PROBING THE LIPOPROTEIN SECRETION PATHWAY IN *BORRELIA BURGDORFERI*

BY

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Date defended: March 21st, 2012

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IN *BORRELIA BURGDORFERI*

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Abstract

The bacterial agent *Borrelia burgdorferi* causes Lyme disease, a debilitating inflammatory disease and a major public health issue in the United States and around the world. Early studies of *B. burgdorferi* pathogenesis determined the major immunogenic factors to be lipid-modified proteins (lipoproteins). The *B. burgdorferi* genome encodes more lipoproteins than any other organism studied to date. The majority of these lipoproteins are tethered to the *B. burgdorferi* cell surface, creating the medically important host-pathogen interface. In addition, many studies have revealed the significance of surface lipoproteins to the *B. burgdorferi* life cycle in both the tick vector and mammalian host. Despite the importance of lipoproteins, little is known about the protein machinery and molecular events responsible for lipoprotein transport to the borrelial outer surface. The goal of this dissertation is to shed light on the mechanism of lipoprotein trafficking in *B. burgdorferi* by (i) investigating borrelial homologs of known lipoprotein transport machinery (the Lol system), (ii) localizing a known lipoprotein, and (iii) investigating the *Borrelia* surface proteome to identify substrates and factors involved in lipoprotein transport.

First, we used genetic manipulation and biochemical analyses to understand the role of Lol pathway homologs in surface and sub-surface lipoprotein transport. Our experiments indicate that the Lol pathway does not play a primary role in transport of surface lipoproteins. Nevertheless, a block in the pathway does impact localization of subsurface lipoproteins as well as OM porins.

Second, we demonstrated inner membrane localization of IpLA7, a major immunogenic protein in Lyme disease. Of the many predicted lipoproteins in the borrelial genome, few have been localized to the periplasm. Third, we constructed reporter surface lipoproteins to identify interacting proteins along the transport pathway and tested several methods to characterize the outer membrane protein composition. Finally, we used *in silico* prediction algorithms to identify novel candidate integral outer membrane proteins, which may be involved in lipoprotein transport.

Together, these data contribute to characterizing the molecular events responsible for subsurface and surface outer membrane lipoprotein secretion in *B. burgdorferi*, an endeavor vital to understanding the regulation and processing of these key virulence factors and thus the pathogenesis of Lyme disease.

Acknowledgements

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Abbreviations

ATc	anhydrotetracycline
CDC	Centers for Disease Control and Prevention
DNA	deoxyribonucleic acid
EM	erythema migrans
IM	inner membrane
LB	Lyme borreliosis
LPS	lipopolysaccharide
OM	outer membrane
OMV	outer membrane vesicle
PC	phosphatidylcholine, protoplasmic cylinder
PE	phosphatidylethanolamine
PG	phosphatidylglycerol

Chapter I: Introduction

Lyme Disease

Lyme disease (Lyme borreliosis) is a multi-system inflammatory syndrome caused by immune reaction to several closely related species of *Borrelia* spirochetes, collectively referred to as *Borrelia burgdorferi* sensu lato. Three genospecies are responsible for human infection in North America, Europe, and Asia: *Borrelia burgdorferi sensu stricto* (the subject of this dissertation and referred to as *B. burgdorferi* hereafter*) Borrelia garinii*, and *Borrelia afzelii.* However, in the United States, Lyme disease is caused exclusively by *B. burgdorferi*. Lyme disease is the most common vector-borne disease in North America and other temperate regions of the Northern hemisphere (Rizzoli, Hauffe et al. 2011). The Centers for Disease Control and Prevention (CDC) reported approximately 30,000 cases of LB in the United States in 2010, making Lyme disease the 5th most common Nationally Notifiable Disease (Patsy A. Baker 2009). The total annual number of cases in the world has been estimated to be as high as 255,000 (Rudenko, Golovchenko et al. 2011).

Borrelia are transmitted to humans by the bite of a tick belonging to the genus *Ixodes*. In the majority of cases (70 to 80 %), the bite of an infected tick results in a red bulls-eye rash or *erythema migrans* (EM) that radiates from the site of the bite (Nowakowski,

Schwartz et al. 2001). In his early-localized stage, EM may be accompanied by general flu-like symptoms (Steere, Coburn et al. 2004). The standard treatment of early Lyme disease is a 2-3 weeks course of doxycycline or amoxicillin (Wormser, Dattwyler et al. 2006). If not treated early, *B. burgdorferi* can disseminate and colonize the joints, heart, muscle, and peripheral and central nervous systems (Steere, Dhar et al. 2003). The resulting arthritis, myocarditis and/or encephalopathy can be severe and even fatal. Antibiotic treatment at these later stages is often unsuccessful in resolving symptoms (Embers, Barthold et al. 2012). Even after early antibiotic treatment, a subset of patients complain of continued or new-onset symptoms such as fatigue, neurological impairment, myalgias and arthralgias. This condition is called post-treatment Lyme disease syndrome and much controversy surrounds its diagnosis and treatment (Feder, Johnson et al. 2007; Lantos 2011). Some patients and physicians advocate the use of long-term antibiotic treatment, although studies have not shown this to provide a health benefit (Klempner, Hu et al. 2001; Klempner 2002).

Although erythema migrans was first described more than one hundred years ago in Europe, not until the 1970s was human borreliosis recognized as a major public health issue in North America. In 1972, Allen Steere, an epidemiologist at the CDC, was sent to investigate an unusually large number of cases of juvenile rheumatoid arthritis in the small town of Lyme, Connecticut. Steere spent two years studying the similarities in the cases of arthritis, which totaled 39 children and 12 adults before concluding that this previously unrecognized epidemic arthritis was due to an infectious agent transmitted by

an arthropod vector. Steere coined the term Lyme arthritis in reference to the town where the outbreak began (Steere, Malawista et al. 1977).

In 1981, Willy Burgdorfer and others at the Rocky Mountain Biological Laboratory isolated a "treponema like organism" from *Ixodes* ticks collected from a Lyme-endemic region. The spirochetes specifically reacted with serum from Lyme disease patients. Furthermore, the researchers were able to see a disease response from rabbits fed on by ticks carrying the spirochete (Burgdorfer, Barbour et al. 1982; Burgdorfer 1984). The following year in New York State, spirochetes morphologically and serologically similar to the organism identified by Burgdorfer *et al*. were isolated directly from the blood of patients with symptoms of Lyme disease (Benach, Bosler et al. 1983). The spirochetes were determined to be of the genus *Borrelia* and were named *Borrelia burgdorferi (Schmid, Steigerwalt et al. 1984)* after their discoverer.

Borrelia burgdorferi

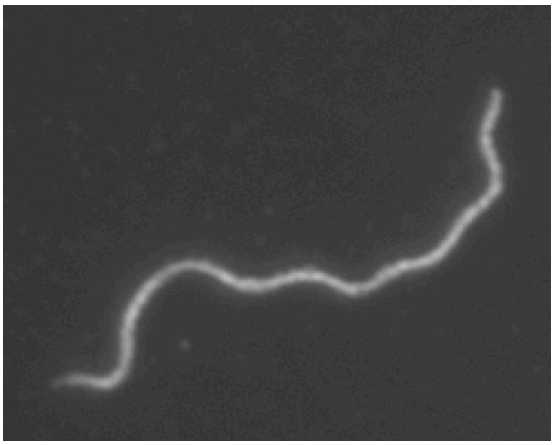
Borrelia burgborferi is a member of the family Spirochaetaceae along with the pathogens *Leptospira* and *Treponema*, which cause leptospirosis and syphilis respectively. *Borrelia* exhibit classic characteristics that set this subset of bacteria apart morphologically; they are long and thin at 0.2-0.5 μm in diameter, are 3-20μm in length, and have a helical shape with several loose coils per cell (Holt 1978; Barbour, Hayes et al. 1986) (Fig. 1).

However, unlike the helical motility of *Treponema* and *Leptospira*, *Borrelia* exhibit flat-wave motility similar to that of eukaryotic flagella (Goldstein, Charon et al. 1994).

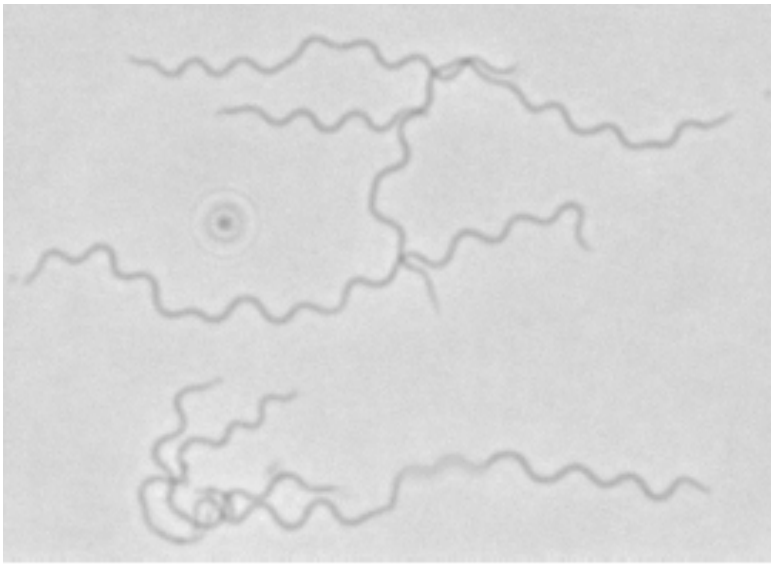
All *Borrelia* species contain an unusual segmented genome consisting of a large linear chromosome and several smaller linear and circular plasmids. *Borrelia burgdorferi* strain B31 was sequenced in 1997 and contains a linear chromosome of 910,725-bp encoding 846 genes; 12 linear plasmids and 9 circular plasmids together provide an additional 6,120-bp (Fraser, Casjens et al. 1997). This small genome lacks genes encoding enzymes crucial for synthesis of many cellular building blocks, which results in an organism highly dependent on its environment for preformed fatty acids, cofactors, amino acids and nucleotides (Fraser, Casjens et al. 1997).

Figure 1. *Borrelia burgdorferi*. (A) Methanol-fixed *B. burgdorferi* cell illuminated by fluorescein isothiocyanate (FITC) conjugated antibodies targeting the periplasmic flagellar protein, FlaB. (B) Phase contrast of *B. burgdorferi* in culture. (Photos: Kristina Bridges)

A



B



Cell Architecture

Borrelia spirochetes are often categorized as Gram-negative since they are diderm (double membrane) bacteria, containing an outer membrane (OM), an inner cytoplasmic membrane (IM), and a periplasmic space in between the two membranes. However, the composition of the borrelial membrane deviates from prototypical Gram-negative bacteria such as *E. coli* in several ways.

First, *B. burgdorferi* outer membranes do not contain a surface layer of lipopolysaccharide (LPS) (Takayama, Rothenberg et al. 1987; Fraser, Casjens et al. 1997). Instead, a thick layer of lipoproteins covers the *B.burgdorferi* surface. Also, *B. burgdorferi* membranes do not contain phosphatidylethanolamine (PE), a phospholipid found in other Gram-negative membranes. Rather, the major lipid components are the two surface-exposed glycolipids cholesteryl-6-*O*-acyl-β-d-galactopyranoside and cholesteryl-β-d-galactopyranoside (more common among monoderm than diderm bacteria), phosphatidylcholine (PC), and phosphatidylglycerol (PG). The *B. burgdorferi* outer membrane contains relatively small amounts of β-barrel outer membrane proteins (OMPs) when compared to typical Gram-negative bacteria (Bergström and Zückert 2010). Further, the peptidoglycan layer is housed between the two membranes and, unlike typical Gram-negatives, is attached to the inner membrane rather than the outer membrane. Also in the periplasm are the flagellar filaments, which are anchored to the inner membrane at each pole and span the length of the cell, conferring not only spiral

morphology, but also an agile, snake-like motility (Barbour and Hayes 1986; Radolf, Bourell et al. 1994).

Electron microscopy of *B. burgdorferi* revealed an amorphous slime layer surrounding the outer membrane, which may be composed of secreted proteins, surface-exposed glycolipids, host/medium-acquired proteins bound to surface proteins, or some combination of these components. Abundant budding vesicles have also been visualized around the outer membrane and may play a role in delivery of immune response modulating antigens (Radolf, Bourell et al. 1994; Kudryashev, Cyrklaff et al. 2009).

Lipoproteins

After the identification of *B.burgdorferi* as the causative agent of Lyme disease, researchers focused their efforts on identifying virulence factors and immunogenic components. When investigators probed *B.burgdorferi* whole cell lysates and detergent soluble fractions with antibodies from the sera of Lyme disease patients, they found the major immunogens to be among the detergent soluble proteins (membrane proteins). Further, protein radiolabelling with [H^3]palmitate revealed that the major *B.burgdorferi* immunogenic proteins were lipoproteins (Brandt, Riley et al. 1990). Nearly ten percent of the small borrelial genome is dedicated to expressing lipoproteins, which represents the highest percentage of lipoproteins in all sequenced bacterial genomes (Setubal, Reis et al. 2006).

Lipoproteins are found in monoderm and diderm bacteria and play myriad roles in cellular physiology and virulence (Kovacs-Simon, Titball et al. 2011). Most lipoproteins are anchored in either the inner membrane or the periplasmic leaflet of the outer membrane. Less commonly, lipoproteins are anchored in the outer leaflet of the outer membrane (cell surface) where they play major roles in cellular adhesion (Jin, Joe et al. 2001), nutrient foraging (Manfredi, Renzi et al. 2011), iron acquisition (Cornelissen, Kelley et al. 1998; Dashper, Hendtlass et al. 2000) and immune evasion (Xu, McShan et al. 2008).

Bacterial lipoproteins contain covalently bound fatty acids at the amino terminus, which act as a lipid tail, anchoring lipoproteins peripherally to the membrane. Lipoproteins are expressed as preprolipoproteins with a signal sequence that directs them to the Sec or Tat machinery, the two major pathways for transport across the bacterial inner membrane (discussed in more detail below) (Inouye, Wang et al. 1977; Tommassen 2010). Prolipoproteins contain a unique motif within their signal sequence termed a lipobox, which ensures subsequent lipid modification (Wu and Tokunaga 1986). The conserved lipobox sequence of typical gram-negative bacteria is Leu-(Ala/Ser)-(Gly/Ala)-Cys; however, sequence specificity for spirochetal lipoboxes is less stringent (Setubal, Reis et al. 2006).

Once on the periplasmic face of the inner membrane, prolipoproteins are transformed into mature lipoproteins by a three-step process. First, thioether linkage of a diacylglycerol occurs at the Cys residue at the end of the lipobox by the enzyme prolipoprotein diacylglyceryl transferase (Lgt). The signal peptide is then cleaved by the type II signal peptidase (Lsp) at the amino end of the partially lipidated Cys. Finally, the amino-terminal Cys residue is N-acylated by apolipoprotein transacylase (Lnt), resulting in a tri-acylated, mature lipoprotein (Hussain, Ichihara et al. 1980; Hussain, Ichihara et al. 1982; Tokunaga, Tokunaga et al. 1982; Yamada, Yamagata et al. 1984).

Once sysnthesis is complete, lipoproteins in *B. burgdorferi* are tethered to either the periplasmic leaflet of the inner membrane, the periplasmic leaflet of the outer membrane, or the outer leaflet of the outer membrane (cell surface), where they are most abundant (Fig. 2) (Bergström and Zückert 2010). As they are positioned at the interface of the bacterium and its host, surface lipoproteins play crucial roles in pathogenesis and adaptation to vector and host environments (Scragg, Kwiatkowski et al. 2000; Ramesh, Alvarez et al. 2003). Importantly, the major inflammatory response apparent in Lyme borreliosis is thought to be triggered in large part by lipoproteins (Bessler, Cox et al. 1985; Radolf, Arndt et al. 1995; Dennis, Dixit et al. 2009; Londono and Cadavid 2010; Batsford, Dunn et al. 2011).

The most abundant surface lipoproteins are the outer surface proteins (Osps). Osps have little sequence homology other than the N-terminal lipidation signal and have varying

functions. Osps play a role in survival and maintenance within the tick (Pal, de Silva et al. 2000), transmission and early infection (Grimm, Tilly et al. 2004), evasion of host immune responses (Kraiczy, Skerka et al. 2001; Stevenson, El-Hage et al. 2002; Alitalo, Meri et al. 2005), and adhesion in host tissues (Coburn, Fischer et al. 2005).

Two of the most abundant Osps, OspA and B, are encoded by a single operon and are highly expressed during *Borrelia* persistence within the tick vector (Barbour 1988; Bergstrom, Bundoc et al. 1989). OspA is an adhesin that mediates attachment to the tick midgut (Pal, de Silva et al. 2000) by specifically binding a tick receptor (tick receptor for ospA: TROSPA) (Pal, Li et al. 2004). OspA also shelters other surface exposed proteins from antibody and protease assault (Bunikis and Barbour 1999) and appears to be required for transmission to mice with humoral immunity, but not for transmission to naïve mice (Battisti, Bono et al. 2008). Therefore, OspA may also act to shield other surface antigens such as the surface-exposed porins P66 and P13 from the blood of mammals with humoral immunity.

The function of OspB is unknown, but it shares structural homology with OspA and may be required for persistence within the tick vector (Yang, Pal et al. 2004). OspC is necessary for transmission from tick to mammal and involved in early mammalian infection (Grimm, Tilly et al. 2004; Tilly, Krum et al. 2006). OspC may function in host immune evasion through specifically binding a tick salivary protein (Salp15) and

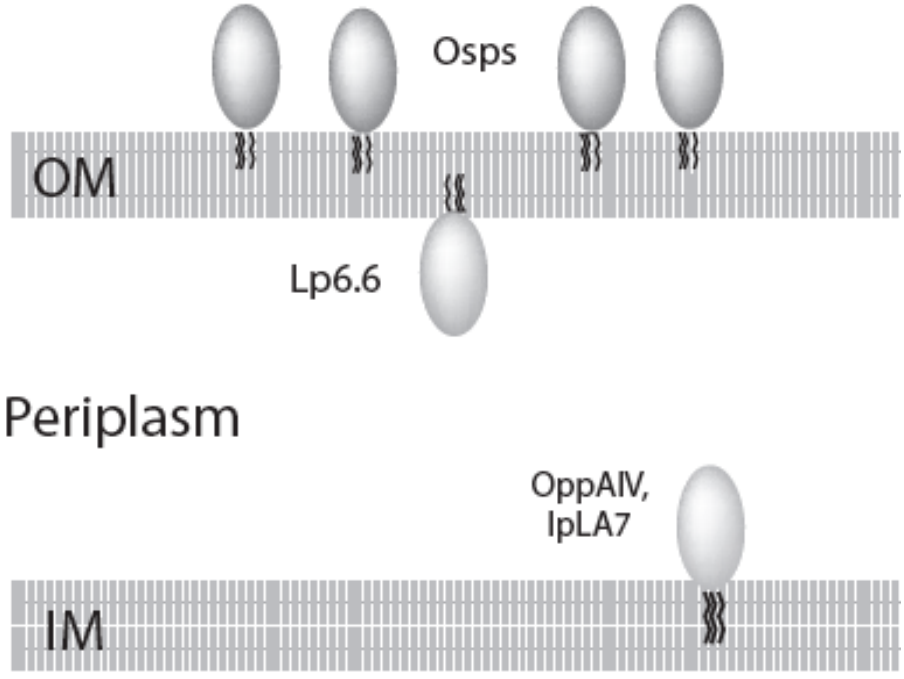
shielding the borrelial surface from host antibodies (Ramamoorthi, Narasimhan et al. 2005).

Lipoproteins are also used by *B. burgdorferi* to evade the host immune response and maintain a persistant infection. This occurs through antigenic variation of surface lipoproteins as well as positive and negative regulation of surface lipoprotein expression. A major strategy of antigenic variation in *B. burgdorferi* is recombination at the *vls* locus. The *vls* locus encodes the 35 kDa surface exposed lipoprotein, *VlsE*, and contains 15 cassettes that can recombine with *vlsE* resulting in variation of the expressed lipoprotein (Zhang, Hardham et al. 1997). A separate mechanism of antigenic variation is variable expression of surface lipoproteins and is best exemplified by the major Osps (A,B,C). OspA and B are highly expressed in the unfed tick, as they are important for adherence and maintenance within the vector. As the tick takes a blood meal and the temperature, pH and nutrient content change within the tick midgut, expression of OspA and B is downregulated and expression of OspC is increased (Schwan, Piesman et al. 1995; Stevenson, von Lackum et al. 2006). After initiation of infection, Borrelia change their surface antigen display again by downregulating OspC (Liang, Jacobs et al. 2002; Liang, Yan et al. 2004).

Subsurface lipoproteins also play roles in survival and pathogenesis in *B. burgdorferi (Haake 2000)*. One lipoprotein tethered to the periplasmic face of the outer membrane is Lp6.6, which is only expressed in the tick vector and required for transmission from tick

to mammal (Promnares, Kumar et al. 2009). Among the few lipoproteins known to be tethered to the periplasmic leaflet of the inner membrane in *B. burgdorferi* are the five homologs (three chromosomally encoded and two plasmid encoded) of the peptide binding protein (OppA) part of the oligopeptide permease (Opp) system (Bono, Tilly et al. 1998; Schulze and Zuckert 2006). Chapter IV describes the localization of the lipoprotein IPLA7, a major immunogen, to the periplasmic leaflet of the inner membrane in *B. burgdorferi* (Von Lackum, Ollison et al. 2007).

Figure 2. Lipoprotein localization in *B. burgdorferi*. Lipoproteins can be anchored in the periplasmic leaflet of the inner membrane (IM) such as OppAIV and IpLA7, in the periplasmic leaflet of the outer membrane (OM) such as Lp6.6, or the surface leaflet of the OM such as OspA-E.


Osps
OM
Lp6.6
Periplasm
OppAIV,
IpLA7
IM

Protein transport in diderm (Gram-negative) bacteria

The bacterial outer membrane is essential to cell structure and interaction with the environment. The proteins localized to the bacterial outer membrane function in structural integrity, selective permeability of nutrients and form pathways for transport of surface-anchored and secreted proteins (Nikaido 2003; Silhavy, Kahne et al. 2010).

To understand lipoprotein transport, it is beneficial to appreciate the context in which lipoproteins exist in the bacterial cell envelope and to be familiar with the known systems of protein transport. All proteins are synthesized in the cytoplasm and must be transported to their final destination to carry out a specific function. For membrane or secreted (extracellular) proteins in diderm bacteria, this may require traversing a cytoplasmic (inner) membrane, an aqueous, energy-devoid periplasm, and an outer membrane. These three cellular compartments contain specialized protein machineries dedicated to protein transport. The following is a brief description of the major concepts in the field of diderm protein transport.

