferi depends on the expression of OspC for its survival during early mammalian infection. The essential role of OspC was demonstrated when OspC knock-out spirochetes were cleared from skin inoculation site by 48h (Tilly *et al.*, 2007). In spite of a myriad of host innate immune responses devised by neutrophils, macrophages, or by toll-like receptors (TLRs), particularly TLR-2 against borrelial lipoproteins (Hartiala *et al.*, 2008, Hirschfeld *et al.*, 1999, Linder *et al.*, 2001, Peterson *et al.*, 1984), one of the mechanisms by

which *Borrelia* are able to evade these responses is through the abundance of expression of

OspC and other lipoproteins on the outer surface of the outer membrane of the spirochete.

Table 2.3. Various types of truncated OspC mutants are unable to aid *Borrelia* evade quick clearance from murine skin [a]

Clone	No. of sites positive/total no. of sites examined at post-inoculation hours		Clone	No. of sites positive/total no. of sites examined at post-inoculation hours	
	24	48		24	48
$\Delta ospC/ospC'/1$	4/4	4/4	$\Delta ospC/\Delta\beta s_1/1$	0/4	0/4
$\Delta ospC/ospC'/2$	4/4	4/4	$\Delta ospC/\Delta\beta s_1/2$	0/4	0/4
$\Delta ospC/\Delta\beta s_2/1$	0/4	0/4	$\Delta ospC/\Delta\alpha H_4/1$	0/4	0/4
$\Delta ospC/\Delta\beta s_2/2$	0/4	0/4	$\Delta ospC/\Delta\alpha H_4/2$	0/4	0/4
$\Delta ospC/\Delta Nt10/1$	0/4	0/4	$\Delta ospC/\Delta Ct13/1$	0/4	0/4
$\Delta ospC/\Delta Nt10/2$	0/4	0/4	$\Delta ospC/\Delta Ct13/2$	0/4	0/4
$\Delta ospC/dbpAmt_{82}/1$	4/4	4/4			
$\Delta ospC/dbpAmt_{82}/2$	4/4	4/4			

[a] Groups of four BALB/c SCID mice each received two intradermal/subcutaneous injections of the clone $\Delta ospC/ospC'/1$, $\Delta ospC/ospC'/2$, $\Delta ospC/\Delta\beta s_1/1$, $\Delta ospC/\Delta\beta s_1/2$, $\Delta ospC/\Delta\beta s_2/1$, $\Delta ospC/\Delta\beta s_2/2$, $\Delta ospC/\Delta\alpha H_4/1$, $\Delta ospC/\Delta\alpha H_4/2$, $\Delta ospC/\Delta Nt10/1$, $\Delta ospC/\Delta Nt10/2$, $\Delta ospC/\Delta Ct13/1$, $\Delta ospC/\Delta Ct13/2$, $\Delta ospC/dbpAmt_{82}/1$ or $\Delta ospC/dbpAmt_{82}/2$. Approximately 10^5 organisms were administered in each inoculation; two inoculations sites were at least 2 cm apart. Two animals from each group were euthanized at 24 and 48 h post-inoculation; skin specimen s were harvested from inoculations sites and cultured for spirochetes in BSK-H complete medium.

OspC has been found to have a dual function: that of protection against host innate

immune responses, which is common to that of other surface lipoproteins and thus can be

overridden by the same, such as DbpA and OspA; and that of dissemination, which is one of the

unique roles of OspC, discovered so far, in the pathogenesis of *B. burgdorferi* (Xu *et al.*, 2008).

Since the crystal structure of OspC has been resolved, many labs have focused on

exploring the structure of OspC for finding epitopes for adaptive immune responses (Earnhart *et al.*, 2005, Jobe *et al.*, 2003). To our knowledge, this is the first study that explored the crystal

structure of OspC for finding its function in protection against innate immune response. Since

deleting several regions of OspC within the core structure (the 2 beta sheets and alpha helix 4)

resulted in non-infectious phenotypes, we decided to test clones with larger deletions of the

OspC termini. The results showed that larger segments in the flexible, non-crystalizable portion

of OspC (the N- and C-terminus) are also involved in protection against host innate immune

responses. Both Δ*ospC/*Δ*Nt10* and Δ*ospC/*Δ*Ct13* clones were included because studies

(described in Chapter 3 and 5, respectively) have shown that these clones were non-infectious at

least at a one month post-inoculation time point and therefore tested in this study to verify that

the loss of infectivity occurred at an earlier time point.

Although numerous studies have attempted to associate infectivity of *B. burgdorferi* with

plasmid content (Caimano *et al.*, 2000, Casjens *et al.*, 2000, Labandeira-Rey and Skare, 2001,

Marconi *et al.*, 1996, Purser and Norris, 2000, Simpson *et al.*, 1990), two of them proved that

lp25 and lp28-1 may be necessary for full virulence (Labandeira-Rey and Skare, 2001, Purser

and Norris, 2000). In this study, because the *B. burgdorferi* strain B31 clone 13A, an ospC

mutant, had lost lp25, the plasmid that carries the gene *bbe22* coding for a nicotinamidase

essential for survival of *B. burgdorferi* in the mammalian environment, the recombinant plasmid

pBBE22, which harbors a copy of *bbe22*, was used as the shuttle vector (Purser *et al.*, 2003). The

clone 13A had also lost lp56 but contained the remaining 19 plasmids (Xu *et al.*, 2007).

However, plasmid lp56 is not associated with loss of infectivity (Purser and Norris, 2000). *B.*

burgdorferi B31 clones that lacked lp28-1 were shown to be of either low or intermediate

infectivity (Purser and Norris, 2000). However, *B. burgdorferi* strains that have lost lp28-1 are

still infectious and persist in immunodeficient mice (Labandeira-Rey *et al.*, 2003). The plasmid

lp28-1 contains the *vlsE* locus that is required for evasion of humoral immune response

(Labandeira-Rey *et al.*, 2003, Labandeira-Rey and Skare, 2001, Purser and Norris, 2000, Zhang

et al., 1997). For that reason, an effort was made in this study to select clones that contained

lp28-1 and to verify the content of those plasmids that can be spontaneously lost. Since one of the $\Delta ospC/\Delta \alpha H_4$ clones had the plasmid lp28-1 while the other had lost the plasmid, and yet both clones were non-infectious, it can be implied that the resulting phenotypes are due to the lack of the alpha helix rather than the lack of the plasmid lp28-1.

The small beta strands may be involved in the stabilization of the OspC dimers (Eicken *et al.*, 2001). Hence, deletion of their beta strands led to complete loss of infectivity, suggesting that the dimer formation may be important in the function of OspC. Interestingly, when the two beta sheets βs_1 and βs_2 of OspC were deleted, the truncated protein expression levels were significantly lower than that of the full length OspC controls. The biological significance of this observation is not yet known. It could be that the beta sheets are involved in the degradation of the lipoprotein.

A previous study showed that OspC-deficient spirochetes showing increased DbpA expression were able to be recovered from skin inoculation site of SCID mice within 24h (Xu *et al.*, 2008). In order to test if mutation of one of the active sites of decorin-binding may compromise the protective role of this lipoprotein, a $DbpAmt_{82}$ was included in the study. The results of this study indicate that in spite of the mutation, spirochetes were still able to be recovered for at least up to 48h. It also indicates that spirochetes lacking OspC but expressing DbpA, although in a mutant form, could protect *B. burgdorferi* against host innate defenses.

This study has shown that a broad range of deletions in the OspC structure can lead to the abolishment of OspC function in protection against host innate responses. Since βs_1 and βs_2 and αH_4 are part of the core structure of OspC, their deletions might have led to a collapse in the structure of the lipoprotein. Deletion of 10-AA of the N-terminus of OspC, on the other hand, might have compromised the anchoring of the lipoprotein to the outer surface of the outer membrane of the spirochete. Finally, deletion of the C-terminus of OspC might have hindered

the interaction of the lipoprotein with the outer environment, since the terminus is not linked to

the borrelial membrane. A further analysis by circular dichroism, which examines protein

structures in solution (Kelly *et al.*, 2005), could demonstrate whether these truncated OspC

proteins cause a collapse of the core structure of OspC. Nevertheless, this study shows that there

is a correlation between the certain regions of OspC and the role of this surface lipoprotein in

evasion of *B. burgdorferi* from host innate defences.

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CHAPTER 3

OUTER SURFACE PROTEIN C IS A DISSEMINATION-FACILITATING FACTOR OF *BORRELIA BURGDORFERI* DURING MAMMALIAN INFECTION

<u>INTRODUCTION</u>

Coordinated production of outer surface proteins (Osp) is crucial for the pathogenic strategy of the Lyme disease spirochete, *Borrelia burgdorferi* (Burgdorfer *et al.*, 1982). In engorged and unfed ticks, *B. burgdorferi* abundantly produces OspA and OspB, but no OspC (Ohnishi *et al.*, 2001, Schwan *et al.*, 1995), but dramatically upregulates OspC in response to a fresh blood meal and prepares itself for infection of mammals (Grimm *et al.*, 2004b, Pal *et al.*, 2004, Stewart *et al.*, 2006). *B. burgdorferi* maintains high OspC synthesis during early mammalian infection (Liang *et al.*, 2002a, Liang *et al.*, 2002b, Liang *et al.*, 2004b). However, OspC is not only a strong immunogen, but also an effective target of protective immunity; its production ultimately induces a robust humoral response that imposes tremendous pressure on the pathogen (Fung *et al.*, 1994, Liang *et al.*, 2004b) To effectively evade the adaptive immune response and establish persistent infection, *B. burgdorferi* must downregulate *ospC* after the specific humoral response has developed (Xu *et al.*, 2006) , suggesting an early role for OspC in mammalian infection.

Inactivation of the *ospC* gene completely abolishes infectivity of *B. burgdorferi* (Grimm *et al.*, 2004a); however, the resulting mutant is able to persist in mammalian tissues, once the initial requirement for OspC is overcome, via introduction of an unstable *ospC* copy, which is readily lost under the immune selection pressure during infection of immunocompetent mice, leading to a conclusion that OspC is required exclusively for initial mammalian infection (Stewart *et al.*, 2006, Tilly *et al.*, 2007, Tilly *et al.*, 2006). In fact, however, this initial requirement for OspC can be overridden by either increasing expression of another Osp (Xu *et*

al., 2008), or simply adapting *ospC* mutants in mammalian hosts (Tilly *et al.*, 2009). The nature

of adaptation is to alter gene expression, and it has been known for years that *B. burgdorferi*

undergoes dramatic changes in its surface lipoprotein expression during mammalian infection

(Liang *et al.*, 2002a, Liang *et al.*, 2002b). Most notably, the downregulation of OspC in response

to the development of the anti-OspC humoral response is always coordinated with an

upregulation of both VlsE and BBF01 (Liang *et al.*, 2004b). Although remaining to be

investigated, the host adaptation process most likely provides OspC-deficient spirochetes with

both time and environment that enable the upregulation of other Osps, such as VlsE and BBF01,

to occur during the course of the disappearance of unstable *ospC* copies. The ability of an Osp to

replace the function of OspC in initial mammalian infection highlights a redundant function of

the Osps, which is to assist *B. burgdorferi* evade innate immune defenses (Xu *et al.*, 2008).

However, increasing expression of an Osp fails to fully restore *ospC* mutants with expected

dissemination ability, leading us to hypothesize that the unique function of OspC is to promote

dissemination (Xu *et al.*, 2008).

As described by some investigators, the protective and dissemination-promoting

functions of OspC are like two sides of the same coin (Radolf and Caimano, 2008), highlighting

the challenge to dissect the functions of OspC. Fortunately, the event of borrelial infection can

be divided into several steps. After inoculated into the dermis of a mammal, *B. burgdorferi* must

first be able to evade innate immune clearance, and then replicate, disseminate and colonize

distal tissues. The 50% infectious dose (ID_{50}) should be the best indication for the ability of *B.*

burgdorferi to evade this initial innate immune elimination. After initial replication, *B.*

burgdorferi disseminates and colonizes distal tissues. Examining the presence of spirochetes in

distal tissues would reveal its dissemination ability. Again, information about tissue bacterial

loads would be another indication of *B. burgdorferi* to evade innate immune clearance. If an

ospC mutant showing the similar ID$_{50}$ value and tissue bacterial load as a wild-type control can be generated, dissecting the protective from dissemination-facilitating functions would become possible.

The goal of the study was to dissect the dissemination-facilitating from protective function of OspC. Our strategy was to generate OspC mutants that can evade innate immunity but are unable to efficiently disseminate via mutagenizing the *ospC* gene. Based on X-ray analyses, OspC is a largely α-helical protein and may be a dimer with a characteristic central four-helical bundle formed by the association of the two longest helices, helices 1 and 5, from each subunit (Eicken *et al.*, 2001, Kumaran *et al.*, 2001). Each subunit consists of five α-helices, two β-sheets and six loops, in addition to the amino (N-) terminal 23-AA linker and the carboxyl (C-) terminal 14-AA stretch. Neither the N-terminal linker nor the C-terminal stretch contributes to the core three-dimensional structure of OspC; instead, the α-helices 1 and 5 bring the two terminal regions in close proximity, where the lipoprotein is anchored to the bacterial outer membrane through lipidation of the first cysteine residue of the N-terminal linker. Within the core, several small secondary structures are present, including α-helix 4 (consisting of 8 amino acids) β-sheet 1 (5 amino acids) and 2 (5 amino acids). We first generated *B. burgdorferi* expressing OspC with each of these small units being deleted. Unfortunately, none of the resulting mutants were infectious, suggesting that these sequences are critical for the functions of OspC (data shown in Chapter 2).

Next, we focused on the N-terminal sequence. One role of this sequence is to carry the sorting signal, which determines the surface location of OspC. In *E. coli* the so-called "+2" rule determines the cellular location of a lipoprotein. However, the sorting signal of *B. burgdorferi* can extend to the +4 position (Schulze and Zuckert, 2006). To create N-terminal deletions, which can be successfully sorted to the outer surface, we intended to generate mutations starting at the

+7 position, and tested the mutants for OspC surface exposure, and then analyzed the mutants for the protective and dissemination-facilitating roles of OspC.

In this study, mutants producing OspC with a 5-AA and a 10-AA N-terminus deletion were generated. Although the N-terminus 10-AA deletion did not affect its surface exposure of the truncated OspC, the deletion mutant was non-infectious and thus could not be used in a dissemination study (data shown in Chapter 2 and here). However, a mutant producing OspC with only 5-AA N-terminal deletion was infectious. This mutant registered a similar ID_{50} value and tissue bacterial load as a control but disseminated to remote tissues at a noticeably slower pace. The study allowed us to confirm that OspC is a dissemination-facilitating factor of *B. burgdorferi*.

MATERIALS AND METHODS

Previously Generated Strains and Constructs Used in The Current Study. The *B. burgdorferi* B31 clone 13A and the *ospC* mutant were generated previously (Xu *et al.*, 2007). The TA cloning vector pNCO1T was constructed in a previous study (Downie *et al.*, 2004). The shuttle vector pBBE22 was a gift from S. Norris (Purser *et al.*, 2003). The features of these constructs and clones are summarized in Table 3.1.

Creation of The Constructs pBBE22-*ospCnt5* and pBBE22-*ospCnt10*. As illustrated in Figure 3.1, to efficiently generate a 5-AA N-terminus deletion, a 1057-bp fragment covering the *ospC* region and the up- and down-stream sequences was amplified from genomic DNA by PCR using primers P1F and P1R (Table 3.2) and cloned into the TA cloning vector pNCO1T (Downie *et al.*, 2004), creating an intermediate vector designated pNCO1T-*ospC*. One large amplicon was generated by inverse PCR using pNCO1T-*ospC* as a template and primers P2F and P2R (Table 3.2). After digestion with *Sap*I and subsequent purification, the amplicon was circularized via ligation and then digested with *Bam*HI and *Xba*I to release *ospCnt5*. This

Table 3.1. Constructs and clones used in the study.

Construct or clone	Description	Source
pNCO1T	homemade T/A cloning vector	(Downie, *et al*., 2004)
pNCO1T-*ospC*	pNCO1T carrying *ospC* gene driven by its native promoter	This study
pNCO1T-*ospCnt5*	pNCO1T carrying *ospC* gene expressing N-terminus 5-AA deletion	This study
pNCO1T-*ospCnt10*	pNOC1T carrying *ospC* gene expressing N-terminus 10-AA deletion	This study
pBBE22	pBSV2 carrying a *bbe22* copy	(Purser, *et al*., 2003)
pBBE22-*ospCnt5*	pBBE22 carrying *ospC* gene expressing N-terminus 5-AA deletion	This study
pBBE22-*ospCnt10*	pBBE22 carrying *ospC* gene expressing N-terminus 10-AA deletion	This study
13A	*B. burgdorferi* B31 clone lacking plasmids lp25 and lp56	(Xu, *et al*., 2007)
ΔospC	*ospC* mutant	(Xu, *et al*., 2007)
*ΔospC/FL/*1	*ospC* mutant expressing *ospC* controlled by *ospC* regulatory elements [a]	(Xu, *et al*., 2007)
*ΔospC/FL/*2	*ospC* mutant expressing *ospC* controlled by *ospC* regulatory elements	(Xu, *et al*., 2007)
ΔospC/Nt5/1	*ospC* mutant expressing N-terminus 5-AA deletion	This study
ΔospC/Nt5/2	*ospC* mutant expression N-terminus 5-AA deletion	This study
ΔospC/Nt10/1	*ospC* mutant expression N-terminus 10-AA deletion	This study
ΔospC/Nt10/2	*ospC* mutant expression N-terminus 10-AA deletion	This study

[a.] The *ospC* regulatory elements include both operator and promoter.

fragment, encoding an OspC variant with N-terminal 5-AA deletion, was cloned into the recombinant plasmid pBBE22 after the vector was digested with *Bam*HI and *Xba*I. The resulting construct was designated pBBE22-*ospCnt5*. The insert and its flanking regions within pBBE22 were sequenced to ensure the construct was as designed. Sequencing was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster city, CA) using a BigDye Terminator kit protocol version 3.1, at the Division of Biotechnology and Molecular Medicine (BioMMed), LSU-School of Veterinary Medicine (LSU-SVM), Baton Rouge, LA. The same strategy was used to generate construct pBBE22-*ospCnt10***.** The details of this construct are found in Chapter 2.

Two constructs, pBBE22-*ospCnt5* (shown in Figure 3.1A) and pBBE22-*ospCnt10* (shown in Chapter 2)*,* with a 5 and 10 amino acid sequence deletion, respectively, were generated. The two constructs were electroporated into the *ospC* mutant, Δ*ospC*, which was generated and characterized in our previous study (Xu *et al.*, 2007). Because Δ*ospC* lacks lp25, the plasmid that carries the gene *bbe22* coding for a nicotinamidase essential for survival of *B.*

burgdorferi in the mammalian environment, the recombinant plasmid pBBE22, which harbors a copy of *bbe22*, was used as the shuttle vector (Purser *et al.*, 2003). Expression of truncated OspC resulting from the introduced construct was confirmed by immunoblot analysis (Figure 3.1B).

Table 3.2. Primers used in the study.

Clone	Primer	Sequences (5' to 3') [a]
FL	P1F	5'-TAGTTGGCTATATTG<u>GGATCC</u>AA-3'
	P1R	5'-TTCC<u>TCTAGA</u>GAAGAGCTTAAAGTTAA-3'
Nt5	P2F	5'-GACT<u>GCTCTTC</u>AGACAATTCTGCTGATGAGTCT-3'
	P2R	5'-CGTAC<u>GCTCTTC</u>AGTCTTTCCCAGAATTATTACAAGA-3'

[a]P1F and P1R contain restriction enzyme sites (underlined) *Bam*HI, and *Xba*I, respectively.
 P2F and P2R contain *Sap*I restriction sites (underlined).

Transformation of *B. burgdorferi* and Selection of Transformants. Constructs were electroporated into Δ*ospC*; resulting transformants were screened and analyzed for plasmid content as described previously (Xu *et al.*, 2005). Restoration of OspC production was verified using immunoblots probed with a mixture of FlaB and OspC MAbs (Figure 3.1B), as described in an earlier study (Xu *et al.*, 2007).

