Chapter from:

LYME DISEASE AND RELAPSING FEVER SPIROCHETES

Genomics, Molecular Biology, Host Interactions and Disease Pathogenesis

Editors:

Justin D. Radolf
Departments of Medicine, Pediatrics,
Molecular Biology and Biophysics,
Genetics and Genome Sciences,
and Immunology
UConn Health
263 Farmington Avenue
Farmington, CT 06030-3715
USA

D. Scott Samuels
Division of Biological Sciences
University of Montana
32 Campus Dr
Missoula MT 59812-4824
USA

Caister Academic Press **www.caister.com**

Chapter 7

Structure, Function, Biogenesis and Maintenance of the *Borrelia* Cell Envelope

Wolfram R. Zückert[1]*, Brandon L. Jutras[2], Alvaro M. Toledo[3] and Sven Bergström[4]

[1]Department of Microbiology, Molecular Genetics and Immunology, University of Kansas School of Medicine, Kansas City, KS, USA
[2]Department of Biochemistry, Virginia Polytechnic Institute, Blacksburg, VA, USA
[3]Department of Entomology, Rutgers University, New Brunswick, NJ, USA
[4]Department of Molecular Biology, Umeå University, Umeå, Sweden
*Corresponding author: wzueckert@kumc.edu

DOI: https://doi.org/10.21775/9781913652616.07

Abstract

Although *Borrelia* spirochaetes are often, but mistakenly, described as Gram-negative bacteria due to their diderm, i.e. double-membrane envelopes, a closer examination reveals significant differences in composition and architecture. Particularly striking is the lack of classical endotoxin/lipopolysaccharide. Instead, glycolipids and surface lipoproteins dominate the the host-pathogen interface, where they play important roles during transmission, persistence and ensuing pathogenic processes. A modified peptidoglycan cell wall is also emerging as a potent pathogenicity determinant, in addition to contributing to cell shape in concert with periplasmic flagella. While surface lipoproteins such as the Osps interact with a variety of ligands in different organ tissues, they are also targets of the immune response and several have emerged as vaccine candidates. Some of the identified periplasmic lipoproteins, i.e., the OppAs, are components of substrate transport complexes. Investigations into integral membrane proteins led to the identification of several *Borrelia* porins: P13, whose structure and function is unknown, DipA, which is specific for dicarboxylates, and P66, which has a dual role as a pore-forming outer membrane protein and adhesin. Tol homologs BesA, -B, and -C appear to form a Type I 'channel' to export exogenous toxic agents such as antibiotics and maintain infectivity by an unknown mechanism. Initial studies on envelope biogenesis pathways and mechanisms based on diderm proteobacterial model organisms have revealed significant deviations from the Gram-negative norm, further bolstering the unique status of *Borrelia* among microbial pathogens.

Introduction

The complex life style of *Borrelia* spirochaetes, i.e. their ability to shuttle between hematophagous arthropods and various vertebrates, exposes them to a variety of niches differing in nutritional content and immunological pressure. Survival, transmission and ultimately pathogenesis, therefore, requires these organisms to possess a large degree of adaptive biological capacity. First, the surface of the pathogen needs to be optimized repeatedly for interaction with environments and tissues as different as the tick midgut, the mammalian skin or joint cartilage. Diverse outer surface-exposed proteins play a critical role in this adaptive process by providing ligands for receptor-mediated adhesion, mechanisms of host immune response avoidance, as well as pathways for the acquisition of nutrients. As they are antigens evoking immune reactions in the mammalian host, they represent pre-eminent vaccine candidates. The spatiotemporal regulation of borrelial outer surface proteins is being understood in ever increasing depth, and structure-function relationships have begun to emerge. Genomics, proteomics, novel genetic tools and sophisticated visualization techniques now aim to reveal a more complete picture of the structure, function, biogenesis and maintenance of the *Borrelia* cell envelope. Of particular interest are (i) transport and assembly of its abundant lipoproteins, (ii) the uptake of essential nutrients and the efflux of toxic and undesired compounds, (ii) the role of glycolipids and other lipid bilayer components in the formation and biological function of lipid rafts, and (iv) the emerging

importance of a unique peptidoglycan cell wall in pathogenesis, cell shape and cell division. In this chapter, we will take an inward journey through the *Borrelia* envelope, using *Borrelia burgdorferi* as a well-studied guide, highlighting common and unique features, reviewing structure-function relationships of instructive examples, and exploring open questions and hypotheses.

Structural features and components of the *Borrelia* envelope

Borrelia has a diderm (i.e. two lipid bilayer) ultrastructure that is distinct from that of gram-negative bacteria, such as *Escherichia coli*. An overview of the *Borrelia* cell envelope is depicted in Figure 1. As with other spirochaetes, a fragile outer membrane surrounds the protoplasmic cylinder, which consists of a peptidoglycan layer, a cytoplasmic (inner) membrane and the enclosed cytoplasmic contents (Johnson et al., 1984; Barbour and Hayes, 1986; Kudryashev et al., 2009). A ribbon of 7-11 flagella is located in the periplasmic space where it is attached to the poles and wraps around the cell cylinder, giving the bacterium its characteristic flat wave shape (see Chapter 8 by Sze *et al.*) (Barbour and Hayes, 1986; Goldstein et al., 1994; Motaleb et al., 2000; Charon et al., 2009).

The outer membrane of all bacteria forms a selective barrier between the cell and the environment that excludes certain molecules due to its physico-chemical properties and simultaneously allows entry of nutrient substances by diffusion through the outer membrane, either via porins (Benz, 2001) or

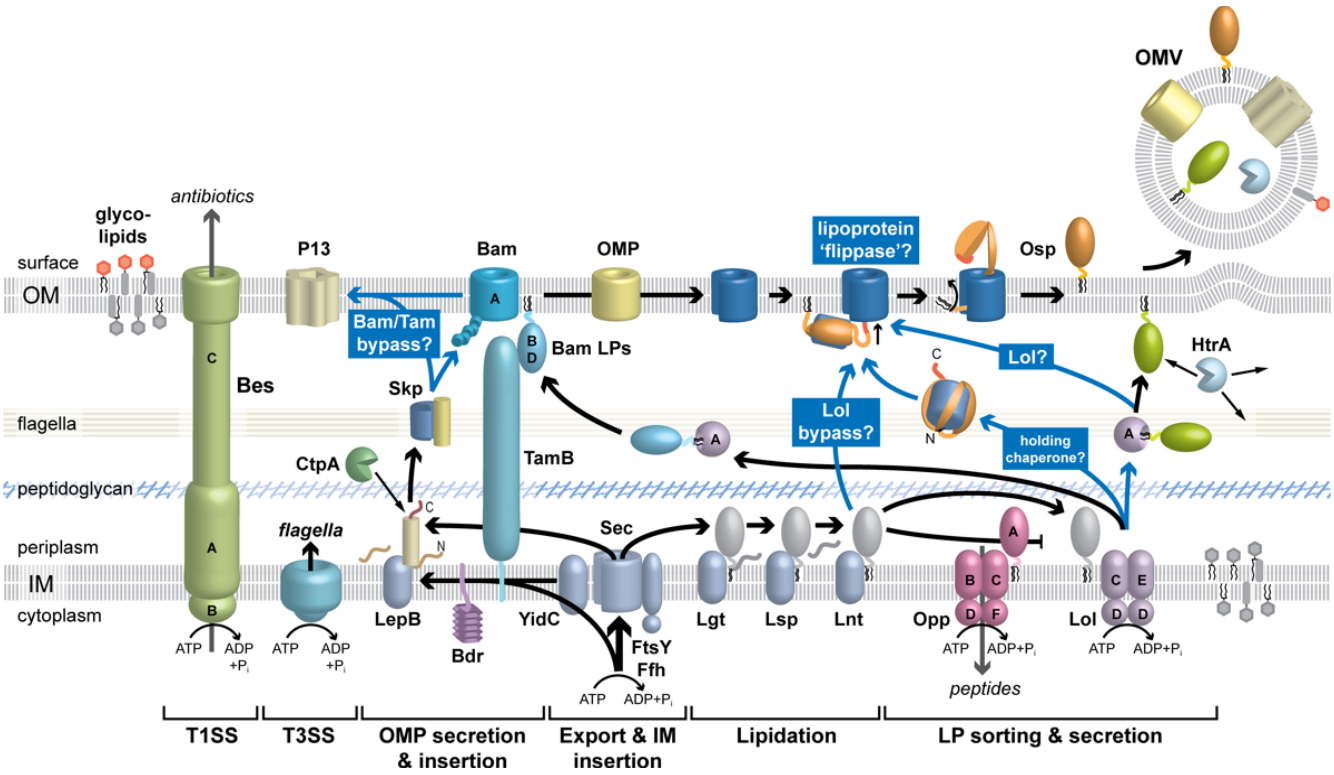

Figure 1. Major components of the diderm *B. burgdorferi* envelope and a model of spirochetal protein secretion. Displayed on the LPS-deficient bacterial surface are abundant and diverse surface lipoproteins, such as the Osps, a variety of glycolipids, and relatively few OM porins (OMPs). A few lipoproteins are anchored in the periplasmic leaflet of the OM. Outer membrane vesicles (OMVs) containing OM and periplasmic contents can be released from bacterial cells. Flagella are confined to the periplasm, atop a thin peptidoglycan layer. Envelope-spanning complexes include the Type I secretion system (T1SS) Bes machinery and the modular Bam/Tam complex involved in OMP assembly. Soluble periplasmic proteins include protein transport chaperones such as Skp and LolA, and proteases like CtpA or HtrA. Shown IM proteins are involved in protein export and processing, e.g., the flagellar T3SS, the general secretory (Sec) pathway components, lipoprotein modification (Lgt, Lsp, Lnt) and localization (Lol) pathways, or peptide import (OppA and OppBCDF) complexes. The IM also contains glycolipids. A depiction of lipid rafts containing glycolipids and membrane proteins has been omitted for clarity. See also text. (Modified from (Zückert, 2019) and used with permission).

receptor-mediated uptake (Nikaido, 2003). Additionally, outer membrane proteins (OMPs) play a critical role in the virulence of bacterial pathogens by responding to different environments, by mediating interactions with the host, or by competing with specific host defense mechanisms, thereby facilitating invasion and colonization of various tissues.

The borrelial outer cell membrane is fluid and consists of 45-62% protein, 23-50% lipid and 3-4% carbohydrate (Barbour and Hayes, 1986). Its composition differs significantly from those of gram-negative bacteria. First, a particularly distinguishing feature is the absence of phosphatidylethanolamine (Belisle et al., 1994) and lipopolysaccharide (LPS) (Takayama et al., 1987) and the presence of non-LPS glycolipid antigens (Eiffert et al., 1991; Wheeler et al., 1993; Belisle et al., 1994). These glycolipids represent about 50% of the total lipids and contain only galactose as the monosaccharide constituent (Berg, 2001; Hossain et al., 2001). It is unclear whether the glycolipids are asymmetrically distributed in the borrelial outer membrane, as with LPS in gram-negative bacteria (Kamio and Nikaido, 1976). Secondly, the *B. burgdorferi* outer membrane exhibits a relatively low density of transmembrane-spanning proteins as initially determined by freeze fracture EM studies (Walker et al., 1991; Radolf et al., 1994; Jones et al., 1995). This may explain why *Borrelia* is more susceptible than Gram negative bacteria to detergents or to disruption by routine physical manipulations such as centrifugation and resuspension. Thirdly, the outer membrane contains an unusually large number of lipoproteins (Brandt et al., 1990) many of which are on the bacterial surface, the host-pathogen interface (Dowdell et al., 2017), where they act as (i) adhesins, (ii) targets for bactericidal antibodies, (iii) or receptors for various molecules.

Outer membrane lipoproteins

An initial analysis of the genome sequence of *B. burgdorferi* sensu stricto predicted 132 open reading frames that contain the signal sequence characteristic for lipoproteins and 32 additional open reading frames that have other similarities to lipoproteins (Hayashi and Wu, 1990; Fraser et al., 1997; Casjens et al., 2000). A re-evaluation using a modified prediction algorithm indicated that the genome encodes up to 127 lipoproteins, corresponding to 7.8% of open reading frames (Setubal et al., 2006), a proportion significantly higher than in other bacterial genomes (Fraser et al., 1997; Fraser et al., 1998; Casjens et al., 2000). A comprehensive *in vitro* spatial assessment of the *B. burgdorferi* lipoproteome indicated that about two thirds of the encoded lipoproteins localize to the surface (Dowdell et al., 2017). This abundance of surface-localized lipoproteins, together with the general importance of outer surface proteins in bacterial adaptation and persistence, has led many researchers to focus their investigations on this class of surface molecules. Furthermore, the unusual membrane architecture of spirochaetes and the ancient phylogeny of these bacteria suggest that the export, structure and function of spirochetal lipoproteins are unique to these organisms.

Borrelial lipoproteins play a major role in the activation of the host inflammatory response and elicitation of inflammation in tissues at sites of infection during Lyme borreliosis. The immuno-potentiating activities of bacterial lipoproteins are related to their N-termini based on two lines of evidence: (i) acylation is essential for their inflammatory activities and (ii) synthetic N-terminal lipopeptides reproduce the proinflammatory activities of the native molecules (Bessler et al., 1985; Lex et al., 1986; Hoffmann et al., 1988; Deres et al., 1989; Erdile et al., 1993; Radolf et al., 1995). While activation of the innate immune response by lipoproteins is crucial for defense against *B. burgdorferi* infection, surface exposed lipoproteins also play essential roles in adaptive responses and pathogenicity of *Borrelia* spirochaetes. Lipoproteins dominate pathogenic mechanisms exploited by *B. burgdorferi,* including those of antigenic variation (Zhang et al., 1997; Zhang and Norris, 1998a), evasion of complement killing (Kraiczy et al., 2001; Alitalo et al., 2002; Stevenson et al., 2002) and adherence mechanisms (Coburn et al., 2005). The role of surface (lipo)proteins in pathogenesis is comprehensively discussed in a recent review by Caine and Coburn (Caine and Coburn, 2016) and chapter 13 by Coburn et al. We will, therefore, discuss only selected lipoprotein examples.

Successful succession: OspA/B and OspC

The reciprocal expression of OspA/B and OspC during *Borrelia* transition from its tick vector to the mammalian host represents the prototypical paradigm of how stage-specific protein expression can contribute to pathogenesis during the natural cycle of spirochetal transmission. In ticks, *Borrelia* expresses large amounts of OspA and OspB and

almost no OspC. The production of OspA and OspB proteins decreases once withiin the mammalian host and, instead, the spirochaete produces OspC (Schwan et al., 1995; Ohnishi et al., 2001). Expression of OspC is induced 36-48 hours into the blood meal, while OspA remains expressed throughout the tick phase and is repressed once the bacteria reach the mammalian host (Barthold et al., 1995; Schwan et al., 1995; de Silva et al., 1996; Fingerle et al., 1998; Schwan and Piesman, 2000; Ohnishi et al., 2001; Iyer et al., 2015; Aranjuez et al., 2019; Caimano et al., 2019).

OspA, whose coding sequence was the first one cloned and sequenced from *B. burgdorferi*, remains one of the most studied proteins in the bacterium (Howe et al., 1986; Bergström et al., 1989). It is encoded by the same operon as OspB on the 54-kb linear plasmid lp54 (Barbour and Garon, 1988; Bergström et al., 1989). OspA consists of four β-sheets, formed by 21 anti-parallel β-strands and a C-terminal α-helix (Li and Lawson, 1995). The crystal structure of the OspB C-terminus (residues 152-296) was identical to that of OspA (Becker et al., 2005). OspA and OspB have been shown to play important roles in the pathogenesis of Lyme borreliosis in a large number of *in vivo* and *in vitro* experiments. OspA is preferentially expressed by the spirochaetes in the unfed tick midgut (Pal et al., 2000) where it mediates adhesion to the midgut epithelium by interacting with a tick receptor, TROSPA (Pal et al., 2004a). The OspA structure revealed a hydrophobic cavity buried in a positively charged cleft in the carboxyterminal domain that might be a binding site the receptor (Li et al., 1997). Providing an example into the emerging multifunctionality of *Borrelia* surface lipoproteins, the tick-specific expression of OspA (and its close homolog OspB) were later shown to shield the bacteria from bactericidal antibodies in the blood meal that are directed against otherwise accessible host-specific *Borrelia* antigens (Battisti et al., 2008; Tilly et al., 2016).