Transport across the inner membrane

Sec

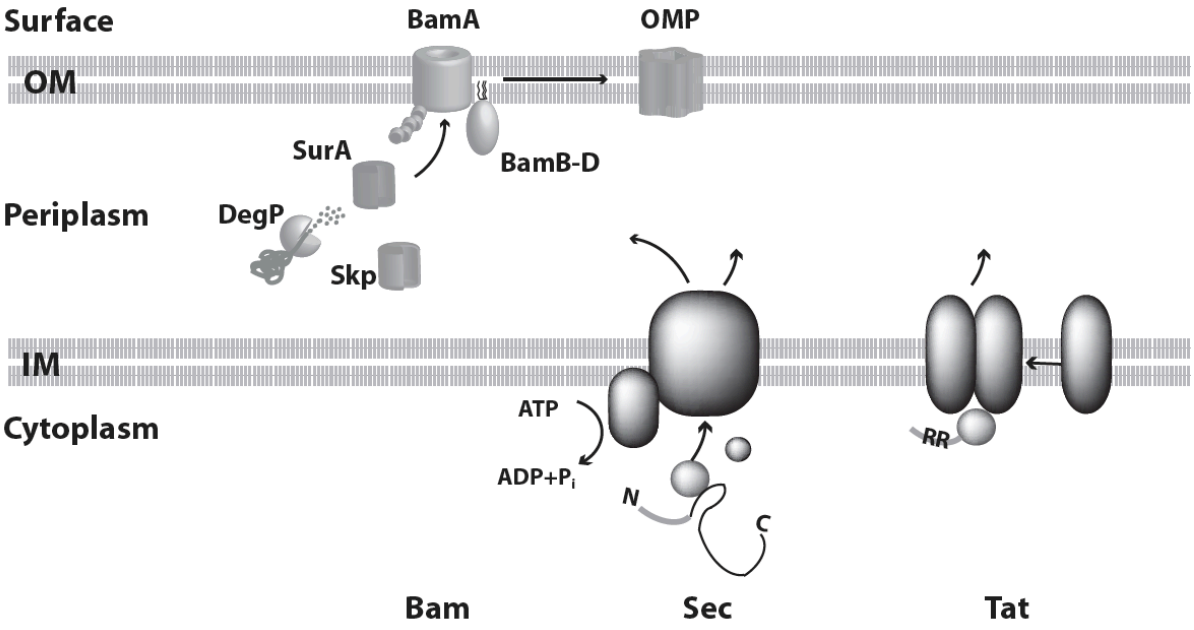
The Sec pathway is the major route by which bacterial protein translocation occurs across the cytoplasmic membrane. The Sec translocon consists of a heterotrimeric pore

(SecYEG), an ATPase (SecA), several chaperones, and accessory proteins. Proteins are targeted to the Sec translocon via cleavable (N)-terminal signal peptides, which range from 20 to 30 amino acids (Papanikou, Karamanou et al. 2007). IM proteins are presented to the Sec translocon co-translationally, whereas OM proteins dissociate from the ribosome prior to translocation and require a chaperone (SecB) to direct them to the Sec translocon. Proteins cross the inner membrane through the SecYEG pore in an unfolded conformation driven by energy from SecA-mediated ATP hydrolysis. Once on the periplasmic side of the inner membrane, proteins can either remain in the membrane, or as in the case of integral OM proteins, surface proteins and exported proteins, can be targeted to one of several Sec-dependent export pathways (Papanikou, Karamanou et al. 2007).

Tat

The twin arginine transport (Tat) pathway differs from the Sec pathway in that it moves folded rather than unfolded proteins across the inner membrane. Substrates are targeted to the Tat pathway by N-terminal signal sequences containing consecutive Arg residues. The Tat pathway consists of three integral membrane proteins (TatABC) and is powered by the transmembrane proton electrochemical gradient. Two of the subunits (BC) recognize and bind substrates; recruitment of the third subunit (A) facilitates formation of a pore capable of transporting large folded proteins across the membrane (Natale, Bruser et al. 2008; Tarry, Schafer et al. 2009).

Figure 3. Illustration of the major inner membrane and periplasmic transport systems in diderm bacteria. The Sec pathway transports proteins bearing an amino-terminal signal sequence across the cytoplasmic membrane in an unfolded conformation. The Tat pathway recognizes substrates bearing a twin arginine motif (RR) and transports folded proteins across the cytoplasmic membrane. The periplasmic chaperones SurA, Skp and DegP transport β-barrel outer membrane proteins (OMPs) through the periplasm to Bam complex (BamA-D) where they are assembled in the outer membrane. Outer membrane, OM. Inner membrane, IM.


Surface
BamA
OMP
OM
SurA
BamB-D
Periplasm
DegP
Skp
IM
Cytoplasm
ATP
ADP+P$_i$
N
C
RR
Bam
Sec
Tat

Transport to the outer membrane

In diderm bacteria, the outer membrane protein components are integral β-barrel outer membrane proteins (OMPs) and lipoproteins (lipoprotein transport will be discussed in the following section).

Transport of OMPs

Bacterial OMPs contain antiparallel amphipathic β-strands that fold into β-barrels in the outer membrane. Many OMPs play structural or enzymatic roles in the outer membrane. OMPs can also function as porins or transporters that control the movement of molecules in and out of the cell (Hagan, Silhavy et al. 2010). After transport across the inner membrane through Sec, OMPs interact with one or a combination of three periplasmic folding chaperones, SurA, Skp and DegP, which transport unfolded OMPs across the periplasm to the β-barrel assembly machinery (Bam) complex for integration into the outer membrane (Rizzitello, Harper et al. 2001; Sklar, Wu et al. 2007; Krojer, Sawa et al. 2008). The Bam complex consists of an essential OMP, BamA, and four accessory lipoproteins (BamB,C,D,E), which have varying roles and essentiality in different organisms (Tommassen 2010). In E.coli, BamA and B form a complex and interact with the separate BamCDE complex. In addition to the membrane embedded β-barrel domain, BamA also contains a large periplasmic domain with five polypeptide transport-associated (POTRA) domains. These POTRA domains are predicted to be the docking

point for substrate Omps and the Bam accessory lipoproteins (Kim, Malinverni et al. 2007; Knowles, Jeeves et al. 2008).

Transport of proteins to the cell exterior (secretion)

Protein secretion systems can be divided into two groups: those that involve the Sec (secretion) pathway, the major route of protein translocation across the inner membrane, i.e. are Sec-dependent, and those that bypass Sec, i.e. are Sec-independent.

Sec-dependent protein secretion

TypeII

The type two secretion system (T2SS) secretes folded proteins, such as hydrolytic enzymes and toxins, across the cell envelope. After Sec-mediated inner membrane transport, T2SS substrates fold in the periplasm. Twelve to fifteen proteins including an ATPase, an inner membrane complex, a periplasmic pilus, and a large gated outer membrane channel work together to transport folded substrate proteins through the outer membrane (Chami, Guilvout et al. 2005; Reichow, Korotkov et al. 2010).

TypeV (autotransporter) and Two-partner Secretion

Type five secretion (T5SS) substrates make up a large family of bacterial virulence factors including many known proteases, toxins and adhesins. A typical T5SS substrate

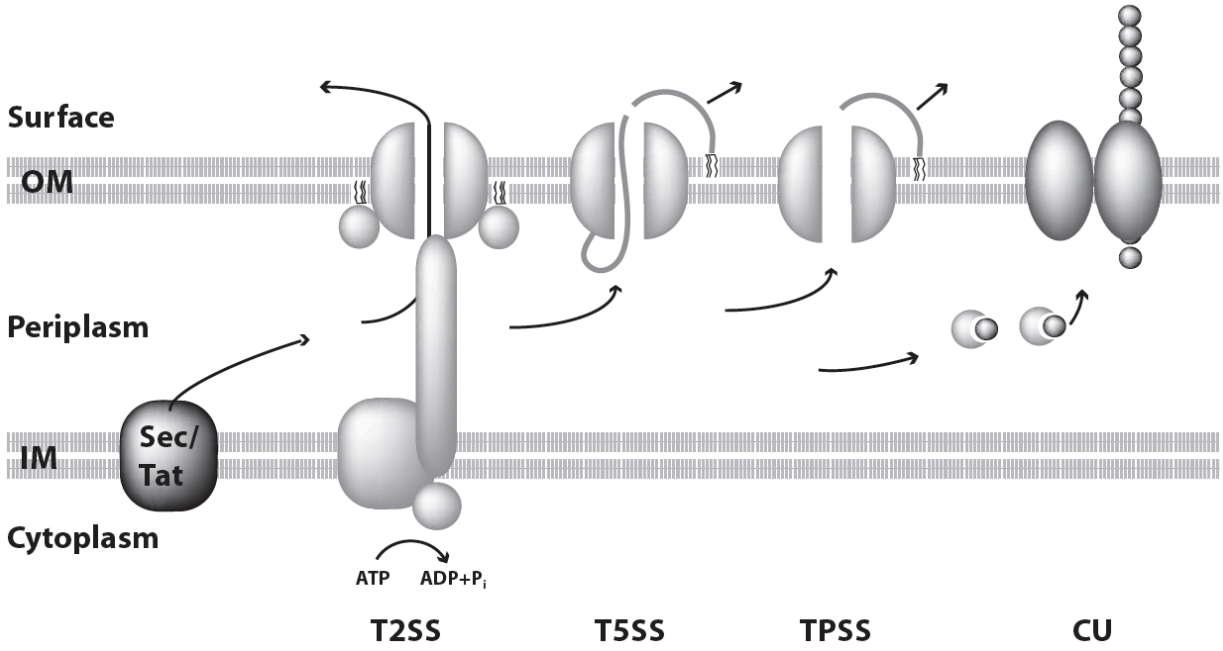
contains a long N-terminal Sec-dependent signal sequence, an internal "passenger" α-domain and a C-terminal β-domain (Nishimura, Tajima et al. 2010). The exceptionally long signal peptides not only target them to the Sec machinery, but prevent misfolding of the passenger domain by transiently tethering them to the periplasmic leaflet of the inner membrane (Szabady, Peterson et al. 2005). It was originally thought that following cleavage of the signal sequence, the C-terminal β-domain would insert into the outer membrane and form a pore through which the passenger domain would be exported to the cell surface, thereby facilitating independent transport (hence, *auto*transporter). However, interaction between an T5SS substrate passenger domain and periplasmic chaperones has been revealed (Ieva and Bernstein 2009). Furthermore, the C-terminal β-domain requires BamA and at least one of the Bam accessory lipoproteins for insertion into the outer membrane (Jain and Goldberg 2007; Sauri, Soprova et al. 2009; Lehr, Schutz et al. 2010; Rossiter, Leyton et al. 2011).

The two-partner secretion system (TPSS) falls under the T5SS substrate family. Instead of a single protein, however, two proteins (typically encoded by one operon) act as translocator and passenger. Both proteins cross the inner membrane via Sec. The translocator protein, which contains an integral membrane β-barrel domain and a periplasmic POTRA domain, is then assembled into the outer membrane by the Bam complex. The passenger protein contains a TPSS signature motif required for translocator recognition and initiation of secretion (Mazar and Cotter 2007; St Geme and Yeo 2009).

Chaperone/ Usher

The chaperone/usher (CU) secretion pathway mediates transport and assembly of surface-anchored virulence factors (fimbriae, pili) which function in host cell adhesion, invasion and biofilm development.(Remaut, Tang et al. 2008). CU substrates cross the inner membrane via the Sec pathway and then interact with the periplasmic chaperone subunit which aids in substrate folding, prevents premature interaction with other substrate molecules, and transports the substrate to the outer membrane usher. The pore-forming usher subunits catalyze the polymerization and translocation of the substrate through the outer membrane (Giraud and de Bentzmann 2011).

Figure 4. Sec-dependent secretion systems in diderm bacteria. Following transport across the inner membrane via the Sec or Tat pathways, proteins can be secreted to the cell exterior by the type two (T2SS), type five (T5SS), two-partner (TPSS), or chaperone/usher (CU) secretion systems. The T2SS requires the energy of adenosine triphosphate (ATP) hydrolysis to adenosine diphosphate (ADP). Outer membrane, OM. Inner membrane, IM.


Surface
OM
Periplasm
IM
Cytoplasm
Sec/
Tat
ATP
ADP+P$_i$
T2SS
T5SS
TPSS
CU

Sec-independent protein secretion

Type I

Direct transport of exoenzymes and toxins from the cytoplasm to the extracellular matrix is mediated by the Type I secretion system (T1SS). T1SS substrates, such as the *E. coli* α-hemolysin (HlyA), contain a non-cleavable C-terminal signal sequence that targets them to the secretion machinery, which consists of an inner membrane ATP-binding cassette (ABC) transporter, a membrane fusion protein, and an outer membrane pore (Holland, Schmitt et al. 2005).

Type III

Many gram-negative pathogens encode a type III secretion system (T3SS, injectisome) to directly inject virulence factors (effector proteins) into eukaryotic host cells. The T3SS complex consists of a pair of rings (one in each membrane), spanning the periplasm with a cylindrical rod. A hollow needle-like structure extends from the bacterial cell into the cytoplasm of the target eukaryotic cell (Matteï, Faudry et al. 2011).

Type IV

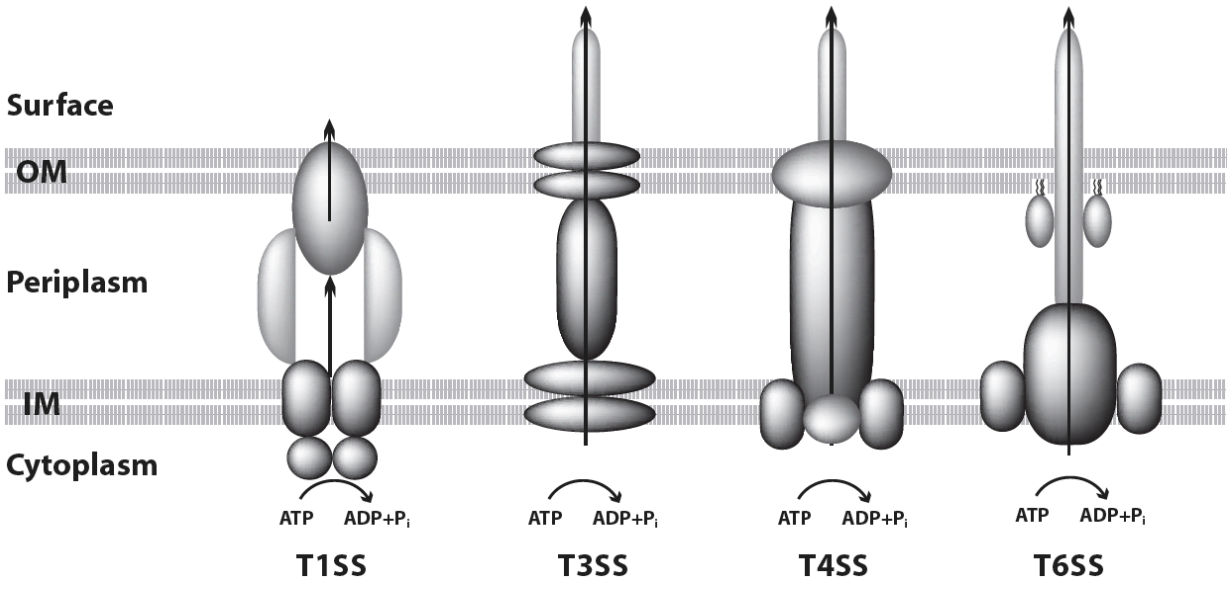
Type four secretion systems directly transport DNA and proteins across the cell envelope and in some cases, straight into host cells. The system has three main functional components: energetic, channel and pilus. Each component varies among different organisms in the number of proteins (twelve or more) or subunits involved. The subunits

of the energetic component act to harness the energy of ATP hydrolysis to power the secretion system, whereas the channel and pilus are involved in transport across each membrane and delivery of substrates to host cells (Alvarez-Martinez and Christie 2009).

Type VI

The large gene cluster (fifteen to twenty genes) encoding type six secretion systems (T6SS) is found in the genomes of many pathogenic bacteria. The model for T6SS is largely speculative at this point, but there are a large number of cytoplasmic subunits, ATPases, few inner membrane proteins, several lipoproteins, and a membrane puncturing pilus. The pilus may be related to the cell-penetrating machinery of tailed bacteriophage, consisting of a long polymer with a trimeric needle-like structure that penetrates host cell membranes (Cascales 2008; Bonemann, Pietrosiuk et al. 2010).

Figure 5. Sec-independent secretion systems in diderm bacteria. Type one (T1SS), type three (T3SS), type four (T4SS), and type six (T6SS) secretion systems transport substrate proteins directly from the cytoplasm to the cell exterior. Each system requires the energy of adenosine triphosphate (ATP) hydrolysis to adenosine diphosphate (ADP). Outer membrane, OM. Inner membrane, IM.


Surface
OM
Periplasm
IM
Cytoplasm
ATP
ADP+Pi
T1SS
ATP
ADP+Pi
T3SS
ATP
ADP+Pi
T4SS
ATP
ADP+Pi
T6SS

Lipoprotein transport

E.coli Lol

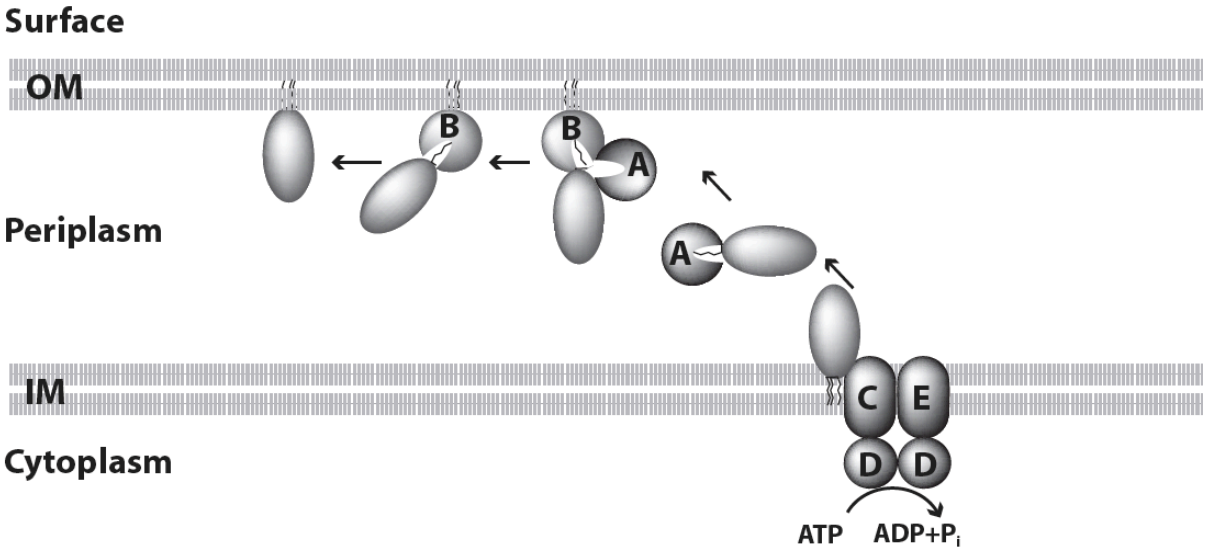
Trafficking of lipoproteins within the *E. coli* periplasm has been well documented (Tokuda and Matsuyama, 2004; Narita and Tokuda 2010). Lipoproteins in *E. coli* are anchored to either the periplasmic leaflet of the inner membrane or the periplasmic leaflet of the outer membrane (Ichihara, Hussain et al. 1981; Yu, Furukawa et al. 1984). Localization is determined by the amino acid immediately following the lipidated Cys, in the +2 position (Yamaguchi, Yu et al. 1988). The "+2 rule" for *E.coli* lipoprotein sorting states that Asp at the +2 position acts as an inner membrane retention signal, while any other amino acid in that position results in outer membrane localization. Although, phenylalanine, tryptophan, tyrosine, glycine or proline at the +2 position do allow inner membrane localization, they are not physiologically relevant retention signals, because they are not found naturally at position +2 in *E.coli* lipoproteins (Seydel, Gounon et al. 1999).

Prolipoproteins are synthesized in the cytoplasm and targeted via an N-terminal signal sequence to the SecYEG translocon or by a twin arginine signal to the TAT pathway, through which they traverse the inner membrane (Okuda and Tokuda 2011) . Once on the

periplasmic face of the inner membrane (IM), mature lipoproteins are formed through processing by the lipidation machinery and Type-II signal peptidase. IM lipoproteins (those with Asp at the +2 position) are anchored in the inner membrane, while those destined for the outer membrane (OM) enter the Lol (Localization of lipoproteins) pathway (Tokuda and Matsuyama, 2004).

The three-step Lol export pathway in *E. coli* begins with lipoprotein interaction with an IM, ABC transporter-like complex, LolCDE (Yakushi, Masuda et al. 2000). OM lipoproteins bind LolCDE and are then handed off in an ATP-dependent manner to a periplasmic chaperone, LolA. The lipid tail of the protein is buried in a water-soluble complex with LolA, which shuttles the lipoprotein through the periplasm to the outer membrane (Matsuyama, Tajima et al. 1995). Finally, the lipoprotein binds the OM receptor, LolB, and is inserted into the inner-leaflet of the outer membrane (Matsuyama, Yokota et al. 1997).

Figure 6. The Lol pathway in *E.coli*. After Sec-mediated transport across the inner membrane, outer membrane lipoproteins are released from the inner membrane via the ATPase binding cassette (ABC) complex, LolCDE, which requires the energy of adenosine triphosphate (ATP) hydrolysis to adenosine diphosphate (ADP). The periplasmic chaperone, LolA receives lipoproteins from LolCDE and shuttles them to the outer membrane receptor, LolB for insertion into the outer membrane. Outer membrane, OM. Inner membrane, IM.


Surface
OM
Periplasm
IM
Cytoplasm
B
B
A
A
C
E
D
D
ATP
ADP+P$_i$

LolCDE

The LolCDE complex is composed of a cytoplasmic ATPase (LolD) dimer bound to the cytoplasmic domains of two transmembrane proteins (LolC and E). This is the classic ATP- binding cassette (ABC) transporter architecture (Dawson and Locher, 2006). Hence LolCDE (known then as YcfUVW) was a proposed ABC transporter even before its function was determined (Linton and Higgins, 1998). However, LolCDE differs from the prototypical ABC transporter in that it is not responsible for transport *across* a membrane, rather it is required for lipoprotein *release* from the periplamsic leaflet of the inner membrane (Yakushi, Masuda et al. 2000).

LolC and E each contain four membrane-spanning domains and a large periplasmic loop (Tokunaga, Tokunaga et al. 1982). LolD contains the canonical sequence elements of all nucleotide-binding proteins: the ABC signature and the Walker A&B motifs. Many Gram-negative bacteria have homologs for LolCDE and they all have a conserved motif in LolD between the Walker A&B motifs termed the LolD motif (Narita and Tokuda 2006).

Lipoproteins with an aspartic acid at the +2 position do not interact with the LolCDE complex. Thus the *E. coli* lipoprotein inner-membrane retention signal functions as a LolCDE avoidance signal (Masuda, Matsuyama et al. 2002). Lol avoidance depends on the presence of negative charge at the +2 position as well as the presence of

phosphatidylethanolamine in the membrane, indicating that steric attraction between Asp at the +2 position and positively charged phospholipids acts to hold lipoproteins in the inner membrane (Hara, Matsuyama et al. 2003). Interaction of an OM lipoprotein with LolCDE increases LolD affinity for ATP. Subsequent ATP hydrolysis by LolD causes a conformational change in LolCE and leads to release of the lipoprotein (Okuda and Tokuda 2011).

LolA and B

Outer membrane lipoproteins are transferred from LolCDE to the periplasmic chaperone, LolA, which transports the lipoprotein in a 1:1 stoichiometry through the periplasm to the periplasmic leaflet of the outer membrane. LolA forms a water-soluble complex with the lipoprotein by burying the lipid tail in its hydrophobic cavity, shielding it from the hydrophilic periplasmic space (Matsuyama, Tajima et al. 1995; Okuda and Tokuda 2009) Based on the LolA crystal structure, the hydrophobic cavity is only large enough to house one of the three lipoprotein acyl chains. However, hydrophobic patches on the surface of LolA are required for optimum LolA function and are thought to accommodate the other two acyl chains (Remans, Pauwels et al. 2010).

The essential outer membrane lipoprotein, LolB, is required for outer membrane specific insertion of lipoproteins in a LolA dependent manner (Matsuyama, Yokota et al. 1997). LolA and LolB have very low sequence identity (8 %), but are structurally homologous, each resembling an open β-barrel with a lid (Takeda, Miyatake et al. 2003). As the LolA-

lipoprotein complex reaches the LolB lipoprotein acceptor, LolA and LolB interact to form a hydrophobic, tunnel-like structure. In the absence of an energy source in the ATP-devoid periplasm, the transfer of lipoproteins from LolA to LolB is facilitated by an affinity gradient (Nakada, Sakakura et al. 2009).