Indirect Immunofluorescence Assay. Spirochetes were grown to stationary phase in BSK-H complete medium. Indirect immunofluorescence was performed on unfixed spirochetes only. Briefly, *B. burgdorferi* was grown to stationary phase (10^8 cells/ml) in BSK-H complete medium at 33 °C. To count the cell, cells were diluted and counted by averaging eight fields under the microscope. It was assumed that one hundred spirochetes/field was equivalent to 3×10^7 spirochetes/ml. For fluorescent labeling, 6×10^7 cells were harvested from 0.6 ml of Δ*ospC*/*FL* control, Δ*ospC*/*nt5* or Δ*ospC*/*nt10* mutant spirochetes by centrifugation at 8,000xg for 10 min,

Fig. 3.1A
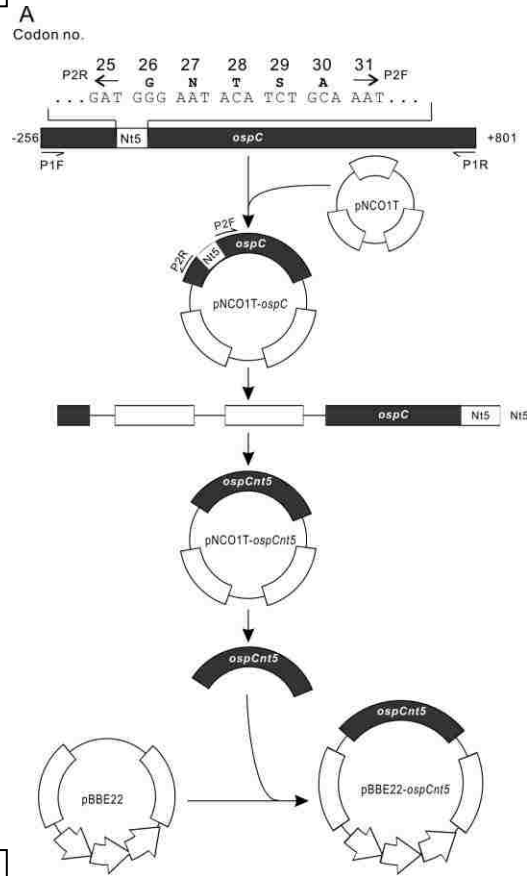
Fig. 3.1B
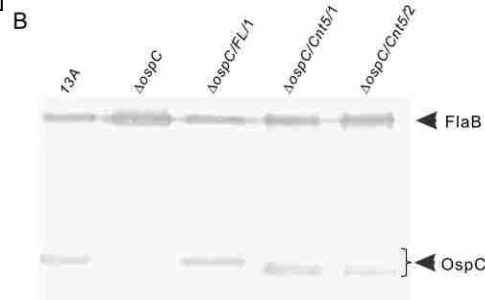
Figure 3.1. Generation of *B. burgdorferi* producing OspC with N-terminal 5-AA deletion. A. Construction of pBBE22-*ospCnt5* coding for OspC with N-terminal 5-AA deletion. The five codons coding for the five amino acid residues (residues 26—30) and their adjacent codons are presented. The amplification starting sites and directions of four primers, P1F, P1R, P2F and P2R, used for plasmid construction are also marked. The long bar represents the 1057-bp fragment, covering the entire *ospC*-coding region and down- (extending to +801 from the transcriptional start site) and up-stream sequences (extending to -256). The detailed construction of the vector was described in Materials and Methods section. **B. Restoration of OspC production.** The parental clone 13A, Δ*ospC*, and the clones Δ*ospC*/*FL*, *ΔospC*/*Cnt5*/1 and Δ*ospC*/*Cnt5*/2 were verified for OspC expression by immunoblot probed with a mixture of FlaB and OspC MAbs.

gently suspended in 100 µl PBS supplemented with 3.6 µl of mouse OspC MAb preparation B5 (Gilmore and Mbow, 1999), and incubated for 1 hour at 33 °C. After one wash with excess volume of PBS by centrifugation at 10,000xg for 10 min, spirochetes were resuspended in 100 µl of PBS containing 1 µg of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Rockford, IL), incubated for 1 hour, washed once with PBS by centrifugation, resuspended in 30µl PBS, applied to microscopic slides, and analyzed using Axio Imager (Carl Zeiss Microimaging, Inc., Thornwood, NY). Differential interference contrast (DIC) images were also taken for the same field to identify all the spirochetes present.

SCID Mice Infectivity Studies. To test for infectivity, groups of three C3H SCID mice were inoculated with 10^4 spirochetes of the clone *ΔospC*/*ΔNt5/*1, *ΔospC*/*ΔNt5/*2, *ΔospC*/*ΔNt10/*1, or *ΔospC*/*ΔNt10/*2. As a control, mice were also inoculated with the clone *ΔospC*/*FL/*1 or *ΔospC*/*FL/*2. The clones *ΔospC*/*FL/*1 and *ΔospC*/*FL/*2 were generated via introduction of a full-length *ospC* gene including both the operator and promoter carried by the shuttle vector pBBE22 into the *ospC* mutant in our previous study (Xu *et al.*, 2007). All mice were sacrificed 1 month post-inoculation; heart, tibiotarsal joint and skin specimens were harvested for spirochete culture in BSK-H complete medium.

Because *ΔospC*/*ΔNt10/*1 and *ΔospC*/*ΔNt10/*2 spirochetes could remain at inoculation sites and not disseminate to other tissues, groups of four SCID mice each received two intradermal/subcutaneous inoculations of 10^5 spirochetes of the clone *ΔospC*/*ΔNt10/*1 or *ΔospC*/*ΔNt10/*2. As a control, an additional eight animals were inoculated with the clone *ΔospC*/*FL/*1 or *ΔospC*/*FL/*2. The two inoculation sites were at least 2 cm apart. Two animals from each group were euthanized at 24 or 48 h later; inoculation site skin specimens were harvested for spirochete culture.

Determination of ID$_{50}$ Values. Spirochetes were grown to late-log phase (10^8 cells/ml) in BSK-H complete medium at 33 °C and 10-fold serially diluted with fresh medium. C3H SCID mice (ages 4-6 week; provided by the Division of Laboratory Animal Medicine at Louisiana State University, Baton Rouge, LA) each received one single intradermal/subcutaneous injection of 100 µl of spirochetal suspension (containing 10^1 to 10^4 organisms). Mice were euthanized one month post-inoculation; heart, tibiotarsal joint, and skin (not from inoculation site) specimens were harvested for bacterial isolation. The ID$_{50}$ value was calculated as described by Reed and Muench (Reed and Muench, 1938). Briefly, mice were inoculated with sequential 10-fold dilutions of spirochetes ranging from 10^4 to 10^1. The ID$_{50}$ value was based on the calculation of an index. The index and a table related to the calculation are shown in Appendix A, along with an example. The values were input into an ID$_{50}$ value calculation tool from www.biorubyrails.com website.

Dissemination Studies in SCID and Wild Type Mice. C3H SCID mice (ages 4-6 weeks; provided by the Division of LSU Laboratory Animal Medicine) each were given a single intradermal/subcutaneous injection of 10^3 spirochetes in the chest and were euthanized at 1, 2, 3 and 4 weeks post-inoculation. Tiobiotarsal joint, inoculation site and remote site skin, ear and heart specimens were harvested for spirochete isolation as described previously (Xu *et al.*, 2005). Because spirochetes were injected into the dermis of the chest, the skin from the back was harvested as a remote site. In a second experiment, BALB/c mice (ages 4-8 weeks; provided by the Division of LSU Laboratory Animal Medicine) each received a single intradermal/subcutaneous injection of 10^5 spirochetes and were euthanized at 1, 2, 3 and 4 weeks post inoculation. The reminder of the experiment was done as previously described for C3H SCID mice.

Quantitation of Tissue Spirochetal Load. C3H SCID mice (4-6 weeks; provided by the Division of LSU Laboratory Animal Medicine) each were intradermally/subcutaneously inoculated with 10^4 spirochetes. Animals were sacrificed one month post-inoculation; heart, joint, and skin specimens were harvested for DNA isolation. DNA was quantified for the copy numbers of *flaB* and murine actin genes by quantitative PCR (qPCR) as described previously (Xu *et al.*, 2005). The tissue spirochete burden was expressed as *flaB* DNA copies per 10^6 host cells (2×10^6 actin DNA copies).

Preparation of *flaB*-actin Fusion Standard. To better normalize the flaB and actin concentration for quantitative PCR (qPCR), an internal sequence from both actin and *flaB* genes were cloned into TA vector pNCO1T (Downie *et al.*, 2004). A primer pair (forward: 5'-AA<u>GG ATCC</u>ATGAGACCACTTTCAACT-3'; reverse: 5'-GGACAGTGAGGCCAGAATG GA-3') was designed to amplify a 231-bp internal fragment of the actin gene using murine DNA as a template. A second primer pair (forward: 5'-CAGCTGAAGAGCTTGGAATGCA-3'; reverse: 5'-AA<u>GGATCC</u>GCCTTGAGAAGGTGCT-3') was used to generate a 260-bp internal fragment of *flaB* with spirochete DNA as a template. The underlined sequences were *Bam*H1 sites.

After digestion with restriction enzyme and purification, the two amplicons were ligated and then amplified by nested PCR with use of a third primer pair (forward: 5'-CCTCTAGAGTTCATGTTGGAGCAAAC-3'; reverse: 5'-ATTCTAGAATGGAGCCACCGA TCCA-3'). The PCR product was cloned into TA vector pNCO1T as described previously (Downie *et al.*, 2004). The *flaB*-actin insert was confirmed by DNA sequencing using the ABI 3130 Genetic analyzer (Applied Biosystems, Foster City, CA) performed at BioMMed, LSU-SVM, Baton Rouge, LA. DNA concentrations were determined by measuring optical density at 650 nm wavelength and converted to copy numbers.

DNA Preparation. Frozen heart, joint, and skin samples were transferred into liquid nitrogen and ground thoroughly with a mortar and pestle. An appropriate amount of tissue powder was transferred into a 1.5 ml microcentrifuge tube for DNA preparation with the use of DNeasy Mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA).

Quantitative PCR (qPCR). qPCR analyses were performed using the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), at BioMMed, LSU-SVM, Baton Rouge, LA. The Platinum Taq DNA polymerase High fidelity kit was purchased from Invitrogen Life Technologies (Carlsbad, CA). The sequences of primers and internal probes were: for *flaB* (forward: 5'-GCAGCTAATGTTGCAAATCTTTTC-3'; reverse: 5'-GCAGGTGCTGGC TGTTGA-3'; probe: 5'-AAACTGCTCAGGCTCCACCGGTTC-3') and actin (forward: 5'-CCATGTACCCAG GCATTGC-3'; reverse: 5'-CCAGAC TGAGTACTTGCGTTC-3'; probe: 5'- GCAGAAGGA GATCACAGCCCTAGCACC-3'). Taqman 6-carboxytetramethylrhodamine (TAMRA) probes were ordered from Applied Biosystems (Foster City, CA). Amplification was performed in a final volume of 10 µl in the ABI PRISM 384-well clear optical reaction plate (Applied Biosystems, Foster City, CA). Two sets of 12 wells were assigned as DNA standards for actin or *flaB*. The standard concentrations ranged from 10^2 to 10^7 copies/well for actin DNA Quantitation, and from 10^0 to 10^5 copies for *flaB*. Both standards and samples were amplified in duplicate wells. A PCR program with the following parameters was used: 50 °C for 30 seconds; 95 °C for 5 min; 50 cycles of 95 °C for 20 seconds; and 60 °C for 1 min. The mean DNA copy numbers of *flaB* and actin of each DNA sample were calculated from duplicate wells. Tissue spirochete burdens were converted to *flaB* DNA copy number per 2×10^6 actin DNA copies (spirochete burden per 10^6 host cells).

Statistical Analysis. A one-way analysis of variance (ANOVA) was used to analyze data, followed by a two-tailed Student *t* test to calculate a *P* value for each two groups. A *P* value ≤ 0.05 was considered to be significant.

RESULTS

Generation of *B. burgdorferi* Producing OspC with Either 5 or 10 Amino Acid Deletion. *B. burgdorferi* B31 Δ*ospC* mutants were transformed with recombinant plasmid pBBE22-*ospCnt5* (shown in Figure 3.1A) and pBBE22-*ospCnt10* (shown in Chapter 2, Figure 2.4). Seven and six transformants, respectively, were obtained receiving each construct; plasmid analyses by PCR led to the selection of four clones, *ΔospC*/*ΔNt5*/1, *ΔospC*/*ΔNt5*/2, *ΔospC*/*ΔNt10*/1 and *ΔospC*/*ΔNt10*/1. These clones shared the same plasmid content as Δ*ospC*, which had lost lp25, lp5, lp21, lp56 and cp9 (Xu *et al.*, 2007). Expression of the truncated OspC proteins was verified by immunoblot analyses (Figure 3.1B). The *in vitro* growth rate of both clones, *ΔospC*/*ΔNt5* and *ΔospC*/*ΔNt10,* was similar to that of Δ*ospC/FL* controls.

Neither The 5- nor The 10-AA Deletion Affects The Surface Location of OspC. Because the deletion was generated close to the N-terminus, which may harbor the sorting signal, it is important to show that truncated OspC can be successfully sorted to the outer surface of *B. burgdorferi*. To this end, we used indirect immunofluorescence to locate truncated OspC. As shown in Figure 3.2, both truncated proteins showed similar fluorescence patterns as the wild-type control protein, indicating that neither deletion influenced the cellular location of OspC.

The 10- but Not 5-AA Deletion Abolishes Infectivity. As shown in Table 3.3, like the two control clones, the *ΔospC*/*ΔNt5/*1 and *ΔospC*/*ΔNt5/*2 spirochetes were grown from each sample of all inoculated mice. In contrast, neither *ΔospC*/*ΔNt10/*1 nor *ΔospC*/*ΔNt10/*2 bacteria were recovered from any specimens, suggesting that the 10-AA deletion may completely abolish the functions of OspC.
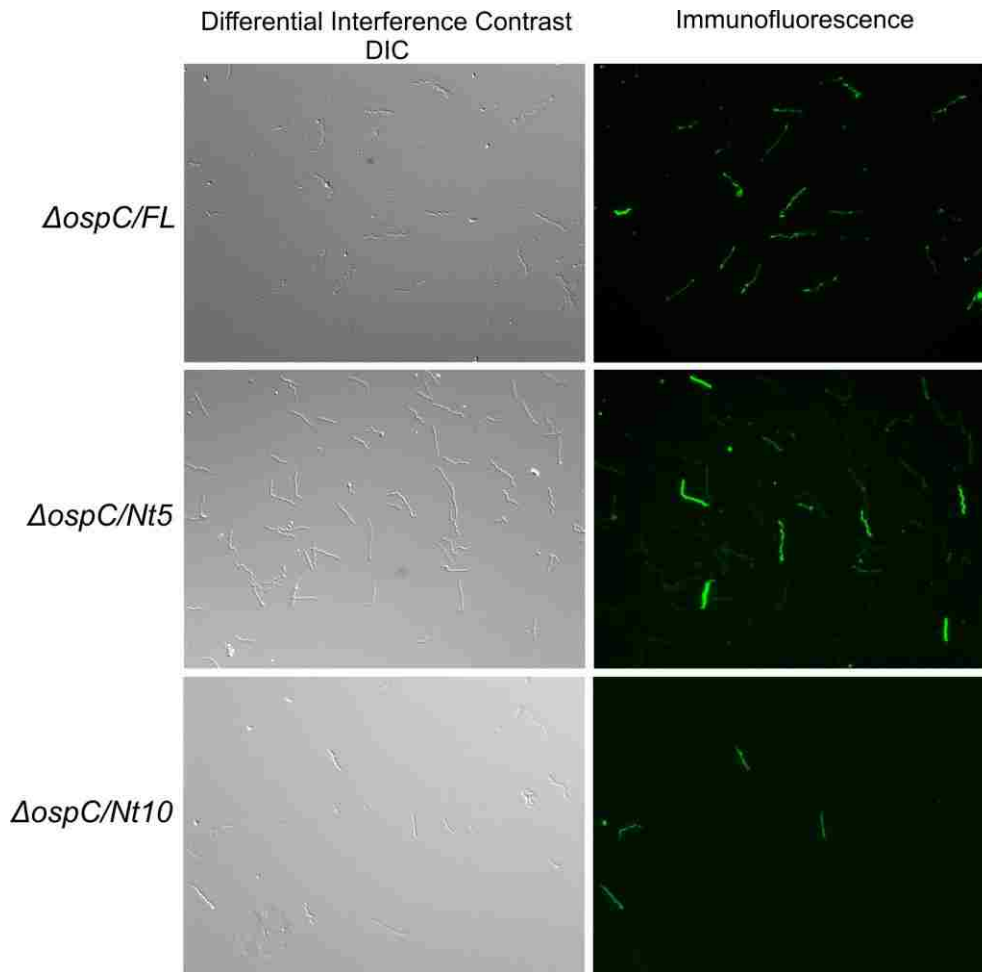
Figure 3.2. Deletion of N-terminus 5-AA or 10-AA does not affect surface exposure of OspC. Δ*ospC/FL*, Δ*ospC/Nt5* or Δ*ospC/Nt10* spirochetes were grown to late log phase (10^8/ml) in BSK-H medium at 33°C. Spirochetes were incubated with mouse mOspC antibodies, washed in PBS, probed with FITC-conjugated goat anti-mouse antibody, washed again before being placed onto slides. Differential interference contrast (left panel) and immunofluorescence images (right panel) were taken from the same field. Magnification: x400.

The *ΔospC/ΔNt10/*1 and *ΔospC/ΔNt10/*2 bacteria might have remained at the inoculation sites without dissemination. To rule out this possibility, C3HS mice were given intradermal/subcutaneous inoculations of *ΔospC/ΔNt10/*1, *ΔospC/ΔNt10/*2, *ΔospC/FL/*1 or *ΔospC/FL/*2 at a total of four sites for each clone and the inoculation sites were harvested at 24h and 48h post-inoculation. Control bacteria were consistently grown from each of the 16

inoculation sites from all eight inoculated mice. In contrast, neither *ΔospC/ΔNt10/*1 nor

*ΔospC/ΔNt10/*2 spirochetes were recovered from any of the 8 sites harvested at each time point,

confirming that the 10-AA deletion completely destroys the functions of OspC and leads to quick

clearance of spirochetes (results shown in Chapter 2, Table 2.3).

The 5-AA Deletion Does Not Affect The ID$_{50}$ Value in SCID Mice. Because the ID$_{50}$

value reflects the smallest number of organisms to sufficiently initiate an infection in 50%

inoculated individuals, it is the best measurable criterion to assess how a pathogen can evade

innate immune elimination, especially when this is measured in SCID mice. To examine

whether the 5-AA deletion reduced the ability of OspC to assist *B. burgdorferi* avoid innate

immunity, groups of three C3H SCID mice each received one single inoculation of 10^1 to 10^4

spirochetes of the clone *ΔospC/ΔNt5/*1, *ΔospC/ΔNt5/*2, Δ*ospC/FL/*1, or Δ*ospC/FL/*2. All animals

were euthanized 1 mo post-inoculation; heart, joint and skin specimens were cultured for

spirochetes. The ID$_{50}$ values of the clones *ΔospC/Nt5/*1 and *ΔospC/Nt5/*2 were 18 and 32

organisms, compared to 18 and 32 organisms determined for the clones *ΔospC/FL/*1 and

*ΔospC/FL/*2, respectively (Table 3.4), indicating that the truncated OspC retains the innate

immunity evasive function.

