# SURFACE LOCALIZATION DETERMINANTS OF *BORRELIA BURGDORFERI* LIPOPROTEINS

BY

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## Abstract

*Borrelia* spirochetes are the causative agents of Lyme disease and relapsing fever, two common vector-borne diseases. Early experimental evidence, gained from development of genetic tools in the Lyme disease spirochete, *Borrelia burgdorferi*, emphasized the importance of outer surface lipo-proteins (Osps) during the infectious cycle. Although the functions of these lipoproteins and the complex mechanism of differential regulation is known in increasing detail, it remains to be understood how these virulence factors reach the spirochetal surface. We observed in previous studies that monomeric red fluorescent protein 1 (mRFP1) fused to specifically mutated outer surface protein A (OspA) lipopeptides could be detected by epifluorescence microscopy in both the periplasm and on the bacterial surface. These findings supported the notion that *Borrelia* spirochetes do not adhere to the +2/+3/+4 sorting rules established in other eubacteria. Rather, borrelial lipoproteins seem to contain a disordered 'tether' peptide located at the extreme N-terminus of the mature lipoprotein that influences sorting within the envelope. One facet of this study utilized an N-proximal tandem negative charge (Glu-Asp) that served as an inner membrane retention signal in OspA20:mRFP1 as a target for mutagenesis. A library of random mutants in the two codons was generated and expressed in *B. burgdorferi*. *In situ* surface proteolysis combined with fluorescence activated cell sorting (FACS) was then used to screen for viable spirochetes expressing subsurface OspA:mRFP1 fusions. We successfully recovered several mutants that mislocalized the lipo-mRFP1 fusions to the periplasm, adding to our database of peptide sequences that are not permissive for surface export. We then broadened our studies to include the structurally and functionally distinct dimeric OspC-Vsp family lipoproteins and identified their requirements for surface localization. As for OspA, tether sequences influence the localization of OspC-Vsp

lipoproteins within the envelope. Interestingly, OspC-Vsp lipoproteins appear to be translocated across the outer membrane as monomers. This suggests that they assume their final oligomeric state only when reaching the spirochetal surface. Additionally, lower molecular weight variants of OspC and Vsp1 were detected indicating cleavage that was exacerbated upon addition of C-terminal epitope tags or mislocalization of the untagged proteins to the periplasm. C-terminal proteolysis of OspC was attributed to a carboxy-terminal protease, CtpA. To date, known substrates of CtpA include the 13-kDa outer membrane porin, P13, and a periplasmic lipoprotein BB0323. C-terminal proteolysis of OspC and Vsp1 suggests CtpA may also function as a periplasmic housekeeping protease. In turn, released C-terminal peptides may play a role in initiation of an envelope stress response. Another aspect of this work examined the subcellular localization pattern of Braun's lipoprotein (Lpp) from *E. coli* using *B. burgdorferi* as a surrogate expression host. Surprisingly, Lpp was localized to the *B. burgdorferi* inner membrane. On the other hand, *B. burgdorferi* OspA mutants were sorted by *E. coli* according to *E. coli* rules. This dataset confirmed that host factors are setting the rules for localization of lipoproteins within the bacterial envelope. Taken together, this work revealed several factors, such as the composition of the lipoprotein tether and the folding state of the lipoprotein, which influences trafficking within the spirochetal cell envelope, and also provided important insights into periplasmic lipoprotein processing of *B. burgdorferi*. These findings will broaden our understanding of spirochetal lipoprotein transport as well as cell envelope biogenesis. Ultimately, this work may lead to novel treatments and/or vaccination strategies that will be extremely helpful in combating Lyme disease and relapsing fever in the years and decades to come.

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## Table of Contents

**Chapter I: Introduction………………………………………………………................. 1**
Lyme disease………………………………………………………………………........... 3
Lyme disease vaccination…………………………………………………………............ 6
Tick Borne relapsing fever……………………………………………………………………9
Antigenic Variation and Tissue Tropism in TBRF *Borrelia*……………………........... 10
Bacteriology……………………………………………………………………………...11
Differential regulation of *B. burgdorferi* lipoproteins during the infectious cycle…………13
OspA and OspB………………………………………………………………............. 14
OspC…………………………………………………………………………………….. 14
Gene regulation via the Rrp2-RpoN-RpoS pathway……………………………............ 16
Lipoprotein anatomy and biosynthesis……………………………………………............ 17
Lipoprotein sorting in *E. coli*…………………………………………………………………18
Surface localization of lipoproteins in other bacteria……………………………………… 20
Lipoprotein sorting in *B. burgdorferi*……………………………………..…………….. 22
The lipoprotein tether…………………………………………………………………… 24
The role of oligomerization in the translocation process……………………………………..25

**Chapter II: Materials and Methods……………………………………………........... 33**
Bacterial strains and growth conditions……………………………………………………34
Site-directed mutagenesis…………………………………………………………......... 35
SDS-PAGE and Immunoblot analysis……………………………………………......... 35
Construction of mutant plasmid library to identify subsurface localization signals………. 36
Fluorescence activated cell sorting (FACS) ……………………………………………… 37
DNA sequence analysis and identity of subsurface retention signals……………….......... 37
Protease accessibility assays……………………………………………………………… 38
Membrane and protein fractionations………………………………………………………38
Densitometry and calculations………………………………………………………………...40
*In situ* crosslinking………………………………………………………………………………40
Salp15 overlay assay……………………………………………………………………..41
Immunoprecipitation………………………………………………………………………..41
Purification of recombinant OspC…………………………………………………………42
Purification of recombinant OspA…………………………………………………………43
Circular dichroism measurements and analysis of thermal unfolding…………………….. 44
RNA extraction and Northern blotting…………………………………………………….. 44

**Chapter III: Development and validation of a FACS-based lipoprotein localization screen in the Lyme disease spirochete *Borrelia burgdorferi*…………………………… 49**
Abstract……………………………………………………………………………………..50
Introduction…………………………………………………………………………………51
Results and Discusssion……………………………………………………………………52
Conclusions………………………………………………………………………........... 57

**Chapter IV: Surface Localization Determinants of *Borrelia* OspC/Vsp Family Lipoproteins……………………………………………………………………… 68**
Abstract……………………………………………………………………………………..69
Introduction…………………………………………………………………………........... 70
Results………………………………………………………………………………………72
Discussion…………………………………………………………………………………..81

**Chapter V: Evidence for an Expanded Role of *Borrelia burgdorferi* CtpA Protease in Envelope Biogenesis……………………………………………………………98**
Abstract……………………………………………………………………………………..99
Introduction………………………………………………………………………….......... 100
Results………………………………………………………………………………........... 101
Discussion…………………………………………………………………………………..103

**Chapter VI: Cross-genus expression and localization of lipoproteins…………………109**
Abstract……………………………………………………………………………………..110
Introduction…………………………………………………………………………........... 111
Results………………………………………………………………………………………112
Discussion…………………………………………………………………………......... 114

**Chapter VII: Conclusions and Discussion…………………………………………........... 120**

**Literature Cited………………………………………………………………………... 129**

## Tables

1.1. Protection of experimental animals from *B. burgdorferi* challenge………………… 8
2.1. Bacterial strains used in this study………………………………………………….. 46
2.2. Antibodies used in this study……………………………………………………... 46
2.3. Plasmids used in this study………………………………………………………... 47
3.1. Phenotypes of OspA20:mRFP1 fusion mutants……………………………………… 67
4.1. Phenotypes of OspC-Vsp1 salt bridge charge swap point mutations………………… 85
6.1. Cross genus localization of Lpp and OspA:mRFP1. ………………………………… 116
7.1. Potential genes that encode the *B. burgdorferi* outer membrane 'flippase'………….. 126

## Figures


1.1. Etiology of Lyme disease. ……27
1.2. Crystal structures of *Borrelia* lipoproteins…… 28
1.3. The Rrp2-RpoN-RpoS pathway……29
1.4. *Borrelia* lipoprotein structure and biogenesis……30
1.5. The Lol sorting machinery in *E. coli* and architecture of the cell envelope…… 31
1.6. The *Borrelia* cell envelope and lipoprotein sorting…… 32
3.1. Screening strategy for subsurface OspA:mRFP1 fusions……59
3.2. FACS plots of OspA:mRFP1 mutant populations…… 61
3.3. Composite phenotypes of lipoprotein mutants…… 62
3.4. Phenotypical analysis of select OspA:mRFP1 fusion mutants……63
3.5. Protease accessibility of OspA:mRFP1 fusion mutants. ……65
3.6. Distribution of OspA:mRFP1 fusion mutants to inner and outer membranes……66
4.1. Genotypes and phenotypes of OspC and Vsp1 mutants. …… 86
4.2. Minimal OspA, OspC and Vsp1 tether sequence requirements for surface display of the mRFPΔ4 reporter. …… 88
4.3. Localization of OspC mutants…… 89
4.4. Localization of Vsp1 mutants…… 90
4.5. Circular dichroism and thermal denaturation data for recombinant OspC and OspA tether deletion mutants……91
4.6. Structural and functional analysis of select OspC and Vsp1 mutants…… 92
4.7. Additional evidence OspC transverses the OM as a monomeric intermediate…… 94
4.8. Structural analysis of epitope-tagged OspC mutants…… 95
4.9. Phenotypes of tetherless OspC and Vsp1 mutants…… 96
4.10. Heterodimerization of Vsp1 and Vsp2…… 97
5.1. Partial peptide sequences of wild type and mutant OspC proteins. ……106
5.2. CtpA-dependent proteolytic processing of OspC. …… 107
5.3. Localization of CtpA-dependent OspC variants……108
6.1. Localization of OspA-mRFP1 fusions in *E. coli*. …… 117
6.2. Peptide sequences of Lpp mutants used in this study. ……118
6.3. Localization of Lpp in *B. burgdorferi*……119

## Abbreviations

LD: Lyme disease
TBRF: Tick borne relapsing fever
GSK: GlaxoSmithKline
BSK: Barbour-Stoenner-Kelly
LB: Lysogeny broth
OMV: Outer membrane vesicle
PC: Protoplasmic cylinder
PE: Phosphatidylethanolamine
PG: Phosphatidylglycerol
PC: Phosphatidylcholine
CL: Cardiolipin
mRFP: monomeric red fluorescent protein
Osp: Outer surface protein
ORF: Open reading frame
CDC: Center for Disease Control and Prevention
TLR: Toll-like receptor
PCR: Polymerase chain reaction
MMP: Matrix metalloproteinase
GAG: Glycosaminoglycan
ECM: Extra-cellular matrix
TNF: Tumor necrosis factor
IL: Interleukin
CD: Circular dichroism
FSC: Forward Scatter
SSC: Side Scatter
CXCL: Chemokine (C-X-C motif) ligand
Form: Formaldehyde
pK: Proteinase K
Trp: Trypsin
Co-IP: Co-Immunopercipitation
Lol: Localization of lipoproteins
EM: Erythema migrans
ABC: ATP-binding cassette
Bam: β-barrel assembly machinery
FACS: Fluorescence activated cell sorting
FCT: Flow cytometry
DFI: Differential fluorescence induction
OMP: Outer membrane protein
LBRF: Louse borne relapsing fever

# Chapter I.

# Introduction

*Borrelia* spirochetes are the causative agents of Lyme disease (LD), tick borne relapsing fever (TBRF) and louse borne relapsing fever (LBRF). Lyme disease is the most common vector borne disease in North America, while TBRF is endemic to Africa. Lyme disease was first described in 1977, when an outbreak of arthritis in children was centered around the town of Old Lyme, Connecticut (Steere et al., 1977), although documentation of LD symptoms date back to the late 19$^{th}$ century (Azfelius, 1910; Lipschutz, 1918). Further investigation revealed the causative agent of LD was a spirochete, *Borrelia burgdorferi* (Burgdorfer et al., 1982), and was transmitted by *Ixodes scapularis* hard ticks (Steere et al., 1983; Benach et al., 1983). The first description of TBRF dates back to the late 18$^{th}$ century and it was later determined transmission occurs via *Ornithodoros* soft ticks (Cutler, 2010; Southern and Sanford, 1969; Dworkin et al., 2008). 'Relapsing fever' was the name first ascribed to the disease following an outbreak in Edinburgh, United Kingdom in the 1840's (Cutler, 2010).

Surface lipoproteins play essential roles in maintaining the tick-mammalian infectious cycle in both diseases. Tick midgut adhesins, immune evasion, and mammalian extra-cellular matrix (ECM) binding proteins are just a few of the functions of surface lipoproteins in *Borrelia* spirochetes. Although an increasing amount is known about the regulation of lipoprotein-coding genes and the functions of the lipoproteins themselves, the basic question of how lipoproteins are localized to the bacterial surface remains elusive. Answering this question is the main focus of our laboratory and the thrust of this dissertation. The answer is far from simple, as more research needs to be completed to unlock the mystery of lipoprotein sorting in *Borrelia*. It is my hope that the contributions of my graduate research will help elucidate decisive rules for lipoprotein sorting in *Borrelia*.

**Lyme disease**

In 2009 there were ~30,000 cases of LD reported to the Centers for Disease Control and Prevention (CDC) with an additional 8,500 probable cases. This represents an increase from ~20,000 in 2006 (Bacon et al., 2008; CDC, 2010). In the United States, LD is most commonly found in the northeast and north-central portions of the country. However, cases have been reported in all 50 states (Bacon et al., 2008). *Borrelia burgdorferi senso stricto* is the most common species of *Borrelia* that causes LD in North America (Baranton et al., 1992), while *B. garinii* and *B. afzelii* are largely responsible for LD in Europe and Asia (Bunikis et al., 2004; Stanek and Strle, 2009; Stanek et al., 2011). With an increasing *Ixodes scapularis* tick population and continued urban sprawl, exposure of human populations to LD has risen significantly. Ticks can acquire and transmit *B. burgdorferi* at any point in their life cycle, but most acquire the spirochetes in the larval stage from feeding on small rodents or birds, which are the natural reservoir (Mather et al., 1989; Donahue et al., 1987). The larvae then molt to nymphs (Fig 1.1B) the following spring and feed on larger mammals. The nymphs mature to adults over the course of the warm season and mate. Eggs are laid in the fall and hatch the following spring, thus completing the life cycle (Bosler et al., 1983; Lane et al., 1991; Anderson, 1989; Matuschka et al., 1992; Schwan et al., 1988).

Human infection commonly occurs during the late spring and summer when outdoor activity is frequent. Ticks are hard to detect due to their small size, and most people do not remember being bitten. *Ixodes* ticks take long blood meals (36-48 hours) that are essential for transmission of *B. burgdorferi* to the mammalian host (Piesman et al., 1987). LD can be treated with a course of antibiotics. Doxycycline is the drug of choice, with amoxicillin, cefuroxime

axetil, and erythromycin as alternatives (Wormser et al., 2006). If left untreated, LD can lead to a serious multisystem disease.

LD is divided into three stages: i) early localized stage, ii) early disseminated, and iii) late persistent. In the early-localized stage, the tick has finished feeding and detaches from the host. A 'bullseye' type rash called Erythema migrans (EM) usually appears at the site of the bite within several days (Fig. 1.1C). The rash grows over time and can increase to 13 cm in diameter. During this stage, the person experiences flu-like symptoms i.e., fever, headache, malaise and muscle aches. A few days later the early-disseminated stage begins when the spirochetes spread hematogenously. Spirochetes can be found in the joints, heart, brain, peripheral nervous system, and skin. Secondary lesions resembling EM can appear on sites throughout the body, but are unrelated to the tick bite. Neurological symptoms can also occur in 10-15% of untreated persons, the most common being Bell's Palsy (Wormser et al., 2006). If left untreated, late disseminated symptoms appear after several months. Arthritis is the most common symptom and usually affects the knees, but can affect other joints as well. About 60% of untreated persons develop arthritis, with children more prone than adults (Steere et al., 1987). Neurologic and cardiac symptoms can also occur at this stage as the spirochetes can invade the peripheral and central nervous systems, with 5% of untreated patients experiencing a condition called neuroborreliosis (Reik et al., 1979; Pachner et al., 1984; Logigian et al., 1990; Pachner and Steiner, 2007).

The immune response to *Borrelia* infection is a double-edged sword. It works to eliminate the spirochetes, but is responsible for inflammation and tissue pathology associated with the disease (Salazar et al., 2003; Hirschfeld et al., 1999). Binding and stabilization of plasmin on the surface of *Borrelia* leads to activation of host matrix-metalloproteinases (MMPs),

which contributes to inflammation and hematogenous spread (Gebbia et al., 2001; Gebbia et al., 2004; Coleman et al., 1995; Coleman et al., 1999; Hu et al., 2001). Toll-like receptor (TLR) 2 is also an important mediator of inflammation that specifically recognizes bacterial lipoproteins (Brightbill et al., 1999). Heterodimerization with TLR1 leads to specific recognition of triacylated lipoproteins (Gram-negative bacteria) while heterodimerization with TLR6 specifically recognized diacylated lipoproteins (Gram-positive bacteria) (Takeuchi et al., 2002; Kang et al., 2009; Schenk et al., 2009). TLR2 activation via borrelial lipoproteins induces production of the pro-inflammatory cytokines TNF-α IL-1, IL-6 and CXCL-1 (Hirschfeld et al., 1999). Contrary to the implication of TLR2 in inflammation, studies in TLR2 and MyD88 (adapter molecule in TLR signaling) knockout mice have shown an increased bacterial load and an increased inflammation when compared to wild type mice (Wooten et al., 2002; Liu et al., 2004; Bolz et al., 2004; Behera et al., 2006; Benhnia et al., 2005). This suggests other mediators of inflammation are also important during infection.

*B. burgdorferi* has evolved mechanisms to persist within infected hosts for several months, or even years, by avoiding clearance by the immune system. The three main mechanisms utilized for immune evasion are antigenic variation, down-regulation of a major surface protein, and binding of host complement factor H. Antigenic variation by the surface lipoprotein VlsE (Vmp-like sequence) occurs at a 10-kb locus on one of the 28 kb linear plasmids (lp28-1) (*vls*) and 15 silent gene cassettes (*vls2-16*) (Zhang et al., 1997). Recombination between the expression site, located downstream of the promoter, and the silent cassettes can occur in as little as four days post infection. No sequence variation was detected in strains that were cultured *in vitro*, suggesting that mammalian factors drive *vlsE* recombination (Zhang and Norris, 1998). *B. burgdorferi* also binds complement factor H, factor H-like protein

1, and/or factor H-related protein, thus resisting attack by the complement system (Kraiczy et al., 2001a; Kraiczy et al., 2001b; Brooks et al., 2005). Outer surface lipoprotein C (OspC, which will be discussed in detail later) is a major surface lipoprotein upregulated during tick feeding, and is required to establish infection (Grimm et al., 2004). In murine infection, *ospC* transcripts are undetectable 2 weeks post infection and anti-OspC antibodies synthesized during this time select for spirochetes that do not produce OspC (Liang et al., 2002). In fact, *Borrelia* cells that constitutively synthesize OspC are rapidly cleared by the immune system, as OspC stimulates an effective humoral immune response (Xu et al., 2006). Thus, down regulation of OspC is essential for persistent infection. With all the mechanisms *B. burgdorferi* employs to evade the immune system and persist within infected hosts for extended periods of time, what is the most effective method to prevent infection?

## Lyme Disease Vaccination

As cases of LD continue to rise prevention of LD via vaccination is an attractive strategy. Other measures such as protective clothing and routine use of tick repellents are ineffective in prevention of LD (Vázquez et al., 2008). These findings, along with the high cost of diagnosis and duration (3-4 weeks) of antibiotic required for treatment make vaccination of high-risk individuals a logical and effective alternative. Currently, no vaccine for LD is available for use in humans as LYMErix (discussed below) was pulled from the market in 2002. Potential vaccine candidates and new strategies will be discussed below.

To date, LYMErix is the first and only vaccine against *B. burgdorferi* licensed for use in humans. It was approved by the FDA in 1997 and was manufactured by GlaxoSmithKline (GSK) (Van Hoecke et al., 1996). LYMErix consists of recombinant OspA (a major surface

lipoprotein) from the ZS7 strain of *B. burgdorferi* that was synthesized in *E. coli* strain AR58 without the lipid moiety. After translation, the lipid was covalently bonded to the N-terminus and absorbed on an aluminum salt adjuvant. The vaccine was buffered with phosphate buffered saline and phenoxy-ethanol was used as a preservative (Steere et al., 1998; Wormser, 1996). LYMErix induced an IgG response against the C-terminal portion of OspA, which corresponds to the protective conformational epitope LA-2 (Sears et al., 1991). LYMErix was protective in ~80% of those vaccinated, although maintenance of high antibody titers was required for the vaccine to be effective (Steere et al., 1998; Sigal et al., 1998). To achieve adequate titers, over the course of one tick transmission season, three boosters were administered (Sigal et al., 1998). This ensured sufficient antibody was taken into the tick midgut with the blood meal. Since OspA antibodies are borreliacidal (de Silva et al., 1996) the vaccine neutralized *B. burgdorferi* and prevented transmission.

Of the 1.4 million doses of the vaccine sold, there were 905 reported adverse side effects with 102 being arthritis. However, the rate of arthritis was below the background occurrence within the general population (Lathrop et al., 2002). Despite this fact, numerous lawsuits were filed including a class action lawsuit in 1999 (Abbott, 2006). Subsequent studies found no conclusive link between development of arthritis and those vaccinated with LYMErix. There was also a concern that sequence homology between an OspA T-cell epitope ($OspA_{163-175}$) and the human leukocyte function antigen 1 ($LFA\text{-}1\alpha_{326-345}$) in persons with HLA haplotype [DRB 0401] led to induction of autoimmune arthritis (Gross and Huber, 2000; Trollmo et al., 2001). However, further studies conclusively proved this was not the case (Chen et al., 1999; Gross and Huber, 2000; Drouin et al., 2008). Despite convincing scientific data to the contrary, the public

was convinced LYMErix was unsafe. The bad press of the lawsuits caused vaccine sales to plummet and GSK had no choice but to discontinue the vaccine in 2002.

Other surface lipoproteins are candidates for the next generation LD vaccine. Possible candidates along with their functions, expression, and immunogenicity are detailed in Table 1.1. OspC is an especially promising candidate as it is expressed at high amounts early in the infections cycle, its genetic locus is stable, it is abundant on the surface and it elicits a robust adaptive and innate immune response. One drawback to OspC vaccination is the great deal of heterogeneity between strains at the *ospC* locus. However, a multivalent OspC vaccine composed of the major serotypes is possible (Wang et al., 1999).

**Table 1.1. Protection of Experimental Animals from *B. burgdorferi* challenge.**

| Immunizing Protein | Protection | Function | Expression |
|---|---|---|---|
| OspA | +++++ | Tick adhesin/Protects against acquired immunity | Tick |
| OspB | ++++ | Tick adhesin | Tick |
| OspC | +++ | Disseminating factor/Establish infection | Tick/Host |
| OspE | - | Complement binding | Host |
| OspF | + | Complement binding | Host |
| FlaB (negative control) | - | Flagella | Constitutive |
| P66 | + | Outer membrane porin | Constitutive |
| DbpA | ++ | Binds decorin | Host |

Data in table is from (A. Plotkin et al., 2008).

Another intriguing possibility for LD vaccination is the use of tick proteins. Arthropod vectors typically transmit a milieu of secreted proteins when a blood meal is taken, which elicits an immune response (Wikel, 1997; Wikel and Bergman, 1997). A study by Dai et al. showed immunization of mice with a tick salivary gland protein, Salp15 (Ramamoorthi et al., 2005; Schuijt et al., 2008), significantly protected mice upon *B. burgdorferi* challenge. The use of a vector protein as a vaccine was also successful in immunizing hamsters against the protozoan parasite *Leishmania* (Gomes et al., 2008). Moving forward, this approach validates vector

proteins as the basis for future LD vaccines. It will be interesting to determine if a vaccine consisting of *I. scapularis* and *B. burgdorferi* antigens will yield a more effective LD vaccine.

**Tick Borne Relapsing Fever**

TBRF is a worldwide disease with most cases occurring in Africa (Goubau, 1984). Although sporadic outbreaks of TBRF occur in the western United States there are only ~25 annual cases ((CDC), 2003; (CDC), 2007). *Ornithodoros* soft ticks, responsible for transmission, take short blood meals lasting 15-90 minutes (E. Sonenshine, 1997), which is significantly shorter than *Ixodes* blood meals. The *Borrelia* species that are transmitted by *Ornithodoros* ticks take their species name by the *Ornithodoros* species responsible for transmission. For example, the two *Borrelia* species that are most common in the United States, *B. hermsii* and *B. turicatae,* are transmitted by *O. hermsi* and *O. turicata*, respectively (Larsson et al., 2009; Dworkin et al., 2008).

TBRF is characterized by recurring episodes of fever, fatigue, headache, malaise, and muscle aches. The incubation period is about 7 days, and is followed by a fever that may or may not accompany the aforementioned symptoms. The fever lasts about 3 days and resolves on its own. About 7 days later, the first relapse occurs with symptoms identical to the initial fever. This cycle can continue in untreated persons up to 10 times (Southern and Sanford, 1969). The person may experience malaise or feel normal between febrile episodes. The causes of febrile symptoms are high levels of spirochetes in the bloodstream ($10^7$-$10^8$ cells/ml blood), which is significantly higher than LD *Borrelia* (Stoenner et al., 1982). A course of antibiotics can treat the infection with chloramphenicol, doxycycline, and erythromycin being the drugs of choice (L.

Kasper and Randolph Harrison, 2005). The mortality rate if left untreated is 4-10% (Dai et al., 2006).

**Antigenic variation and tissue tropism in tick borne relapsing fever *Borrelia***

The continued recurrence/cessation of febrile symptoms is a direct result of antigenic variation. Seroconversion effectively neutralizes 90% of the bacterial population while the remaining 10% undergo an antigenic switch via the variable membrane-like large (~36kDa) and variable membrane-like small (~20kDa) proteins or Vlp and Vsp, respectively (Stoenner et al., 1982; Barbour, 1990; Schwan and Hinnebusch, 1998). The mechanism of antigenic variation is different than the switching of silent gene cassettes that occurs at the *vlsE* locus in *B. burgdorferi* (described above). In *B. hermsii*, antigenic variation is governed by gene conversion involving recombination at upstream and downstream homology sequences (UHS and DHS) that replaces the *vsp* or *vlp* genes at the expression site. The UHS and DHS are located on a storage plasmid and recombination occurs downstream of the promoter on the expression plasmid. This results in a new Vsp or Vlp protein that is not recognized by the antibodies synthesized in response to the first antigen (Rich et al., 2001; Barbour et al., 2000). The spirochetes are one step ahead of the adaptive immune system as they multiply in the bloodstream and cause a relapse in febrile symptoms. The cycle continues until seroconversion eliminates all the spirochetes, or until the infected person is treated with antibiotics (Dai et al., 2006; Barbour et al., 2000; Burman et al., 1990; Kitten and Barbour, 1992).

Two distinct serotypes of *B. turicatae* Vsp (Vsp1 and Vsp2) exhibit differential tissue tropism. *Borreliae* that express Vsp1 preferentially invade the central nervous system of the host, while expression of Vsp2 results in spirochetemia and systemic illness (Cadavid et al.,

1997; Cadavid et al., 1994; Pennington and Cadavid, 1999; Cadavid et al., 1993; Pennington et al., 1997). The Vsps also bind glycosaminoglycans (GAGs), which enhances invasiveness (Magoun et al., 2000; Zückert et al., 2001). Vsp1, and to a lesser extend Vsp2, can disseminate from the periphery and inflame the brain (Londoño and Cadavid, 2010). Furthermore, interaction with the brain endothelium occurs via the lipid moiety and the variable dome region of Vsp1 (Gandhi et al., 2010)

**Bacteriology**

*Borrelia spp* belong to an atypical phylum of bacteria called the spirochetes, named for their wavelike morphology (Fig 1.1A). Members include *Treponema pallidum* (Syphilis) and *Leptospira interrogans* (Leptospirosis) (Hyde and Johnson, 1984; Haake and Matsunaga, 2010). *Borrelia* cells are 3-25 μm in length, 0.2-0.5 μm wide, and are diderm i.e., they contain two membranes that sandwich a periplasmic space. Double membrane envelope architecture is common among Gram-negative bacteria. However, *Borrelia* possesses distinct characteristics that make it difficult to classify. Unlike most bacteria the morphology of *Borrelia* cells is dependent on the periplasmic flagella and not peptidoglycan, although peptidoglycan does play a role in determining the overall rod shape of the cell cylinder (Motaleb et al., 2000).

The fragmented genome of *B. burgdorferi* also contains unusual characteristics such as high A/T content (~70%), a 0.9 MB linear chromosome and ~600 kb in linear and circular plasmids (Casjens and Huang, 1993; Baril et al., 1989; Ferdows and Barbour, 1989; Stewart et al., 2005). Interestingly, most of these plasmids are unnecessary for *in vitro* growth (and are readily cured when grown *in vitro*) yet contain gene products that are indispensable during the course of mammalian infection (Schwan et al., 1988; Skare et al., 1999; Skare et al., 1995).

Unlike *Treponema pallidum*, *Borrelia* can be cultivated in vitro using a complex, undefined medium developed by Alan Barbour, Herbert Stoenner, and Richard Kelly (BSK) (Zückert, 2007; Barbour, 1984; Kelly, 1971; Stoenner, 1974). The doubling time of *Borrelia* cells is relatively long (5-18 hours) when compared to *E. coli* and other bacteria, which can be as short as 20-30 minutes (Zückert, 2007; Stevenson et al., 1995).

Although *Borrelia* stains weakly negative when subjected to gram staining (Aberer and Duray, 1991), the spirochete is not considered gram negative due to the absence of lipopolysaccharide (LPS) in the cell envelope (Takayama et al., 1987). Glycolipids, termed BbGL-1 and BbGL-II, comprise 36% of the total lipid mass in *Borrelia* and may function as a substitute for LPS. Since *Borrelia* glycolipids share certain characteristics with LPS, i.e., are surface exposed, conform to a three-domain structure, and are abundant they could play a role in stabilizing the envelope. Thus, these glycolipids could function analogous to the role of LPS in Gram-negative bacteria (Schröder et al., 2003; Ben-Menachem et al., 2003; Östberg et al., 2007). Phospholipid composition and high lipoprotein content are other distinctive features of the *Borrelia* cell envelope. The phospholipid composition in the *E. coli* envelope is phosphatidylglycerol (PG) (65-70%), cardolipin (CL) and phosphatidylethanolamine (PE) (30-35%). On the other hand, *B. burgdorferi* lipid bilayers contain only phosphatidylcholine (PC) (~80%) and PG (~20%) (Belisle et al., 1994; Wang et al., 2004). The *E. coli* proteome contains ~90 lipoproteins that are largely localized to the periplasmic face of outer membrane and function to maintain envelope integrity (Gennity and Inouye, 1991), although a subset of Braun's lipoprotein seems to have a surface exposed domain (Cowles et al., 2011). The *B. burgdorferi* proteome contains ~127 lipoproteins (~7.8% of all ORFs) (Setubal et al., 2006; Eggers et al., 2000; Fraser et al., 1997) that are largely localized to the outer surface and function as virulence

factors (Skare et al., 1995). Surface localization of lipoproteins is absolutely essential in establishing and maintaining infection. Thus, a complex regulatory network that ensures proper expression of lipoproteins adapted for specific stages of the infectious process is necessary.

**Differential regulation of *B. burgdorferi* lipoproteins during the infectious cycle**

Over the past decade vigorous research has been conducted to elucidate the molecular mechanisms that govern differential gene regulation as *B. burgdorferi* progresses through the arthropod-mammalian infectious cycle. Schwan and Piesman made the initial observation that there was reciprocal regulation of two surface lipoproteins, OspA and OspC, at 23°C and 35°C (Schwan and Piesman, 2000), respectively. More recently, Srivastava and Silva have more precisely defined reciprocal expression of OspA and OspC at the single cell level (Srivastava and Silva, 2008). The 23°C to 35°C temperature shift mimics the change in environment from the arthropod vector to the mammalian host. Whole genome array analysis determined 200 ORFS were differentially regulated at 23°C and 35°C. One hundred-thirty ORFs were upregulated at 35°C, while 82 were upregulated at 23°C (Ojaimi et al., 2003). A complex global regulatory pathway, the Rrp2-RpoN-RpoS pathway, is responsible for differential regulation of lipoprotein coding genes during the infectious process. *ospA, ospB, ospC, dbpA, bbk32* and *vleE* are a few examples of lipoprotein encoding genes regulated by this pathway (Ouyang et al., 2008; Hubner et al., 2001; Caimano et al., 2004; Caimano et al., 2005; Fisher et al., 2005). Most experimental data is based on the relationship between OspA/B and OspC and will be the focus of discussion.