Surface lipoprotein transport

Though the majority of lipoproteins in *E.coli* are subsurface (Tokuda 2009), a few surface-exposed lipoproteins have been reported (Drummelsmith and Whitfield 2000; Robinson, Ashman et al. 2006). For example, the most abundant and best-characterized lipoprotein of *E. coli*, Lpp, exists in a peptidoglycan-bound form and a free form; recently, the C-terminus of the free form has been shown to be surface-exposed (Cowles, Li et al. 2011). Unlike *Borrelia* surface lipoproteins, which are completely extracellular and only attached to the outer membrane via their lipid tail (Schulze, Chen et al. 2010), surface-exposed lipoproteins in *E.coli* are thought to span the outer membrane and achieve only partial surface exposure (Bernstein 2011; Cowles, Li et al. 2011).
To date, the only known mechanisms for lipoprotein transport to the bacterial cell surface are through the type II secretion system (T2SS) and Autotransporter pathways (Pugsley 1993; Coutte, Willery et al. 2003).

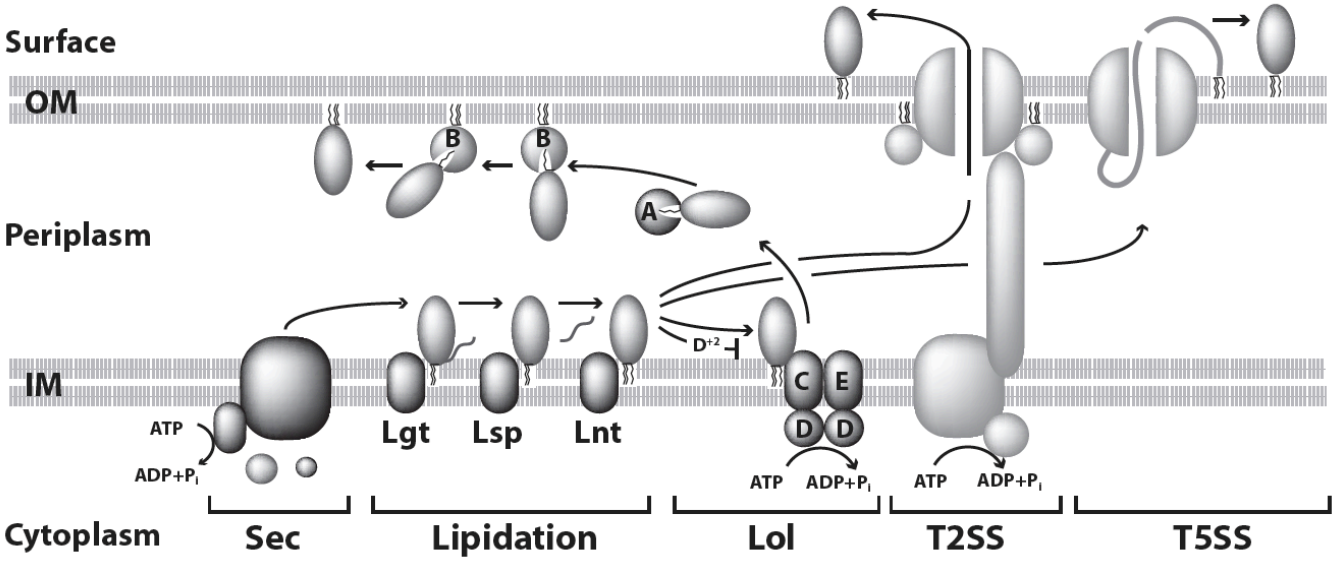
To date, the only known mechanisms for lipoprotein transport to the bacterial cell surface are through the type II secretion system (T2SS) and Autotransporter pathways (Pugsley 1993; Coutte, Willery et al. 2003).

The Pullulanase (PulA) lipoprotein, PulA, is a 116 kDa, surface-exposed isoamylase expressed by the Gram-negative bacterium *Klebsiella oxytoca*. PulA is exported across the inner membrane by the Sec translocon. On the periplasmic side of the inner membrane, the PulA signal sequence is cleaved, lipidation occurs at the N-terminal Cys residue and PulA folds into a stable intermediate. The folded PulA intermediate is then secreted to the bacterial surface by the type II secretion system (T2SS) (Pugsley 1993). A discontinuous secretion signal composed of three non-adjacent regions approximately 80 amino acids each is required for proper transport of PulA (Sauvonnet and Pugsley 1996; Francetic and Pugsley 2005). *K. oxytoca* transports lipoproteins to the periplasmic leaflet of the outer membrane via a complete Lol pathway (Collin, Guilvout et al. 2011). In the absence of the T2SS, PulA remains in the inner membrane due to its +2 Asp. However, mutation of the PulA +2 Asp results in transport to the periplasmic leaflet of the outer membrane via the Lol pathway (Pugsley, Kornacker et al. 1990).

The *Bordetella pertussis* lipoprotein, SphB1, which is a surface-anchored maturation protease. SphB1 is an autotransporter and mediates its own outer membrane translocation through a C-terminal translocator domain (Coutte, Willery et al. 2003). Similarly, the *Neisseria meningitidis* surface protease, NalP, is an autotransporter-lipoprotein (van

Ulsen, van Alphen et al. 2003).

Figure 7. Modes of lipoprotein transport. All lipoproteins cross the inner membrane (IM) through the Sec translocon. IM lipoproteins (in many diderm bacteria, those with Asp at the +2 position, D^{+2}) remain tethered to the inner membrane. The Lol pathway directs lipoprotein localization within the periplasm, resulting in lipoprotein insertion in the periplasmic leaflet of the outer membrane (OM). The only two know systems to transport lipoproteins to the surface of diderm bacteria are the T2SS and the T5SS (autotransporter).


Surface
OM
Periplasm
IM
Cytoplasm
B
B
A
Lgt
Lsp
Lnt
D+2
C
E
D
D
ATP
ADP+Pi
ATP
ADP+Pi
ATP
ADP+Pi
Sec
Lipidation
Lol
T2SS
T5SS

B. burgdorferi lipoprotein transport

The protein composition of the *B. burgdorferi* outer membrane is predominantly surface lipoproteins, followed by subsurface lipoproteins, and few integral membrane proteins (OMPs). Of the protein transport systems described above, the *B. burgdorferi* genome encodes only the Sec machinery, the Bam complex, a T1SS and a partial Lol pathway (Bergström and Fraser, Casjens et al. 1997; Bunikis, Denker et al. 2008; Lenhart and Akins 2010; Zückert 2010).

B. burgdorferi encodes all components of the Sec machinery except the chaperone, SecB (Fraser, Casjens et al. 1997). The omission of SecB from the *B. burgdorferi* Sec apparatus is due to its ancient phylogeny, as the Sec machinery appears to have evolved in a stepwise manor, and SecB does not show up in bacterial genomes until the proteobacteria (van der Sluis and Driessen 2006).

Borrelia spirochetes express a partial Bam complex for assembly of OMPs in the outer membrane (Lenhart and Akins 2010). The *B. burgdorferi* Bam complex consists of the essential OMP, BamA, and two accessory lipoproteins, a homolog of the essential BamD protein and a protein with no homology to other Bam proteins, BB0028 (Lenhart 2010).

The *B. burgdorferi* genome contains homologs for four of the five components of the Lol pathway. As in *E. coli*, the homologs of LolCDE form one operon. The open reading

frame BB0078/79 has 19 % amino acid identity to LolC, BB0080 has 40 % identity to LolD and BB0081 is 22 % identical to LolE, and BB0346 has a 20 % amino acid identity to LolA (Chapter 3) (Fraser, Casjens et al. 1997). We originally hypothesized that the *Borrelia* homologs form a partial Lol pathway and function similarly to their *E. coli* counterparts in the transport of periplasmic outer membrane lipoproteins. In the absence of a Type II secretion system, autotransporter homology, or any other proposed mechanism for surface lipoprotein transport in *B. burgdorferi*, we further hypothesized that the partial Lol pathway is responsible for surface lipoprotein transport as well. The working model for lipoprotein transport in *B. burgdorferi* is similar to that of *E. coli* except it has been shown that an Asp immediately following the lipidated Cys does not act as an inner membrane retention signal (Schulze and Zückert 2006). Outer membrane insertion is also different, since *Borrelia* do not have a LolB homolog and since lipoproteins localize to the surface of the outer membrane, in addition to the periplasmic leaflet of the outer membrane.

Like all lipoproteins, *B. burgdorferi* surface lipoproteins have a tether region, comprising an N-terminal region that lacks secondary structure between the lipidated Cys and the globular protein. The tether region contains information for proper transport to the outer membrane as well as translocation to the outer leaflet of the outer membrane, as deletion or mutation of amino acid residues in the tether region led to mislocalized lipoproteins (Schulze and Zückert 2006; Kumru, Schulze et al. 2010).

B. burgdorferi surface lipoproteins appear to cross the outer membrane C-terminus first, in an unfolded state (Schulze, Chen et al. 2010). Maintenance of this unfolded, translocation-competent state is likely mediated by an N-terminal tether-dependent interaction with a periplasmic "holding" chaperone. The final step in surface lipoprotein transport could involve a "flippase" to take the lipid anchor from the periplasmic leaflet of the outer membrane to the outer leaflet of the outer membrane (Chen, Kumru et al. 2011; Chen and Zuckert 2011). Subsequent functional folding of lipoproteins and assembly into multimeric complexes occurs on the bacterial surface (Chen, Kumru et al. 2011).

To test the function of the *B. burgdorferi* Lol homologs, we analyzed the effects of inactivating the LolD ATPase. Expression of inactive LolD caused a growth defect in cells, but did not block transport of lipoproteins to the surface of the cell, indicating that surface lipoprotein transport is independent of the Lol pathway in *B. burgdorferi*. Expression of the LolD mutant did alter abundance of a subsurface lipoprotein and two outer membrane porins. Additionally, protein-protein interactions revealed that the periplasmic chaperone homolog, LolA, specifically binds lipoproteins (Chapter III). Together these data indicate a role for the partial Lol pathway in subsurface lipoprotein transport. However, future research is needed to determine the protein machinery responsible for transport of surface lipoproteins in *B. burgdorferi*.

Borrelia rely heavily on lipoproteins, primarily those on the cell surface, for survival in varying environments as well as establishment and maintenance of pathogenesis. Continued investigation of the processing, regulation and transport of lipoproteins in *B. burgdorferi* is necessary to understanding the disease causing mechanisms of pathogenic spirochetes.

Chapter II: Materials and Methods

Bacterial strains and culture conditions

Plasmids were propagated in *E. coli* strains DH5α, JS238 or Top10 and were grown in Luria-Bertani (LB) liquid or solid medium (Difco). *Borrelia burgdorferi* B31-e2, a clone of type strain B31 (ATCC 35210), was used for expression of recombinant *B. burgdorferi* Lol pathway homologs and OspA-MalE fusion proteins. Spirochetes were transformed by electroporation with 5-20 μg plasmid DNA. Transformants were plated on solid BSK-II and selected with 250 μg/ml kanamycin. Clones were grown in liquid BSK-II with selection at 34 °C under 5 % CO_2 (Barbour 1984; Zückert 2007). Plasmids were verified by DNA sequencing (Center for Genetic Medicine, Genomics Core Facility, Northwestern University Medical Center, Chicago, IL.) Spirochetes harboring $LolD_{G41D}$ were grown to a density of 10^5 cells/ml in BSKII liquid medium containing 250 μg/ml kanamycin and induced with anhydrotetracycline hydrochloride (ATc, IBA GmbH, Göttingen, Germany) at a concentration of 0.002 – 2 μg/ml. Spirochetes were counted at various time points with a Petroff-Hausser chamber and phase-contrast microscopy.

Production of recombinant Lol proteins

Derivatives of the *E.coli* - *Borrelia* shuttle vector pBSV2 (Stewart, Thalken et al. 2001) were used to express recombinant Lol proteins. *B. burgdorferi* ORFs BB0080 (the *B. burgdorferi lolD* homolog) and BB0346 (*lolA*) were amplified by PCR using primers

pairs Nde_BB0080-fwdN/ BspEI_BB0080his-rev and NdeI_BB0346_fwd / BspEI_BB0346his-rev. The BB0078/79, 80, 81 (*lolCDE*) locus was amplified by primers NdeI-HA-BB0078-79-fwdN and Xba_BB0081his_Rev (Table 1). Amplified gene products were cut with NdeI and BspEI or XbaI (New England BioLabs) and ligated into pBSV2 cut with the same restriction enzymes. Point mutations were introduced using the QuikChange-II XL site-directed mutagenesis kit (Stratagene) using primers BB0080 (G41D)_Fwd and BB0080 (G41D)_Rev. $LolD_{G41D}$ and GFP expression was driven by the ATc inducible, hybrid P_{ost} promoter (Whetstine, Slusser et al. 2009). The LolCDE locus as well as C-terminally histidine-tagged LolD and LolA were expressed under the constitutive *B. burgdorferi* flagellin *flaB* promoter (P_{flaB}).

OspA-MalE gene fusions were created by sequence overlap extension PCR (SOE-PCR) (Ho, Hunt et al. 1989) using platinum Taq polymerase (Invitrogen). The coding region for the first 17, 20, 22, or 28 amino acids of the *ospA* gene was amplified from pRJS1009 (Schulze and Zückert 2006) using primer pair OspAN28, S22, N20, or C17_fwd respectively and OspAMalE_rev. The *E.coli* maltose binding protein E gene (*malE*) was amplified from pJC90 (Mukherjee, Cao et al. 1998) using the primer pair KpnPflaB-fwd and SphMalE-rev. The resulting *ospA* and *malE* PCR fragments were fused using the primer pairKpnPflaB-fwd and SphMalE-rev. *ospA-malE* gene fusions were digested with *Kpn*I and *Sph*I and ligated into pBSV2 (Stewart, Thalken et al. 2001). Expression of OspA-MalE fusions was driven by P_{flaB}.

Protein localization assays

Mid-exponential phase cultures of *B. burgdorferi* were harvested, washed with phosphate buffered saline with 5 mM magnesium chloride (PBS-Mg), and incubated in 100 mg/ml proteinase K (pK, Invitrogen) for 1 h at room temperature. Cells were gently washed with PBS containing 1 mM bovine serum albumin (BSA) and placed back into growth medium with kanamycin and ATc. Cultures were incubated at 34 °C for 12 h prior to a second round of proteolysis with 100 mg/ml pK for 1 h at room temperature. Proteolysis was stopped by addition of phenylmethylsulfonylfluoride (PMSF, Sigma) to a final concentration of 1 mM. Spirochetes were washed with PBS, and resuspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer for subsequent electrophoresis and immunoblot analysis.

Protoplasmic cylinder and outer membrane fractions of *B. burgdorferi* P_{ost} $LolD_{G41D}$ under induced or uninduced conditions were isolated by isopycnic centrifugation as previously described (Skare, Shang et al. 1995; Schulze and Zückert 2006). Proteins of each fraction were then separated by SDS-PAGE (12 %) and analyzed by immunoblotting.

Triton X-114 phase partitioning was carried out as described (Brandt, Riley et al. 1990; Nally, Timoney et al. 2001). Briefly, log phase *B. burgdorferi* cells were solubilized overnight with 2 % Triton X-114 in PBS-Mg. Insoluble material was removed by

centrifugation. Aqueous and detergent-soluble fractions were separated by phase separation at 37 °C for 15 min followed by centrifugation. Both fractions were washed three times with 2 % Triton X-114 (aqueous) or PBS-Mg (detergent) and phase separated as above. Proteins were concentrated by acetone precipitation.

Protein radiolabeling

B31e2 cells were incubated with 2 μg/ml ATc for 36 h to induce P_{ost}-driven expression of $LolD_{G41D}$. Subsequently, 5×10^8 early-log-phase spirochetes, expressing $LolD_{G41D}$, were harvested, washed once with PBS+Mg, and resuspended in 0.5 ml protein-free RPMI 1640 medium (pH 7.5; Sigma-Aldrich) containing a cysteine- and methionine-free amino acid mixture and appropriate antibiotics. Final concentrations of 2 μg/ml ATc and 100 μCi/ml [^{35}S]Cys-Met (EasyTag Express ^{35}S protein labeling mix; Perkin-Elmer) were added to maintain P_{ost} promoter-driven expression of $LolD_{G41D}$ and induce metabolic radiolabeling of cellular proteins. After incubation for 2 h at 34 °C, the cells were pelleted, resuspended in RPMI 1640 medium containing 1.5 mg/ml cold Cys and Met, and incubated for 10 min at 34 °C. The spirochetes were then harvested, washed twice with PBS+Mg, and subjected to in situ proteolysis. SDS-PAGE (12 %) gels containing whole-cell proteins were stained with 0.05 % Coomassie brilliant blue R-250 (Sigma) in 40 % methanol and 10 % acetic acid, destained with 20 % methanol and 10 % acetic acid, and immersed in Amplify fluorographic reagent (Amersham Biosciences) for 30 min. After drying under a vacuum for 1 h at 60 °C, the gels were exposed overnight to Kodak (Rochester, NY) XAR-5 film.

Immunoblot analyses

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, blocked with 5 % milk in Tris-Buffered Saline and Tween 20 (TBST), then incubated with an appropriate antibody/serum diluted in TBST. Custom antibodies were generated against select C-terminal peptides from BB0080 (LolD; 1:500) (CDSKYEFKDRTLKKL) and BB0081 (LolE; 1:100) (LNIVSNLKEKEILR) (Open Biosystems). Additional antibodies used. Bound antibodies were detected by chemiluminescence using the alkaline phosphatase substrate CDP-Star (Amersham Biosciences) or 1-Step NBT/BCIP (Nitro-blue tetrazolium chloride/5-bromo-4-chloro-39- indolyl phosphate p-toluidine) colorimetric substrate (Pierce). Digital images were acquired with a Fuji LAS-4000 luminescent image analyzer and were processed using Adobe Photoshop CS4 for Macintosh. Immunoblot signal intensities were analyzed using ImageJ version 1.440 (USA National Institutes of Health). All protein loading was normalized to the control FlaB signal.

Rabbit antiserum raised against P22-A (IPLA7), the protein product of pSPR37 (Simpson, Schrumpf et al. 1991), was provided by Tom Schwan (Rocky Mountain Laboratories, NIH, Hamilton, MT). Monoclonal antibodies recognizing *B. burgdorferi* FlaB, OspA and OspC were produced from hybridomas H9724, H5332 and B5, respectively (Barbour, Barrera et al. 1983; 1986; Mbow, Gilmore et al. 2002). Polyclonal rabbit antiserum specific for *B. burgdorferi* OppA-IV (Bono, Tilly et al. 1998) was

provided by Patricia Rosa.

Osmotic sensitivity assay

B. burgdorferi cells conditionally expressing $LolD_{G41D}$ under P_{ost} promoter control were grown to mid log phase in BSK II medium and diluted to a cell density of $2x10^5$ cells/ml in fresh BSK II supplemented with glycerol (7 % vol/vol final) or ethanol (1.5 % vol/vol final) and ± 2μg/ml ATc. Cells were counted 24 and 48 h after induction of $LolD_{G41D}$ expression with ATc using a Petroff-Hausser chamber under phase-contrast microscopy.

Cobalt affinity chromatography

B. burgdorferi w.t. or $LolA_{His}$ overexpressing cells were cultured to late exponential phase, washed in PBS and resuspended in binding buffer (50 mM Tris, 400 mM NaCl, 10 mM imidazole, 1 mM PMSF). Cells were disrupted by sonication and remaining intact cells were removed by centrifugation at 10,000 x *g*. The cleared cell lysate was incubated for 30 min at 25 °C with 2 ml of TALON resin (Clontech), which had been equilibrated with binding buffer. The resin was washed with 30 ml binding buffer in batch and then loaded on a column and washed with another 30 ml binding buffer. $LolA_{His}$ was eluted with binding buffer containing 250 mM imidazole. The eluate was concentrated by acetone precipitation and reconstitution in SDS PAGE loading buffer.

Cellular localization of IpLA7

Localization was first addressed by immunofluoresence assay (IFA) determination of antibody accessibility to IpLA7 in intact *B.burgdorferi* (Zuckert, Meyer et al. 1999). Briefly, methanol-fixed or intact spirochetes were incubated with antibodies specific for IpLA7, the major surface lipoproteins OspA and OspC, or the periplasmic protein, FlaB. Cells were then incubated with either fluorescein isothiocyanate (FITC) labeled goat-anti-rabbit IgG (whole molecule) (Sigma-Aldrich) or goat-anti-mouse IgG (H+L) (Kierkegaard & Perry Laboratories). Cells were analyzed by microscopy using a Nikon Eclipse E600 microscope fitted with a FITC HYQ filter block and a Q-Imaging Micropublisher Digital CCD color camera. In a second series of experiments, intact, live *B. burgdorferi* were treated by surface proteolysis (Bunikis and Barbour, 1999; El-Hage, Babb et al. 2001). Briefly, mid-exponential phase cultures of *B. burgdorferi* were harvested, washed, and incubated in 200 mg/ml proteinase K for 1 h at room temperature. Proteolysis was stopped by addition of phenylmethylsulfonylfluoride (PMSF, Sigma) to a final concentration of 1 mM. Bacteria were lysed by immersion in boiling water, and proteins separated by SDS-PAGE followed by transfer to nitrocellulose membranes. Protease degradation of specific proteins was assessed by immunoblot analyses using antibodies specific for IpLA7 and control proteins OppA-IV, OspA, OspC and FlaB.

Inner- and outer-membrane fractions of *B. burgdorferi* were isolated by isopycnic centrifugation as previously described (Skare, Shang et al. 1995; Carroll and Gherardini 1996; Carroll, El-Hage et al. 2001). Proteins of each fraction were separated by SDS-

PAGE and analyzed by immunoblot.

Surface proteolysis

Mid-exponential phase cultures of *B. burgdorferi* were harvested, washed, and incubated in 200 mg/ml proteinase K (pK) for 1 h at room temperature as described (Bunikis and Barbour, 1999; El-Hage *et al.* 2001). Proteolysis was stopped by addition of PMSF (Sigma) to a final concentration of 1 mM. Bacteria were lysed by immersion in boiling water, and proteins separated by SDS-PAGE followed by transfer to nitrocellulose membranes. Protease degradation of specific proteins was assessed by immunoblot analyses using antibodies specific for MalE and control proteins OspA and FlaB.

Maltose binding protein (MalE) affinity purification

Magnetic amylose beads

50 ml cultures of mid-exponential growth B31-e2 cells or B31-e2 cells expressing OspAN28-MalE were harvested and washed twice with PBS-Mg. Cells were resuspended in 0.5 ml amylose binding buffer (200 mM NaCl, 20 mM Tris, 1 mM EDTA, pH 7.4) and lysed using a Branson Sonifier cell disruptor. Unbroken cells were removed by centrifugation for 10 min at 15,000 × g and 4 °C. 100 μl washed magnetic amylose beads (New England BioLabs) were added to the cell lysate and incubated, rotating at 4 °C for 1 h. Magnetic amylose beads were separated from unbound proteins by magnetic

separation and washed three times with binding buffer. Proteins were eluted by 10 mM maltose in binding buffer. Purification fractions were analyzed by SDS PAGE.