The 5-AA Deletion Does Not Reduce Spirochete Burdens in Joint or Skin of SCID

Mice. The tissue bacterial load reflects the balance between the replication and death rates so

that it is another measurable criterion to assess how well a pathogen is protected from

elimination by innate immunity (Figure 3.3). To examine the influence of the 5-AA deletion on

the tissue bacterial load, subgroups of five SCID mice each received a single

intradermal/subcutaneous inoculation of 10^4 spirochetes of the clone *ΔospC/Nt5/*1, *ΔospC/Nt5/*2,

*ΔospC/FL/*1, or *ΔospC/FL/*2. In 10 mice that were inoculated with the *ΔospC/FL/*1 or

*ΔospC/FL/*2, joint swelling evolved around 10 d post-inoculation and developed into severe

month post-inoculation; DNA was extracted from heart, joint and skin specimens and quantified

Table 3.3. The 10-AA deletion abolishes infectivity of *B. burgdorferi* [a]

	No. of cultures positive/total specimen examined				No. of mice infected/total mice inoculated
Clone	Heart	Joint	Skin	All sites	
*ΔospC/FL/*1	3/3	3/3	3/3	9/9	3/3
*ΔospC/FL/*2	3/3	3/3	3/3	9/9	3/3
*ΔospC/ΔNt5/*1	3/3	3/3	3/3	9/9	3/3
*ΔospC/ΔNt5/*2	3/3	3/3	3/3	9/9	3/3
*ΔospC/ΔNt10/*1	0/3	0/3	0/3	0/9	0/3
*ΔospC/ΔNt10/*2	0/3	0/3	0/3	0/9	0/3

[a] Groups of three C3H SCID mice were inoculated with 10^4 spirochetes of the clone *ΔospC/FL/*1, *ΔospC/FL/*2, *ΔospC/ΔNt5/*1, *ΔospC/ΔNt5/*2, *ΔospC/ΔNt10/*1, or *ΔospC/ΔNt10/*2. Mice were sacrificed 1 mo post-inoculation; heart, tibiotarsal joint and skin specimens were harvested for spirochete culture in BSK-H complete medium.

for bacterial burden. Although the *ΔospC/Nt5* spirochete burden was 6.7-fold lower than that of

the *ΔospC/FL* ($P = 2.83 \times 10^{-10}$) in the heart tissue, there was no significant difference detected

either in joint ($P = 0.52$) or skin tissue ($P = 0.13$). The study indicated that the 5-AA deletion

does not reduce the ability of OspC to protect *B. burgdorferi* against innate immunity.

The 5-AA Deletion Leads to Slowed Dissemination During Infection of SCID Mice.

Groups of 20 SCID mice each received a single intradermal/subcutaneous inoculation of 10^4

spirochetes of the clone Δ*ospC/FL*/1 or *ΔospC*/ΔNt5/1. Five animals from each group were

euthanized at 1-wk intervals; inoculation site and remote site skin, ear, heart, and joint specimens

were harvested for spirochete isolation. Bacteria were injected into the dermis of the chest so the

skin from the back was harvested as remote sites. As a positive control, the *ΔospC/FL*/1 bacteria

were grown from all of the skin, joint and heart specimens but from none of the ear samples at

first week; all sites became culture positive at 2 wk after initial inoculation (Table 3.5).

Table 3.4. **Deletion of the N terminus 5-AA sequence of OspC does not significantly affect the ID_{50} value in immunodeficient mice [a]**

Clone and dose (no. of organisms)	No. of cultures positive/total no. of specimens examined				No. of mice infected/total no. of mice inoculated	ID_{50} (No. of organisms)
	Heart	Joint	Skin	All sites		
ΔospC/FL/1						18
10^4	3/3	3/3	3/3	9/9	3/3	
10^3	3/3	3/3	3/3	9/9	3/3	
10^2	3/3	3/3	3/3	9/9	3/3	
10^1	1/3	1/3	1/3	3/9	1/3	
ΔospC/FL/2						32
10^4	3/3	3/3	3/3	9/9	3/3	
10^3	3/3	3/3	3/3	9/9	3/3	
10^2	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/9	0/3	
ΔospC/ΔNt5/1						18
10^4	3/3	3/3	3/3	9/9	3/3	
10^3	3/3	3/3	3/3	9/9	3/3	
10^2	3/3	3/3	3/3	9/9	3/3	
10^1	1/3	1/3	1/3	3/9	1/3	
ΔospC/ΔNt5/2						32
10^4	3/3	3/3	3/3	9/9	3/3	
10^3	3/3	3/3	3/3	9/9	3/3	
10^2	2/3	2/3	2/3	6/9	2/3	
10^1	1/3	1/3	1/3	3/9	1/3	

[a] The *ΔospC/FL/1, ΔospC/FL/2, ΔospC/ΔNt5/1and ΔospC/ΔNt5/2* spirochetes were grown to late-log phase (10^8 cells/ml) and 10-fold serially diluted with BSK-H medium. Approximately 100 μl of bacterial suspension was intradermally/subcutaneously inoculated into each C3HS mouse. Animals were sacrificed 1 month later; heart, tibiotarsal joint and skin specimens were harvested for bacterial isolation. The ID_{50} values were calculated by the method of Reed and Muench (Reed and Muench, 1938).

In contrast, the *ΔospC/ΔNt5/*1 spirochetes were grown only from inoculation sites at a week

post-inoculation. Although the mutant disseminated to most of the joint, remote skin and heart

specimens at 2 wk, the majority of ear tissues were not colonized until 3 wk. These data

indicated that OspC is important for efficient dissemination.
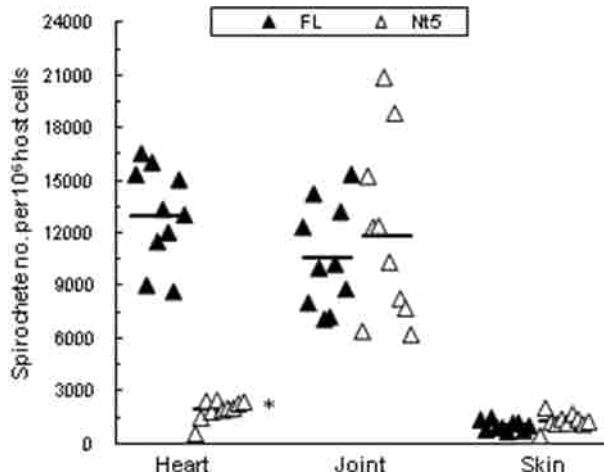
Figure 3.3. The N-terminal 5-AA deletion does not reduce the ability of *B. burgdorferi* to colonize joint or skin but heart tissues of SCID mice. Subgroups of five SCID mice were inoculated with 10^5 spirochetes of the clone *ΔospC*/FL/1, *ΔospC*/FL2, *ΔospC*/ΔNt5/1, or *ΔospC*/ΔNt5/2, and euthanized a month later. DNA was prepared from heart, joint and skin specimens and analyzed for spirochete *flaB* and murine actin DNA copies by qPCR. Data are expressed as spirochete numbers per 10^6 host cells and presented in four groups by combining the subgroups *ΔospC/FL*/1 and *ΔospC/FL*/2, *ΔospC/ΔNt5/1* and *ΔospC/ΔNt5*/2 and named as FL and Cnt5 respectively.*, *P* < 0.05 between FL and Cnt5.

The 5-AA Deletion Causes Severely Impaired Dissemination During Infection of

Immunocompetent Mice. More severely impaired dissemination was noted during infection of

immunocompetent mice. Groups of 24 BALB/c mice each received a single

intradermal/subcutaneous inoculation of 10^5 spirochetes of the clone Δ*ospC*/*FL*/1 or

ΔospC/ΔNt5/1. Six animals from each group were euthanized at 1-wk intervals; inoculation site

and remote site skin, ear, heart, and joint specimens were harvested for spirochete isolation.

Bacteria were injected into the dermis of the chest so the skin from the back was harvested as

remote sites. As a positive control, the *ΔospC*/*FL*/1 bacteria were grown from all of the remote

tissues but the ear at first week; all sites became culture positive at 2 wk after initial inoculation

(Table 3.6). In contrast, the *ΔospC*/ΔNt5/1 spirochetes were not grown from joint tissues until 4

weeks after inoculation, and no heart, remote skin or ear tissues became culture positive during

the period. These data further highlights that OspC is required for efficient dissemination.

DISCUSSION

Like typical Gram-negative bacteria, *B. burgdorferi* possesses inner and outer

membranes, between which is a periplasmic space (Cullen *et al.*, 2004, Steere, 2001). Gram-

negative pathogens typically make a thick lipopolysaccharide (LPS) coat to provide a broad

array of crucial protection (Raetz and Whitfield, 2002). However, *B. burgdorferi* does not

produce any LPS but instead abundantly expresses lipoproteins and anchors them to the outer

membranous surface through lipidation (Cullen *et al.*, 2004, Radolf *et al.*, 1994, Takayama *et al.*,

1987). This unique surface antigenic architecture makes the host-pathogen interactions more

complicated and may contribute to the pathogenic strategy of *B. burgdorferi*. As protein is a

direct product of gene expression, regulation of surface antigen expression can quickly result in

modification of the surface antigenic architecture. *B. burgdorferi* indeed takes this advantage and

vigorously modifies its surface lipoprotein expression to constantly reformulate its overall

surface antigenic architecture during the enzootic life cycle traveling between the tick vector and

a mammal, and during the course of mammalian infection (Yang *et al.*, 2004). The pathogen

abundantly expresses outer surface proteins (Osps) A and B in the unfed tick (Schwan and

Piesman, 2000, Schwan *et al.*, 1995), suggesting a critical role for these surface antigens while

residing in the tick vector, which has been confirmed by series of subsequent studies (Fikrig *et

al.*, 2004, Neelakanta *et al.*, 2007, Ohnishi *et al.*, 2001, Yang *et al.*, 2004). A fresh blood meal

induces the down-regulation of OspA/B and the up-regulation of OspC and other lipoproteins, a

process that prepares *B. burgdorferi* for infection of mammals (Fingerle *et al.*, 2007, Grimm *et

al.*, 2004b, Pal *et al.*, 2004, Stewart *et al.*, 2006). Abundant OspC expression ultimately induces a

robust early humoral response that imposes tremendous pressure on the pathogen (Fung *et al.*,

1994, Xu *et al.*, 2006). To evade the specific humoral response and cause persistent infection, *B. burgdorferi* down-regulates OspC and dramatically upregulates other surface lipoproteins, including VlsE and BBF01 (Crother *et al.*, 2004, Liang *et al.*, 2004a, Liang *et al.*, 2002a, Liang *et al.*, 2002b). This demonstrated *ospC* expression pattern strongly suggested its early role in mammalian infection. This initial requirement has been confirmed as genetic manipulation of *B. burgdorferi* became possible (Stewart *et al.*, 2006, Tilly *et al.*, 2007, Tilly *et al.*, 2006).

Recent studies showed that this initial requirement for OspC can be overridden by either increasing expression of another Osp (Xu *et al.*, 2008), or simply adapting *ospC* mutants in mammalian hosts (Tilly *et al.*, 2009) . The adaptation process apparently is to increase expression of other Osps, which replace lost OspC functions. Apparently, increasing expression of Osps, either via genetic modification or adaptation in mammalian host, can at least partially replace OspC. This raises a question about OspC functions. These studies led us to hypothesize that the borrelial lipoproteins may have a common function, which is to protect *B. burgdorferi* against innate immunity, while the unique function of OspC is to facilitate dissemination, which can be only partially replaced by other Osps. In the current study, we successfully separated protective function and dissemination-facilitating function. We successfully generated a truncated OspC, which can effectively protect *B. burgdorferi* against innate elimination but fails to make spirochetes able to disseminate as efficiently as the wild-type control.

Our previous study showed that increasing production of one of four randomly chosen but well-investigated surface lipoproteins can effectively replace OspC to protect *B. burgdorferi* against innate immunity, clearly suggesting that any surface lipoprotein apparently can substitute OspC for this function. What strikes us is that not all truncated OspC mutants are able to protect *B. burgdorferi* against innate immunity while any other wild-type surface lipoprotein can replace OspC for this specific function. Although the 5-AA deletion retains the full

Table 3.5. N-terminal 5-AA deletion of OspC impairs the ability of *B. burgdorferi* to disseminate to remote tissues in immunodeficient mice. [a]

Clone	No. of specimens positive/total specimens examined at post-inoculation weeks														
	1 wk					2 wks					3 wks				
	I.S.	R.S.	Ear	Heart	Joint	I.S.	R.S.	Ear	Heart	Joint	I.S.	R.S.	Ear	Heart	Joint
ΔospC/ospC	5/5	5/5	0/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	ND[b]	ND	ND	ND	ND
ΔospC/ΔNt5	5/5	0/5	0/5	0/5	0/5	5/5	4/5	1/5	4/5	5/5	5/5	5/5	5/5	5/5	5/5

[a] Groups of 10 to 15 C3HS SCID mice each received a single intradermal/subcutaneous injection of 10^3 organisms of the clone *ΔospC/ospC* or *ΔospC/ΔNt5*. Five animals from each group were euthanized at 1, 2, or 3 wk post-inoculation; inoculation site (I.S.) and remote site (R.S.) skin, ear, heart, and joint specimens were harvested for spirochete isolation. The I.S. site was at the chest; therefore the R.S. site was at the back of mice. b. ND, not determined.

Table 3.6. N-terminal 5-AA deletion of OspC severely impairs the ability of *B. burgdorferi* to disseminate to remote tissues in immunocompetent BALB/c mice. [a]

Clone	No. of specimens positive/Total specimens examined at post-inoculation weeks																			
	1 wk					2 wks					3 wks					4 wks				
	I.S.	R.S.	Ear	Heart	Joint	I.S.	R.S.	Ear	Heart	Joint	I.S.	R.S.	Ear	Heart	Joint	I.S.	R.S.	Ear	Heart	Joint
ΔospC/ospC	6/6	6/6	0/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
ΔospC/ΔNt5	6/6	0/6	ND	0/6	1/6	1/6	0/6	0/6	0/6	0/6	2/6	0/6	0/6	0/6	6/6	6/6	0/6	0/6	0/6	6/6

[a] Groups of 24 BALB/c mice each received a single intradermal/subcutaneous injection of 10^5 organisms of the clone *ΔospC/ospC* or *ΔospC/ΔNt5*. Six animals from each group were euthanized at 1, 2, 3 or 4 wk post-inoculation; inoculation site (I.S.) and remote site (R.S.) skin, ear, heart, and joint specimens were harvested for spirochete isolation. The I.S. site was at the chest; therefore the R.S. site was at the back of mice.

capacity of OspC to protect *B. burgdorferi* against innate elimination, the 10-AA deletion completely destroys its functions. Interestingly, a previous study showed that any of the four Osps tested can replace OspC to protect *B. burgdorferi* from innate elimination. However, OspC itself, after 10-AA deletion, failed to protect *B. burgdorferi*. This suggests that each Osp may protect *B. burgdorferi* against innate immunity in a unique method.

After inoculated into murine skin, *B. burgdorferi* must first be able to evade initial innate immune elimination and gain a foothold. This ability is best represented by the ID_{50} value because this criterion measures the smallest number of organisms that is sufficient to escape initial innate elimination. Regardless of how large the inoculum is, OspC-deficient spirochetes are quickly cleared after inoculation into murine skin (Tilly *et al.*, 2007, Xu *et al.*, 2008) . Production of OspC with 5-AA deletion conferred OspC deficient *B. burgdorferi* with ID_{50} values similar to the control, indicating that the truncated molecule has the full protective function of OspC.

Although tissue colonization is an extremely complicated event carried out by an extracellular bacterial pathogen during infection and can be affected by many factors, one can predict that the ability of the pathogen to evade immune clearance should be one of the major determinants. *B. burgdorferi* producing OspC with 5-AA deletion was able to generate similar bacterial loads in both joint and skin tissues of SCID mice as the control, indicating that the truncated OspC can effectively assist the pathogen evade innate immune clearance at least in these tissues. Because many other determinants, such as the interactions of host cells and extracellular matrices with the pathogen mediated with its surface antigens, may significantly influence tissue colonization, the lower *ΔospC/ΔNt5* spirochete load in the heart does not necessarily suggest that the truncated OspC cannot effectively provide evasion from host innate immune defenses. Alternatively, OspC may be an important factor contributing to the ability of

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B. burgdorferi to colonize the heart tissue. This explanation is also supported by previous studies showing that treatment with OspC antibody induces the downregulation of *ospC* and more effectively reduces the bacterial load in the heart more than other tissues (Liang *et al.*, 2004a, Liang *et al.*, 2004b).

Both ID_{50} and tissue bacterial load data demonstrated that the 5-AA deletion does not affect the innate immune evasive function of OspC, allowing us to dissect the evasive from dissemination-facilitating functions. OspC-deficient *B. burgdorferi* producing OspC with 5-AA deletion disseminated to remote tissues, especially to the heart and ear at a significantly slower pace in immunodeficient mice. In immunocompetent mice, the defect caused by deletion was much more severe. Four weeks after inoculation, the mutant was not able to colonize heart or ear tissues, while the control disseminated to all tissues examined within 2 weeks. Given the difference between immunodeficient and immunocompetent mice, infection in the latter induces an anti-OspC humoral response, which in turn may further slow dissemination. The observation highlights the importance of quick dissemination of *B. burgdorferi* during infection of immunocompetent animals. Infection induces immune responses that slow dissemination. If *B. burgdorferi* fails to colonize remote tissues and establish a systemic infection before effective immune responses have developed, its dissemination would be dramatically slowed.

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CHAPTER 4

OUTER SURFACE PROTEIN C IS INVOLVED IN SELF-REGULATION IN *BORRELIA BURGDORFERI* DURING MURINE INFECTION

<u>INTRODUCTION</u>

Outer surface protein C (OspC) is a critical virulence factor of the Lyme disease spirochete *Borrelia burgdorferi*. Timely expression of OspC is essential for the survival of the spirochete as it transits between a tick vector and a mammalian host (Burgdorfer *et al.*, 1982). The pathogen abundantly produces OspC during initial infection (Liang *et al.*, 2002a, Liang *et al.*, 2002b), when the antigen is required for efficient dissemination (Xu *et al.*, 2008b) (and, as shown in Chapter 3), but represses its production to a baseline level after specific humoral responses have developed and distal tissues have been colonized (Liang *et al.*, 2004b, Xu *et al.*, 2006). The downregulation of OspC allows *B. burgdorferi* to not only effectively evade the immune system but also preserve the integrity of the crucial gene for the subsequent enzootic cycle, because a failure to downregulate *ospC* would lead to either clearance of infection or selection of *ospC* mutants (Xu *et al.*, 2006). OspC production is driven by an RpoS-dependent promoter (Yang *et al.*, 2005) and its repression appears to be achieved through the interaction of the operator with an unknown repressor (Xu *et al.*, 2007). Here we show that deletion of an N-terminal 5-amino acid sequence of OspC diminishes the ability of *B. burgdorferi* to repress *ospC* expression during murine infection and, as a result, dramatically reduces its ability to evade the immune system. Further investigation may lead to disclosure of how OspC can be involved in self regulation.

Coordinated production of OspC is crucial for the pathogenic strategy of the Lyme disease spirochete, *B. burgdorferi* (Burgdorfer *et al.*, 1982). In unfed ticks, *B. burgdorferi* does not express OspC (Ohnishi *et al.*, 2003, Schwan *et al.*, 1995); a fresh blood meal upregulates

OspC, a process that prepares the pathogen for infection of a mammal (Grimm *et al.*, 2004b, Pal *et al.*, 2004, Stewart *et al.*, 2006). *B. burgdorferi* abundantly expresses OspC during early infection when the antigen is required for efficient dissemination as shown in Chapter 3 of this dissertation and in Liang, *et al*., 2004, Tilly, *et al*., 2006, and Xu *et al*., 2008 (Liang *et al.*, 2004a, Tilly *et al.*, 2006, Xu *et al.*, 2008a). However, OspC is not only a strong immunogen, but also an effective target of protective immunity; its production ultimately induces a robust humoral response that imposes tremendous pressure on the pathogen (Fung *et al.*, 1994, Liang et al., 2004a). If *B. burgdorferi* fails to repress *ospC* expression or undergo escape mutations on the *ospC* gene, infection would be cleared (Xu *et al.*, 2006). While mutation is an effective way to evade the immune system, it may cause the functional loss of affected genes. Although the spirochetes with lost OspC function may persist during chronic infection (Tilly *et al.*, 2006), they are unable to initiate an infection (Stewart *et al.*, 2006), thus leading to a discontinuation of the enzootic cycle. Therefore, the downregulation of OspC allows the pathogen to not only effectively evade the specific immune response but also to preserve the integrity of the *ospC* gene for subsequent need during its life cycle (Liang *et al.*, 2004a, Liang *et al.*, 2002a). It is also critical for *B. burgdorferi* to maintain an OspC downregulation status after acquired by the tick vector as specific antibodies in bloodmeal can efficiently kills spirochetes (Gilmore and Piesman, 2000). Overall, when OspC is required, *B. burgdorferi* up-regulates it; when its presence confers disadvantage to the bacteria, the pathogen down-regulates it.