OspC is encoded on the 26-kb circular plasmid cp26 (Fraser et al., 1997) and forms a genus-wide, structurally conserved protein family with the variable small proteins (Vsps) in RF *Borrelia*. The common OspC/Vsp structural fold consists of α-helices, organized as dimer of five parallel α-helices and two short β-strands (Carter et al., 1994; Eicken et al., 2001; Kumaran et al., 2001; Zückert et al., 2001; Lawson et al., 2006). OspC initially was thought to facilitate the migration of *Borrelia* to tick salivary glands, which is initiated by a blood meal promoting the transmission of spirochaetes to the mammalian host (Gilmore and Piesman, 2000; Pal et al., 2004b). Yet, in subsequent studies, OspC was dispensable for localization or migration within the tick but was strictly required to efficiently infect mice (Grimm et al., 2004; Tilly et al., 2006; Dunham-Ems et al., 2012; Tilly et al., 2013). Further studies consolidated OspC's impressive role as a multifunctional essential early host colonization and dissemination factor, showing binding of both an immunosuppressive tick salivary protein (Ramamoorthi et al., 2005) and host complement component C4b (Caine et al., 2017), as well as protection from phagocytosis by macrophages (Carrasco et al., 2015).

Initial evaluation of the *ospC* genes from different *Borrelia* genospecies revealed a large degree of sequence polymorphism (Jauris-Heipke et al., 1995; Livey et al., 1995), resulting in the classification of strains into 19 major OspC groups (Wang et al., 1999). Molecular analysis suggested that recombination between different *ospC* alleles occurs frequently and that this genetic exchange is mediated by lateral transfer of *ospC* sequences within the same genospecies or between different *Borrelia* species (Livey et al., 1995; Seinost et al., 1999; Barbour and Travinsky, 2010). Of particular interest for the further functional definition of OspC was the finding that certain invasive *Borrelia* isolates belonged to defined OspC groups, implying that the OspC protein was also an important driver of invasiveness and dissemination of Lyme borreliosis (Seinost et al., 1999; Lagal et al., 2003). Indeed, OspC variants were later shown to differ in their affinity for plasminogen and fibrinogen (Lagal et al., 2006; Bierwagen et al., 2019), and a recent well-controlled study demonstrated that differential OspC binding to fibronectin and/or dermatan sulfate directly affects tissue tropism in an OspC variant-dependent manner (Lin et al., 2020b).

Adhesin and anti-complement factors: multifunctionality and redundancy

Attachment to tissues and cellular matrix is an important prerequisite for colonization of the host by pathogenic bacteria. Therefore, it is not surprising that in addition to OspA/B and OspC, additional borrelial lipoproteins have been shown to function as adhesins (see also Chapter 13). Decorin-binding proteins A and B (DbpA/B) are early-identified examples of this functional group of lipoproteins, facilitating the colonization of collagenous tissues

and tissue tropism to the joints by adhering to the collagen-associated proteoglycan decorin (Guo et al., 1995; Guo et al., 1998; Hanson et al., 1998; Brown et al., 2001; Fischer et al., 2003). Antibodies against DbpA can prevent infection via needle infection only (Hagman et al., 1998; Hanson et al., 1998), nevertheless illustrating the importance of adhesion in the pathogenesis of Lyme disease.

Bgp (*Borrelia* glycosaminoglycan-binding protein) is a *B. burgdorferi* hemagglutinin with the capacity to bind heparan sulphate, dermatan sulphate, and aggrecan (Parveen and Leong, 2000; Russell and Johnson, 2013). Bgp is surface exposed, but its classification as a lipoprotein remains unresolved (Parveen and Leong, 2000). Bgp production was upregulated during conditions that mimicked tick feeding (Ramamoorthy and Philipp, 1998), while secretion of Bgp into extracellular environment also has been observed, indicating a possible role as a immune response decoy (Cluss et al., 2004). Since the adaptation of spirochaetes to the mammalian host environment leads to enhanced binding of glycosaminoglycan (Parveen et al., 2003) and aggrecan is a major proteoglycan found in joints, Bgp is likely contributing to efficient dissemination and colonization.

A diverse family of surface lipoproteins contributes to the bacterium's ability to evade clearance by the complement cascade, a system of plasma proteins that can be activated by antibody or lectins bound to the bacterial surface or by the pathogen surface itself (Medzhitov and Janeway, 2000). Several of the lipoproteins recruit complement regulatory factors to the bacterial surface and are therefore called CRASPs, for complement regulatory factor acquiring surface proteins. CspA (CRASP-1), expressed in the unfed tick environment, binds Factor H (FH), Factor H-like 1 (FHL-1) as well as complement components C7 and C9; CspZ (CRASP-2), expressed in the mammalian environment, binds FH and FHL-1; and some of the the OspE/F-related proteins, ErpA (CRASP-5), ErpC (CRASP-4) and ErpP (CRASP-3), bind FH, several FHL protein variants, as well as plasminogen (reviewed in (Lin et al., 2020a). CspA is a homodimer that forms a novel α-helical fold (Cordes et al., 2005) and binds FH, C7 and C9 at distinct sites (Kenedy et al., 2009; Hallstrom et al., 2013; Hammerschmidt et al., 2014), indicating that serum resistance remains important in the tick environment.

Another example of a multifunctional surface lipoprotein is BBK32. BBK32 (P35) initially was shown to bind fibronectin (Probert and Johnson, 1998) and later also to promote attachment of *Borrelia* to GAGs. Fibronectin binding proved to be important for initiating microvascular interactions, while binding of GAG via a discrete domain was shown to drive joint colonization (Fischer et al., 2006; Norman et al., 2008; Lin et al., 2015). A recent study demonstrated BBK32-mediated binding to complement factor C1r, thereby blocking the classical complement pathway. Thus, BBK32 further contributes to the bacterium's serum resistance and hematogenous spread (Garcia et al., 2016).

Multiphasic antigenic variation and persistence

The VlsE proteins are of particular interest because they were shown to be essential for persistence of *B. burgdorferi* in the natural enzootic cycle (Rogovskyy et al., 2015). VlsE belongs to the VMP (variable major proteins) family also found in RF *Borrelia* (Barbour, 1990; Burman et al., 1990; Barbour, 1993) (see Chapter 14 by Lopez et al.). VlsE is encoded on lp28-1 of *B. burgdorferi* and undergoes antigenic vajriation through an elaborate unidirectional gene conversion mechanism (Zhang et al., 1997; Zhang and Norris, 1998a; Coutte et al., 2009) via a mechanism similar to that responsible for recombination between the expressed pilin gene *pilE* and silent copies of *pilS* in *Neisseria gonorrhoeae* (Hagblom et al., 1985; Haas and Meyer, 1986; Zhang et al., 1992; Koomey, 1997; Zhang et al., 1997). Fifteen silent, non-expressed *vls* cassettes can be recombined into a functional, expressed *vlsE* gene, resulting in multiphasic antigenic variation of the expressed lipoprotein (Zhang and Norris, 1998a, b). The Holliday junction RuvAB helicase was shown to play a central role in the required recombinatory processes (Dresser et al., 2009; Lin et al., 2009).

VlsE contains invariable N- and C-terminal domains and a central variable domain that includes six variable and six invariable regions. Little is known about the signals that trigger recombination events at the *vls* locus**.** Recombination occurs *in vivo* as soon as four days after experimental infection of mice but not *in vitro*, suggesting that the mammalian host provides the signal for Vls recombination (Zhang and Norris, 1998b). The role of the mammalian host is important since the VlsE recombination rate of *B. burgdorferi* s.l. was found to be lower in IFN-γ R-deficient mice than in control animals. These results suggest that the murine immune response can

promote the *in vivo* adaptation of *B. burgdorferi* s.l. (Anguita et al., 2001). The solved 3-D structure of VlsE is unlike OspA-C, consisting of eleven α-helices and four short β-strands (Eicken et al., 2002). This is in spite of the fact that VlsE and OspC/Vlps share overall structural features, such as the close proximity of their N and C termini and a hypervariable sequence corresponding to a surface exposed region (Cullen et al., 2004; Lawson et al., 2006; Radolf and Caimano, 2008; Zückert, 2013). A recent report indicates that VlsE masks epitopes of another surface protein, Arp (Lone and Bankhead, 2020), a phenomenon that may be similar to OspA blocking access to the outer membrane porin P66 and other surface proteins (Bunikis and Barbour, 1999; Tilly et al., 2016).

Another protein important for host adaptation is Lp6.6, a small but abundant 6.6-kDa lipoprotein localizing to the periplasmic face of the outer membrane in large protein complexes (Katona et al., 1992; Lahdenne et al., 1997; Promnares et al., 2009). Lp6.6 was shown to be important for transmission from the tick to the vertebrate host (Promnares et al., 2009) and is significantly downregulated during mammalian infection (Lahdenne et al., 1997). This downregulation is initiated during tick feeding through an RpoS-dependent mechanism (Yang et al., 2000; Caimano et al., 2005).

Outer membrane porins

The molecular sieving properties of bacterial cell envelopes are the result of channel forming proteins in their outer membranes, called porins. Porins are integral membrane proteins that form large water filled pores through the outer membrane (Benz, 1994; Achouak et al., 2001). Their function is mainly for uptake of substances from the environment and can be subdivided into two classes. The porins of the first class can be described as general diffusion pores. These porins sort mainly according the molecular mass of the solutes and show a linear relation between translocation rate and solute concentration gradient. The second class contains pores with a binding site inside the channel. These specific porins are responsible for the rapid uptake of classes of solutes such as carbohydrates, phoshate or nucleosides (Ferenci et al., 1980; Benz et al., 1986; Hancock and Benz, 1986; Benz et al., 1988).

Borrelia are limited in their metabolic and biosynthetic capacities and, therefore, highly dependent on nutrients provided by their hosts (Fraser et al., 1997; Casjens et al., 2000; Thein et al., 2008) (see Chapter 6 by Gherardini et al.). Consequently, these bacteria need to have an efficient regulation of the nutrient uptake across the cell envelope. The first indication of porins in *B. burgdorferi* came through investigation of channel-forming activities in the planar lipid bilayer assay of outer membrane vesicles (OMV). Two porin activities of 0.6 nS and 12.6 nS were found (Skare et al., 1995). Subsequent work characterized three possible porins in *Borrelia burgdorferi*: P13 (Östberg et al., 2002; Pinne et al., 2004; Barcena-Uribarri et al., 2014), P66 (Skare et al., 1997; Pinne et al., 2007; Barcena-Uribarri et al., 2010; Barcena-Uribarri et al., 2013), and Oms38/Dip A (Thein et al., 2008).

There are also indications of uncharacterized channel-forming activities present in *B. burgdorferi* as determined by black lipid bilayer analysis of outer membrane fractions (Östberg et al., 2002). Using computer-based algorithms, Kenedy and coworkers identified two novel borrelial OMPs with porin activities, BB0405 and BB0406 (Kenedy et al., 2016). BB0405 and BB0406 are cotranscribed and share a high degree of amino acid homology. Yet, BB0405 is accessible to proteases whereas BB0406 is not (Kenedy et al., 2016). Further analysis of these putative porins revealed that BB0405 appears to be essential for infection and that BB0406 is interacting with laminin and important for the dissemination into distant organs (Bista et al., 2020). In contrast to LD *Borrelia* species, knowledge of porin activities in RF *Borrelia* is rather limited. There are, however, indications of several pore-forming activities in outer membrane preparations of RF spirochaetes (Shang et al., 1998; Thein et al., 2008), and genes with a high degree of homology to *B. burgdorferi p13*, *p66,* and *dipA* can be found in the published genomes of the RF agents *B. duttonii*, *B. recurrentis* and *B. hermsii*.

The P13 porin family

The outer membrane protein P13 is encoded by ORF BB0034 which belongs to the paralogous gene family (PF) 48. PF48 has eight additional plasmid-encoded genes or pseudo-genes (Fraser et al., 1997; Noppa et al., 2001; Pinne et al., 2006). P13 was discovered in a high passage derivative of *B. burgdorferi* strain B31, B313, which lacked major Osps (OspA-D and DbpA/B) (Sadziene et al., 1995). Mice infected with B313 were used to generate MAbs against P13, which, in turn, inhibited the growth of the Osp-deficient strain but not the wild-type strain (Sadziene

et al., 1995). This implied that surface exposed lipoproteins must hide the P13 epitope, in a similar manner to OspA protecting P66 (Bunikis and Barbour, 1999) and VlsE shielding Arp (Lone and Bankhead, 2020). Surface exposure of P13 was later confirmed by a variety of techniques, while the presence of an N-terminal signal sequence, a signal peptidase I cleavage site and three transmembrane spanning α-helices were predicted by computer analysis (Noppa et al., 2001). Furthermore, peptide sequencing revealed that P13 is processed at the C-terminus, but the N-terminal sequence was blocked by possible modification at the N-terminus (Noppa et al., 2001). Combined mass-spectrometry revealed this modification to be pyroglutamylation, i.e., pyroglutamate formation at an N-terminal glutamine (Nilsson et al., 2002). Pyroglutamate modification is either catalysed by the enzyme glutamine cyclotransferase or can occur spontaneously (Busby et al., 1987; Khandke et al., 1989; Awade et al., 1994). The latter might occur in P13 since no glutamine cyclotransferase homologue was found in the *B. burgdorferi* genome. Although formation of pyroglutamate at N-terminal glutamine residues has been suggested to protect some proteins from proteolytic degradation (Strobl et al., 1997), the role of pyroglutamylation of P13 remains to be elucidated.

The surface exposure and predicted transmembrane spanning domains of P13 indicated that it might possess channel-forming properties. The pore-forming activity of P13 was first described in 2002 using protein purified by FPLC from an outer membrane protein preparation. In a black lipid bilayer (BLB) analysis, it displayed an average single channel conductance (SCC) of 3.5 nS (Östberg et al., 2002). However, in a subsequent study using a different purification method, the SCC was revised and confirmed to be 0,6 nS (Barcena-Uribarri et al., 2014). Most porins exist in either open or closed states, depending on the transmembrane potential, a phenomenon known as voltage gating. Whether this is physiologically significant is questionable, since porin voltage dependence measurements in planar lipid bilayer assays suggest that the critical voltage (*Vc*), above which general porins close far exceeds the naturally occurring Donnan potential across the outer membrane (Koebnik et al., 2000). However, this strategy is still widely used to characterize porins. The channel formed by P13 was not influenced by voltage increases, reflecting a pore stably incorporated in the lipid bilayer. Since substrate-specific channels tend to be voltage-independent (Koebnik et al., 2000), perhaps P13 is a specific porin. Single channel and selectivity measurements showed that P13 had no preference for either cations or anions and showed no voltage-gating up to +/- 100 mV (Barcena-Uribarri et al., 2014).

Blue-native PAGE using P13 in its native state revealed that the P13 complex, consisting only of P13 monomers, has a molecular mass of roughly 300 kDa (Barcena-Uribarri et al., 2014). Using non-electrolytes to estimate channel diameter, the P13 homo-oligomer was estimated to be approximately 1,4 nM with a 400-Da molecular mass cut-off (Barcena-Uribarri et al., 2014). While numerous substrates have been tested, no substrate specificity for the P13 channel has yet been demonstrated.

The channel-forming activity of P13 also was confirmed by genetic experiments. The *p13* gene was inactivated by allelic-exchange mutagenesis, this being the first reported example of disruption of a gene encoding an integral outer membrane protein of *B. burgdorferi.* The porin activities of OM protein preparations derived from knock-out and wild-type strains were compared using the planar lipid bilayer assay. The SCC activity corresponding to P13 was eliminated in the *p13* knock-out strain. Evidence of additional porin activities present in OM of *B. burgdorferi*, distinct from the activities of P66, P13 and DipA, have been suggested (Östberg et al., 2002; Pinne et al., 2007; Thein et al., 2008).
Several interesting characteristics of P13, such as its small size, unusual amounts of subunits in an oligomer, hydrophobicity and predicted membrane-spanning α-helices are all in disagreement with common features of conventional porins (Östberg et al., 2002; Pinne et al., 2006; Barcena-Uribarri et al., 2014).

To further investigate the unusual processing of P13, an attempt to identify the C-terminal protease was performed. Using the amino acid sequence of the known carboxyl-terminal protease A (CtpA) from *Bartonella bacilliformis* (Mitchell and Minnick, 1997) to BLAST search the *B. burgdorferi* B31 MI genome (Fraser et al., 1997; Casjens et al., 2000), a homologous gene (*bb0359*) was found (Östberg et al., 2004). To elucidate if the activities of P13 and/or other *Borrelia* proteins are affected by CtpA, the *ctpA* gene was inactivated by allelic exchange mutagenesis (Östberg et al., 2004). Immunoblot analysis revealed that P13 was larger and had a more acidic pI in the *ctpA* knock-out, consistent with

the theoretical size and pI of P13 if it were not processed at the C-terminus. 2-D gel electrophoresis of *B. burgdorferi* total proteins revealed several proteins present only in the *ctpA* mutant. Mass spectroscopy analysis of these potential CtpA substrates revealed that P13 and BB0323 are processed by CtpA, while BBA74 is up-regulated and produced in multiple isoforms in the absence of CtpA. These effects were due to loss of CtpA because complementation with a wild type copy of *ctpA* restored wild type-like protein expression profiles (Östberg et al., 2004). However, BBA74 is not a substrate for CtpA, so that its up-regulation is probably a secondary effect of *ctpA* inactivation (Östberg et al., 2004). Interestingly, the expression level of CtpA in the complemented strain is higher than in wild type, reducing the levels of BBA74 below its detection limit (Östberg et al., 2004). Thus, CtpA has pleiotropic effects, processing P13 and influencing the appearance of several other proteins. The importance of this unusual C-terminal processing of P13 is not fully known, but it may play a role in translocation of the protein *in vivo*. In a study by Kumru and coworkers, the P13 C-terminal peptide was altered to investigate its function (Kumru et al., 2011a). These studies suggests that the C-terminal peptide likely acts as a safeguard against misfolding or mislocalization in the outer membrane prior to its removal by CtpA (Kumru et al., 2011a). The connection of this C-terminal processing to the channel forming activity needs to be elucidated.