Magnetic MBP monoclonal antibody beads

Cultures were harvested and washed as above, resuspended in 0.5 ml of 0.1 M Na phosphate buffer (pH 8.0) and lysed using a Branson Sonifier cell disruptor. Unbroken cells were removed by centrifugation for 10 min at 15,000 × g and 4 °C. 40 µl washed magnetic MBP monoclonal antibody beads were a added to the cell lysate and incubated, rotating at 4 °C for 1 h. Magnetic beads were separated from unbound proteins by magnetic separation and washed three times with 0.1 M Na phosphate buffer (pH 8.0). Proteins were eluted with SDS sample buffer at 70 °C for 5 min. Purification fractions were analyzed by SDS PAGE.

Immunoprecipitation

Cells were harvested and washed as above and resuspended in 1 ml ice-cold lysis buffer (150 mM NaCl, 1 % octylphenoxypolyethoxyethanol (IGEPAL CA-630), 50 mM Tris, 1 mM PMSF, pH 8.0) for 15 min on ice. Lysates were cleared of cellular debris by centrifugation for 10 min at 15,000 × g and 4 °C. Polyclonal rabbit α-MBP antibodies (Bono, Tilly et al. 1998) were added at a dilution of 1:200 and samples were incubated, rotating at 4 °C for 1 h. 50 µl washed magnetic protein A beads were added. Samples were washed and antibody-protein complexes were eluted with 50 µl SDS sample buffer and boiled for 5 min. Samples were analyzed by SDS PAGE.

In silico identification of candidate OMPs

Online versions of bioinformatic software was used to identify candidate OMPs in the *B. burgdorferi* strain, B31e2. Candidate OMPs were identified by three independent algorithms based on the presence of predicted signal sequence, membrane-spanning regions, and protein fold, using PSortdb (http://db2.psort.org/search), TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), and PRED-TMBB (http://biophysics.biol.uoa.gr/PRED-TMBB/). Conserved domains were identified using the National Center for Biotechnology Information Conserved Domain Database (NCBI-CDD, http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Surface biotinylation

50 ml cultures of *B. burgdorferi* strains B31-e2, B31-3 (strain lacking the most abundant surface lipoproteins), and B31-5A3 (low-passage, infectious strain) were grown to a density of 10^8 cells/ml, harvested, and washed twice with PBS-Mg. Cells were resuspended in PBS-Mg (pH 7.2) and half of the sample was lysed using a Branson Sonifier cell disruptor. Unbroken cells were removed by centrifugation for 10 min at 15,000 × g. 10 μl of Sulfo-NHS-LC-Biotin EZ link was added to each sample and incubated for 30 min at room temperature. Tris-HCl (pH 7.2) was added to a final concentratio of 50 mM. Samples were washed twice with PBS-Mg and resuspended in SDS PAGE sample buffer. Samples were analyzed by SDS PAGE followed by coomassie staining and detection with NeutrAvidin Biotin-Binding Protein (Invitrogen).

Cell surface proteolysis and membrane fractionation

1 L cultures of late exponential phase *B. burgdorferi* strains B31-e2 and B31-3 were harvested and washed twice with PBS-Mg. Each strain was split into two samples. One sample was incubated for 1 h with 75-200 mg/ml pK. Proteolysis was stopped with 1 mM PMSF. Protoplasmic cylinder (PC) and outer membrane vesicle (OMV) fractions were isolated by membrane fractionation as previously described (Skare, Shang et al. 1995; Schulze and Zückert 2006). Briefly, cells were treated with a hypotonic citrate buffer (25 mM final, pH 3.2) + 0.1 % bovine serum albumin (BSA) and incubated at room temperature for 2 h with vigorous shaking. Unbroken cells were removed by centrifugation for 20 min at 15,000 × g. Cells were resuspended in citrate buffer and applied to a discontinuous sucrose density gradient (25 %, 42 %, and 56 % sucrose in citrate buffer). PC and OMV fractions were separated by ultracentrifugation (Beckman L8-80M centrifuge). Fractions were washed and stored in PBS+PMSF for subsequent analysis by SDS PAGE and silver staining (BIO-RAD silver stain plus).

Table 1. Primers used in this study.

Primer name	Sequence 5’ → 3’
BB0080(G41D)_Fwd	GTGAATTTATTTCAATTCAAGACAAAAGTGGTTGTGGAAAATC
BB0080(G41D)_Rev	GATTTTCCACAACCACTTTTGTCTTGAATTGAAATAAATTCAC
NdeI_BB0080-fwdN	GGAATTCCATATGGAGAATATATTAATTATAAAAAATC
BspEI_BB0080his-rev	CTCCTCCGGAAGCCACAAGAGGCGACAGACATCATCAGTGATGATGATG
NdeI_BB0346_fwd	TGGAGGAATGACATATGATAAAAACAAT
BspEI_BB0346his-rev	CTCCTCCGGAAGCCACAAGAGGCGACAGACATCATCAGTGATGATGATG

Table 2: Antibodies used in this study.

Antibody	Type	Dilution	Source	Reference
MBP	Rabbit pAb	1:200	P. Rosa (NIH/NIAID Rocky Mountain Laboratories, Hamilton, MT)	(Bono, Tilly et al. 1998)
OppA-IV	Rabiit pAb	1:500	P. Rosa (NIH/NIAID Rocky Mountain Laboratories, Hamilton, MT)	(Bono, Tilly et al. 1998)
OspA (H5332)	Mouse mAb	1:100		(Barbour, Tessier et al. 1983)
OspC	Mouse mAb	1:50	B. Stevenson (University of Kentucky, Lexington, KY)	(Mbow, Gilmore et al. 2002)
FlaB	Rat pAb	1:4000	M. Caimano (University of Connecticut Health Center, Farmington, CT)	(Akins, Caimano et al. 1999)
IpLA7	Rabbit pAb	1:500	T. Schwan (Rocky Mountain Laboratories, NIH, Hamilton, MT)	(Simpson, Schrumpf et al. 1991)
His-tag	Mouse mAb	1:1000	Sigma-Aldrich	
HA-tag	Mouse mAb	1:1000	Sigma-Aldrich	
BamA	Rat pAb	1:4000	D. Akins (University of Oklahoma Health Sciences Center, Oklahoma City, OK)	(Lenhart and Akins 2010)
P66	Rabbit pAb	1:500		(Schmid, Steigerwalt et al. 1984)
P13	Rabbit pAb	1:1000	S. Berström (Umeå University, Umeå, Sweden)	(Noppa, Ostberg et al. 2001)
Lp6.6	Mouse mAb	1:500		Katona, Beck et al. 1992)

Chapter III: Functional analysis of the *B. burgdorferi* Lol pathway homologs

Abstract

The surface of the Lyme disease spirochete, *Borrelia burgdorferi*, is populated by lipoproteins, many of which have been identified as major virulence factors. The mechanism of lipoprotein secretion in *B. burgdorferi* is unclear. In *Escherichia coli*, lipoproteins that are ultimately anchored in the periplasmic leaflets of either the inner or outer membrane are sorted by the Lol pathway. Based on sequence analysis, *B. burgdorferi* encode homologs for four of the five proteins in the Lol pathway: an inner-membrane ABC transporter LolCDE and a lipoprotein-specific periplasmic carrier chaperone LolA. Here we analyze the ATPase component of the LolCDE complex, LolD. Consistent with its predicted inner membrane association, the LolD homolog was found primarily in the inner membrane fraction of a *B. burgdorferi* lysate along with LolC and E. Expression of a dominant-negative LolD mutant ($LolD_{G41D}$), containing a point mutation in the conserved Walker A motif, resulted in a growth defect in *B. burgdorferi.* $LolD_{G41D}$ expression reduced abundance of the subsurface outer membrane lipoprotein, Lp6.6, and two integral outer membrane proteins, P66 and P13, but did not detectably

affect the localization or abundance of the major surface lipoprotein, OspA. This research suggests transport of *B. burgdorferi* surface lipoproteins is independent of the Lol pathway.

Introduction

The *Borrelia burgdorferi* genome encodes more putative lipoproteins than any other bacterial genome studied (Setubal, Reis et al. 2006) and the majority are localized to the bacterial surface. Like all lipoproteins, *B. burgdorferi* surface lipoproteins have a tether region, comprising an N-terminal unordered region between the lipidated cysteine and the globular protein. This region contains information for proper transport to the outer membrane as well as translocation to the outer leaflet of the outer membrane (Schulze and Zückert 2006; Kumru, Schulze et al. 2010). *B. burgdorferi* surface lipoproteins appear to cross the outer membrane C-terminus first, in an unfolded state (Schulze, Chen et al. 2010). Maintenance of this unfolded, translocation-compentent state is likely mediated by an N-terminal tether-dependent interaction with a periplasmic "holding" chaperone. Subsequent functional folding of lipoproteins and assembly into multimeric complexes occurs on the bacterial surface (Chen, Kumru et al. 2011).

The three-step Lol export pathway in *E.coli* begins with lipoprotein interaction with an IM, ATP-binding cassette (ABC) transporter complex, LolCDE (Yakushi, Masuda et al.

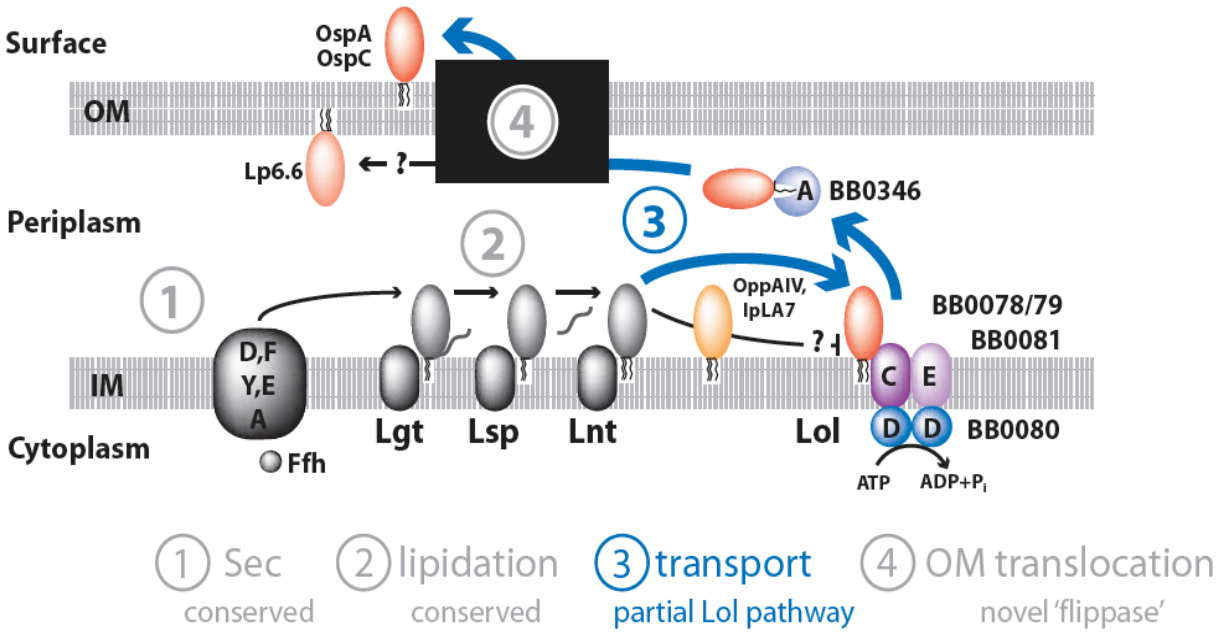
2000). OM lipoproteins bind LolCDE and are handed off in an ATP-dependent manner to a periplasmic chaperone, LolA. The lipid tail of the protein is buried in a water-soluble complex with LolA, which shuttles the lipoprotein through the periplasm to the OM (Matsuyama, Tajima et al. 1995). Finally, the lipoprotein binds the OM receptor, LolB, and is inserted into the periplasmic-leaflet of the OM (Matsuyama, Yokota et al. 1997).

The *Borrelia* genome contains homologs for four of the five Lol pathway proteins: the IM releasing LolCDE complex and the periplasmic chaperone, LolA (Fraser, Casjens et al. 1997). Due to the absence of a T2SS, autotransporter capability or any other proposed mechanism for surface lipoprotein transport in *B. burgdorferi*, we earlier hypothesized that these homologs form a partial Lol pathway and function in IM release and periplasmic transport of both subsurface and surface lipoproteins (Bergström and Zückert 2010). Therefore, the working model for lipoprotein transport in *B. burgdorferi* is similar to that of *E. coli*, except that the absence of a LolB homolog in *Borrelia* as well as the predominant surface localization of *B. burgdorferi* lipoproteins suggests a novel step in lipoprotein secretion at the *Borrelial* outer membrane (Fig. 8).

In this study, we analyzed the effect of inactivating the LolD homolog on surface and subsurface OM lipoprotein transport as well as OM composition. Surprisingly, expression of the inactive mutant, $LolD_{G41D}$, had no detectable effect on surface lipoprotein localization. Instead, expressing the mutant LolD altered the abundance and localization of sub-surface lipoproteins and integral outer membrane porins. Additionally, we

investigated the protein-protein interaction of the *B. burgdorferi* LolA homolog. LolA was found to specifically bind lipoproteins.

Figure 8. Model for lipoprotein transport in *B. burgdorferi*. Lipoproteins are transported across the inner membrane and lipidated by the conserved *Borrelia* homologs of the Sec and lipidation machinery respectively. Inner membrane lipoproteins remain in the inner membrane due to an unknown sorting signal. Outer membrane lipoproteins are predicted to rely on a partial Lol pathway for extraction from the inner membrane and transport through the hydrophilic periplasm. Outer membrane lipoproteins are either inserted into the periplasmic leaflet of the outer membrane or translocated to the outer surface by unknown mechanisms. Borrelia burgdorferi homologues of the partial Lol pathway are indicated by their TIGR ORF number. OM, outer membrane. IM, inner membrane.


Surface
OspA
OspC
OM
4
Lp6.6
?
A BB0346
Periplasm
3
2
1
OppAIV,
lpLA7
BB0078/79
BB0081
D,F
Y,E
A
IM
C
E
Lgt
Lsp
Lnt
Lol
D
D
BB0080
Cytoplasm
Ffh
ATP
ADP+P$_i$
1 Sec
conserved
2 lipidation
conserved
3 transport
partial Lol pathway
4 OM translocation
novel 'flippase'

Results

In *E.coli*, following Sec-mediated translocation of prolipoproteins through the IM, signal sequence removal and lipidation, OM lipoproteins are released from the IM by the LolCDE ABC transporter complex (Yakushi, Masuda et al. 2000). Lipoproteins interact with the integral membrane subunits LolC and LolE, while the ATPase subunit, LolD, homodimer provides energy for transfer of lipoproteins from LolC and E to the periplasmic chaperone, LolA (Narita and Tokuda 2010).

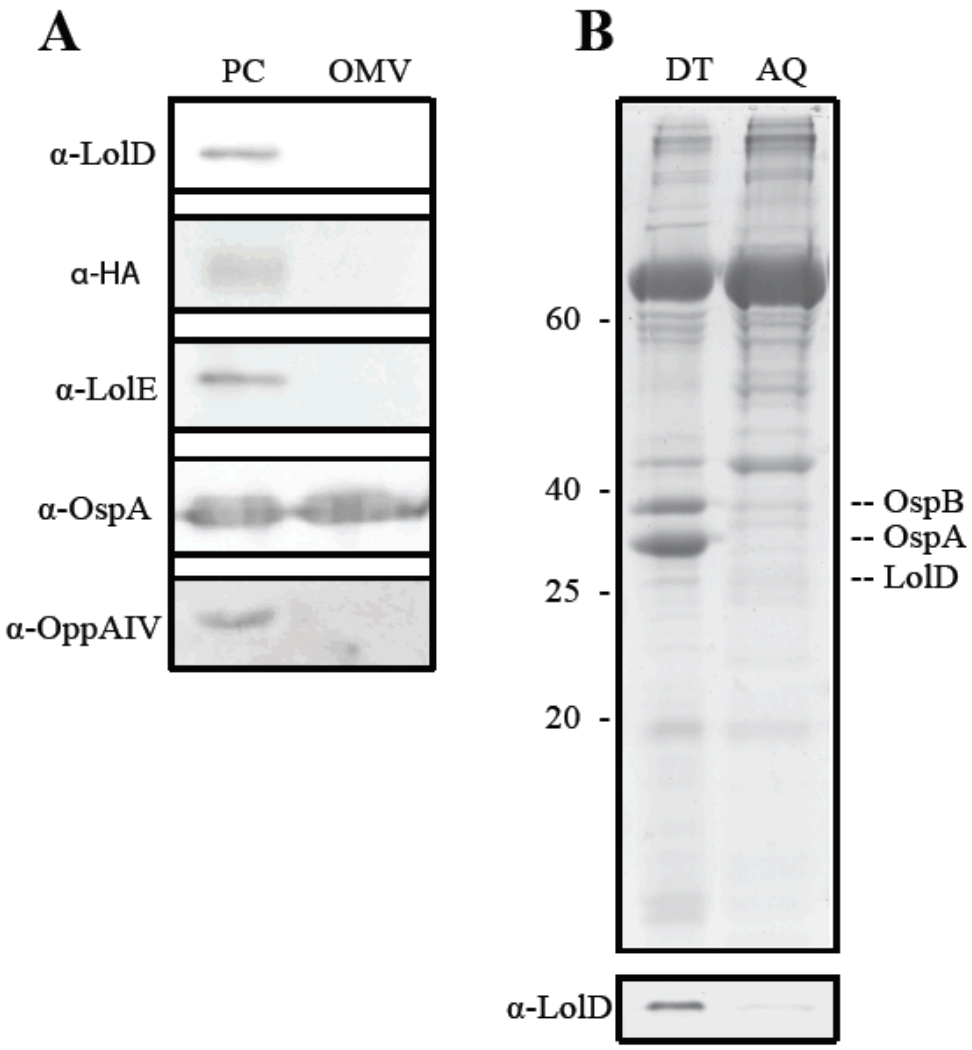
Identification of the B. burgdorferi Lol homologs

Using PSI-BLAST search algorithms, we identified a *B. burgdorferi* strain B31 chromosomal locus spanning ORFs BB0078/79, BB0080, and BB0081 as potentially encoding for spirochetal LolC, -D, and –E homologs, respectively. Interestingly, *E. coli* LolC showed N-terminal and C-terminal similarity to the potential BB0078 and BB0089 polypeptides, respectively. We therefore re-sequenced the locus and found that this ORF split was caused by an erroneous frame-shift in an A stretch in the published *B. burgdorferi* B31 genome sequence (Fraser, Casjens et al. 1997) (GenBank accession number AE000783) and that BB0078 and -79 indeed form a single open reading frame (J. Liu and W.R. Zückert, unpublished; GenBank accession number AF492471). *B. burgdorferi* LolCDE (BB0078/79, BB0080, BB0081) share 19% , 40% and 22% amino acid identity with their respective *E. coli* homologs.

Consistent with their homology to integral membrane subunits of an IM ABC transporter, *B. burgdorferi* LolC and E are both predicted to have multiple transmembrane domains, while LolD has no predicted transmembrane helices (TMHMM version 2.0, www.cbs.dtu.dk/services/ TMHMM/). Therefore, LolD membrane association was analyzed by triton X-114 phase partitioning in cells overexpressing the LolCDE locus under the control of the constitutive *B. burgdorferi* FlaB promoter. Detergent soluble and aqueous fractions were separated by SDS-PAGE. Subsequent immunoblot analysis revealed that LolD fractionated predominantly with the detergent soluble proteins (Fig. 9B), consistent with its homology to the cytoplasmic subunit of an IM complex.

To determine the cellular localization of the LolCDE homologs, *B. burgdorferi* B31e2 cells overexpressing the LolCDE locus were subjected to membrane fractionation resulting in protoplasmic cylinder (PC; cytoplasm, inner membrane and whole cells) and outer membrane vesicles (OMV). Cells were treated with a hypotonic citrate buffer to release OMVs, and OMV and PC fractions were subsequently fractionated on an isopycnic sucrose gradient. LolC, D, and E were all found exclusively in the PC fraction (Fig. 9A). Together, these data suggest IM localization of the *B. burgdorferi* LolCDE homologs. Attempts to purify the complex using metal affinity chromatography and immunoprecipitation were unsuccessful.

Figure 9. Membrane localization of the *B. burgdorferi* LolCDE homologs. (A) Immunoblot analysis of protoplasmic cylinder (PC) and outer membrane vesicle (OMV) fractions in *B. burgdorferi* B31e2 cells overexpressing LolCDE. LolC was detected using an antibody specific for an amino-terminal hemagglutinin (HA) tag (Sigma). (B) Coomassie-stained SDS-PAGE and immunoblot analysis of aqueous (AQ) and detergent (DT) of *B. burgdorferi* cells. Size markers indicated in kilodaltons.


A
PC
OMV
α-LolD
α-HA
α-LolE
α-OspA
α-OppAIV
B
DT
AQ
60 -
40 -
25 -
20 -
-- OspB
-- OspA
-- LolD
α-LolD

Inducible expression of a B. burgdorferi LolD mutant

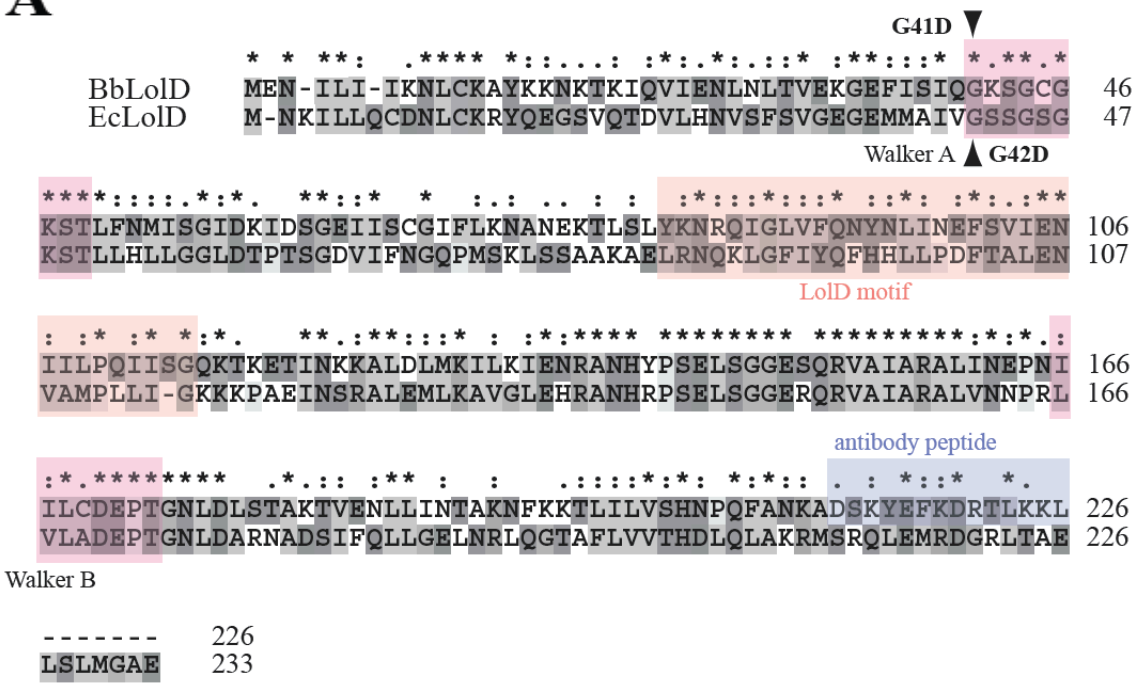
Based on gene similarity of BB0078/79, BB0080, BB0081 with *E. coli* LolCDE, we decided to examine whether the *B. burgdorferi* homologs have similar function in lipoprotein trafficking. BB0080, like all LolD homologs, contains the Walker A, Walker B, and ABC signature motifs characteristic of ABC transporter proteins, as well as the highly conserved LolD motif (Yakushi, Masuda et al. 2000) (Fig. 10A). Previous studies in *E. coli* showed that changing the first glycine of the LolD Walker A motif to an aspartic acid resulted in a dominant-negative block in the Lol pathway. *E. coli* $LolD_{G42D}$ was not able to bind ATP, and therefore was not functional in releasing lipoproteins from proteoliposomes (Yakushi, Masuda et al. 2000).