**OspA and OspB**

*ospA* and *ospB* are two lipoprotein-encoding genes that make up the *ospAB* operon located on the 54-kb linear plasmid (lp54) (Howe et al., 1986). Although both lipoproteins are synthesized in abundance during in vitro culture, expression during the course of mammalian infection is low. However, in the midguts of unfed ticks OspA and OspB are expressed at significantly higher levels (Stevenson et al., 1995; Liang et al., 2004). OspA facilitates adhesion to the tick midgut via the tick receptor for outer surface protein A, or TROSPA (Pal et al., 2000; Pal et al., 2001; Pal et al., 2004a). However, a study by Battisti et al., showed OspA deficient *B. burgdorferi* cells still bound tick midgut extracts, persisted within the midgut, and were able to infect mice. The authors propose OspA functions to protect *B. burgdorferi* from acquired host immunity upon the influx of immune blood from the host and is dispensable for midgut adhesion (Battisti et al., 2008). OspB serves a similar function as OspA as it binds to tick midgut extracts and *ospB* knockout strains are unable to colonize ticks (Neelakanta et al., 2007; Fikrig et al., 2004). Both lipoproteins are monomeric and consist of an open β-sheet with the C-terminus confined to a short α-helix (Fig. 1.2D) (Li et al., 1997; Becker et al., 2005). OspA and OspB are rapidly downregulated once the tick takes a blood meal and environmental conditions change (temperature, pH, oxygen, and $CO_2$). This mechanism facilitates detachment from the midgut, migration to the salivary glands and OspC synthesis (Liang et al., 2004; Brooks et al., 2003; Schwan et al., 1995; Schwan and Piesman, 2000).

**OspC**

OspC is a α-helical dimer (Eicken et al., 2001; Kumaran et al., 2001; Zückert et al., 2001) (Fig. 1.2A) encoded on the 26-kb circular plasmid (cp26). Cp26 cannot be cured under normal

circumstances (Jewett et al., 2007). OspC shares significant sequence and structural homology to the *B. turicatae* neurotropic lipoprotein Vsp1 (Fig. 1.2B) and *B. burgdorferi* VlsE, (Fig 1.2C) (Lawson et al., 2006; Zückert et al., 2001; Eicken et al., 2002). Other than the aforementioned lipoproteins, OspC only shares structural homology with the periplasmic domain of the *Salmonella typhimurium* aspartate receptor (15% amino acid identity) (Eicken et al., 2001; Kumaran et al., 2001; Yeh et al., 1996).

OspC is synthesized upon tick feeding and during the initial stages of infection. Further investigation revealed OspC is required for the establishment of mammalian infection (Grimm et al., 2004; Stewart et al., 2006; Tilly et al., 2006; Tilly et al., 2007), which appears to be further enhanced by binding to Salp15, an immune-modulating tick salivary gland protein (Anguita et al., 2002; Garg et al., 2006; Ramamoorthi et al., 2005). Salp15 is thought to coat the surface of *B. burgdorferi* and provide protection against antibody-mediated lysis (Schuijt et al., 2008). OspC has also been show to bind plasminogen, which presumably enhances invasiveness (Lagal et al., 2006). Despite providing these seemingly essential functions, Xu et al. showed that unrelated lipoproteins (DbpA, OspA, OspE), as well as VlsE were able to restore infectivity to strains that lack OspC. This calls into question the importance of OspC and emphasizes the composition of the borrelial surface and the general function of lipoproteins in stabilization of the outer membrane during infection (Xu et al., 2008). Indeed, the precise function of OspC is still the subject of considerable debate. The mechanism of differential regulation of OspA/B and OspC is a central paradigm of *B. burgdorferi* research and will be discussed in detail below.

**Gene regulation via the Rrp2-RpoN-RpoS pathway**

Changes in temperature, pH, cell density, $CO_2$ and oxygen are thought to be some of the external cues *B. burgdorferi* utilizes to down regulate genes associated with arthropod persistence, and upregulate genes involved in mammalian infection (Schwan et al., 1995; Indest et al., 1997; Carroll et al., 1999; Yang et al., 2000; Stevenson et al., 1995; Hyde et al., 2007). These cues lead to the induction of the Rrp2-RpoN-RpoS pathway that eventually leads to upregulation of *ospC* and other genes required for host adaptation. Phosphorylation and subsequent activation of Rrp2, which is the transcriptional activator of RpoN ($\sigma^{54}$), occurs by the small molecular weight high-energy phosphoryl-donor acetyl phosphate (acetyl~P) (Xu et al., 2010a). RpoN then binds the $\sigma^{54}$ dependent promoter of RpoS ($\sigma^{S}$) (Elias et al., 2000; Yang et al., 2000; Fisher et al., 2005; Hübner et al., 2001; Smith et al., 2007) and RpoS binds $\sigma^{S}$ dependent promoters. As an additional level of post-transcriptional control, a small regulatory RNA ($DsrA_{Bb}$) binds the upstream region of the *rpoS* mRNA at 37°C, but not 23°C, permitting the ribosome to access the ribosome-binding site and translation to proceed (Lybecker and Samuels, 2007). The proposed role of $DsrA_{Bb}$ is that of a molecular thermometer, as it only regulates *rpoS* mRNA levels in response to temperature and not in response to pH and cell density (Lybecker and Samuels, 2007) (Fig. 1.3).

The ultimate result of this complex signaling cascade is upregulation of OspC and other gene products involved in mammalian infection, while the tick associated genes (Caimano et al., 2005) are downregulated. An elegant study used flow cytometry to characterize OspA and OspC expression at the single cell level (Srivastava and Silva, 2008). Srivastava and Silva showed OspA and OspC expression is binary, i.e., the same cells that downregulate OspA are the same ones that upregulate OspC. An independent study by He et al. showed cells expressing OspC at

high levels do not express OspA, and abrogation of the *ospAB* operon leads to constitutive activation of the RrpS-RpoN-RpoS pathway (He et al., 2008). The authors proposed activation of the RpoN-RpoS pathway in conjunction with downregulation of OspA forms a positive feedback loop that further upregulates the Rrp2-RpoN-RpoS pathway. This mechanism insures the adequate production of OspC and other lipoproteins involved in mammalian infection (He et al., 2008) (Fig 1.3). Indeed, detailed information is available on the subject of differential regulation of OspA and OspC, but how do these lipoproteins reach the bacterial surface? Before we can attempt to answer this question it is important to understand how lipoproteins synthesized are trafficked in other bacteria.

**Lipoprotein structure and biosynthesis**

Lipoproteins are synthesized in the cytoplasm with an N-terminal signal peptide (~20 residues in length) that directs the unfolded polypeptide to the periplasm via the SecYEG translocon located in the cytoplasmic membrane (Manting and Driessen, 2000). Once Sec-dependent translocation of the pre-lipoprotein is complete, two acyl chains are added to a conserved N-terminal cysteine residue by lipoprotein diacylglycerol transferase (Lgt). The N-terminal signal peptide is then proteolytically removed by the lipoprotein signal peptidase (Lsp), more commonly known as signal peptidase II. Lipidation and signal peptide cleavage are determined by the presence of the lipobox motif at the C-terminal end of the signal peptide (Figure 1.4) (Choi et al., 1987; Sankaran and Wu, 1994; Qi et al., 1995). The spirochetal lipobox is 4 amino acids long and defined as follows: Leu(Ala,Ser,Val,Phe,Ile)$_{-4}$-Leu(Val,Phe,Ile)$_{-3}$-Ile(Val,Gly)$_{-2}$-Ala(Ser,Gly,Asn,Cys)$_{-1}$-↓Cys$_{+1}$ (Haake, 2000; Setubal et al., 2006). Compared to the *E. coli* lipobox: Leu(Ala,Val)$_{-4}$-Leu$_{-3}$-Ala(Ser)$_{-2}$-Gly(Ala)$_{-1}$-↓Cys$_{+1}$ (↓ denotes the Lsp cleavage site

and $Cys_{+1}$ is lipidated Cys) (Wu and Tokunaga, 1986), the spirochetal lipobox exhibits a higher degree of plasticity, presumably to tolerate the difference in active site specificities in the lipidiation and signal peptidase machinery (Paetzel et al., 2002). The final acyl chain is then added by the lipoprotein N-acyl transferase (Lnt) yielding the mature lipoprotein (Figure 1.4). All these steps occur while the lipoprotein is associated with the inner membrane and the corresponding enzymes are conserved across most bacterial species (Qi et al., 1995; Jackowski and Rock, 1986; Tokunaga et al., 1982). Lipoproteins are either retained in the inner membrane or sorted via the Lol (localization of lipoproteins) machinery across the periplasm to the outer membrane. There, the lipoproteins are either anchored to the periplasmic face of the outer membrane or translocated across the outer membrane to the surface of the cell (Zückert et al., 2004; Schulze and Zückert, 2006).

**Lipoprotein sorting in *E. coli***

Once lipidation and signal peptide cleavage is complete, the mature lipoprotein is shunted to the LolCDE machinery (stoichiometry $C_1D_2E_1$) located in the cytoplasmic membrane. LolCDE is an essential ABC transporter dedicated to the transport of lipoproteins, thus playing a vital role in outer membrane biogenesis (Yakushi et al., 2000; Ruiz et al., 2006; Silhavy et al., 2010). LolCDE releases lipoproteins, by hydrolysis of ATP, to LolA which functions as a periplasmic lipoprotein transit chaperone (Matsuyama et al., 1997). LolA binds to the lipoprotein via the lipid moiety, creates a water-soluble complex and delivers the lipoprotein to the outer membrane lipoprotein receptor LolB (Yokota et al., 1999; Yakushi et al., 1998). Finally, the lipoprotein is anchored to the periplasmic face of the outer membrane (Okuda and Tokuda, 2009; Tsukahara et al., 2009) (Fig. 1.5). Most lipoproteins in *E. coli* and other enterobacteriaceae are localized to

the outer membrane and are essential to maintain integrity of the cell envelope e.g., Lpp and Pal (Ruiz et al., 2006; Cascales et al., 2002). However, there are some lipoproteins anchored to the inner membrane that avoid interaction with LolCDE (Fig. 1.5). These lipoproteins share a common aspartic acid in the +2 position immediately downstream of the lipidated cysteine. This is illustrated as follows:

| | Lipobox | | | ↓ | | | | |
|---|---|---|---|---|---|---|---|---|
| | -4 | -3 | -2 | -1 | +1 | +2 | +3 | +4 | Envelope Localization |
| Lpp | L | L | A | G | C | S | S | N | Outer membrane |
| Lipoprotein-28 | L | L | A | G | C | D | Q | S | Inner membrane |

↓denotes the signal peptidase II (Lsp) cleavage site.

The +2 Asp functions as a LolCDE avoidance signal, and is commonly known as the +2 rule. If any other amino acid is present in the +2 position, the lipoprotein is permissive for interaction with LolCDE and sorted to the outer membrane (Yamaguchi et al., 1988). Interestingly, a study by Seydel et al. found the amino acids phenylalanine, tryptophan, tyrosine, glycine, and proline could also act as Lol avoidance signals (Seydel et al., 1999). Upon further investigation, it was determined that the aforementioned amino acids are never present in the +2 position of native lipoproteins and do not represent true inner membrane retention signals.

The mechanism of Lol avoidance is dependent on the distance between the negative charge of the Cα of the +2 residue (2.72-3.5 Å) and phosphatidylethanolamine (PE). When Asp is in the +2 position, the negative charge of the side chain forms a tight complex with PE that contains five acyl chains and is unable to interact with LolCDE. Glu cannot satisfy this requirement because the negative charge is too far (4.24-4.93 Å) from the Cα of the +2 residue, but oxidation of Cys to cysteic acid can act as an avoidance signal because the negative charge is within range (2.87-3.95 Å) (Hara et al., 2003; Robichon et al., 2005). Inner membrane retention is enhanced when Asp, Glu, and Gln are in the +3 position (Terada et al., 2001). The positive

charge of PE was also found to be crucial, as amine specific modification of PE or replacement with cardiolipin abolished LolCDE avoidance despite the presence of Asp in the +2 position (Hara et al., 2003). Since *Borrelia* lack PE (Belisle et al., 1994) this is a possible explanation on why the +2 rule does not apply. The reason Phe, Trp, Pro, Gly, and Tyr act as inner membrane retention signals is not explained by the +2 rule and is likely due to a different mechanism that has yet to be elucidated. The +2 rule also extends to other members of *Enterobacteriaceae* (*Shigella flexneri, Salmonella enterica, Yersinia pseduotouberulosis,* and *Erwinia carotovora)* (Lewenza et al., 2006). Variations of the +2 rule also exist, as Pseudomonas aeruginosa lipoprotein sorting signals are located at positions +3 and +4 (Naritia and Tokuda, 2007). Overall, surface exposure of lipoproteins is rare in bacteria, although surface presentation occurs via the autotransporter (Type V), Type II (T2SS) secretion systems. In addition to secretion system mediated surface localization, a subset of an abundant *E. coli* 'outer membrane' lipoprotein seems to be surface exposed (Cowles et al., 2011).

**Surface localization of lipoproteins in other bacteria**

Autotransporters represent a large superfamily of bacterial transporters that are quite diverse in function and size (<20 to >400 kDa), but their basic structure is composed of an N-terminal signal peptide, an N-terminal extracellular passenger domain and a C-terminal β-barrel (β domain) (Pohlner et al., 1987; Pallen et al., 2003). It was once widely accepted that the β domain assembles a pore in the outer membrane that mediates translocation of the passenger domain to the surface after SecYEG dependent secretion to the periplasm. 'Autotransporters' was termed such because of the perceived self-contained nature of the protein translocation

system (Pohlner et al., 1987). This notion has since been called into question, and the exact mechanism of how autotransporters cross the outer membrane remains a subject of debate.

A recent report by Ieva and Bernstein, shows direct interaction between the *E. coli* EspP autotransporter passenger and β domains with BamA (β-barrel assembly machinery, also known as Omp85/YaeT) (Gentle et al., 2005; Ieva and Bernstein, 2009). BamA functions to insert integral outer membrane proteins (OMPs) into the outer membrane (Gentle et al., 2005). This data strongly supports the model of insertion of the β domain in the outer membrane by BamA and subsequent translocation of the passenger domain. Some autotransporters are lipoproteins, including *Bordetella pertussis* subtilisin SphB1 and *Neisseria meningitidis* serine protease NalP (Coutte et al., 2003; van Ulsen et al., 2003). These lipoproteins represent some of the rare exceptions of surface exposed lipoproteins that occur in bacteria other than *Borrelia*. However, their functions as proteases are distinct in comparison to the functions of *Borrelia* lipoproteins.

Other examples of surface exposed lipoproteins are Pullulanase (PulA) in *Klebsiella oxytoca,* and MtrC, and OmcA in *Shewanella oneidensis.* These lipoproteins reach the surface via the Type II Secretion System (T2SS) after SecYEG dependent secretion to the periplasm (d'Enfert et al., 1987; Shi et al., 2008). In the absence of the T2SS, localization of PulA is dependent on the +2 rule. Only when the T2SS is present is PulA localized to the bacterial surface (Pugsley, 1993). Another example of a surface exposed lipoprotein is free-form Braun's lipoprotein (Lpp) (Cowles et al., 2011). Lpp is an extremely abundant outer membrane lipoprotein that exists in two forms: the bound form that is bound to peptidoglycan (Fig 1.5) and the free form that is not (Braun and Rehn, 1969; Braun and Bosch, 1972; Braun and Wolff, 1970). Cowles et al. recently showed that the free form of Lpp is surface exposed (Cowles et al., 2011). The free form Lpp trimer is likely anchored to the periplasmic face of the outer

membrane and spans the outer lipid bilayer in such a way that the C-terminus is surface exposed (Bernstein, 2011).

**<u>Lipoprotein sorting in *B. burgdorferi*</u>**

The *B. burgdorferi* cell envelope contains many distinct features (see bacteriology section). However, the pathways involved in insertion of β-barrel proteins in the outer membrane as well as the essential periplasmic chaperones seem to be conserved (Bergström et al., 2010), e.g., DegP, SurA, and Skp. Additionally, CtpA is another periplasmic protease that is not commonly found in the proteomes of eubacteria. With the exception of CtpA, these proteins have not been characterized in *B. burgdorferi*. CtpA is an unusual serine-like C-terminal protease with homologues in *T. pallidum* and other bacteria. Östberg et al. made the observation that inactivation of *ctpA* in *B. burgdorferi* resulted in an altered protein expression profile, but did not significantly affect *in vitro* growth (Östberg et al., 2004). Two of the proteins identified in this study were the lipoprotein BB0323 and P13, an outer membrane porin. Both of these proteins had a C-terminal processing defect, but it did not seem to influence localization within the envelope (Östberg et al., 2004). Previous studies showed inactivation of *bb0323* caused significant membrane blebbing that led to considerable defects in envelope stability (Zhang et al., 2009; Stewart et al., 2004). It is possible that CtpA modifies other lipoproteins in the process of outer membrane biogenesis and maintenance of envelope homeostasis.

Our knowledge of *Borrelia* lipoprotein sorting is still in its infancy. *B. burgdorferi* contains homologues of all the proteins of the Lol machinery with the exception of the outer membrane lipoprotein receptor, LolB. Although existence of a LolB-like protein with little sequence homology is certainly possible no protein has been assigned this function, to date.

From preliminary studies, LolA has been shown to interact with outer membrane and surface lipoproteins, but not inner membrane lipoproteins, which is consistent with the *E. coli* Lol system (Bridges and Zückert, unpublished data). *B. burgdorferi* Lol homologues share significant sequence homology with the *E. coli* Lol proteins, so it is reasonable to postulate that they function in a similar way.

What are the requirements for proper localization of *Borrelia* lipoproteins? Schulze and Zückert first began to address this question when they made N-terminal fusions of OspA to a monomeric red fluorescent reporter protein (mRFP1) and found that mRFP1 localized to the *B. burgdorferi* cell surface. A minimum of five amino acids of the mature lipoprotein was sufficient for proper surface localization, as fusions of less than five amino acids were localized to the periplasm. Negative charges could also act to retain these OspA lipo-mRFP1 fusions in the inner membrane when they were present in a certain context. Alanine mutagenesis of $OspA_{28}$:mRFP1 had no effect on surface localization of mRFP1. Surprisingly, a fusion of an inner membrane lipoprotein, OppAIV, to mRFP1 was also localized to the cell surface. Taken together, these results led to the model of *B. burgdorferi* lipoproteins localized to the surface by default (Schulze and Zückert, 2006). This study also was important in showing lipoproteins of *B. burgdorferi* do not adhere to the established +2 rule in *E. coli,* or the +2/+3/+4 rule that other bacteria follow (Narita and Tokuda, 2007).

In a follow up study again using OspA as a model lipoprotein, Schulze et al. found four N-terminal amino acids of mRFP1 (ASSE) artificially increased the length of the N-terminal mature region of the lipo-RFP fusion. Removal of these four residues resulted in a new reporter (mRFPΔ4) (Schulze et al., 2010), and localization of OspA:mRFPΔ4 fusions and mutants were consistent with localization of OspA mutants not fused with mRFPΔ4. More importantly, this

study also identified a tetrapeptide in OspA ($V_{21}S_{22}S_{23}L_{24}$) that was essential in localization of OspA to the surface. Single amino acid deletions of any of these four residues led to mislocalzation of OspA to the periplasmic face of the outer membrane. Further analysis using C-terminal epitope tagged variants of OspA in conjunction with C-terminal destabilizing mutations revealed that secretion of OspA across the outer membrane requires an unfolded confirmation that initiates at the C-terminus (Schulze et al., 2010). Finally, the mechanism of lipoprotein transport and outer membrane biogenesis is likely conserved across all species of *Borrelia* as *B. burgdorferi* correctly localizes the *B. turicatae* lipoproteins Vsp1 and Vsp2 to the spirochetal surface (Zückert et al., 2004).

**The lipoprotein tether**

All *Borrelia* lipoproteins seem to contain a disordered stretch of amino acids located at the extreme N-terminus of the mature lipoprotein. Crystallization data indicates that these N-terminal residues are either void of election density or lack any secondary structure. We envision a model in which the lipoprotein is anchored to the membrane via the lipid moiety and the N-terminal disordered amino acids acts as a flexible linker (Fig 1.4). This linker functions to 'tether' the structurally confined portion of the lipoprotein to the lipid bilayer. Thus, we have termed this portion of the lipoprotein the 'tether'. We hypothesize that the tether residues must adopt a certain degree of flexibility necessary for interaction with a periplasmic chaperone. In support of this hypothesis, replacement of the OspA VSSL tetrapeptide with alanines was permissive for surface translocation, while replacement with glycines was not.

The primary sequence of the tether is highly variable (Schulze et al., 2010) with the exception of the absolutely conserved +1 Cys. Primary sequence alignment of all known and

predicted *B. burgdorferi* lipoproteins from the +1 Cys onward failed to identify a canonical motif or sorting 'signal' (Schulze et al., 2010). Therefore, it is difficult to determine if sorting rules are based on amino acid identity alone. A random mutagenesis approach would be a viable strategy in ascertaining the answer to this question. Our current hypothesis states that regions of the tether must interact with a periplasmic chaperone and subsequently an outer membrane lipoprotein translocon or 'flippase'. This 'flippase' would function to translocate surface lipoproteins across the outer membrane and to the bacterial surface. To date, all attempts to identify this protein using classic protein-protein interaction techniques, such as co-immunoprecipitation, epitope tag pulldowns, yeast two-hybrid screens, and biotinylation assays have yielded inconsistent results. Future approaches will make use of conditional knockout mutants of chromosomally encoded integral outer membrane proteins that have not been characterized (Table 7.1) (Whetstine et al., 2009). One such conditional knockout has already been characterized using the *B. burgdorferi* BamA homologue (BB0795) (Lenhart and Akins, 2010). Depletion of BB0795 resulted in a reduction of lipoproteins in the outer membrane, although this effect might be secondary (Lenhart and Akins, 2010) as BB0795 may be responsible for insertion of the aforementioned outer membrane lipoprotein flippase.

## The role of oligomerization in the translocation process

Eubacteria have evolved a diverse number of mechanisms to transport proteins across cell membranes. Although explanation of each secretion mechanism is beyond the scope of this dissertation, a brief comparison of two systems: the Sec and the Tat translocons are important. The Sec translocase exports unfolded proteins across the cytoplasmic membrane via hydrolysis of ATP and cannot tolerate folded substrates. The Tat translocase also exports proteins across

the cytoplasmic membrane, but can only tolerate folded substrates and utilizes the proton motive force as an energy source (Manting and Driessen, 2000; DeLisa et al., 2003). The oligomeric state of lipoproteins during translocation is one of the questions addressed in this dissertation. Are lipoproteins translocated across the outer membrane in their final, oligomeric conformation or is a monomer necessary for export? In the case of the Sec translocon, oligomerization occurs in the periplasm and premature oligomerization leads to a block in secretion (Natale et al., 2008). On the other hand, failure of Tat substrates to oligomerize prior to translcoation results in a translocation block (Jack et al., 2004). What are the oligomeric requirements for lipoprotein sorting through the outer membrane? Answering this question would provide important information regarding the nature of the outer membrane 'flippase.'

The experimental work presented in this dissertation has focused on elucidating the requirements that govern localization of lipoproteins. We have used many approaches in an attempt to answer this question and further define the rules of lipoprotein sorting in *B. burgdorferi*. Our first approach used random mutagenesis in combination with FACS to identify novel periplasmic localization signals (Chapter III). We then shifted the focus of our studies to the dimeric OspC-Vsp lipoprotein family to further define the role of oligomerization during the translocation process (Chapter IV) and to directly compare factors that influence surface localization to OspA. During the course of these two main projects we further defined the role of a periplasmic C-terminal protease, CtpA, which proteolytically cleaves certain lipoproteins (Chapter V). This finding gave us some insight to the role of CtpA in envelope biogenesis. Finally, we expressed a lipoprotein native to *E. coli* in *B. burgdorferi* and localized it within the envelope (Chapter VI).

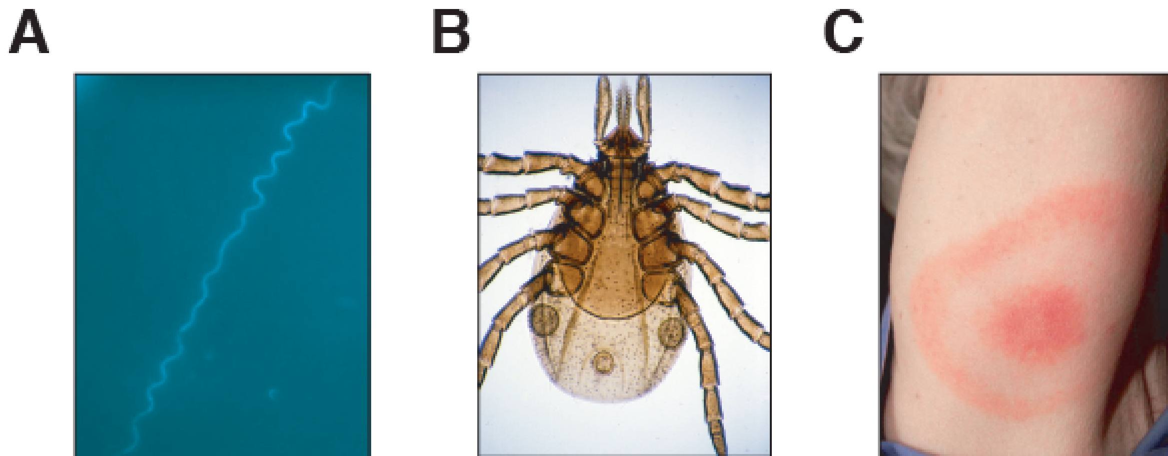



**Figure 1.1. Etiology of Lyme disease.**

**(A)** A single *B. burgdorferi* cell stained with DAPI under 40X magnification. Image was taken and processed by Ozan Kumru. **(B)** Whole mount of an *Ixodes scapularis* nymph. Image kindly provided, with permission, by Dr. Steve Upton, Kansas State University Parasitology (Upton, 1999). **(C)** Erythema migrans rash that commonly develops at the site of the tick bite and is a telltale sign of Lyme disease. Image kindly provided by James Gathany of the CDC with permission.

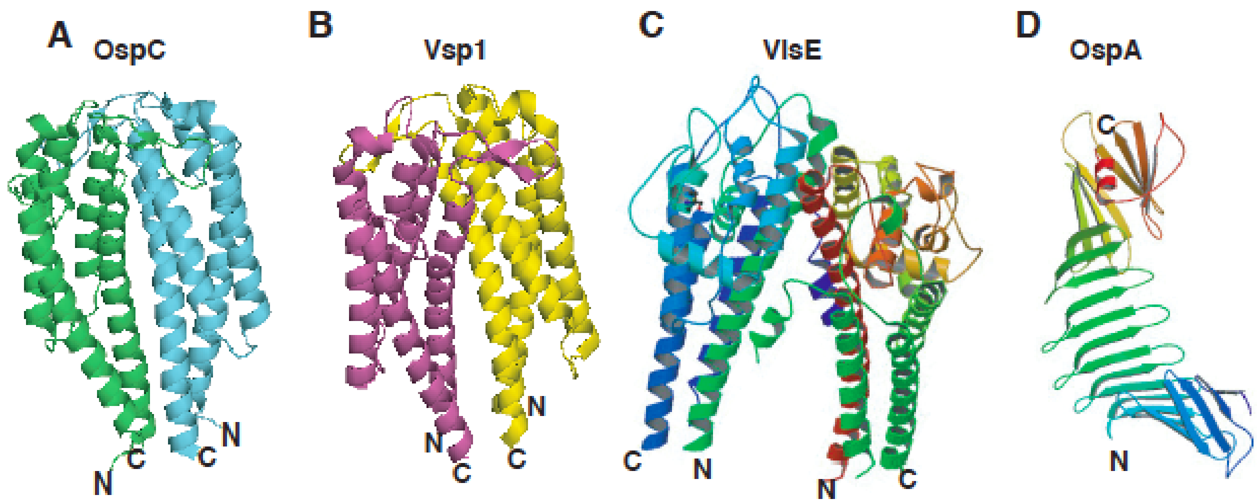



**Figure 1.2. Crystal structures of *Borrelia* lipoproteins.**

Crystal structures of the *Borrelia* lipoproteins **(A)** OspC, **(B)** Vsp1, **(C)** VlsE and **(D)** OspA. N and C termini are indicated. PDB accession numbers are 1GGQ, 1YJG, 1L8W, and 3EEX respectively.

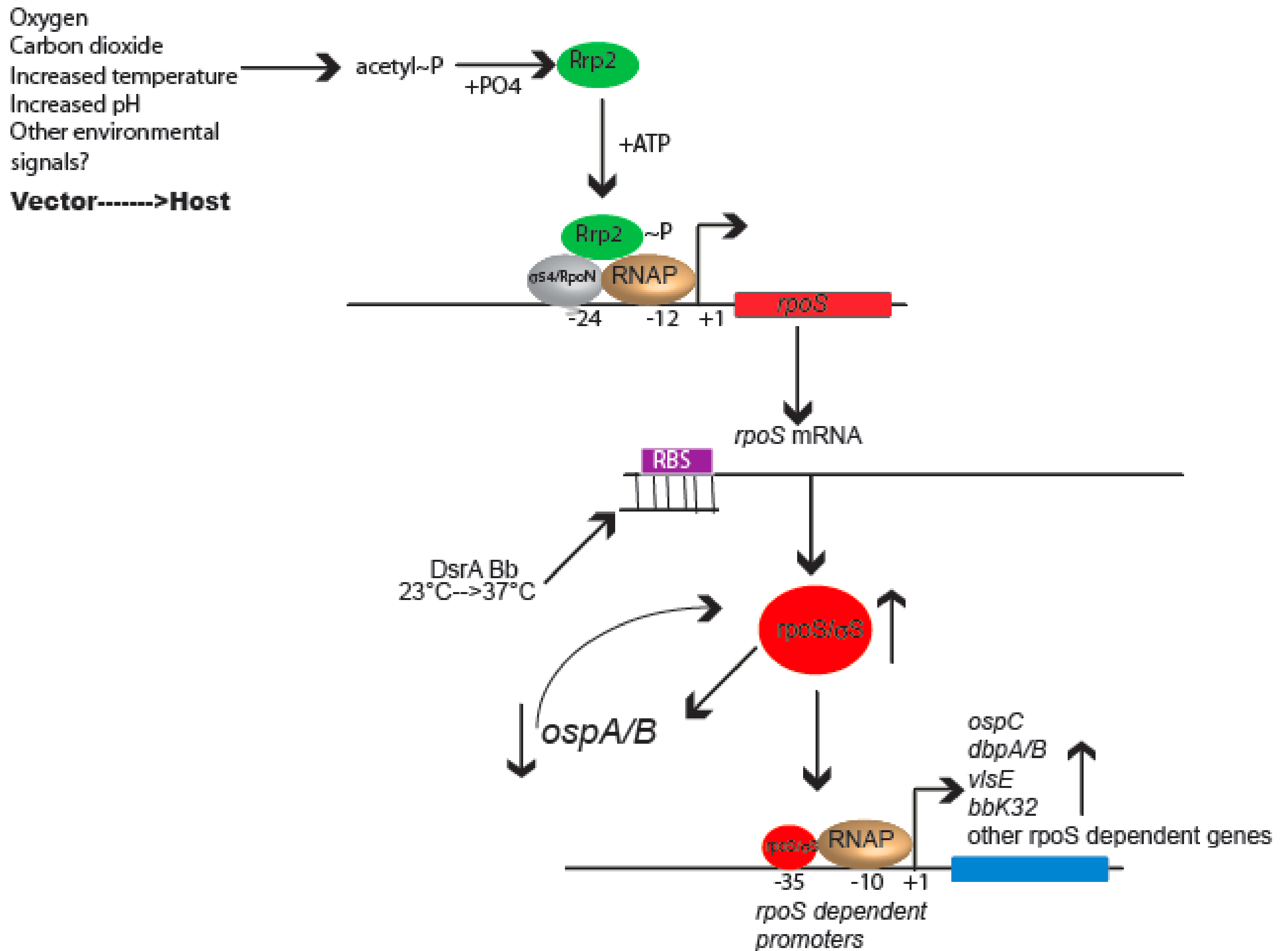



**Figure 1.3. The Rrp2-RpoN-RpoS pathway.**

Environmental changes activate a signaling cascade that culminates in the activation of *rpoS* ($\sigma^S$) that binds the promoters of genes whose products are involved in mammalian infection. Many of the gene products are lipoproteins, including OspC (Burtnick et al., 2007; He et al., 2008; Lybecker and Samuels, 2007; Xu et al., 2010a; Caimano et al., 2007). Figure is not drawn to scale. See text for details.