While many gaps remain to be filled, recent advances in borrelial genetic study provide a relatively clear picture of how *ospC* is regulated during the course of the enzootic cycle. *ospC* expression uses a well-defined RpoS-dependent promoter (Eggers *et al.*, 2004, Yang *et al.*, 2005). In response to a fresh bloodmeal, *B. burgdorferi* upregulates the alternative σ factor, which in turn initiates expression of many genes important for mammalian infection, including

ospC (Caimano *et al.*, 2007). Because RpoS controls the expression of multiple crucial virulence determinants, *B. burgdorferi* cannot afford to shut off *rpoS* in order to realize *ospC* downregulation during mammalian infection. Instead, the pathogen is most likely to induce expression of an unknown repressor, which specifically interacts with the *ospC* operator and shuts the gene off without affecting expression of other RpoS-dependent genes in response to the development of specific humoral responses (Xu *et al.*, 2007). Once acquired by the tick vector, *B. burgdorferi* turns *rpoS* off, and consequently *ospC* and other RpoS-dependent genes (Caimano *et al.*, 2007), regardless of whether the *ospC* operator-repressor system is involved.

Either down- or up-regulation of RpoS is initiated in the tick vector (Caimano *et al.*, 2007), while the *ospC* operator-repressor system is needed only in the response to the specific humoral response during mammalian infection (Xu *et al.*, 2007). A series of seminal studies by Norgard and colleagues showed that *rpoS* expression is dependent on the response regulator protein, Rrp2, which along with RpoN regulates expression of RpoS (Hubner *et al.*, 2001, Smith *et al.*, 2007, Yang *et al.*, 2003). However, nothing is known about how the environmental cues are converted into regulatory events. In our previous study, we noticed that the *ospC* regulatory element did not function well when it controlled *ospA* expression in an *ospC* knockout mutant, leading us to hypothesize that OspC may be involved in self-regulation. The current study focused on this issue.

Structurally, OspC is predominantly alpha-helical and forms a dimer in solution. The lipoprotein is made up of five alpha-helices, two beta sheets, six loops and a carboxy (C-) terminus and an amino (N)-terminus (Eicken *et al.*, 2001, Kumaran *et al.*, 2001). The amino-terminus anchors the lipoprotein to the outer surface of the outer membrane of *Borrelia* by a lipidated cystein (Hayashi and Wu, 1990, Pugsley and Possot, 1993). Since the N-terminus is the only portion of the crystal structure of OspC that connects the rest of the lipoprotein to the outer

membrane, this region was chosen for a minor amino acid deletion mutation and then tested for resulting changes in *ospC* regulation.

MATERIALS AND METHODS

Strains and Constructs Generated Previously and Used in This Study. The *B. burgdorferi* B31 clone 13A and the ospC mutant were generated previously (Xu *et al.*, 2007). The shuttle vector pBBE22 was a gift from S. Norris (Purser *et al.*, 2003). The features of the constructs and clones used in this study are summarized in Table 4.1

Table 4.1. Constructs and clones used in the study.

Construct or clone	Description	Source
pNCO1T	homemade T/A cloning vector	(Downie, *et al.*, 2004)
pNCO1T-*ospC*	pNCO1T carrying *ospC* gene driven by its native promoter	This study
pNCO1T-*ospCnt5*	pNCO1T carrying *ospC* gene expressing N-terminus 5-AA deletion	This study
pBBE22	pBSV2 carrying a *bbe22* copy	(Purser *et al.*, 2003)
pBBE22-*ospCnt5*	pBBE22 carrying *ospC* gene expressing N-terminus 5-AA deletion	This study
13A	*B. burgdorferi* B31 clone lacking plasmids lp25 and lp56	(Xu *et al.*, 2007)
ΔospC	*ospC* mutant	(Xu *et al.*, 2007)
*ΔospC/FL/*1	*ospC* mutant expressing *ospC* controlled by *ospC* regulatory elements[a]	(Xu *et al.*, 2007)
*ΔospC/FL/*2	*ospC* mutant expressing *ospC* controlled by *ospC* regulatory elements	(Xu *et al.*, 2007)
*ΔospC/Nt5/*1	*ospC* mutant expressing N-terminus 5-AA deletion	This study
*ΔospC/Nt5/*2	*ospC* mutant expression N-terminus 5-AA deletion	This study

[a.] The *ospC* regulatory elements include both operator and promoter.

Generation of *B. burgdorferi* Producing OspC with N-terminus 5-AA Deletion. As illustrated in Figure 4.1A, to efficiently generate an N-terminus deletion, a 1057-bp fragment covering the *ospC* region and the up- and down-stream sequences was amplified on genomic DNA by PCR using primers P1F and P1R (Table 4.2) and cloned into the TA cloning vector pNCO1T (Downie *et al.*, 2004), creating an intermediate vector designated pNCO1T-*ospC*. One linear and large amplicon was generated by inverse PCR using pNCO1T-*ospC* as a template and primers P2F and P2R (Table 4.2). After digestion with *Sap*I and subsequent purification, the amplicon was circularized via ligation and then digested with *Bam*H1 and *Xba*I to release *ospCnt5*. This fragment, encoding an OspC variant with N-terminal 5-AA deletion, was cloned

into the recombinant plasmid pBBE22 after the vector was digested with *Bam*H1 and *Xba*I. The resulting construct was designated pBBE22-*ospCnt5*. The insert and its flanking regions within pBBE22 were sequenced to ensure the construct was as designed.

The complementation plasmid was then electroporated into an ospC mutant ($\Delta ospC$), which was generated in our previous study (Xu *et al.*, 2007); the seven resulting transformants were first screened for the presence of lp28-1, a plasmid essential for persistent infection in immunocompetent hosts (Grimm *et al.*, 2004a, Xu *et al.*, 2005). Two of the transformants that contained lp28-1 were further analyzed for the content of those plasmids that tend to be spontaneously lost during in vitro propagation, as described previously (Xu *et al.*, 2005). The plasmid contents for both these clones were identical with the presence of lp28-2, lp28-4 and cp32-3. Efficient production of OspC protein by the clones was verified by immunoblots probed with a mixture of FlaB and OspC monoclonal antibodies (MAbs) (Figure 4.1B) as previously described (Xu *et al.*, 2006). The clone 13A, which was generated in our previous study was included as a control (Xu *et al*., 2007).

Indirect Immunofluorescene Assay (I-IFA): Indirect immunofluorescence was performed on unfixed and fixed spirochetes. Briefly, *B. burgdorferi* was grown to late log phase (10^8 cells/ml) in BSK-H complete medium at 33 °C. For fluorescent labeling of unfixed spirochetes, 6×10^7 cells were harvested from 0.6 ml of ΔospC/*FL* or ΔospC/*Nt5* mutant spirochetes by centrifugation at 8,000xg for 10 min, gently suspended in 100 μl PBS supplemented with 3.6 μl of mouse OspC or FlaB MAb preparation, and incubated for 1 hour at 33 °C. After one wash with excess volume of PBS by centrifugation at 10,000xg for 10 min, spirochetes were resuspended in 100 μl of PBS containing 1 μg of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Rockford, IL), incubated for 1 hour, washed once with PBS by centrifugation, resuspended in 30μl PBS, applied to
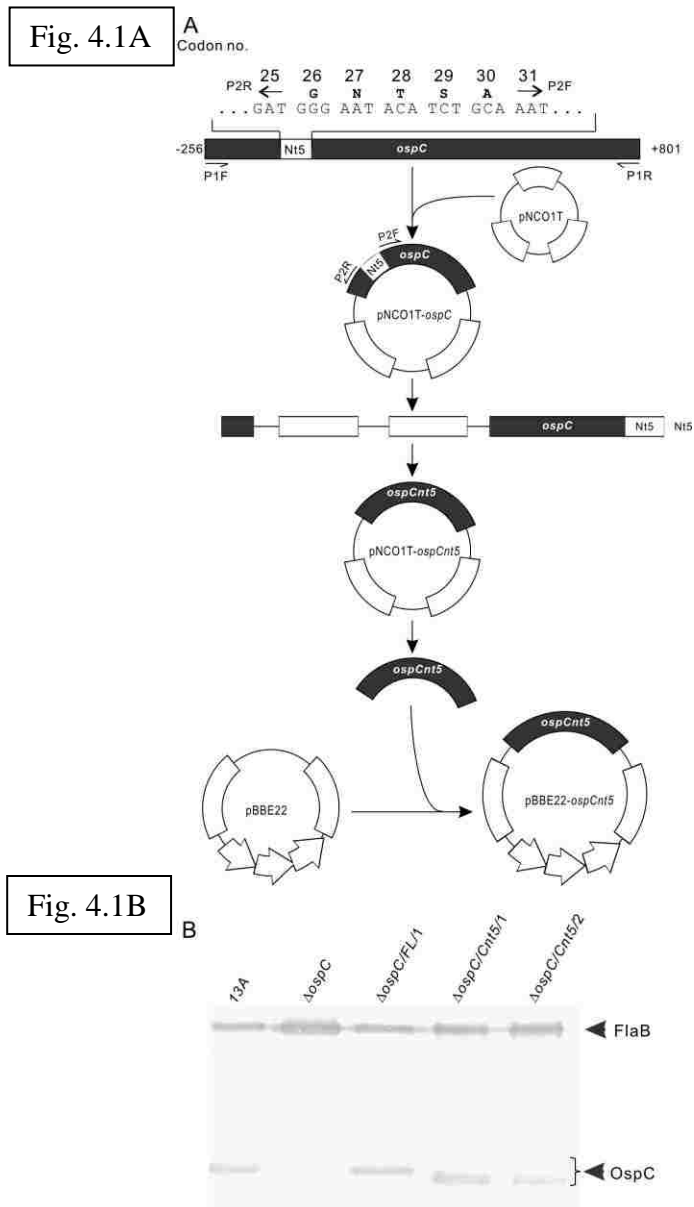
Figure 4.1. Generation of *B. burgdorferi* producing OspC with N-terminal 5-AA deletion.
A. Construction of pBBE22-*ospCNt5* coding for OspC with N-terminal 5-AA deletion. The
five codons coding for the five amino acid residues (residues 26—30) and their adjacent codons
are presented. The amplification starting sites and directions of four primers, P1F, P1R, P2F and
P2R, used for plasmid construction are also marked. The long bar represents the 1057-bp
fragment, covering the entire *ospC*-coding region and down- (extending to +801 from the
transcriptional start site) and up-stream sequences (extending to -256). The detailed construction
of the vector was described in Materials and Methods section. **B. Restoration of OspC
production.** The parental clone 13A, Δ*ospC*, and the clones Δ*ospC*/*FL*, *ΔospC*/*Nt5*/1 and
Δ*ospC*/*Nt5*/2 were verified for OspC expression by immunoblot probed with a mixture of FlaB
and OspC MAbs.

Table 4.2 Primers used in this study:

_____

Construct:	Primer name	Primer sequence (5'-3')[a]

_____

FL	P1F	5'-TAGTTGGCTATATTG<u>GGATCC</u>AA-3'
	P1R	5'-TTCC<u>TCTAGA</u>GAAGAGCTTAAAGTTAA-3'
ΔNt5	P2F	5'-GACT<u>GCTCTTC</u>AGACAATTCTGCTGATGAGTCT-3'
	P2R	5'-CGTAC<u>GCTCTTC</u>AGTCTTTCCCAGAATTATTACAAG-3'

_____

[a] The underlined sequences are restriction enzyme sites. P1F contains a *Bam*HI site; P1R has an *Xba*I site; P2F and P2R have a *Sap*I site.

microscopic slides, and analyzed using Axio Imager (Carl Zeiss Microimaging, Inc., Thornwood, NY).

For fluorescent labeling of fixed spirochetes, organisms were harvested from 0.6 ml culture by centrifugation, suspended in 50 μl of PBS, followed by addition of 700 μl acetone (Mallincrodt Baker, Phillipsburg, NJ), incubated at room temperature for 10 min, and then centrifuged at 10,000xg for 10 min. After being washed once with excess volume of PBS, fixed spirochetes were processed as described above for fluorescent labeling of unfixed bacteria.

Quantitation of *ospC* Gene Expression. C3H SCID mice (ages 4-6 weeks; provided by the Division of LSU Laboratory Animal Medicine) each were intradermally/subcutaneously inoculated with 10^4 spirochetes. Animals were sacrificed one month post-inoculation; heart, joint, and skin specimens were harvested for RNA extraction. RNA was quantified for *flaB* and *ospC* mRNA copy numbers by reverse transcription qPCR (RT-qPCR) as described below. Data are presented as *ospC* transcripts per 1,000 *flaB* mRNA copies.

RNA Preparation. Frozen heart, joint, and skin samples were transferred in liquid nitrogen and ground thoroughly with a mortar and pestle. RNA was isolated with the use of Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). Briefly, 1ml of Trizol was added to the sample when the mortar became wet. The solution containing the powder was gently mixed

and transferred to a microcentrifuge tube by pipetting, and the sample was incubated for 5 minutes at room temperature. 0.2 ml of chloroform:isoamyl alchohol (24:1) was added to the tube and mixed vigorously and incubated again for 3 minutes. Samples were spun at 12400 rpm for 15 minutes at 4 °C. The upper colorless aqueous phase was transferred to a fresh microfuge tube. 0.5 ml of Isopropanol was added, incubated at room temperature for 10 minutes and centrifuged for 10 minutes at 12400xg at 4 °C. The supernatant was removed by pipetting. The RNA pellet was washed with 1 ml of fresh 75% cold Ethanol, vortexed and centrifuged at 10,000 rpm for 5 minutes at 4 °C. Ethanol was removed as much as possible. Samples were stored at -20 °C for a short period.

cDNA Preparation. The DNA-free RNA preparation was first annealed with reverse oligonucleotide mixture of *flaB* (5'-ATTCCAAGCTCTTCAGCTG-3') and *ospC* (5'-CAGCCATCAGTAACACCTTC-3') genes at 65, 60, 55, 50, and 45 °C each for 1 min, in the presence of reverse transcription (RT) buffer (Invitrogen). Deoxynucleoside triphosphates and SuperScript II RNase H⁻ reverse transcriptase (Invitrogen) were added, RT was conducted at 42 ° C for 1 h, and then reaction mixture was inactivated at 70 °C for 15 min.

Quantitative RT-PCR (qRT-PCR). qRT-PCR analyses were performed by using the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The Platinum Taq DNA polymerase high fidelity kit was purchased from Invitrogen Life Technologies (Carlsbad, CA). The sequences of primers and internal probes were: for *flaB* (forward: 5'-GAGTTTCTGGTAA GATTAATGCTC-3'; reverse: 5'-CATTTAAATTCCCTTCTGTTG TCTGA-3'; probe: 5'-AGAGGTTTGTCACAAGCTTCTAGAAATACTTCAAAGGC-3') and for *ospC* (forward: 5'-TACGGATTCTAATGCGGTTTTAC-3'; reverse: 5'-GTGATTATT TTCGGTATCCA AACCA-3'; probe: 5'-TGAAGCGTTGCTGTCATCTATAGATGAAATTG CTGCT-3').

Passive Immunization of SCID Mice with Anti-OspC Monoclonal Antibody. C3HS

SCID mice (ages 4-6 weeks; provided by the Division of LSU Laboratory Animal Medicine)

each were given a single intradermal/subcutaneous injection of 10^5 $\Delta ospC/FL$ or $\Delta ospC/Nt5$

spirochetes. At one month post-inoculation, OspC monoclonal antibody B5 (Mbow *et al.*, 1999)

or purified normal mouse IgG2a isotype (Sigma Aldrich) as a control was given at 100 µg. Three

days later, mice were killed and heart, joint, skin (remote), and ear specimens were collected for

bacterial culture.

Evaluation of Anti-*Borrelia* Specific Immune Response in Wild Type Mice. BALB/c

mice (ages 4-8 weeks; provided by the Division of LSU Laboratory Animal Medicine) each

received a single intradermal/subcutaneous injection of 10^5 $\Delta ospC/FL$ or $\Delta ospC/Nt5$ spirochetes

and were euthanized at 1, 2, 3 and 4 weeks post inoculation. Tiobiotarsal joint, inoculation site

and remote site skin, ear and heart specimens were harvested for spirochete isolation as

described previously (Xu *et al.*, 2005). Spirochetes were injected into the dermis of the chest, so

the skin from the back was harvested as remote site.

Statistical Analysis. A one-way analysis of variance (ANOVA) was used to analyze

data, followed by a two-tailed Student *t* test to calculate a *P* value for each two groups. $P \le 0.05$

was considered to be significant.

RESULTS

Generation of *B. burgdorferi* Producing OspC with N-terminal 5-AA Deletion.

One construct, namely pBBE22-*ospCnt5*, was electroporated into the *ospC* mutant $\Delta ospC$, which

was generated and characterized in our previous study (Xu *et al*., 2007). pBBE22-*ospCnt5* was

constructed as illustrated in Figure 4.1A. Because $\Delta ospC$ had lost lp25, the plasmid that carries

the gene *bbe22* coding for a nicotinamidase essential for survival of *B. burgdorferi* in the

mammalian environment, the recombinant plasmid pBBE22, which harbors a copy of *bbe22*,

was used as the shuttle vector (Purser *et al.*, 2003). Thirteen transformants were obtained; plasmid analyses led to selection of two clones, namely *ΔospC*/*CNt5*/1 and *ΔospC*/*CNt5*/2. Both clones shared the same plasmid content as Δ*ospC*, which had lost lp25, lp5, lp21, lp56 and cp9 (Xu et al., 2007). Expression of truncated OspC resulting from the introduced construct was confirmed by immunoblot analysis (Figure 4.1B).

The N-terminal Truncation Does Not Affect The Surface Location of OspC. The cellular location of borrelial lipoprotein is primarily determined by the first few residues following the signal peptide (Schulze and Zuckert, 2006). To investigate whether removal of the N-terminal 5 residues affects the surface location of OspC, indirect immunofluorescence was performed using mOspC (Figure 4.2A). Since flagellin is a periplasmic protein, FlaB MAb was used as an internal control (Figure 4.2B). Both Δ*ospC/FL* and Δ*ospC/ΔNt5* spirochetes fluoresced after incubation with anti-OspC antibodies (Figure 4.2A), indicating that the truncation does not affect the cellular location of the lipoprotein. FlaB MAb could not bind its antigen in Δ*ospC/FL* unfixed spirochetes but resulted in strong fluorescence on all fixed bacteria (Figure 4.2B), indicating that our indirect immunofluoresence procedure did not expose internal antigens of unfixed spirochetes.

Deletion of The N-terminal 5-AA Sequence Severely Affects The Ability of *B. burgdorferi* to Downregulate *ospC* During Infection of SCID Mice. Aimed at investigating whether deletion of N-terminal truncation affects *ospC* regulation, *ospC* mRNA accumulation was analyzed. RNA samples were prepared from the heart, joint, and skin specimens of the 10 infected mice used for the determination of spirochetal loads, and quantified for *flaB* and *ospC* expression by RT-qPCR. The results are shown in Figure 4.3.

There was no significant difference in the *ospC* mRNA accumulation between *ΔospC*/*Nt5* and Δ*ospC/FL* spirochetes in the heart tissue ($P =0.3145$). In the joint tissue, *ospC* mRNA levels

were significantly higher in the *ΔospC*/*Nt5* spirochetes when compared to Δ*ospC*/*FL* spirochetes (*P* <.0001). In the skin, there was no significant difference in the levels of *ospC* mRNA when *ΔospC*/*Nt5* spirochetes were compared to Δ*ospC*/*FL* spirochetes (*P* = 0.1064).

The results indicate that, in the joint, operator of the spirochetes expressing truncated *ospC* was unable to repress *ospC* mRNA expression, when compared to wild type controls. Taken together, these data indicated that deletion of the N-terminal 5-AA sequence led to significant changes in *ospC* mRNA accumulation in the joints by *B. burgdorferi* during murine infection.