To analyze the effect of an inactive Lol pathway on surface and subsurface lipoprotein localization in *Borrelia*, the corresponding point mutation was made in the *B. burgdorferi* LolD homolog to create $LolD_{G41D}$ (Fig. 10A). Consistent with the essential function of Lol proteins, expression of $LolD_{G41D}$ from the constitutive *B. burgdorferi* FlaB promoter did not result in viable transformants (data not shown), suggesting that constitutive overexpression of the mutant protein was toxic to the spirochetes. Therefore, $LolD_{G41D}$ was expressed in *B. burgdorferi* under the control of the tetracycline-inducible, hybrid *B. burgdorferi* ospA-tetO expression system, P_{ost} (Whetstine, Slusser et al. 2009). *B. burgdorferi* B31e2 cells harboring P_{ost}–driven $LolD_{G41D}$ were cultured in the presence or absence of anhydrotetracycline (ATc). Western blot analysis reveled no expression of

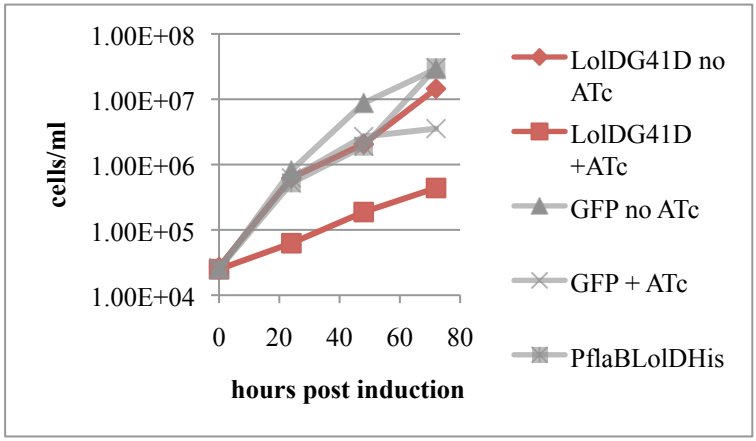
His-tagged $LolD_{G41D}$ in the absence of ATc. In the presence of 2 μg/ml ATc, expression of $LolD_{G41D}$ was detected at w.t. LolD levels at 30 min post-induction, and at five-fold higher than w. t. levels at 24 h post-induction (Fig. 10B and data not shown).

Figure 10. Effect of $LolD_{G41D}$ expression on *B. burgdorferi* cell growth. (A) Alignment of *E.coli* LolD and *B. burgdorferi* LolD (BB0080). The conserved Walker A, Walker B, and LolD motifs are indicated as well as the C-terminal sequence used to generate a peptide antibody against BB0080. Identical (*), very similar (:) and similar (.) amino acid residues are indicated. (B) Growth curve of *B. burgdorferi* expressing $LolD_{G41D}$ from the anhydrotetracycline (ATc)-inducible P_{ost} promoter. pCRK53 expressing P_{ost}-driven GFP (Whetstine, Slusser et al. 2009) was used as a control. **(C)** Average change in growth of $LolD_{G41D}$ expressing cells relative to uninduced cells. Error bars represent standard deviation. n = 11.

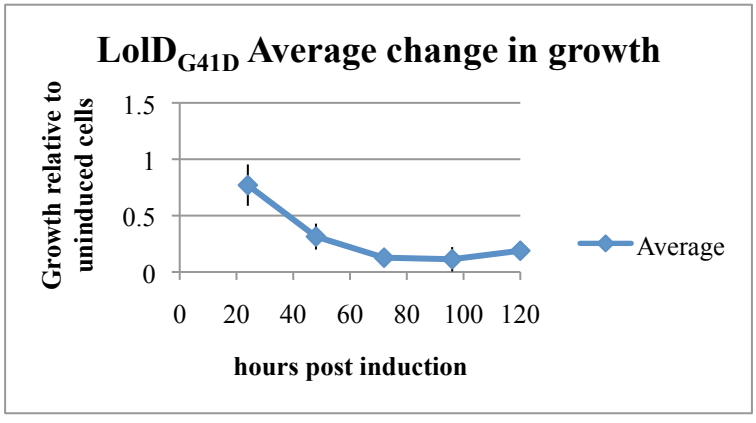
A


G41D
BbLolD MEN-ILI-IKNLCKAYKKNKTKIQVIENLNLTVEKGEFISIQGKSGCG 46
EcLolD M-NKILLQCDNLCKRYQEGSVQTDVLHNVSFSVGEGEMMAIVGSSGSG 47
Walker A
G42D
KSTLFNMISGIDKIDSGEIISCGIFLKNANEKTLSLYKNRQIGLVFQNYNLINEFSVIEN 106
KSTLLHLLGGLDTPTSGDVIFNGQPMSKLSSAAKAELRNQKLGFIYQFHHLLPDFTALEN 107
LolD motif
IILPQIISGQKTKETINKKALDLMKILKIENRANHYPSELSGGESQRVAIARALINEPNI 166
VAMPLLI-GKKKPAEINSRALEMLKAVGLEHRANHRPSELSGGERQRVAIARALVNNPRL 166
antibody peptide
ILCDEPTGNLDLSTAKTVENLLINTAKNFKKTLILVSHNPQFANKADSKYEFKDRTLKKL 226
VLADEPTGNLDARNADSIFQLLGELNRLQGTAFLVVTHDLQLAKRMSRQLEMRDGRLTAE 226
Walker B
226
LSLMGAE 233

B


1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
cells/ml
0
20
40
60
80
hours post induction
LolDG41D no ATc
LolDG41D +ATc
GFP no ATc
GFP + ATc
PflaBLolDHis

C


LolD$_{G41D}$ Average change in growth
Growth relative to uninduced cells
1.5
1
0.5
0
0
20
40
60
80
100
120
hours post induction
Average

Expression of LolD$_{G41D}$ causes a growth defect in B. burgdorferi

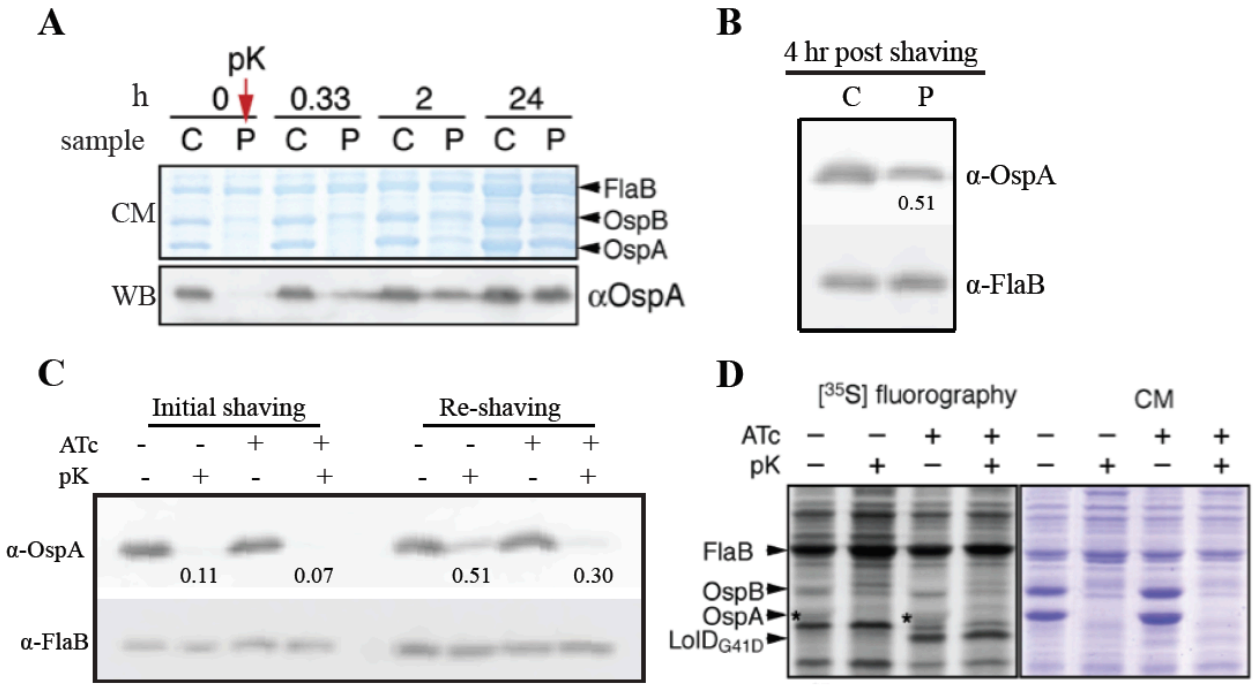
In *E.coli*, expression of an inactive LolD mutant resulted in a growth defect (Yakushi, Masuda et al. 2000). To analyze the effect of conditional LolD$_{G41D}$ expression on cell viability, *B. burgdorferi* B31e2 cells carrying LolD$_{G41D}$ under the control of the P*ost* promoter were grown to 1x10^5 cells/ml and incubated in the presence or absence of 2 μg/ml ATc. Induction of LolD$_{G41D}$ mutant expression resulted in a growth defect relative to uninduced cells (Fig. 10B). The growth defect was evident 24 h post-induction and further increased between 36 and 72 h post-induction. As controls, we determined that neither P$_{ost}$-driven expression of green fluorescent protein (GFP) nor P$_{flaB}$-driven constitutive expression of w.t. LolD affected growth (Fig. 10B).

Inactivation of LolD has no detectable effect on OspA localization and abundance

In *E. coli,* the growth defect resulting from expression of a LolD mutant was due to the toxic inner membrane accumulation of the most abundant OM lipoprotein, Lpp (Yakushi, Masuda et al. 2000). Due to the mislocalization of OM lipoproteins in an *E.coli* LolD mutant, we sought to determine what effect expression of a LolD mutant would have on the protein composition of the *B. burgdorferi* OM. One of the most abundant OM lipoproteins in *B. burgdorferi* is outer surface protein A (OspA). If a functional Lol pathway is necessary for OspA transport, then expression of LolD$_{G41D}$ should cause IM build up of OspA and less OspA should be found on the surface.

Surface proteolysis of intact cells was used to determine the effect of an inactive Lol pathway on surface localization of OspA. Preliminary studies using cells expressing $LolD_{G41D}$ for 12 h showed that protease accessibility of OspA was identical in induced and uninduced cells. However, since $LolD_{G41D}$ expression was induced in the context of an intact membrane with properly localized lipoproteins, it may be difficult to distinguish between lipoproteins transported before and after $LolD_{G41D}$ expression. Therefore, to examine surface localization of lipoproteins transported exclusively under $LolD_{G41D}$ inducing conditions, we employed a "pre-shaving" proteolysis technique: First, intact cells were treated with protease to remove surface exposed proteins including OspA. Second, cells were washed and allowed to recover in growth medium under either inducing or non-inducing conditions. Finally, the cells were treated again with protease to probe for reappearance of proteins on the bacterial surface. In wild type cells, whole cell OspA levels were restored to 50 % at 2 h and to 95 % at 24 h post shaving (Fig. 11A). Surface OspA levels were restored to 50 % at 4h post shaving (Fig. 3B). Therefore, cells carrying $P_{ost}LolD_{G41D}$ were induced for 36 h with ATc, treated with protease, allowed to recover for 4 to 24 h after the initial shaving and then treated to a second round of proteolysis. To our surprise, OspA protease accessibility in cells expressing $LolD_{G41D}$ was indistinguishable from that in uninduced cells (Fig. 11C). This suggested that LolD was not required for surface lipoprotein localization in *B. burgdorferi*.

Figure 11. Secretion of OspA in the presence of LolDG41D. (A) Kinetics of Osp secretion. Cells were proteolytically shaved at t =0 with proteinase K (pK), washed extensively and allowed to recover in BSK-II growth medium. Samples were assayed for the reappearance of OspA at the indicated time points. Whole cell proteins were fractionated by SDS-PAGE, stained with Coomassie Blue (CM) or immunoblotted (WB). P, pK treated sample; C, untreated control sample. (B) Protease accessibility assay to determine OspA surface localization in wild type cells 4 hours after initial shaving. Western immunoblots of OspA and FlaB. The constitutively expressed, periplasmic protein, FlaB, acts as a loading control. (C) Protease accessibility of OspA in the presence of inactive LolD. Western immunoblots of OspA and FlaB. B31-e2 cells carrying P_{ost}lolD$_{G41D}$ induced with ± ATC for 36 h and subsequently treated with proteinase K for one hour (initial-shaving) to remove existing surface OspA. pK treated cells were washed and incubated in growth medium +/- ATc for 12hrs and treated a second time with pK (re-shaving). OspA remaining after pK treatment in the presence of LolD$_{G41D}$, relative to untreated (pK-) cells and adjusted to FlaB loading, is indicated. (D) Cellular proteins were metabolically labeled for 2 h with [35S]-Met/Cys at 36 h post-induction of LolD$_{G41D}$ expression and then subjected to surface proteolysis. The fluorograph and corresponding Coomassie-stained gel (CM) are shown. Asterisks indicate the weakly labeled OspA band.


A
pK
h
0
0.33
2
24
sample
C P C P C P C P
CM
WB
FlaB
OspB
OspA
αOspA
B
4 hr post shaving
C P
α-OspA
0.51
α-FlaB
C
Initial shaving
Re-shaving
ATc - - + + - - + +
pK - + - + - + - +
α-OspA
0.11 0.07 0.51 0.30
α-FlaB
D
[35S] fluorography
CM
ATc − − + + − − + +
pK − + − + − + − +
FlaB
OspB
OspA
LolDG41D

To confirm this finding, we employed a pulse-labeling assay. B31-e2 cells carrying P_{ost}LolD$_{G41D}$ were induced for 36 h with ATc. Cells were gently washed and reconstituted in media containing [^{35}S]-Cys/Met to metabolically label newly translated proteins. After 2 h of labeling, cells were subjected to surface proteolysis, and whole cell protein preparations were analyzed by SDS-PAGE and fluorography. As expected, overexpression of the 27 kDa LolD$_{G41D}$ was visible in induced cell lysates only. Confirming our previous experimental data, newly translated OspA remained protease accessible in the presence of LolD$_{G41D}$ (Fig. 11D). Interestingly, protein bands migrating at molecular masses of about 42 kDa and 28 kDa were reduced or missing when LolD$_{G41D}$ was expressed. Further analysis is ongoing to determine the significance of this observation.

LolD$_{G41D}$ expression decreases abundance of the periplasmic OM lipoprotein Lp6.6 and two outer membrane porins, P66 and P13

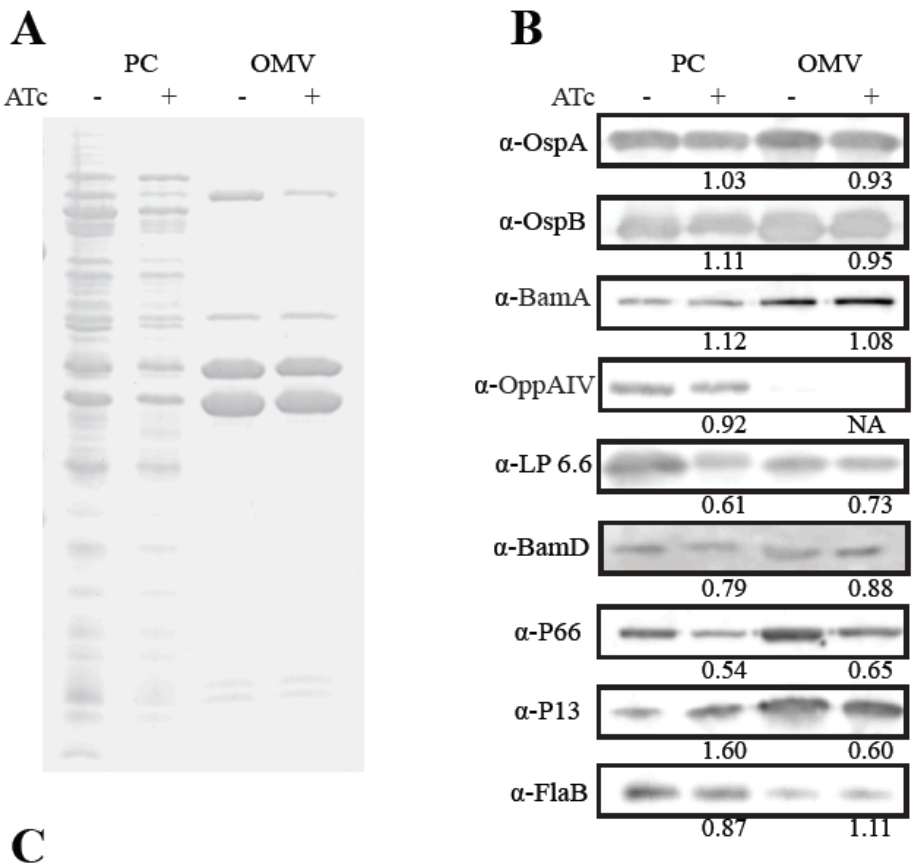
To assess whether expression of an inactive LolD mutant has an effect on subsurface OM lipoprotein transport, we examined protoplasmic cylinder (PC) and outer membrane vesicle (OMV) fractions of conditional LolD$_{G41D}$ mutant cells grown in the presence or absence of ATc. Consistent with our previous results, OspA and another major surface lipoprotein, OspB, were detected in similar proportions in the OMV fractions of induced and uninduced cells. Our model subsurface lipoprotein, Lp6.6, was decreased in both the

PC and OMV fractions. As expected, abundance of the inner membrane lipoprotein, OppAIV, was not altered (Fig. 12 B and C).

We probed samples with antibodies specific for the outer membrane porin, P66, after noticing a decrease in a protein in the induced fraction between 60 and 70 kD, visible in the coomassie stained gel (Fig. 12A). Western analysis indicated that P66 was decreased in PC and OMV fractions of $LolD_{G41D}$ expressing cells. We then probed samples for the only other known *B. burgdorferi* outer membrane porin, P13. Interestingly, P13 was enriched in the PC fraction, but appeared depleted from the OMV fraction under $LolD_{G41D}$-expressing conditions (Fig. 12 B and C).

One explanation for porins being affected by disruption of the Lol pathway is their dependence on the β-barrel assembly machinery (Bam) complex for proper insertion into the outer membrane. The Bam complex contains subsurface OM lipoproteins that may be affected by expression of the LolD mutant, therefore, indirectly affecting porin insertion. So, we next examined the affect of $LolD_{G41D}$ expression on the Bam complex. The β-barrel outer membrane protein BamA was unaffected by expression of $LolD_{G41D}$, but the subsurface Bam accessory lipoprotein, BamD, was decreased in both fractions (Fig. 12 B and C).

Figure 12. Effect of LolD mutant expression on cellular protein levels. (A) Coomassie stained SDS PAGE of protoplasmic cylinder (PC) and outer membrane vesicle (OMV) fractions of cells expressing P_{ost}-driven $LolD_{G41D}$. Size markers in kilodaltons. (B) Immunoblots of protoplasmic cylinder (PC) and outer membrane vesicle (OMV) fractions of cells expressing P_{ost}-driven $LolD_{G41D}$. Ratios of protein levels in +ATc ($LolD_{G41D}$ +) cells compared to –ATc ($LolD_{G41D}$ –) cells are indicated below the respective immunoblots. Ratios have been adjusted to FlaB loading. (C) Average change in membrane protein levels in PC and OMV fractions between +ATc ($LolD_{G41D}$ +) cells and –ATc ($LolD_{G41D}$ –) cells (n=3). Error bars represent standard deviation.

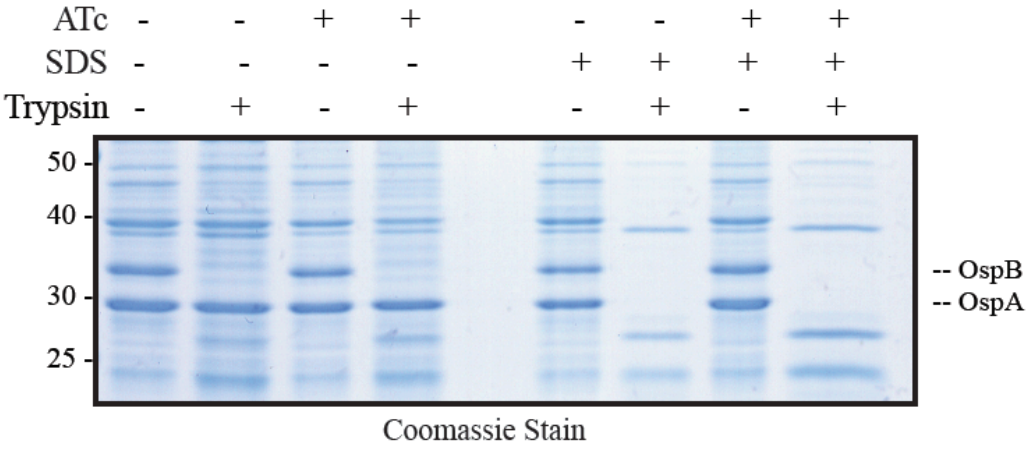

A
PC
OMV
ATc - + - +
B
PC
OMV
ATc - + - +
α-OspA
1.03 0.93
α-OspB
1.11 0.95
α-BamA
1.12 1.08
α-OppAIV
0.92 NA
α-LP 6.6
0.61 0.73
α-BamD
0.79 0.88
α-P66
0.54 0.65
α-P13
1.60 0.60
α-FlaB
0.87 1.11
C
Fold-change in protein abundance
LolDG41D induced vs. uninduced
2
1
0.5
* = p-value < 0.05
OspA-PC
OspB-PC
BamA-PC
OppAIV-PC
Lp6.6-PC
BamD-PC
P66-PC
P13-PC
OspA-OMV
OspB-OMV
BamA-OMV
Lp6.6-OMV
BamD-OMV
P66-OMV
P13-OMV

Expression of $LolD_{G41D}$ does not cause general membrane instability

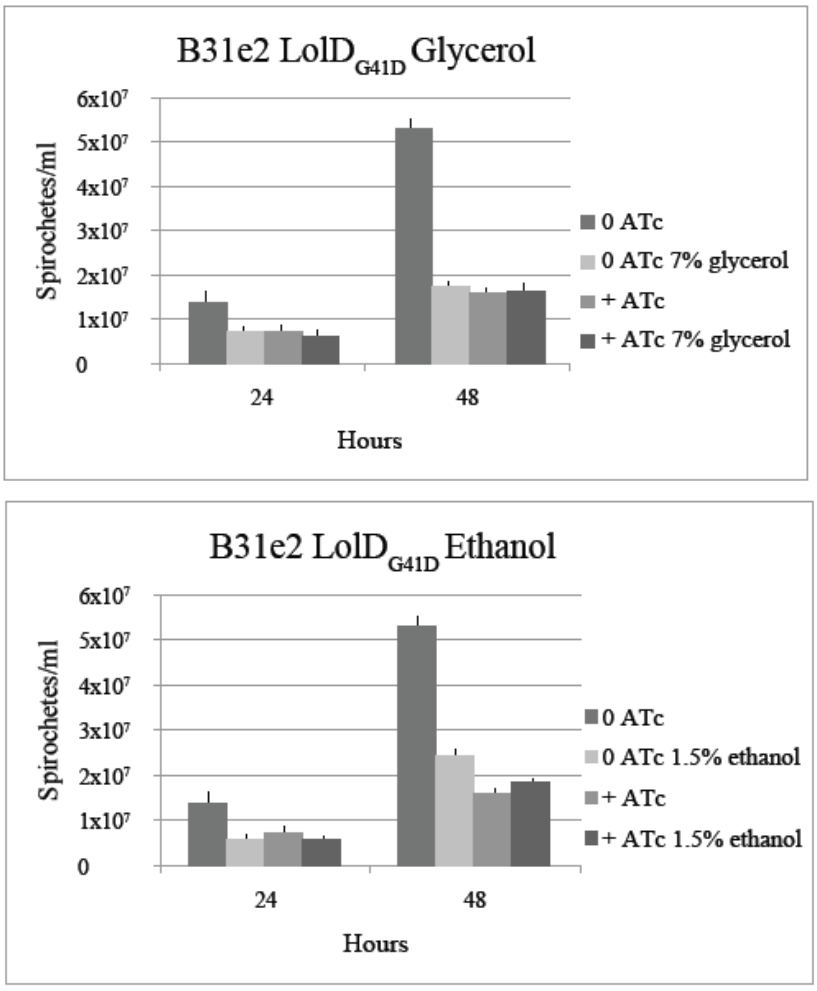
Since a change in the OM protein composition could be due to general membrane stress, we assessed the effect of $LolD_{G41D}$ on membrane integrity. OspA is resistant to trypsin cleavage in an intact membrane, but becomes accessible once cells are treated with detergent, whereas another major surface lipoprotein, OspB, is trypsin accessible (Bunikis and Barbour 1999). We used this selective trypsin accessibility to test whether expression of $LolD_{G41D}$ results in membrane disruption leading to trypsin cleavage of ospA even in the absence of detergent. Cells were treated with trypsin in the presence or absence of ATc and proteolysis of surface lipoproteins was compared in these and detergent treated cells. OspA accessibility to trypsin did not increase in the presence of an inactive LolD (Fig. 13A). To further test membrane integrity, the effect of high osmolarity under $LolD_{G41D}$ induction was analyzed. In the presence of 7 % glycerol or 1.5 % ethanol, *B. burgdorferi* growth rate was lower than that in standard BSK-II growth medium, however, induction of $LolD_{G41D}$ did not show increased sensitivity to high osmolarity (Fig. 13B).