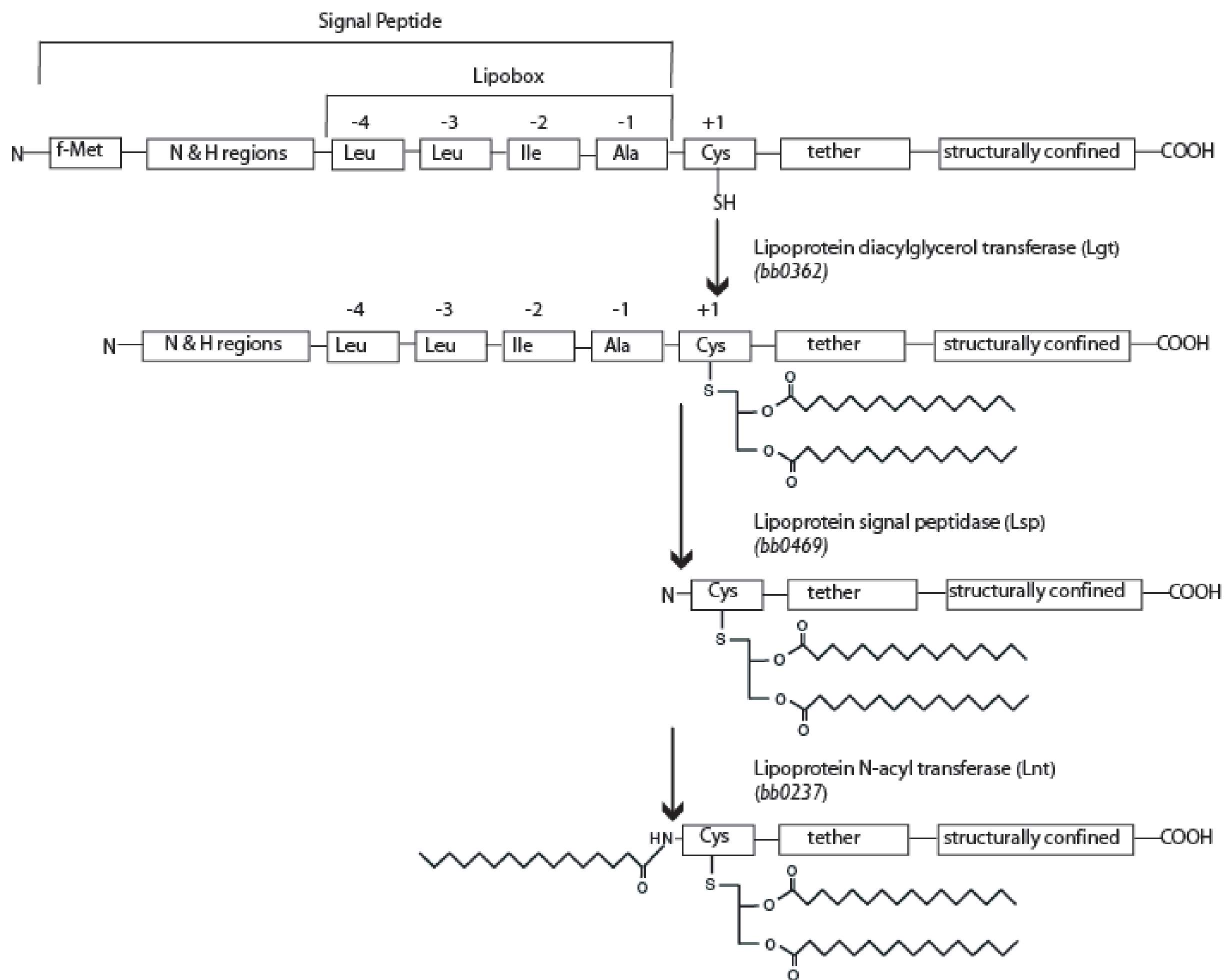



**Figure 1.4.** ***Borrelia*** **lipoprotein structure and biogenesis.**

*Borrelia* lipoproteins are synthesized in the cytoplasm and directed to the inner membrane SecYEG translocon by the N-terminal signal peptide. The lipobox motif ensures the protein is diacylated at the +1 Cys by Lgt, followed by proteolytic cleavage of the signal peptide by Lsp. Finally, Lnt adds the third acyl chain via an amine linkage at the +1 Cys. The lipoprotein is now fully mature and is then sorted through the Lol machinery depending on the composition of the lipoprotein tether (see text for details). Figure is not drawn to scale. Figure was inspired by (Kovacs-Simon et al., 2010).

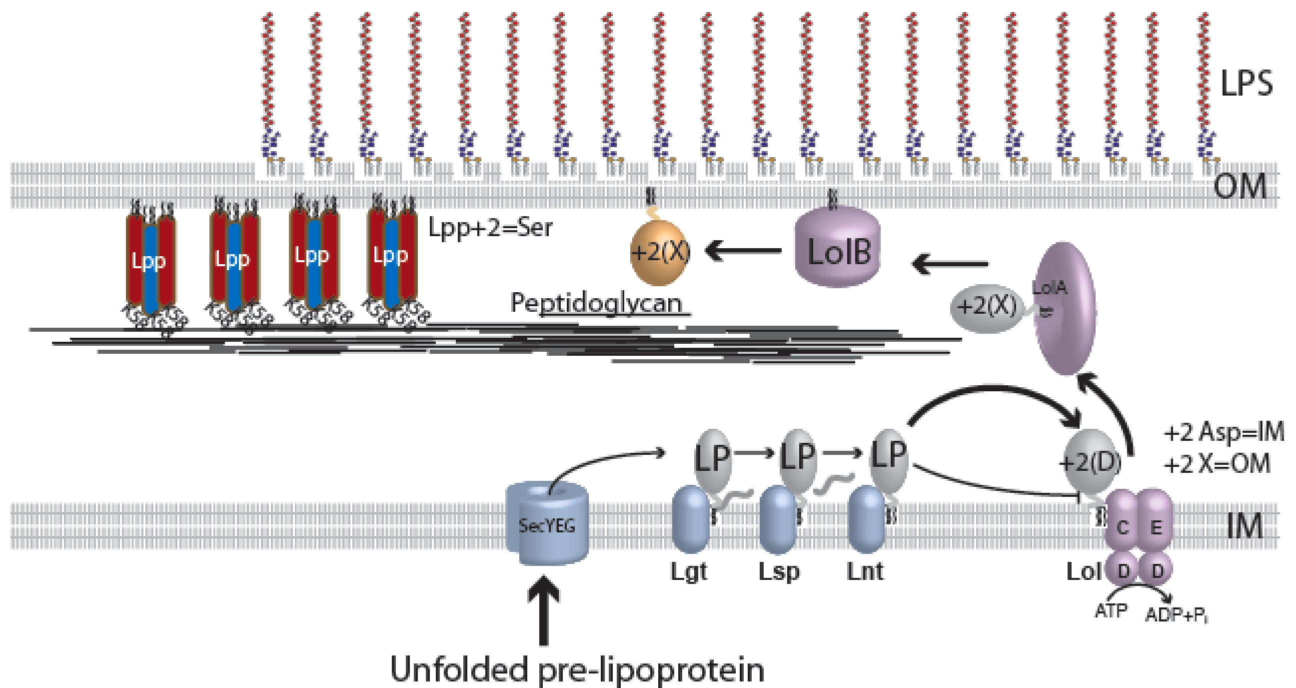



**Figure 1.5. The Lol sorting machinery in *E. coli* and architecture of the cell envelope.** LolCDE is an ABC transporter that functions to release lipoproteins destined to the outer membrane and creates a water-soluble complex with LolA. In turn, LolA transfers the lipoprotein to LolB, which inserts the lipoprotein into the periplasmic side of the outer membrane. Outer membrane translocation only occurs if there is not an Asp in the +2 position of the mature lipoprotein. If an Asp is in the +2 position, the lipoprotein creates a complex with the phospholipid PE that possesses five acyl chains, which fails to interact with LolCDE and the lipoprotein is retained in the inner membrane (Tokuda and Matsuyama, 2004). Lpp contains a Ser in the +2 position and is sorted to the outer membrane and bound to peptidoglycan via Lys58. The C-terminus of free-form Lpp is surface exposed (not pictured) (Cowles et al., 2011; Bernstein, 2011). See text for details.

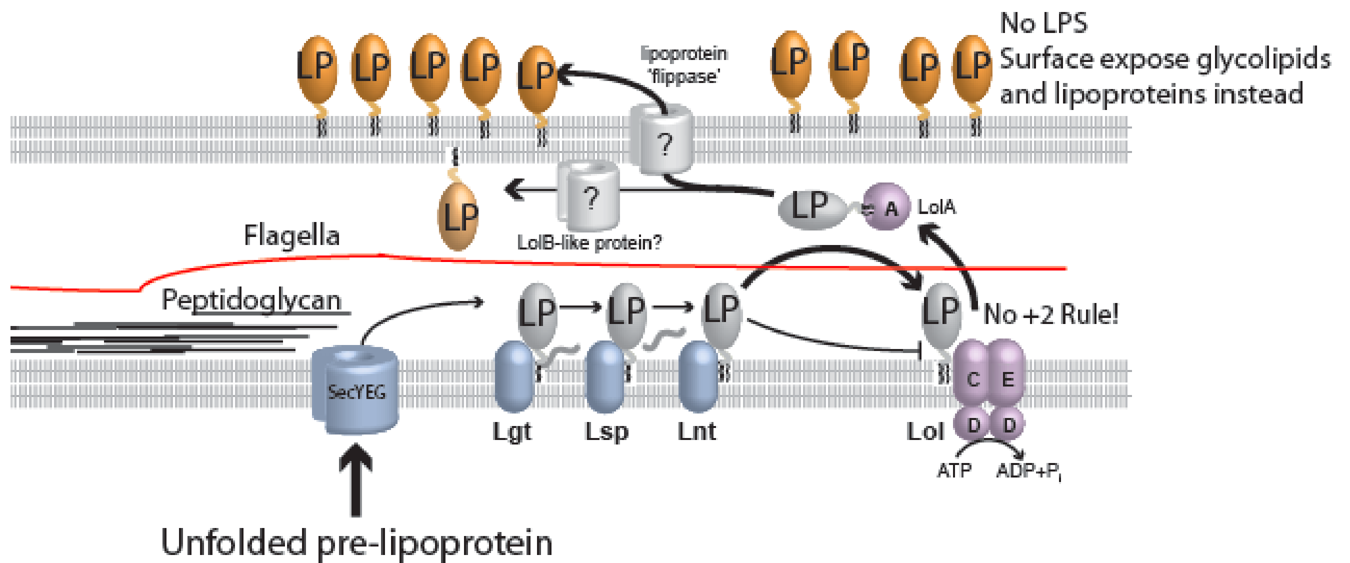



**Figure 1.6. The *Borrelia* cell envelope and lipoprotein sorting.**

The *B. burgdorferi* proteome contains homologues of all the localization of lipoproteins (Lol) machinery with the exception of the outer membrane lipoprotein receptor protein, LolB. Presumably, the lipoproteins are anchored into the periplasmic face of the outer membrane by a LolB-like protein before an unidentified lipoprotein-specific 'flippase' transfers the lipoprotein from the periplasmic face of the outer membrane to the surface (Schulze and Zückert, 2006; Schulze et al., 2010; Bergström and Zückert 2010). See text for details.

# Chapter II.

# Materials and Methods

**Bacterial Strains and growth conditions**

*Borrelia burgdorferi* B313 (Sadziene et al., 1993) B31 A3 *ospC::kanR* (provided by Patti Rosa, NIH/NIAID Rocky Mountain Laboratories, Hamilton, MT), B31-A (Bono et al., 2000), B31-A *ctpA::kanR* (provided by Sven Bergström, Umeå University, Umeå, Sweden)(Östberg et al., 2004), and B31-e2 (provided by B. Stevenson, University of Kentucky, Lexington, KY; (Babb et al., 2004) are all derivatives of strain B31 (ATCC 35210). B313 contains plasmids cp26, cp32-1, cp32-2/7, cp32-3, and lp17 (Zückert et al., 1999; Zückert et al., 2004). B31-e2 contains plasmids cp26, cp32-1, cp32-3, cp32-4, lp17, lp38 and lp54 (Babb et al., 2004). Using PCR with plasmid-specific primer sets (Labandeira-Rey and Skare, 2001; Caimano et al., 2004), B31-A and B31-A *ctpA::kanR* were determined to harbor plasmids cp26, cp32-1, cp32-2/7, cp32-3, cp32-9, lp17, lp28-1, lp28-2, lp28-3, lp36, lp54, and lp56; B31-A3 *ospC::kanR* is a low-passage, transformable clone lacking lp25 (not shown). This information is detailed in Table 2.1. *B. burgdorferi* were cultured in liquid or solid BSK-II medium at 34ºC under a 5% $CO_2$ atmosphere (Barbour, 1984; Zückert, 2007). Selective BSK-II media were supplemented where needed with 200 $\mu g\ ml^{-1}$ of kanamycin, 40 $\mu g\ ml^{-1}$ gentamicin, or 50 $\mu g\ ml^{-1}$ of streptomycin (Sigma). *E. coli* strains TOP 10 (Invitrogen) and XL-10 Gold (Stratagene) were used for plasmid construction and propagation, and BL21(DE3) pLysS for recombinant protein expression. Unless noted otherwise, *E. coli* cultures were grown at 37ºC in Lysogeny broth (LB) (Bertani, 2004) or LB agar (Difco) supplemented with 30 $\mu g\ ml^{-1}$ of kanamycin, 15 $\mu g\ ml^{-1}$ gentamicin, or 100 $\mu g\ ml^{-1}$ spectinomycin (Sigma).

**Site-directed mutagenesis**

Plasmids carrying mutant genes were constructed either by splicing overlap extension PCR (SOE-PCR) (Ho et al., 1989) with Pfx Platinum (Invitrogen) or Phusion Hotstart (New England Biolabs) thermostable proofreading DNA polymerases or by following the Quick-Change site-directed mutagenesis protocol (Stratagene). Sequences were verified by DNA sequencing (ACGT Inc., Wheeling, IL or Northwestern University, Chicago, IL). The plasmids used are shown in Table 2.2.

**SDS-PAGE and Immunoblot analysis**

Proteins were separated by sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie blue or silver staining. For immunoblots, proteins were electrophoretically transferred to Immobilon-NC nitrocellulose membranes (Millipore) using a Transblot semi-dry transfer cell (Bio-Rad). Membranes were washed in 20 mM Tris, 500 mM NaCl, pH 7.5 (TBS). TBS with 0.05% Tween 20 (TBST) containing 5% dry milk was used for membrane blocking and subsequent incubation with primary and secondary antibodies; TBST alone was used for the intervening washes. Antibodies used are detailed in Table 2.2. Alkaline phosphatase substrates were 1-Step NBT/NCIB (Pierce) for colorimetric and CDP-Star (GE Healthcare Life Sciences) for chemiluminescent detection. Restore Western blot stripping reagent (Pierce) was used to remove bound antibodies from immunoblots to allow for reprobing of membranes. Proteins tagged with a hexahistidine epitope tag were detected directly with a nickel-activated HisProbe-HRP conjugate and SuperSignal HRP chemiluminescent detection substrate (Pierce)

**Construction of mutant plasmid library to identify subsurface localization signals**

First, translationally silent restriction endonuclease sites for *Bsa*I and *Bst*BI were engineered into plasmids pRJS1009 and pRJS1016 (Schulze and Zückert, 2006) using the QuickChange II XL site-directed mutagenesis kit (Stratagene) and oligonucleotide primers (IDT Integrated DNA Technologies, Coralville, IA) BsaImut-fwd and –rev and Bstmut-fwd and -rev, respectively (Figure 2.1). OspA codon L10 (CTA) was changed to CTC using primers, and mRFP1 codons V15 (GTG) and R16 (CGC) were changed to CTT and CGA, resulting in pOSK1. Next, a 114-mer random mutagenesis oligonucleotide, Rmut-oligo, was synthesized and purified by polyacrylamide gel electrophoresis (PAGE, Integrated DNA Technologies, Coralville, IA). In Rmut-oligo, the mRFP-1 E4 and D5 codons are replaced by NNK. K, i.e., G or T in the third position allows for any amino acid, but is biased against stop codons. Only UAG "amber" had to be allowed to cover all amino acids. Rmut-oligo was converted into a double-stranded DNA molecule using oligonucleotide Rmut-rev and the large fragment of DNA polymerase I (Invitrogen). The fill-in reaction was terminated using a MinElute reaction cleanup kit (Qiagen). pOSK1 and the double-stranded Rmut linker were then both digested with *Bsa*I and *Bst*BI (New England Biolabs). The cut vector was treated with shrimp alkaline Phosphatase (Invitrogen) before ligation to the Rmut DNA linker with a Quick Ligation kit (NEB). Chemically competent *E. coli* Top10 were transformed with approximately 10 ng of the ligation reaction and transformants were grown in batch in 500 ml of LB broth for 18 hours at 37°C with aeration. Plasmid DNA was then isolated using a Biotech Spin Doctor BAC prep kit (Midwest Scientific) following the manufacturer's protocol. *Borrelia* cells were transformed by electroporation with 2 µg of plasmid DNA using established protocols (Stewart et al., 2001; Samuels, 1995) and grown in liquid BSK-II media at 34°C and 5% $CO_2$.

**Fluorescence activated cell sorting (FACS)**

$2x10^6$ spirochetes were harvested as described (Schulze and Zückert, 2006), washed twice with phosphate buffered saline containing 5 mM $MgCl_2$ (PBS+Mg), and incubated with a final concentration of 50 $\mu$g $ml^{-1}$ proteinase K (Invitrogen) for one hour at room temperature. Mock-treated cells were incubated in PBS+Mg only. Cells were then washed three times with PBS containing 0.1% bovine serum albumin (PBS+BSA) and resuspended in 1 ml of PBS+BSA at a density of 1 to $1.5x10^6$ cells $ml^{-1}$. Spirochetes retaining red fluorescence were then sorted by FACS on a BD FACSAria (BD Biosciences) at a flow rate of 200 events $s^{-1}$ and 55 psi through a 70 $\mu$m nozzle. Excitation, long pass, and band pass wavelengths were 488 nm, 635 nm, and 695 $^{+/-}$ 40 nm, respectively. Upon completion of FACS, the volume of the sorted cells (about 1 ml) was immediately adjusted to 12 ml with BSK-II and incubated at 34°C. The FlowJo program suite, version 7.2.2 (Treestar), was used for data analysis.

**DNA sequence analysis and identity of subsurface retention signals**

Spirochetes were counted using a Petroff-Hauser counting chamber, adjusted to 200 cells $ml^{-1}$, plated on solid BSK II media (Zückert, 2007), and incubated at 34°C and 5% $CO_2$. Individual colonies were picked using sterile toothpicks and cultured in 200 $\mu$l of BSK-II complete media in a sterile 96-well tissue culture plate (Corning). The mutated *ospA-mrfp1* region was amplified from 1 $\mu$l of 1:10 diluted culture in sterile water using primers Mutscreen-fwd and –rev (Fig. 3.1). PCR products were purified using a PCR purification kit (Qiagen) and sequenced (AGCT Inc., Wheeling, IL) using primer Mutscreen-seq. Each sequenced mutant was cultured in liquid BSK-II culture for further analysis.

**Protease accessibility assays**

To assess protein surface exposure by protease accessibility, intact *B. burgdorferi* cells were treated *in situ* with proteinase K as described (Bunikis and Barbour, 1999; Schulze and Zückert, 2006). Briefly, cells were harvested, washed twice in PBS+Mg and treated with 200 μg $ml^{-1}$ proteinase K (Invitrogen) or treated with buffer as a control. To test the sensitivity of proteins to trypsin, cells were incubated with 200 μg $ml^{-1}$ of trypsin (Sigma) as described (Bunikis and Barbour, 1999). To gain access to periplasmic proteins, cells were also treated with 0.1% SDS to permeabilize the OM (Sigma; (Jewett et al., 2007)). All protease reactions were terminated with PMSF (Sigma).

**Membrane and protein fractionations**

For *B. burgdorferi*, outer membrane vesicles were isolated and purified by treatment of cells with low pH, hypotonic citrate buffer followed by isopycnic sucrose gradient centrifugation as described (Skare et al., 1995). Briefly, late exponential phase *B. burgdorferi* cells were washed in 1× PBS containing 0.1% BSA, resuspended and incubated under vigorous shaking for 2 h in 25 mM citrate buffer, pH 3.2, containing 0.1% BSA. OMVs and PCs were fractionated by ultracentrifugation in a discontinuous gradient of 56%, 42% and 25% (w/w) sucrose in citrate buffer using a Beckman L8-80M centrifuge, SW28 rotor and 25×89mm Ultra-Clear ultracentrifuge tubes. Fractions were washed and resuspended in 1× PBS containing 1 mM PMSF.

Membrane proteins were extracted by detergent solubilization using a protocol modified from (Brandt et al., 1990) and (Nally and Timoney, 2001). Briefly, harvested *B. burgdorferi* cells were solubilized overnight in ice-cold PBS-Mg containing 2% (v/v) Triton X-114 with rotation

at 4°C. Insoluble PC material was removed by centrifugation and the supernatant was phase-separated at 37°C for 15 min and centrifuged to obtain the aqueous periplasmic and detergent-soluble membrane fractions. Both the aqueous and detergent fractions were washed three times by addition of ice-cold Triton X-114 to the aqueous phase at 2% final concentration, or ice-cold PBS+Mg to detergent phase and phase-separated as above. Proteins were concentrated by acetone precipitation.

For *E. coli*, a modified protocol from (Robichon et al., 2005) was followed. Briefly, A 1:100 dilution of an overnight culture grown at 37°C in LB containing 30 μg $ml^{-1}$ kanamycin was used to inoculate a 200 ml culture of selective LB broth. After cultivation at 37°C to an $OD_{600}$ = 0.8-1.0, cells were pelleted, washed with PBS, and resuspended in 5 ml of 25mM HEPES (Sigma) pH 7.4. Cells were lysed by three passages through a French pressure cell. The lysate was then supplemented with 10 μg $ml^{-1}$ DNase I (Invitrogen) and pancreatic RNase A (Qiagen) then centrifuged for 10 min at 4,000 rpm remove unbroken cells. Membranes were then collected by ultracentrifugation at 160,000 × *g* for 1 h at 4 °C, resuspended and saturated at 60% (w/w) of sucrose in 200 μl of 25 mm HEPES (pH 7.4), and then placed at the bottom of an ultracentrifuge tube. Steps (600 μl) were created using 56.2, 53.2, 50.2, 47.1, 44.2, 41.2, 38.1, and 35.9% (w/w) sucrose solutions, and the tubes were centrifuged in Sw-55Ti swinging bucket rotor (Beckman) for 36 h at 230,000 × *g* at 10 °C. Twenty fractions (250 μl) were collected from the top of the tubes and analyzed by SDS-PAGE and immunoblotting.

**Densitometry and calculations**

Densitometry of Coomassie blue-stained protein bands and Western blot signals acquired with a Fuji LAS-4000 fluorescence imager with a linearity of 4 orders of magnitude was done using the Image J image analysis software (http://rsb.info.nih.gov/ij/). The percentage of surface-localized protein was calculated using the following formula: % surface = 100 - [($Exp_{+pK}$ x $FlaB_{-pK}$) ÷ ($mExp_{-pK}$ x $FlaB_{+pK}$)] x 100, where experimental (Exp) and FlaB indicate the raw Western immunoblot densitometry data in absence (–pK) or presence (+pK) of proteolysis. Negative % surface were set to zero. The OM/PC distribution ratio using the following formula: $ratio_{OM/PC}$ = ($mExp_{OM}$ ÷ $mExp_{PC}$) ÷ [($OspA_{OM}$ ÷ $OspA_{PC}$) – ($OppAIV_{OM}$ ÷ $OppAIV_{PC}$)], where experimental (Exp), OspA and OppAIV represent the raw Western immunoblot densitometry data in either the OM or PC fractions. Genomic *B. burgdorferi* strain B31 (GenBank Accession # NC_001318) codon usage data were acquired from the Georgia Tech Codon Usage Database (http://exon.gatech.edu/GeneMark/metagenome/CodonUsageDatabase/).

***In situ* crosslinking**

Assays were carried out as described (Bunikis and Barbour, 1999; Zückert et al., 2001). Briefly, cells were grown to a density of ~$5x10^7$ cells $ml^{-1}$, harvested, and washed twice in PBS+Mg. Proteins were crosslinked with 1% (v/v) formaldehyde (Sigma) at room temperature for 30 minutes. Cells were washed twice with PBS+Mg and resuspended in SDS-PAGE loading dye with 50 mM DTT and incubated at 37ºC for 10 minutes.

**Salp15 overlay assay**

The *Ixodes scapularis* Salp15 overlay assay was performed as described (Ramamoorthi et al., 2005). Briefly, *B. burgdorferi* lysates separated by SDS-PAGE and blotted to a nitrocellulose membrane were first incubated in blocking buffer (1% BSA, 3% dry milk in Tris Buffered Saline 0.05% Tween-20) at 4ºC overnight. Then, the membrane was incubated with 1 $\mu g\ ml^{-1}$ of recombinant, V5-epitope-tagged *I. scapularius* Salp15 (a gift from E. Fikrig, Yale University, New Haven, CT) in blocking buffer. The membrane was then washed, incubated with a 1:2000 dilution of anti-V5 HRP conjugated antibody (Invitrogen), washed again, and developed using the SuperSignal HRP chemiluminescent detection kit (Pierce).

**Immunoprecipitation**

$2.5x10^9$ *B. burgdorferi* cells grown to late exponential phase ($5x10^7$ cells $ml^{-1}$) were harvested, washed three times with PBS+Mg and lysed in IGEPAL lysis buffer (150mM NaCl, 50mM Tris pH 8.0, 1% IGEPAL, 1mM PMSF) or RIPA lysis buffer (300mM NaCl, 50mM Tris pH 7.4, 1% TritonX-100, 0.1% SDS, 1% deoxycholic acid, 1mM PMSF). After preclearing by incubation with protein A beads (GE) for one hour at 4ºC with end-over-end rotation, the lysate was incubated with 10 µg of purified anti-OspC monoclonal antibody (Gilmore and Mbow, 1999; Mbow et al., 1999), a gift from R. Gilmore, CDC-Fort Collins, CO), anti-FLAG monoclonal antibody (Sigma), or anti-HA monoclonal antibody (Sigma) for one hour at 4ºC. Protein A beads were added to the lysate at 4ºC for one hour. The beads were washed three times with IGEPAL or RIPA lysis buffer and once with 50mM Tris (pH 8.0). Beads were incubated in SDS PAGE loading dye with 50 mM DTT, boiled, and the supernatant analyzed by SDS-PAGE and immunoblotting.

**Purification of recombinant OspC**

DNA fragments corresponding to N-terminally truncated, soluble OspC were amplified by PCR from pOSK307 (Table 2.3) with oligonucleotide primers including 5' NdeI and 3' BamHI extensions. The three different 5' oligonucleotides primers placed the N20, N31, and V37 codons immediately after the $^{f}$Met start codon, respectively. The PCR products were gel-purified, digested with NdeI and BamHI and ligated with a pET29b (Novagen) backbone previously linearized with NdeI and BamHI. The resulting expression plasmids were sequenced and used to transform BL21 (DE3) pLysS (Novagen). A 1:100 dilution of an overnight culture grown at 37°C in LB containing 30 µg $ml^{-1}$ kanamycin and 100 µg $ml^{-1}$ chloramphenicol was used to inoculate a larger culture of selective Terrific Broth. After cultivation at 37°C to an $OD_{545}$ = 0.3-0.4, recombinant protein expression was induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside (Invitrogen) for 5 hours at 30°C. Cells were harvested and lysed by sonication, and cell debris was removed by centrifugation at 12,000 x *g* for 20 min at 4°C. The cell-free lysate was then applied to a Talon cobalt column (Clontech) equilibrated with loading buffer (300 mM NaCl, 50mM $NaPO_4$ pH 7.0), and washed with loading buffer containing 10 mM imidazole. Recombinant OspC at a purity of about 90% was eluted in buffer containing 100 mM imidazole (Sigma). For further purification, we followed the protocol of (Kumaran et al., 2001) with some modifications. The cobalt column eluate was dialyzed twice against 20mM $NaPO_4$, 5mM NaCl, pH 7.7, and then applied to a Hi-Trap Q anion exchange column (GE Healthcare). The flow-through containing OspC was dialyzed twice against 10mM $NaPO_4$, 5 mM NaCl, pH 6.0 and applied to a Hi-Trap SP cation exchange column (GE Healthcare). OspC eluted quantitatively at 500mM NaCl at a purity of about 98%, was dialyzed twice against 10mM

$NaPO_4$ buffer, pH 7.0, and concentrated using Amicon Ultra centrifugal filters with a 3 kDa cutoff (Millipore). Protein concentrations were determined by Bradford assay (Bio-Rad).

**Purification of recombinant OspA**

DNA fragments corresponding to N-terminally truncated, soluble OspA were amplified by PCR from pCSY1000 with oligonucleotide primers including 5' NdeI and 3' BamHI extensions. The two different 5' oligonucleotides primers placed the A17 and S29 codons immediately after the $^{f}$Met start codon, respectively. The PCR products were gel-purified, digested with NdeI and BamHI and ligated with a pET29b (Novagen) backbone previously linearized with NdeI and BamHI. The resulting expression plasmids were sequenced and used to transform BL21(DE3) pLysS (Novagen). A 1:100 dilution of an overnight culture grown at 37°C in LB containing 30 $\mu g\ ml^{-1}$ kanamycin and 100 $\mu g\ ml^{-1}$ chloramphenicol was used to inoculate a larger culture of selective Terrific Broth. After cultivation at 37°C to an $OD_{545}$ = 0.3-0.4, recombinant protein expression was induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside (Invitrogen) for 5 hours at 30°C. Cells were harvested and lysed by sonication, and cell debris was removed by centrifugation at 12,000 x *g* for 20 min at 4°C. The cell-free lysate was then applied to a Talon cobalt column (Clontech) equilibrated with loading buffer (300 mM NaCl, 50mM $NaPO_4$ pH 7.0), and washed with loading buffer containing 10 mM imidazole. Recombinant OspA at a purity of about 95% was eluted in buffer containing 100 mM imidazole (Sigma). OspA was dialyzed twice against 10mM $NaPO_4$ buffer, pH 7.0, and concentrated using Amicon Ultra centrifugal filters with a 3 kDa cutoff (Millipore). Protein concentrations were determined by Bradford assay (Bio-Rad).

**Circular dichroism measurements and analysis of thermal unfolding**

Circular dichroism measurements were performed using an upgraded Jasco-720 spectropolarimeter (Japan Spectroscopic Company, Tokyo). 10-20 scans were recorded between 190 and 260 nm with a 1 nm step at +20°C, using a 1 mm optical path cuvette. protein concentrations of 3-5 μM, determined by UV absorbance measurements using a coefficient of molar extinction of 2400 $M^{-1}*cm^{-1}$, were used in the experiments. All spectra were corrected for background. Temperature dependencies of unfolding were measured at 222 nm (rOspC) or 212 nm (OspA) with 1 degree/min scan rate. Thermal unfolding was analyzed as described (Eftink et al., 1994) to obtain transition temperature ($T_m$) and enthalpy ($\Delta H$). The free energy ($\Delta G$) stabilizing native structure at room temperature was estimated using standard assumptions on the value of heat capacity according to (Robertson and Murphy, 1997).

**RNA extraction and Northern Blotting**

Total RNA was isolated from 14-ml cultures at a density of ~$5x10^7$ spirochetes/ml using an RNeasy Mini Kit (Qiagen). Prior to isolation, the RNAs were fixed using RNA Protect reagent (Qiagen). RNA concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Total RNA (2.0 μg) was fractionated in a 1.2% formaldehyde-agarose gel and transferred to an Immobilon-NY membrane (Millipore) by upward capillary transfer (Sambrook and William Russell, 2001). RNA ladders (0.24 to 9.5 kb; Invitrogen) served as size standards. A DNA probe was generated by PCR using primer pairs complementary to the *ospC* and *vsp1* coding regions and pOSK200 and pVsp1 as templates, respectively (Table 2.3). Probe labeling and Northern blot hybridizations were performed using the Gene Images AlkPhos Direct

Labeling and Detection System with CDP-Star (GE Healthcare) according to the manufacturer's instructions. A Fujifilm LAS-4000 Luminescent Image Analyzer was used for data acquisition and analysis.