Being extremely hydrophobic (hydropathicity index, 0.47) and spanning the membrane with α-helices, one would have assumed that P13 would insert into the inner membrane by the conventional SRP-dependent protein secretion machinery. Nevertheless, the unusual feature of C-terminal cleavage CtpA led to the hypothesis that this event initiates translocation to the outer membrane (Östberg et al., 2004). Yet, P13 is present in OM protein preparations from the *ctpA* knock-out mutant. Therefore, the 28-amino acid C-terminal extension of P13 appears not to be required for transportation to the outer membrane, but cleavage may be necessary for correct P13 assembly. This mechanism is supported by the function of CtpA in photosynthetic organisms. A substrate for CtpA in photosystem II, the hydrophobic peptide D1 that spans the thylakoid membranes of chloroplasts (Hankamer et al., 2001), must be cleaved at its C-terminus for correct assembly and translocation and to ensure a functional photosystem II (Nixon et al., 1992; Ivleva et al., 2000). The C-terminus of P13 is processed directly after the alanine residue removing the extreme C-terminal 28 aa (Noppa et al., 2001). Interestingly, the C-terminal extension removed in D1 from different organisms varies from between 8 to 16 aa but is always cleaved after an alanine (Ivleva et al., 2000). Clearly, similarities between P13 and D1 exist and imply that CtpA might play a critical role in the translocation and assembly of functional P13. Moreover, CtpA of *B. burgdorferi* contains a predicted N-terminal signal sequence reminiscent of the need to transport CtpA across the thylakoid membrane in chloroplasts (Karnauchov et al., 1997) . By analogy, we interpret that CtpA is transported through the *Borrelia* inner membrane and that C-terminal processing of P13 occurs in the periplasmic space. In summary, P13 is able to form channels in the borrelial outer membrane despite its small molecular mass and α-helical secondary structure (Östberg et al., 2002; Barcena-Uribarri et al., 2014). This homooligomeric porin with a 400 kDa cut-off possibly acts as a general diffusion channel for small molecules into *Borrelia.*

P66 (Oms66)

P66 is a well-studied dual-function, integral outer membrane protein that is encoded by a chromosomal gene in both Lyme disease and relapsing fever spirochaetes. Its function as a pore-forming outer membrane protein has been clearly demonstrated by studies in planar lipid membranes and liposomes (Skare et al., 1997; Pinne et al., 2007; Thein et al., 2008; Barcena-Uribarri et al., 2010; Kenedy et al., 2014). Skare and colleagues initially showed that P66 forms pores in artificial membranes with an atypical huge single channel conductance of 9.6 nS in 1 M KCl (Skare et al., 1997). In later experiments by Pinne et al. (Pinne et al., 2007) using FPLC-purified protein, P66 exhibited voltage-dependent closure of the channels although at higher voltages. The zero-current membrane potential was –5 mV for an five-fold KCl-gradient, which means that the permeability ratio *P*K/*PCl* was about 0.77 as calculated from the Goldman–Hodgkin–Katz equation (Benz et al., 1979). This suggests that P66 is permeable to anions and cations. Incubation of purified P66 with corresponding polyclonal rabbit antiserum resulted in total loss of the 11 nS channel-forming activity (Pinne et al., 2007).

A channel sizing analysis, examining single-channel conductance in black lipid bilayers in the presence of different non-electrolytes with known hydrodynamic

radii, showed that non-electrolytes with hydrodynamic radii smaller than 0.34 nm entered the pore, whereas non-charged molecules with a greater radius were non-permeable. The effective diameter of the P66 channel lumen was determined to be ~1.9 nm, with a central constriction of about 0.8 nm (Barcena-Uribarri et al., 2013). As derived from single- and multichannel experiments, the P66-induced membrane conductance could be blocked by 80–90% after addition of the non-electrolytes PEG 400, PEG 600 and maltohexaose in the low millimolar range (Barcena-Uribarri et al., 2013). The blockage of one P66 single-channel conductance unit of 11 nS occurred by seven or eight subconducting states, indicating a heptameric or octameric constitution of P66. This could be confirmed by Blue native PAGE and immunoblot analysis which revealed that P66 forms a complex with a corresponding mass of approximately 440 kDa (Barcena-Uribarri et al., 2013). Of note, other *Borrelia* species, including the relapsing fever spirochaetes *B. duttonii*, *B. recurrentis* and *B. hermsii*, also carry P66 homologs (Lescot et al., 2008) with inter-species amino acid identities of 41% but similar physicochemical properties; all form large pores with single-channel conductances between 9 and 11 nS (Barcena-Uribarri et al., 2010).

The second known function of P66, as a ligand for β3-chain integrins, was initially revealed using a phage display library of total genomic *B. burgdorferi* DNA (Coburn et al., 1999; Antonara et al., 2007). In follow-up studies, a synthetic peptide corresponding to P66 amino acids 203–209, i.e, within the region panned from the phage display library, inhibited *B. burgdorferi* attachment to integrin αIIbβ3 (Defoe and Coburn, 2001), while targeted mutagenesis of the same domain led to P66 mutants that were deficient in binding to αvβ3 integrin (Coburn and Cugini, 2003). About a decade later, P66-mediated integrin binding was shown to play a role in bacterial escape from the site of entry and further dissemination into the tissues in the mammalian host (Ristow et al., 2012; Ristow et al., 2015), likely by interactions with integrins found on different immune cells, blood platelets and endothelial cells. Most recently, intravital imaging of *B. burgdorferi* in a mouse model of infection demonstrated the importance of P66 for vascular transmigration (Kumar et al., 2015). Together, these studies firmly establish the role of P66 in vascular spread of the bacterium. Of note, site-directed mutagenesis of the P66 integrin binding domain revealed that binding activity is distinct from porin activity (Ristow et al., 2015).

The precise structure of P66 remains undetermined, although available data point to the formation of a large beta-barrel. Early experiments already predicted and then confirmed surface-exposed epitopes (Bunikis et al., 1995; Probert et al., 1995), with a major antigenic surface loop being protected by Osp lipoproteins, in particular OspA, from access of both antibodies and trypsin (Bunikis and Barbour, 1999) - whether the role of this interaction extends beyond this shielding function remains to be determined. Structural modeling based on the surface accessibility of this loop and circular dichroism spectrometry suggests that P66 forms a β-barrel with either 22 or 24 transmembrane domains (Kenedy et al., 2014).

DipA

Borrelia spirochaetes are – due to their small genomes – metabolically and biosynthetically deficient, thereby making them highly dependent on nutrients provided by their various hosts (see Chapter 6 by Gherardini et al.). With neither P13 or P66 displaying any substrate specificity, the search for nutrient-specific porins continued. The OM porin Oms38 was discovered in the OM fractions of RF *Borrelia* and is highly conserved in *B. duttonii*, *B. hermsii* and *B. recurrentis* (Thein et al., 2008). In black lipid bilayers, purified Oms38 formed water-filled pores of small conductance (80 pS in 1 M KCl) that were selective for anions. Further analyses of analogous proteins in LD borreliae revealed a 36-kDa protein (BB0418) with sequence and functional similarity to Oms36 in RF *Borrelia* (Thein et al., 2012). The protein, designated DipA (for dicarboxylate-specific porin A), has an average single-channel conductance of 50 pS in 1 M KCl. DipA is selective for anions with a ratio of permeability for cations over anions of 0.57 in KCl and does not show voltage-dependent closure. The permeation of KCl through the channel could be partly blocked by titrating the DipA-mediated membrane conductance with increasing concentrations of different organic di- and tricarboxylic anions. Particular high stability constants up to 28,320 l/mol (in 0.1 M KCl) were obtained among the 11 tested anions such as oxaloacetate, 2-oxoglutarate and citrate. The obtained results imply that DipA does not form a general diffusion pore, but a porin with a binding site specific for dicarboxylates which play important key roles in the deficient

metabolic and biosynthetic pathways of *Borrelia* species (Thein et al., 2008; Thein et al., 2012).

Outer membrane glycolipids

Lipids constitute roughly 25-30 % of *B. burgdorferi* dry weight, of which >36% correspond to glycolipids (Hossain et al., 2001; Ben-Menachem et al., 2003). Early studies on glycolipids focused on their antigenic role in Lyme disease (Eiffert et al., 1991; Wheeler et al., 1993) while their structural analysis (Hossain et al., 2001; Ben-Menachem et al., 2003; Schroder et al., 2003; Stubs et al., 2009; Stubs et al., 2011) and biophysical properties (Huang et al., 2016) were later studied.

While lipoproteins are significant contributors to the immune response in Lyme disease patients and therefore, useful markers for serological diagnostic tests, several studies also noted the contribution of *Borrelia* lipids to the immune response. For example, *Borrelia* fatty acids and carbohydrates strongly reacted with sera containing anti-*B. burgdorferi* antibodies (Eiffert et al., 1991), which supported that *B. burgdorferi* lipids could mediate in antibody responses. Further studies showed that two lipid fractions from *B. burgdorferi* were recognized by sera collected from patients with Lyme disease (Wheeler et al., 1993) and some of the components of these antigenic fractions were identified as glycolipids (Wheeler et al., 1993). Interestingly, a subset of Lyme disease patients with neuroborreliosis had IgM antibodies that reacted to gangliosides with a Gal (β1-3) GalNac terminal sequence (Garcia Monco et al., 1993). Subsequent studies in rats proved that purified lipid fractions of *B. burgdorferi* elicit high levels of IgM antibodies that cross-reacted with asialo-GM1 and GM1 (Garcia-Monco et al., 1995). Conversely, sera of rats immunized with asialo-GM1 produced antibodies that cross-reacted with *B. burgdorferi* antigens (Garcia-Monco et al., 1995). These studies showed the antigenic potential of *Borrelia* lipids and opened the possibility, not yet resolved, that the pathogenesis of Lyme disease could be driven, at least in part, by cross-reaction between *Borrelia* glycolipids and host lipids. More recently, it was shown that glycolipids also produce a strong IgG antibody response in patients with Lyme arthritis (Jones et al., 2009), which supports that host antibody responses to glycolipids are not limited to Lyme disease patients with central nervous involvement. Several studies have purified, characterized and confirmed their immunogenicity (Hossain et al., 2001; Ben-Menachem et al., 2003; Schroder et al., 2003; Stubs et al., 2009; Stubs et al., 2011).

It was not until the beginning of the 21st century that several studies shed light on the structure of lipids in *B. burgdorferi*. A study using high-performance thin liquid chromatography (TLC) revealed the presence of 11 components, including two phospholipids, phosphatidylcholine (PC) and phosphatidylglycerol (PG), as well as a glycolipid, mono α-galactosyl-diacylglycerol (MGalD), which contains galactose and two fatty acids (Hossain et al., 2001). Shortly thereafter, two additional glycolipids containing cholesterol were identified, cholesteryl-β-D-galactopyranoside (CGal) and cholesteryl 6-O-palmitoyl-β-D-galactopyranoside (ACGal) (Ben-Menachem et al., 2003; Schroder et al., 2003). Both cholesterol glycolipids are very similar in their structure, with ACGal having an extra acyl chain bound to the galactose group (Ben-Menachem et al., 2003; Schroder et al., 2003). Glycolipids MGalD, CGal, and ACGal are ubiquitous within the *B. burgdorferi* sensu lato complex and are present in both pathogenic and non-pathogenic genospecies. (Stubs et al., 2009; Stubs et al., 2011). The cholesterol glycolipid ACGal is immunogenic and recognized by sera from patients with Lyme disease but not syphilis (Stubs et al., 2011). Other glycolipids that can be found in *B. burgdorferi* in trace amounts are CGlu and ACGlu (Stubs et al., 2009), which are the only cholesterol glycolipids present in the relapsing fever species *Borrelia hermsii* (Livermore et al., 1978). Although both glycolipids are very similar and only differ in the sugar group, ACGal is recognized by sera from Lyme disease patients while ACGlu is not (Stubs et al., 2011). The specificity and immunogenicity of *Borrelia* glycolipids MGalD and ACGal led to their synthesis and evaluation as potential vaccine antigens as well as diagnostic applications (Pozsgay et al., 2011; Twibanire et al., 2012; Jager et al., 2019). Glycolipids are present in both the outer and inner membranes of *Borrelia* (Toledo et al., 2018a). Nonetheless, the relative amount of the non-cholesterol glycolipid MGalD is higher in the inner membrane, whereas ACGal and CGal are relatively more abundant in the outer membrane (Toledo et al., 2018b). A proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed that the three glycolipids had an equivalent double bond content, which supports that the structure and biophysical properties of *Borrelia* glycolipids in the outer and inner membranes are similar (Toledo et al., 2018b)

Outer membrane lipid rafts

Lipid rafts are membrane microdomains that are rich in cholesterol and sphingolipids and have a subset of specific proteins associated with them (London, 2002). These microdomains are highly dynamic and float freely within the liquid-disorder bilayer of cellular membranes but also can cluster together to form larger ordered domains (Simons and Ehehalt, 2002). Until recently, lipid rafts were associated with eukaryotic cells since sphingolipids and cholesterol are rare among prokaryotes (London, 2002; Bieberich, 2018). Pioneer studies on *Bacillus subtilis* and *B. burgdorferi* showed that prokaryotes also have membrane domains that resemble eukaryotic lipid rafts (LaRocca et al., 2010; Lopez and Kolter, 2010; LaRocca et al., 2013). The presence of lipid rafts in *B. burgdorferi* was first identified while studying the mechanism of action of CB2, a complement-independent bactericidal antibody (LaRocca et al., 2009; LaRocca et al., 2010). The CB2 antibody has a very high affinity for its antigen, OspB protein, and disrupts the membrane of the spirochaete leading to an osmotic shock and subsequent death of the bacterium (LaRocca et al., 2009). The bactericidal action of CB2 is specific to *Borrelia* and depends on the presence of cholesterol (LaRocca et al., 2010). Partial depletion of cholesterol from the membrane using methyl-β-cyclodextrin protects the spirochaete from the bactericidal effect of the antibody. The bactericidal CB2 antibody also recognizes, but does not lyse, an *E.coli* strain that expresses a surface-exposed OspB.

Transmission electron microscopy showed the presence of cholesterol-rich microdomains from *in vitro*- and *in vivo*-derived spirochaetes after tagging cholesterol glycolipids with 6 nm colloidal gold particles (LaRocca et al., 2010; LaRocca et al., 2013; Toledo et al., 2014). One of the characteristics of lipid rafts is that they form fluid microdomains enriched in cholesterol and sphingolipids that are more ordered and tightly packed than the surrounding bilayer (London, 2002; Simons and Ehehalt, 2002). The properties of *Borrelia* cholesterol-rich domains were studied using biophysical approaches, including anisotropy and fluorescence resonance energy transfer (FRET). Anisotropy experiments conducted on vesicles made out of total lipid extracts, as well as on live spirochaetes, confirmed the presence of temperature-dependent ordered domains. (LaRocca et al., 2010; LaRocca et al., 2013; Toledo et al., 2014). Similarly, weak FRET, measured as the ratio of fluorescence intensity with acceptor to that without acceptor (F/F_0), supported the co-existence of temperature-dependent ordered (rafts) and disordered domains in the membrane of the spirochaete (LaRocca et al., 2010; LaRocca et al., 2013; Toledo et al., 2014)

In contrast to eukaryotic cells, *Borrelia* does not have sphingolipids. Sphingolipids are critical components of eukaryotic lipid rafts (Brown and London, 2000; Bieberich, 2018), which raised questions about the biophysical properties of *Borrelia* microdomains and whether the same principles of lipid raft formation apply to prokaryotic and eukaryotic organisms. Cholesterol substitution experiments performed in live spirochaetes using sterols with different biophysical properties, ranging from raft-supporting to raft-inhibiting when mixed with eukaryotic sphingolipids, proved that sterols are necessary and sufficient for the formation of lipid rafts in the spirochaete (Crowley et al., 2013). Raft-supporting sterols also formed detergent-resistant membranes, and saturated acyl chains were required for biotinylated probes to partition within lipid rafts, supporting that cholesterol-rich domains in *Borrelia* have the hallmarks of eukaryotic lipid rafts (LaRocca et al., 2013).