OspC Monoclonal Antibodies Clear *Borrelia* Expressing N-terminus 5-AA Deleted OspC. The *ospC* promoter is RpoS-dependent (Eggers *et al.*, 2004, Yang *et al.*, 2000, Yang *et al.*, 2005). Because RpoS is constitutively expressed during mammalian infection, the downregulation of *ospC* is realized through the interaction of the *ospC* operator with its specific, but as-yet unidentified, repressor (Xu *et al.*, 2007). If the coding region of OspC is involved in self-regulation, spirochetes containing a deletion of the N-terminus 5-AA sequence of OspC would be affected adversely by anti-OspC borreliacidal antibodies.

To examine whether specific anti-OspC antibodies in the absence of T and B cells are able to kill Δ*ospC*/*Nt5 Borrelia* for their inability to downregulate *ospC*, a passive immunization study was conducted (Table 4.3). C3H SCID mice were infected with Δ*ospC*/*FL* and Δ*ospC*/*Nt5* *B. burgdorferi* for one month, a time point at which *Borrelia* would still express OspC, and then passively immunized either mouse IgG2a as a control or OspC monoclonal antibody B5. This monoclonal antibody was shown to passively protect mice from a tick-transmitted challenge infection (Mbow *et al.*, 1999). Previous studies have shown that *B. burgdorferi* persistently
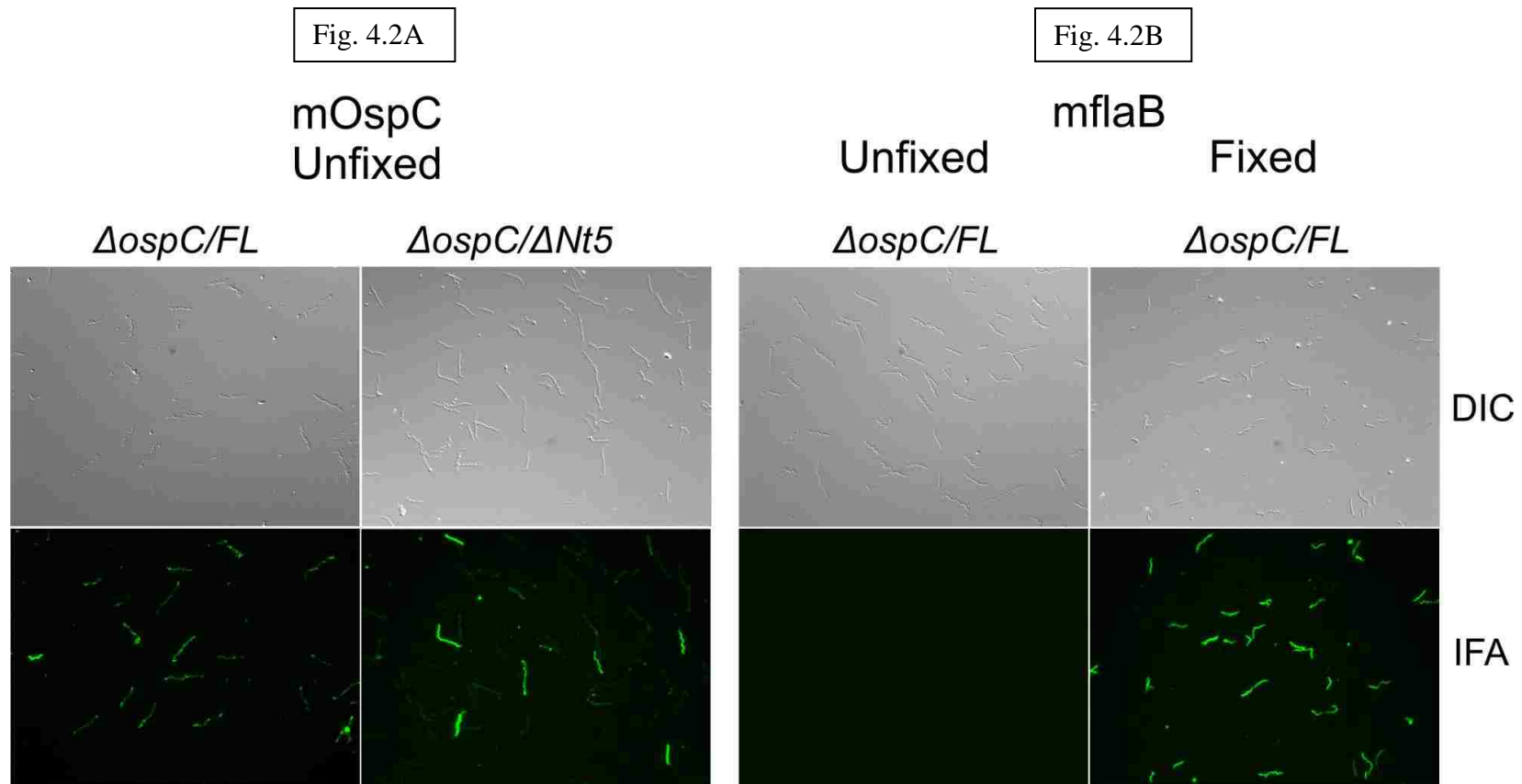
Figure 4.2. The N-terminal 5-AA deletion does not affect the surface exposure of OspC. The clones Δ*ospC/FL* and Δ*ospC/Nt5* were grown to late-log phase and analyzed by indirect immunofluorescence. Each pair of images (top and bottom) was taken from the same field under differential interference contrast (DIC-top) and immunofluorescence (IFA-bottom) microscopy. (**A**) Unfixed Δ*ospC/FL* and Δ*ospC/Nt5* spirochetes were probed with OspC MAb. (**B**) Unfixed and fixed Δ*ospC/FL* spirochetes were probed with FlaB MAb. Images were obtained at x400 magnification.
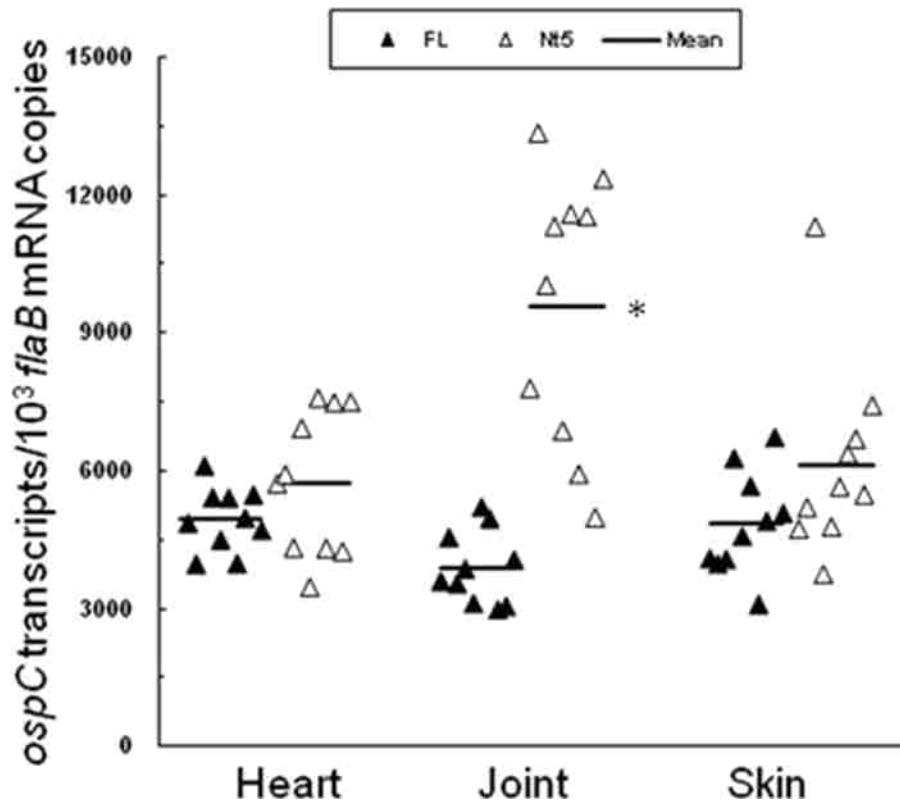
Figure 4.3. Deletion of N-terminus 5-AA residues leads to significant changes in *ospC* mRNA accumulation in the joint tissues. RNA samples were prepared from heart, joint, and skin specimens and quantified for *flaB* and *ospC* expression by RT-qPCR. The data are presented as *ospC* transcripts per 1,000 *flaB* mRNA copy numbers. *, $P < 0.05$ between FL and Nt5.

expresses *ospC* during infection of immunodeficient mice (Liang *et al.*, 2002a, Liang *et al.*, 2002b). However, the pathogen downregulates *ospC* expression when placed under immune selection pressure (Liang *et al.*, 2002b).

Whereas both Δ*ospC/FL* and Δ*ospC/Nt5* spirochetes were able to be recovered from all sites examined upon control IgG2a treatment, Δ*ospC/FL* spirochetes were able to be recovered from the skin (remote), joint, ear, and most of the heart tissues upon OspC MAb treatment. However, Δ*ospC/Nt5* spirochetes were cleared from all sites examined, except for the joint

tissues, upon OspC MAb treatment. These results indicate that Δ*ospC/Nt5* spirochetes are cleared due to the borreliacidal nature of the anti-ospC antibodies.

Anti-*Borrelia* Specific Immune Responses Clear Δ*ospC/Nt5* Spirochetes in Immunocompetent Mice. *B. burgdorferi* abundantly expresses OspC during initial mammalian infection, when the antigen is required. Its continued presence, however, poses a threat to the pathogen since anti-ospC antibodies are borreliacidal (Fung *et al.*, 1994, Liang *et al.*, 2004b). To evade the specific immune response *B. burgdorferi* reduces *ospC* expression through the interaction of an as yet unidentified repressor to the *ospC* operator (Xu *et al.*, 2007).

We used immunocompetent mice to investigate if the deletion of the N-terminus 5-AA of OspC affects the regulation of *ospC* expression under broader immune pressure. The results are shown in Table 4.4. Control Δ*ospC/FL* spirochetes were grown from the inoculation site (I.S.) and remote site (R.S.) skin, joint, and heart specimen but from none of the ear samples at first

Table 4.3. Monoclonal antibodies clear spirochetes expressing N terminus 5-AA deleted OspC in SCID mice. [a]

		No. of cultures positive/no. examined 1 month				
Clone	Antibody treatment	Skin	Joint	Heart	Ear	All sites
Δ*ospC/FL*	mAb	5/5	5/5	3/5	5/5	18/20
	IgG2a	5/5	5/5	5/5	5/5	20/20
Δ*ospC/Nt5*	mAb	0/6	6/6	0/6	0/6	6/24
	IgG2a	6/6	6/6	6/6	6/6	24/24

[a] Groups of 5 or 6 C3H SCID mice were inoculated with either Δ*ospC/FL* or Δ*ospC/Nt5* at $10^5/100$ μl. At one month post inoculation, OspC monoclonal antibody or purified normal mouse IgG2a as a control was given at 100 μg. Three days later, mice were killed and heart, joint, skin and ear specimens were collected for bacterial culture.

week; the Δ*ospC/Nt5* bacteria were grown only from all of the skin inoculation sites and one of six joint samples at a week post-inoculation. Whereas all sites became culture positive with control Δ*ospC/FL* spirochetes by 2 weeks after initial inoculation, the Δ*ospC/Nt5* bacteria were cleared from all sites, except from one inoculation site skin. At 3 and 4 weeks post infection, the Δ*ospC/Nt5* spirochetes were cleared from all sites examined except inoculation site (I.S.) and joint tissues. These data demonstrate that Δ*ospC/Nt5* spirochetes were initially (at 1 week post-inoculation) unable to disseminate to remote tissues. Furthermore, upon development of specific humoral responses at 2 weeks post-inoculation, the Δ*ospC/Nt5* spirochetes began to be cleared from all remote sites except the joint by 3 to 4 weeks, which might serve as an immune-privileged site. This is an indirect indication that the Δ*ospC/Nt5* spirochetes were unable to downregulate *ospC* in response to the development of specific anti-OspC borrelicidal antibodies in an immunocompetent environment.

DISCUSSION

B. burgdorferi upregulates the alternative sigma factor *rpoS* in response to a fresh blood meal, which in turn upregulates OspC, a lipoprotein required for initial mammalian infectivity (Caimano *et al.*, 2007). The pathogen then selectively reduces the expression of this antibody-targeted antigen in response to immune pressure through an unknown trans-acting repressor (Crother *et al.*, 2003, Liang *et al.*, 2002a, Liang *et al.*, 2002b, Liang *et al.*, 2004b, Xu *et al.*, 2007). Constitutive expression of OspC leads to clearance of infection, if not survival of spirochetes expressing OspC escape mutants (Xu *et al*., 2006). Interestingly, when *ospC* regulatory elements were placed upstream of OspA coding region in an ospC mutant background, OspA was unable to be downregulated under immune pressure (data not shown). This led us to investigate whether OspC is involved in self-regulation.

Table 4.4. Spirochetes expressing the N-terminus 5-AA deleted OspC are unable to evade specific immune response in wild type mice.[a]

Clone	No. of specimens positive/no. of specimens examined at post-inoculation week																			
	1					2					3					4				
	I.S.	R.S.	Ear	Heart	Joint	I.S.	R.S.	Ear	Heart	Joint	I.S.	R.S.	Ear	Heart	Joint	I.S.	R.S.	Ear	Heart	Joint
Δ*ospC/FL*	6/6	6/6	0/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Δ*ospC/Nt5*	6/6	0/6	ND	0/6	1/6	1/6	0/6	0/6	0/6	0/6	2/6	0/6	0/6	0/6	6/6	6/6	0/6	0/6	0/6	6/6

[a.] Groups of 12 to 24 BALB/c Wt mice each received a single intradermal/subcutaneous injection of 10^5 organisms of the clone Δ*ospC/FL* and Δ*ospC/Nt5*. Six animals from each group were euthanized at 1, 2, 3 and 4 wk post-inoculation; inoculation site (I.S.) and remote site (R.S) skin, ear, heart, and joint specimens were harvested for spirochete isolation. The I.S. site was at the chest; therefore the R.S. site was at the back of the mice. **b.** ND, not determined.

Structurally, OspC is a predominantly alpha-helical lipoprotein; in its dimer

conformation, the C- and N-terminus of OspC come close together and while the C-terminus of

OspC is exposed to the outer environment, the N-terminus anchors the lipoprotein to the outer

membrane of *B. burgdorferi* by a lipidated cystein (Eicken *et al.*, 2001, Kumaran *et al.*, 2001).

This study addressed the role of the N-terminus of OspC in *ospC* mRNA regulation when a small

5-AA region of OspC downstream of the lipidated cystein was deleted. Deletion of N-terminal 5-

AA did not affect the surface exposure of OspC as observed by indirect immunofluorescence

assay. Sorting of major lipoproteins in the *E.coli* Lol pathway is based on properties of the amino

acid following the N-terminal, acylated cystein, i.e., the so-called '+2' rule (Yamaguchi *et al.*,

1988). Although *Borrelia* spirochetes have Lol homologues (Masuda *et al.*, 2002), sorting signal

in these pathogens are different from those found in other diderm bacteria. Hence, the '+2' rule

established for *E.coli* inner membrane (IM) and outer membrane (OM) lipoproteins does not

apply to borrelial lipoproteins. It also known that for the outer surface lipoprotein OspA, the first

five aminoacids from the N-terminus cystein are required for surface localization (Schulze and

Zuckert, 2006). In this study, the 5-AA deletion was made seven amino acids downstream of the

lipidated cystein, allowing for the translocation of the lipoprotein to the outer surface of the outer

membrane of *B. burgdorferi*.

The infectivity and innate immunity evasive roles of OspC were preserved in spite of the

N-terminal 5-AA deletion as reflected in the 50% infectious dose values at one month post-

infection in SCID mice. Measurements of spirochete burdens showed that the deletion did not

reduce the spirochete burden in joint or skin, but did reduce in the heart, compared to control

spirochetes. A recent study has shown that OspC-deficient spirochetes over expressing

heterologous lipoproteins generated significantly lower bacterial loads when compared to

controls, indicating that OspC is required for the colonization of the heart (Xu *et al.*, 2008b). In

addition, OspC may serve as an adhesion molecule and was shown to colonize the heart in high frequency (Antonara *et al.*, 2007). Nevertheless, the deletion did not decrease spirochete burden in the skin and, on the contrary, recorded an increase in the joint tissue when compared to FL control spirochetes. The phenotype that resulted from these initial studies (explained in detail in Chapter 3) allowed us to use this strain to further investigate the effect of such deletion on the regulation of *ospC*.

Studies have shown that in the absence of adaptive immune pressure, the tissue microenvironment influences the spirochetal mRNA expression. In the joint, *ospC* is usually expressed at low levels (Liang *et al.*, 2004b). Our data related to upregulation of *ospC* expression in the joints in 5-AA deleted spirochetes is consistent with previous studies that also showed an upregulation of *ospC* mRNA in spirochetes lacking the *ospC* operator region comprising the two inverted repeats (Xu *et al.*, 2007) indicating that both the *ospC* operator and the coding region of OspC may be involved in the repression of *ospC* mRNA by spirochetes in this tissue and therefore, that OspC may be involved in self-regulation.

Wild type *B. burgdorferi* downregulates OspC under immune pressure (Liang *et al.*, 2004b). In this study, the specific monoclonal OspC antibody treatment was probably unable to downregulate OspC, hence leading to clearance of the bacteria from all the sites tested, except joint. These results are in agreement with previous data that shows monoclonal antibody treatment led to clearance of spirochetes expressing OspC lacking the operator region from the sites tested, including joint tissue, whereas ear was not tested in their study (Xu *et al.*, 2007). In wild type mice, the data indicate that spirochetes expressing N-terminus 5-AA deleted ospC were cleared from most sites except the inoculation site and the joint tissues. It could be that the deletion led to an initial dissemination defect (in the first week, as also shown in Chapter 3) that could be a confounding factor to the study of self-regulation of OspC. OspC has been shown to

be a dissemination-facilitating factor of *B. burgdorferi* (Xu *et al.*, 2008b). In addition, the fact that the spirochetes could not be cleared from the joint tissues either under monoclonal antibody treatment or under an anti-*Borrelia* immunocompetent environment denotes that the joint is an immune-privileged site and that although *ospC* levels are high this tissue, it is able to harbor the spirochetes and avoid host adaptive immune pressure. The persistent finding of spirochetes at the site of inoculation may signify a high density of spirochetes in this particular location, and thus the inability of OspC monoclonal antibodies to clear the spirochetes from this area. On the other hand, it may signify a dissemination defect due to the N-terminus sequence deletion.

Researchers have shown previously that the majority of IgM and IgG OspC antibodies produced after infection with *B. burgdorferi* were complement-dependent borreliacidal antibodies that killed the spirochetes without the necessity of scavenging by phagocytic cells (Callister *et al.*, 1996, Jobe *et al.*, 2003, Rousselle *et al.*, 1998). The OspC MAb B5 is located at the C-terminus of OspC and has a conformational-dependent epitope (Gilmore and Mbow, 1999, Mbow *et al.*, 1999). Therefore, since overall fold of OspC monomer brings the adjacent location of the N- and C-terminal residues in the membrane proximal region of OspC, this explains how the N-termini can be recognized by this B5 OspC monoclonal antibody (Eicken *et al.*, 2001).