**Table 2.1. Bacterial Strains Used in this Study**

| Strain | Description | Source/Reference |
|---|---|---|
| Strains | | |
| *Borrelia burgdorferi* | | |
| B313 | Clone of B31 ATCC 35210 (cp26, cp32-1, cp32-2/7, cp32-3 and lp17). | (Sadziene et al., 1995) |
| B31e2 | Clone of B31 ATCC 35210 (cp26, cp32-1 cp32-3, cp32-4, lp17, lp38 and lp54). | (Babb et al., 2004) |
| B31-A | Clone of B31 ATCC 35210 (cp26, cp32-1, cp32-2/7, cp32-3, cp32-9, lp17, lp28-1, lp28-2, lp28-3, lp36, lp54, lp56). | (Bono et al., 2000) |
| B31-A *(ΔctpA)* | C-terminal protease A (*ctpA*) knockout, $P_{flaB}$-*kan* insertion in *ctpA* (plasmid content is identical to B31-A) | (Östberg et al., 2004) |
| B31-A3 *(ΔospC)* | Outer surface protein C (*ospC*) knockout, $P_{flaB}$-*kan* insertion in *ospC* ($lp25^{-}$) | (K. Tilly and P. Rosa unpublished) |
| *Escherichia coli* | | |
| Top10 | $F^{-}$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L ($Str^{R}$) *end*A1 *nup*G | Invitrogen |
| XL-10 Gold | $Tet^{r}$ D*(mcrA)183* D*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* The [F' *proAB lacI*$^{q}$Z*DM15 Tn10* ($Tet^{r}$) Amy $Cam^{r}$ | Stratagene |
| BL21(DE3) pLysS | F- ompT $hsdS_{B}(r_{B}- m_{B}-)$ gal dcm λ(DE3) tonA pLysS ($Cam^{r}$) | Novagen |

**Table 2.2. Antibodies Used in this Study**

| Antibody | Source Animal | Dilution | Source/Reference |
|---|---|---|---|
| anti-OspA (monoclonal) | Mouse | 1:50 | (Barbour et al., 1983) |
| anti-OspC (monoclonal) | Mouse | 1:50 | (Gilmore and Mbow, 1999) |
| anti-OspC (polyclonal) | Rabbit | 1:200 | (Gilmore and Mbow, 1999) |
| anti-Vsp1 (monoclonal) | Mouse | 1:25 | Alan Barbour |
| anti-Vsp1 (polyclonal) | Rabbit | 1:500 | Alan Barbour |
| anti-Vsp2 (polyclonal) | Rabbit | 1:500 | Alan Barbour |
| anti-His Tag (monoclonal) | Mouse | 1:500 | Sigma |
| anti-HA Tag (monoclonal) | Mouse | 1:500 | Sigma |
| anti-FLAG Tag (monoclonal) | Mouse | 1:10,000 | Sigma |
| anti-CtpA (polyclonal) | Rabbit | 1:100 | (Östberg et al., 2004) |
| anti-OppAIV (polyclonal) | Rabbit | 1:100 | Patricia Rosa (Bono et al., 1998) |
| anti-mRFP1 (polyclonal) | Rabbit | 1:5000 | Patrick Viollier (Chen et al., 2005) |
| anti-FlaB (polyclonal) | Rabbit | 1:1000 | Darrin Akins (Akins et al., 1999) |
| anti-FlaB (polyclonal) | Rat | 1:2000 | Melissa Caimano |
| anti-Mouse IgG (H+L)* | Rabbit | 1:30,000 | Sigma |
| anti-Rabbit IgG (H+L)* | Goat | 1:30,000 | Sigma |
| anti-Rat IgG(γ-chain specific)* | Goat | 1:30,000 | Sigma |

.
*Conjugated to Alkaline Phosphatase.

**Table 2.3. Plasmids Used in this Study**

| Plasmid | Description | Source/Reference |
|---|---|---|
| pET29b | Expression vector for protein purification | Novagen |
| pVsp1 | pBSV2:PflaB-vsp1 | (Zückert et al., 2001) |
| pBSV2 | Shuttle vector (Kan$^R$) | (Stewart et al., 2001) |
| pKFSS1 | Shuttle vector (Str$^R$) | (Frank et al., 2003) |
| pBSV2.2 | Shuttle vector (Gm$^R$) | (Elias et al., 2002) |
| pBSVG+ctpA | pBSV2G:ctpA | (Östberg et al., 2004) |
| pRJS1091 | pBSV2:PflaBospA22-mRFPΔ4 | This study |
| pRJS1090 | pBSV2:PflaBospA25-mRFPΔ4 | This study |
| pOSK240 | pBSV2:PflaBospC29-mRFPΔ4 | This study |
| pOSK258 | pBSV2:PflaBospC30-mRFPΔ4 | This study |
| pOSK257 | pBSV2:PflaBvsp1-31-mRFPΔ4 | This study |
| pOSK256 | pBSV2:PflaBvsp1-32-mRFPΔ4 | This study |
| pOSK200 | pKFSS1:PflaB-ospC | This study |
| pOSK273 | pKFSS1:PflaBospC(ΔN20-A30) | This study |
| pOSK274 | pKFSS1:PflaBospC(ΔN31-N41) | This study |
| pOSK299 | pKFSS1:PflaBospC(ΔS22) | This study |
| pOSK300 | pKFSS1:PflaBospC(ΔN21) | This study |
| pOSK287 | pKFSS1:PflaBospC(ΔN20-S22) | This study |
| pOSK288 | pKFSS1:PflaBospC(ΔG23-D25) | This study |
| pOSK301 | pKFSS1:PflaBospC(ΔN21-S22) | This study |
| pOSK294 | pKFSS1:PflaBospC(ΔA33-N41) | This study |
| pOSK302 | pKFSS1:PflaBospC(Δ34-N41) | This study |
| pOSK309 | pBSV2:PflaBospC(Ala)21-22 | This study |
| pOSK310 | pBSV2:PflaBospC(Gly)21-22 | This study |
| pOSK268 | pBSV2:PflaBvsp1(ΔN20-S25) | This study |
| pOSK269 | pBSV2:PflaBvsp1(ΔG23-D28) | This study |
| pOSK262 | pBSV2:PflaBvsp1(ΔN20-S22) | This study |
| pOSK263 | pBSV2:PflaBvsp1(ΔG23-S25) | This study |
| pOSK275 | pBSV2:PflaBvsp1(ΔN20-A32) | This study |
| pOSK276 | pBSV2:PflaBvsp1(ΔK33-I39) | This study |
| pOSK279 | pBSV2:PflaBvsp1(ΔN21-S25) | This study |
| pOSK278 | pBSV2:PflaBvsp1(ΔS22-S25) | This study |
| pOSK284 | pBSV2:PflaBvsp1(ΔN20-G23) | This study |
| pOSK285 | pBSV2:PflaBvsp1(ΔN20-T24) | This study |
| pOSK289 | pBSV2:PflaBvsp1(ΔS22-S25) | This study |
| pOSK291 | pBSV2:PflaBvsp1(ΔN21-G23) | This study |
| pOSK292 | pBSV2:PflaBvsp1(ΔS22-T24) | This study |
| pOSK313 | pBSV2:PflaBvsp1(ΔN20-S25)P26A | This study |
| pOSK351 | pET29b:pT7ospCN20-His | This study |
| pOSK352 | pET29b:pT7ospCN31-His | This study |
| pOSK353 | pET29b:pT7ospCV37-His | This study |
| pOSK248 | pBSV2:PflaB-vsp1D60K/D87K/D150K | This study |
| pOSK307 | pKFSS1:PflaB-ospC-linker-his tag | This study |
| pOSK312 | pKFSS1:PflaB-ospC-(ΔS22)-linker-his tag | This study |
| pOSK326 | pKFSS1:PflaB-ospC-A204X | This study |
| pOSK260 | pKFSS1:PflaB-ospC-linker | This study |
| pOSK277 | pKFSS1:PflaB-ospC-internal his tag | This study |
| pOSK308 | pKFSS1:PflaB-ospC-L2-FLAG tag | This study |
| pOSK308.1 | pBSV2.2:PflaB-ospC-L2-FLAG tag | This study |
| pOSK374 | pKFSS1:PflaB-ospC(Δ2̃0-30̆)L2-HA tag | This study |
| pOSK384 | pET29b:pT7ospA-A17-His | This study |

| | | |
|---|---|---|
| pOSK385 | pET29b:pT7ospA-S29-His | This study |
| pOSK101 | pBSV2:PflaB-lpp-His | This study |
| pOSK102 | pBSV2:PflaB-lpp(ΔK58)-His | This study |
| pOSK103 | pBSV2:PflaB-lpp(N22A/S23A/S24A)-His | This study |
| pOSK104 | pBSV2:PflaB-lpp(ΩKQNV/N22)-His | This study |
| pOSK260 | pKFSS1:PflaB-ospC(Δtether) | This study |
| pOSK267 | pBSV2:PflaB-vsp1(Δtether) | This study |
| pCSY1000 | pBSV2:pFlaB-ospA-link-his | Shyiong Chen |
| pOSK344 | pBSV2:pFlaB-vsp1D60K/D87K/D150K-HA-L5 | This study |
| pOSK208 | pBSV2:pFlaB-vsp2-FLAG-L5 | This study |
| pOSK208.1 | pBSV2.2:pFlaB-vsp2-FLAG-L5 | This study |
| pOSK364 | pBSV2:pFlaB-vsp1-HA-L5 | This study |
| pOSK3 | pBSV2:pFlaB-ospA20mRFP1(E4ND5N)(pRJS1016 based) | (Kumru et al., 2010) |
| pOSK4 | pBSV2:pFlaB-ospA20mRFP1(E4ND5N)(pRJS1009 based) | (Kumru et al., 2010) |

# Chapter III.

# Development and validation of a FACS-based lipoprotein localization screen in the Lyme disease spirochete *Borrelia burgdorferi*

**Abstract**

Surface lipoproteins of *Borrelia burgdorferi*, the causative agent of Lyme borreliosis, are major virulence factors in the establishment and persistence of infection within the tick vector and the human host. We previously showed that *B. burgdorferi* lipoproteins are transported to the spirochetal surface by default and are not subject to the '+2' sorting rule. Yet, we identified an N-proximal tandem negative charge (Asp-Glu) in a fusion of outer surface protein A to monomeric red fluorescent protein 1 (OspA20:mRFP1), which served as an inner membrane retention signal. Here, we asked whether other amino acid combinations could function as subsurface localization signals in the same context. Random mutagenesis was performed on the OspA20:mRFP1 Asp-Glu codons to generate a library of 400 potential amino acid combinations. *In situ* proteolysis of intact *Borrelia* cells combined with fluorescence activated cell sorting (FACS) was then used to screen for viable spirochetes expressing subsurface OspA:mRFP1 fusions. Analysis of 104 individual clones identified a total of 44 distinct mutants. Only 15 of these mutants were predominantly surface-localized, which validated this fluorescence-based screen for further application. An Asp-Glu mutant identical to OspA19:mRFP1 was re-isolated and remained the only one restricted to the inner membrane; all other mutants were released from the inner membrane, but impaired to different degrees in translocating the outer membrane. This suggests a rather specific inner membrane retention mechanism involving N-proximal negative charge patches in this model *B. burgdorferi* lipoprotein system.

## Introduction

Temporally and spatially regulated expression of surface-exposed lipoproteins such as OspA, OspC and VlsE enables the Lyme disease spirochete *Borrelia burgdorferi* to adapt to changing environmental conditions and allows for maintenance of the organism within an enzootic tick-mammal cycle (Pal et al., 2000; Grimm et al., 2004; Bankhead and Chaconas, 2007). Yet, we are only beginning to understand the factors that govern accurate localization of these important virulence factors to the bacterial cell surface, thereby generating the pathogen-host interface. In prior studies, we demonstrated a role for the 'tether' region, i.e., the disordered N-terminus of these lipoproteins in the localization process. Fusion of the first five residues of the mature outer surface lipoprotein OspA was sufficient to target the red fluorescent reporter protein mRFP1 to the surface of the Borrelia cell (Schulze and Zückert, 2006). The same study also revealed that previously identified lipoprotein sorting rules identified in *Enterobacteriaceae* and *Pseudomonas* (Yamaguchi et al., 1988; Silva-Herzog et al., 2008; Narita and Tokuda, 2007) did not apply to *Borrelia* lipoproteins. An alignment of *B. burgdorferi* lipoprotein tether peptide sequences failed to reveal any apparent primary sequence conservation. Trafficking may thus depend on specific biophysical properties of the tether polypeptide such as hydrophobicity, charge, or secondary structure propensity, rather than strict amino acid identity alone (Haake, 2000; Cullen 2004).

In the present study, we designed and tested an experimental approach that might help in elucidating these still obscure sorting signals. Based on an existing OspA tether-mRFP1 fusion with a characterized inner membrane (IM) release defect, we generated a partially randomized fluorescent lipopeptide library in *B. burgdorferi*. A fluorescence-activated cell sorting (FACS)-based screen was then used to enrich for mutants localizing to the periplasm. Our results

indicate that this approach can become an important tool to detect general patterns in peptides mediating surface or subsurface localization.

## Results & Discussion

**Design of a fluorescence-based screen for lipoprotein localization in *B. burgdorferi.***

In our recent studies, the use of fusions of red fluorescent mRFP1 to various N-terminal fragments and point mutants of *B. burgdorferi* surface lipoprotein OspA led to an initial assessment of the sequence requirements for proper surface display (Schulze and Zückert, 2006; Schulze et al., 2010). To complement this step-wise, targeted mutagenesis approach, we set out to develop a random mutagenesis screen. Our starting point was a previously described OspA-mRFP1 fusion, OspA20:mRFP1, which could be redirected from the IM to the bacterial surface by mutagenesis of two adjacent negatively charged amino acids (Glu-Asp) to two Ala residues. We therefore hypothesized that (i) additional mutagenesis in this OspA20:mRFP1 dipeptide would reveal the specificity of periplasmic, particularly IM retention signals in this model lipoprotein, and that (ii) periplasmically localized fusion protein mutants could be enriched by a combination of *in situ* surface proteolysis and fluorescence-activated cell sorting (FACS). The approach is detailed in Chapter II and shown in Fig 3.1.

Two plasmid libraries were generated from two different starting materials, pRJS1009 and pRJS1016 (Schulze and Zückert, 2006). pRJS1009 carried a fusion of the full-length signal peptide and tether of OspA to mRFP1 (OspA28:mRFP1), which was targeted to the bacterial surface. In pRJS1016, the OspA tether sequence was truncated to 4 amino acids (OspA20:mRFP1), which led to significant retention of the fusion in the inner spirochetal membrane. In both plasmids, a fragment containing the 5' *ospA:mrfp1* sequence was swapped

for a DNA fragment randomized at the Glu-Asp codons. After library expansion in *E. coli* and electroporation of *B. burgdorferi*, transformants were grown in liquid medium selecting for the library plasmids. To eliminate any non-expressers, we subjected the populations to a first round of FACS, collecting only cells with a clear red fluorescent signal (not shown). Gating was determined by plotting logs of forward scatter (FSC) versus side scatter (SSC) as described (Whetstine et al., 2009) (Fig. 3.2). After presorting, cells were allowed to recover in liquid BSK-II medium and then subjected to proteolytic shaving using proteinase K. We surmised that treated cells would remain fluorescent only if they expressed a subsurface mutant of the OspA:mRFP1 fusion.

**Genotypic and phenotypic analysis of pre- and post-sorting cell populations.**

Compared to mock-treated cells, the fluorescent population post-treatment decreased for both libraries, suggesting that proteolytic shaving indeed resulted in a reduction of surface-associated fluorescence. Interestingly, the reduction was more significant in the pRJS1009-based library (from 50% to 7%) than the pRJS1016-based library (from 82% to 64%) (Fig. 2). We initially attributed this to the potential of bleed-through of the original plasmid in the pRJS1016-derived library. Yet, further analysis showed that this effect was negligible as only three Glu-Asp clones were recovered post-sorting (see below and Fig. 3.3).

A total of 172 random clones, 38 from an unsorted population and 134 from a sorted population, were analyzed by DNA sequencing. 63 mutants were identified, 8 being unique to the unsorted population, 40 unique to the sorted population, and 15 common to both populations. Within the sorted population, the majority of the mutants (40 out of 55, i.e., 73%) were recovered repeatedly, e.g. 11 times for Ser-Gly (Fig. 3.3). This suggested that we were

approaching saturation in this experimental setting. As predicted, sorting for fluorescent cells significantly selected against the presence of non-expressing cells: the incidence of "amber" stops within the two mutated codons was reduced 18-fold, from 5 clones in the unsorted to 1 in the sorted population.

We randomly chose 93 clones from the sorted population for further analysis. This cohort covered 43 individual mutants, 11 of which were also identified in the presorted population (Fig. 3.3). The mutants were assessed for (i) protein levels and (ii) protein localization within the spirochetal cell envelope by *in situ* proteolysis and membrane fractionation. The observed protein levels provided a measure of protein stability *in vivo*, as an identical promoter drove expression of all mutant proteins and no correlation between the wild type genomic frequency of the introduced codons and protein levels was observed.

For OspA:mRFP1 fusion lipoprotein stability analysis, *B. burgdorferi* whole cell lysates were probed by Western immunoblot analysis using polyclonal antisera against mRFP1 and FlaB (Fig. 4). FlaB served as a loading control. The signals from the OspA:mRFP1 fusion proteins were quantified by densitometry of digital fluorometric images and normalized to the FlaB signals. All experiments were done in triplicate. Fig. 3.4 shows a representative dataset. The signals from OspA20:mRFP1 (labeled as ED) on each blot were used to normalize between individual replicates. Expression/*in vivo* stability levels were calculated in percent compared to OspA28:mRFP1. Surface localization of the OspA:mRFP1 mutants was assessed by proteolytic shaving with proteinase K followed by Western immunoblotting (Fig. 3.5). OspA served as a surface control while FlaB served as both a loading and periplasmic control. Accordingly, densitometry data were normalized to both OspA and FlaB signals. Localization of proteins to the IM or OM was assessed by Western immunoblots of PC and OM membrane fractions, using

OspA and OppAIV as membrane-specific controls and normalization standards (Fig. 3.6). The specific formulas used to calculate both the percentage of surface-localized protein and the OM/PC distribution ratios are described in Chapter II.

## Classification of phenotypes.

Based on the *in situ* proteolysis assay data, the characterized 43 mutant lipoproteins were classified according to their surface exposure phenotype (Fig. 3.3 and Table 3.1): 14 mutants or 31 clones were grouped as predominantly surface-exposed (class +++), 13 mutants or 42 clones had an intermediate phenotype (class ++), and 10 mutants or 22 clones localized largely to a subsurface compartment (+). 6 mutants represented by 19 clones were indistinguishable in their proteinase K accessibility phenotype from the original OspA20:mRFP1$_{ED}$ fusion (class –). Although we observed a continuum of phenotypes from IM-retained to surface-localized lipoprotein mutants, there was an appreciable enrichment of subsurface phenotypes in the sorted population (Fig. 3.3). Quantifying this shift towards periplasmic localization, the median surface percentage dropped from 54% in the unsorted population to 35% in the sorted population (Fig. 3B). The median expression levels and OM/PC ratios were 34% and 0.7 for both the unsorted and sorted populations. This indicated that the screen did not exert a pleiotropic, but rather a specific and intended selective pressure on the surface phenotype.

Surface exposure of lipoproteins in diderm bacteria can be affected by defects in either the release from the bacterial IM or a defect in translocation through the OM. To our surprise, most mutants, including the newly identified class – and + mutants localized in significant ratios to the OM (Fig. 3.3A and Table 3.1). One standout mutant in that respect is OspA20:mRFP1$_{KR}$: The fusion protein fractionated to the OM comparable to the surface-exposed OspA28:mRFP1,

but 99% of the total protein was protected from proteinase K. This indicated that this and most other mutant proteins were significantly impaired in "flipping" through the OM. Two aspects of this finding are particularly intriguing. First, we recently observed a similar predominance of OM translocation defects when disrupting a Val-Ser-Ser-Leu tetrapeptide within the tether of otherwise wild type OspA. These defects were overcome when the mutant OspA tethers were fused to mRFP1, which contains a similar N-terminal Ala-Ser-Ser-Glu tetrapeptide (Schulze and Zückert, 2006; Schulze et al., 2010). The mutations introduced in this study tangentially affect this mRFP1-derived tetrapeptide by altering the Glu residue, with similar results. For example, the introduction of Gly residues as in the GG mutant led to a defect while the previously described replacement by two Ala residues did not (Schulze and Zückert, 2006). This supports our earlier speculation that the mRFP1 tetrapeptide could functionally offset an OspA tether defect (Schulze et al., 2010). Second, the original OspA20:mRFP1$_{ED}$ retains the most profound IM-release defect phenotype. The Cys-Lys mutant, although comparable in membrane localization, is significantly less stable *in vivo* than OspA20:mRFP1$_{ED}$. Confirming our earlier site-directed mutagenesis data (Schulze and Zückert, 2006), single negative charges as in the Asp-Tyr or Glu-Leu mutants were insufficient to quantitatively restrict a lipoprotein to the borrelial IM. Therefore, small patches of negative charges continue as the only identified IM retention signal for lipoproteins expressed in *Borrelia* cells, albeit in an artificial model lipoprotein setting. Further studies will be needed to identify IM retention signals of natural *B. burgdorferi* lipoproteins such as OppAIV (Schulze and Zückert, 2006; Bono et al., 1998).

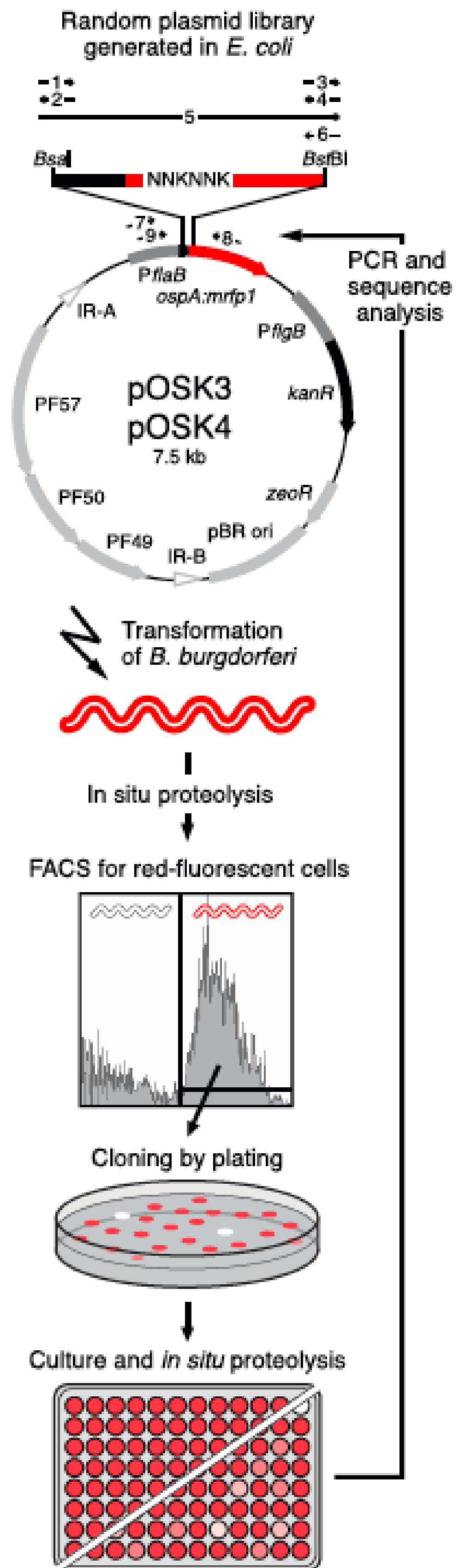
With the exception of the Gly-Ser and Gly-Val substitutions, most mutants were detected at significantly lower levels than both OspA28:mRFP1 and OspA20:mRFP1 (Figs. 3.3A and 3.4), despite being expressed from an identical promoter. Interestingly, this phenotype tended to

cluster with class +++ surface-localized proteins, e.g. OspA20:mRFP1$_{VR}$ or OspA20:mRFP1$_{WI}$ (Fig. 3.3 and Table 3.2). Based on structural data on the mRFP1 parent molecule DsRed, the mutated residues coincide with the transition from the fusion protein's flexible tether to the structurally confined red fluorescent protein β-barrel (Yarbrough et al., 2001). Amino acid substitutions, particularly with large bulky amino acids such as Trp or Phe therefore may compromise the protein fold. Based on our recent discovery that translocation of OspA through the borrelial OM requires an unfolded conformation (Schulze et al., 2010), we propose that the structural instability of mutants contributes to their ultimate surface localization.

**Conclusions**

Since their inception, fluorescence-based analytical and preparative methods such as flow cytometry (FCT) and FACS have reached beyond the realm of immunology. FCT already has seen several applications in spirochetal systems, predominantly in deciphering gene regulation mechanisms (Whetstine et al., 2009; Eggers et al., 2006; Srivastava and de Silva, 2008) but also in probing membrane characteristics (Cox and Radolf, 2001). Various FACS-based methods such as differential fluorescence induction (DFI; ref (Valdivia, 1997)) have been used in different bacterial systems to identify virulence factors important for different pathogenic processes such as invasion and intracellular survival (reviewed in (Rediers et al., 2008)) Building on the earlier development of recombinant DNA technology (Stewart et al., 2001) and fluorescent reporter genes (Schulze and Zückert, 2006; Carroll et al., 2003; Campbell et al., 2002) this study expands the application of FACS to the study of protein transport mechanisms. Similar FACS-based approaches are perceivable to study secretion of other microbial proteins localizing to the host-pathogen interface. The demonstrated ability to sort live *B. burgdorferi*

cells for a particular fluorescent phenotype also opens the door to DFI studies, i.e., the trapping of promoters that are active during different stages in the complex multi-host life cycle of this medically important spirochete.



Random plasmid library
generated in E. coli
1
2
3
4
5
6
BsaI
BsiBI
NNKNNK
7
9
8
PCR and
sequence
analysis
PflaB
ospA:mrfp1
IR-A
PflgB
PF57
pOSK3
pOSK4
7.5 kb
kanR
PF50
zeoR
PF49
pBR ori
IR-B
Transformation
of B. burgdorferi
In situ proteolysis
FACS for red-fluorescent cells
Cloning by plating
Culture and in situ proteolysis

**Figure 3.1. Screening strategy for subsurface OspA:mRFP1 fusions.** A random mutagenesis oligo was synthesized to change mRFP1 codons E4 and D5 in OspA20:mRFP1 to any amino acid, with a bias against stop codons (except for amber UAG, see text). The oligo was converted to a double-stranded linker and ligated with a shuttle vector carrying the 5' and 3' portions of the OspA20:mRFP1 fusion gene. The resulting library was amplified in *E. coli* and used to transform *B. burgdorferi*. A presorted population of red fluorescent spirochetes was incubated with proteinase K, washed, and sorted again for red fluorescence. Clones grown from individual colonies were grown in 96-well plates and subjected to a confirmatory *in situ* proteolysis assay. PCR and DNA sequence analysis revealed the mutant genotypes. Numbered arrows indicate specific oligonucleotides used. For details, see the Chapter II.

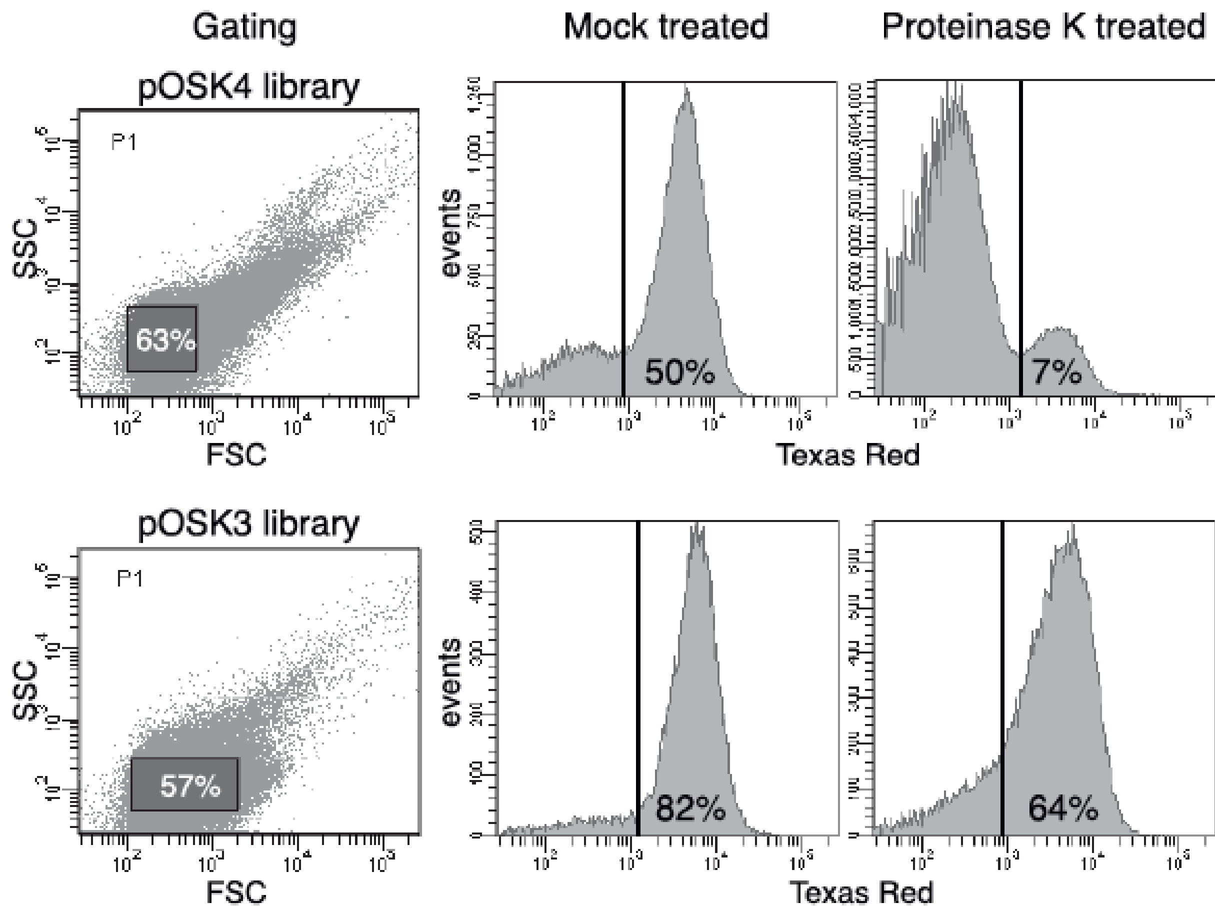



**Figure 3.2. FACS plots of OspA:mRFP1 mutant populations.** Both pRJS1009- and pRJS1016-based *B. burgdorferi* libraries were assayed. The two panels to the left indicate the gating used. Forward scatter (FSC) is plotted against side scatter (SSC). The percentage of events, i.e., cells inside the gated population (shaded rectangles) is indicated. The four panels to the right show the distribution of presorted, i.e., OspA:mRFP1-expressing fluorescent cells upon treatment with proteinase K. Mock treated cells were incubated in buffer only. Fluorescence measured via a Texas Red filter is plotted against number of events, i.e., cells. The vertical line indicates the cut-off fluorescence for sorting. The percentage of events within the fluorescent population is indicated.

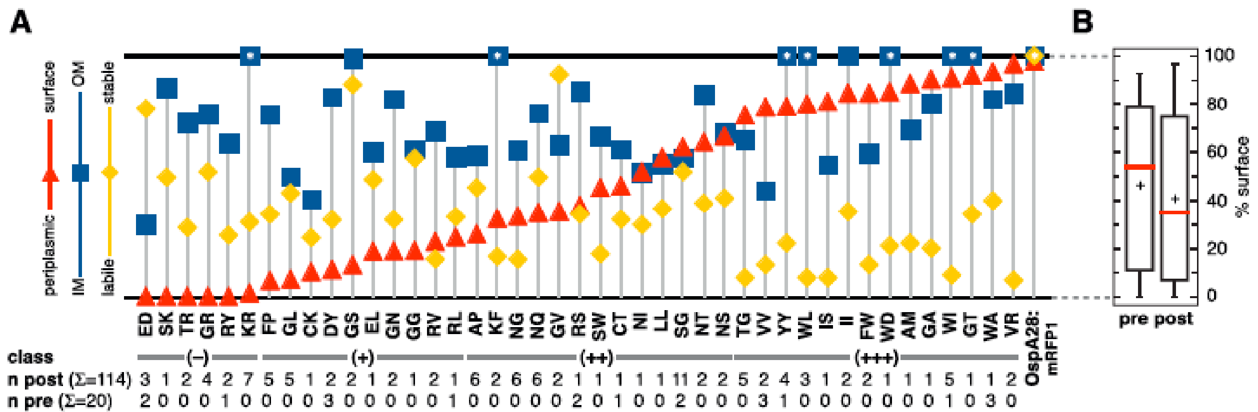
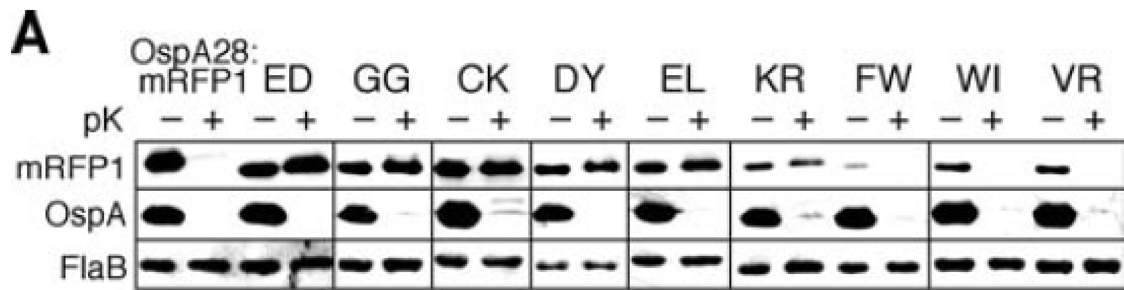



**Figure 3.3. Composite phenotypes of lipoprotein mutants.**

**(A)** Expression, surface exposure and membrane fraction ratio values are plotted for each of the 43 identified mutants, including OspA20:mRFP1 (ED), as well as the OspA28:mRFP1 control are plotted. Data were derived from independent duplicate or triplicate Western immunoblot experiments. Representative data are shown in Figs. 3.4, 3.5 and 3.6. Numerical data are listed in Table 3.5. Y-axis ranges were 0-100% for expression/stability levels (yellow diamonds) and surface exposure (red triangles), and 0 to 1.0 for the OM/PC ratio (blue squares). Data points with asterisks (*) lay beyond the y-axis ranges (Table 3.1). Mutant-specific amino acid sequences are listed in single letter code on the x-axis. n indicates the number of times a particular mutant was isolated from the unsorted (pre) and sorted (post) population. Unanalyzed mutants are listed in Table 3.1. **(B)** Boxplots of surface percentage values of the unsorted (pre) and sorted (post) populations. For each dataset, the box outlines the first and third quartiles, the horizontal red line indicates the median, the cross (+) denotes the mean, and the vertical lines extend to the minimum and maximum values.