The role of different lipids in the formation of rafts is crucial in understanding the organization of the outer membrane of *B. burgdorferi*. The most abundant lipids in *Borrelia*, including ACGal, MGalD, and PC, were purified and used to make vesicles in order to study the principles that govern raft-formation. Experiments using vesicles made of ACGal, MGalD and PC and their mixtures, analyzed by FRET and anisotropy, proved that ACGal was the primary lipid responsible for order (raft) domain formation and that its role was similar to that observed for cholesterol in eukaryotic rafts (Huang et al., 2016). In contrast to unsaturated PC or *Borrelia* MGalD, the level of saturation detected in *Borrelia* PC by NMR was relatively high, which facilitates domain formation by taking a similar role as to sphingolipids in eukaryotic rafts (Huang et al., 2016).

The first evidence that lipid rafts in *Borrelia* were molecular platforms that contain a subset of specific proteins came from co-localization experiments using transmission electron microscopy. Cholesterol glycolipids formed discrete domains on the surface of the spirochaete associated with lipoproteins OspA and OspB (LaRocca et al., 2010). Depletion of cholesterol from the spirochetal outer membrane

using methyl-β-cyclodextrin disrupted lipid rafts as well as the proteins associated with them (LaRocca et al., 2010; Toledo et al., 2015). The protein content of lipid rafts was determined by mass spectrometry (Toledo et al., 2015; Toledo et al., 2018a).

The proteome of outer membrane lipid rafts provided a consistent set of selected lipoproteins, proteases and sensing molecules, some of which are prokaryotic homologs of eukaryotic lipid raft markers (Toledo et al., 2015; Toledo et al., 2018b). It is not surprising that the proteome was enriched in acylated proteins since fatty acylation is one mechanism for targeting proteins to lipid rafts (Moffett et al., 2000). Two of the most abundant proteins found in rafts were OspA and OspB. Both lipoproteins are predominantly expressed during the tick phase (Fingerle et al., 1995; Schwan et al., 1995) and mediate in the adhesion of the spirochaete to the tick midgut (Yang et al., 2004; Battisti et al., 2008). Nonetheless, a lipid tail is not sufficient to drive proteins to rafts since some lipoproteins (*e.g.*, OspC) are not associated with these microdomains. The raft proteome also included protease HtrA. The Deg/HtrA protein family is involved in adaptation to stress responses acting either as a chaperone or protease to maintain tigh protein quality control (Clausen et al., 2011). The periplasmic serine protease HtrA is present in different subcellular locations, including in the IM and OM (Coleman et al., 2013; Russell et al., 2013; Toledo et al., 2018b) where it is associated with lipid rafts (Toledo et al., 2018b). In the periplasm, the enzyme degrades unfolded proteins such as flagellin (Zhang et al., 2019). Nonetheless, *in vitro* experiments showed that a recombinant BbHtrA also degrades membrane proteins including P66 and BB0323 that co-localize with the protease in outer and inner membrane rafts, respectively (Kariu et al., 2015; Toledo et al., 2015; Coleman et al., 2016). In fact, a *B. burgdorferi* strain that overexpresses HtrA showed a two-fold decrease in the levels of P66 (Coleman et al., 2016), suggesting that co-localization of proteases and their substrates in rafts could facilitate protein degradation. Other proteins with important biological functions that were enriched in outer membrane rafts included plasminogen binding protein ErpA (Brissette et al., 2009) and Factor H binding protein CspA (Kenedy et al., 2009).

The selective association of lipoproteins and their contributions to raft formation was assessed using OspA and OspB mutants. Single OspA and OspB mutants showed no defect in lipid raft formation compared to the WT strain. However, *B. burgdorferi* B313, a strain that lacks OspA and OspB as well as most of the linear and circular plasmid, showed lower fluorescence values by fluorescence resonance energy transfer (FRET), which is compatible with less or smaller lipid rafts. The WT phenotype was recovered when B313 was transformed with a plasmid containing OspA but not when it was transformed by a plasmid expressing OspC, which is not found on lipid rafts. Collectively, these findings support a selective association of lipoproteins and lipid rafts that favors the formation of these microdomains (Toledo et al., 2014).

The peptidoglycan cell wall

Sandwiched between outer and inner membranes of the *B. burgdorferi* cell envelope is the peptidoglycan (PG) cell wall. PG is a mesh-like, stress-bearing, biological sac (i.e. sacculus). The primary role of PG is to act as an osmoprotectant, a dynamic bio-fabric that prevents the cell from bursting due to the high solute concentration in the cytoplasm (Holtje, 1998). Apart from combating turgor, PG has additional functions. PG is, in essence, the exoskeleton of most bacteria as well as the major cell envelope component that imparts cell shape (Young, 2006). Cell envelope organization is largely dictated by PG in that the sacculus often acts as a scaffold and provides continuity between layers (Cava and de Pedro, 2014). Because the PG layer is porous, it acts as a nondiscriminatory sieve for molecules up to 24kDa (Demchick and Koch, 1996). Apart from playing a structural role, PG organization is also central to the sub-cellular organization of many cell cycle events (Turner et al., 2014). Fragments of PG, or muropeptides, are even capable of acting as a chemical means of communication between bacteria and even kingdoms (Dworkin, 2014). Despite these exciting and versatile functions, until recently, the PG of *B. burgdorferi* has been largely ignored. Here we consider the unique chemical and structural features of *B. burgdorferi* PG and how these peculiarities may be involved in the biology and pathogenesis of Lyme disease.

The PG mesh is composed of two salient features – roughly parallel (Verwer et al., 1978) glycan strands that are connected by short peptides. Each glycan strand contains the repeating disaccharide *N*-Acetyl Glucosamine (Glc*N*Ac) and *N*-Acetyl Muramic acid (Mur*N*Ac). The latter is a sugar that is only found in bacteria (Vollmer et al., 2008). Disaccharides are joined by a ß-1,4 glycosydic bond. Virtually all

bacteria follow this glycan strand organization; however, modifications that make the linkage resistant to lysozyme degradation, such as *O*-acetylation, are known to occur in several pathogens (Vollmer, 2008). While glycan organization and structure are well-conserved among all bacteria, some variability exists in the bridging peptides. The most common amino acids in the first and second position are L-Alanine and D-Glutamine, respectively. Variations occur at the third position, but chemical constraints require that this position is occupied by a dibasic amino acid to complete the cross-bridge with the neighboring peptide chain. Most gram-negative bacteria incorporate diaminopimelic acid (DAP) as the diamine, in the third position, while gram-positive bacteria carry Lysine (Lys) at the analogous position (Desmarais et al., 2013). Deviations from this dichotomy are rare (Vollmer, 2008). Di-D-Alanine terminate the pentapeptide and are the substrate(s) for Penicillin-binding protein (PBP) transpeptidase reactions, reactions that are required for remodeling the overall PG structure during sacculus elongation.

B. burgdorferi PG has several unusual features that deviate from convention. For instance, L-Ornithine (L-Orn) occupies the third position of the stem peptide, linked to a single Glycine (Gly) residue (Beck et al., 1990; Jutras et al., 2019). Several earlier reports show that some spirochaetes, including the relapsing fever agent *Borrelia hermsii* and treponemes incorporate L-Orn into PG (Azuma et al., 1975; Yanagihara et al., 1984; Caimano et al., 1999). The amidation of DAP – a common PG modification (Vollmer, 2008) – has the exact same mass as L-Orn-Gly. Thus, a more indepth analysis may be required to validate earlier findings ((Azuma et al., 1975; Yanagihara et al., 1984). Nevertheless, recent radiolabeling studies, coupled with tandem liquid chromatography/liquid scintillation (Jutras et al., 2019), confirmed the presence of L-Orn-Gly in *B. burgdorferi* PG. The same study uncovered a surprising alteration in glycan strand composition, providing evidence for a yet to be identified *N*-acetylated Hexose (Hex*N*Ac). Our high-resolution analysis of *B. burgdorferi* PG has both confirmed the presence of a third Hex*N*Ac, while identifying amidated Glutamine as another modification (DeHart and Jutras, unpublished data). The biological relevance of these uncommon PG alterations remains to be determined.

The structural architecture of *B. burgdorferi* PG is beginning to be elucidated. Composition and abundance of muropeptide profiles indicate that, on average, *B. burgdorferi* glycan strands are 30 disaccharides long (Jutras et al., 2019), which equates to ~30nm. In comparison, this is roughly similar to the glycan strand length of *Escherichia coli* grown under normal conditions. Glycan strands lie perpendicular to the long axis (Gan et al., 2008). When we consider the diameter of the spirochetal cell— 5 times smaller than *E. coli*— the number of strands required to cover the *B. burgdorferi* cell circumference (~20) is miniscule relative to other PGs previously analyzed (Vollmer and Seligman, 2010). Of the peptides present in *B. burgdorferi* PG, approximately 40% are participating in cross-linking glycan strands (Jutras et al., 2019). In line with this observation, free stem peptides are devoid of terminal D-Alanine(s); their detection required a high-resolution, targeted MS approach (Jutras et al., 2016). The degree of cross-linking may be involved in the seemingly non-uniform distribution of *B. burgdorferi* PG. Cryo-electron tomography images of intact *B. burgdorferi* cells show that the thickness of the PG layer varies from ~6 nm at mid-cell to ~40 nm at the poles (Figure 3A). Preliminary analysis of purified sacculi suggests that poles may be reinforced with multiple layers of PG (Brock, Liu, and Jutras, unpublished data) – yet another unusual feature and paradigm shift from the ~1-layer model for diderms (Gan et al., 2008).

Why do *Borrelia*, and seemingly other spirochaetes (DeHart and Jutras, unpublished data), produce PG with unusual chemical and structural features? Unlike most bacteria, *Borreliae* and *Treponema* possess periplasmic flagella form a ribbon that wrap around the cell cylinder (Charon et al., 2009) and are inserted, sub-terminally, at each pole. The natural flat-wave morphology is created by the inherent helicity of the collective flagellar filaments (Motaleb et al., 2000). Together, many studies support an unusual paradigm as it relates to PG and cell morphology – PG doesn't impart cell shape in *Borrelia* and treponemes (Charon et al., 2012). Instead, the PG – a straight rod when purified (Jutras et al., 2016) – pushes against the helical pitch created by the filaments, indicating that *Borrelia* flagella are the actual cytoskeletal structure. Flagellar motors are anchored into the PG layer and their rotation (and thus movement) produces immense torque on the cell cylinder. To protect cytoplasmic contents, PG produced by most spirochaete must be exceptionally flexible in order to exert counter force against the torque of the flagellar filaments (Charon

et al., 2009). PG chemistry and structure are likely to be important in the elasticity of the biopolymer. Several lines of evidence support this notion. First, in order to create flat-wave morphology, mathematical modeling suggests that the PG of *B. burgdorferi* be more flexible than other, helical-shaped spirochaetes that do not possess L-Orn-type PG (Wolgemuth et al., 2006; Dombrowski et al., 2009; Slamti et al., 2011). Treating *B. burgdorferi* with vancomycin, an inhibitor of PG transpeptidation and cell wall architecture, alters both PG stiffness and bacterial motility (Harman et al., 2017). Finally, recent data indicate that mutations in PG synthesis enzymes that result in altered PG peptide composition cause a dramatic change in cell morphology (MecClune and Jutras, unpublished data), which raises the notion that that *B. burgdorferi* PG may have evolved to strike the perfect biophysical balance with the spirochaete motility machinery (DeHart and Jutras, unpublished data).

There may be unintended consequences of having atypical PG. *B. burgdorferi* is considered by many to be a stealth pathogen. Many of the ill effects during the course of LD infection are propagated by the human host response. As *B. burgdorferi* elongates its PG sacculus, it sheds muropeptides – 45% of all PG inside the cell is released per generation (Jutras et al., 2019). Released muropeptides are known to act as host immune modulatory molecules in other pathogens. For instance, *Neisseria gonorrhoeae* release many different muropeptides (Sinha and Rosenthal, 1980; Garcia and Dillard, 2008), most of which are cytotoxic and play a critical role in pathogenesis (Melly et al., 1984; Cloud-Hansen et al., 2008; Chan and Dillard, 2016; Schaub et al., 2016; Chan and Dillard, 2017; Lenz et al., 2017). Another gram negative, *Helicobacter pylori* both releases and injects PG breakdown products during mammalian infection to modulate host response (Viala et al., 2004; Gorrell et al., 2013; Irving et al., 2014). Tracheal Cytotoxin (TCT), one of the first bacterial molecules to demonstrate toxin activity (Goldman et al., 1982) is indeed a released muropeptide produced by *Bordetella pertussis* involved in whooping cough pathology (Goldman et al., 1982; Heiss et al., 1993; Heiss et al., 1994; Flak and Goldman, 1999).

Transport proteins and complexes

Transport proteins catalyze the translocation of solutes by various systems that differ with respect to energy coupling, substrate specificity and transmembrane topology. So far, four distinct types of functionally characterized transport systems have been described. These are (i) bacterial channel proteins, (ii) secondary transporters that utilize chemo-osmotic energy, i.e. proton gradient, (iii) primary transporters utilizing chemical energy, typically in the form of ATP, and (iv) group translocators that phosphorylate their substrates during transport (PTS). From a comparative genome analysis of transporters from 18 different prokaryotes by Paulsen and co-workers (Paulsen et al., 2000), it appeared that, relative to genome size, the spirochaetes *B. burgdorferi* and *T. pallidum* have few transporters, particularly secondary transporters (Saier and Paulsen, 2000). Interestingly, both spirochaetes contained the lowest numbers of transmembrane segments and transporters as compared to other bacteria (Paulsen et al., 2000). In another comparative study of 215 protein-translocation outer membrane porins of Gram-negative bacteria, only the TolC homologue (BB0142) that belongs to the outer membrane factor (OMF) family was identified in *B. burgdorferi* (Yen et al., 2002). Among the bacteria compared, *B. burgdorferi* has the largest percentage of phosphotransferase system (PTS) permeases. Notably *B. burgdorferi* also encodes a large number of peptide transporters (Paulsen et al., 2000).

Within the OMF family are the so called channel-tunnels which are engaged in exporting substances across the membranes of Gram- negative cell envelopes. This family incorporates the efflux pumps, which confer resistance to various harmful substances like antibiotics, heavy metals, dyes, bile salts and detergents. Efflux pumps consist of an inner membrane transporter, a periplasmic accessory protein and an outer membrane channel-tunnel. The well-studied *E. coli* channel-tunnel, TolC, is involved in protein secretion and can also be part of diverse efflux pumps (Andersen et al., 2000). The RND (resistance-nodulation division) transporters (Li and Nikaido, 2004) exist in all kingdoms of living organisms but seem to be involved in drug resistance, especially in Gram-negative bacteria, in which they export toxic substances across the cell envelope in a single energy-coupled step (Koronakis et al., 2000). The TolC crystal structure has been solved and consists of channel, equatorial, and tunnel domains (Koronakis et al., 2000). The channel domain is embedded in the outer membrane and consists of non-polar residues directed towards lipids and polar residues that locate to the interior resulting

in typical amphipathic β-strands. The β-barrel of channel-tunnel is different from those of typical porins formed by single peptide chain. To form a TolC channel domain, three protomers are necessary in order to generate a single 12-stranded β-barrel. The tunnel domain of TolC consists entirely of α-helices assembled in an α-barrel, representing a unique structure. In the *B. burgdorferi* outer membrane, a channel-forming activity corresponding to a TolC homologue, BesC, has been identified, which is part of a putative export system comprising the components BesA, BesB and BesC (Bunikis et al., 2008). BesC forms channels in planar lipid bilayers. A structural model of the efflux apparatus was described showing the putative spatial orientation of BesC with respect to the AcrAB homologues BesAB. This structural modelling showed both similarities and differences between TolC of *E. coli* and BesC of *B. burgdorferi* (Bunikis et al., 2008). For TolC, it is well known that residues lining the periplasmic entrance are critical for the electrophysiological behaviour. A ring consists of six aspartate residues at the TolC tunnel entrance, viewed from the periplasm, is believed to be responsible for its high cation selectivity (Andersen et al., 2002). In BesC, the aspartate ring is replaced by positively charged lysine residues (K366) and negatively charged aspartate residues (D363), resulting in no net charge at the tunnel entrance and, thus, a non-selective BesC channel (Bunikis et al., 2008). Another difference between BesC and TolC is that the BesC channel has a nearly four-fold higher conductance in 1 M KCl than TolC (80 pS in 1 M KCl) (Benz et al., 1993). Bunikis and coworkers further demonstrated that the BesC protein was necessary for *B. burgdorferi* to establish infection in mice and also is involved in antibiotic resistance. This efflux system might be part of a Type I secretion machinery for maintenance of cellular homeostasis or export of exogenous toxic agents, perhaps also necessary for survival in vastly different host environments (Bunikis et al., 2008). An additional finding from the structural modelling of BesA is the lack of the α-hairpin domain creating a different adaptor structure as compared to *E. coli* (Bunikis et al., 2008). This modelling was subsequently verified by a high resolution structure analysis of BesA that confirmed the missing α-hairpin domain of the periplasmic adaptor protein (Greene et al., 2013). Furthermore, a primary sequence comparison revealed that this paradigm shift of adaptor structure is not unique to *Borrelia* as it also was found in other spirochaetes, including *Treponema* and *Spirochaeta* (Greene et al., 2013).