Figure 13. Effect of $LolD_{G41D}$ on membrane integrity (A) OspA accessibility to trypsin. Coomassie-stained SDS-PAGE of B31-e2 cells carrying $P_{ost}LolD_{G41D}$ treated with trypsin in the presence or absence of SDS after 24 h induction with ± ATc. Size markers indicated in kilodaltons. (B) Susceptibility of B31e2 cells, carrying P_{ost}-driven $LolD_{G41D}$ ± ATc, to high osmolarity. Number of spirochete cells/ml of culture after growth for 24 and 48 h in the presence or absence of an osmotic stressor (7 % glycerol or 1.5 % ethanol).

A


ATc - - + + - - + +
SDS - - - - + + + +
Trypsin - + - + - + - +
50
40
30
25
-- OspB
-- OspA
Coomassie Stain

B

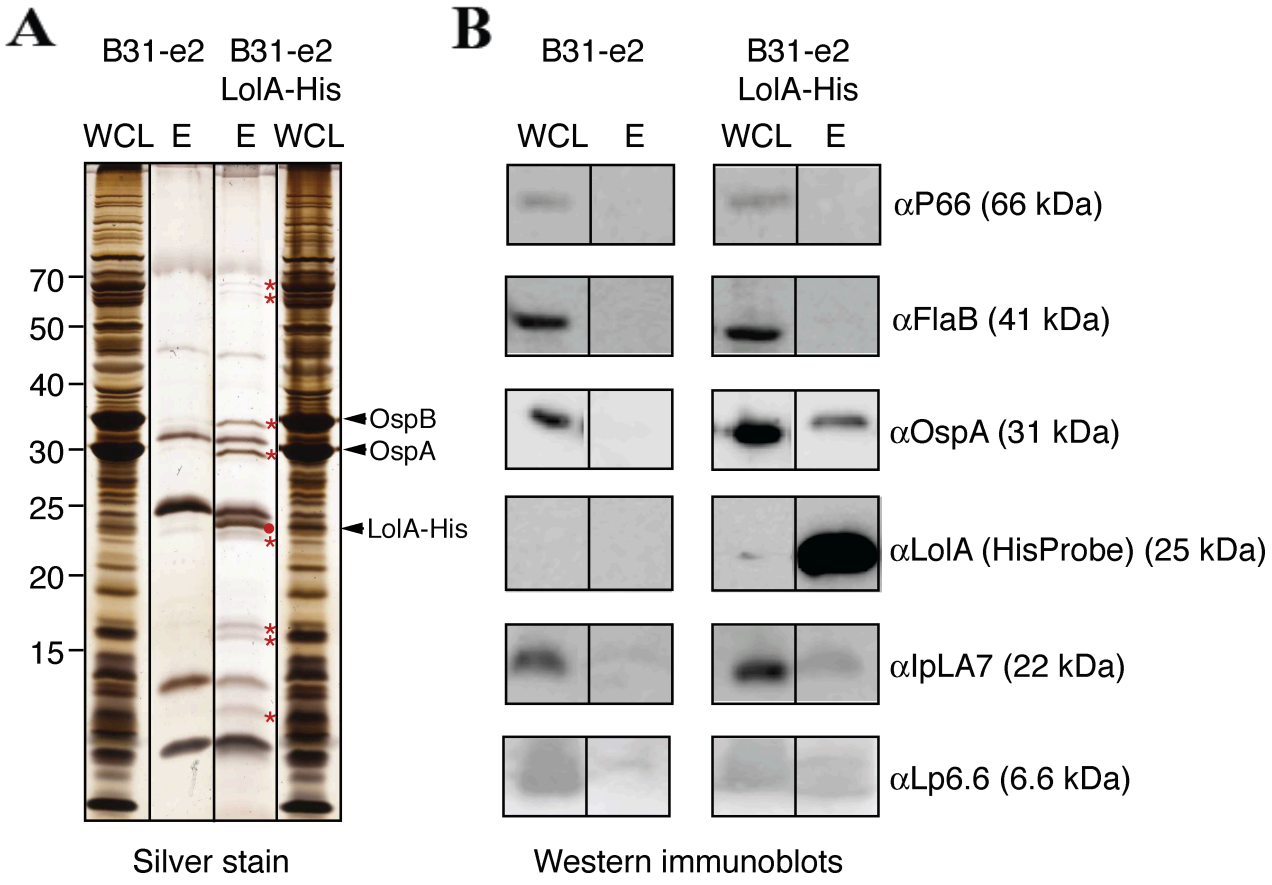

B31e2 LolD$_{G41D}$ Glycerol
Spirochetes/ml
6x10^7
5x10^7
4x10^7
3x10^7
2x10^7
1x10^7
0
24
48
Hours
0 ATc
0 ATc 7% glycerol
+ ATc
+ ATc 7% glycerol
B31e2 LolD$_{G41D}$ Ethanol
Spirochetes/ml
6x10^7
5x10^7
4x10^7
3x10^7
2x10^7
1x10^7
0
24
48
Hours
0 ATc
0 ATc 1.5% ethanol
+ ATc
+ ATc 1.5% ethanol

B. burgdorferi LolA specifically interacts with lipoproteins

In addition to homologs of the LolCDE complex, *B. burgdorferi* also encodes a homolog of the periplasmic chaperone, LolA. The *B. burgdorferi* LolA, BB0346, has 20 % amino acid identity to *E. coli* LolA. Affinity chromatography studies in *E. coli* have demonstrated that LolA specifically binds periplasmic OM lipoproteins in a 1:1 ratio and transports them to the OM for insertion (Matsuyama, Tajima et al. 1995).

To examine protein-protein interaction of the putative periplasmic chaperone, C-terminally his-tagged *B. burgdorferi* LolA was engineered and expressed in B31-e2 cells under the control of the constitutive P_{flaB} promoter. Affinity chromatography pulldown experiments exposed several proteins that co-eluted with $LolA_{His}$. Contrary to *E. coli* lolA which only binds OM lipoproteins, immunoblot analysis of the *B. burgdorferi* LolA elution fraction revealed the presence of several known inner and outer membrane lipoproteins. The OM porin P66 and the soluble periplasmic flagellar protein, FlaB did not co-elute with LolA (Fig. 14B), suggesting that the *B. burgdorferi* LolA homolog interacts specifically with lipoproteins.

Figure 14. Affinity chromatography experiments using LolA-His. Silver stained SDS-PAGE of *B. burgdorferi* B31e2 cells expressing LolA-His. The B31-e2 expression host strain was used as a background control. WCL, whole cell lysate; E, eluate from cobalt column at 250 mM imidazole, 400 mM NaCl. Red asterisks indicate protein bands specific to to the LolA-His sample. A red dot indicates the LolA-His band. Size markers indicated in kilodaltons. (B) Western immunoblots of samples shown in panel A. LolA was detected using a HisProbe (Pierce). All other proteins were detected using protein-specific antibodies. IpLA7 served as an inner membrane control. FlaB and P66 served as non-lipoprotein controls.


A
B31-e2
B31-e2 LolA-His
WCL E E WCL
70
50
40
30
25
20
15
OspB
OspA
LolA-His
Silver stain
B
B31-e2
B31-e2 LolA-His
WCL E WCL E
αP66 (66 kDa)
αFlaB (41 kDa)
αOspA (31 kDa)
αLolA (HisProbe) (25 kDa)
αlpLA7 (22 kDa)
αLp6.6 (6.6 kDa)
Western immunoblots

Discussion

We previously hypothesized that a partial Lol pathway consisting of LolCDE and LolA directs all *B. burgdorferi* OM lipoproteins to the outer membrane (Bergström and Zückert 2010). However, our most recent data challenge this assumption. P_{ost}-driven conditional overexpression of a $LolD_{G41D}$ ATPase Walker A motif mutant in *B. burgdorferi* led to a significant growth defect, predicted to be due to a dominant negative effect on the function of the LolCDE complex in releasing OM lipoproteins from the inner membrane. The fate of newly expressed OspA in the presence of $LolD_{G41D}$ was tracked by lipoprotein localization assays after prior proteolytic removal of already surface-exposed OspA or through radiolabeling with [^{35}S]- Cys/Met. To our surprise, both approaches failed to detect any appreciable OspA localization defect. Instead, we observed significant mislocalization and/or destabilization of the periplasmic leaflet OM lipoprotein Lp6.6 and the porins P66 and P13.

P66 and P13 are two of the few β-barrel outer membrane proteins (OMPs) in *B. burgdorferi* and the only two confirmed OM porins (Bergström 2010). P66 is a well characterized porin, which also acts as an adhesin with β-3 integrin binding capability (Skare, Mirzabekov et al. 1997; Coburn, Chege et al. 1999). P13 is an essential porin with unknown substrate specificity, for which there are eight paralogs in *B. burgdorferi* (Pinne, Denker et al. 2006; Bergström 2010).

Total cellular P66 levels declined during $LolD_{G41D}$ expression, likely due to periplasmic degradation of misfolded intermediates. This observation is consistent with the fate of the *Pseudomonas aeruginosa* porin, OprF, in response to depletion of the Lol pathway (Wada, Matsuyama et al. 2004). Interestingly, overall P13 levels rose in $LolD_{G41D}$ expressing cells, possibly in an attempt to compensate for the mislocalization of this essential protein (Ostberg, Pinne et al. 2002; Pinne, Ostberg et al. 2004). It is possible that $LolD_{G41D}$ mediated OM lipoprotein mislocalization could lead to general membrane instability, causing disruption of OM porins. However, no defect in overall membrane integrity was observed in assays for cell sensitivity to osmotic stressors.

The effect of the conditional LolD mutant on OM porins could be indirect, since lipoproteins are known to play a role in periplasmic transport and outer membrane insertion of OMPs (Collin, Guilvout et al. 2011). *B. burgdorferi* OMPs likely depend on a functional β-barrel assembly machinery (Bam) complex for proper outer membrane insertion, as shown for P66 (Lenhart and Akins 2010). The Bam complex consists of an integral β-barrel protein, BamA, and four accessory lipoproteins, BamBCDE (Vanini, Spisni et al. 2008). Disruption of the Lol pathway in other organisms has been shown to disrupt localization of Bam lipoproteins (Wu, Malinverni et al. 2005; Malinverni, Werner et al. 2006; Sklar, Wu et al. 2007). Therefore, $LolD_{G41D}$ mediated disruption of *B. burgdorferi* OM porin localization may be due to mislocalization/destabilization of periplasmic OM Bam lipoprotein homologs that are predicted to be associated with the Bam complex (Lenhart and Akins 2010).

We detected a decrease in the abundance of BB0324 (the proposed BamD homolog) in PC and OMV fractions of $LolD_{G41D}$ expressing cells, although not to the same degree as the subsurface lipoprotein Lp6.6. BamD is essential in *E.coli* and proposed to be essential in *B. burgdorferi* based on transposon mutagenesis studies. Therefore, expression of the LolD mutant may have a greater effect on BB0324 than is apparent, but *B. burgdorferi* may be compensating by an increase in expression of the potentially essential protien.

The $LolD_{G41D}$ phenotype appears to be at odds with the observed interaction of C-terminally his-tagged LolA with OspA. However, we are inclined to attribute this finding to the relative abundance of OspA in whole cell lysates, which might lead to the non-specific binding of OspA's acyl chains to the overexpressed LolA. The observed background of IM lipoprotein IpLA7 in the pulldown supports this conclusion. Yet, LolA appears to interact specifically with lipidated proteins, as indicated by the lack of interaction with FlaB and P66.

Here we demonstrate that the *B. burgdorferi* LolCDE homologs all localize to the IM as predicted. However, were unable to purify the complex using protocols previously demonstrated for *E. coli* (Yakushi, Masuda et al. 2000; Ito, Kanamaru et al. 2006). The level of LolCDE overexpression from promoters available for use in *Borrelia* (pFlaB) is lower than that available in *E. coli* studies (pBad, tacPO). This resulted in low abundance

of bait protein, likely contributing to the ineffectiveness of pulldown assays. Perhaps differences in *Borrelia* membrane composition (Bergström 2010) also hampered our efforts, making protein solubility optimization unsuccessful.

Our results indicate that the IM LolCDE complex is not involved in transport of lipoproteins to the surface of *B. burgdorferi*. This is consistent with the previous finding that, in contrast to other gram-negative bacteria, an aspartic acid immediately following the lipidated Cys does not act as an inner membrane retention (Lol avoidance) signal for *B. burgdorferi* lipoproteins (Schulze and Zückert 2006). Furthermore, *B. burgdorferi* OspA expressed in *E. coli* is localized to the inner membrane even in the absence of a Lol avoidance signal, suggesting that OspA is not recognized by the Lol pathway (Zückert 2010). In contrast, the *Klebsiella pneumoniae* surface lipoprotein, PulA, which is secreted by the T2SS, is transported by the Lol pathway when expressed in *E. coli* and follows established Lol lipoprotein sorting signals (Pugsley and Kornacker 1991). Future research is needed to determine how surface lipoproteins are extracted from the inner membrane in a LolCDE independent manner in *Borrelia*. Inner membrane release and outer membrane translocation of *B. burgdorferi* surface lipoproteins appear to be mediated by separate amino-terminal determinants (Schulze, Chen et al. 2010). The mechanism of subsurface lipoprotein insertion into the outer membrane in the absence of a LolB homolog remains unclear.

Chapter IV: Localization of IpLA7 in *B. burgdorferi*

Published in collaboration with studies on the regulation of IpLA7 expression (von Lackum et al., 2007).

Abstract

The *Borrelia burgdorferi* lipoprotein IpLA7 is a major immunogenic protein identified in late-stage Lyme disease. The function of IpLA7 is unknown and previous studies reported mixed results as to its cellular localization. Here, we show that IpLA7 is not surface-exposed. Instead, the proteins localizes primarily to the borrelial inner membrane. These data are consistent with the ability of these bacteria to produce IpLA7 throughout mammalian infection despite the protein's ability to elicit a robust immune response.

Introduction

Throughout their life cycle, *Borrelia burgdorferi* express a large number of lipoproteins that play major roles in survival and pathogenesis (Steere, Malawista et al. 1977; Haake 2000). Many of the predominant immunogens identified from sera of Lyme disease patients are lipoproteins (Brandt, Riley et al. 1990), including the 22 kDa lipoprotein,

IpLA7. IpLA7 was named due to its recognition by the monoclonal antibody, LA7 (Wallich, Simon et al. 1993) and has also been called 'P22' and 'LA7' in the literature (Lam, Nguyen et al. 1994; Nowalk, Nolder et al. 2006) *B. burgdorferi* express IpLA7 throughout mammalian infection, as well as during transmission both from feeding ticks to mice and from infected mice to feeding ticks (Von Lackum, Ollison et al. 2007). IpLa7 is encoded on the *B. burgdorferi* linear chromosome by open reading frame (ORF) BB0365. Homologues of BB0365 are conserved among Lyme disease spirochetes, but not found in other borrelial species (Simpson, Schrumpf et al. 1991).

IpLA7 was determined to be a lipoprotein based on the presence of a cleavable N-terminal signal sequence, the lipoprotein consensus sequence (lipobox) and lipid modification (Lam, Nguyen et al. 1994). The function of IpLa7 is unknown, and previous reports listed conflicting results of localization studies of the lipoprotein within the bacterium (Grewe & Nuske, 1996; Simpson et al., 1991; Wallich et al., 1993). Lipoproteins in *B. burgdorferi* are anchored in the periplasmic leaflet of the inner membrane, periplasmic leaflet of the outer membrane, or surface leaflet of the outer membrane.

Here, we found IpLA7 to be localized to the periplasmic space based on immunofluoresence assay (IFA) of intact and permeabilized cells as well as by protease susceptibility assays. Subsequent membrane fractionation and immunoblot blot analysis of inner and outer membrane fractions using anti-IpLA7 antibodies revealed that IpLA7

is localized to the inner membrane. These results were published in collaboration with studies on the regulation of IpLA7 expression (von Lackum *et al*., 2007).

Results

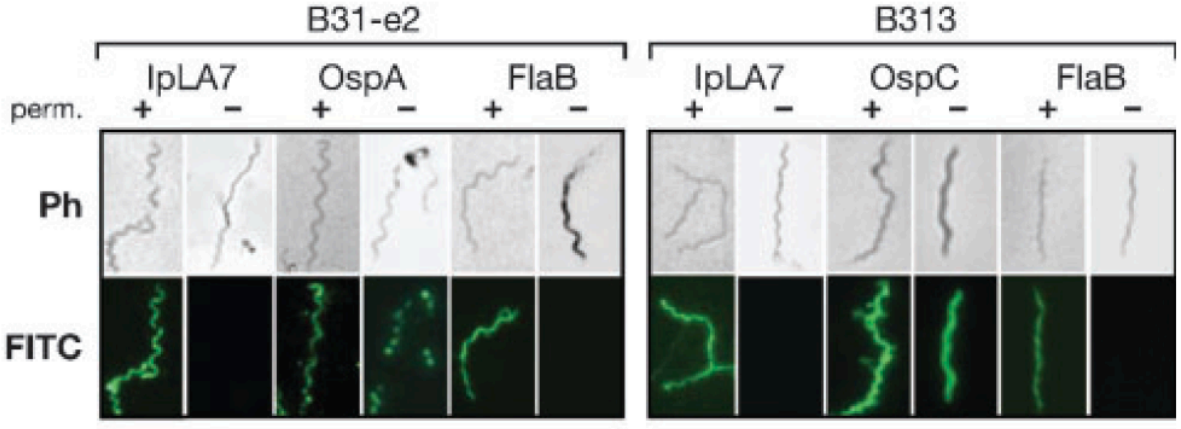
IpPLA7 is not exposed to the borrelial outer surface

Accurate knowledge of the cellular localization of IpLA7 is extremely important for studies of its function. Given the contradictory results of previous studies, we sought to answer the question of IpLA7 localization using several different, established lipoprotein localization techniques.

As a first step, intact *B. burgdorferi* were examined for their ability to bind IpLA7-directed antibodies. Binding would be indicative of IpLA7 surface-exposure. Some abundant *B. burgdorferi* surface proteins physically block interactions between antibodies and other *borrelial* surface proteins (Sadziene, Thomas et al. 1995; Bunikis and Barbour 1999; Noppa, Ostberg et al. 2001). Therefore, one complication to this assay could be the masking of IpLA7 epitopes by other surface proteins. For that reason, we examined both B31-e2, a subculture of strain B31 that expresses abundant surface proteins such as outer surface protein A (OspA), and B313, which produces very few surface proteins. Intact and permeabilized *B. burgdorferi* were examined by IFA using antibodies directed

against IpLA7, the periplasmic FlaB protein, and either of two surface-exposed proteins, OspA and OspC. Regardless of the strain examined, unfixed, non-permeabilized bacteria were unable to bind IpLA7 directed antibodies (Fig. 15) FlaB was also inaccessible, while surface OspA or OspC were readily detected. As a control, bacteria permeabilized by methanol were also examined by IFA, which confirmed that the examined spirochetes did indeed produce IpLA7 and the other proteins.

Figure 15. **Subsurface localization of IpLA7**. IFA of *B. burgdorferi* B31 clones B31-e2 and B313, either unpermeabilized (-) or permeabilized with methanol (+), using polyclonal antiserum specific for IpLA7. As controls, similarly treated bacteria were examined by IFA using antibodies specific for the periplasmic FlaB and the surface-exposed OspA or OspC proteins. Ph, phase-contrast; FITC, epifluorescence using a FITC filter.


B31-e2
B313
IpLA7
OspA
FlaB
IpLA7
OspC
FlaB
perm.
+ − + − + − + − + − + −
Ph
FITC

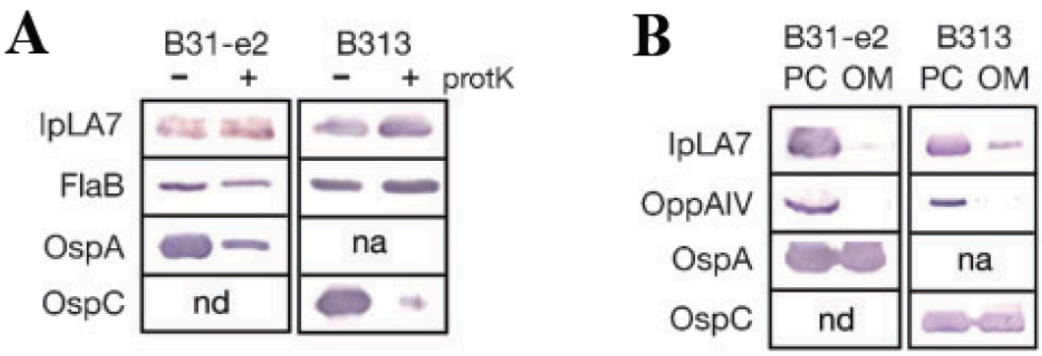
Next, we examined whether IpLA7 was susceptible to in situ proteolysis. Surface-exposed proteins of intact bacteria are degraded by proteases, whereas subsurface proteins are protected from proteolysis. As reported previously (Wallich, Simon et al. 1993), proteinase K (pK) was unable to degrade IpLA7 from intact B31 bacteria (Fig. 16A). However, to ensure that IpLA7 is not simply shielded from proteases by abundant surface proteins, we also tested *B. burgdorferi* strain B313 (lacking many surface proteins) for susceptibility of its IpLA7 to protease degradation. Proteases were also unable to digest IpLA7 from intact, live B313 bacteria (Fig. 16A), further strengthening the conclusion that IpLA7 is not exposed to the borrelial outer surface.

IpLA7 is localized to the periplasmic leaflet of the inner membrane in B.burgdorferi.