A
OspA28:
mRFP1
ED
GG
CK
DY
EL
KR
FW
WI
VR
pK
− + − + − + − + − + − + − + − + − + − +
mRFP1
OspA
FlaB

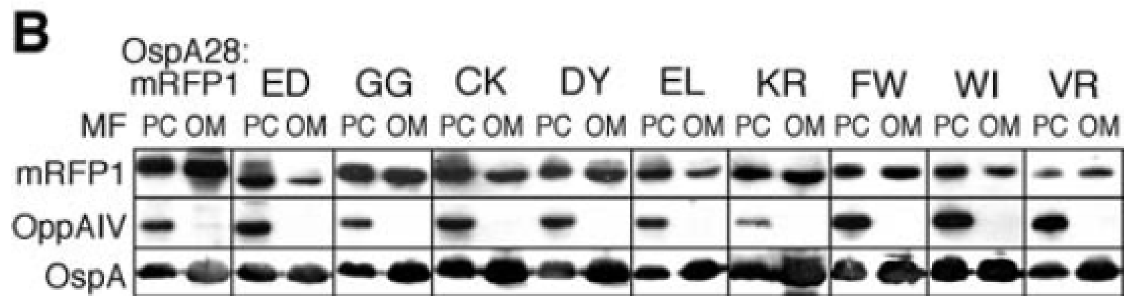

B
OspA28:
mRFP1
ED
GG
CK
DY
EL
KR
FW
WI
VR
MF
PC OM PC OM PC OM PC OM PC OM PC OM PC OM PC OM PC OM PC OM
mRFP1
OppAIV
OspA

**Figure 3.4. Phenotypical analysis of select OspA:mRFP1 fusion mutants.**

Representative Western blots of select mutants are shown (Figures 3.5 and 3.6 for full data set). Mutant-specific amino acid sequences are listed in single letter code above the blots. OspA28:mRFP1 and OspA20:mRFP1 (ED) were included as controls. **(A)** Protein expression and protease accessibility. Whole cell lysates of *B. burgdorferi* expressing mutant OspA:mRFP1 fusions from an identical PflaB promoter (Figure 3.1) were obtained before (-) or after (+) in situ treatment with proteinase K (pK). A polyclonal antiserum against mRFP1 was used to detect the OspA:mRFP1 fusions. Constitutively expressed periplasmic FlaB was used as a control for loading (to normalize signals within samples) as well as for subsurface localization (negative control). OspA served as a surface control. Untreated (-pK) samples were used to assess protein expression/in vivo stability of OspA:mRFP1 fusions. **(B)** Distribution of proteins to inner or outer membranes. Protoplasmic cylinder (PC) and outer membrane vesicle (OM) fractions from *B. burgdorferi* expressing mutant OspA:mRFP1 fusions were probed with a polyclonal antiserum against mRFP1 to detect the OspA:mRFP1 fusions. IM-localized lipoprotein OppAIV was used as a PC-specific control. Surface lipoprotein OspA was used as an outer membrane control. Note that the PC fraction also contains intact cells, i.e., also contains OM proteins.

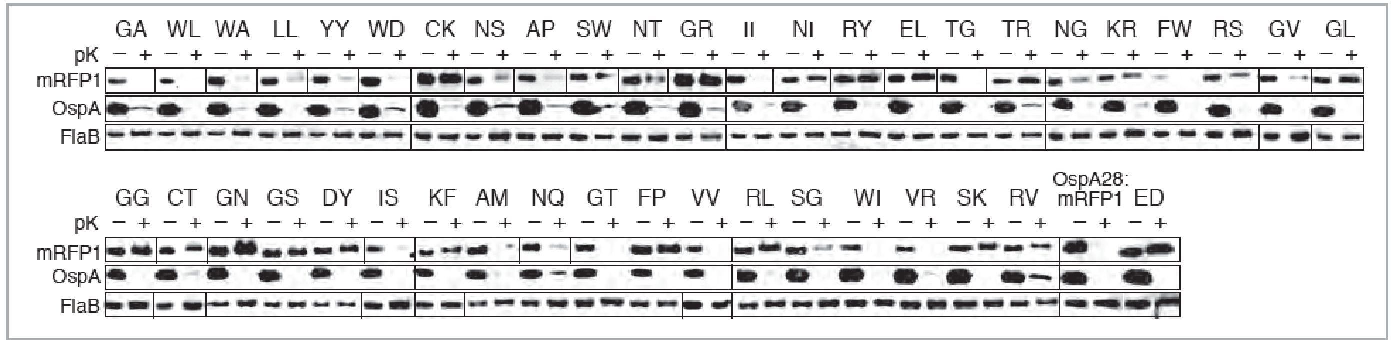



**Figure 3.5. Protease accessibility of OspA:mRFP1 fusion mutants.** Representative Western immunoblots of *B. burgdorferi* whole cell lysates expressing mutant OspA:mRFP1 fusions from an identical *flaB* promoter (Fig. 3.1) before (–) or after (+) in situ treatment with proteinase K (pK). A polyclonal antiserum against mRFP1 was used to detect the OspA:mRFP1 fusions. Constitutively expressed periplasmic FlaB was used as a control for loading (to normalize signals within samples) as well as for subsurface localization (negative control). OspA served as a surface control. Mutant-specific amino acid sequences are listed in single letter code above the blots. OspA28:mRFP1 and OspA20:mRFP1 (ED) were included as controls.

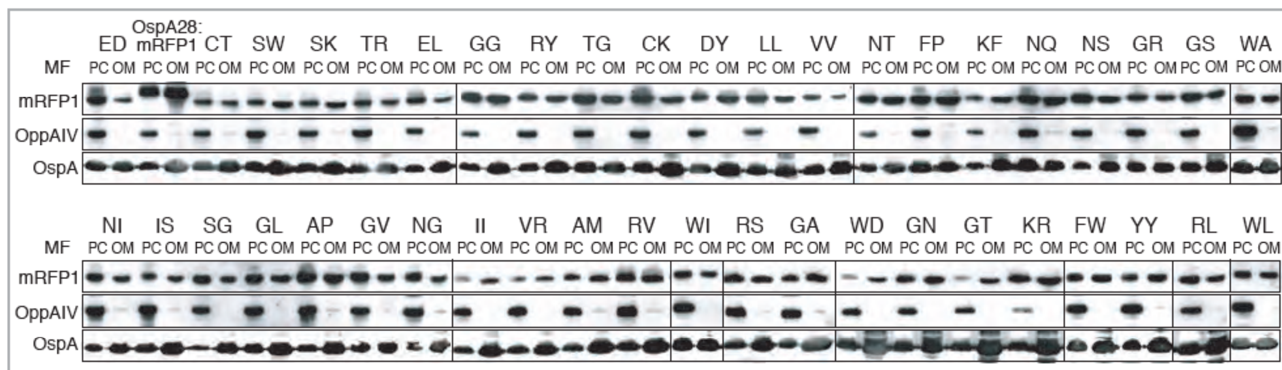



**Figure 3.6. Distribution of OspA:mRFP1 fusion mutants to inner and outer membranes.**

Representative Western immunoblots of protoplasmic cylinder (PC) and outer membrane vesicle (OM) fractions from *B. burgdorferi* expressing mutant OspA:mRFP1 fusions. A polyclonal antiserum against mRFP1 was used to detect the OspA:mRFP1 fusions. IM-localized lipoprotein OppAIV was used as a PC-specific control. Surface lipoprotein OspA was used as an outer membrane control. Note that the PC fraction also contains intact cells, i.e., also contains OM proteins. Mutant-specific amino acid sequences are listed in single letter code above the blots. OspA28:mRFP1 and OspA20:mRFP1 (ED) were included as controls.

## Table 3.1. Phenotypes of OspA20:mRFP1 fusion mutants

| Peptide Sequence[a] | Codons | | n Isolated | Surface Exposure Class | % Surface | | % Expression | | OM/PC Ratio | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Mean | SD | Mean | SD | Mean | SD |
| ED | GAG | GAC | 3 | – | 0 | 18 | 78 | 0 | 0.30 | 0.06 |
| SK | AGT | AAG | 1 | – | 0 | 7 | 50 | 17 | 0.87 | 0.10 |
| TR | ACG | AGG | 2 | – | 0 | 10 | 29 | 6 | 0.72 | 0.06 |
| GR | GGG | AGG | 4 | – | 0 | 3 | 52 | 4 | 0.76 | 0.12 |
| | GGG | CGG | | | | | | | | |
| RY | AGG | TAT | 2 | – | 0 | 14 | 26 | 10 | 0.64 | 0.08 |
| KR | AAG | AGG | 7 | – | 1 | 5 | 31 | 0 | 1.37 | 0.30 |
| FP | TTT | CCG | 5 | + | 6 | 18 | 34 | 6 | 0.76 | 0.34 |
| GL | GGT | TTG | 5 | + | 7 | 2 | 43 | 5 | 0.50 | 0.08 |
| | GGG | TTG | | | | | | | | |
| CK | TGT | AAG | 1 | + | 10 | 38 | 24 | 7 | 0.40 | 0.05 |
| DY | GAT | TAT | 2 | + | 11 | 14 | 33 | 13 | 0.83 | 0.05 |
| GS | GGT | TCG | 3 | + | 13 | 33 | 88 | 21 | 0.99 | 0.36 |
| EL | GAG | TTG | 1 | + | 19 | 33 | 49 | 0 | 0.60 | 0.16 |
| GN | GGT | AAT | 1 | + | 19 | 12 | 32 | 10 | 0.82 | 0.20 |
| GG | GGT | GGG | 1 | + | 19 | 5 | 57 | 36 | 0.61 | 0.12 |
| RV | CGT | GTG | 2 | + | 23 | 2 | 16 | 8 | 0.69 | 0.14 |
| RL | CGG | TTG | 1 | + | 25 | 4 | 34 | 10 | 0.58 | 0.17 |
| AP | GCT | CCG | 6 | ++ | 26 | 33 | 45 | 1 | 0.59 | 0.13 |
| KF | AAG | TTT | 2 | ++ | 32 | 17 | 17 | 7 | 1.14 | 0.18 |
| NG | AAT | GGT | 6 | ++ | 33 | 5 | 15 | 8 | 0.61 | 0.12 |
| NQ | AAT | CAG | 6 | ++ | 35 | 16 | 50 | 11 | 0.76 | 0.02 |
| GV | GGT | GTC | 2 | ++ | 35 | 16 | 92 | 15 | 0.63 | 0.07 |
| | GGG | GTG | | | | | | | | |
| RS | AGG | AGT | 1 | ++ | 38 | 19 | 35 | 7 | 0.85 | 0.09 |
| SW | AGT | TGG | 1 | ++ | 45 | 4 | 18 | 0 | 0.66 | 0.17 |
| CT | TGT | ACG | 1 | ++ | 46 | 4 | 32 | 5 | 0.61 | 0.11 |
| NI | AAT | ATT | 1 | ++ | 52 | 40 | 30 | 12 | 0.51 | 0.13 |
| LL | CTT | CTG | 1 | ++ | 58 | 17 | 36 | 7 | 0.55 | 0.10 |
| SG | AGT | GGT | 11 | ++ | 62 | 18 | 52 | 2 | 0.58 | 0.09 |
| | TCT | GGG | | | | | | | | |
| | TCC | GGT | | | | | | | | |
| NT | AAT | ACG | 2 | ++ | 64 | 17 | 39 | 3 | 0.84 | 0.14 |
| NS | AAT | TCG | 2 | ++ | 66 | 24 | 41 | 6 | 0.68 | 0.17 |
| TG | ACG | GGG | 5 | +++ | 75 | 19 | 8 | 5 | 0.65 | 0.05 |
| VV | GTG | GTT | 2 | +++ | 79 | 4 | 13 | 2 | 0.44 | 0.11 |
| YY | TAT | TAT | 4 | +++ | 79 | 15 | 22 | 7 | 1.18 | 0.12 |
| WL | TGG | TTG | 3 | +++ | 80 | 6 | 7 | 2 | 1.19 | 0.07 |
| IS | ATT | TCT | 1 | +++ | 81 | 4 | 8 | 1 | 0.55 | 0.08 |
| II | ATT | ATT | 2 | +++ | 84 | 3 | 35 | 15 | 1.05 | 0.23 |
| FW | TTT | TGG | 2 | +++ | 85 | 13 | 13 | 0 | 0.59 | 0.02 |
| WD | TGG | GAT | 1 | +++ | 85 | 9 | 21 | 7 | 1.08 | 0.35 |
| AM | GCG | ATG | 1 | +++ | 88 | 5 | 22 | 3 | 0.69 | 0.13 |
| GA | GGT | GCG | 1 | +++ | 90 | 7 | 20 | 2 | 0.80 | 0.22 |
| WI | TGG | ATT | 5 | +++ | 91 | 1 | 9 | 2 | 1.23 | 0.51 |
| GT | GGG | ACG | 1 | +++ | 92 | 4 | 34 | 12 | 1.11 | 0.27 |
| WA | TGG | GCG | 1 | +++ | 93 | 9 | 39 | 13 | 0.82 | 0.07 |
| VR | GTG | CGG | 2 | +++ | 97 | 4 | 7 | 2 | 0.84 | 0.16 |
| N28 | N/A | N/A | N/A | +++ | 98 | 1 | 100 | 25 | 2.03 | 0.40 |

[a]mutants not analyzed in this study (n post/n pre): EI (1/0), ET (1/0), GL (1/0), LD (1/0), NR (1/0), RM (1/0), RR (1/0), SL (1/0), VT (6/1), GQ (2/1), LR (2/2), IL (1/1),ES (0/1),NW (0/1), RI (0/1), SE (0/1), TW (0/1),VA (0/1),YF (0/1), YT (0/1).

# Chapter IV

## Surface Localization Determinants of *Borrelia* OspC/Vsp Family Lipoproteins

**Abstract**

The dimeric OspC/Vsp family surface lipoproteins of *Borrelia* spirochetes are crucial to the transmission and persistence of Lyme borreliosis and tick-borne relapsing fever. Yet, the requirements for their proper surface display remains undefined. In previous studies, we showed that localization of *Borrelia burgdorferi* monomeric surface lipoprotein OspA was dependent on residues in the N-terminal 'tether' peptide. Here, site-directed mutagenesis of the *B. burgdorferi* OspC tether revealed two distinct regions affecting either release from the inner membrane or translocation through the outer membrane. Determinants of both of these steps appear consolidated within a single region of the *Borrelia turicatae* Vsp1 tether. Periplasmic OspC mutants still were able to form dimers. Their localization defect could be rescued by addition of an apparently fold-destabilizing C-terminal epitope tag, but not by co-expression with wild type OspC. Furthermore, disruption of intermolecular Vsp1 salt bridges blocked dimerization, but not surface localization of the resulting Vsp1 monomers. Together, these results suggest that oligomeric *Borrelia* surface lipoproteins traverse the periplasm and the outer membrane as unfolded monomeric intermediates and assemble into their functional multimeric folds only upon reaching the spirochetal surface.

## Introduction

Since the original description of a prokaryotic lipoprotein in the cell envelope of *Escherichia coli* over four decades ago (Braun and Rehn, 1969), this class of peripherally anchored membrane proteins has been increasingly appreciated. In diderm bacteria, lipoproteins are routed via the general secretory pathway through and to the inner membrane (IM), where they are post-translationally modified by acylation at a conserved Cys residue (Hantke and Braun, 1973). Sorting within the periplasm depends on variations of an N-terminal signal first identified in *E. coli* (Yamaguchi et al., 1988; Gennity and Inouye, 1991; Seydel et al., 1999; Lewenza et al., 2006; Narita and Tokuda, 2007; Silva-Herzog et al., 2008) and is carried out by the Lol system, consisting of the IM ABC transporter-like sortase complex LolCDE (Yakushi et al., 2000), the periplasmic lipoprotein carrier LolA (Matsuyama et al., 1995), and the outer membrane (OM) lipoprotein receptor LolB (Matsuyama et al., 1997; Yokota et al., 1999). Established pathways of lipoprotein translocation through the OM involve either a Type II or Type V secretion system (Pugsley et al., 1990; Pugsley, 1993; Francetic and Pugsley, 2005; Sauvonnet and Pugsley, 1996; Coutte et al., 2003; van Ulsen et al., 2003)

Beyond the involvement of Braun's lipoprotein Lpp in bacterial cell envelope stability (Braun and Wolff, 1970), lipoproteins were shown to play roles in a variety of cellular and pathogenic processes most recently reviewed by (Kovacs-Simon et al., 2010). In *Borrelia* spirochetes, the etiologic agents of arthropod-borne Lyme disease and relapsing fever, surface lipoproteins are particularly abundant and constitute the predominant class of known virulence factors at the vector/host-pathogen interface (Brandt et al., 1990; Kudryashev et al., 2009; Bergström et al., 2010; Norris et al., 2010; Barbour and Travinsky, 2010). Outer surface protein A (OspA), e.g., is expressed by the Lyme disease spirochete *Borrelia burgdorferi* during the

vector phase to facilitate adhesion to the tick midgut (Barbour et al., 1983; Pal et al., 2000; Pal et al., 2004). Upon tick feeding and transmission to a new mammalian host, complex regulatory mechanisms lead to the replacement of OspA by OspC (Schwan et al., 1995; Stevenson et al., 1995; Schwan and Piesman, 2000; Pal et al., 2001; Pal et al., 2004; Srivastava and de Silva, 2008). OspC is required for the establishment of mammalian infection (Grimm et al., 2004), via interaction with the *Ixodes scapularis* salivary gland protein, Salp15 (Anguita et al., 2002; Ramamoorthi et al., 2005). OspC also binds plasminogen, which further enhances invasiveness (Lagal et al., 2006). Variable small proteins (Vsp) are expressed by tick-borne relapsing fever spirochetes such as *Borrelia turicatae* and are phylogenetically and structurally related to OspC (Zückert et al., 2001; Lawson et al., 2006; Kumaran et al., 2001; Eicken et al., 2001). They contribute to chronic infection of mammalian hosts by participating in an elaborate scheme of multiphasic antigenic variation designed to repeatedly evade the host's immune response (Dworkin et al, 2008). Vsps have also been shown to be the determinants of *B. turicatae* tissue tropism (Cadavid et al., 1994; Cadavid et al., 1997; Pennington et al., 1997; Pennington and Cadavid 1999; Gandhi et al., 2010) and may enhance invasion of tissues by binding to glycosaminoglycans (Magoun et al., 2000).

Our previous investigations into the secretion of the major *Borrelia* surface lipoproteins led to some intriguing discoveries. We first noticed that any known OM lipoprotein secretion modules, i.e., LolB, Type II or Type V systems, were missing from *Borrelia* genomes. At the same time, relapsing fever *Borrelia* lipoproteins such as Vsp1 were compatible with the *B. burgdorferi* lipoprotein secretion machinery (Zückert et al., 2004). This implied a novel genus-wide mechanism for *Borrelia* OM lipoprotein targeting and translocation. Using OspA as a first model lipoprotein, we subsequently showed that the established eubacterial sorting rules

(Yamaguchi et al., 1988; Gennity and Inouye, 1991; Seydel et al., 1999; Lewenza et al., 2006; Narita and Tokuda, 2007; Silva-Herzog et al., 2008) did not apply to borrelial lipoproteins (Schulze and Zückert, 2006). Next, we discovered that a specific region in the OspA tether region is required for efficient OM translocation, and that C-terminal epitope tags of periplasmic OspA mutants were selectively displayed on the bacterial surface. Additional OspA mutants indicated that the above described tether mutations lead to premature folding of OspA in the periplasm (Schulze et al., 2010). This suggested that lipoprotein translocation through the outer spirochetal membrane requires an unfolded conformation of the substrate protein and can initiate at the C-terminus, yet is independent of a specific C-terminal targeting peptide.

In this report, we expanded our studies to the OspC-Vsp family of proteins, which form dimeric α-helical bundles (Eicken et al., 2001; Kumaran et al., 2001; Lawson et al., 2006; Zückert et al., 2001) and therefore are structurally distinct from the OspA β-sheet monomer (Li et al., 1997; Becker et al., 2005). The data now allow us to compare and contrast the secretion requirements of two different *Borrelia* surface lipoprotein folds. Even more significantly, they permit us for the first time to establish the oligomeric state of lipoproteins throughout secretion.

## Results

### OspC-Vsp1 localization determinants are also confined to the tether, but minimal tether requirements differ from OspA.

In two previous studies, we determined the N-terminal lipopeptides required for surface localization of monomeric OspA in fusions to the red-fluorescent reporter protein mRFP1. Using a standard protocol, which combined proteolytic ‘shaving’ to distinguish between surface and periplasmic lipoproteins along with the analysis of OM vesicle fractions to localize

periplasmic lipoproteins to either the IM or OM (see Chapter II), we originally concluded that five N-terminal residues of the mature OspA lipoprotein were sufficient for surface localization of mRFP1. OspA20:mRFP1, providing only four OspA tether residues (Fig. 4.1A), was protected from proteolytic shaving with proteinase K, i.e., localized largely to the periplasm, while fusions of mRFP1 to OspA21 and longer lipopeptides were protease accessible, i.e., surface-displayed (Schulze and Zückert, 2006). However, we later determined that four N-terminal amino acids of mRFP1 contributed to the process and switched to a truncated mRFP1 reporter, mRFPΔ4 (Schulze et al., 2010). To enable direct comparison of OspA and OspC/Vsp1 data, we revisited the OspA tether requirements and fused OspA20, OspA21, OspA22 and OspA25 (Fig. 4.1A) to mRFPΔ4. As expected, the requirement shifted to longer OspA-derived lipopeptides: in contrast to the mRFP1 fusions, OspAV21 and OspAS22 tethers no longer were sufficient for surface exposure of mRFPΔ4; only OspA25:mRFPΔ4 remained surface exposed (Fig. 4.2A). This indicated that nine N-terminal residues of mature OspA are sufficient for surface exposure.

As for OspA, fusions with OspC and Vsp1 full-length tether peptides were able to guide mRFPΔ4 properly through the spirochetal cell envelope and to the surface (Figs. 4.1, 4.2B and 4.2C). Yet, fusions to truncated OspC and Vsp1 tethers revealed some interesting differences. First, the minimal surface localization requirements were extended to tethers 12 and 14 amino acids in length, respectively. OspC30:mRFPΔ4 and Vsp1.32:mRFPΔ4 were displayed on the surface while OspC29:mRFPΔ4 and Vsp1.31:mRFP1Δ4 localized to the periplasm (Figs. 4.2B and 4.2C). Longer and shorter tether fusion data were consistent with these surface-to-subsurface transitions (data not shown). In context with the previously published OspA-derived data (Schulze and Zückert, 2006; Schulze et al., 2010) these experiments corroborated a common

tether-dependent secretion pathway for *Borrelia* surface lipoproteins, which yet appears to tolerate significant primary sequence diversity.

**Tether mutagenesis reveals two separate OspC domains required for OM and surface targeting.**

Based on the fluorescent protein fusion data, we next deleted the N-terminal OspC and Vsp1 peptide sequences deemed dispensable or essential for surface localization. Tetherless $OspC_{\Delta 20\text{-}41}$ and $Vsp1_{\Delta 20\text{-}39}$ mutants had a null phenotype i.e., no protein was detected. Upon further examination, no mRNA was detected in cells expressing $OspC_{\Delta 20\text{-}41}$ and $Vsp1_{\Delta 20\text{-}39}$ as determined by northern blotting using DNA probes complementary to the *ospC* and *vsp1* coding regions (Fig. 4.9). The simplest explanation for this phenomenon is that site-directed mutagenesis resulted in the creation of a transcription termination sequence.

As expected, deletion of the 'essential', anchor-proximal OspC tether peptide led to a defect, localizing $OspC_{\Delta 20\text{-}30}$ to the periplasmic leaflet of the OM. Surprisingly, the deletion of the 'dispensable' anchor-distal $OspC_{\Delta 31\text{-}41}$ peptide also resulted in a sorting defect, largely retaining the construct in the IM. Expansion of the tether by three residues in $OspC_{\Delta 34\text{-}41}$ was required to restore surface localization. The smallest alterations leading to mislocalization of OspC were either single or double amino acid deletions in the +3 and +4 positions. $OspC_{\Delta N21}$, $OspC_{\Delta S22}$ and $OspC_{\Delta N21/S22}$ localized to the periplasmic leaflet of the OM. Replacement of the two residues with either Gly or Ala dipeptides led to phenotypes already observed with OspA (Schulze et al., 2010): Ala-Ala in $OspC_{N21A/S22A}$ was permissive for surface exposure while Gly-Gly in $OspC_{N21G/S22G}$ prevented proper translocation through the OM. With the exception of a

triple +2/+3/+4 position residue deletion in $OspC_{\Delta 20\text{-}22}$, deletions elsewhere within the tether did not affect OspC surface localization (Fig. 4.1 and Fig 4.3).

Vsp1 tether deletion data tracked the mRFP1Δ4 fusion data (Fig. 4.4): $Vsp1_{\Delta 33\text{-}39}$ remained surface exposed while $Vsp1_{\Delta 20\text{-}32}$ was retained in the periplasm. A six-residue region (Asn20-Ser25) proximal to the N-terminal cysteine proved important for proper localization. Deletion of at least four of these six residues led to an OM translocation defect, localizing the respective mutants to the periplasmic leaflet of the OM. Its full deletion in $Vsp1_{\Delta 20\text{-}25}$ led to retention in the IM. Yet, replacing the Pro residue in the +2 position with Ala in $Vsp1_{\Delta 20\text{-}25/P26A}$ restored release from the IM to the periplasmic leaflet of the OM. Other deletions within the Vsp1 tether did not affect the surface phenotype (Fig. 4.4B). Together, these experiments suggested that functional surface display of *Borrelia* lipoproteins required a subset of common tether amino acid residues, albeit with no stringent positional constraints relative to the N-terminal cysteine.

**Tether peptides do not affect overall protein thermal stability.**

Several prior studies using maltose binding protein had shown that its signal peptide retarded protein folding to favor interactions with the SecB chaperone, thereby ensuring efficient secretion through the inner membrane Sec machinery (Park et al., 1988; Liu et al., 1989; Beena et al., 2004). We therefore decided to test whether tethers of surface lipoproteins had similar intrinsic destabilizing capabilities. Recombinant non-lipidated OspC variants were purified and their folded state was monitored over a temperature range from 25°C to 95°C by circular dichroism (CD) spectroscopy. Three recombinant OspC (rOspC) variants were analyzed: $rOspC_{N20}$ contained the full-length tether, replacing the N-terminal Cys with an $^{f}$Met. $rOspC_{N31}$

and rOspC$_{V37}$ lacked 11 or 17 N-terminal amino acids, respectively; a deletion identical to the one in rOspC$_{N31}$ had resulted in the mislocalization of OspC$_{D20-30}$ to the periplasm *in vivo* (Fig. 4.3). Circular dichroism spectra did not reveal any significant variations in the predominantly α-helical secondary structure between the three proteins (Fig. 4.5A). Thermal denaturation curves of all three proteins were virtually identical and had a single transition state at about 50°C (Fig. 4.5B). This indicated that mutations within the tether, in the absence of other cellular proteins, resulted in only marginal changes in thermodynamic stability.

To verify these findings, we repeated the thermal unfolding experiments using an unrelated protein, OspA. As mentioned before, OspA is monomeric and primarily composed of an open β-sheet. Non-lipidated OspA with the full-length tether peptide (rOspA$_{A17}$) and tetherless OspA (rOspA$_{S29}$) were purified and thermal unfolding experiments performed as with OspC. As with OspC, the CD spectra of OspA did not reveal any differences of the overall β-sheet secondary structure (Fig. 4.5C). However, rOspA is more thermal stable than rOspC, as there were only minor differences in secondary structure at 25°C compared to 80°C, which makes comparison of unfolding profiles between the two proteins difficult (data not shown).

**OspC mislocalized to the periplasm folds and dimerizes.**

We previously found that C-terminal fold-destabilizing mutations in OspA were able to overcome a tether-based mislocalization defect (Schulze et al., 2010) and interpreted this as a requirement for OspA to remain at least partially unfolded prior to translocation through the OM. Consequently, we surmised that premature folding leads to periplasmic retention of otherwise surface-displayed lipoproteins. To further test this hypothesis, we determined the folding status of two periplasmic OspC mutants, OspC$_{\Delta 20-30}$ and OspC$_{\Delta 31-41}$. A first approach built on earlier

observations that the tight α-helical bundles of wild type OspC and Vsp1 left only the proteins' N– and C-termini vulnerable to trypsinolytic attack (Eicken et al., 2001; Kumaran et al., 2001; Lawson et al., 2006; Zückert et al., 2001). The maintenance of trypsin-resistant OspC/Vsp 'core' proteins could therefore serve as a hallmark for a proper structural conformation. To gain access to the periplasmic $OspC_{\Delta 20\text{-}30}$ and $OspC_{\Delta 31\text{-}41}$ proteins, we were required to permeabilize the borrelial envelope with 0.1% SDS (Jewett et al., 2007) prior to trypsin treatment. In the presence of 0.1% SDS, $OspC_{\Delta 20\text{-}30}$ and $OspC_{\Delta 31\text{-}41}$ became trypsin-susceptible like $OspC_{wt}$ (Fig. 4.6A). Based on densitometry of Western immunoblot signals, we observed an approximately 2- to 3-fold decrease in $OspC_{\Delta 20\text{-}30}$ and $OspC_{\Delta 31\text{-}41}$ compared to $OspC_{wt}$. The trypsin resistance of periplasmic OspC was comparable to that of surface OspA in the presence of detergent and significantly higher than that of periplasmic OM lipoprotein Lp6.6 (Fig. 4.6A). In a second experiment, we probed the oligomeric state of periplasmic OspC by formaldehyde crosslinking and proteolytic shaving. Upon addition of formaldehyde, we detected a 46-kDa band corresponding to the OspC dimer (Fig. 4.6B; (Bunikis and Barbour, 1999)). The $OspC_{\Delta 20\text{-}30}$ and $OspC_{\Delta 31\text{-}41}$ dimers were protected from proteinase K, indicating their subsurface localization. Finally, a Far-Western protein overlay assay (Fig 4.6C) showed that $OspC_{\Delta 20\text{-}30}$ and $OspC_{\Delta 31\text{-}41}$ were able to form functional epitopes that could interact with the *Ixodes scapularis* salivary gland protein Salp15 (Dai et al., 2009; Ramamoorthi et al., 2005). Based on this evidence, we concluded that mislocalized OspC tether mutants were not blocked from assuming a proper conformation within the periplasm.

**Fold destabilization of periplasmic OspC stimulates OM translocation.**

We previously found that C-terminal epitope tags of the periplasmic $OspA_{\Delta S22}$ mutant were selectively surface exposed (Schulze et al., 2010). To determine if an identically tagged OspC protein would have the same phenotype, we added a C-terminal His-tag to $OspC_{\Delta 22}$. To our surprise, not only the C-terminal tag, but the entire $OspC_{\Delta S22}$-His became surface localized (Fig. 4.7A). Surface proteolysis with trypsin tested for the maintenance of the OspC trypsin-resistant core (Eicken et al., 2001; Kumaran et al., 2001; Zückert et al., 2001). Cell-associated $OspC_{wt}$, OspC-His and $OspC_{\Delta S22}$-His proteins showed the expected proteolytic pattern, i.e., a removal of the C-terminus (Fig. 4.7B). The trypsin-resistant core protein released from the cell into the reaction supernatant was clearly detectable for $OspC_{wt}$, but not for the OspC-His and $OspC_{\Delta S22}$-His proteins. This indicated that addition of a C-terminal epitope tag sufficiently destabilized the OspC fold to stimulate the mutant's release from the periplasm to the spirochetal surface. To exclude the possibility of an artifact introduced by the His tag these experiments were repeated using C-terminal FLAG tag with identical results (data not shown). Together, these experiments further supported our earlier conclusions that trapping of surface lipoproteins within the periplasm is avoided by maintaining unfolded translocation intermediates.