The genome of *B. burgdorferi* encodes a large amount of homologous carbohydrate transporters that belong to the PTS group (Fraser et al., 1997). A number of studies have attempted to characterize some of these transporters. Chitobiose is a component of the tick cuticle and peritrophic matrix. The products of *chbA*, *chbB*, and *chbC* allow *Borrelia* to transport and utilize chitobiose in the tick environment (Tilly et al., 2001; Tilly et al., 2004). Different *chb* genes may respond differently to various temperatures – *chbB* transcription is up-regulated at 23°C, whereas *chbC* transcription increases at 35°C (Ojaimi et al., 2003). Glucose is the major energy source for *B. burgdorferi* involving several PTS glucose transporters (von Lackum and Stevenson, 2005). BBB29 is one of the components of a glucose transporter system of *B. burgdorferi* (Byram et al., 2004). BBB29 expression is elevated in a *B. burgdorferi* non-disseminating clinical isolate compared to a disseminating isolate (Ojaimi et al., 2005) and at 23°C versus 35°C (Ojaimi et al., 2003). Mannose-modified host proteins are also a likely carbohydrate source for *B. burgdorferi,* with the BB0408 and BB0629 proteins suggested to be mannose transporters (von Lackum and Stevenson, 2005).

The members of major intrinsic protein (MIP) group consists of essential channel forming proteins that maintain an osmotic cell equilibrium and include aquaporins and glycerol facilitators (GlpF) (Paulsen et al., 2000; Froger et al., 2001). The GlpF homologue in *B. burgdorferi* (BB0240) is involved in glycerol uptake, which can either be used as an energy source or for phospholipid and lipoprotein biosynthesis (Schwan et al., 2003). The process of glycerol uptake (*glpF*) and utilization (*glpK/bb0241* and *glpA/bb0243*) might be required when *Borrelia* resides in its arthropod host since these *glp* genes are significantly up-regulated in *B. burgdorferi* grown at 23°C versus 35°C (Ojaimi et al., 2003).

Inner membrane transmembrane proteins

The oligopeptide permeases (Opps) belong to the family of ATP-binding cassette (ABC) transporters used for transportation of peptides into bacteria to be subsequently used as carbon and nitrogen sources (Lazazzera, 2001). Although the major role of bacterial Opp is in nutrient uptake, they may also be involved in sensing environmental signals in the form of peptides leading to diverse responses, including expression of virulence determinants, quorum sensing, chemotaxis, sporulation, conjugation, and

competence (Perego et al., 1991; Rudner et al., 1991; Leonard et al., 1996). It is not known if the oligopeptide permease has a sensing capability in *B. burgdorferi s.l.* similar to other bacteria although an interesting feature to note is that Opp-like proteins are involved in expression of virulence determinants in other bacteria (Payne and Gilvarg, 1968; Goodell and Higgins, 1987; Finlay and Falkow, 1989; Gominet et al., 2001). The Opp complex in *B. burgdorferi* consists of an oligopeptide-binding lipoprotein OppA, a heterodimeric cytoplasmic membrane permease (OppB and OppC), and the nucleotide binding domain (NBD) heterodimer OppD and OppF that drives the transport of oligopeptides using ATPases (Das et al., 1996; Bono et al., 1998; Kornacki and Oliver, 1998; Monnet, 2003; Groshong et al., 2017). Interestingly, *B. burgdorferi* has five *oppA* genes each controlled by promoters which respond differently to various environmental signals (Wang et al., 2002; Ojaimi et al., 2003). However, *oppA* genes can also be coordinately up-regulated in *B. burgdorferi* clinical isolates with different capacities for haematogenous dissemination (Ojaimi et al., 2005). Perhaps each OppA possesses a different substrate binding affinity that is active during different stages of *B. burgdorferi* life cycle (Wang et al., 2004a), which would attribute to the targeting and penetration of specific tissues in invertebrate and vertebrate hosts (Ojaimi et al., 2005). The Opp system is particularly interesting in *B. burgdorferi s.l.* since it was evident from the genome sequence that only rudimentary machinery for synthesis or transport of amino acids exists. In fact, only a single intact peptide transporter operon could be found. From the genome analysis of *B. burgdorferi s.l.* it is also apparent that the *oppA* operon differs from that of *E. coli* as it has three separate chromosomally encoded substrate binding proteins (Lin et al., 2001; Wang et al., 2002), OppA-1, 2 and 3 and two additional plasmid encoded homologues OppA-4 and 5 (Wang et al., 2002). Interestingly, all of the *B. burgdorferi s.l.* OppA proteins were found to complement *E. coli* OppBCDF to form a functional peptide transport system for nutritional purposes (Lin et al., 2001). All five *oppA* genes have their own active promoters, and the apparent difference in regulation of gene expression implies that the *oppA* genes respond by different environmental signals for the adaptation of the *Borrelia* spirochaetes to various hosts (Wang et al., 2002). There was also a considerable difference in the substrate specificities of the five different OppA proteins, which suggest that they may have separate functions in the spirochaete (Wang et al., 2004a). These findings led to an in depth investigation of the factors that regulate the different *oppA* promoters. Thus, the alternative sigma factors, RpoS and RpoN, regulate the expression of *oppA5* (Caimano et al., 2007), the BosR/Fur homologue of *Borrelia* interacts with the *oppA4* promoter, and another candidate transcription factor, EbfC, interacts with the *oppA5* promoter (Medrano et al., 2007) (see chapter five by Samuels et al.). In a very elegantly executed study by Groshong and coworkers, structural analyses and modeling explain how the five oligopeptide binding proteins (OBPs) interact with variable peptide fragments. Transcriptional analyses of the different OBPs further indicated that they are both independently regulated and sensing different signals whereas the permeases and NBDs are constitutively expressed (Groshong et al., 2017). This study further showed the importance of peptide uptake for virulence and physiology of *Borrelia* spirochaetes.

Inner membrane lipid rafts

The presence of inner membrane proteins, including HflC, HflC, FtsH, among others, in the proteome of lipid rafts, was the first indirect evidence that these microdomains exist in both membranes (Toledo et al., 2015). The lipid profiles of the inner and outer membranes are very similar and contain the same lipid species, including cholesterol, cholesterol esters, ACGal, CGal, MGalD, PC and PG (Toledo et al., 2018b). The main difference between the lipid composition of the two membranes is the relative amount of glycolipids. Cholesterol glycolipids, ACGal and CGal, are relatively more abundant in the outer membrane than in the inner membrane, whereas the non-cholesterol glycolipid, MGalD, is more abundant in the inner membrane. Multilamellar vesicles (MLVs) prepared from isolated lipids from both membranes showed ordered (rafts) and disordered domains, as measured by FRET and anisotropy. However, they exhibited different levels of membrane order and domain segregation (Toledo et al., 2018b). These differences could be attributed, at least in part, to the presence of higher quantities of ACGal in the outer membrane, since ACGal is the primary lipid implicated in raft formation (Huang et al., 2016). Another factor that might contribute to the differences observed between both membranes is the level of saturation of PC, which is significantly higher in the outer membrane compared to the inner membrane (Toledo et al., 2018b).

The proteome of inner membrane lipid rafts is enriched in proteins involved in trafficking, signaling

and contains proteases, including HtrA and FtsH, as well as lipid rafts markers. The proteome included HflC/K proteins, which have Stomatin Prohibitin Flotillin HflC/K (SPFH) domains (Rivera-Milla et al., 2006). Proteins with SPFH domains are very diverse and have different functions, but they partition in lipid rafts and are commonly used as lipid raft markers (Browman et al., 2007). In contrast to HtrA, FtsH is only associated with the inner membrane, although it is unclear whether FtsH partition in lipid rafts or is recruited by HflC/K, which are transmembrane regulators of FtsH (Kihara et al., 1997). FtsH is essential for the viability of the spirochaete *in vitro* and *in vivo* and its independent of regulators HflC/K (Chu et al., 2016). The substrates for FtsH in *Borrelia* are unknown, but in *E. coli* FtsH degrades membrane-embedded proteins such as SecY and SecD as well as soluble substrates (Kihara et al., 1995; Chiba et al., 2002). Interestingly, Sec proteins, including SecA and SecD, are enriched in lipid rafts in the inner membrane (Toledo et al., 2018b) and are potential substrates of FtsH. Similarly, inner membrane lipid rafts also were enriched in ABC transporters (BB0330, BB0334, BB0335, BB0466, BB0754 and BBB16, among others) and methyl chemotaxis proteins (BB0596, BB0597), core sensing elements in prokaryotes (Salah Ud-Din and Roujeinikova, 2017). In summary, inner membrane lipids rafts, based on their protein content, are associated with protein trafficking and signaling processes, whereas outer membrane lipid rafts discussed earlier are rich in proteins associated with adaptation and survival to tick and vertebrate milieus.

The cell cycle and cell division

The bacterial cell cycle – the life of a single cell from birth to division – is riddled with challenging events that need to be coordinated in both time and space. If one were to think of the cell like a water balloon - the envelope being the elastic balloon, and the cytoplasmic contents the water - we can start to understand the challenges. To grow, the balloon needs to physically expand and divide, not just stretch, while controlling the distribution and displacement of the water. To divide, the balloon needs to be cut in half, and each new end needs to be fabricated, all without spilling the water. This seems like an insurmountable feat, and yet, all bacteria perform these tasks rapidly and virtually mistake free. The key is controlling the timing and spatial arrangement of enzymes involved in cell envelope elongation with the machinery necessary for division. Here, we discuss these processes, highlight some of the recent findings in understanding these events, and discuss the implications in the context of Lyme disease treatment.

The PG sacculus is akin to the aforementioned balloon – a big molecular bag that surrounds the cytoplasm. Cell growth requires PG elongation. Cell division means that the *sac* is cut and PG poles are formed. Thus, by tracking PG synthesis we can understand all facets of the *B. burgdorferi* cell cycle. Recent advances in chemical synthesis have made tracking PG biogenesis possible. The covalent attachment of fluorophores, with different physical and spectral properties (Hsu et al., 2017), to the ubiquitous PG component D-Alanine (D-Ala), has furthered our understanding of *how* bacteria grow (Kuru et al., 2012). While there are always incredible exceptions in nature (Caccamo and Brun, 2018), most bacteria grow using one of the three following strategies. (i) Many rod-shaped bacteria elongate their cell wall by lateral synthesis, which involves near homogenous growth throughout the cell cylinder prior to septal synthesis at division (Figure 2A). (ii) Some rod-shaped bacteria, like *Agrobacterium tumefaciens* and *Mycobacterium tuberculosis*, have an alternative mode of growth – elongating from either one or both poles, respectively (Figure 2B) (Kuru et al., 2012; Garcia-Heredia et al., 2018). (iii) Most coccoids replicate by simply producing two halves of each daughter cell, also known as septal synthesis (Figure 2C). Once again, *B. burgdorferi* has deviated from convention. Feeding *B. burgdorferi* fluorescent D-Ala resulted in an unmistakably unique pattern of PG synthesis that seems to combine several forms of growth, simultaneously. With the exception of 2-4 um at the poles, *B. burgdorferi* elongates by both lateral synthesis and discrete zones of intense growth (Jutras et al., 2016). Zones are organized in both time and space – a central zone of growth is followed by secondary and tertiary sites. Despite distances that can exceed 30 um in length, each zone precisely forms at the ¼, ½, and ¾ positions along the cell length (Figure 2D). The primary zone at the ½ position becomes the site of cell division in the F_0 generation, while the ¼ and ¾ positioned zones are the site of cell division in the F_1 generation. In essence, how the mother cell grows determines where the daughter cells will divide. Multi-zonal growth appears to be a unique feature to the *Borrelia* genus. All *Borrelia spp.* tested, including those responsible for relapsing fever, display a similar PG growth pattern. Other spirochaetes, such as *Treponema denticola* and *Leptospira interrogans,*

grow through lateral PG synthesis. The mechanism(s) responsible for zone placement, across a distance 30-50 times longer than the average rod-shaped cell, requires exquisite spatial and temporal balance between the elongation and division machinery.

We know very little about multi-zone, PG synthesis in *B. burgdorferi*. Co-incubation with fluorescent D-Ala and D-cycloserine (Kuru et al., 2019), a putative inhibitor of cytoplasmic D-Ala-D-Ala synthesis (Strominger et al., 1960), leads to the disappearance of growth zones in *B. burgdorferi,* unlike in other bacteria. Together, these findings suggest that zones of growth are in essence PG synthesis machinery hubs that are pre-established. These focal points extend into the periplasm, and orchestrate the incorporation of muropeptides into nascent PG, since there is a near perfect correlation between zones of PG synthesis and the localization of Penicillin-Binding Proteins (PBPs) in *B. burgdorferi* (Jutras et al., 2016). In depth analysis of D-Ala signal, relative to PBP signal, suggests that active sites of incorporation are more finite and lie inside the expanded area of each zone (Brock and Jutras, unpublished data, and Figure 3B). These observations provide clues as to a possible mechanism for zone placement (see below). Wu and colleagues made a case for an alternative mode of *B. burgdorferi* growth, which only occurs in stationary phase cultures, whereby cells grow by polar synthesis (Wu et al., 2018). In exponential, or stationary-phase growth, vancomycin – a glycopeptide inhibitor of periplasmic PG incorporation – eliminated both zonal and *polar* synthesis (Wu et al., 2018). These findings weaken the argument for polar growth in stationary phase and provide for an alternative, more parsimonious explanation for their observation. Late-stage cultures grow slowly, due to a depletion of nutrients in the culture media. Recently divided bacteria have a single zone of growth and polar signal from the previous division event (Figure 2E). As stationary phase and vancomycin treatment continue, slow lateral synthesis continues without further zonal growth. Upon reaching the total cessation of growth, the previously established zone becomes the site of division, which is forced by washing and centrifugation prior to image acquisition. The resulting cells have either a single pole labeled, or both poles, but one with more recent/intense signal (Figure 2E). In short, zonal growth and experimental conditions, can explain the recent observation without inferring a secondary strategy for PG synthesis.

The mechanism(s) that drive zonal growth and its placement are not known. Absolute cell length is not the impetus since different strains, which achieve average lengths that vary by >20um still form zones at the same relative ¼, ½, and ¾ positions (Jutras et al., 2016). Researchers in the field have long recognized the association between motility and cell division, as if the forceful torque applied by flagella on the cell cylinder is required for final cell separation (Motaleb et al. 2000). Be that as it may, a mutant that is unable to produce the major flagellar filament protein FlaB still faithfully positions zones that are correctly placed along the long axis of the cell. *B. burgdorferi* PG zones are truly finite, but extended regions of elongation. Photobleaching experiments demonstrated that the *B. burgdorferi* cytoplasm is contiguous in cells with three zones, indicating that these wide regions (2-4um) are not septa, produced by three discrete cells that are chained together (Jutras et al., 2016). That said, zones clearly become sites of cell division, implicating the cell division machinery in zone establishment and placement.

FtsZ is the most likely candidate for determining both the sites of zonal growth, and septal PG synthesis, in *B. burgdorferi.* FtsZ is a tubulin homologue that is essential for cell division in virtually all bacteria (Du and Lutkenhaus, 2019). FtsZ forms a ring-like structure and coordinates the placement and activity of nearly 20 proteins in most bacteria; many of which are essential for viability under typical growth conditions (Egan et al., 2020). The dynamic treadmilling behavior of FtsZ (Yang et al., 2017) coordinates the location and activity of PG septal synthesis machinery (Bisson-Filho et al., 2017), and ultimately, cell division. Many of the factors that influence FtsZ activity, placement, and cell division related processes are present in the *B. burgdorferi* genome, including putative homologues of *fts* genes A, B, E, H, I, K, Q, W, Y, Z. However, the negative regulators, whose role is to prevent FtsZ ring formation, too early or in erroneous places (e.g. Min and SlmA/Noc systems, EzrA, MipZ, etc) (Levin et al., 1999; Errington et al., 2003; Wu and Errington, 2004; Thanbichler and Shapiro; Cho et al., 2011) are conspicuously absent. Homologues of so-called positive regulators of Z-ring assembly, such as MapZ, PomZ, and SsgA/B (Willemse et al., 2011; Treuner-Lange et al., 2013; Fleurie et al., 2014), are also not found in the *B. burgdorferi* genome. Given the

complex genome, in combination with the absolute cell length of *B. burgdorferi*, there are likely analogous systems yet to be identified.