To determine whether subsurface IpLA7 is tethered to the periplasmic leaflet of the inner or outer membrane, we subjected *Borrelia* to membrane fractionation. Inner and outer membranes of cultured *B. burgdorferi* were purified and analyzed for presence of IpLA7. Immunoblots of subcellular fractions showed that the protein was primarily associated with the inner-membrane-enriched/protoplasmic cylinder fractions, although some was evident in outer-membrane fractions (Fig. 16B). A control marker for purity of membrane preparations, OppAIV (Bono, Tilly et al. 1998) was found only in inner-membrane fractions. The outer-surface proteins OspA or OspC were found in both the outer-membrane- and inner-membrane-enriched/ protoplasmic cylinder fractions, probably due to inclusion of intact bacteria in the latter fraction (Radolf, Goldberg et al. 1995). These

data lead us to conclude that the IpLA7 lipoprotein is primarily tethered to the inner membrane.

Figure 16. Subcellular localization of lpLA7. (a) Immunoblot analyses of lysates of *B. burgdorferi* incubated in situ with (+) or without (-) proteinase K (protK). (b) Immunoblot analyses of protoplasmic cylinder (PC) and outer membrane vesicle (OM) fractions of *B. burgdorferi*. OspA or OspC served as outer-membrane controls, and OppA-IV served as the inner-membrane control (Schulze and Zückert 2006). Note that PC fractions also contain some intact cells, which explains significant amounts of OspA and OspC in those fractions. na, Not applicable: clone B313 lacks the plasmid encoding OspA (Sadziene et al., 1995); therefore OspC was used as surface control for that strain. nd, Not detected.


A
B31-e2
B313
- + - + protK
lpLA7
FlaB
OspA
OspC
nd
na
B
B31-e2
B313
PC OM PC OM
lpLA7
OppAIV
OspA
OspC
nd
na

Discussion

The 22kDa lipoprotein IpLa7 is unique to the Lyme disease *Borrelia* genome and may play a role important to the pathogenesis of Lyme disease. The function of IpLA7, however, is unknown. In order to aid in the characterization of this immunogenic protein, we studied its cellular localization. IpLA7 was found to be subsurface, and localized to the *B. burgdorferi* IM. Subsurface localization is consistent with the ability of *B. burgdorferi* to continually express this antigenic protein while maintaining a persistent infection.

Our results are consistent with two previous studies that described a periplasmic localization for IpLA7 (Wallich, Simon et al. 1993; Lam, Nguyen et al. 1994). On the other hand, a third study found IpLA7 to be in the outer membrane and accessible to immunogold labeling of intact spirochetes, suggesting surface exposure of the protein (Grewe and Nuske 1996). An analysis of the methods of the latter study revealed the absence of controls for specificity and cross-reactivity of antibody-conjugated gold beads with other *Borrelia* proteins. In addition, outer membrane fractions in the Grewe and Nuske study were attained by crude cellular lysis by SDS followed by centrifugation and also lacked proper controls for known inner and outer membrane proteins. This fractionation technique is less specific than the well-referenced sucrose density gradient used here (Skare, Shang et al. 1995; Carroll, Dorward et al. 1996; El-Hage, Babb et al.

2001). Taken together, this evidence points to the validity of our conclusion that IpLA7 is an IM anchored, periplasmic lipoprotein.

Lipoproteins of *B. burgdorferi* appear to be secreted to the outer membrane by default, while some lipoproteins are retained in the inner membrane. As *B. burgdorferi* do not follow the sorting rules described for other diderm bacteria (Schulze and Zückert 2006; Tokuda 2009), the mechanism for inner membrane retention is unclear. Therefore, lipoprotein localization in the inner or outer membrane is not easily predicted by amino acid sequence and must be experimentally determined. Few inner membrane lipoproteins have been identified in *B. burgdorferi*. Among the known inner membrane lipoproteins are the five homologs (three chromosomally encoded and two plasmid encoded) of the peptide binding protein (OppA), which is part of the oligopeptide permease (Opp) system (Bono, Tilly et al. 1998). Localization of IpLA7 to the inner membrane proves useful in our understanding of its role in *Borrelia* and will be useful as an inner membrane control for localization studies of other lipoproteins.

Despite being a subsurface lipoprotein, antibodies against IpLa7 are found abundantly in serum from Lyme patients. *Borrelia*, like other spirochetes, have a fragile outer membrane (Barbour 1986; Narita and Tokuda 2010). Perhaps *Borrelia* are lysed readily in mammalian blood, exposing abundant subsurface proteins to the host immune system. Triggering of an antibody response to proteins not present on the surface of intact cells would afford live cells with the ability to go unrecognized.

Chapter V: Investigation of the *B. burgdorferi* outer membrane proteome

Abstract

Surface-anchored lipoproteins are not common in bacteria, although pathogenic spirochetes of the genus *Borrelia* have evolved to employ an abundance of surface lipoproteins throughout their life cycle. Several lipoproteins on the *Borrelia* surface are known virulence factors in Lyme disease and Relapsing Fever. However, the molecular events responsible for lipoprotein transport to the bacterial cell surface are unknown in *Borrelia*. To elucidate the protein machinery involved in lipoprotein transport, we have generated lipoprotein fusions with a carrier protein, the *E. coli* maltose binding protein (MalE), to short N-terminal lipopeptides of the surface lipoprotein, outer surface protein A (OspA). OspA-MalE fusions were targeted to the spirochaetal surface in *Borrelia burgdorferi*. We evaluated the efficacy of lipidated MalE as a protein bait in the study of lipoprotein trafficking in *B. burgdorferi*. To identify additional exported lipoproteins and integral outer membrane proteins (OMPs), which may represent important factors in the export process, we also generated a list of novel OMPs using *in silico* prediction and tested several methods to characterize the *B. burgdorferi* surface proteome.

Introduction

Surface-anchored lipoproteins in *B. burgdorferi* are necessary for transmission and persistence of Lyme disease. However, the protein machinery involved in transporting lipoproteins to the surface in *B. burgdorferi* is unknown. Lipoproteins are synthesized in the cytoplasm and targeted for export across the inner membrane via an N-terminal signal sequence that contains a lipoprotein-specific motif termed a lipobox. On the periplasmic side of the inner membrane, the signal sequence is cleaved just before the last Cys residue of the lipobox. It is upon this new N-terminal Cys residue that lipid modification occurs (Wu and Tokunaga 1986). Between the lipidated Cys and the globular protein, there is an unstructured region termed the 'tether.' The tether region varies in length and has been shown to contain all the information necessary for lipoprotein transport in *B. burgdorferi* (Schulze and Zückert 2006; Kumru, Schulze et al. 2010; Kumru, Schulze et al. 2011).

Subsurface outer membrane lipoproteins may rely on a partial Lol (localization of lipoproteins) pathway (Narita and Tokuda 2010), consisting of an inner membrane extraction complex and a periplasmic chaperone, for delivery to the outer membrane, whereas surface lipoproteins seem to follow a different pathway (Chapter III). The protein machineries and molecular mechanisms required for insertion of subsurface lipoproteins into the outer membrane as well as for translocation of lipoproteins to the

surface remain unknown. An outer membrane "flippase" is proposed to facilitate the translocation of lipoproteins from the periplasm to the cell surface (Chen and Zuckert 2011).

Herein we describe attempts to elucidate the protein machinery responsible for lipoprotein transport to the cell surface by generating lipoprotein tether fusions with the *E. coli* maltose binding protein (MalE) (OspA-malE). OspA-malE was shown to be surface exposed in *B. burgdorferi*. We evaluated lipidated malE as a protein bait in the study of lipoprotein trafficking. OspA-MalE was purified from *B. burgdorferi* cells both by two methods: affinity purification with monoclonal anti-MalE conjugated magnetic beads and affinity purification with amylose-conjugated magnetic beads. OspA-malE pull-down samples from both techniques were unsuitable for protein identification. Efforts to optimize purification conditions did not result in a specific enrichment of proteins. We also describe *in silico* identification of candidate integral outer membrane proteins. Finally, attempts to identify novel outer membrane and surface proteins of *B. burgdorferi* through protein labeling and combined surface proteolysis and membrane fractionation are described as well.

Results

Construction of lipidated MalE

To facilitate identification of outer membrane and/or surface proteins involved in *B. burgdorferi* surface lipoprotein transport, we set out to construct a reporter protein that would be processed (lipidated and transported) as a native surface lipoprotein, but that would be a useful tool for protein-protein interaction studies.

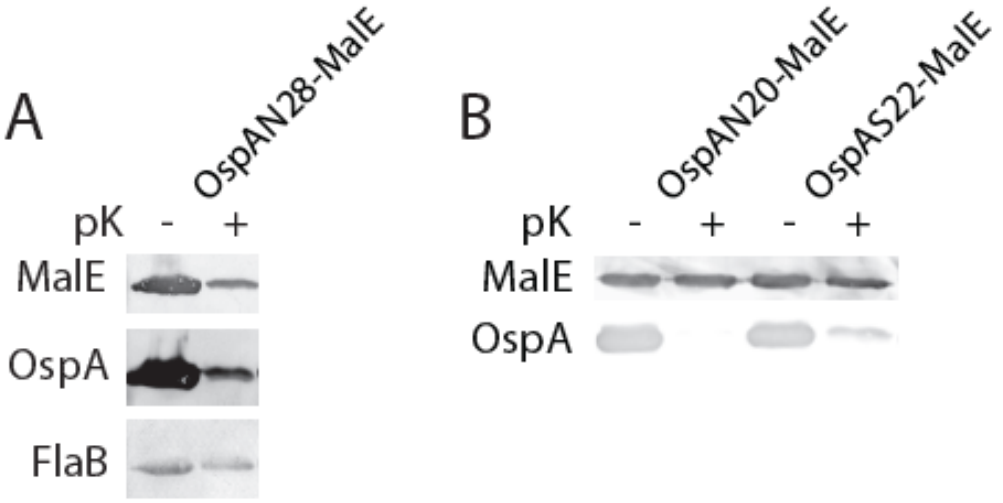
Maltose binding protein (MalE) is a commonly used carrier protein for purification of proteins. Target proteins fused to MalE can be purified from complex samples via MalE interaction with immobilized amylose or monoclonal anti–MalE antibodies (Pryor and Leiting 1997). Studies in *E.coli* (Seydel, Gounon et al. 1999) showed that fusion of the signal sequence and first four amino acids of a lipoprotein, PulA, with MBP E (MalE) was lipidated (lipoMalE) and localized to the periplasmic leaflet of the inner membrane. If the amino acid immediately following the lipidated Cys (+2 position) was mutated from Asp to Ser, lipoMalE was mislocalized to the periplasmic leaflet of the outer membrane. Indicating that lipoMalE was processed properly by all lipoprotein lipidation and transport machinery.

Successful lipoprotein-reporter fusions in *B. burgdorferi* using a major surface lipoprotein, outer surface protein A (OspA) and monomeric red fluorescent protein (mRFP) have been described. OspA-mRFP, consisting of either full length OspA or varying N-terminal tether peptides, was used to study lipoprotein sorting signals in *B.*

burgdorferi. Five N-terminal OspA residues were required to send mRFP to the surface of the cell (Schulze and Zückert 2006).

We constructed gene fusions by sequence overlap extension (SOE) PCR with varying lengths of OspA N-terminal tether peptides linked to the full length *E. coli* MalE. *ospA-malE* fusions were cloned into an *E. coli* - *B. burgdorferi* shuttle vector under the control of the constitutive *B. burgdorferi* flagellin *flaB* promoter (P_{flaB}). OspAN20, S22 and N28 contain the lipidated Cys residue (C17) plus three (N20), five (S22), or eleven (N28) additional residues respectively. To test transport of the fusion proteins, we treated whole cells, expressing either OspAN20, S22, or N28-MalE, with a membrane-impermeable, broad-spectrum protease, proteinase K (pK). As expected based on OspA-mRFP localization studies (Schulze and Zückert 2006), OspAN28-MalE was protease accessible to the same degree as wild-type (w.t.) OspA (Fig 17A), indicating successful transport to the cell surface. Also as expected, OspAN20-MalE was not surface exposed. However, contrary to OspA-mRFP, five N-terminal OspA residues were not sufficient to transport OspAS22-MalE to the *Borrelia* cell surface (Fig 17B.).

Figure 17. Surface localization of OspA-MalE fusion proteins. (A) Immunoblots of *Borrelia* B31-e2 cells expressing OspAN28-MalE, (B) OspAN20-MalE or OspAS22-MalE treated with proteinase K (pK +) or mock treated (-). OspA is used as a surface control and FlaB as a subsurface control.

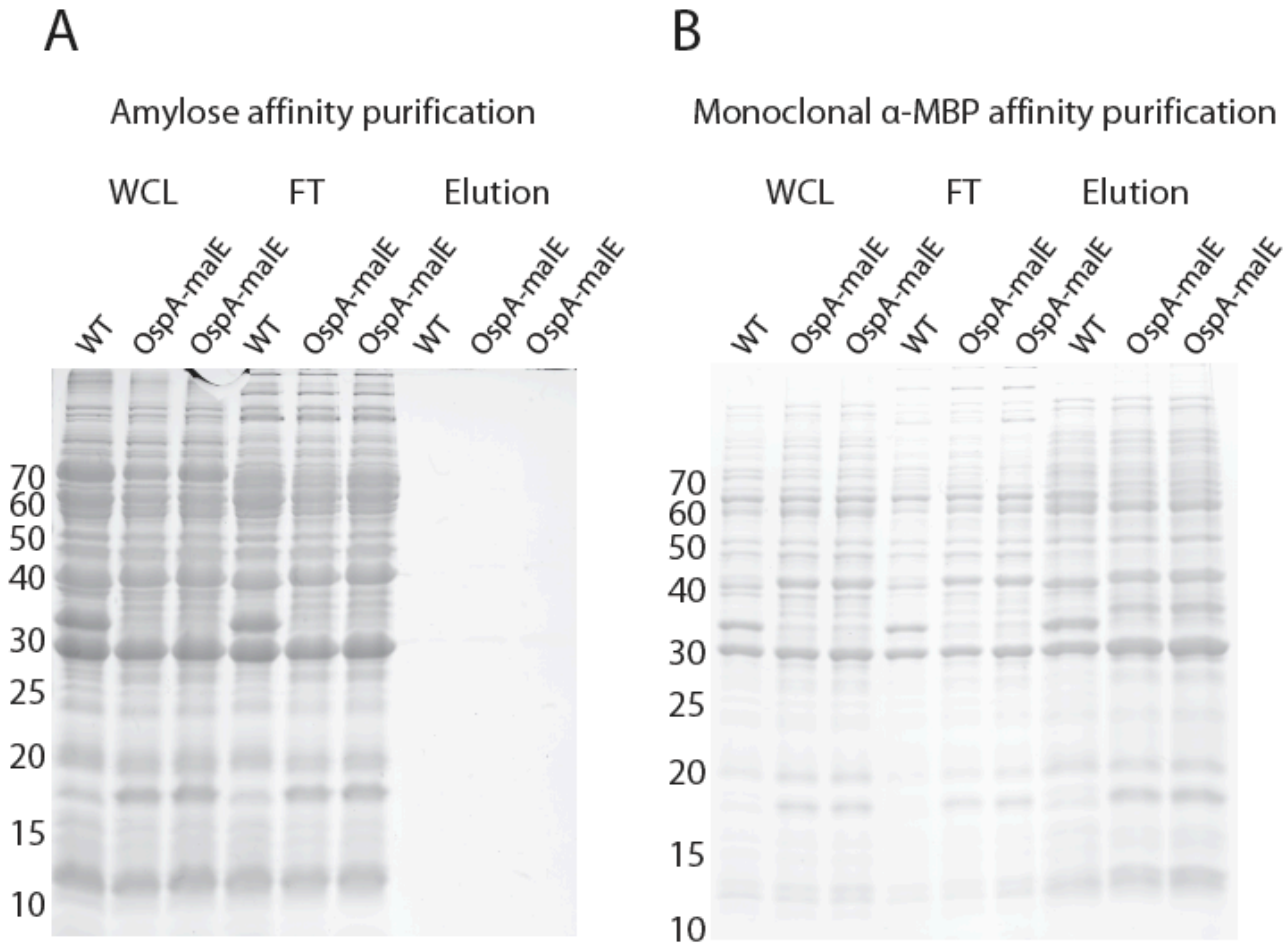

A
OspAN28-MalE
pK - +
MalE
OspA
FlaB
B
OspAN20-MalE
OspAS22-MalE
pK - + - +
MalE
OspA

OspA-MalE protein interaction

Surface-exposed OspAN28-MalE (referred to here on as OspA-MalE) was used as protein bait to detect proteins that interact with surface lipoproteins during transport from the cytoplasm to the cell surface. We attempted to isolate OspA-MalE based on its affinity for amylose and planned to subsequently analyze co-eluting proteins by mass spectrometry (MS).

B. burgdorferi cells expressing OspA-MalE were lysed and mixed with amylose-conjugated resin. OspA-MalE was eluted from the amylose resin with maltose, for which MalE has a higher affinity. Purification fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and Coomassie staining. Unexpectedly, the OspA-MalE elution fraction contained no protein, indicating that MalE was not properly adhering to the amylose resin (Fig. 18A). Altering binding buffer stringency or adding a detergent solubilization step prior to binding did not increase elution yield (data not shown). We next attempted to isolate OspA-MalE using monoclonal α-MalE antibody-conjugated resin. Cell lysates were mixed with affinity resin as before and purification fractions were analyzed by SDS PAGE. Under these conditions, the OspA-MalE elution fraction contained an abundance of non-specific background binding (Fig. 18B). We were unable to optimize conditions to specifically enrich for OspA-MalE binding.

Figure 18. OspA-MalE affinity purification. Coomassie stained SDS PAGE of whole cell lysate (WCL), flow through (FT) and elution fractions from MalE affinity purification with (A) amylose affinity resin or (B) Monoclonal maltose bininding protein (α-MBP) antibody resin. Size markers indicated in kilodaltons.


A
Amylose affinity purification
WCL
FT
Elution
WT
OspA-malE
OspA-malE
WT
OspA-malE
OspA-malE
WT
OspA-malE
OspA-malE
70
60
50
40
30
25
20
15
10
B
Monoclonal α-MBP affinity purification
WCL
FT
Elution
WT
OspA-malE
OspA-malE
WT
OspA-malE
OspA-malE
WT
OspA-malE
OspA-malE
70
60
50
40
30
25
20
15
10

Investigation of the B. burgdorferi outer membrane proteome

Next, we broadened our scope to characterize surface and subsurface proteins in the outer membrane of *B. burgdorferi* in hopes to identify candidate proteins that may play a role in lipoprotein trafficking.

We began investigating the *B. burgdorferi* outer membrane proteome by *in silico* identification of integral outer membrane proteins (OMPs). Using online versions of bioinformatic software (Gardy, Spencer et al. 2003; Bagos, Liakopoulos et al. 2004), we identified candidate OMPs based on the presence of an N-terminal signal sequence and multiple predicted transmembrane domains. Our search criteria resulted in a list containing experimentally confirmed OMPs, such as BamA , BesC, P66 and P13, as well as novel candidate OMPs with unknown functions (Table 2). Conserved domains in the candidate OMPs were identified using the NCBI Conserved Domain Database (Marchler-Bauer, Lu et al. 2011). OMPs were checked against a database of *B. burgdorferi* transposon insertion mutants to determine essentiality (Botkin, Abbott et al. 2006). We also compared our list to preliminary MS analysis of outer membrane fractions of *B. burgdorferi* (Schulze and Zückert, unpublished).

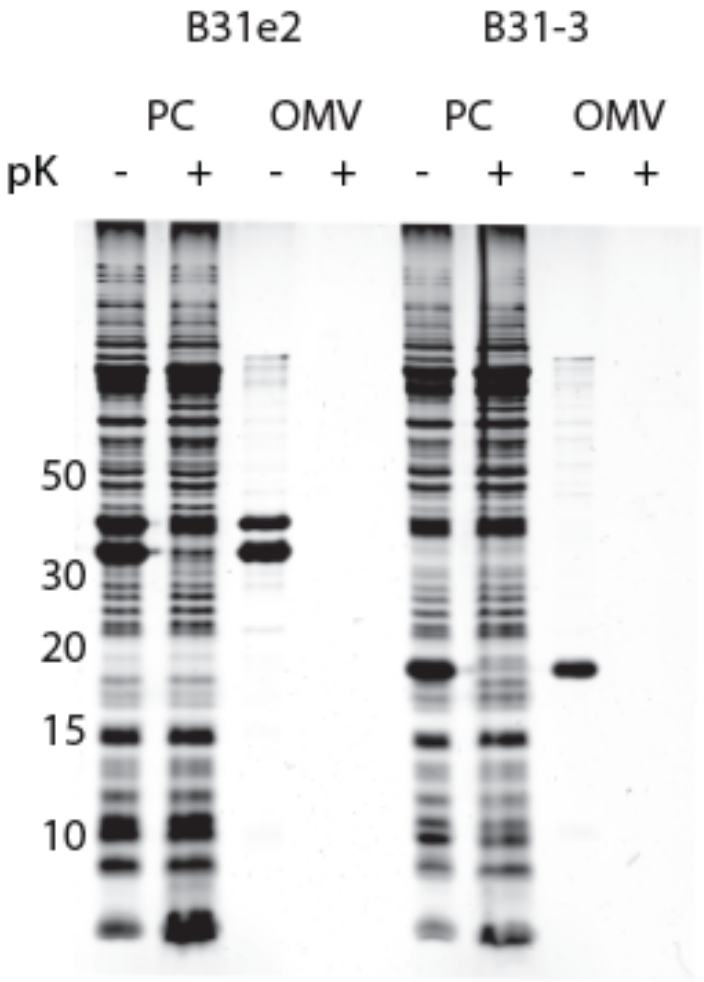
Table 3. Predicted outer membrane proteins with two or more transmembrane domains. Proteins are listed by their open reading frame (ORF) designation. The number of predicted transmembrane (TM) domains is noted. Proteins found in preliminary analysis of *B. burgdorferi* outer membrane vesicles (OMV) are designated with OMV.

ORF	Predicted TM domains	Membrane fraction	Essential	Known function or conserved domain
bb0019	3	Not detected	Yes	None
bb0032	2	Not detected	Yes	Peptidase, Sulfatase
bb0034 (P13)	5	OMV	Yes	Porin
bb0039	2	Not detected	Yes	Rotamase
bb0089	3	Not detected		None
bb0106	2	Not detected	Yes	Tetratricopeptide repeat
bb0110	5	Not detected		None
bb0125	3	OMV	Yes	None
bb0142 (BesC)	4	OMV		TolC
bb0161	2	Not detected		None
bb0170	6	Not detected	Yes	Tetratricopeptide repeat
bb0236	9	Not detected	Yes	Tetratricopeptide repeat
bb0308	3	Not detected	Yes	None
bb0322	3	Not detected		None
bb0326	2	Not detected	Yes	Tetratricopeptide repeat
bb0345	2	Not detected	Yes	Helix-turn-helix-XRE
bb0405	3	OMV		Domain of unknown function
bb0406	3	OMV		None
bb0418	5	Not detected		None
bb0526	3	Not detected		None
bb0543	5	Not detected	Yes	None
bb0554	9	Not detected		None
bb0600	4	PC Fraction	Yes	Immunoglobulin-like fold, Plexins, Transcription factors
bb0603 (P66)	6	OMV		Porin, integrin binding
bb0759	8	OMV		Domain of unknown function
bb0790	3	Not detected		None
bb0795 (BamA)	8	OMV	Yes	OMP assembly
bb0838	4	Not detected		Organic solvent tolerance protein
bb0840	2	OMV		None
bbb28	3	Not detected		Ankyrin repeats

B. burgdorferi have abundant surface lipoproteins, which dominate MS analysis of outer membranes (Schulze and Zückert, unpublished). To characterize subsurface OMPs of *B. burgdorferi* by MS, we devised a strategy to reduce sample complexity by removal of surface proteins by protease degradation followed by isolation of outer membrane fractions. In addition to allowing detection of less abundant proteins, this technique would allow differentiation of surface and subsurface proteins.