The current study highlighted the involvement of the coding sequence of OspC in the regulation of the gene. The fact that a gene expressing the coding region of a heterologous lipoprotein (OspA) was unable to be regulated by the *ospC* operator led us to analyze the coding region of OspC itself for self-regulation. This study showed that there was a clear defect in the regulation of OspC when a segment of the N-terminus of OspC was deleted. This may be a partial defect, since some tissues still harbored the variant spirochetes. The mechanistic details of the self-regulation of OspC are also yet to be deciphered. A long-term study may allow us to

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make stronger conclusions. Further studies with deletion of more amino acids from the N-terminus of OspC or studies of *ospC* expression levels in double knock-out spirochetes in which both the *ospC* operator and the N-terminus 5-AA sequence are deleted can provide additional insights into the regulation of this lipoprotein that is crucial in the enzootic life cycle of *B. burgdorferi.*

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CHAPTER 5

THE C-TERMINAL 9-AA STRETCH OF OSPC IS NOT IMPORTANT FOR VIRULENCE OF *BORRELIA BURGDORFERI* BUT FOR BALANCING *OSPC* EXPRESSION DURING MURINE INFECTION

INTRODUCTION

The Lyme disease spirochete, *Borrelia burgdorferi*, is one of the most invasive bacterial pathogens, causing persistent infection despite the development of vigorous immune responses (Schwan *et al.*, 1995, Seiler and Weis, 1996, Steere, 2001). Both timely up- and down-regulations of the outer surface protein C (OspC) are crucial for its pathogenic strategy. In unfed ticks, *B. burgdorferi* does not express OspC (Ohnishi *et al.*, 2001, Schwan *et al.*, 1995); a fresh blood meal upregulates OspC and prepares the pathogen for infection of a mammal (Grimm *et al.*, 2004, Pal *et al.*, 2004, Stewart *et al.*, 2006). *B. burgdorferi* abundantly expresses OspC during early infection when the antigen is required (Liang *et al.*, 2004, Tilly *et al.*, 2006) and its expression ultimately induces a robust humoral response that imposes tremendous pressure on the pathogen (Fung *et al.*, 1994, Liang *et al.*, 2004). If *B. burgdorferi* fails to repress OspC expression or undergo escape mutations, infection is cleared (Xu *et al.*, 2007, Xu *et al.*, 2006). Although mutation is an effective way to evade the immune system, it may cause the functional loss of affected genes. *B. burgdorferi* with lost OspC function may eventually persist during chronic infection (Tilly *et al.*, 2006); however, it would lose the ability to initiate an infection (Stewart *et al.*, 2006), leading to discontinuation of the enzootic cycle. *B. burgdorferi* is able to down-regulate OspC, thus allowing the pathogen to effectively evade specific humoral immunity and preserve the integrity of the *ospC* gene for subsequent need during its life cycle (Liang *et al.*, 2002a, Liang *et al.*, 2004).

In terms of regulation, *ospC* is probably has been the most investigated among all borrelial genes. Its expression is driven by an RpoS-dependent promoter (Eggers *et al.*, 2004, Gilbert *et al.*, 2007, Yang *et al.*, 2005). The alternative δ factor, RpoS, not only positively regulates several other important borrelial genes, such as decorin-binding protein A (*dbpA*) and *ospF* (Eggers *et al.*, 2004, Fisher *et al.*, 2005, Hubner *et al.*, 2001), but may also indirectly repress other lipoprotein genes, including *ospA* and *lp6.6* (Caimano *et al.*, 2005, Caimano *et al.*, 2007), making it unfeasible for *B. burgdorferi* to turn off *rpoS* to achieve *ospC* down-regulation. To selectively repress *ospC* in response to specific immune pressure during mammalian infection, *B. burgdorferi* may express a repressor to interact with the newly identified operator, a large palindromic sequence consisting mainly of two 20 bp inverted repeats located immediately upstream of the *ospC* promoter (Xu *et al.*, 2007).

The *ospC* gene, like other microbial lipoprotein genes, encodes a leader peptide of 18-AA residues, which is cleaved off during post-translational modification, and protein portion (Figure 5.1A). The mature OspC, a largely α-helical protein, may be a dimer with a characteristic central four-helical bundle formed by the association of the two longest helices from each subunit (Eicken *et al.*, 2001, Kumaran *et al.*, 2001). Neither the N-terminal 23-AA linker nor the C-terminal 14-AA stretch contributes to the core three-dimensional structure of OspC; instead, the α-helices 1 and 5 bring the two terminal regions in close proximity, where the lipoprotein is anchored to the bacterial outer membrane through lipidation of the first cystein residue of the N-terminal linker (Figure 5.1B). Compared to the N-terminal linker, the C-terminal stretch is highly conserved among borrelial isolates (Figure 5.1C) and highly immunogenic (Mathiesen *et al.*, 1998).

One essential role of the N-terminal linker is to position the lipoprotein on the bacterial outer membrane, with the essential role of the C-terminal stretch remaining elusive in its
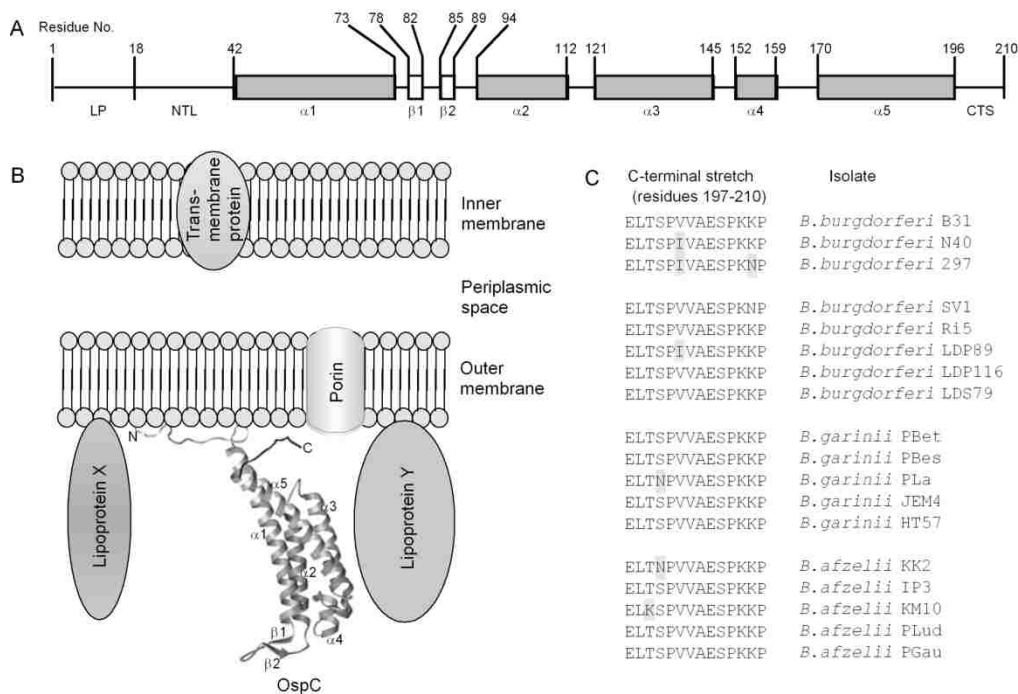
Figure 5.1. The tertiary structure and possible cellular location of OspC. A. Fragmentation of the secondary structure of OspC (Eicken *et al.*, 2001, Kumaran *et al.*, 2001). The *ospC* gene of *B. burgdorferi* B31 codes for a total of 210 amino acids. The first 18-AA sequence is a leader peptide (LP) that is cleaved off before a mature OspC molecule is translocated to the spirochete outer surface, followed by the N-terminal linker (NTL) that includes the residues 19 – 41. The residues 42 – 73, 94 – 112, 121 – 145, 152-159, and 170 – 196 form the α-helices 1, 2, 3, 4, and 5, respectively; 78 – 82 and 85 – 89 make up the β-strands 1 and 2, respectively. The residues 197 – 210 form the C-terminal stretch (CTS). **B. The tertiary structure and possible cellular location of OspC.** *B. burgdorferi* possesses inner and outer membranes that are separated by a periplasmic space. As a surface lipoprotein, OspC is anchored to the outer membrane through lipidation of the cystein residue 19. Neither the N-terminal 23-AA linker nor the C-terminal 14-AA stretch contributes to the OspC core structure, which consists mainly of five α-helices. The helices 1 and 5 bring the two terminal sequences in close proximity, where the NTL anchors the protein to the bacterial outer membrane, thus positioning the CTS to the membrane. **C. Sequence comparison of the C-terminal 14-AA stretch among borrelial isolates.** The sequences (top) are from the three most investigated *B. burgdorferi* sensu stricto strains, followed by 15 sequences randomly selected from *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* isolates. The highlighted letters indicate amino acid substitutions.

contribution to the overall function of OspC. In this study, *B. burgdorferi* expressing OspC with

different C-terminal truncations was generated and investigated for infectivity, tissue

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colonization, pathogenicity, and gene expression in a murine model. The study allowed us to

conclude that the C-terminal stretch of OspC is not required for virulence of *B. burgdorferi*, but

may be involved in a yet unidentified event that either directly or indirectly regulates *ospC*

expression in the murine host. Interestingly, the N-terminal linker was also shown to be involved

in the regulation of *ospC* expression (Chapter 4).

<div align="center">

MATERIALS AND METHODS

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Generation of *B. burgdorferi* Expressing OspC with Different C-terminal Deletions.

As illustrated in Figure 5.2A, to efficiently generate constructs containing various versions of C-

terminal deletions, a 1057-bp fragment covering the *ospC*-coding region and up- and down-

stream sequences was amplified by PCR using the primers P1F and P1R (Table 5.1) and cloned

into the TA cloning vector pNCO1T (Shi *et al.*, 2006), creating an intermediate vector,

designated pNCO1T-*ospC*. Three large amplicons, namely, *ct6*, *ct9* and *ct13*, were generated by

PCR using pNCO1T-*ospC* as a template and one common forward primer, P2F, and each of the

three reverse primers, P2R, P3R, and P4R (Table 5.1). After digestion with *Afl*II and subsequent

purification, the amplicons were circularized via ligation and then digested with *Bam*HI and *Xba*I

to release *ospCct6*, *ospCct9*, and *ospCct13*. These three fragments, encoding an OspC variant

with the C-terminal 6-, 9- or 13-AA deletion, were cloned into the recombinant plasmid pBBE22

(a gift from S. Norris) after the vector was digested with *Bam*HI and *Xba*I. Resulting

complementation plasmids were designated pBBE22-*ospCct6*, pBBE22-*ospCct9* and pBBE22-

ospCct13, respectively. The inserts and their flanking regions within pBBE22 were sequenced to

ensure the constructs were as designed.

Complementation plasmids were electroporated into an *ospC* mutant, namely, *ospCΔ*,

which was generated in our previous study (Xu *et al.*, 2007); resulting transformants were

screened as described previously (Xu *et al.*, 2005). Identified transformants were first surveyed

for the presence of lp28-1, a plasmid essential for infection of immunocompetent hosts

(Labandeira-Rey *et al.*, 2003, Purser and Norris, 2000). Only clones containing lp28-1 were

further analyzed for plasmid content as described previously (Xu *et al.*, 2005). Restoration of

OspC expression was verified using immunoblots probed with a mixture of FlaB and OspC

monoclonal antibodies (MAbs), or of the FlaB MAb and mouse anti-OspC sera (Figure 5.2B), as

described previously (Xu *et al.*, 2006). The clone *ospCΔ*/*FL*/1, which was generated in our

previous study (Xu *et al.*, 2007) and expressed wild-type OspC, was included as a control.

Table 5.1. Primers used in the study

Primer	Sequences (5' to 3') [a]
P1F	TTG<u>GGATCC</u>AAAATCTAATACAAGT
P1R	TTCC<u>TCTAGA</u>GAAGAGCTTAAAGTTAA
P2F	GCG<u>CTTAAG</u>ATCAAATTAATATTATAAGA
P2R	ATC<u>TTAAG</u>CCACAACAGGGC
P3R	AG<u>CTTAAG</u>GGCTTGTAAGCTCTTTAAC
P4R	ACAT<u>CTTAAG</u>TTACTCTTTAACTGAATTAGCA

[a] The underlined sequences are the restriction enzyme site *Bam*HI (P1F), *Xba*I (P1R), or *Afl*II
(P2F, P2R, P3R, and P4R).

Infection Study in SCID Mice. SCID mice on a C3H background, ages $4 - 8$ weeks,

(provided by the Division of Laboratory Animal Medicine at Louisiana State University, Baton

Rouge, LA) were given one single intradermal/subcutaneous injection of 10^4 spirochetes.

Animals were examined for the development of arthritis at 2-day intervals, starting at day 7, and

sacrificed one month post-inoculation. Joint, heart, and skin specimens were collected for

spirochete isolation and histopathological examination as described previously (Xu *et al.*, 2005).

The harvested samples were also used for DNA and RNA preparation as described below.

Determination of ID$_{50}$ Values. Spirochetes were grown to late-log phase (10^8 cells/ml)

at 33°C and 10-fold serially diluted with Barbour-Stoenner-Kelly H (BSK-H) complete medium
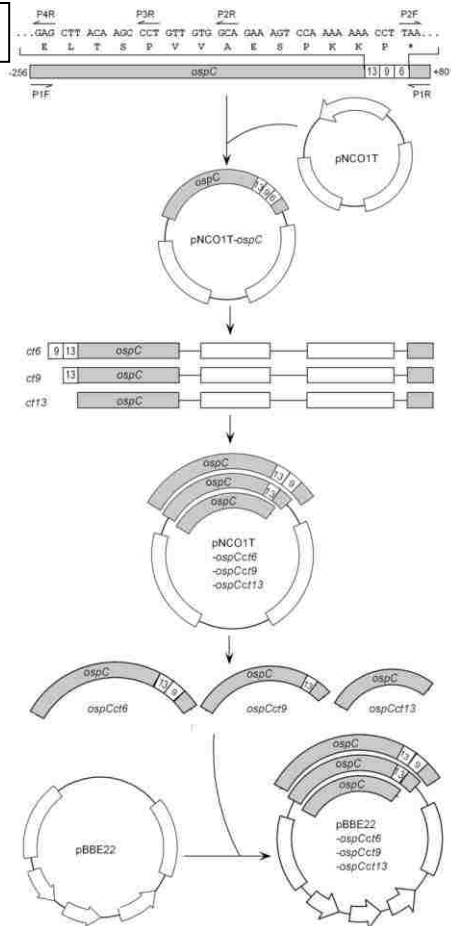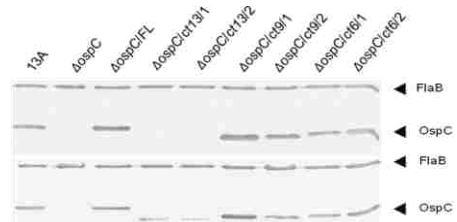
Figure 5.2. Generation of *B. burgdorferi* expressing OspC with C-terminal deletions.
A. Construction of complementation plasmids coding for OspC with C-terminal truncations. The last 15 codons, including the stop one (marked with an asterisk), and their encoded 14-AA sequence of OspC are presented (on top). The amplification starting sites and directions of four primers, P2F, P2R, P3R, and P4R, used for plasmid construction are also marked. The long bar represents the 1057-bp fragment, covering the entire *ospC*-coding region and down- (extending to +801 from the transcriptional start site) and up-stream sequences (extending to -256). **B. Restoration of OspC expression.** The parental clone 13A, *ospCΔ*, and the *ospCΔ/FL*, *ospCΔ/ospCct13*/1, *ospCΔ/ospCct13*/2, *ospCΔ/ospCct9*/1, *ospCΔ/ospCct9*/2, *ospCΔ/ospCct6*/1 and *ospCΔ/ospCct6*/2 spirochetes were verified for OspC expression by immunoblots probed with a mixture of FlaB and OspC MAbs (top panel), or of the FlaB MAb and mouse antisera raised against recombinant OspC (bottom panel).

(Sigma Chemical Co., St. Louis, MO). C3H SCID mice or BALB/c mice, ages 4 to 6 weeks,

(provided by the Division of Laboratory Animal Medicine at Louisiana State University, Baton

Rouge, LA) each received one single intradermal/subcutaneous injection of 100 μl of spirochetal

suspension. SCID mice, but not wild-type, were examined for the development of arthritis as described above. Mice were euthanized one month post-inoculation; heart, tibiotarsal joint, and skin (not from inoculation site) specimens were harvested for bacterial culture as described above (Xu *et al*., 2005). The ID_{50} value was calculated as described by Reed and Muench (Reed and Muench, 1938). SCID mouse specimens were also used for DNA and RNA preparation as described below.

Quantification of Tissue Spirochetal Load and *ospC* Gene Expression Level. DNA was extracted for heart, joint, and skin specimens of infected SCID mice and quantified for the copy numbers of *flaB* and murine actin genes by quantitative PCR (qPCR) as described previously (Xu *et al*., 2005). The tissue spirochete burden was expressed as *flaB* DNA copies per 10^6 host cells (2×10^6 actin DNA copies). RNA was prepared from heart, joint, and skin tissues, then quantified for the mRNA copy numbers of *flaB* and *ospC* by reverse transcription qPCR (RT-qPCR) as described previously (Xu *et al*., 2006). The data are presented as *ospC* transcripts per 1,000 *flaB* mRNA copy numbers.

Statistical Analysis. A two-tailed Student *t* test was used to compare each two groups and calculate *P* values. A *P* value ≤ 0.05 was considered to be significant.

RESULTS

Generation of *B. burgdorferi* Expressing OspC with Different C-terminal Truncations. Three *trans*-complementation plasmids, pBBE22-*ospCct6*, pBBE22-*ospCct9* and pBBE22-*ospCct13*, were constructed as diagrammed in Figure 5.2A. These constructs encoded OspC with C-terminal 6-, 9- or 13-AA deletion, respectively. Because the *ospC* mutant, *ospCΔ*, had lost lp25, the plasmid that carries the gene *bbe22* coding for a nicotinamidase essential for survival of *B. burgdorferi* in the mammalian environment, all the complementation plasmids were derived from the recombinant plasmid pBBE22, which harbors a copy of *bbe22* (Purser *et*

al., 2003). Between six and fourteen transformants were obtained from transformation of the

mutant *ospCΔ* with each construct. Plasmid analyses identified two clones receiving each

complementation plasmid, namely, *ospCΔ/ospCct13*/1, *ospCΔ/ospCct13*/2, *ospCΔ/ospCct9*/1,

ospCΔ/ospCct9/2, *ospCΔ/ospCct6*/1 and *ospCΔ/ospCct6*/2. These clones shared the same

plasmid content as the mutant *ospCΔ* (Xu *et al.*, 2007): they all lost cp9, lp5, lp21, lp25 and lp56.

Restoration of OspC expression was confirmed by immunoblot analysis (Figure 5.2B), in

which the clone *ospCΔ/FL*/1 was included as a control. This clone was generated from

transformation of the mutant *ospCΔ* with a complementation plasmid, pBBE22-*FL*, which was

constructed by cloning a 1057-bp fragment covering the *ospC*-coding region and up- and down-

stream sequences into pBBE22, as described in our previous study (Xu *et al*., 2007). Similar to

the parental clone 13A, the *ospCΔ/FL*/1 spirochetes expressed full-length OspC (Figure 5.2B).

In contrast, the *ospCΔ/ospCct6*/1, *ospCΔ/ospCct6*/2, *ospCΔ/ospCct9*/1, *ospCΔ/ospCct9*/2,

ospCΔ/ospCct13/1, and *ospCΔ/ospCct13*/2 bacteria expressed shorter versions of OspC

respective to their C-terminal deletions. The truncated OspC expressed by the clones

ospCΔ/ospCct13/1 and *ospCΔ/ospCct13*/2 was no longer recognized by OspC MAb (Figure

5.2B, top panel) but reacted well with antisera raised against recombinant OspC (Figure 5.2B,

bottom panel).

Deletion of the C-terminal 6- but Not 9-AA Sequence Abrogates Arthritis Virulence

in SCID Mice. Groups of three C3H SCID mice were inoculated with the clone

ospCΔ/ospCct13/1, *ospCΔ/ospCct13*/2, *ospCΔ/ospCct9*/1, *ospCΔ/ospCct9*/2, *ospCΔ/ospCct6*/1,

or *ospCΔ/ospCct6*/2. Additionally, six animals received the clone *ospCΔ/FL*/1 as a control,

which had been extensively examined for infectivity and *ospC* expression in our previous study

(Xu *et al*., 2007). In these six mice, joint swelling evolved 10 to12 days post-inoculation and

developed into severe arthritis within a week (Figure 5.3A). In the six mice that received either

clone *ospCΔ/ospCct9*/1 or *ospCΔ/ospCct9*/2, joint swelling developed following the same course as described above, indicating that deletion of the C-terminal 9-AA stretch does not reduce infectivity and arthritis virulence in immunodeficient mice. However, none of the 12 animals inoculated with the *ospCΔ/ospCct13*/1, *ospCΔ/ospCct13*/2, *ospCΔ/ospCct6*/1, or *ospCΔ/ospCct6*/2 bacteria showed joint swelling during the course of 1 month, indicating that truncation of either the C-terminal 6- or 13-AA sequence completely diminished arthritis virulence, and probably infectivity as well. Histopathological examination confirmed that intensive inflammatory responses appeared in and around the joint space of mice receiving the *ospCΔ/FL*/1, *ospCΔ/ospCct9*/1 or *ospCΔ/ospCct9*/2 spirochetes but not in those inoculated with the clone *ospCΔ/ospCct13*/1, *ospCΔ/ospCct13*/2, *ospCΔ/ospCct6*/1, or *ospCΔ/ospCct6*/2 (Figure 5.3B).