Efforts to produce bacteria with fluorescent protein fusions to *B. burgdorferi* FtsZ, including in *cis* and in *trans*, have been futile (Pichoff, Zückert and Lutkenhaus; Brock and Jutras, unpublished data). This includes a strategy whereby a fluorescent protein is sandwiched between disordered domains in FtsZ (Moore et al., 2017), a hybrid fusion that is fully functional in *E. coli*. These observations suggest that *B. burgdorferi* FtsZ may have unique properties, which could explain both the unusual mode of *Borrelia* growth and the unsuccessful attempts to localize the protein in live cells. Indeed, unpublished data indicate that *B. burgdorferi* produces two isoforms of FtsZ (Brock and Jutras, unpublished data). Analysis of the *B. burgdorferi ftsZ* locus provides clues. A primary Ribosome Binding Site (RBS), upstream of the annotated Methionine (Met) start codon, is followed by an internal RBS and an additional Met in the same reading frame. Thus, *in vitro* cultured bacteria produce two variants of FtsZ.

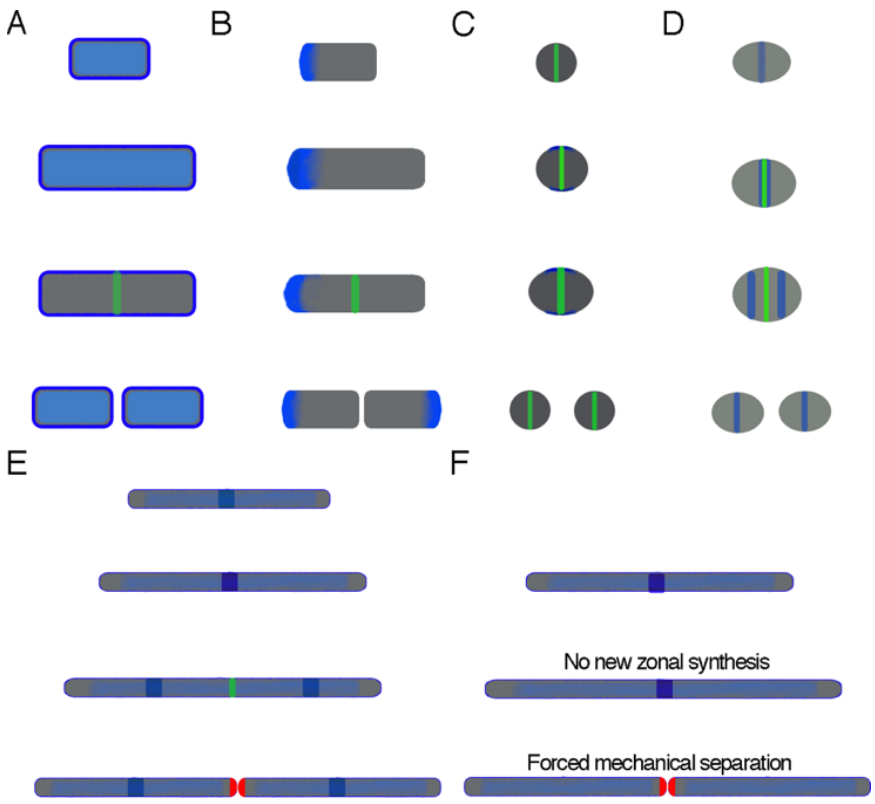

Figure 2. Different modes of bacterial growth. New fluorescent probes that track PG synthesis have illuminated different bacterial strategies for self-replication. (A) Lateral growth involves near ubiquitous PG synthesis (blue) throughout the cell cylinder, prior to septal PG synthesis (green) at division. (B) Polar growth occurs at one or both ends, only, prior to division. (C) Many spherical bacteria grow by producing two halves of a circle, almost exclusively synthesizing a septum. Note some pre-septal (blue) growth, adjacent to the septum, does occur in some species. (D) Ovoccoid bacteria (e.g. *Streptococcus pneumonia*) simultaneously elongate by producing pre-septal zones of PG synthesis, while producing a septum. Prior to septation, pre-septal zones are apparent in a mother cell. (E) In all *Borreliae* tested thus far, PG synthesis occurs by both lateral synthesis, and discrete zones of elongation. A single zone, at mid-cell, becomes the site of septal PG synthesis. Prior to membrane invagination, secondary and tertiary zones are asynchronously established, which will become the sites of division in the following generation. Note that lateral synthesis in *B. burgdorferi* does not occur (gray) at the poles, but post-division, residual signal becomes trapped (red). (F) An alternative explanation for apparent polar growth in *B. burgdorferi.* Lateral signal, at any stage of growth, has never been observed at poles. Upon vancomycin treatment, in stationary phase, primary zonal growth is reduced, no other zones are great, and lateral synthesis slowly continues. Both the zonal and lateral PG synthesis are, eventually, halted. After washing and mechanical manipulation of the cells, cell separation occurs, producing two daughter cells with trapped signal (red).

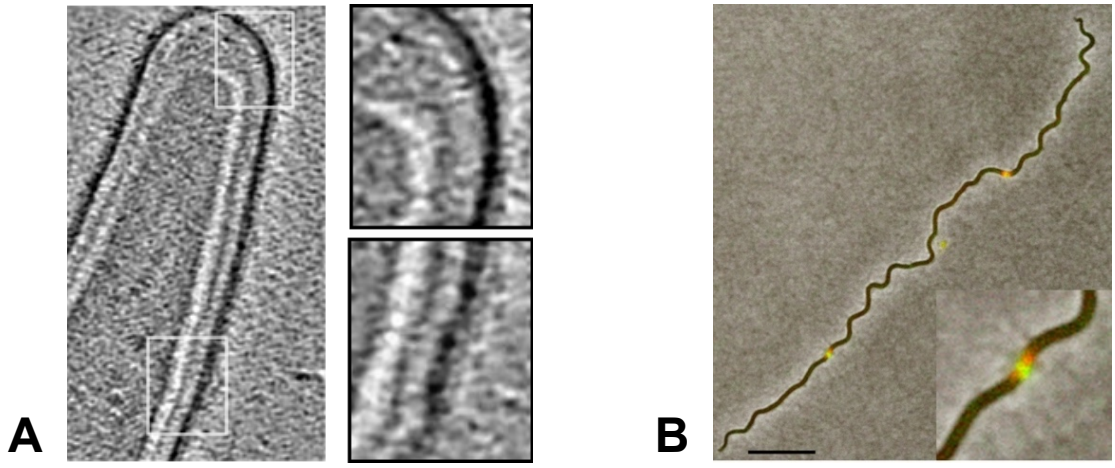

Figure 3. A. Cryo-electron tomography images of intact *B. burgdorferi* cells. PG thickness from mid cell to pole changes from ~6nm to ~40nm. **B. Penicillin-Binding Protein probes identify foci within PG synthesis zones.** Merged phase contrast and epifluorescence micrograph of *B. burgdorferi* co-labeled with fluorescent D-Alanine HADA (red) and PBP probe bocillin (green) suggest that the PG synthesis machinery is positioned in finite positions, inside zones of growth. Scale bar 10 µm, inset 4X.

The consequences of producing two isoforms of FtsZ are not known.

The pattern of *B. burgdorferi* PG synthesis, and the priming of division sites, bares resemblance to the cell cycle of the ovacoccoid bacterium *Streptococcus pneumoniae.* Three discrete zones of PG synthesis are apparent in *S. pneumoniae* prior to cell division (Peters et al., 2014). Each zone is equidistant and delineate future division sites (Peters et al., 2014; Tsui et al., 2014). These similarities are contrasted by some notable differences that truly make *B. burgdorferi* growth unique. For instance, *S. pneumoniae* PG zones are peripheral to the site of septal synthesis and both modes of growth occur simultaneously but do so in the absence of lateral synthesis. The latter can be attributed to *S. pneunmonia* not producing MreB, the central player in lateral PG elongation (Vollmer et al., 2019). Peripheral and septal PG zones are driven by Pbp2a and Pbp2x, respectively (Pinho et al., 2013). In contrast, *B. burgdorferi* zonal and lateral PG biogenesis occur simultaneously, but are discrete from septal synthesis. *B. burgdorferi* does not appear to encode a Pbp2a homologue but does produce an unusual Pbp analogous to FtsI with an additional PASTA (penicillin-binding protein and serine/ threonine kinase associated) domain –a domain found within Pbp2x (Beilharz et al., 2012) and important regulator cell division (Fleurie 2014}.

Lipid acquisition/biosynthesis and modification

Phospholipids are the main components of cellular membranes; they form permeable bilayers that act as a barrier and support critical biological processes, including sensing and signaling. Most bacteria possess anionic phospholipids such as PG and cardiolipin (CL) and the zwitterionic phosphatidyl-ethanolamine (PE). Like most bacteria, *B. burgdorferi* has PG, but it also possesses PC (Belisle et al., 1994), which is the most common phospholipid in eukaryotic membranes but is far less common among prokaryotes (Sohlenkamp and Geiger, 2016). The levels of PC in the membrane of prokaryotes range from small amounts in *Pseudomonas aeruginosa* to up to 73% of total membrane lipids in *Acetobacter aceti* (Sohlenkamp et al., 2003). In *Borrelia*, PC is the most abundant phospholipid (68%-74%), while PG constitutes 26-32% of the membrane phospholipids. The structures of PE and PC are very similar, and the only difference is the methylation of the nitrogen in the choline of PC. Nonetheless, PC is a bilayer forming phospholipid while PE prefers to form lipid structures such as inverse hexagonal phase (Popova and Hincha, 2011). There are two biosynthesis pathways in bacteria that produce PC, the methylation pathway and the phosphatidylcholine synthase (PCs) pathway. The methylation pathway is a stepwise process that converts phosphatidylethanolamine (PE) to PC and involves one or more phospholipid N-

methyltransferases (Geiger et al., 2013). The second pathway present in bacteria is the PCs pathway, in which choline condenses directly with CDP-diacylglycerol to form PC and CMP (Geiger et al., 2013; Sohlenkamp and Geiger, 2016). Some bacteria species possess both pathways, including *Legionella pneumophila* and *Agrobacterium tumefaciens* (Wessel et al., 2006; Geiger et al., 2013), but the majority rely on one of these pathways. The genome of *B. burgdorferi* is one of the smallest genomes among bacteria and lacks homologs of N-methyltransferase genes (Fraser et al., 1997; Geiger et al., 2013). In addition, PE is required to produce PC via the methylation pathway, but it is not found in the membrane of *Borrelia* (Belisle et al., 1994). The lack of PE in *Borrelia*, which is required in many prokaryotes, suggests that other lipids, possibly glycolipids, could compensate for it (Wang et al., 2004b). The spirochaete possesses a phosphatidyl-choline synthase, encoded in the gene *bb0249*, that is involved in the production of PC via the PCs pathway (Wang et al., 2004b). *bb0249* was cloned in *Escherichia coli*, which does not contain PC, resulting in the production of PC in the presence of exogenous choline (Wang et al., 2004b). Choline is readily available in the host and is likely transported into the cell through the ATP dependent transporter system ProU (Bontemps-Gallo et al., 2016)

The second phospholipid present in the membrane of *B. burgdorferi* is PG. The spirochaete encodes a phosphatidylglycerol synthase (PGs) gene (*bb0721*) in the chromosome that is critical for the production of PG from CDP-DAG. *E. coli* HD38/pHD102 was transformed with a plasmid containing *bb0721* (pTAC56) to assess the PGs activity of this gene. *E. coli* HD38 lacks a PGs, which is essential for growth, and therefore requires a functional copy expressed by the temperature-sensitive plasmid pHD102. As a result, at temperatures above 30°C, the growth of *E. coli* HD38/pHD102 stops. In contrast, transformed *E. coli* HD38/pTAC56 grew at 43°C, supporting the role of BB0721 as a PGs. This was further confirmed by analyzing the contribution of PG to the total phospholipid composition in both strains. The PG content in HD38/pTAC56 did not change, whereas in HD38/pHD102 dropped from 22% at 30°C to less than 5% at 43°C. (Wang et al., 2004b)

Overall, the presence of functional PCs and PGs indicates that PC and PG are synthesized in a pathway(s) involving CDP-DAG. The spirochaete has a *cdsA* homolog (*bb0119*) that mediates in the transfer of CMP from CTP to phosphatidic acid (PA) to form CDP-DAG, which is used by PCs that condense choline directly with CDP-DAG to form PC and releases CMP (Wang et al., 2004b). An unusual trait of phospholipids in *Borrelia* is the low double bond/acyl chain ratio (~0.25) that results in saturated acyl chain levels close to 75% (Hossain et al., 2001; Huang et al., 2016). Thus, at least 50% of PC molecules have two saturated acyl chains since some of the remaining PC molecules may have two unsaturated or one polyunsaturated acyl chains (Huang et al., 2016). Nonetheless, *B. burgdorferi* has outer and inner membranes. The phospholipid composition in both membranes is similar, but the saturated acyl chain levels of PC from the inner membrane is significantly lower (Toledo et al., 2018b). It is important to note that differences in the saturation of acyl chains confer different biophysical properties to lipids and is vital for the formation of tightly packed ordered domains (rafts) in eukaryotic cells (London, 2002) and *B. burgdorferi* (Huang et al., 2016). Therefore, this difference suggests that IM PC has a lower ability to support rafts relative to PC in the OM. (Toledo et al., 2018b).

As mentioned above, *B. burgdorferi* has three glycolipids. ACGal, CGal, and MGalD. The presence of glycolipids, similar to MGalD in Gram-negative organisms, is unusual. In contrast, Gram-negative bacteria possess LPS, a glycolipid that is absent in *Borrelia*. On the other hand, the presence of glycolipids similar to MGalD is frequent in Gram-positive bacteria, where major glycolipids are usually di-hexose DAG. The monogalacto-syldiacylglycerol synthase from *B. burgdorferi* (bbMGS) was identified by homology using the amino acid sequence of the monoglucosyl diacyglycerol synthase from the *Acholeplasma laidlawii* (alMGS) (Berg et al., 2001; Östberg et al., 2007). The activity of bbMGS was addressed by expressing the recombinant protein in *E. coli* and supplementing the cell extract with potential acceptor and donor substrates. The addition of radiolabeled Gal generated MGalD, whereas the addition of radiolabeled glucose did not render any product, which supports that the glycosylation is galactose-specific (Östberg et al., 2007).

Interestingly, the activity of bbMGS was influenced by the phospholipid composition in the mixture. The activity of bbMGS increased 10-fold in the presence of PG compared to PC (Östberg et al., 2007). In Gram-positives, glycosyltransferases generally yield di-hexose and sometimes tri-hexose DAG products.

Nonetheless, bbMGS only produce a mono-hexose DAG, MGalD, which is the only hexose DAG present in the membrane of Lyme disease and relapsing fever *Borrelia* (Livermore et al., 1978; Hossain et al., 2001). Two other glycolipids in *B. burgdorferi*, CGal and ACGal, require a glycosyltransferase. However, bbMGS is not involved in the glycosylation of these lipids since no product was detected when dyacylglycerol was substituted with cholesterol. Nevertheless, the addition of UDP-Gal/UDP-Glu to *Borrelia* cell extracts confirmed the synthesis of a cholesteryl galactoside (Östberg et al., 2007). There are three other glycosyltransferases in *B. burgdorferi* (Fraser et al., 1997), and the most suitable candidate involved in the glycosylation of cholesterol is *bb0572*, a putative inverted (α→β) galactosyltransferase since both cholesterol glycolipids have β-glycosidic linkages between the galactose residue and cholesterol (Ben-Menachem et al., 2003; Schroder et al., 2003).

Cholesterol is present in the membranes of *B. burgdorferi* as cholesterol, cholesterol esters, and also, it is an essential component of cholesterol glycolipids (Ben-Menachem et al., 2003; Schroder et al., 2003). The spirochaete is auxotrophic for cholesterol and relies on the host to acquire the sterol. *In vitro* experiments demonstrated that *B. burgdorferi* takes up cholesterol directly from HeLa cells to make cholesterol glycolipids (Crowley et al., 2013). During the transfer, the spirochaete exchanges lipids and antigens with HeLa cells, including cholesterol glycolipids. In addition, *B. burgdorferi* can also produce outer membrane vesicles that mediate in the antigen transfer to HeLa cells (Crowley et al., 2013). The implications of this mechanism in the pathogenesis of Lyme disease has not been elucidated yet, but the transfer of antigens from the spirochaete to vertebrate cells could contribute to an exacerbated and long-lasting immune response.