Intact spirochetes of *Borrelia* strains B31e2 and B313 (strain lacking many surface exposed lipoproteins) were treated with pK and washed. Outer membrane vesicles (OMV) were subsequently isolated by incubation in a hypotonic citrate buffer followed by ultracentrifugation on a sucrose density gradient. Resulting OMVs and protoplasmic cylinder (PC) fractions (enriched inner membrane and cytoplasm as well as some whole cells) from pK treated and untreated cells were compared by SDS PAGE and silver staining (Fig. 19). No protein was detected in OMV fractions from pK treated cells, precluding MS analysis.

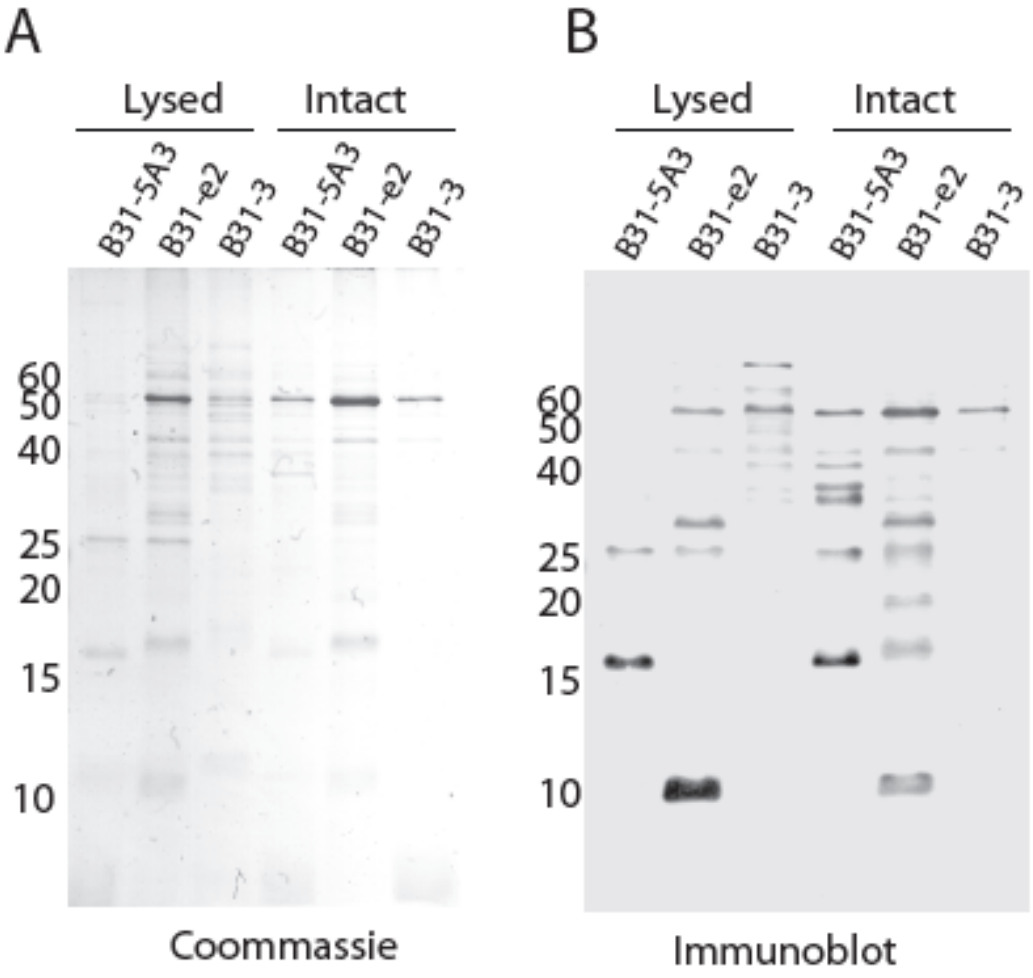
Figure 19. Membrane fractionation plus surface proteolysis. Silver stained SDS PAGE of protoplasmic cylinder (PC) and outer membrane vesicle (OMV) fractions from *Borrelia* strains B31e2 and B313 (strain lacking many surface proteins). Cells were either treated with proteinase K (pK +) or mock treated (-) prior to fractionation. Size markers indicated in kilodaltons.


B31e2
B31-3
PC
OMV
PC
OMV
pK
-
+
-
+
-
+
-
+
50
30
20
15
10

Next, we attempted to identify surface-exposed proteins of *B. burgdorferi* through surface biotinylation. *N* -Hydroxysulfosuccinimide (sulfo-NHS) esters of biotin react spontaneously to form amide bonds with primary amine groups present as lysine side chains. Sulfo-NHS-biotin is specifically used for surface protein labeling, as it does not penetrate the cell surface (Scheurer, Rybak et al. 2005; Gatlin, Pieper et al. 2006).

We chose three different Borrelia strains for comparison of surface biotinylation: B31-e2, the low-passage, infectious strain, B31-5A3, and a strain lacking abundant surface proteins, B313. Intact and lysed *Borrelia* cells were labeled with Sulfo-NHS-biotin. Samples were denatured in SDS loading buffer and analyzed by SDS PAGE followed by Coomassie staining and biotin-specific immunoblot. Contrary to what was expected, biotin-specific detection of labeled proteins was similar for intact and lysed cells (Fig 20). The absence of a specific subset of labeled proteins for intact cells versus lysed cells indicated that Sulfo-NHS-biotin was not specifically labeling surface proteins.

Figure 20. Biotin labeling of *B. burgdorferi*. (A) Coomassie stained SDS PAGE of whole cell lysates from *B. burgdorferi* strains B31-5A3 (low-passage, infectious strain), B31-e2 and B313 (strain lacking abundant surface proteins) after biotinylation with Sulfo-NHS-biotin. (B) Biotinylation samples transferred to nitrocellulose membrane and probed with NeutrAvidin Biotin-Binding Protein. Size markers indicated in kilodaltons.


A
Lysed
Intact
B31-5A3
B31-e2
B31-3
B31-5A3
B31-e2
B31-3
60
50
40
25
20
15
10
Coommassie
B
Lysed
Intact
B31-5A3
B31-e2
B31-3
B31-5A3
B31-e2
B31-3
60
50
40
25
20
15
10
Immunoblot

Discussion

Much attention has been paid to *B. burgdorferi* surface lipoproteins as they play major roles in the pathogenesis of Lyme disease. Less is known about the integral outer membrane proteins (OMPs) of *B. burgdorferi*, some of which may be involved in the as of yet uncharacterized surface lipoprotein transport pathway.

Here we describe construction and expression of a lipidated MalE reporter protein (OspA-MalE). Consistent with previous reports of lipidated reporter proteins in *B. burgdorferi*, fusion of variable lengths of the OspA N-terminal flexible tether region resulted in varying localization of the reporter (surface vs. subsurface). Initial localization studies of OspA fusions to monomeric red fluorescent protein (mRFP) concluded that five N-terminal OspA residues were sufficient for mRFP surface transport. However, we show here that fusion of the first five residues of OspA to MalE resulted in subsurface OspA-MalE. Our results are consistent with subsequent OspA-mRFP localization studies, which revealed that a four amino acid flexible linker region just before the structured globular domain of mRFP caused artifactual surface localization (Schulze, Chen et al. 2010). Wild-type MalE has no N-terminal flexible region (Lecroisey, Martineau et al. 1997), and appears to be a more faithful lipoprotein reporter than mRFP.

Attempts to use surface localized OspA-MalE as a protein bait to purify interacting proteins along the lipoprotein transport pathway were unsuccessful. Our affinity

purification techniques resulted in either no purified proteins or purification samples that were too complex for analysis by Mass Spectrometry (MS). Mass spectrum from a complex mixture is difficult to analyze due to the overwhelming number of components. Sample complexity is intensified during the MS identification process by enzymatic digestion of protein samples into a large number of peptide products. Protein identification by MS is much more successful using samples with limited complexity to minimize the signal suppression of less-abundant proteins by that of high-abundance species (Biemann 1992). Perhaps production of MalE fusion proteins with additional accessory tags would aid in increasing purification efficiency as described previously (Fox, Routzahn et al. 2003).

We attempted to generate outer membrane samples suitable for protein MS analysis to identify *B. burgdorferi* OMPs. Removal of surface proteins prior to membrane fractionation resulted in undetectable protein levels. The extremely fragile *Borrelia* membrane (Barbour 1984) may not tolerate techniques appropriate for classic Gram-negative bacteria. Further, biotinylation of intact *Borrelia* did not result in surface-specific labeling as was expected, indicating that biotin may penetrate the borrelial outer membrane. These unexpected complications prevented application of surface-labeling and surface-proteolysis/membrane fractionation assays to the characterization of the *B. burgdorferi* outer membrane.

We used *in silico* prediction algorithms to create a list of novel *B. burgdorferi* OMPs. Our search criteria identified several experimentally determined OMPs that serve as true positive controls. Several of the OMPs on our list have conserved domains that indicate a potential role in protein-protein interactions. For example, four OMPs, BB0106, BB0170, BB0236, and BB0326 contain Tetratricopeptide repeat (TPR) domains. TPR domains are found in the accessory lipoproteins of the Bam OMP assembly complex (Dong, Hou et al. 2012). BBB28 contains ankyrin repeats, which is another domain known to mediate protein-protein interactions (Li, Mahajan et al. 2006).

Some of the OMPs on our list were also detected in preliminary MS analysis of *B. burgdorferi* OMV fractions. Due to the complexity of the OMV fractions, only the most abundant proteins were detected (Schulze and Zückert, unpublished), therefore the proteins on our list that were detected in OMVs by MS likely represent abundant outer membrane proteins. Conversely, proteins that were not detected in the membrane fraction MS analysis may still be present in the outer membrane.

Three candidate OMPs from our list, BB0405, BB0418, and BB0543, were detected in outer membrane multiprotein complexes (Yang, Promnares et al. 2011). BB0405 and BB0543 were found independently and together in multiple complexes. The function of these outer membrane complexes is unknown, but they support our prediction of candidate OMPs.

Future structural and functional studies of the candidate OMPs we have identified are needed to determine their role in the *B. burgdorferi* outer membrane. Perhaps one or more of the proteins identified here are involved in novel protein transport pathways, such as transport of lipoproteins to the *Borrelia* cell surface.

Chapter VI: Discussion and future directions

In the past 30 years of Lyme disease research, it has become clear that the causative agent, *Borrelia burgdorferi,* has a unique reliance on lipid-anchored proteins (lipoproteins) for survival and pathogenesis. In particular, the lipoproteins tethered to the bacterial surface, creating the medically important host-pathogen interface, have been the subject of much research, not only as diagnostic markers and vaccine targets, but also as models for bacterial antigenic variation and virulence mechanisms (Schwan, Piesman et al. 1995; Pal, de Silva et al. 2000; Liang, Yan et al. 2004; Londono and Cadavid 2010; Livey, O'Rourke et al. 2011; Steere, Drouin et al. 2011).

Lipoproteins are essential to survival and pathogenesis of *B. burgdorferi*. Accordingly, lipoproteins make up nearly 10% of the borrelial genome, which is the largest percentage of lipoproteins in any sequenced bacterial genome (Setubal, Reis et al. 2006). However, little is known about the regulation, maturation and transport of these important virulence factors. How are lipoproteins transported to their final destination? What is the protein machinery necessary for transport and is it similar to other known protein transport systems?

To answer questions surrounding lipoprotein transport in *B. burgdorferi*, we adopted several approaches. First, we localized the lipoprotein, IpLA7, to the inner membrane

(Chapter IV). Localization of IpLA7 put to rest conflicting localization reports and provided a useful inner membrane control for lipoprotein studies. Second, we attempted to characterize the surface and subsurface outer membrane proteins in *B. burgdorferi* and produced a list of candidate integral outer membrane proteins (OMPs) based on *in silico* analyses (Chapter V). Many of the identified OMPs contain conserved domains important to protein-protein interaction and may play a role in protein transport. Finally, we analyzed the function of the *B. burgdorferi* Lol pathway homologs in lipoprotein transport (Chapter III). Our data suggest that periplasmic (subsurface) lipoproteins may rely on the Lol pathway, but that surface lipoprotein secretion appears to be independent of the Lol pathway in *B. burgdorferi*.

Likely due to their ancient phylogeny and small genome, *B. burgdorferi* do not encode many of the known bacterial protein transport pathways (Fraser, Casjens et al. 1997). Of the few transport systems that are present in the *B. burgdorferi* genome, Sec, Bam, T1SS, and Lol, most are limited or partial (Fraser, Casjens et al. 1997; Bunikis, Denker et al. 2008; Lenhart and Akins 2010).

The Lol system controls lipoprotein transport within the periplasm of diderm bacteria (Narita and Tokuda 2010). *B. burgdorferi* encodes homologs for four of the five proteins of the Lol pathway, LolCDE/A, but lacks a homolog of the outer membrane lipoprotein receptor, LolB. Partial Lol pathways aren't rare, however, as LolB homologs are found in only half of the bacterial organisms predicted to contain LolCDE and LolA homologs

(Okuda and Tokuda 2011). Therefore, the outer membrane insertion stage is less conserved than the inner membrane release stage. *B. burgdorferi* LolCDE/A may represent a more ancient version of the Lol pathway than that of *E.coli.* We hypothesized that perhaps the *B. burgdorferi* partial Lol pathway is involved in transport of surface lipoproteins.

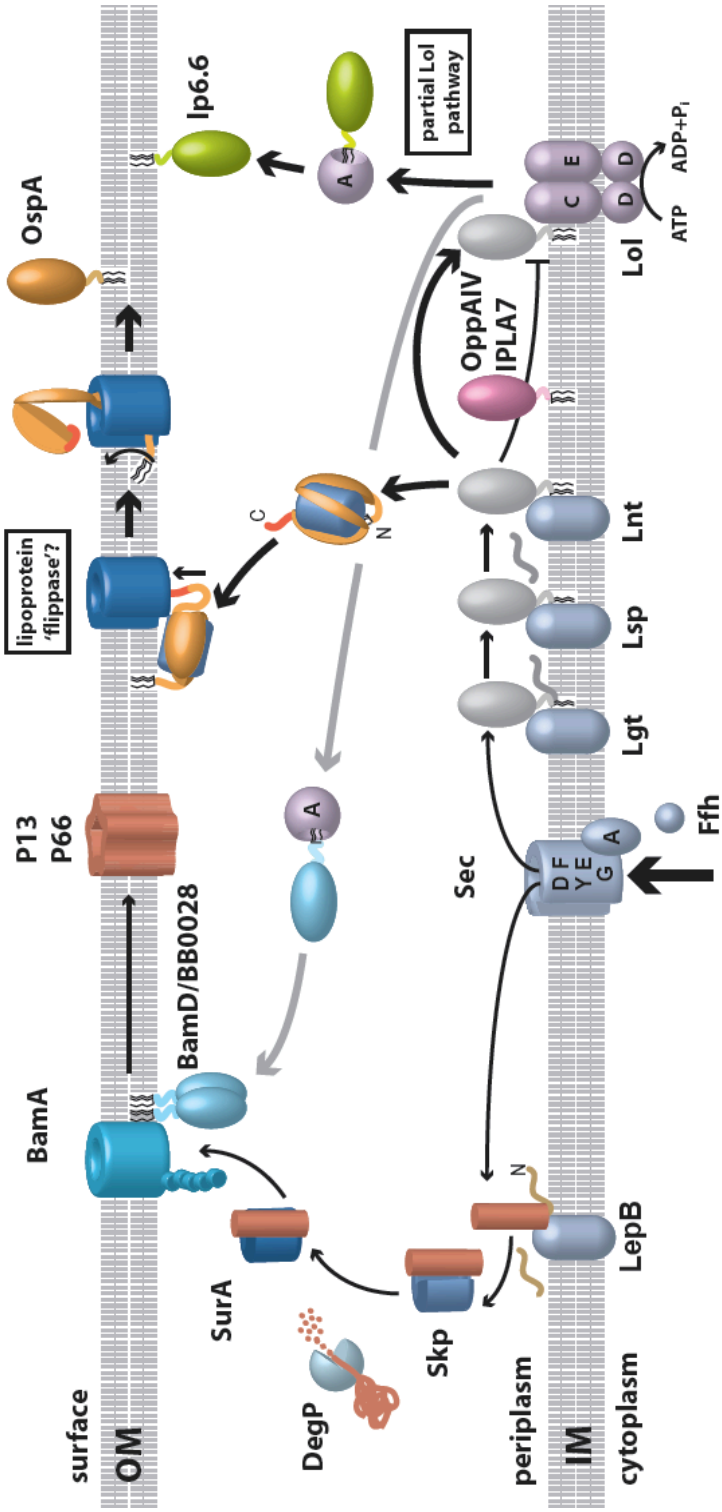
It is unclear whether there exists a correlation between the number of Lol components expressed by an organism and the presence of surface lipoproteins. Though, spirochetes of the genus *Leptospira* also have surface lipoproteins and a partial Lol pathway (LolB is lacking, as in *Borrelia*). *Leptospira* do, however, have an intact T2SS, which may be responsible for transport of lipoproteins to the outer leaflet of the outer membrane (Haake and Matsunaga 2010). Alternatively, treponemal spirochetes have no identified surface lipoproteins and also encode a partial Lol pathway (Fraser, Norris et al. 1998; Liu, Howell et al. 2010). Perhaps bioinformatic analysis of closely related organisms with surface lipoproteins (*Borrelia* and *Leptospira*) and without (*Treponema*) may identify differences in the genome that will help identify the protein machinery responsible for surface lipoprotein transport.

The main finding of this dissertation is that the partial Lol pathway encoded by *B. burgdorferi* does not appear to have a role in surface lipoprotein transport as we had hypothesized. Rather, the Lol pathway is involved only in transport of subsurface lipoproteins to the outer membrane (Chapter III). This finding has led us to modify our

working model of lipoprotein secretion, wherein the Lol pathway primarily is involved in sorting and transport of periplasmic lipoproteins including the Bam associated lipoproteins (Fig. 21).

Figure 21. Modified model of lipoprotein transport in *Borrelia burgdorferi*.

Lipoproteins are synthesized in the cytoplasm and traverse the inner membrane via the conserved Sec machinery. On the periplasmic face of the inner membrane, lipoproteins are processed by conserved signal sequence cleavage and lipidation components. Inner membrane (IM) lipoproteins like IpLA7 (Chapter IV) are retained in the inner membrane by an unknown mechanism. Subsurface outer membrane lipoproteins such as Lp6.6 and the β-barrel assembly (Bam) complex associated lipoproteins, BamD and BB0028, are extracted from the IM and transported through the periplasm by LolCDE and LolA respectively and inserted into the outer membrane (OM) by an unknown mechanism. After lipidation, surface lipoproteins like OspA do not interact with LolCDE (Chapter III), but instead are shuttled through the periplasm and maintained in an unfolded state by a holding chaperone. An unidentified integral OM protein likely acts as a flippase to transport surface lipoproteins to the cell exterior.


surface
OM
periplasm
IM
cytoplasm
BamA
BamD/BB0028
P13
P66
SurA
DegP
Skp
LepB
Sec
D F
Y E
G A
Ffh
Lgt
Lsp
Lnt
Lol
C E
D D
ATP
ADP+P$_i$
partial Lol pathway
lp6.6
OspA
lipoprotein 'flippase'?
OppAIV
IPLA7
A
C
N

Disruption of the *B. burgdorferi* Lol pathway showed an effect on the stability of outer membrane porins, P66 and P13. However, this effect is proposed to be indirect. Outer membrane porins are integral β-barrel proteins (OMPs) and rely on a functional β-barrel assembly (Bam) complex for insertion into the outer membrane. The Bam complex requires accessory subsurface outer membrane lipoproteins to function, therefore, disruption of the Lol pathway impairs formation of the Bam complex and downstream effects can be seen in OMP stability.

The outer membrane component of the surface lipoprotein transport pathway in *B. burgdorferi* is likely an integral outer membrane β-barrel protein, as depletion of the Bam complex has been shown to disrupt outer membrane transport of two major surface lipoproteins, OspA and CspA (Lenhart and Akins 2010). Dependence of surface lipoprotein localization on the Bam complex is proposed to be indirect rather than direct since neither OspA nor CspA are β-barrel proteins. However, a major question remains regarding the interplay of the Bam and Lol pathways in *B. burgdorferi*. If *Borrelia* surface lipoprotein transport is (indirectly) dependent on the Bam complex, and Bam is dependent on the Lol pathway for transport of stabilizing accessory lipoproteins (Ricci and Silhavy 2011; Rigel, Schwalm et al. 2011), why doesn't disruption of the *Borrelia* Lol pathway have an (indirect) affect on surface lipoprotein localization as it appears to have on P66 and P13? One possible explanation is that some OMPs are more dependent on the Bam complex than others or are dependent on certain components of the complex more than others, as described for *P. aeruginosa* OMPs (Hoang, Nickerson et al. 2011).

Perhaps P66 and P13 are more dependent on the Bam complex accessory lipoproteins for folding and stability than are other OMPs. Alternatively, P66 and P13 may rely more directly on the Lol pathway. Periplasmic lipoproteins play a role in Bam-independent transport of OMPs (Koo, Burrows et al. 2012), such as in the transport of the OM channel forming protein, PulD, of the Klebsiella T2SS. PulD requires a lipoprotein chaperone, PulS, for traversal of the periplasm. PulD binds a helical region of PulS while PulS is bound to LolA (Collin, Guilvout et al. 2011), and together they are shuttled to the outer membrane. Future research is needed to fully understand the interplay between bacterial cell envelope biogenesis pathways such as Bam and Lol.

The requirements for inner membrane release, periplasmic transport and outer membrane translocation are unknown for surface lipoproteins in *B. burgdorferi*. Requirements such as lipidation and tether length may differ from those of subsurface lipoproteins. In *E.coli*, lipidation is required for inner membrane release of lipoproteins by LolCDE. A non-lipidated version of the major *E.coli* lipoprotein, Lpp, was created in *E.coli* by replacing the Lpp signal peptide with that of the integral outer membrane protein, OmpF. Non-lipidated Lpp was localized entirely in the periplasm (Yu, Furukawa et al. 1984). However, non-lipidated LolB still goes to the outer membrane in *E.coli*, even though lipidation is required for LolCDE release (Tsukahara, 2009). Lipidation may be required for outer membrane translocation of lipoproteins, but this remains unknown in *Borrelia*.

Lipidation is not required for the transport of *Klebsiella oxytoca* surface lipoprotein, PulA, which is transported by a T2SS (d'Enfert, Chapon et al. 1987; Poquet, Kornacker et al. 1993). A non-lipidated mutant of PulA was generated by fusion of the signal peptide of the periplasmic, *E.coli* protein, MalE, to the amino-terminus of PulA. Non-lipidated PulA was transported through the inner membrane of *E.coli* via the SEC pathway, processed by the type I signal peptidase, LepB, and transported through the outer membrane, although, transport was less efficient at 30-70% compared to 95-99% for wild-type PulA. In the absence of a lipid anchor, however, PulA was secreted into the growth medium (Poquet, Faucher et al. 1993). Non-lipidated mutants of *B. burgdorferi* surface lipoproteins may be useful tools to advance understanding of the mechanism of lipoprotein translocation through the outer membrane.

Continued exploration of the molecular events and identification of the proteins involved in *B. burgdorferi* surface lipoprotein transport will provide insight into the unusual dependence on lipoproteins for survival and pathogenesis. Further, based on its small genome and the absence of almost all known protein transport pathways, yet a large number of secreted proteins, *B. burgdorferi* is a prime model organism for identification of novel protein transport mechanisms.

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