To rule out the possibility that the loss of infectivity disabled the four clones from inducing arthritis, the heart, joint, and skin samples were cultured for spirochetes. Similar to the clones *ospCΔ/FL*/1, *ospCΔ/ospCct9*/1, and *ospCΔ/ospCct9*/2, *ospCΔ/ospCct6*/1 and *ospCΔ/ospCct6*/2 bacteria were recovered from each specimen of all 12 inoculated mice (Table 5.2), indicating that deletion of the C-terminal 6-AA stretch does not abrogate infectivity. Neither *ospCΔ/ospCct13*/1 nor *ospCΔ/ospCct13*/2 spirochetes could be grown from any of the six inoculated mice, indicating that deletion of the C-terminal 13-AA sequence abrogates the infectivity of *B. burgdorferi*. The loss of infectivity in this clone occurs as early as 24h post-inoculation as shown in Chapter 2.

Deletion of the C-terminal 6- or 9-AA Sequence Does Not Lead to an Increase in The ID$_{50}$ Values in SCID or Immunocompetent Mice. Groups of two or three C3H SCID mice each received one single inoculation of 10^1 to 10^4 spirochetes of the clone *ospCΔ/FL*/1, *ospCΔ/ospCct9*/1, *ospCΔ/ospCct9*/2, *ospCΔ/ospCct6*/1, or *ospCΔ/ospCct6*/2. Animals were

Figure 5.3. Deletion of the C-terminal 6- but not 9-AA sequence diminishes arthritis virulence in SCID mice. A. SCID mice were inoculated with the *ospCΔ/FL*/1, *ospCΔ/ospCct13*/1, *ospCΔ/ospCct9*/1 or *ospCΔ/ospCct6*/1 spirochetes, and sacrificed one month later. Severe joint swelling was noted only in mice challenged with *ospCΔ/FL* or *ospCΔ/ospCct9*/1, but not with *ospCΔ/ospCct13*/1or *ospCΔ/ospCct6*/1. **B. Intensive inflammatory responses (pointed with black arrows) were noted in and around the joint space of mice inoculated with *ospCΔ/FL* or *ospCΔ/ospCct9*/1, but not with *ospCΔ/ospCct13*/1or *ospCΔ/ospCct6*/1.** Tissue sections were stained with hematoxylin and eosin. *js*: joint space; *sm*: synovial membrane; *tb*: tibial bone; *tr*: tarsal bone.

euthanized one month later; heart, joint and skin specimens were cultured for spirochetes for

ID_{50} determination. The ID_{50} values were 18 and 32 organisms for the clone *ospCΔ/FL*/1 (in two

Table 5.2. Deletion of the C-terminal 13- but not 6- or 9-AA sequence abrogates infectivity in SCID mice[a]

Inoculum	No. of cultures positive/total no of specimens examined				No. of mice infected/total no. of mice inoculated
	Heart	Joint	Skin	All sites	
ospCΔ/*FL*/1	6/6	6/6	6/6	18/18	6/6
ospCΔ/*ospCct13*/1	0/3	0/3	0/3	0/9	0/3
ospCΔ/*ospCct13*/2	0/3	0/3	0/3	0/9	0/3
ospCΔ/*ospCct9*/1	3/3	3/3	3/3	9/9	3/3
ospCΔ/*ospCct9*/2	3/3	3/3	3/3	9/9	3/3
ospCΔ/*ospCct6*/1	3/3	3/3	3/3	9/9	3/3
ospCΔ/*ospCct6*/2	3/3	3/3	3/3	9/9	3/3

[a] Groups of three or six C3H SCID mice were inoculated with the clone *ospCΔ*/*FL*/1, *ospCΔ*/*ospCct13*/1, *ospCΔ*/*ospCct13*/2, *ospCΔ*/*ospCct9*/1, *ospCΔ*/*ospCct9*/2, *ospCΔ*/*ospCct6*/1, or *ospCΔ*/*ospCct6*/2, and sacrificed one month later. Heart, tibiotarsal joint, and skin specimens were harvested and cultured for spirochetes in BSK-H complete medium.

separate experiments), 56 and 178 for the clones *ospCΔ*/*ospCct6*/1 and *ospCΔ*/*ospCct6*/2, and 32 and 32 for the clones *ospCΔ*/*ospCct9*/1 and *ospCΔ*/*ospCct9*/2, respectively (Table 5.3).

The ID_{50} values were also determined in immunocompetent mice. The values were 32 and 32 organisms for the clone *ospCΔ*/*FL*/1 (in two separate experiments), 32 and 56 for the clones *ospCΔ*/*ospCct6*/1 and *ospCΔ*/*ospCct6*/2, and 316 and 56 for the clones *ospCΔ*/*ospCct9*/1 and *ospCΔ*/*ospCct9*/2, respectively (Table 5.4). Taken together, the study indicated that either C-terminal deletion leads to a decrease in infectivity in both immunodeficient and immunocompetent mice.

Deletion of The C-terminal 9-AA Sequence Does Not Affect Tissue Colonization in Heart or Joint Tissue but Increases Colonization in The Skin of SCID Mice. The spirochetal burden was analyzed to further assess whether truncations affected tissue colonization. Heart, joint and skin specimens were harvested from SCID mice that were infected with *ospCΔ*/*FL*/1,

Table 5.3. Deletion of neither C-terminal 6- nor 9-AA sequence significantly affects the ID_{50} value in SCID mice[a]

Inoculum & dose	No. of cultures positive/total no. of specimens examined				No. of mice infected/total no. of mice inoculated	ID_{50} (No. of organisms)
	Heart	Joint	Skin	All sites		
ospCΔ/FL/1						18
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	3/3	3/3	3/3	9/9	3/3	
10^1	1/3	1/3	1/3	3/9	1/3	
ospCΔ/FL/2						32
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/9	0/3	
ospCΔ/ospCct6/1						56
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	2/3	1/3	2/3	5/9	2/3	
10^1	0/3	0/3	0/3	0/9	0/3	
ospCΔ/ospCct6/2						178
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	1/3	0/3	1/3	2/9	1/3	
10^1	0/3	0/3	0/3	0/9	0/3	
ospCΔ/ospCct9/1						32
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/9	0/3	
ospCΔ/ospCct9/2						32
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/9	0/3	

[a] The *ospCΔ/FL*/1, *ospCΔ/ospCct6*/1, *ospCΔ/ospCct6*/2, *ospCΔ/ospCct9*/1 and *ospCΔ/ospCct9*/2 spirochetes were grown to late-log phase (10^8 cells/ml) and 10-fold serially diluted with BSK-H medium. Approximately 100 µl of bacterial suspension with cell densities at $10^2 – 10^5$ ml^{-1} was intradermally/subcutaneously inoculated into each C3H SCID mouse. Animals were sacrificed one month later; heart, tibiotarsal joint, and skin specimens were harvested for bacterial isolation. The ID_{50} values were calculated by the method of Reed and Muench.

Table 5.4. Deletion of neither C-terminal 6- nor 9-AA sequence significantly affects the ID_{50} value in immunocompetent mice[a]

Inoculum & dose	No. of cultures positive/total no. of specimens examined				No. of mice infected/total no. of mice inoculated	ID_{50} (No. of organisms)
	Heart	Joint	Skin	All sites		
ospCΔ/FL/1						32
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/9	0/3	
ospCΔ/FL/2						32
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/9	0/3	
ospCΔ/ospCct6/1						32
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	3/3	3/3	3/3	9/9	3/3	
10^1	0/3	0/3	0/3	0/9	0/3	
ospCΔ/ospCct6/2						56
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	2/3	2/3	2/3	6/9	2/3	
10^1	0/3	0/3	0/3	0/9	0/3	
ospCΔ/ospCct9/1						316
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	0/3	0/3	0/3	0/9	0/3	
10^1	0/3	0/3	0/3	0/9	0/3	
ospCΔ/ospCct9/2						56
10^4	2/2	2/2	2/2	6/6	2/2	
10^3	2/2	2/2	2/2	6/6	2/2	
10^2	1/3	2/3	2/3	5/9	2/3	
10^1	0/3	0/3	0/3	0/9	0/3	

[a] The *ospCΔ/FL*/1, *ospCΔ/ospCct6*/1, *ospCΔ/ospCct6*/2, *ospCΔ/ospCct9*/1 and *ospCΔ/ospCct9*/2 spirochetes were grown to late-log phase (10^8 cells/ml) and 10-fold serially diluted with BSK-H medium. Approximately 100 μl of bacterial suspension with cell densities at 10^2 - 10^5 ml^{-1} was intradermally/subcutaneously inoculated into each BALB/c mouse. Animals were sacrificed one month later; heart, tibiotarsal joint, and skin specimens were harvested for bacterial isolation. The ID_{50} values were calculated by the method of Reed and Muench.

*ospC*Δ/*ospCct9*/1, *ospC*Δ/*ospCct9*/2, *ospC*Δ/*ospCct6*/1, or *ospC*Δ/*ospCct6*/2. A total of 30 mice,

which had been used for Table 5.2 or 5.3, were used, including 10 mice infected with the

*ospC*Δ/*FL*/1, 10 mice with either clone *ospC*Δ/*ospCct6*/1 (n = 5) or *ospC*Δ/*ospCct6*/2 (n = 5),

and 10 mice with clone *ospC*Δ/*ospCct9*/1 (n =5) or *ospC*Δ/*ospCct9*/2 (n = 5). DNA samples

were extracted and quantified for tissue spirochete loads. The *ospC*Δ/*FL*/1 and *ospC*Δ/*ospCct9*

spirochetes generated similar bacterial loads in the heart ($P = 0.26$) and joint tissues ($P = 0.57$)

(Figure 5.4A). In contrast, the *ospC*Δ/*ospCct6* load was 3.6-, and 1.8-fold lower than that of the

*ospC*Δ/*FL*/1 spirochetes in the heart ($P = 1.8 \times 10^{-7}$) and joint tissues ($P = 4.8 \times 10^{-4}$),

respectively, but both bacteria generated similar burdens in skin ($P = 0.14$). The *ospC*Δ/*ospCct6*

load was also 3.0-, and 2.1-fold lower than that of the *ospC*Δ/*ospCct9* bacteria in the heart ($P = 6.0 \times 10^{-4}$) and joint tissues ($P < 0.03$), respectively, but they registered similar burdens in skin

($P = 0.17$). Interestingly, deletion of the C-terminal 9-AA sequence led to a two-fold increase in

the bacterial load in the skin over the control ($P < 0.05$). Taken together, deletion of the C-

terminal 9-AA stretch did not affect tissue colonization in either heart or joint but led to a

significant increase in the bacterial load in the skin of SCID mice.

Deletion of the C-terminal 9-AA Sequence Leads to Significant Changes in *ospC*

mRNA Accumulation in All Tissues of SCID Mice. Aimed at explaining how truncations

affected tissue colonization, *ospC* mRNA accumulation was analyzed. Heart, joint, and skin

specimens were randomly selected from 15 mice that were infected with the *ospC*Δ/*FL*/1 (n = 5),

*ospC*Δ/*ospCct6*/1 (n = 4), *ospC*Δ/*ospCct6*/2 (n = 1), *ospC*Δ/*ospCct9*/1 (n = 1), or

*ospC*Δ/*ospCct9*/2 (n = 4) spirochetes. These 15 mice had been used either for Table 5.3 or 5.4.

RNA samples were prepared and quantified for *flaB* and *ospC* expression by RT-qPCR. No

significant differences in *ospC* mRNA accumulation were noted between the *ospC*Δ/*FL* and

*ospC*Δ/*ospCct6* bacteria in either heart ($P = 0.25$) or skin tissue ($P = 0.81$), but deletion of the 6-

AA sequence led to a seven-fold decrease in *ospC* mRNA density in the joint ($P = 0.0002$)

(Figure 5.4B). The *ospCΔ/ospCct9* spirochetes accumulated *ospC* transcripts 61% and 84%

more than the clone *ospCΔ/FL*/1 in the heart ($P = 0.001$) and skin tissues ($P = 0.002$),

respectively, but 3.4-fold less in the joint ($P = 0.0007$). *B. burgdorferi* expressing OspC with the

9-AA deletion accumulated *ospC* mRNA 37%, 109%, and 92% more than the *ospCΔ/ospCct6*

spirochetes in the heart ($P = 0.03$), joint ($P = 0.04$), and skin tissues ($P = 0.0003$), respectively.

Taken together, these data indicated that deletion of the C-terminal residues led to significant

changes in *ospC* mRNA accumulation by *B. burgdorferi* during murine infection.

DISCUSSION

OspC is a well-defined virulence factor of *B. burgdorferi* in mammalian hosts (Grimm *et

al.*, 2004, Stewart *et al.*, 2006, Tilly *et al.*, 2006). In immunocompetent mice, OspC is

abundantly expressed only during early infection when it is required (Liang *et al.*, 2004, Tilly *et

al.*, 2006), and its expression ultimately induces a robust, protective humoral response (Fung *et

al.*, 1994, Liang *et al.*, 2004). To evade the specific immune response, *B. burgdorferi* must

repress OspC expression (Liang *et al.*, 2002a, Liang *et al.*, 2002b, Liang *et al.*, 2004, Xu *et al.*,

2006). To investigate how the C-terminal sequence contributes to the overall function of OspC,

B. burgdorferi expressing OspC with different deletions were generated. The study showed that

deletion of the C-terminal 13- but not the 6- or 9-AA sequence completely abolishes infectivity

as tested at one month post-inoculation. This loss in protection occurs as early as 24h post-

inoculation (as shown in Chapter 2). Although truncation of the 6-AA region did not

significantly increase the ID_{50} values in SCID or immunocompetent mice, *B. burgdorferi*

expressing OspC lacking the six residues generated lower bacterial loads in both heart and joint

tissues and was unable to induce arthritis in SCID mice. However, removal of the C-terminal 9-

AA sequence did not increase the ID_{50} value in SCID or immunocompetent mice, or reduce the
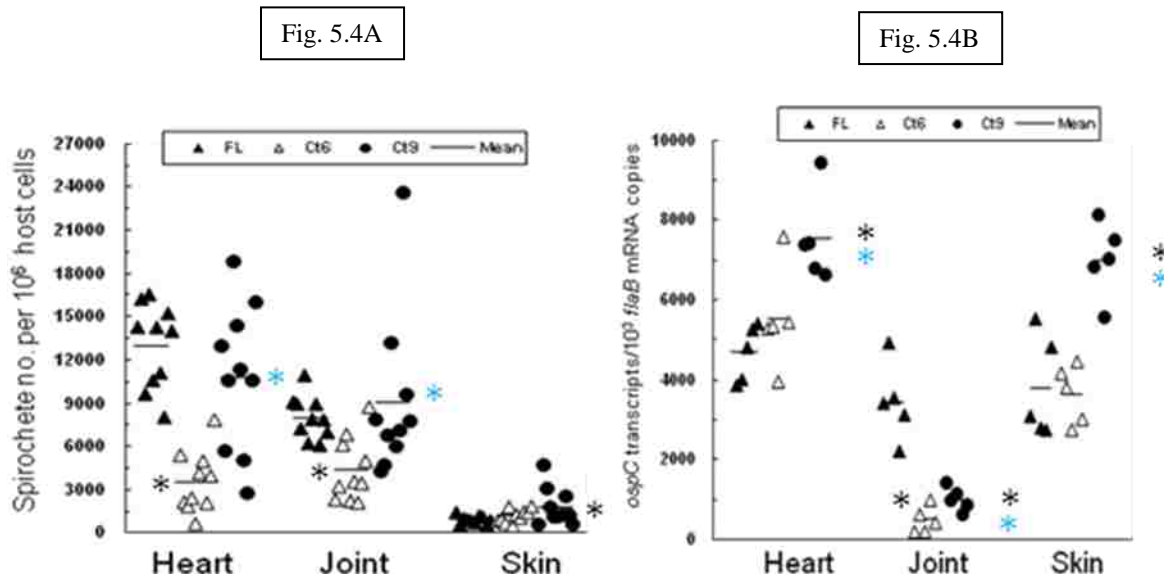
Figure 5.4. Influence of deletion of the C-terminal 6- and 9-AA sequences on the tissue bacterial burden and *ospC* expression. A. Deletion of the C-terminal 6- but not 9-AA sequence reduces bacterial burdens in both heart and joint tissues but not skin. Heart, joint and skin specimens were harvested from 10 mice infected with the clone *ospCΔ/FL*/1, 10 mice with either clone *ospCΔ/ospCct6*/1 (n = 5) or *ospCΔ/ospCct6*/2 (n = 5), and 10 mice with clone *ospCΔ/ospCct9*/1 (n = 5) or *ospCΔ/ospCct9*/2 (n = 5); the three groups are labeled as FL, ct6 and ct9, respectively. These 30 mice had been used in Tables 5.2 and 5.3. **B. Deletion of the C-terminal residues leads to significant changes in *ospC* mRNA accumulation.** RNA samples were prepared from heart, joint, and skin specimens and quantified for *flaB* and *ospC* expression by RT-qPCR. The data are presented as *ospC* transcripts per 1,000 *flaB* mRNA copy numbers. Because of a smaller variation in *ospC* expression compared with the tissue bacterial load, the size of five samples was large enough to generate sufficient statistical power to show a significant difference. *, $P < 0.05$ (Ct6 or Ct9 compared with FL controls) *, $P < 0.05$ (Ct6 compared with Ct9).

tissue bacterial load either in heart or joint tissue, but did significantly increase the burden in skin

while retaining arthritis virulence in immunodeficient mice. Deletion of the 6-AA region led to a

decrease in *ospC* mRNA accumulation only in the joint. Likewise, the removal of additional

three residues significantly reduced mRNA accumulation in the joints, as it increased in both

heart and skin tissues of SCID mice.

As a lipoprotein, OspC repeatedly has been shown to be essential for initial mammalian

infection (Grimm *et al*., 2004, Stewart *et al*., 2006, Tilly *et al*., 2006). It likely contributes to the

virulence of *B. burgdorferi* by interacting with host components and protecting the pathogen

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against hostile mammalian environments. Portions of some of its five helices are exposed to the environment (Buckles *et al.*, 2006, Eicken *et al.*, 2001, Kumaran *et al.*, 2001), so they potentially may play such a protective role. In contrast, neither the N-terminal 23-AA linker nor the C-terminal 14-AA stretch composes the core structure of OspC; instead, these terminal sequences are brought together by the α-helices 1 and 5, therefore allowing the terminal regions to directly interact with the components of the spirochetal outer membrane. Although the last 14-AA sequence of the C-terminus does not contribute to the core structure, some of the residues, especially those that are close to the fifth helix, may play a critical role in stabilizing the core structure. The removal of the last 13 residues indeed abolished the reactivity with the MAb. It is unknown whether deletion of these residues causes a collapse of the core structure and, as a result, abolishes infectivity. Alternatively, the residues, especially those that are close to the helix, may mediate crucial interactions of OspC with the bacterial outer membrane, essential for the function of OspC during mammalian infection.

Deletion of the C-terminal 6-AA region did not significantly reduce infectivity, in terms of ID_{50}, in either immunodeficient or immunocompetent mice, but impaired the ability of *B. burgdorferi* to colonize both heart and joint tissues and diminished arthritis virulence in SCID mice. In the heart tissue, the C-terminal truncation did not affect *ospC* expression, but significantly reduced the bacterial load, providing evidence that the integrity of OspC is crucial for its full function and that the lipoprotein is important for maintaining a high bacterial load in this specific tissue. In joints, removal of the six residues significantly reduced *ospC* mRNA accumulation and the bacterial load, while diminishing arthritis virulence. The reduced *ospC* expression level could be the primary reason for this chain of defects, as the OspC density on the spirochetal surface may be crucial for maintaining the bacterial load, which, in turn, may be important for the induction of arthritis. Alternatively, the OspC expression level may be less

important than the integrity of OspC for these capacities. In skin, the lack of the 6-AA sequence affected neither *ospC* mRNA accumulation nor tissue colonization, indicating that the integrity of OspC is not important for its full function; or alternatively, the lipoprotein has no significant contribution to borrelial virulence in this tissue. In addition, these data indicate that the effect resulting from the deletion of the C-terminal 6-AA sequence is tissue-dependent, in both *ospC* mRNA accumulation and bacterial load.