Protein export and secretion

The biogenesis of the *Borrelia* cell envelope is still very much a mystery. Inference based on known mechanisms and pathways in other diderm bacteria provides some intriguing clues, but few of these clues have been experimentally tested. The working model of *Borrelia burgdorferi* envelope biogenesis that we develop below (see also Figure 2) is, therefore, largely speculative in nature. It should serve as a working model to delineate how these two important components of the borrelial envelope are synthesized and transported to the sites where they are biologically active.

Lipoprotein transport

As already stated above and also evidenced by other chapters in this book, lipoproteins represent a class of membrane-anchored proteins that are crucial for the natural maintenance, transmission and virulence of the spirochaetes. In contrast to integral membrane proteins, lipoproteins are only peripherally tethered to the lipid bilayer leaflets of the inner (IM) or outer membranes (OM) via their acylated amino terminal cysteine residues. This not only mirrors their variable functions as flexible components of the periplasmic space or the host-pathogen interface, but also has implications for their thermodynamically different translocation through both membranes and the aqueous periplasmic space in between. Detailed studies on lipoprotein transport in Gram-negative bacteria have revealed a general three-step process: (i) Sec-dependent transport of prolipoproteins through the IM, (ii) lipid modification on the periplasmic face of the IM and cleavage of the signal peptide by signal peptidase II, and (iii) retention or release of acylated proteins and transport to the inner or outer leaflet of the OM. The latter sorting step is currently known to be mediated by either the Lol pathway (Tokuda and Matsuyama, 2004), a type II (T2SS) (Pugsley, 1993) or a type V/autotransporter (T5SS/AT) secretion system (Coutte et al., 2003; van Ulsen et al., 2003).

The enzymes responsible for lipoprotein maturation were first discovered in *E. coli* (Sankaran et al., 1995). The modification of lipoproteins occurs in three steps. First, the signal sequence LXYC specifies the addition of diacyl glycerol in a thioether linkage to the sulfur of the cysteine side chain. Then signal peptidase II cleaves at the amino side of the cysteine residue and a third fatty acid is added in an amide linkage to the new amino terminus (Hayashi and Wu, 1990). Homologues for these three enzymes have been identified in the *B. burgdorferi* genome (Fraser et al., 1997), suggesting that spirochetal lipoproteins are processed in an identical way.

Lipoproteins entering the *Escherichia coli* Lol pathway (Tokuda and Matsuyama, 2004) first interact with an IM ABC transporter-like complex, LolCDE, which releases OM-targeted lipoproteins from the IM in an ATP-dependent manner (Yakushi et al., 2000). Released lipoproteins then form a water-soluble

complex with the periplasmic chaperone LolA (Yokota et al., 1999). After crossing the periplasm, this complex interacts with the OM receptor LolB, which mediates anchoring of lipoproteins to the inner leaflet of the OM (Yokota et al., 1999). The initial '+2 lipoprotein sorting rule' established in *E. coli* indicated that Asp at the penultimate (+2) N-terminal position of the mature lipoprotein leads to IM retention (Yamaguchi et al., 1988), while Ser or other amino acids at the +2 position allow the lipoproteins to be released to the OM. One potential mechanistic explanation is that the interaction of the negatively charged aspartate with the zwitterionic phosphatidyl-ethanolamine blocks an interaction with the LolCDE sortase (Masuda et al., 2002; Harada et al., 2003). Yet, its general applicability remains to be determined as it has become increasingly clear that the '+2 rule' is far from universal. In *E. coli*, amino acids at position +3 can also affect localization (Seydel et al., 1999; Terada et al., 2001; Masuda et al., 2002; Lewenza et al., 2006). Furthermore, lipoprotein sorting in other Gram-negative pathogens such as *Pseudomonas aeruginosa* (Lewenza et al., 2008) and *Yersinia pestis* (Silva-Herzog et al., 2008) appears to rely on N-terminal signals at positions +3/+4 and beyond.

While the Lol machinery operates within the periplasm, established pathways of lipoprotein translocation through the OM involve either a T2SS or T5SS/AT mechanism. The prototypical T2SS, the *Klebsiella oxytoca* Pul secretion (Pugsley, 1993), secretes the surface-anchored enzyme PulA. Its secretion signal is located within at least three non-adjacent regions of the structural protein (Sauvonnet and Pugsley, 1996; Francetic and Pugsley, 2005). In the absence of a type II secretion system, PulA's penultimate Asp residue functions as IM retention/Lol avoidance signal (Pugsley et al., 1990). Lipoproteins exported via a T5SS/AT are translocated through the OM via a C-terminal autotransporter domain and include the *Bordetella pertussis* subtilisin SphB1 (Coutte et al., 2003) and the *Neisseria meningitidis* serine protease NalP (van Ulsen et al., 2003).

A database homology search for analogous spirochetal lipoprotein export pathways showed that orthologs of all essential components of the Sec translocase complex and the three enzymes required for lipoprotein modification are present in *Borrelia* (Figure 1), *Leptospira* and *Treponema* (Fraser et al., 1997; Fraser et al., 1998; Haake, 2000; Nascimento et al., 2004; Seshadri et al., 2004). Yet, further Sec-dependent or -independent bacterial protein secretion pathways are missing in spirochaetes, with the exception of the described Type I Bes secretion system in *Borrelia burgdorferi* (Bunikis et al., 2008) or potential twin-arginine translocation (Tat) and type II secretion orthologs in *Leptospira* (Nascimento et al., 2004). Lol pathway orthologs were identified as well.

In diderm bacteria, the Lol pathway is responsible for ensuring proper sorting of lipoproteins within the periplasm. LolCDE forms an IM ABC transporter-like complex that uses the energy from ATP hydrolysis by LolD to release fully modified lipoproteins from the IM via LolCE to the periplasmic carrier protein LolA (Matsuyama et al., 1995; Matsuyama et al., 1997; Tajima et al., 1998; Yakushi et al., 1998; Yakushi et al., 2000; Miyamoto et al., 2001; Tanaka et al., 2001; Fukuda et al., 2002; Masuda et al., 2002; Hara et al., 2003; Takeda et al., 2003; Taniguchi et al., 2005; Ito et al., 2007; Oguchi et al., 2008; Okuda et al., 2008; Watanabe et al., 2008; Okuda and Tokuda, 2009; Tsukahara et al., 2009; Yasuda et al., 2009; Remans et al., 2010; Tao et al., 2010; Narita and Tokuda, 2011; Okuda and Tokuda, 2011; Mizutani et al., 2013; Hayashi et al., 2014; Zückert, 2014; Grabowicz and Silhavy, 2017). Orthologs of LolCDE (Yakushi et al., 2000), LolA (Yokota et al., 1999), but not the OM lipoprotein receptor LolB (Yokota et al., 1999), were detected in the *B. burgdorferi* genome. The absence of LolB is not surprising, as LolB, an OM periplasmic lipoprotein and structural homolog of LolA (Takeda et al., 2003) is found only in β- and γ-proteobacteria (Wilson et al., 2015).

Conditional *B. burgdorferi* type strain B31 mutants in the LolD and the periplasmic lipoprotein carrier LolA were shown to have a significant growth defect, indicating that the pathway is essential (Bridges, Dowdell, Chen, Kueker, Liu, Blevins & Zückert, unpublished results). A working model of the *Borrelia* lipoprotein transport pathway therefore includes LolCDE (BB0078-81) and LolA (BB0346) orthologs, but diverges from that of other diderm bacteria by utilizing a so far unidentified additional OM module to facilitate translocation, or 'flipping', of surface lipoproteins through the OM (Figure 1). This mechanism might be analogous to the Slam machinery in *Neisseria meningitidis* (Hooda et al., 2016), although those proteins have restricted substrate specificities, and Slam homologs appear absent from *Borrelia* genomes. Genetically engineered *B. burgdorferi* express and present on their surface biologically active VMPs of RF *Borrelia*

(Zückert et al., 2004). This indicates that lipoprotein export machineries in different species of the genus *Borrelia* are functionally conserved.

Several independent lines of evidence point toward a periplasmic mechanism that maintains the large and diverse set of *B. burgdorferi* surface lipoproteins (Dowdell et al., 2017) in a translocation-competent conformation by preventing them from folding prematurely in the periplasm. (Schulze et al., 2010; Chen et al., 2011; Chen and Zückert, 2011; Kumru et al., 2011b). Site-directed mutagenesis studies of model surface lipoproteins OspA, OspC and Vsp1, including fusions to a faithful localization reporter variant of monomeric red fluorescent protein mRFP1 or conditionally folding calmodulin, also have repeatedly implied that the N-terminal tether peptides of the lipoproteins are intimately involved in this process - tether deletion and substitution mutants were specifically deficient in OM translocation (Schulze and Zückert, 2006; Kumru et al., 2010; Schulze et al., 2010), and surface localization of lipoprotein-calmodulin fusions required either the presence of a wild-type tether or calcium chelation-induced unfolding of calmodulin (Chen and Zückert, 2011). One potential mechanism would be that surface-targeted pro-lipoprotein peptides interact with a periplasmic "holding" chaperone (Figure 1) as they emerge tether-first from the IM Sec complex on the periplasmic side of the IM. This would be analogous to the "high affinity, low specificity" interaction of the proteobacterial chaperone SecB with the diverse peptides released from the ribosome (Park et al., 1988; Liu et al., 1989; Randall and Hardy, 2002; Lilly et al., 2009). The holding chaperone would then deliver surface lipoproteins in an at least partially unfolded conformation to an OM lipoprotein "flippase", which facilitates translocation through the OM and leads to the demonstrated ultimate anchoring of surface lipoproteins in the surface leaflet of the OM (Schulze et al., 2010; Chen et al., 2011; Chen and Zückert, 2011) (Figure 1).

The *B. burgdorferi* version of the Lol pathway may function either like a purely periplasmic lipoprotein sorting pathway as in other diderm bacteria, albeit with a LolA that can operate in absence of a cognate LolB. This would require an additional branch within a dichotomous lipoprotein secretion machinery where surface lipoproteins bypass the Lol machinery altogether to reach the surface. Alternatively, *B. burgorferi* LolA could have evolved to become fully integrated into the surface lipoprotein secretion pathway, potentially taking on the added role of a "holding" chaperone (Figure 1)

Integral membrane protein transport

While other bacteria commonly ensure correct localization of their membrane proteins by including transmembrane-spanning domains, *Borrelia* seemingly prefer N-terminal lipidation as a membrane anchoring method. Proteins containing transmembrane-spanning domains appear to be rare compared to Gram-negative bacteria. Biosynthetic labeling of *Borrelia* cultures with [^{14}C] amino acids identified only a few amphiphilic polypeptides that did not co-migrate with lipoproteins (Brandt et al., 1990) and freeze-fracture electron microscopy visualized only few outer membrane pore structures (Walker et al., 1991; Radolf et al., 1994).

Integral transmembrane proteins (TMPs) are important components of both the cytoplasmic inner (IM) and outer membrane (OM) lipid bilayers and required for their selective permeability. Studies on the mechanisms of processing, folding and membrane insertion in model systems have identified several IM, periplasmic and OM components involved in the assembly of TMPs in both the IM and OM. Figure 1 shows potential functional *B. burgdorferi* orthologs identified in sequence homology searches. The long-standing textbook dogma on membrane protein structures stated that α-helical transmembrane (TM) domains span the IM and β-barrels span the OM. Yet, recent data suggest that this might be an oversimplification. The Wza translocon for *E. coli* polysaccharides forms an α-helical barrel in the OM (Dong et al., 2006). P13, a multimeric *B. burgdorferi* OM porin, is predicted to have largely α-helical secondary structure and might form similar tertiary structure in the OM (Noppa et al., 2001; Nilsson et al., 2002).

TMPs, like LPs, are also predominantly targeted to the Sec translocon, but their signal peptides are cleaved by a separate periplasmic enzyme, signal peptidase I. Intriguingly, the *B. burgdorferi* genome encodes for three potential signal I pepdidases that – like sets of multiple LepBs in some gram-positive bacteria (Bonnemain et al., 2004) – may have different substrate specificities. *B. burgdorferi* LepB2 (BB0031), is closest to a canonical signal I peptidase, with an N-terminal two-TM anchor domain and an appropriately localized periplasmic active site. LepB3 (BB0263) similarly is predicted to have all enzymatically relevant residues located in the

periplasm, anchored by a single N-terminal TM domain. LepB1 (BB0030) immediately upstream of LepB2, however, appears to be a signal peptidase I-like protein with a four-TM domain topology that lacks the canonical active site residues. A conditional knockout of *lepB1* led to a marked growth defect with signs of significant envelope instability (You, Whetstine and Zückert, unpublished results), but the protein's precise function remains unknown.

Subsequent to being threaded through, the TM domains of IM-localizing TMPs exit the Sec channel through a lateral gate into the hydrophobic environment of the lipid bilayer. This process is catalyzed by YidC, an IM insertase (Xie and Dalbey, 2008). Interestingly, YidC can also mediate a Sec-independent insertion of IM TMPs, particularly those with only one to two TM domains (Samuelson et al., 2000). The Bdr proteins, a family of bitopic type II membrane proteins with cytoplasmic N-termini and single C-terminal TM domains (Zückert et al., 1999), might, therefore, insert into the IM in a YidC/BB0442-dependent, but Sec-independent manner.

TMPs destined to the OM somehow avoid IM insertion and enter the periplasmic space where they interact with two chaperones, Skp and SurA. A double *skp surA* mutation is synthetically lethal in *E. coli* (Rizzitello et al., 2001). Yet, it remains unresolved whether Skp and SurA act in two parallel, redundant pathways, or rather sequentially. In the parallel scenario, SurA is the primary chaperone involved in periplasmic transit of OM TMPs, Skp attempts to rescue proteins that fall off the SurA pathway, and DegP degrades proteins abandoned by Skp (Sklar et al., 2007). In the sequential scenario, Skp prevents aggregation of nonnative TMPs, and SurA then promotes structural folding (Bos et al., 2007). A Skp homolog (BB0796) is expressed downstream from BamA, but its function remains to be determined (Iqbal et al., 2016). The *B. burgdorferi* HtrA/DegP chaperone/protease homolog (BB0104) appears to be a multifunctional protein with activity in the *Borrelia* periplasm, at the OM and as a secreted protein (Gherardini, 2013). Together with CtpA, HtrA is involved in the proteolytic maturation of BB0323, a periplasmic OM lipoprotein that is required for envelope stability and contains a C-terminal peptidoglycan-binding LysM domain (Stewart et al., 2004; Kariu et al., 2013). Additional HtrA substrates are *Borrelia* chemotaxis and motility factors (Coleman et al., 2013). Secreted HtrA has been shown to degrade components of host extracellular matrix components (Russell et al., 2013).

The insertion of OM TMPs is catalyzed by the Bam complex (Hagan et al., 2011; Konovalova et al., 2017). The OM β-barrel TMP BamA (formerly YaeT or Omp85) (Voulhoux et al., 2003) displays several periplasmic polypeptide-transport-associated (POTRA) domains (Sanchez-Pulido et al., 2003). These N-terminal domains have been shown to recognize C-terminal motifs of OM TMPs and might initiate the structural assembly process of TMPs (Robert et al., 2006). They are also thought to provide a scaffold for the interaction with four associated lipoproteins, BamB (YfgL), BamC (NlpB), BamD (YfiO) and BamE (SmpA) (Kim et al., 2007). The exact roles of these periplasmic OM lipoproteins in OM biogenesis and their interaction with BamA and substrate proteins are under intense investigation. So far, only BamD appears to be essential (Onufryk et al., 2005; Malinverni et al., 2006), indicating that BamAD forms the core Bam machinery in *E. coli*.

The *B. burgdorferi* BamA homolog (BB0795) is associated with only two structural Bam lipoprotein homologs, BamB (BB0324) and BamD (BB0028). Depletion of BamA led to a noticeable decrease of OMPs and OM lipoproteins (Lenhart and Akins, 2010). Of note, that study did not assess surface exposure, but the data are consistent with a likely indirect Bam complex dependency of the surface lipoprotein flipping process (Figure 1). BamB and BamD are the only two of the 8 lipoproteins anchored in the periplasmic leaflet of the OM (Dowdell et al.) that could not be disrupted by transposon mutagenesis (Lenhart et al., 2012; Lin et al., 2012; Lin et al., 2014); as mentioned above, a deletion mutant of BB0323 remains viable, albeit with a severe envelope structure defect (Stewart et al., 2004). *B. burgdorferi* mutants lacking BamB and BamD exhibited altered membrane permeability and enhanced sensitivity to various antimicrobials. BamB mutants also exhibited significantly impaired *in vitro* growth (Dunn et al., 2015). Thus, in deviation from the gram-negative/diderm dogma, BamB might replace BamD in the core Bam complex of *B. burgdorferi*.