**OspC and Vsp1 likely traverse the periplasm as monomeric intermediates.**

If unfolded periplasmic translocation intermediates were universal for surface lipoproteins, oligomerization interfaces of proteins such as the OspC/Vsp homodimers would likely be disrupted. Therefore, these proteins would remain monomeric within the periplasm before assuming their final tertiary and quaternary structures on the spirochetal surface. We used two approaches to test this hypothesis. First, we asked whether periplasmic heterodimerization with

a wild type OspC monomer could 'rescue' a mutant subsurface OspC monomer to the bacterial surface. We transformed *B. burgdorferi* strains B31-e2 and B313, which endogenously express wild-type OspC, with a plasmid that encodes for the periplasmic $OspC_{\Delta 31-41}$ and $OspC_{\Delta 20-30}$ mutants, respectively. Based on densitometry of Western blots, there was no shift in the protease accessibility of the mutant OspC proteins in the presence of wild type OspC and vice versa (Figure 4.6D). This indicated that mutant and wild type OspC proteins failed to interact with each other in the periplasm.

In a variation of this approach, we marked the periplasmic $OspC_{\Delta 20-30}$ with a HA-tag (YPYDVPDYA) and wild type surface OspC with a FLAG tag (DYKDDDDK) and attempted to co-immunoprecipitate the proteins with antibodies against their respective tags. Cells expressing two surface-localizing FLAG or HA-tagged OspC copies served as control. No significant heterodimer levels were detected (Fig. 4.7C). However, introduction of an HA tag into OspC generated a membrane localization artifact, as localization of $OspC_{\Delta 20-30-HA}$ is largely localized to the inner membrane, while $OspC_{\Delta 20-30}$ is largely localized to the outer membrane (Fig 4.7B and 4.3B). Regardless of the localization artifacts introduced by the HA tag, the data clearly show no heterodimerization between a periplasmic OspC and a surface OspC (Fig 4.7).

Second, we set out to generate monomeric mutants of OspC and Vsp1 by disrupting intermolecular salt bridges by charge swapping. All OspC mutants either still dimerized or had a null phenotype (Table 4.1). However, an obtained triple Vsp1 D60K/D87K/D150K mutant (Fig. 1B) was instructive. $Vsp1_{D60/87/150K}$ was likely destabilized, as it was detected at a lower level than $Vsp1_{wt}$. *In situ* formaldehyde crosslinking and protease accessibility experiments showed that $Vsp1_{D60K/D87K/D150K}$ failed to dimerize, yet still reached the *B. burgdorferi* surface (Fig. 4.6E). This showed that dimerization was not required for Vsp1 surface localization. Together,

these three experiments provided evidence for monomeric periplasmic intermediates of oligomeric surface lipoproteins.

**Vsp1 and Vsp2 form surface heterodimers when expressed in the same cell.**

Relapsing fever *Borrelia* express two different serotypes of Vsp lipoproteins during mammalian infection: Vsp1 and Vsp2. Both of these lipoproteins undergo antigenic variation during the course of infection and exhibit differential tissue tropism (Cadavid et al., 1997; Cadavid et al., 1994; Pennington and Cadavid, 1999; Cadavid et al., 1993; Pennington et al., 1997). We wished to determine if Vsp1 and Vsp2 could form heterodimers *in vivo*. Heterodimerization between these two serotypes could function as an additional mechanism for antigenic variation. To test this hypothesis, we introduced an HA tag into Vsp1 and a FLAG tag into Vsp2 (like with OspC above). First, we expressed Vsp1-HA and Vsp2-FLAG in the same *B. burgdorferi* cell from separate plasmids. We then performed co-immunopercipitation (Co-IP) using anti-HA and anti-FLAG antibodies. Vsp1-HA and Vsp2-FLAG were immunoprecipitated irrespective of which antibody was used as bait (Fig 4.10). As a control, we repeated the experiment using the Vsp1 monomer mutant ($Vsp1_{D60/87/150K}$-HA) and Vsp2-FLAG. As expected $Vsp1_{D60/87/150K}$-HA was immunoprecipitated only with anti-HA antibodies and Vsp2-FLAG was only immunoprecipitated with anti-FLAG antibodies (Fig 4.10). Vsp1-HA, $Vsp1_{D60/87/150K}$-HA, and Vsp2-FLAG and all were surface exposed (data not shown).

## Discussion

While major lipoproteins of diderm bacteria generally localize to the periplasmic leaflets of either the IM or OM depending on N-terminal sorting signals recognized by the Lol machinery, the sorting of major lipoproteins in *Borrelia* is inherently more complex due to the requirement of surface lipoproteins to cross the OM. Our previous studies focused on the secretion requirements of the monomeric surface lipoprotein OspA (Schulze and Zückert, 2006; Schulze et al., 2010). In the present study, we turned our attention to the *Borrelia* OspC/Vsp lipoproteins, a family of functionally diverse, but structurally conserved dimeric surface lipoproteins. This represented an important next step toward our ultimate goal of defining canonical sorting rules for *Borrelia* lipoproteins. It also provided first hints at how *Borrelia* cells cope with an additional layer of complexity during lipoprotein secretion: the oligomerization of dimeric lipoproteins.

Although OspC and Vsp1 share the same protein fold, their overall peptide sequence identity is only about 40% (Eicken et al., 2001; Kumaran et al., 2001; Lawson et al., 2006; Zückert et al., 2001). This heterogeneity extends into the membrane-distal tether portions and may explain most of the distinct secretion determinants for the two surface lipoproteins, e.g. the lack of a phenotype for the $Vsp_{\Delta 33\text{-}39}$ mutant. The first five tether residues, however, are conserved between OspC and Vsp1. It is therefore puzzling that the deletion of three residues internal to this pentapeptide yields a subsurface phenotype for $OspC_{\Delta 20\text{-}22}$, but not for $Vsp1_{\Delta 20\text{-}22}$. Deletion of the subsequent tripeptides does not affect surface localization of either $OspC_{\Delta 23\text{-}25}$ or $Vsp1_{\Delta 23\text{-}25}$. This suggests that Vsp1 the $Gly^{23}/Thr^{24}/Ser^{25}$ tripeptide is functionally redundant to $Asn^{20}/Asn^{21}/Ser^{22}$. Yet, we cannot exclude that some of the observed variances between OspC and Vsp1 are due to the heterologous expression of Vsp1 in the *B. burgdorferi* surrogate host.

While overall lipoprotein sorting mechanisms appear to be conserved within the genus *Borrelia* (Zückert et al., 2004), they might have undergone additional fine-tuning within individual species. Unfortunately, the absence of a *B. turicatae* genetic system currently prevents further exploration of this issue.

Several common attributes are emerging from a comparison of the now known *Borrelia* surface lipoprotein secretion requirements. First, there is the confinement of lipoprotein targeting information to the N-terminal tether peptide. This is not entirely surprising as the sorting rules previously identified in other diderm bacteria also implicate the N-termini of the mature lipoproteins (Yamaguchi et al., 1988; Silva-Herzog et al., 2008; Seydel et al., 1999; Narita and Tokuda, 2007; Lewenza et al., 2006; Gennity and Inouye, 1991). Vsp1/OspC-derived peptides required for the proper secretion of the mRFPΔ4 reporter are at least 3 to 5 residues longer than the OspA minimal tether. This may be a consequence of the above-mentioned optimization of different substrates for a common lipoprotein secretion machinery, and the significance of these length differences may be exaggerated due to the currently limited dataset. Second, the essential tether 'motifs' of OspA, OspC and Vsp1 (shaded in blue in Fig. 4.1A) commonly contain at least one Ser residue. The significance of this apparent conservation remains to be elucidated. Also conserved is the tolerance for Ala, but not Gly substitutions within these 'motifs' of OspA and OspC (Fig. 4.3;(Schulze et al., 2010)). This further supports our earlier conclusions that a defined degree of flexibility within a critical tether segment is required for proper function.

It is worth reiterating that the above described essential tether 'motifs' are otherwise quite variable in sequence, extent and spacing relative to the N-terminal acylated cysteine '+1' residue. This further bolsters our OspA-based conclusions regarding the absence of a positional

'+2/+3/+4' rule for *Borrelia* lipoproteins. On first sight, the Vsp1$_{\Delta 20\text{-}25}$ and Vsp1$_{\Delta 20\text{-}25/P26A}$ mutants may provide a counter-argument, as they conclusively show that a Pro residue at position '+2' specifically leads to lipoprotein mislocalization to the *B. burgdorferi* IM. Yet, secondary structure-disrupting prolines are found throughout the tethers of *B. burgdorferi* lipoproteins, except in the '+2' position (Setubal et al., 2006; Schulze et al., 2006). Therefore, a '+2' Pro should be considered a non-native lipoprotein IM retention signal, which interestingly is shared across genus barriers with *E. coli* (Schulze et al., 2010; Setubal et al., 2006). As such, it might be of questionable biological relevance, but may hint at common molecular mechanisms. In the context of our earlier identification of borrelial LolCDE and LolA homologs, as well as basic amino acids serving as borrelial IM retention signals (Schulze et al., 2010; Zückert et al., 2004; Kumru et al., 2010), we therefore propose that the lipoprotein sorting mechanisms in the IM of diderm bacteria are conserved on a general level, albeit with variations in the exact nature and placement of the IM retention or Lol avoidance signals.

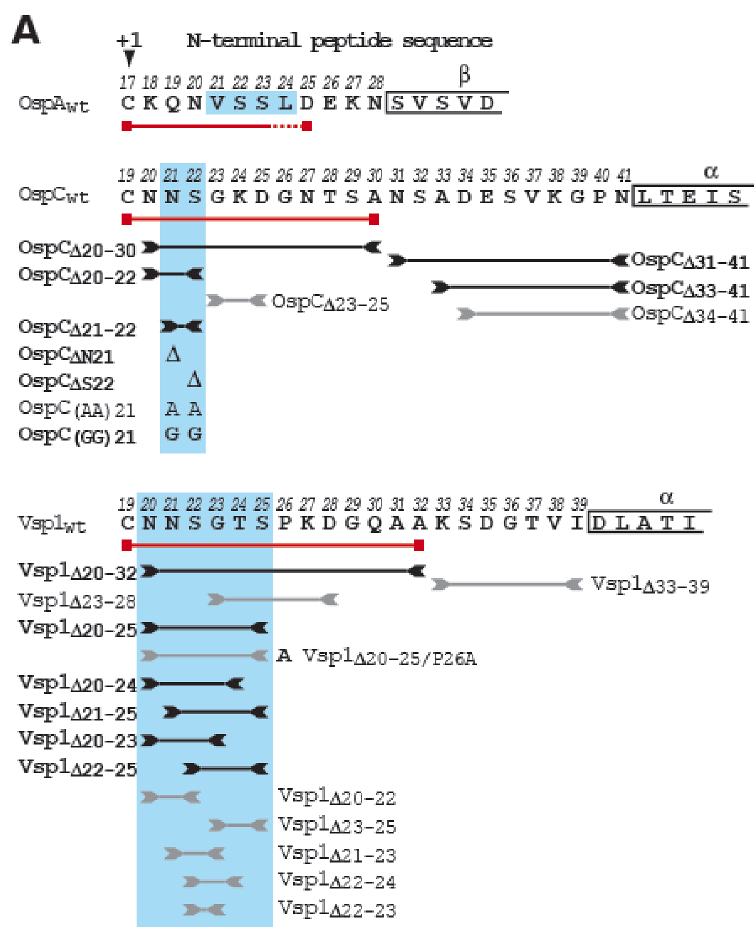
The current OspC/Vsp1 data also corroborate the previously established OspA-derived requirements for lipoprotein translocation through the OM. First, the ability of a Vsp1 monomer and a fold-destabilized, otherwise periplasmic OspC mutant to reach the bacterial surface further supports the requirement of the OM lipoprotein translocation machinery for at least partially unfolded substrates (Schulze et al., 2010). The apparent differences in the phenotypes of C-terminally tagged, otherwise periplasmic OspA and OspC mutants may be explained by the structural differences between the two proteins. In OspA, C-terminal tags are distal from the N-terminal membrane tether and likely will act as separate protein domains. In OspC, however, the proximity of both protein termini may cause the C-terminal tags to sterically interfere with the formation of a tight $\alpha$-helical bundle. Second, OspC and Vsp1 tether mutants mislocalizing to

the periplasm were not prevented from folding and assembling into quaternary structures. Yet, wild type OspC molecules failed to rescue mutant mislocalized OspC molecules to the spirochetal surface. This might be due to sequestration of the wild type protein from the mutant isotype. In light of the other data, however, it is best explained by the failure of wild type lipoprotein dimer subunits to form proper intermolecular interfaces in the periplasm. Third, the *in vitro* studies of recombinant OspC proteins with various tether deletions demonstrated that the tether peptide did not significantly affect the thermal stability of the OspC fold. This suggests that tether peptides of surface lipoproteins such as OspC do not possess intrinsic fold-destabilizing properties, i.e., most likely require binding to a 'holding' chaperone to prevent premature folding in the periplasm and thereby exclusion from the bacterial surface.

Future studies will continue to define sorting determinants for other mono- and multimeric lipoproteins targeted to different subcellular compartments, test the involvement of the Lol machinery in the secretion of surface lipoproteins, and aim to identify additional lipoprotein secretion pathway components, including the proposed OM lipoprotein flippase complex. Together, these studies will continually refine our working model of how *B. burgdorferi* targets its most important class of virulence factors to the host-pathogen interface.

**Table 4.1. Phenotypes of OspC-Vsp1 salt bridge charge swap point mutations**

| Protein | Point Mutations | Phenotype |
|---|---|---|
| OspC | | |
| | E61K | Dimer |
| | E61K/E90K/H93K | Null |
| | E61K/E90K/H93K/E148K | Null |
| | E61K/K111A | Dimer |
| | E61K/E90K/H93K/E148K/K111A | Null |
| | E90K/H93K/E148K | Dimer |
| Vsp1 | | |
| | D60K | Dimer |
| | D60K/D87K | Dimer |
| | D60K/D87K/D150K | Monomer |
| | D60K/D87K/D150K/E191K | Null |


A
+1
N-terminal peptide sequence
17 18 19 20 21 22 23 24 25 26 27 28
β
OspAwt
C K Q N V S S L D E K N S V S V D
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
α
OspCwt
C N N S G K D G N T S A N S A D E S V K G P N L T E I S
OspCΔ20-30
OspCΔ20-22
OspCΔ23-25
OspCΔ31-41
OspCΔ33-41
OspCΔ34-41
OspCΔ21-22
OspCΔN21
OspCΔS22
OspC (AA) 21
OspC (GG) 21
Δ
Δ
A A
G G
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
α
Vsp1wt
C N N S G T S P K D G Q A A K S D G T V I D L A T I
Vsp1Δ20-32
Vsp1Δ33-39
Vsp1Δ23-28
Vsp1Δ20-25
A Vsp1Δ20-25/P26A
Vsp1Δ20-24
Vsp1Δ21-25
Vsp1Δ20-23
Vsp1Δ22-25
Vsp1Δ20-22
Vsp1Δ23-25
Vsp1Δ21-23
Vsp1Δ22-24
Vsp1Δ22-23

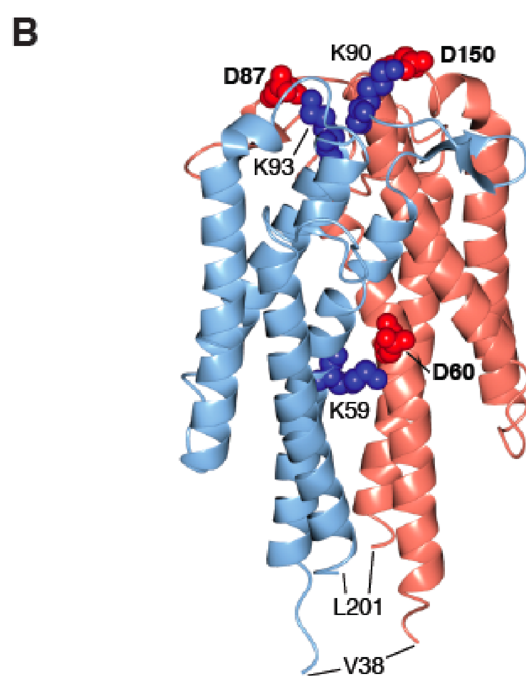

B
D87
K90
D150
K93
D60
K59
L201
V38

**Fig. 4.1. Genotypes and phenotypes of OspC and Vsp1 mutants.**

**(A)** N-terminal sequences of mature lipoproteins OspA, OspC and Vsp1 are shown in single letter amino acid code. The '+1' position Cys residue is marked with an arrowhead. Numbers above the residues indicate their position in the pro-lipoprotein including the cleaved signal peptide. Greek letters above boxed residues indicate secondary structure elements as determined by X-Ray crystallography (Eicken et al., 2001; Kumaran et al., 2001; Lawson et al., 2006; Li et al., 1997). Red lines with boxed ends underline the minimum tether sequences required for surface localization of mRFPΔ4. Lines flanked by inverted arrowheads span the peptides deleted in the respective tether mutants. Black lines/bold letters mark mutants with non-wild type phenotypes. Gray lines/regular letters mark mutants with a wild type phenotype. Boxed shaded in light blue indicate the essential tether 'motifs' of OspA, OspC and Vsp1. **(B)** A ribbon representation of the Vsp1 tertiary structure (PDB ID 2GA0;(Lawson et al., 2006)) was generated using the CCP4 software for Macintosh (version 2.4.3; (Potterton et al., 2002)). The two Vsp1 chains are colored light blue and pink, respectively. Residues involved in salt bridging of the monomers are highlighted as red (Asp) or blue (Lys) spheres representing the Cα and side chain atoms. The bolded residues were mutated to yield the Vsp1 monomer. $Val^{38}$ and $Leu^{201}$ are the first and last residues visible in the crystal structure.

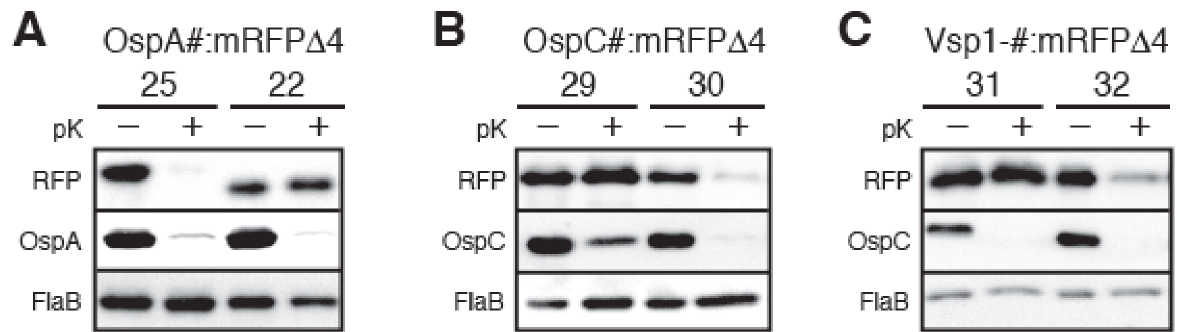



**Fig. 4.2. Minimal OspA, OspC and Vsp1 tether sequence requirements for surface display of the mRFPΔ4 reporter.** Proteinase K (pK) accessibility immunoblots of OspA **(A)**, OspC **(B)** and Vsp1 **(C)** tether fusions to mRFPΔ4 compared with $OspC_{wt}$. FlaB is used as a periplasmic, protease-resistant control. OspA and OspC are used as surface, protease sensitive controls.

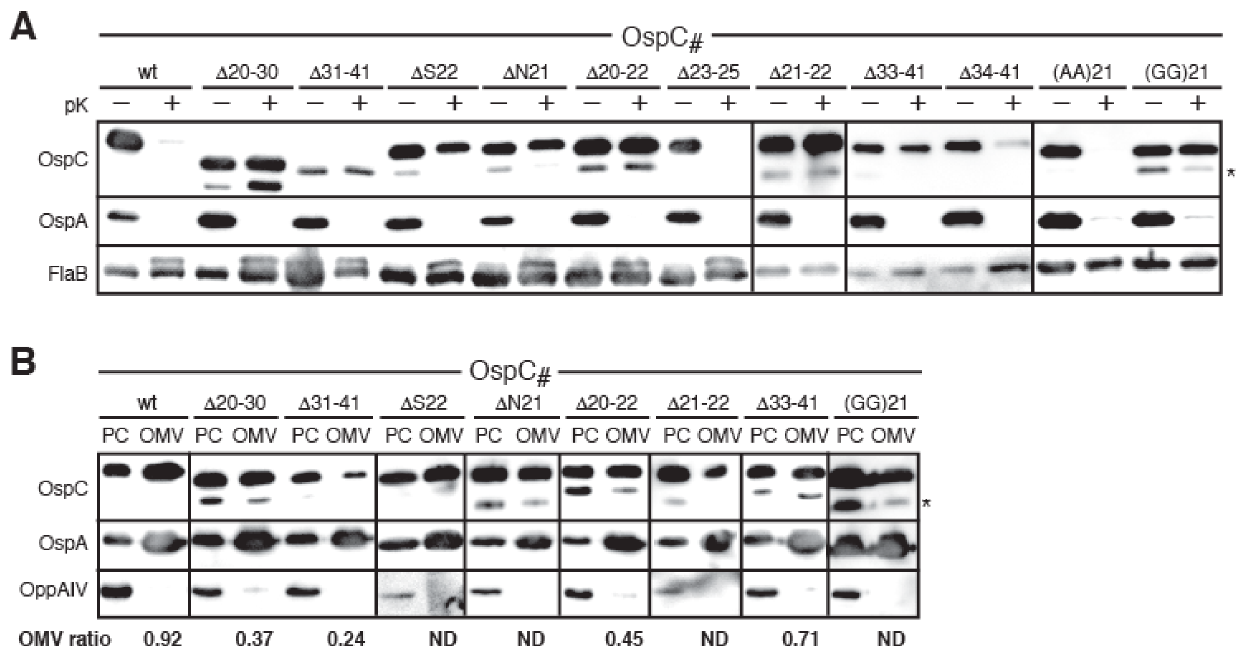



**Fig. 4.3. Localization of OspC mutants.**

**(A)** Proteinase K (pK) accessibility immunoblots of OspC tether mutants compared with $OspA_{wt}$. FlaB was used as a periplasmic, protease-resistant control. **(B)** Membrane fractionation immunoblots of proteinase K-resistant, i.e., periplasmic OspC tether mutants compared with $OspA_{wt}$. OppAIV served as IM control. OMV, outer membrane vesicle fraction; PC, protoplasmic cylinder fraction (also containing intact cells; (Schulze and Zückert, 2006; Skare et al., 1995)). The OMV ratio was calculated from densitometry data that were normalized to both OspA and OppAIV as described (Kumru et al., 2010). An asterisk (*) in both panels indicates an OspC degradation product dependent on the periplasmic CtpA protease (Östberg et al., 2004, Chapter V); cleavage of OspC by CtpA is stimulated by periplasmic retention of the substrate or by addition of a C-terminal epitope tag (see also Figs. 4.4, 4.6 and 4.7; Chapter V).

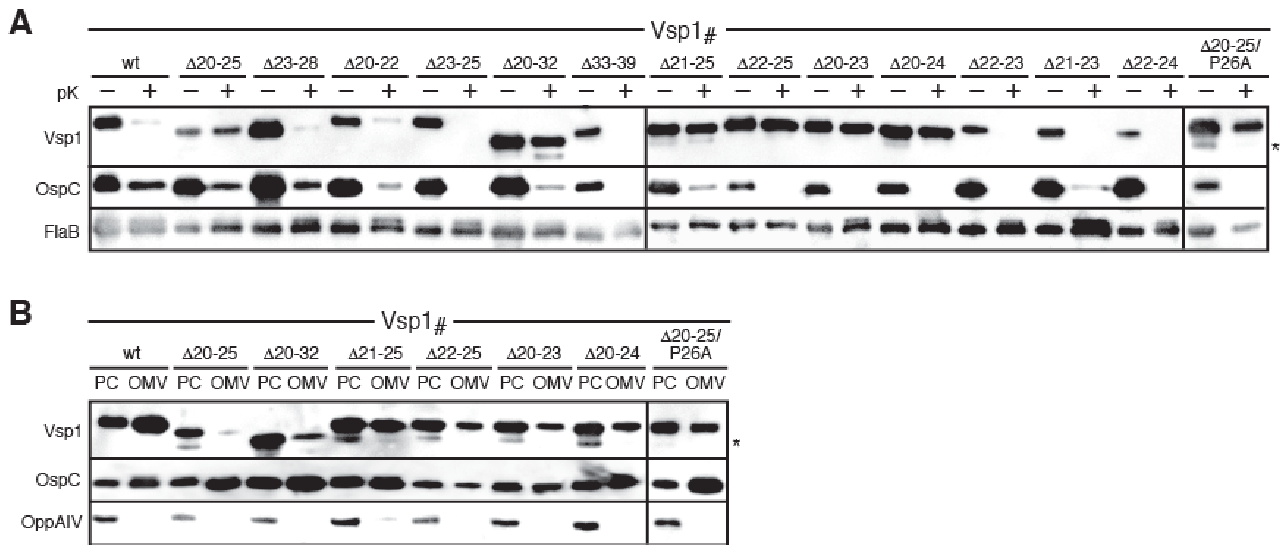



**Fig. 4.4. Localization of Vsp1 mutants.**

**(A)** Proteinase K (pK) accessibility immunoblots of Vsp1 tether mutants compared with $OspC_{wt}$. FlaB was used as a periplasmic, protease-resistant control. **(B)** Membrane fractionation immunoblots of proteinase K-resistant, i.e., periplasmic Vsp1 tether mutants compared with $OspC_{wt}$. OppAIV served as IM control. OMV, outer membrane vesicle fraction; PC, protoplasmic cylinder fraction (also containing intact cells; (Schulze and Zückert, 2006; Skare et al., 1995). An asterisk (*) in both panels indicates a Vsp1 degradation product dependent on the periplasmic CtpA protease (Chapter V; (Östberg et al., 2004))

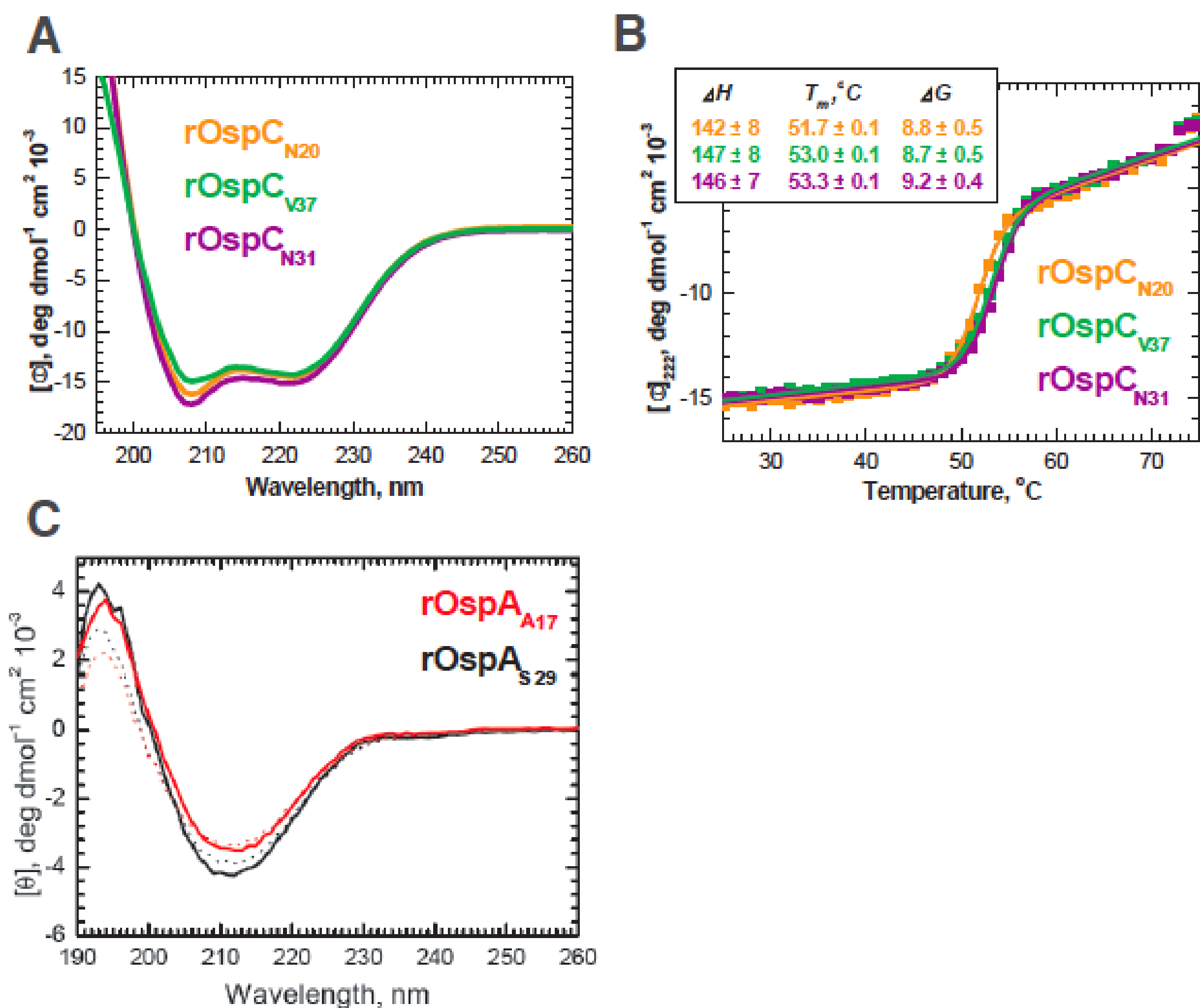
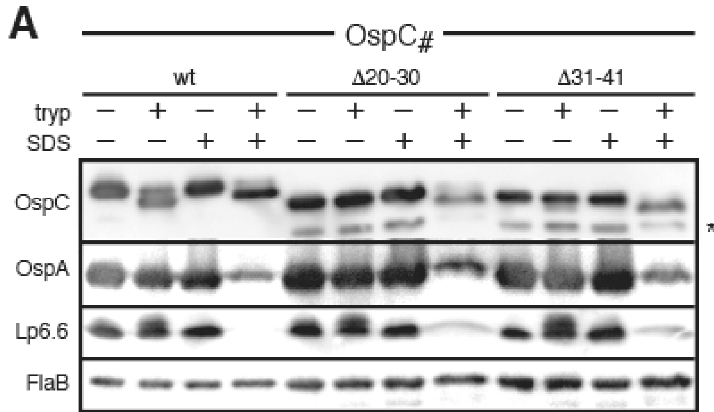
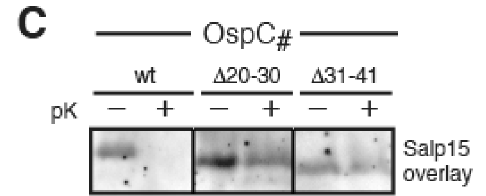
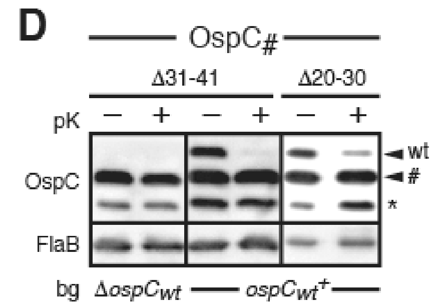
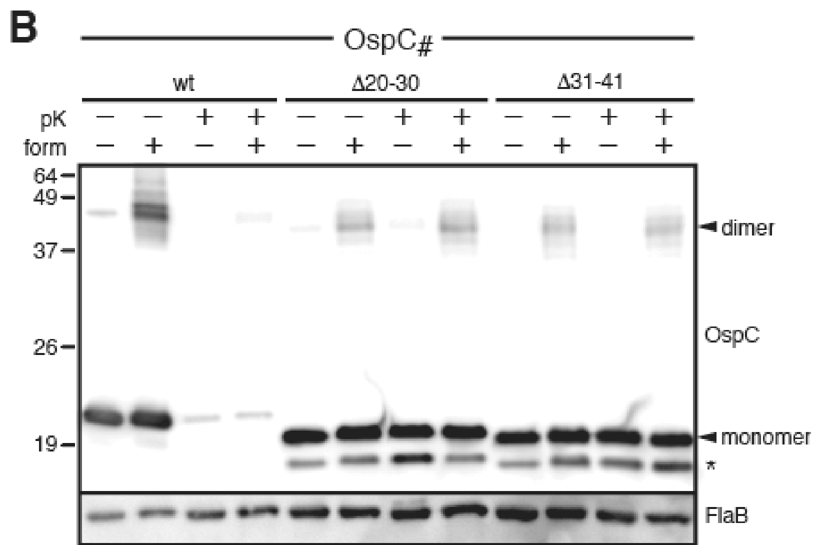



**Fig. 4.5. Circular dichroism and thermal denaturation data for recombinant OspC and OspA tether deletion mutants.** Circular dichroism spectra of recombinant **(A)** OspC and **(C)** OspA proteins. Per-residue ellipticity [Θ] plotted as a function of wavelength indicates that all mutants have similar structure dominated by an **(A)** α-helical or **(C)** β-sheet conformation. All spectra were obtained at 25º C in 10mM $NaPO_4$ buffer. Thermal unfolding of rOspC proteins determined as change in ellipticity at 222 nm with solid lines represented fitting to a two-state transition model. **(B)** The values for the transition enthalpy ΔH and the free energy of the native state ΔG (both in kcal/mol) are shown in the insert, along with the transition temperature.