No significant defects were noted, in terms of ID_{50}, tissue colonization and arthritis virulence, due to deletion of the C-terminal 9-AA sequence; instead, the truncation elevated the tissue bacterial load in skin. An analysis of *ospC* expression revealed that the deletion significantly increased *ospC* mRNA accumulation in both heart and skin tissues. As discussed above, the integrity of OspC may be critical for OspC function in the heart tissue. The increased OspC expression resulting from the removal of the 9-AA sequence apparently compensated for the structural defects caused by the truncation, therefore allowing the mutant to maintain a bacterial burden at the control level. In the joint, the lack of the 9-AA sequence led to a decrease in *ospC* expression but maintained the bacterial load and retained arthritis virulence, indicating that neither OspC expression level nor structural integrity is crucial for the virulence of *B. burgdorferi* in this tissue. However, a threshold level of OspC expression may be required for these two capacities, as the removal of the 6-AA sequence led to a reduced *ospC* mRNA accumulation, a lower bacterial load, and diminished arthritis virulence. In skin, the integrity of OspC may not be important for tissue colonization as evidenced by the dramatically elevated tissue bacterial load resulting from the 9-AA deletion. It also provides evidence that OspC is an important virulence factor in colonization of the skin tissue, although its integrity is not critical in this specific tissue. Again, the study highlights tissue-dependent effects resulting from deletion of the C-terminal sequence.

Rosa and colleagues repeatedly showed that OspC is not required for the enzootic cycle of *B. burgdorferi* in the tick vector (Grimm *et al*., 2004, Stewart *et al*., 2006); in contrast, neither Pal *et al*. nor Fingerle *et al*. could make OspC mutants cross the salivary gland barrier to a murine host (Fingerle *et al.*, 2007, Pal *et al.*, 2004). The first group used fully competent *ospC* mutants in their studies, while the *ospC* mutants generated by the latter two groups could not be restored to infectivity. Remaining reasons for the disparity could be techniques used by different groups. A fresh blood meal leads to the upregulation of OspC and other antigens, a process that prepares *B. burgdorferi* for mammalian infection. The requirement for OspC is temporal; once *B. burgdorferi* has adapted to the mammalian environment, the lipoprotein becomes unessential (Tilly *et al*., 2006). If OspC plays a role in the tick, it remains to be addressed whether the C-terminal stretch is negligible for *B. burgdorferi* during the life cycle in the vector.

The presence of the C-terminal stretch influences *ospC* mRNA accumulation, which is determined by both transcriptional initiation and RNA turnover. Deletion of the 6-codons affected *ospC* mRNA accumulation only in the joint, while removal of three more influenced all tissues. The 6-codon deletion unlikely affected mRNA turnover; nevertheless, removal of an additional three codons increased *ospC* mRNA accumulation in both heart and skin tissues, arguing against faster mRNA turnover. For these reasons, the reduced mRNA accumulation originated most likely from lowered transcriptional initiation. The *ospC* expression is driven by an RpoS-dependent promoter (Eggers *et al.*, 2004, Yang *et al.*, 2005) and repressed through the interaction of the newly identified operator with a unknown repressor (Xu *et al*., 2007). Reducing RpoS expression and/or inducing the repressor would lead to the down-regulation of OspC, unless other unidentified regulatory layers exist. It is well known that *B. burgdorferi* adapts to diverse environments, in part, by selective gene expression. How environmental cues, such as temperature (Brooks *et al.*, 2003, Ojaimi *et al.*, 2003, Ramamoorthy and Scholl-Meeker,

2001, Stevenson *et al.*, 1995), pH (Carroll *et al.*, 1999, Yang *et al.*, 2000), nutrients or chemicals

(Yang *et al.*, 2001), and mammalian environments and specific immune responses (Caimano *et al.*, 2007, Liang *et al.*, 2002a, Liang *et al.*, 2004), are decoded to specific signals, which

ultimately influence gene expression in *B. burgdorferi*, remains unknown. The C-terminal 9-AA

stretch does not contribute to the core structure of OspC (Eicken *et al.*, 2001, Kumaran *et al.*,

2001) and is not required for virulence of *B. burgdorferi*; given its special membranous location,

future research should address how the C-terminal deletion affects *ospC* expression during

murine infection. It is interesting to find that while deletion of N-terminus 5-AA of OspC led to

an upregulation of *ospC* (Chapter 4), deletion of C-terminus of up to 9-AA of OspC led to a

down-regulation of *ospC*, further suggesting a role for the termini of OspC in self-regulation of

this enigmatic lipoprotein of *B. burgdorferi*.

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CHAPTER 6

GENERAL CONCLUSIONS

Lyme disease is included in the current list of emerging and re-emerging infectious diseases posted online by the National Institute of Allergy and Infectious disease (NIAID) (http://www3.niaid.nih.gov/topics/emerging). The disease is caused by at least three species of bacteria belonging to the genus *Borrelia* (Johnson *et al.*, 1984). *Borrelia burgdorferi* sensu lato is the major cause of Lyme disease in the United States (Baranton *et al.*, 1992). *Borrelia* is transmitted to humans by the bite of an infected tick of the genus *Ixodes* (the so-called hard ticks) (Cooley and Kohls, 1945). The pathogen accomplishes some remarkable feats during its enzootic life cycle. Not only does it adjust physiologically to two markedly different host environments, the arthropod vector and the mammalian reservoir, but on a molecular level the pathogen changes its surface lipoprotein expression in order to maintain its enzootic life cycle.

The outer surface protein C (OspC) is a major lipoprotein of *B.burgdorferi* that is induced within the tick during blood-feeding and is maintained in mammals only during the early stages of infection (Grimm *et al.*, 2004). Using improved genetic and molecular methodologies, investigators have begun to slowly decipher the various functions of OspC. Some of these functions are specific while others are non-specific to OspC. Table 6.1 summarizes these functions of OspC within the tick and the mammalian host (with their respective references), some of which are already well characterized, while others remain controversial.

Within the tick vector, OspC was shown not to be required for either tick colonization or transmission from the tick vector to a mammal at least in *B. burgdorferi* (Grimm *et al.*, 2004, Tilly *et al.*, 2006) . However, OspC was shown to be crucial for the dissemination of the European species *B. afzelii,* transmitted by the European vector *Ixodes ricinus* (Fingerle *et al.*,

2007). OspC also serves as a ligand for tick salivary gland protein Salp15, and thus facilitates the survival of the spirochete after inoculation into the mammal (Ramamoorthi *et al.*, 2005).

Table 6.1: Functions of OspC within the tick vector and the mammalian host.

Tick vector	References:
Crucial for dissemination of *B.afzelii* in *I. ricinus*	(Fingerle *et al.*, 2007)
Not required for migration to salivary glands or transmission to the mammal of *B. burgdorferi* in *I. scapularis*	(Grimm *et al*., 2004) (Tilly *et al*., 2006)
Interacts with Salp15 tick salivary gland protein	(Ramamoorthi *et al.*, 2005)
Mammalian host	
Protection against host innate defenses (non-specific)	(Tilly *et al.*, 2006) (Xu *et al*., 2008)
Dissemination-facilitating factor of *B. burgdorferi* (specific)	(Xu *et al*., 2008) This research project
Not required for dissemination of host-adapted *B.burgdorferi*	(Tilly *et al*., 2009)
Adhesion for heart colonization	(Antonara *et al*., 2007)
Plasminogen-binding protein	(Lagal *et al.*, 2006)
Self-regulation	This research project

Within the mammalian host, OspC is absolutely required in the early stages of infections initiated by tick bite or needle inoculation. OspC deletion mutants were shown to be cleared within 48h. Eight days are required for the wild type spirochetes to disseminate. OspC then becomes dispensable as infection proceeds (Tilly *et al.*, 2007), because a product produced during persistent infection can perform the early essential function normally performed by OspC

(Tilly *et al.*, 2009). The requirement for OspC ends by 28 days post-inoculation (Tilly *et al.*, 2006). The essential function of OspC in evasion from host innate defences can be replaced by other lipoproteins, if properly regulated (Xu *et al*., 2008).

One function that is uniquely attributed to OspC is that of facilitating the dissemination of *B. burgdorferi*. OspC-deficient spirochetes over-expressing heterologous lipoproteins were shown not to be as efficient in disseminating to distal tissues as those over-expressing OspC itself (Xu *et al.*, 2008). Although OspC as a whole was shown to be involved in assisting the dissemination of *B. burgdorferi* to remote tissues, this research work was the first of its kind being able to delineate this particular function of OspC to at least 5-AA of the N-terminus of OspC. This was also the first study to dissect the dissemination-facilitating function from the protection-promoting role of OspC. A study by the Tilly, K. *et al*., however, reports contradictory findings (Tilly *et al.*, 2009). Their study suggests that OspC plays no role in dissemination since spirochetes lacking OspC could disseminate and efficiently colonize distal tissues within 3 weeks of infection. However, the spirochetes used in their study were host-adapted, and thus, it is not possible to discriminate between the role that OspC performs from the role that a presumptive OspC surrogate (produced by the transferred host-adapted bacteria) performs in facilitating dissemination of *B. burgdorferi*. Further experiments directed towards solving this controversy are required.

Yet another function for OspC in the mammalian host is that of an adhesion to endothelial cells for the colonization of the heart (Antonara *et al.*, 2007). *ospC* transcripts were shown to be more abundant in the heart than in any other tissues (Hodzic *et al.*, 2003). Finally, the role of OspC as a plasminogen receptor was confirmed by using ELISA and surface plasmon resonance (Lagal *et al.*, 2006). The results of this study suggest that the correlation between

OspC polymorphism and *Borrelia* invasiveness is attributed in part to differences in OspC affinity for plasminogen.

In spite of recent advances in borrelial genetics that led to the development of selectable markers and shuttle vectors ((Bono *et al.*, 2000, Eggers *et al.*, 2002, Elias *et al.*, 2003, Stewart *et al.*, 2001), targeted gene inactivation (Rosa *et al.*, 2005), and identification of virulence factors such as OspC, OspA/B and PncA (Grimm *et al*., 2004, Purser *et al.*, 2003, Yang *et al.*, 2004), the use of these methodologies alone cannot help investigators elucidate the functions of these virulence factors. The analysis of secondary structures and three-dimensional crystal structures of solved proteins may lead to a more thorough understanding of the functions of these virulence factors. However, the resolution of OspC in 2001 did not help much in elucidating the function of this lipoprotein. Although a similarity between OspC and the aspartate receptor of Salmonella was found (Kumaran *et al.*, 2001), it did not have further implications. One reason for this gap in knowledge being that research groups have so far been focusing on the OspC structure as it relates to epitopes for the purposes of vaccine production and characterization of monoclonal antibodies (Earnhart *et al.*, 2007, Earnhart and Marconi, 2007, Yang *et al.*, 2006).

The overall goal of this research project was to investigate how the secondary structure of OspC relates to its functions and/or regulation of the *ospC* mRNA expression. This goal was accomplished by deleting certain amino acid sequences of OspC and studying their effects on infectivity, dissemination, spirochete burden, pathogenicity and *ospC* mRNA expression. A summary of how the diverse amino acid sequences affect the function of the OspC protein, its regulation of the *ospC* transcript, and other parameters are shown in Table 6.2.

The studies in this dissertation have shown that deletion of AA sequences within the core structure of OspC led to complete loss of infectivity of *B. burgdorferi*, whereas deletions towards the N-terminus or the C-terminus of the lipoprotein had different effects: from loss of infectivity,

depending on the number of amino acids deleted, to inefficient dissemination of the spirochetes to distal tissues, or changes in pathogenicity, to changes in *ospC* mRNA accumulation. Thus, our hypothesis that phenotypic changes related to the function(s) of OspC in *B. burgdorferi* can be attributed to certain structures of OspC was proven to be true.

It is interesting that the roles that OspC plays in innate immune evasion and in dissemination of *B.burgdorferi* have significance in the light of its regulation. While OspC aids *B. burgdorferi* to evade early innate host defences at the site of the tick bite, its role in facilitating dissemination aids the spirochetes to spread to distal tissues within two weeks, at which crucial period anti-OspC borreliacidal antibodies have been developed. However, since the pathogen has already disseminated to and colonized the remote tissues, it can then afford to downregulate *ospC* expression. Although other lipoproteins can substitute the role of OspC in dissemination to various extents, they are not as efficient and as fast in facilitating the dissemination as OspC (Xu *et al*., 2008). One study has shown that OspC is not needed for persistence in the mammalian host. It is hypothesized that other lipoproteins may to be able to substitute for the role of OspC after a certain period (Tilly et al., 2009). VlsE, a lipoprotein that is required for persistence of *B. burgdorferi,* may take over the function of OspC, which only remains a possibility.

It is interesting that the roles that OspC plays in innate protection and in dissemination of *B.burgdorferi* have significance in the light of its regulation. While OspC assists *B. burgdorferi* in evasion of host innate defences at the site of the tick bite, its role in facilitating dissemination aids the spirochete to spread to distal tissues within two weeks, at which crucial period anti-OspC borreliacidal antibodies have been developed. However, since the pathogen has already disseminated to and colonized the remote tissues, it can then afford to downregulate *ospC* expression. Although other lipoproteins can substitute the role of OspC in dissemination to various extents, they are not as efficient and as fast in facilitating the dissemination as OspC

Table 6.2. The effect of OspC amino-acid sequence deletions on OspC function, regulation of *ospC* transcript, and other parameters.[a]

OspC AA-sequence deleted	Function related to OspC
αH4 *βs1* *βs2* *Nt10* *Ct13*	Loss of protection against host innate defenses
Nt5	No loss of protection against host innate defenses Inefficient dissemination of spirochetes

Regulation of *ospC* transcript (SCID mice)

	Heart	Joint	Skin
Nt5	—	↑	—
Ct6	—	↓	—
Ct9	↑	↓	↑

Spirochete burden (SCID mice)

	Heart	Joint	Skin
Nt5	↓	↑	—
Ct6	↓	↓	—
Ct9	—	—	↑

Arthritis virulence (SCID mice)

Nt5 and Ct6	Loss
Ct9	Preserved

[a] Symbols: — : no change   ↑ : Significantly increased   ↓ : Significantly decreased

(Xu *et al*., 2008). One study has shown that OspC is not needed for persistence in the mammalian host. It is hypothesized that other lipoproteins may to be able to substitute for the role of OspC after a certain period (Tilly et al., 2009). VlsE, a lipoprotein that is required for

persistence of *B. burgdorferi,* may take over the function of OspC, which only remains a possibility.

OspC is among the major lipoproteins regulated by the RpoN-RpoS pathway (Hubner *et al.*, 2001). The two-component response regulator Rrp2, along with RpoN, plays a role in activating the transcription of *rpoS* (Yang *et al.*, 2003). An operator region containing two inverted repeats has been identified upstream of the RpoS-dependent promoter of *ospC* (Xu *et al.*, 2007). This operator is critical for the immune evasion of *B. burgdorferi*. A repressor for ospC has not yet been identified. A recent study has shown that spirochetes lacking lp281 exhibit a sustained and higher production of OspC, suggesting that *ospC* gene expression is downregulated during murine infection by a repressor encoded on lp28-1 and that the absence of the repressor in spirochetes lacking lp28-1 plasmid led to constitutive OspC production (Embers *et al.*, 2008).

OspC protein, on the other hand, is involved in self-regulation. This hypothesis was confirmed by the results of this research project have shown that there was an either down- or up-regulation of *ospC* mRNA transcripts in spirochetes expressing truncated OspC. When the C-terminus 6-AA and 9-AA sequence of OspC was deleted, there was a down-regulation of *ospC* transcript, whereas when the N-terminus 5-AA sequence of OspC was deleted, there was an up-regulation of *ospC* transcript compared to the transcripts of control spirochetes. However, we cannot exclude the possibility that another molecule could be binding to these C-terminus or N-terminus sites and thus causing the effect in regulation of *ospC* transcript.

In conclusion, OspC is a virulence factor of *B.burgdorferi* that has multiple functions. In deciphering the functions of this lipoprotein one can appreciate the pathogenicity of *Borrelia* within the mammalian host. This research project was able to relate the structure of OspC to its function and transcript regulation among other phenotypic changes. Since in biological solutions,

OspC forms dimers, further studies of mutagenesis and crystal structure of truncated versions of OspC will be helpful. Further studies are also needed to better understand the regulation of *ospC*. Since OspC is involved in facilitating the dissemination of *Borrelia* in the mammalian host, prevention of disease progression from an early stage to a disseminated stage can be possible. In terms of regulation, molecular control over *ospC* regulation while the spirochetes remain in the mammalian host could mean a discontinuation of the enzootic life cycle of the Lyme disease spirochete, *B. burgdorferi*.

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APPENDIX A

CALCULATION OF ID$_{50}$ VALUE BASED ON REED AND MUENCH MATHEMATICAL TECHNIQUE

Doses	No. of infected	No. of uninfected	Accumulated Number			Percentage of infected
			Infected (A)	Not infected (B)	Total No. of tested(A+B)	
10^5	10	0	31	0	31	31/31=100%
10^4	10	0	21	0	21	21/21=100%
10^3	8	2	11	2	13	11/13=85%
10^2	3	7	3	9	12	3/12=25%
10^1	0	10	0	19	19	0/19=0%

Calculation of the index

```
                   (% infected at dilution immediately above 50%) − 50%
Index = _____
               (% infected immediately above 50%) − (% infected immediately below 50%)
```

This is the application for calculating ID50, please prepare your data as shown above, then fill out the form below

Please input your number of dilution factor (it is 10 in the table above)

Please input the series of dilution from low to high (e.g. 1, 2, 3, 4, 5 in the table above)

Please input numbers of positive treatments (infected in the above table) in the series of dilution from low to high (0, 3, 8, 10, 10 in the table above)

Please input numbers of negative treatments (uninfected in the above table) in the series of dilution from low to high (10, 7, 2, 0, 0 in the table above)

Submit query

APPENDIX B

COMPARISION OF ARTHRITIS VIRULENCE BETWEEN MICE INOCULATED WITH CONTROL SPIROCHETES AND MICE INOCULATED WITH SPIROCHETES EXPRESSING THE N-TERMINUS 5-AA DELETED OSPC

VITA

Miss Sunita Venkata Seemanapalli was born in India in March 1971 to her parents Dr. Sarma V. Seemanapalli, Ph.D. and Mrs. Santa K. Seemanapalli, M.P.A. (USA). She earned a medical degree from the Federal University of Paraiba (UFPB), Campina Grande, Paraiba, Brazil, in 1994. She then pursued her master's degree from Southern University (SU), Baton Rouge, Louisiana. Under the supervision of Dr. Perpetua Muganda, her thesis work was focused on human cytomegalovirus and tumor suppressor protein p53. After obtaining her degree in 1998, she worked as a lecturer for a brief period at the Baton Rouge Community College (BRCC) and then she joined as a research associate at the Hansen's Disease Center, Baton Rouge, Louisiana for almost a year. At the Center, she was responsible for screening plant-derived compounds for activity against multi-drug resistance *Mycobacterium tuberculosis* strains. She continued her research work at the Pennington Biomedical Research Center, Baton Rouge, Louisiana, for about 5 years where she was involved in human genomics related projects. In order to deepen her knowledge of science and to improve her skills in biomedical research, she joined the doctoral program in the Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University (LSU), Baton Rouge, Louisiana, in the fall of 2004. Under the guidance of Dr. Fang-ting Liang, she studied the role of the outer surface protein C (OspC) in self-regulation and in facilitating the dissemination of *Borrelia burgdorferi* during murine infection. In May of 2010, Sunita finished her dissertation research and completed the requirements for the degree of Doctor of Philosophy. She will be joining a *Borrelia* research laboratory as a post-doctoral fellow at the Texas A&M University-Health Science Center (TAMU-HSC), College Station, Texas.