A
OspC#
wt
Δ20-30
Δ31-41
tryp
SDS
OspC
OspA
Lp6.6
FlaB
*


C
OspC#
wt
Δ20-30
Δ31-41
pK
Salp15
overlay


D
OspC#
Δ31-41
Δ20-30
pK
wt
#
OspC
*
FlaB
bg
ΔospCwt
ospCwt+


B
OspC#
wt
Δ20-30
Δ31-41
pK
form
64
49
37
26
19
dimer
OspC
monomer
*
FlaB

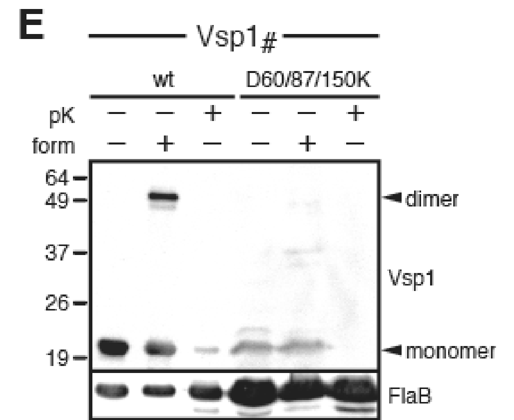

E
Vsp1#
wt
D60/87/150K
pK
form
64
49
37
26
19
dimer
Vsp1
monomer
FlaB

**Figure 4.6. Structural and functional analysis of select OspC and Vsp1 mutants.** **(A)** Trypsin (tryp) resistance immunoblots of periplasmic OspC tether mutants compared to $OspC_{wt}$, Surface $OspA_{wt}$ and periplasmic lipoprotein Lp6.6 were used as OM lipoprotein controls. FlaB was used as a loading control. 0.1% SDS was used gain access of trypsin to the periplasm. Note that OspA becomes more susceptible to trypsin in the presence of detergent. **(B)** Dimerization and proteinase K (pK) accessibility immunoblots of periplasmic OspC tether mutant compared to $OspC_{wt}$. Formaldehyde (form) crosslinking was used to stabilize OspC dimers (Bunikis and Barbour, 1999). FlaB was used as both periplasmic, protease-resistant and loading control. **(C)** Far-Western overlay blot of periplasmic OspC tether mutants with recombinant Salp15 tick salivary gland protein. Whole cell lysates of an $OspC_{wt}$-expressing *B. burgdorferi* strain obtained before and after proteolytic shaving with proteinase K (pK) were used as control. **(D)** Proteinase K (pK) accessibility immunoblots of OspC tether mutants ectopically expressed in an $OspC_{wt}$-deficient (B31-A3 *ospC::kan*; Δ$ospC_{wt}$) or $OspC_{wt}$-expressing (B313; $ospC_{wt}^{+}$) backgrounds (bg). Note that there is no reduction in the mutant OspC protein band marked by a pound sign (#) upon protease treatment, independent of background. FlaB served as a periplasmic, protease-resistant and loading control. **(E)** Dimerization and proteinase K (pK) accessibility immunoblots of the Vsp1 triple salt bridge mutant compared to $Vsp1_{wt}$. Formaldehyde (form) crosslinking was used to stabilize any existing Vsp1 dimers (Bunikis and Barbour, 1999). FlaB was used as both periplasmic, protease-resistant and loading control. Note that the mutant Vsp1 samples had to be overloaded to sufficiently visualize the Vsp1 monomer. An asterisk (*) in panels A, B and D indicates an OspC degradation product dependent on the periplasmic *B. burgdorferi* CtpA protease (Chapter V (Östberg et al., 2004))

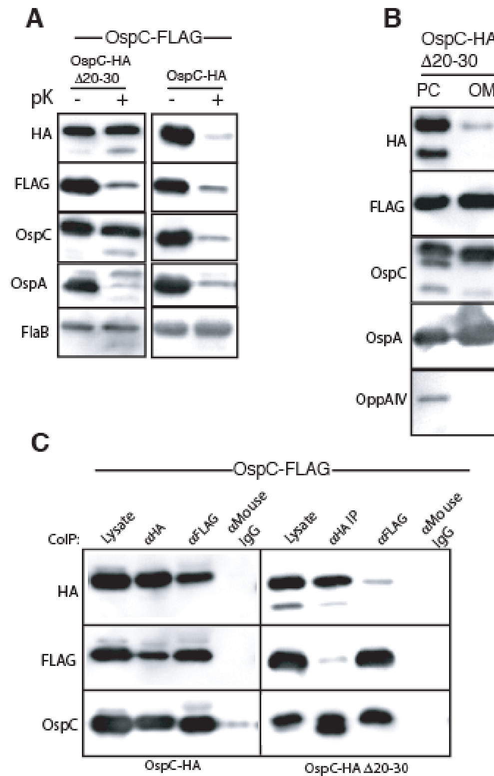



**Figure 4.7. Additional evidence OspC transverses the OM as a monomeric intermediate.** (**A)** Proteinase K (pK) accessibility immunoblots of B31-A3 *ospC::kanR* cells that express OspC$_{\Delta 20\text{-}30\text{-HA}}$ and OspC-FLAG indicate OspC-HA and OspC-FLAG, but not OspC$_{\Delta 20\text{-}30\text{-HA}}$ are surface exposed. FlaB was used as a periplasmic, protease-resistant control and OspA was used as a protease sensitive control. (**B)** Membrane fractionation immunoblots of proteinase K-resistant, i.e., periplasmic OspC$_{\Delta 20\text{-}30\text{-HA}}$ compared with OspA$_{wt}$. OppAIV served as IM control. OMV, outer membrane vesicle fraction; PC, protoplasmic cylinder fraction (also containing intact cells; (Schulze and Zückert, 2006; Skare et al., 1995)). Note the artifact introduced by the HA tag (see Fig 4.3 for OspC$_{\Delta 20\text{-}30}$) **(C)** Co-immunoprecipitation indicates heterodimerization of OspC occurs only when OspC-FLAG and OspC-HA are localized to the same cellular compartment. All immunoprecipitation were repeated using anti-mouse IgG as a negative control.

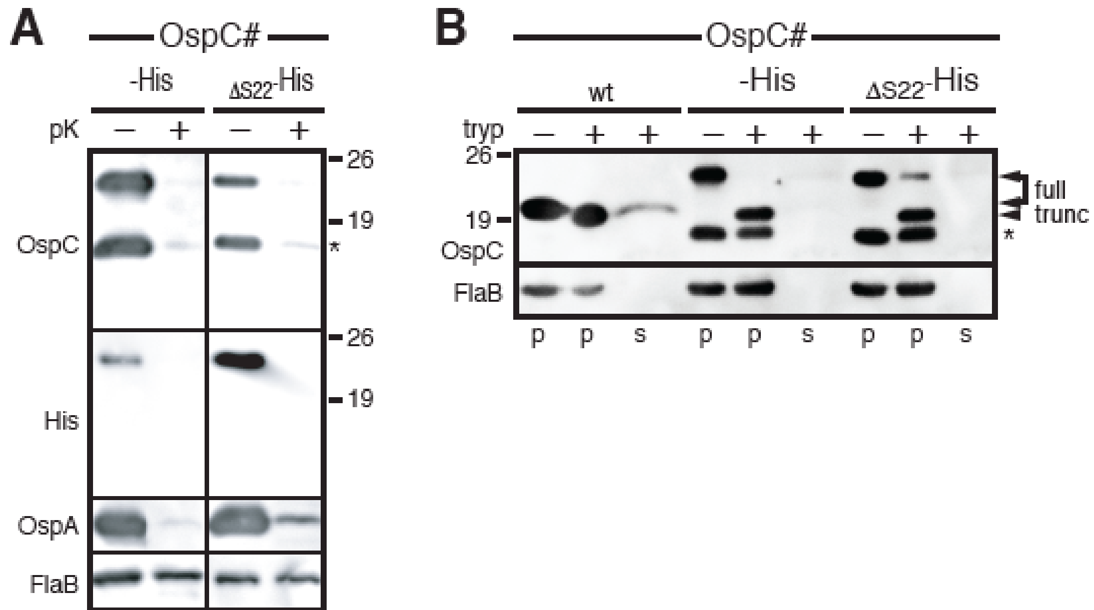



**Figure 4.8. Structural analysis of epitope-tagged OspC mutants. (A)** Proteinase K (pK) accessibility immunoblots of C-terminally histidine-tagged OspC tether mutant $OspC_{\Delta S22}$ compared with to a histidine-tagged $OspC_{wt}$. OspA was used as a surface control, and FlaB was used as a periplasmic, protease-resistant and loading control. A HisProbe-HRP ($Ni^{2+}$-HRP) conjugate was used to confirm the full-length protein band. **(B)** Trypsin (tryp) resistance immunoblots of C-terminally histidine-tagged OspC tether mutant $OspC_{\Delta S22}$ compared with to a histidine-tagged $OspC_{wt}$ and untagged $OspC_{wt}$. FlaB was used as a loading control. Arrowheads mark the bands corresponding to full-length proteins (full) and trypsin-resistant core proteins (trunc) released from the cell into the reaction supernatant (Zückert et al., 2001). An asterisk (*) in both panels indicates an OspC degradation product dependent on the periplasmic CtpA protease (Chapter V (Östberg et al., 2004)).

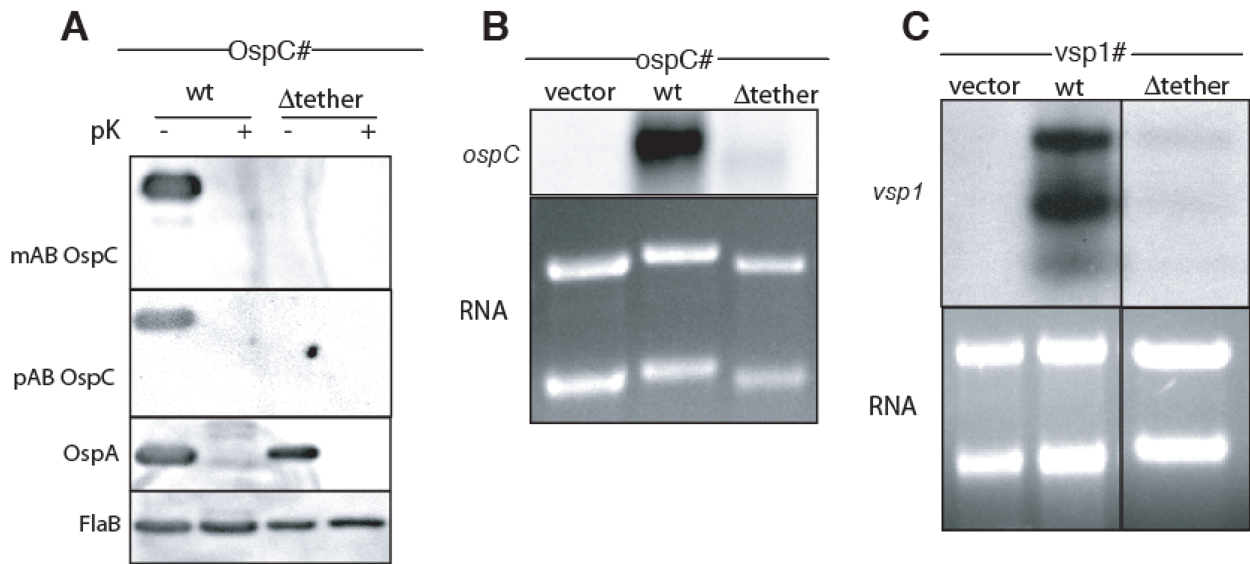



**Figure 4.9. Phenotypes of tetherless OspC and Vsp1 mutants.**

**(A)** Western immunoblots using monoclonal and polyclonal OspC antibodies indicate no OspC is synthesized. Northern blots using DNA probes complementary to **(B)** *ospC* and **(C)** *vsp1* coding regions indicate no *ospC or vsp1* mRNA is present. Total RNA from cells harboring wild type plasmids and empty vectors were used as positive and negative controls, respectively. RNA gels stained with EtBr are shown as a loading control.

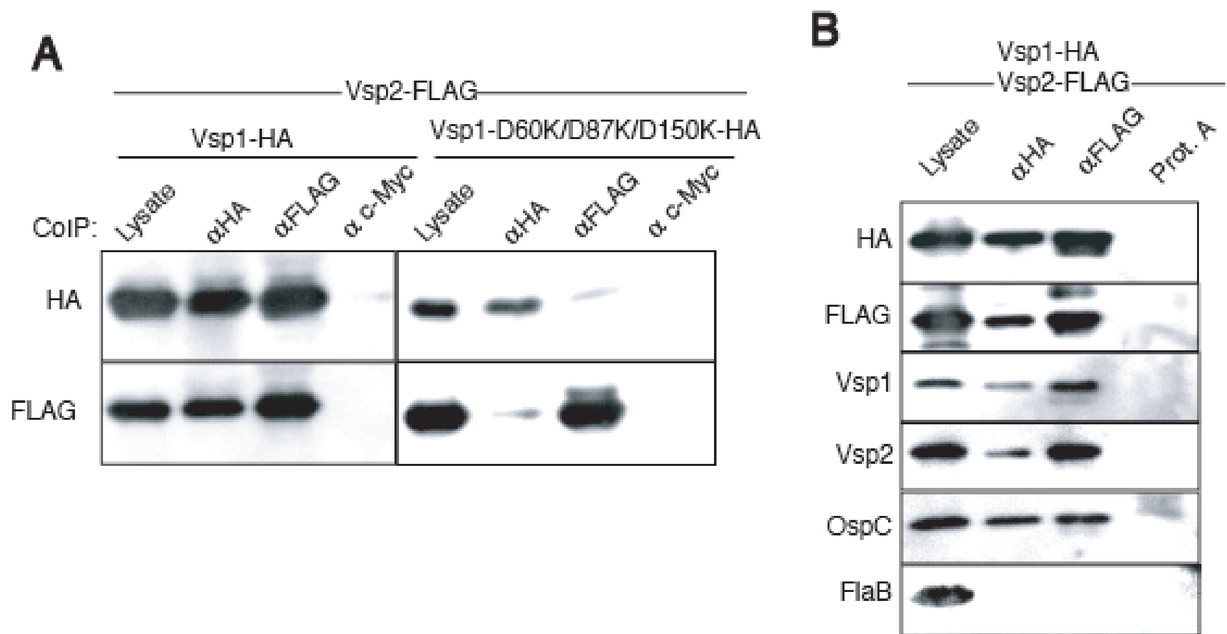



**Figure 4.10. Heterodimerization of Vsp1 and Vsp2.**

**(A)** Co-immunoprecipitation of Vsp1-HA/Vsp2-FLAG expressing cells and $Vsp1_{D60K/D87K/D150K\text{-}HA}$/Vsp2-FLAG expressing cells using anti-HA and anti-FLAG tag antibodies. Introduction of Vsp1 salt bridge mutations in Vsp1-HA abrogates heterodimerization with Vsp2. Co-immunoprecipitations were repeated using anti-c-Myc antibodies as a negative control. **(B)** An independent experiment as in **(A)** but, probing the immunopercipitate for HA, FLAG, Vsp1, Vsp2, OspC, and FlaB antibodies. Protein A alone was used as a negative control. For details of the different antibodies used see Table 2.2.

# Chapter V.

# Evidence for an Expanded Role of *Borrelia burgdorferi* CtpA Protease in Envelope Biogenesis

**Abstract**

Proteolytic processing of secreted proteins commonly occurs at the N-terminus, where signal peptides are removed after Sec-dependent translocation through the cytoplasmic membrane. C-terminal proteolytic processing, e.g., by the Sortase system in gram-positive bacteria also occurs, but overall is poorly understood. Recently, an unusual serine-like periplasmic protease, CtpA, was discovered in *Borrelia burgdorferi*, the spirochetal agent of Lyme disease. Inactivation of *ctpA* in *B. burgdorferi* did not affect growth *in vitro*, but had a pleiotropic processing defect. Known substrates of CtpA include the 13-kDa outer membrane porin P13 and a periplasmic lipoprotein that contains a LysM domain, which presumably functions to bind peptidoglycan to maintain integrity of the envelope. While investigating lipoprotein sorting rules and analyzing several mutants of *B. burgdorferi* outer surface lipoprotein C (OspC), we discovered a lower molecular weight variant of OspC. Ectopic expression of OspC with a C-terminal histidine tag in a *ctpA* knockout strain showed that CtpA is responsible for C-terminal cleavage of OspC. C-terminal processing in *ctpA*$^{+}$ and *ctpA*$^{-}$ strains was about 50% and <1%, respectively. Interestingly, a higher molecular weight variant of OspC was detected in the *ctpA*$^{-}$ strain that could be a result of incomplete removal of the signal peptide at the N-terminus. Taken together, these results suggest that CtpA can also function as a periplasmic housekeeping protease.

## Introduction

Proteolytic processing performs essential physiological functions in all bacteria. N-terminal proteolysis of Sec substrates via signal peptidases is the most common and well characterized example (Manting and Driessen, 2000). Overall C-terminal processing is rare in gram-negative bacteria and limited information is available concerning this phenomenon. However, C-terminal processing commonly occurs in gram-positive bacteria via the Sortase system. The sortase system functions to attach proteins to the cell surface after cleavage of the C-terminal sorting signal (LPXTG). The Sortase substrate is then anchored to the peptidoglycan layer via transpeptidation (Cossart, 2000).

As part of our analysis of the requirements for proper surface display of OspC, we introduced mutations within the N-terminal OspC tether peptide that led to mislocalization of the lipoprotein to the periplasm. A common trait of the periplasmic OspC (and Vsp1) mutants was the presence of an additional protein band (labeled OspC*) that reacted with an OspC mouse monoclonal antibody (MAb) (Mbow et al., 1999), but at about 19-kDa had a lower apparent molecular mass than the 21-kDa wild type OspC (Chapter IV, Figs 4.3, 4.4). Expression of a C-terminally hexahistidine-tagged OspC (OspC-His) also yielded an OspC* band, which lacked reactivity with a HisProbe $Ni^{2+}$-horse radish peroxidase conjugate (Chapter IV, Fig 4.8). We therefore concluded that OspC* resulted from C-terminal cleavage (see Chapter IV, Fig 4.3).

An obvious candidate for mediating this process was CtpA, a recently identified *B. burgdorferi* serine-like C-terminal protease involved in processing of both outer membrane porins such as P13 or periplasmic lipoproteins such as BB0323 (Stewart et al., 2004; Noppa et al., 2001; Östberg et al., 2004). Homologues of CtpA are mostly found in cyanobacteria, but are also found in pathogenic bacteria (Mitchell and Minnick, 1997; Ivleva et al., 2000; Bandara et

al., 2005; Bandara et al., 2008; Hoge et al., 2010). 2D gel analysis revealed potential substrates for CtpA, although the identity of some of these substrates remains unclear (Östberg et al., 2004). We therefore decided to investigate CtpA's potential role in OspC processing.

## Results

### C-terminal processing of OspC is stimulated by addition of a C-terminal linker.

We first set out to further define the substrate requirements for OspC C-terminal processing and modified the recombinant OspC expression plasmid pOSK200 (Table 2.3) by oligonucleotide-mediated site directed mutagenesis yielding recombinant plasmids pOSK360 and pOSK277. pOSK360 encoded for OspC-link, an OspC tagged with a C-terminal PGGSGA linker peptide also present in OspC-His (Fig. 5.1). pOSK277 encoded for OspC-L2his, an OspC with a His tag inserted between residues $Lys^{116}$ and $Asn^{117}$ in loop 2 (Table 2.3, Fig.5.1, ref. (Kumaran et al., 2001). Using established protocols (Samuels, 1995; Stewart et al., 2001), we transformed B31-A3 (Δ*ospC*), an $OspC_{wt}$-deficient background strain (Table 2.1) with both pOSK360 and pOS307. The results showed that addition of the 6-amino-acid linker peptide alone in OspC-link was sufficient to stimulate cleavage and resulted in the OspC* band. Conversely, OspC-L2His did not yield an OspC* band i.e., was not a substrate for processing (Fig. 5.1 and Fig 5.2A). This showed that C-terminal processing of OspC was specifically stimulated by the addition of unordered peptides to the lipoprotein's C-terminus. The intensity of the OspC and OspC* bands were consistently similar. Therefore, we reasoned processing efficiency was about 50%. As additional controls, we also fused a FLAG tag and full-length mRFP1 to the C-terminus and introduced them into B31-A3 (Δ*ospC*). As expected, both the FLAG tag and mRFP1 were cleaved from the C-terminus at an efficiency of about 50% (data not shown).

**CtpA is responsible for C-terminal proteolysis of OspC.**

To probe CtpA's involvement in processing of OspC, we transformed the *ctpA* knockout mutant B31-A (Δ*ctpA*), its complemented derivative B31-A(Δ*ctpA*)/pBSV2G+*ctpA* (ref. (Östberg et al., 2004) ; gifts from S. Bergström), as well as its parent strain B31-A (ref.(Bono et al., 2000); a gift from P.A. Rosa) with pOSK307 encoding OspC-His (Fig. 5.1, Table 2.3). In Western immunoblot assays, the OspC* band was not detectable in the *ctpA* knockout strain, but present in the CtpA-expressing B31-A background as well as the complemented *ctpA* deletion strain (Fig. 5.2B), which confirmed CtpA's involvement in this process.

We wished to confirm OspC with an unmodified C-terminus was a suitable substrate for CtpA. When we overloaded immunoblots of cells expressing wild type OspC we detected OspC*, albeit at significantly lower levels (Fig. 5.2A). Since expression of wild type OspC is low in B31-A (Δ*ctpA*), we transformed this strain with a plasmid that expresses OspC from the constitutive *flaB* promoter (pOSK200, Table 2.3), which greatly increased OspC expression. As expected, OspC* was not detected in the *ctpA* knockout strain that expressed wtOspC (Fig. 5.2C). This confirmed wild type surface OspC is a CtpA substrate, albeit at a relatively low incidence.

**CtpA-dependent proteolysis of OspC occurs at or around $Ala^{204}$.**

To map the extent of the C-terminal OspC peptide removed by CtpA, we truncated OspC beyond the C-terminal α-helix (Kumaran et al., 2001) by replacing $Ala^{204}$ with a stop codon by site directed mutagenesis of pOSK200 as described above (Tables 2.3, Fig. 5.1). The $OspC_{A204X}$ and OspC* proteins co-migrated on SDS-PAGE, as detected by Western immunoblotting with the

OspC MAb (Fig. 2A). Thus we concluded that CtpA removed OspC's disordered C-terminal residues, cleaving at or close to $Ala^{204}$. Such a proteolytic site would be similar, if not identical to the site identified in P13, where CtpA cleaves after an Ala residue (Noppa et al., 2001). We also observed a minor band derived from wild type OspC that co-migrated with OspC*, suggesting CtpA dependent proteolysis of wild type OspC occurs at the same residue as OspC-His and OspC-link (Fig. 2A).

**CtpA inactivation results in detection of a higher molecular weight species of OspC.** In addition to the OspC* and full-length OspC-His protein bands, three higher molecular weight variants with molecular masses around 26 kDa (labeled $OspC^{‡}$) were prominent in immunoblots of the *ctpA* knockout strain with the anti-OspC MAb (Fig. 5.2B). Only the largest $OspC^{‡}$ band reacted with an anti-His MAb (HIS-1, Sigma), suggesting that the variants originated from both $OspC_{wt}$ and OspC-His expressed by the B31-A derivatives (Fig. 5.2B). Triton X-114 partitioning and proteolytic shaving assays indicated that both OspC* and $OspC^{‡}$ protein species were associated with the membrane (Fig. 5.3A) and were surface exposed (Fig. 5.3B).

**Discussion**

This study shows that *B. burgdorferi* CtpA is responsible for the lower molecular weight variants observed when OspC is modified with a C-terminal epitope tag or mislocalized to the periplasm. Interestingly, OspC-His expressed in a *ctpA* knockout strain resulted in a higher molecular weight variant of OspC at about 26 kDa. Östberg and colleagues had previously observed higher molecular mass variants of P13 in the *ctpA* knockout and speculated that they may result from the incomplete removal of its N-terminal type I signal peptide by signal peptidase I (Östberg et

al., 2004). Analogously, treatment of *Borrelia* cells with the signal peptidase II inhibitor globomycin was shown to yield higher molecular mass lipoprotein species (Carter et al., 1994). Therefore, incomplete N-terminal OspC processing may best explain the OspC$^{‡}$ variants. Yet, it remains perplexing that such a defect would not impact the variants' surface localization. It is conceivable that (i) the partial acylation of the N-terminal cysteine, which generally occurs before signal peptide cleavage (Fig 1.1 and 1.6), is sufficient for membrane anchoring, and that (ii) the accumulating non-mature lipoproteins eventually overwhelm secretion substrate checkpoints and ultimately reach the bacterial surface via the standard borrelial lipoprotein transport pathway. Steady-state analyses as the ones performed here would be unlikely to detect the expected differences in secretion efficiencies.

Our observation of CtpA-mediated cleavage of the surface lipoprotein OspC raises some intriguing questions regarding the protease's functional range. Processing of wild type OspC was detectable, but was significantly stimulated by disordered C-terminal extensions described here or by mislocalization of OspC and the related *B. turicatae* Vsp1 to the periplasm described in Chapter IV. CtpA may therefore act as a promiscuous housekeeping protease that removes structurally unconstrained C-termini of proteins in the periplasm. In that case, OspC may be a mere target of opportunity that becomes particularly attractive if its escape from the periplasm is hindered. However, the observed low-frequency C-terminal cleavage of wild type OspC may also represent evidence for a quality control mechanism that senses proper translocation of surface lipoproteins by detecting the release of C-terminal peptides. Disruption of such a mechanism may elicit a bacterial envelope stress response, which in part could be responsible for the secondary effects of *ctpA* deletion observed by Östberg et al. (Östberg et al., 2004).

We previously did not notice any C-terminal processing of *B. burgdorferi* OspA periplasmic mutants (Schulze and Zückert, 2006; Schulze et al., 2010). This simply might be due to the confinement of OspA's C-terminus in α-helical secondary structure (Li et al., 1997) or point to a more limited checkpoint role for CtpA. The role CtpA plays in the infectious process is still unknown. Interestingly, inactivation of *ctpA* in *Burkholderia mallei* and *Brucella suis* significantly attenuates the bacteria in macrophages and mice, respectively (Bandara et al., 2005; Bandara et al., 2008). Given the number of potential substrates observed via 2D gel analysis (Östberg et al., 2004) and the identification of OspC as a substrate, full or partial attenuation of *B. burgdorferi* in the mouse model by inactivation of *ctpA* remains a possibility. If CtpA plays a role in activation of an envelope stress response, then gene inactivation of *ctpA* could lead to OM permeabilization and/or other envelope defects during the course of mammalian infection. Thus, making clearance by the immune system a far simpler task. Based on these findings, we advocate additional studies defining the proteolytic specificities and precise biological role(s) of CtpA in envelope biogenesis and host-pathogen interactions of this globally important spirochetal pathogen.

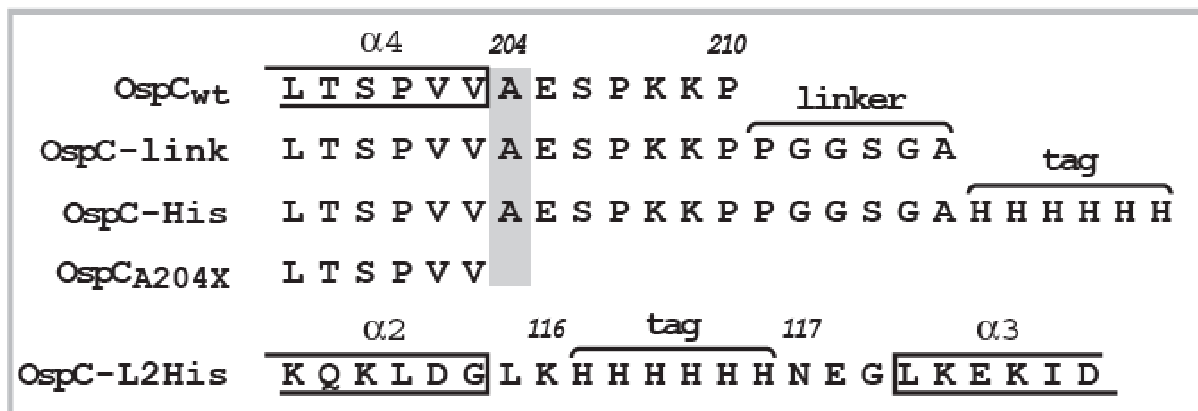



**Figure 5.1. Partial peptide sequences of wild type and mutant OspC proteins.**

The residues confined to the α-helices of OspC are boxed and numbered. Residue numbers of the signal peptide-containing pro-OspC lipoprotein are shown above the sequences. Labeled brackets mark the linker and epitope tag sequences. A gray box indicates the predicted CtpA cleavage site; Note that the C-terminus of P13 is cleaved after an Ala residue (ref. (Noppa et al., 2001) also see text).

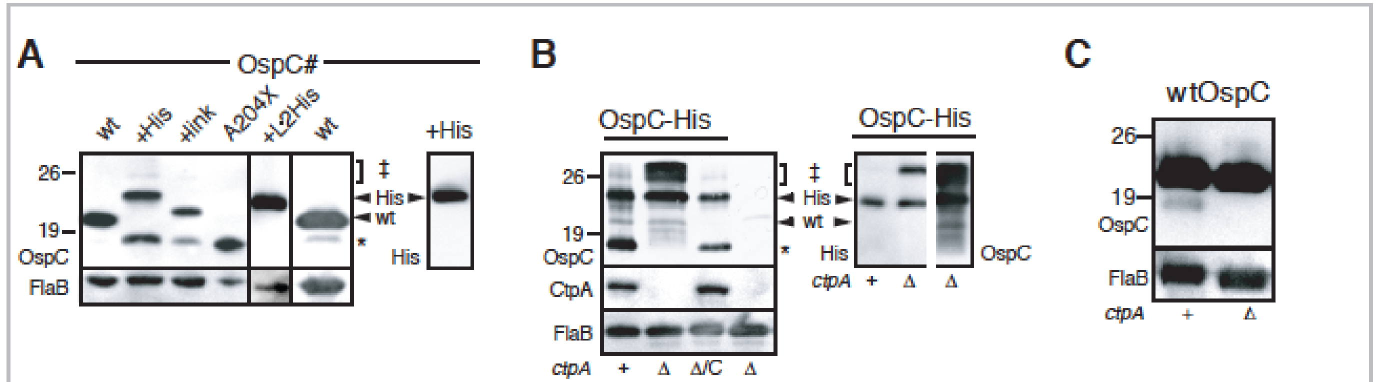



**Figure 5.2. CtpA-dependent proteolytic processing of OspC.**

Western immunoblots of whole cell protein preparations obtained from cells expressing wild type (wt) or mutant OspC proteins in CtpA-expressing or (–) deficient strain backgrounds. Positions of molecular mass markers (in kilodaltons) are indicated to the left of each panel. A bracket denotes the three higher molecular weight OspC$^{‡}$ bands; arrowheads point to the $OspC_{wt}$ or OspC-His protein bands; an asterisks marks the OspC* band. **(A)** $OspC_{wt}$ and all OspC mutants are expressed in the B31-A3 (Δ*ospC*) background (Table 2.2). Left panel: The right-most lane contains an overloaded $OspC_{wt}$ sample to visualize the $OspC_{wt}$-derived OspC* band. FlaB was used as a loading control. Right panel: The HisProbe $Ni^{2+}$-HRP conjugate (Pierce) was used to confirm the mobility of the OspC-His band. **(B)** Strain backgrounds are indicated by *ctpA* phenotype below the panel as follows: +, B31-A; Δ, B31-A (Δ*ctpA*); Δ/C, B31-A (Δ*ctpA*)/pBSV2G+*ctpA* (Table 2.2). Left panel: CtpA was detected by a polyclonal rabbit antibody (Östberg et al., 2004). FlaB served as a loading control. Right panel: Whole cell protein preparations separated SDS-PAGE in adjacent lanes, blotted and probed with His and OspC antibodies. Note that anti-His only reacts with the uppermost OspC$^{‡}$ band. Other labels are identical to Fig. 5.2A. **(C)** OspC* is detected in *ctpA*$^{+}$ strains expressing wtOspC. OspC* is dependent on CtpA and independent of C-terminal modification.

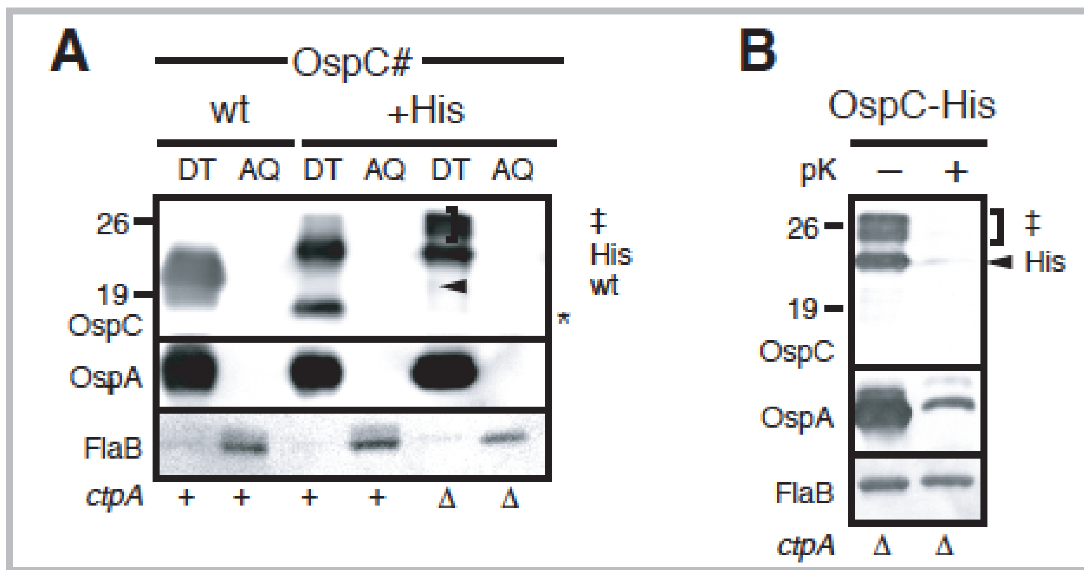



**Figure 5.3. Localization of CtpA-dependent OspC variants.**

Western immunoblots of protein preparations obtained from cells expressing wild type (wt) or OspC-His proteins in CtpA-expressing or deficient strain backgrounds. Positions of molecular mass markers (in kilodaltons) are indicated to the left of each panel. A bracket denotes the three higher molecular weight OspC$^{‡}$ bands; arrowheads point to the OspC$_{wt}$ or OspC-His protein bands; an asterisks marks the OspC* band. Strain backgrounds are indicated by *ctpA* phenotype below the panel as follows: +, B31-A3 (Δ*ospC*); Δ, B31-A (Δ*ctpA*). **(A)** Triton X-114 detergent (DT) and aqueous (AQ) protein fractions were probed. OspA and FlaB were used as membrane-associated and soluble controls, respectively. **(B)** Protease-accessibility assay of the OspC-His derived variant protein bands produced in a CtpA-deficient background. – pK, untreated cells; + pK, cells treated with 200 μg ml$^{-1}$ proteinase K. OspA and FlaB were used as surface and subsurface controls, respectively.

# Chapter VI.

## Cross genus expression and localization of lipoproteins

**Abstract**

Bacterial lipoproteins are a distinct class of peripheral membrane proteins that serve a diverse number of functions. In *E. coli* lipoproteins usually function to maintain integrity of the cell envelope as the majority of lipoproteins are localized to the periplasmic face of the outer membrane. However, lipoproteins in the Lyme disease spirochete, *Borrelia burgdorferi,* are mostly localized to the bacterial surface and function as virulence factors. Lipoproteins are sorted through the LolCDE machinery, a dedicated lipoprotein ABC transporter. In *E. coli*, interaction with LolCDE is dependent on the residue immediately downstream of the tri-acyl modified cysteine and is known as the +2 rule. *B. burgdorferi* lipoproteins do not adhere to the +2 rule or any other established lipoprotein sorting rules. Recently, the question was posed if *Borrelia* lipoproteins could be correctly localized within the envelope independent of the *Borrelia* cell envelope. In this study we expressed *B. burgdorferi* OspA fused to mRFP1 in *E. coli* and expressed Braun's lipoprotein (Lpp) in *B. burgdorferi*. Localization of OspA:mRFP1 in *E. coli* was dependent on the +2 rule while Lpp expressed in *B. burgdorferi* was localized to the inner membrane. These results indicate that expression of a lipoprotein alone is not a sufficient mediator for proper localization. Furthermore, lipoproteins ectopically expressed in a non-native surrogate hosts adhere to the lipoprotein sorting rules of the host. Finally, this data argues against spontaneous membrane insertion as a mechanism for translocation of bacterial lipoproteins.

**Introduction**

Lipoprotein sorting rules have been well established in *E. coli* and other *Enterobacteriaceae*. Yet, we are only beginning to elucidate the mechanisms that govern lipoprotein sorting in *Borrelia burgdorferi*. Although, *B. burgdorferi* contains homologues to several proteins that make up the Lol machinery (LolACDE), it lacks an obvious LolB homologue that functions to insert lipoproteins in the outer membrane in other eubacteria.

The question was posed if expression of lipoproteins alone is sufficient to facilitate proper localization to their respective subcellular compartments. Perhaps *B. burgdorferi* lipoproteins are self-contained translocation machines that can spontaneously insert into and across the outer membrane? This possibility is unlikely due to the lack of a C-terminal translocator domain (β domain), like those found in autotransporters (Type V secretion system). Regardless, little experimental data is available to disprove this hypothesis. Localization of *B. burgdorferi* OspA in *E. coli* reveals that it is not surface exposed, but rather associated with the inner membrane (Dunn et al., 1990). To further answer these questions we used previously localized fusions of *B. burgdorferi* Outer Surface Protein A (OspA) to monomeric red fluorescent protein 1 (mRFP1). We used fusions that localized to the inner membrane and surface in *B. burgdorferi* and expressed them in *E. coli*. In a variation of this approach we also expressed and localized Braun's lipoprotein (Lpp) from *E. coli* (Braun and Rehn, 1969; Braun and Wolff, 1970) in *B. burgdorferi* and determined its subcellular localization.

## Results

### OspA:mRFP1 fusions that are differentially localized in *B. burgdorferi* follow the +2 rule in *E. coli.*

Previous studies using varying lengths of *B. burgdorferi* OspA fused with mRFP1 indicated OspA20:mRFP1 localized to the inner membrane, while OspA28:mRFP1 localized to the outer surface (Schulze and Zückert, 2006). We wished to determine if the same cellular localization pattern would be present if we expressed these fusions in *E. coli*. Therefore, we transformed TOP 10 (HB101) *E. coli* with *B. burgdorferi-E. coli* shuttle vectors expressing *ospA20:mRFP1*, *ospA28:mRFP1*, and *ospA28*$_{K19D}$*:mRFP1* from the same constitutive *flaB* promoter. After confirmation of red fluorescence (data not shown) we performed sucrose density gradient ultracentrifugation using a modified protocol (Chapter II and (Robichon et al., 2005)). As expected, these lipoprotein fusions followed the +2 rule as OspA28$_{K19D}$:mRFP1 fractionated with the inner membrane and the other two fusion proteins fractionated with the outer membrane (Fig 6.1, Table 6.1).

### Lpp expressed in *B. burgdorferi* is localized to the inner membrane

Using the inverse approach to the above, we wished to determine the cellular localization pattern of an *E. coli* lipoprotein using *B. burgdorferi* as a surrogate host. Therefore, we amplified the *lpp* gene from *E. coli* HB101 and placed it under the control the constitutive *flaB* promoter. A hexahistidine tag was placed at the C-terminus to facilitate detection. Preparation of whole cell lysates from *B. burgdorferi* strains B313 and B31-e2 (Table 2.2) cells expressing Lpp-His yielded a band of ~7.2 kDa that reacted with anti-His tag antibodies and a $Ni^{2+}$-HRP conjugate. Lpp was resistant to proteolysis via treatment of intact cells with proteinase K indicating Lpp is

not localized to the surface (Fig. 6.3A). Surprisingly, when we performed membrane fractionations we failed to detect any Lpp associated with the outer membrane vesicle fraction (Fig. 6.2B, Table 6.1). Triton X-114 phase partitioning revealed Lpp-His fractionated with the detergent phase, suggesting Lpp is properly lipidated and localized to the periplasm (Fig. 6.3C).

**Mutagenesis of Lpp does not influence inner membrane localization in *B. burgdorferi***

The peptidoglycan (murien) layer in *B. burgdorferi* is associated with the inner membrane, rather than the outer membrane as is in *E. coli* (Rosa et al., 2005; Motaleb et al., 2000). We hypothesized Lpp might be bound to peptidoglycan causing Lpp to be sequestered in the inner membrane before it has an opportunity to interact with LolCDE. To test this hypothesis we used site directed mutagenesis to delete the C-terminal lysine that covalently binds peptidoglycan and designated the mutant LppΔK58-His (Zhang and Wu, 1992; Zhang et al., 1992; Braun and Wolff, 1970; Choi et al., 1987). Proteolytic shaving and membrane fractionations revealed no difference between Lpp-His and LppΔK58-His (Fig 6.2, Fig. 6.3A and 6.3B center lanes).

We have previously noted the importance of the lipoprotein tether in proper surface/membrane localization (Schulze and Zückert, 2006; Schulze et al., 2010; Kumru et al., 2010). The N-terminus of Lpp has only three amino acids that are disordered (Shu et al., 2000) and could function as a tether in *B. burgdorferi*. To test the hypothesis that these residues influence Lpp localization, we used alanine mutagenesis and domain swapping. First, we replaced the $S_{22}S_{23}N_{24}$ tri-peptide with alanines and tested their surface and membrane localization (Fig 6.2). The phenotype of LppS22A/S23A/N24A-His was identical to that of the Lpp-His and the LppΔK58-His (Fig 6.3A and 6.3B right lanes). Next, we inserted the N-terminal KQNV tetrapeptide from OspA upstream of the SSN tripeptide of Lpp (Fig. 6.2). We

reasoned since this tetrapeptide (with the addition of the lipid modified Cys) was sufficient for surface localization of mRFP1 (Schulze and Zückert, 2006) that it might be sufficient for surface localization of Lpp-His. However, addition of this tetrapeptide to the N-terminus of Lpp, resulted in a null phenotype, i.e., no protein was detected by western blotting using the $Ni^{2+}$-HRP conjugate or anti-His tag antibodies. Additionally, the protein band corresponding to Lpp-His was not detected on coomassie blue stained SDS-PAGE gels (data not shown).

**Discussion**

Lipoprotein sorting rules are well established in *E. coli* and other enterobacteriaceae (Robichon et al., 2005; Narita and Tokuda, 2007; Lewenza et al., 2006). Yet, *B. burgdorferi* does not adhere to these sorting rules (Schulze and Zückert, 2006; Schulze et al., 2010; Kumru et al., 2010). The question has been raised if lipoproteins expressed in surrogate hosts follow the sorting rules of said host. This study shows expression of lipoproteins themselves is not sufficient to correctly localize them in surrogate hosts. Although the overall conclusions of this study are not surprising, the observation that Lpp expressed in *B. burgdorferi* localized to the inner membrane is intriguing. We were unsure that the *B. burgdorferi* lipid modification and signal peptidase II enzymes properly processed Lpp, but Triton X-114 phase partitioning clearly shows Lpp is associated with the detergent soluble fraction. This data, taken with the fact that the lipobox of *B. burgdorferi* is more relaxed than *E. coli* (Haake, 2000; Setubal et al., 2006) and thus would be more likely to tolerate substrates from other genera of bacteria, is a good indication Lpp is localized to the inner membrane.

Deletion of Lys 58, which abrogates the covalent linkage to peptidoglycan (Zhang and Wu, 1992), has no effect on localization of Lpp in *B. burgdorferi*. These results suggest

localization is not a consequence of Lpp being bound to peptidoglycan. We cannot fully rule out the possibility that the His tag at the C-terminus in causing a localization artifact or in complementing the K58 mutation in peptidoglycan binding. However, there are two lines of evidence that suggest this is not the case. Previous studies have shown the amino acid substitution K58R abolishes the ability of Lpp to bind peptidoglycan (Yakushi et al., 1997; Zhang and Wu, 1992), suggesting covalent linkage to peptidoglycan is specific for lysine. Second, addition of a FLAG tag to the C-terminus of Lpp does not impact localization of Lpp nor the ability to bind peptidoglycan, indicating Lpp with a non-native C-terminus is still physiologically functional (Cowles et al., 2011).

The +2, +3 and +4 residues of Lpp are disordered and thus could function as the Lpp 'tether' in *B. burgdorferi*. Alanine mutagenesis of these residues did not affect localization within the envelope. A possible explanation is the three amino acid tether is too short to be recognized by the *B. burgdorferi* lipoprotein sorting machinery and a longer tether is necessary. *Borrelia* lipoproteins have tethers ranging from 12-46 residues, although only portions of these tethers seem to be necessary for proper localization to the surface (Schulze and Zückert, 2006; Schulze et al., 2010; Kumru et al., 2010) (Chapter III/IV).

Recently, the question was raised if lipoproteins by themselves are sufficient to determine localization. Until now, limited experimental data was available to disprove this hypothesis. This study shows lipoproteins expressed in surrogate hosts follow said host's lipoprotein sorting rules. *B. burgdorferi* lipoprotein fusions to mRFP1 expressed in *E. coli* were localized according to the +2 rule, while Lpp was localized to the inner membrane in *B. burgdorferi* independent of the +2 rule. The data presented here suggests Lpp is localized to the inner membrane according

to the imprecisely defined lipoprotein sorting rules of *B. burgdorferi*. Further characterization of this phenomenon might help in elucidating lipoprotein sorting rules in *B. burgdorferi.*

**Table 6.1. Cross genus localization of Lpp and OspA:mRFP1.**

| Lipoprotein | *E. coli* | *B. burgdorferi* |
|---|---|---|
| Lpp-His | OM, periplasm | IM, periplasm |
| OspA20:mRFP1 | OM, periplasm | IM, periplasm |
| OspA28:mRFP1 | OM, periplasm | OM, surface |
| $OspA28_{K19D}$:mRFP1 | IM, periplasm | OM, periplasm |

**Figure 6.1. Localization of OspA-mRFP1 fusions in *E. coli*.**

Membranes were separated by sucrose density gradient ultracentrifugation. Each fraction was collected and analyzed by SDS-PAGE and western immunoblotting using antibodies against mRFP1. **(A)** OspA28:mRFP1 was localized to the outer membrane, while OspA28$_{K19D}$:mRFP1 was retained in the inner membrane. **(B)** OspA20:mRFP1, which is inner membrane localized in *B. burgdorferi*, is localized to the *E. coli* outer membrane.

```
                signal peptide        +1 tether
                                       ▼ +2+3+4
Lpp-wt           M K A T K L V L G A V I L G S T L L A G C S S N A K I D Q L S S D V Q T L N A K V
Lpp-His          M K A T K L V L G A V I L G S T L L A G C S S N A K I D Q L S S D V Q T L N A K V
LppΔK58-His      M K A T K L V L G A V I L G S T L L A G C S S N A K I D Q L S S D V Q T L N A K V
LppSSN(AAA)-His  M K A T K L V L G A V I L G S T L L A G C A A A A K I D Q L S S D V Q T L N A K V
Lpp(KQNV)-His    M K A T K L V L G A V I L G S T L L A G C K Q N V S S N A K I D Q L S S D V Q T L N A K V
```

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                                                                              PG
                                                                              ▼
Lpp-wt           D Q L S N D V N A M R S D V Q A A K D D A A R A N Q R L D N M A T K Y R K  his tag
Lpp-His          D Q L S N D V N A M R S D V Q A A K D D A A R A N Q R L D N M A T K Y R K H H H H H H
LppΔK58-His      D Q L S N D V N A M R S D V Q A A K D D A A R A N Q R L D N M A T K Y R - H H H H H H
LppSSN(AAA)-His  D Q L S N D V N A M R S D V Q A A K D D A A R A N Q R L D N M A T K Y R K H H H H H H
Lpp(KQNV)-His    D Q L S N D V N A M R S D V Q A A K D D A A R A N Q R L D N M A T K Y R K H H H H H H
```

**Figure 6.2. Peptide sequences of Lpp mutants used in this study.**

Signal peptide, tether residues and His tag sequences are marked. The +1 Cys residue is marked with an arrowhead and the C-terminal Lys that binds peptidoglycan is indicated in red. The mutated residues are shaded in blue.

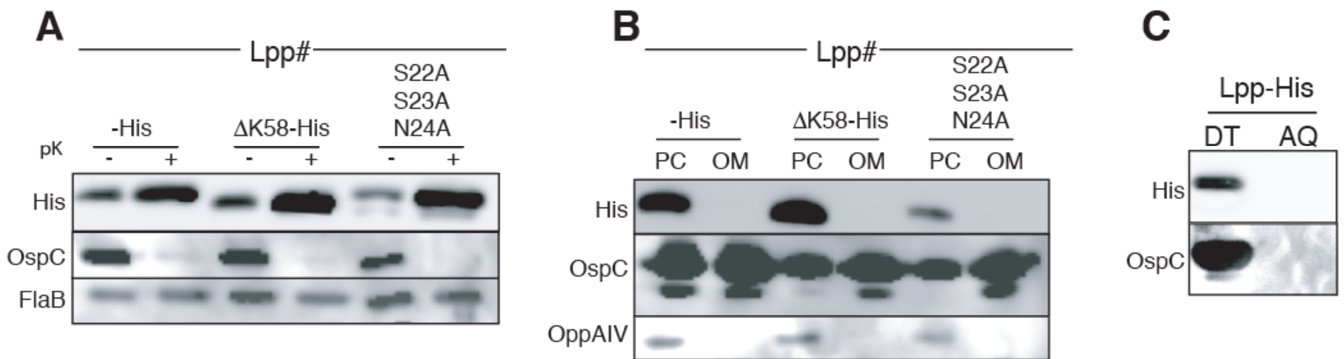


**Figure 6.3. Localization of Lpp in *B. burgdorferi*.**

**(A)** Proteinase K (pK) accessibility western blots of Lpp-His, ΔK58, and the S22A/S23A/N24A tether mutant. OspC was used as a protease susceptible, surface control. FlaB was used as a periplasmic, protease-resistant control.

**(B)** Membrane fractionation western blots of proteinase K-resistant, i.e., periplasmic Lpp-His, ΔK58, and the S22A/S23A/N24A tether mutant. OppAIV served as IM control, while OspC served as the outer membrane control. OM, outer membrane vesicle fraction; PC, protoplasmic cylinder fraction (also containing intact cells; (Schulze and Zückert, 2006; Skare et al., 1995).

**(C)** Whole *B. burgdorferi* cells expressing Lpp-His were subjected to Triton X-114 phase partitioning, detergent (DT) and aqueous (AQ) phases were analyzed by SDS-PAGE and western blotting using $Ni^{2+}$-HRP specific for the His tag and OspC as a detergent phase control.

# Chapter VII.

# Conclusions and Discussion

Considering the data presented in (Schulze and Zückert, 2006; Schulze et al., 2010), *B. burgdorferi* does not seem to sort lipoproteins based on amino acid identity. Further, the results of the FACS based screen argue against any biophysical properties of the amino acids themselves in being permissive for surface localization. Aromatic and bulky residues tended to cluster with the class of mutants that are completely sensitive to proteinase K treatment. This data suggest aromatic and bulky residues may compromise the overall fold of the OspA:mRFP1 fusion protein, leading to surface localization (Chapter II). The screen identified two Gly residues in these positions were not permissive for surface localization, while two Ala residues were permissive (Schulze and Zückert, 2006). This phenomenon has been observed in OspA (Schulze et. al. 2010) and OspC (Chapter IV). Therefore, we feel it is compelling that a certain limited degree of flexibility in this region is required that is afforded by alanine, but not glycine. Glycines are unrestricted in their backbone rotational freedom and may introduce excessive entropy, which could affect protein folding and protein-protein interactions (Chakrabartty *et al.*, 1991; Brady and Sharp, 1997; Schulze et al., 2010)

The screen also identified several amino acid combinations that localized the OspA:mRFP1 mutants to the periplasm in lieu of the Asp-Glu residues, but none that could retain the reporter in the inner membrane to the same degree (Chapter III). To our surprise, substitution of a Pro in the +2 position of Vsp1 caused complete retention in the inner membrane, a phenomenon yet to be observed in *Borrelia* (Chapter IV). This finding was reminiscent of a study by Seydel et al. where Pro, Trp, Phe, Gly, and Tyr could also act as LolCDE avoidance signals in the +2 position of *E. coli* lipoproteins (Seydel et al., 1999). Yet, these amino acids are never present in the +2 position of native lipoproteins. In *B. burgdorferi,* Cys, His, Pro, and Trp are never found in the +2 position of native lipoproteins, with His, Trp,

and Cys rarely found in the tether (with the exception of the +1 Cys) (Schulze et al., 2010). Interestingly, proline is frequently found throughout lipoprotein tethers and has the property to disrupt secondary structure and is commonly found as the first residue of a α-helix or β-turn (Kabsch and Sander, 1983; MacArthur and Thornton, 1991). Taken together, these data suggests there are different mechanisms of inner membrane retention observed when a Pro is in the +2 position and placement of two tandem negative charges in certain contexts.

This work also presented an important expansion to the structurally and functionally distinct virulence factors: OspC in *B. burgdorferi* and Vsp1 of *B. turicatae*. Previously, all our data was collected using monomeric OspA. By investigating surface localization determinants of OspC and Vsp1 it enabled us to directly compare and contrast the factors responsible for surface localization across different lipoprotein families. As expected, proper surface display of OspC-Vsp1 lipoproteins was dependent on the amino acids in their respective tethers. A phenomenon so far unique to OspC is that two distinct portions of the tether are necessary for different steps in surface localization. Deletion of residues 20-30 results in a defect of flipping the lipoprotein across the outer membrane, while deletion of residues 31-41 significantly impairs release from the inner membrane. Similar mutations made in dimeric lipoproteins Vsp1 (Fig. 3.3) and CspA (data not shown) did not produce this phenotype. Additional evidence, gained from localization of Vsp1 N-terminal signal peptide and tether fused to the structurally-confined portion of OspC revealed precisely the same residues were responsible for localization of Vsp1 and $\text{Vsp}_{\text{tether}}$-OspC fusion (Chapter IV/data not shown). This suggests this phenomenon occurs independent of the structurally confined portion of OspC.

What could be the reason for two distinct regions of OspC that are necessary for surface localization? A great deal of heterogeneity exists at the *ospC* locus, as there are 19 different

major groups of *ospC* based on DNA sequence homology (Wang et al. 1999). Yet, the tether residues are conserved between these groups, with the exception of a few point mutations (Kumaran et al. 2001). Interestingly, there is absolute conservation of peptide sequence from residue N31 to N41 across all 19 of these groups (Kumaran et al. 2001; Fig. 4.1). This implicates the importance of maintaining this specific tether peptide sequence during the course of evolution.

Concurrent with preparing this dissertation a study was published further investigating the function of OspC during murine infection. Several truncated OspC proteins were generated, but most notably mutations corresponding to tether residues $OspC_{\Delta 26-30}$ and $OspC_{\Delta 26-35}$ (Seemanapalli et al., 2010). Both of these mutants were surface localized, which is consistent with our findings (Chapter IV). The $OspC_{\Delta 26-30}$ mutant was of particular interest as, $OspC_{\Delta 26-30}$ takes weeks longer to disseminate to remote tissues (skin and joints) compared to $OspC_{wt}$. However, there were no significant differences between $OspC_{wt}$ and $OspC_{\Delta 26-30}$ in terms of $ID_{50}$ and spirochetal burden in the skin or joints (Seemanapalli et al., 2010). Seemanapalli et al., concluded that deletion of 5 tether amino acids of OspC impairs *B. burgdorferi* in dissemination to remote tissues. This data suggests the length of the tether may influence the function of OspC, perhaps in reduced ligand binding efficiency *in vivo*.

Deletion of 11 or 17 tether residues of OspC had a negligible effect on thermodynamic stability as measured by CD spectroscopy. These experiments have disproved the hypothesis that tether residues are inherently destabilizing *in vitro*. Mutation of amino acids in the tether most likely abrogates a protein-protein interaction site between a periplasmic chaperone and the lipoprotein, rather than retarding protein folding, as is the case with Sec signal peptides (Park et al., 1988; Beena et al., 2004). However, it is important to note that the recombinant OspC

proteins used in the thermal unfolding experiments were non-lipidated. It remains possible the lipid moiety could sufficiently destabilize the protein and promote interaction with a periplasmic chaperone needed for translocation. Taken together, this data strongly implicates that a periplasmic chaperone(s) must be involved in the translocation process and that the tether is most likely a site for this protein-protein interaction. This best explains why no canonical 'motif' has been identified despite extensive random and site-directed mutagenesis (Chapter III/Chapter IV).

Characterization of potential chaperones involved in lipoprotein transport is currently underway in our laboratory. We are in the process of developing a Bimolecular Fluorescence Complementation (BifC) system using a split mRFP1 (Jach et al., 2006) with the objective of identifying lipoprotein-chaperone interactions *in vivo*. Preliminary experiments using OspC fused to the N and C terminal halves of mRFP1 indicate proper surface display of both constructs, although there are still CtpA-dependent cleavage products (data not shown, see Chapter V). If co-expression of both halves of OspC:mRFP1 results in restoration of red fluorescence, this system could be used to probe protein-protein interactions between lipoproteins and candidate chaperones hypothesized to the involved in the translocation process. Mutants of OspC and Vsp1 localized to distinct cellular compartments (Chapter IV) could become even more important in determining how the lipoprotein sorting machinery operates and to identify the outer membrane translocation machinery required for surface export.

We also determined the role of dimerization during the translocation process. All experimental evidence supports the notion that dimerization of OspC and Vsp1 can occur in the periplasm in mislocalized mutant proteins, but that the wild type lipoproteins are translocated through the outer membrane as monomers (Chapter IV). Considering OspA surface translocation requires an unfolded conformation (Schulze et al., 2010), it is reasonable to

postulate that OspC and Vsp1 monomers are also translocated across the outer membrane in an unfolded conformation. Further, this data suggests the outer membrane lipoprotein 'flippase' does not tolerate folded substrates.

Potential genes that code for the outer membrane 'flippase' must fulfill certain characteristics. First, the gene is most likely essential. Therefore, the gene is probably encoded on the chromosome since *B. burgdorferi* can be cured of virtually all plasmids, including cp26 when the essential genes are complemented elsewhere. However, even the most highly passaged strains contain cp26 and at least one plasmid from the cp32 family (Sadziene et al., 1993; Casjens et al., 1997; Jewett et al., 2007). Though unlikely, the possibility remains components of the outer membrane secretion complex could be encoded on these plasmids. Second, it is obvious the protein must be an integral outer membrane protein so it must possess both an N-terminal signal peptide and transmembrane domains.

A crude bioinformatic analysis was performed using chromosomal ORFs that did not contain a transposon insertion from a sufficiently saturated genome-wide transposon library. Genes that did not contain an insertion were assumed to be essential (transposon library constructed by Norris et al., unpublished data). Using the translated sequences of the essential genes, probable N-terminal signal peptides were identified using the SignalP prediction algorithm (available at http://www.cbs.dtu.dk/services/SignalP). A membrane prediction algorithm was then performed on signal peptide positive sequences using the SPLIT membrane protein prediction algorithm (available at http://split.pmfst.hr/split/4/). Finally, the data was analyzed by BLAST and is shown in Table 7.1. Twenty-five potential genes were identified using this approach, most with little homology to other genes and/or with unknown functions. The tools are now available to define the roles of each of these gene products by using

conditional knockout systems (Whetstine et al., 2009; Gilbert et al., 2007). We foresee this approach to be the most viable and decisive in identification and characterization of the outer membrane lipoprotein 'flippase.'

**Table 7.1. Potential genes that code for the *B. burgdorferi* outer membrane 'flippase'**

| Gene | Predicted function/BLAST results |
|---|---|
| *bb0038* | predicted lipoprotein, highly conserved |
| *bb0058* | tetratricopeptide repeat domain protein |
| *bb0155* | predicted lipoprotein, highly conserved |
| *bb0171* | predicted lipoprotein, highly conserved |
| *bb0174* | aerotolerance-related exported protein |
| *bb0192* | putative membrane protein |
| *bb0199* | putative membrane protein |
| *bb0236* | tetratricopeptide repeat domain protein |
| *bb0238* | TPR Domain containing protein |
| *bb0259* | lytic transglycosolase |
| *bb0507* | conserved among spirochetes, possible lipoprotein, shares homology with spore germination proteins |
| *bb0535* | conserved among *Borrelia* species, shares similarity with *Plasmodium* proteins of unknown function |
| bb0539 | predicted inner membrane protein |
| *bb0562* | predicted sec-independent protein translocase |
| *bb0602* | chaperonin, putative |
| *bb0624* | putative 27.7 kDa hydrolase |
| *bb0673* | putative OrfX protein |
| *bb0708* | divergent polysaccharide deacetylase superfamily protein |
| *bb0709* | putative periplasmic solute-binding protein |
| *bb0770* | divergent polysaccharide deacetylase superfamily protein |
| *bb0776* | signal peptide protein |
| *bb0783* | membrane-bound protein LytR |
| *bb0794* | conserved hypothetical protein |
| *bb0838* | predicted organic solvent tolerance protein |
| *bb0843* | arginine-ornithine antiporter |

During the course of studying OspC and Vsp1 surface localization we recognized a smaller molecular weight variant of both proteins that became even more pronounced upon addition of a C-terminal linker and/or epitope tags (Chapter IV/V). These lower molecular weight variants are a result of C-terminal processing by CtpA (Chapter V). Only a handful of CtpA substrates have been identified using proteomics (Östberg et al., 2004), and OspC was not among them. There are three possible reasons why the study by Östberg et al. did not detect any

difference in the molecular weight and isoelectric point of OspC in their analysis. First, Östberg et al. used the strain B31-A, which expresses OspC poorly (Chapter V; Kumru and Zückert, unpublished observation). Second, surface OspC is processed by CtpA at significantly higher levels when the C-terminus is modified by a linker and/or epitope tags (Chapter V). Finally, wild type OspC must be overexpressed to detect the CtpA dependent band (OspC*) (Fig. 5.2C).

Perhaps CtpA targets the C-termini of unfolded outer membrane proteins to initiate an envelope stress response? Such a system is reminiscent of how another periplasmic protease, DegS, functions in activation of the envelope stress response via the alternative sigma factor, $\sigma^E$ in *E. coli* (Walsh et al., 2003). DegS recognizes unfolded OMPs by their periplasmically exposed C-terminal peptides and initiates a complex signaling cascade that results in upregulation of envelope stress response genes (Walsh et al., 2003). Mislocalization of OspC and Vsp1 (or any other lipoprotein with a disordered C-terminus) to the periplasm could result in CtpA-dependent recognition of the unfolded C-termini. The released peptides could then serve as substrates in induction of an envelope stress response. CtpA could function as a periplasmic 'quality control' protein to detect mislocalized surface proteins and cleave C-terminal peptides to activate this response. As stated in Chapter V, a possible reason CtpA fails to recognize mislocalized OspA is the C-terminus of OspA is structurally confined in a short $\alpha$-helix. The data presented in this dissertation implicate a broader function for CtpA as a protease involved in envelope biogenesis.

This research has revealed several determinants involved in localization of lipoproteins in *B. burgdorferi*. More research is necessary to identify and characterize the lipoprotein sorting machinery. Also, characterization of the localization requirements for native inner membrane lipoproteins (OppAI, OppAII, OppAIV, OppAV, IpLA7) is an important step in determining the

native mechanism of inner membrane retention.  Theses experiments, in conjunction with the data in this dissertation will be invaluable in understanding lipoprotein sorting and envelope biogenesis in *B. burgdorferi*.  Eventually, this could lead to novel treatments and/or vaccination strategies that will be extremely helpful in combating Lyme disease and relapsing fever in the years and decades to come.

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