Recently, the *B. burgdorferi* Bam machinery was shown to interact with a TamB (BB0794) homolog (Iqbal et al., 2016) that in other bacteria is part of a parallel OMP Translocation and Assembly Module

(TAM) pathway. This indicates that *B. burgdorferi* has adapted modules of separate secretion machineries to function in a single hybrid pathway that allows OMPs to efficiently traverse the periplasm (Stubenrauch et al., 2016). It is tempting to speculate that the *Borrelia* Bam/Tam machinery has evolved to also catalyze the insertion of outer membrane porins with predicted α-helical TM domains such as the outer membrane porin P13 (Sadziene et al., 1995; Noppa et al., 2001). If this were indeed the case, P13 would have to somehow avoid recognition and sorting to the IM by the Sec/YidC complex, possibly through the 28 amino acid C-terminal domain that is ultimately removed by CtpA (BB0359) (Östberg et al. 2004).

Conclusions and future directions

Since the discovery of the first outer membrane components, the borrelial cell envelope has given up many secrets, but many more await unraveling. The completed genome sequences of several species have also aided in the understanding of the common biology of *Borrelia* spirochaetes. In future studies, structure-function relationships of lipoprotein virulence factors need to be determined beyond their antibody epitopes. Recent advances in protein structure determination need to be leveraged to reveal the likely unusual conformations of *Borrelia* OM transmembrane proteins and to further understand the adaptive capacity of the bacterium in the various ecological niches it encounters. Envelope biogenesis pathways have to be further tested experimentally. A better understanding of the *Borrelia* cell cycle and envelope homeostasis are paramount to a better understanding and improved treatments for Lyme disease and relapsing fever. For example, quantitative, high-resolution reporters for PG synthesis can address issues related to so called persister organisms, and the overall status of an active infection. Identification and characterization of essential structural proteins and enzymes that can be targeted by of small molecule inhibitors can lead to novel therapeutics. We continue to discover that this phylogenetic ancient spirochetal microorganism has developed solutions of its own and often does not conform to the dogmatic structure and function of the cell envelope of Gram-negative bacteria - be it with a bacterial surface dominated by lipoproteins, the formation of lipid rafts in both membranes, an intriguing interplay of its unique peptidoglycan with the nearby motility apparatus, and the modular shuffling and adaptive salvaging of proven molecular machineries to maintain an envelope under conditions where change is constant. We continue to anticipate a bright future with many challenges and unsolved mysteries for several generations of *Borrelia* researchers.

Acknowledgements

This work was supported by grants from the National Institutes of Health (NIH) to WRZ (R21 AI144624) and AMT (R21 AI125806), by the Steven & Alexandra Cohen Foundation to BLJ, and by the Swedish Research Council to SB (VR-MH 01885). WRZ wishes to dedicate his contributions to the chapter to his parents.

References

Achouak, W., Heulin, T., and Pages, J.M. (2001). Multiple facets of bacterial porins. FEMS Microbiol Lett *199*, 1-7. https://doi.org/10.1111/j.1574-6968.2001.tb10642.x

Alitalo, A., Meri, T., Lankinen, H., Seppala, I., Lahdenne, P., Hefty, P.S., Akins, D., and Meri, S. (2002). Complement inhibitor factor H binding to Lyme disease spirochetes is mediated by inducible expression of multiple plasmid-encoded outer surface protein E paralogs. J Immunol *169*, 3847-3853. https://doi.org/10.4049/jimmunol.169.7.3847

Andersen, C., Hughes, C., and Koronakis, V. (2000). Chunnel vision. Export and efflux through bacterial channel-tunnels. EMBO Rep *1*, 313-318. https://doi.org/10.1093/embo-reports/kvd075

Andersen, C., Koronakis, E., Hughes, C., and Koronakis, V. (2002). An aspartate ring at the TolC tunnel entrance determines ion selectivity and presents a target for blocking by large cations. Mol Microbiol *44*, 1131-1139. https://doi.org/10.1046/j.1365-2958.2002.02898.x

Anguita, J., Thomas, V., Samanta, S., Persinski, R., Hernanz, C., Barthold, S.W., and Fikrig, E. (2001). *Borrelia burgdorferi*-induced inflammation facilitates spirochete adaptation and variable major protein-like sequence locus recombination. J Immunol *167*, 3383-3390. https://doi.org/10.4049/jimmunol.167.6.3383

Antonara, S., Chafel, R.M., LaFrance, M., and Coburn, J. (2007). *Borrelia burgdorferi* adhesins identified using in vivo phage display. Mol Microbiol *66*, 262-276. https://doi.org/10.1111/j.1365-2958.2007.05924.x

Aranjuez, G.F., Kuhn, H.W., Adams, P.P., and Jewett, M.W. (2019). *Borrelia burgdorferi* bbk13 Is Critical for Spirochete Population Expansion in the Skin during Early Infection. Infect Immun *87*. https://doi.org/10.1128/IAI.00887-18

Awade, A.C., Cleuziat, P., Gonzales, T., and Robert-Baudouy, J. (1994). Pyrrolidone carboxyl peptidase (Pcp): an enzyme that removes pyroglutamic acid (pGlu) from pGlu-peptides and pGlu-proteins. Proteins *20*, 34-51. https://doi.org/10.1002/prot.340200106

Azuma, I., Taniyama, T., Yamamura, Y., Yanagihara, Y., and Hattori, Y. (1975). Chemical studies on the cell walls of *Leptospira biflexa* strain Urawa and Treponema pallidum strain Reiter. Jpn J Microbiol *19*, 45-51. https://doi.org/10.1111/j.1348-0421.1975.tb00846.x

Barbour, A.G. (1990). Antigenic variation of a relapsing fever *Borrelia* species. Annual Reviews in Microbiology *44*, 155-171. https://doi.org/10.1146/annurev.mi.44.100190.001103

Barbour, A.G. (1993). Linear DNA of *Borrelia* species and antigenic variation. Trends Microbiol *1*, 236-239. https://doi.org/10.1016/0966-842x(93)90139-i

Barbour, A.G., and Garon, C.F. (1988). The genes encoding major surface proteins of *Borrelia burgdorferi* are located on a plasmid. Ann N Y Acad Sci *539*, 144-153. https://doi.org/10.1111/j.1749-6632.1988.tb31847.x

Barbour, A.G., and Hayes, S.F. (1986). Biology of *Borrelia* species. Microbiol Rev *50*, 381-400.

Barbour, A.G., and Travinsky, B. (2010). Evolution and distribution of the *ospC* gene, a transferable serotype determinant of *Borrelia burgdorferi*. mBio *1*. https://doi.org/10.1128/mBio.00153-10

Barcena-Uribarri, I., Thein, M., Barbot, M., Sans-Serramitjana, E., Bonde, M., Mentele, R., Lottspeich, F., Bergström, S., and Benz, R. (2014). Study of the protein complex, pore diameter, and pore-forming activity of the *Borrelia burgdorferi* P13 porin. J Biol Chem *289*, 18614-18624. https://doi.org/10.1074/jbc.M113.539528

Barcena-Uribarri, I., Thein, M., Maier, E., Bonde, M., Bergström, S., and Benz, R. (2013). Use of nonelectrolytes reveals the channel size and oligomeric constitution of the *Borrelia burgdorferi* P66 porin. PLoS One *8*, e78272. https://doi.org/10.1371/journal.pone.0078272

Barcena-Uribarri, I., Thein, M., Sacher, A., Bunikis, I., Bonde, M., Bergström, S., and Benz, R. (2010). P66 porins are present in both Lyme disease and relapsing fever spirochetes: a comparison of the biophysical properties of P66 porins from six *Borrelia* species. Biochim Biophys Acta *1798*, 1197-1203. https://doi.org/10.1016/j.bbamem.2010.02.011

Barthold, S.W., Fikrig, E., Bockenstedt, L.K., and Persing, D.H. (1995). Circumvention of outer surface protein A immunity by host-adapted *Borrelia burgdorferi*. Infect Immun *63*, 2255-2261. https://doi.org/10.1128/IAI.63.6.2255-2261.1995

Battisti, J.M., Bono, J.L., Rosa, P.A., Schrumpf, M.E., Schwan, T.G., and Policastro, P.F. (2008). Outer surface protein A protects Lyme disease spirochetes from acquired host immunity in the tick vector. Infect Immun *76*, 5228-5237. https://doi.org/10.1128/IAI.00410-08

Beck, G., Benach, J.L., and Habicht, G.S. (1990). Isolation, preliminary chemical characterization, and biological activity of *Borrelia burgdorferi* peptidoglycan. Biochem Biophys Res Commun *167*, 89-95. https://doi.org/10.1016/0006-291x(90)91734-a

Becker, M., Bunikis, J., Lade, B.D., Dunn, J.J., Barbour, A.G., and Lawson, C.L. (2005). Structural investigation of *Borrelia burgdorferi* OspB, a bactericidal Fab target. J Biol Chem *280*, 17363-17370. https://doi.org/10.1074/jbc.M412842200

Beilharz, K., Novakova, L., Fadda, D., Branny, P., Massidda, O., and Veening, J.W. (2012). Control of cell division in *Streptococcus pneumoniae* by the conserved Ser/Thr protein kinase StkP. Proc Natl Acad Sci U S A *109*, E905-913. https://doi.org/10.1073/pnas.1119172109

Belisle, J.T., Brandt, M.E., Radolf, J.D., and Norgard, M.V. (1994). Fatty acids of *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins. J Bacteriol *176*, 2151-2157. https://doi.org/10.1128/jb.176.8.2151-2157.1994

Ben-Menachem, G., Kubler-Kielb, J., Coxon, B., Yergey, A., and Schneerson, R. (2003). A newly discovered cholesteryl galactoside from *Borrelia burgdorferi*. Proc Natl Acad Sci U S A *100*, 7913-7918. https://doi.org/10.1073/pnas.1232451100

Benz, R. (1994). Uptake of solutes through bacterial outer membranes. In Bacterial Cell Wall, M. Ghuysen, and R. Hackenbek, eds. (Amsterdam: Elsevier), pp. 397 - 423. https://doi.org/https://doi.org/10.1016/S0167-7306(08)60422-6

Benz, R. (2001). Porins - structure and function. In: Microbial Transport Systems. (Winkelmann, G, ed), WILEY-VCH Verlag GmbH, Weinheim/Germany, pp. 227-246.

Benz, R., Janko, K., and Lauger, P. (1979). Ionic selectivity of pores formed by the matrix protein (porin) of *Escherichia coli*. Biochim Biophys Acta *551*, 238-247. https://doi.org/10.1016/0005-2736(89)90002-3

Benz, R., Maier, E., and Gentschev, I. (1993). TolC of *Escherichia coli* functions as an outer membrane channel. Zentralbl Bakteriol *278*, 187-196. https://doi.org/10.1016/s0934-8840(11)80836-4

Benz, R., Schmid, A., Maier, C., and Bremer, E. (1988). Characterization of the nucleoside-binding site inside the Tsx channel of *Escherichia coli* outer membrane. Reconstitution experiments with lipid bilayer membranes. Eur J Biochem *176*, 699-705. https://doi.org/10.1111/j.1432-1033.1988.tb14333.x

Benz, R., Schmid, A., Nakae, T., and Vos-Scheperkeuter, G.H. (1986). Pore formation by LamB of *Escherichia coli* in lipid bilayer membranes. J Bacteriol *165*, 978-986. https://doi.org/10.1128/jb.165.3.978-986.1986

Berg, S. (2001). The glucolipid pathway in *Acholeplasma laidawii*: Functional and structural properties of three consequetively-acting enzymes and similarities to orthologs in prokaryotes. Doctoral thesis, Umeå University, Umeå, Sweden ISBN 91-7305-025-3.

Berg, S., Edman, M., Li, L., Wikstrom, M., and Wieslander, A. (2001). Sequence properties of the 1,2-diacylglycerol 3-glucosyltransferase from *Acholeplasma laidlawii* membranes. Recognition of a large group of lipid glycosyltransferases in eubacteria and archaea. J Biol Chem *276*, 22056-22063. https://doi.org/10.1074/jbc.M102576200

Bergström, S., Bundoc, V.G., and Barbour, A.G. (1989). Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB, of the Lyme disease spirochaete *Borrelia burgdorferi*. Mol Microbiol *3*, 479-486. https://doi.org/10.1111/j.1365-2958.1989.tb00194.x

Bessler, W.G., Cox, M., Lex, A., Suhr, B., Wiesmuller, K.H., and Jung, G. (1985). Synthetic lipopeptide analogs of bacterial lipoprotein are potent polyclonal activators for murine B lymphocytes. J Immunol *135*, 1900-1905.

Bieberich, E. (2018). Sphingolipids and lipid rafts: Novel concepts and methods of analysis. Chem Phys Lipids *216*, 114-131. https://doi.org/10.1016/j.chemphyslip.2018.08.003

Bierwagen, P., Szpotkowski, K., Jaskolski, M., and Urbanowicz, A. (2019). *Borrelia* outer surface protein C is capable of human fibrinogen binding. FEBS J *286*, 2415-2428. https://doi.org/10.1111/febs.14810

Bisson-Filho, A.W., Hsu, Y.P., Squyres, G.R., Kuru, E., Wu, F., Jukes, C., Sun, Y., Dekker, C., Holden, S., VanNieuwenhze, M.S., *et al.* (2017). Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. Science *355*, 739-743. https://doi.org/10.1126/science.aak9973

Bista, S., Singh, P., Bernard, Q., Yang, X., Hart, T., Lin, Y.P., Kitsou, C., Singh Rana, V., Zhang, F., Linhardt, R.J., *et al.* (2020). A novel laminin-binding protein mediates microbial-endothelial cell interactions and facilitates dissemination of Lyme disease pathogens. Journal of Infectious Diseases *221*, 1438-1447. https://doi.org/10.1093/infdis/jiz626

Bonnemain, C., Raynaud, C., Reglier-Poupet, H., Dubail, I., Frehel, C., Lety, M.A., Berche, P., and Charbit, A. (2004). Differential roles of multiple signal peptidases in the virulence of *Listeria monocytogenes*. Mol Microbiol *51*, 1251-1266. https://doi.org/10.1111/j.1365-2958.2004.03916.x

Bono, J., Tilly, K., Stevenson, B., Hogan, D., and Rosa, P. (1998). Oligopeptide permease in *Borrelia burgdorferi*: putative peptide-binding components encoded by both chromosomal and plasmid loci. Microbiology *144* 1033-1044.

Bontemps-Gallo, S., Lawrence, K., and Gherardini, F.C. (2016). Two different virulence-related regulatory pathways in *Borrelia burgdorferi* are directly affected by osmotic fluxes in the blood meal of feeding *Ixodes* ticks. PLoS Pathog *12*, e1005791. https://doi.org/10.1371/journal.ppat.1005791

Bos, M.P., Robert, V., and Tommassen, J. (2007). Biogenesis of the gram-negative bacterial outer membrane. Annual Reviews in Microbiology *61*, 191-214. https://doi.org/10.1146/annurev.micro.61.080706.093245

Brandt, M.E., Riley, B.S., Radolf, J.D., and Norgard, M.V. (1990). Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. Infect Immun *58*, 983-991. https://doi.org/10.1128/IAI.58.4.983-991.1990

Brissette, C.A., Haupt, K., Barthel, D., Cooley, A.E., Bowman, A., Skerka, C., Wallich, R., Zipfel, P.F., Kraiczy, P., and Stevenson, B.

(2009). *Borrelia burgdorferi* infection-associated surface proteins ErpP, ErpA, and ErpC bind human plasminogen. Infect Immun *77*, 300-306. https://doi.org/10.1128/IAI.01133-08

Browman, D.T., Hoegg, M.B., and Robbins, S.M. (2007). The SPFH domain-containing proteins: more than lipid raft markers. Trends Cell Biol *17*, 394-402. https://doi.org/10.1016/j.tcb.2007.06.005

Brown, D.A., and London, E. (2000). Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J Biol Chem *275*, 17221-17224. https://doi.org/10.1074/jbc.R000005200

Brown, E.L., Wooten, R.M., Johnson, B.J., Iozzo, R.V., Smith, A., Dolan, M.C., Guo, B.P., Weis, J.J., and Hook, M. (2001). Resistance to Lyme disease in decorin-deficient mice. J Clin Invest *107*, 845-852. https://doi.org/10.1172/JCI11692

Bunikis, I., Denker, K., Östberg, Y., Andersen, C., Benz, R., and Bergström, S. (2008). An RND-type efflux system in *Borrelia burgdorferi* is involved in virulence and resistance to antimicrobial compounds. PLoS Pathog *4*, e1000009. https://doi.org/10.1371/journal.ppat.1000009

Bunikis, J., and Barbour, A. (1999). Access of antibody or trypsin to an integral outer membrane protein (P66) of *Borrelia burgdorferi* is hindered by Osp lipoproteins. Infect Immun *67*, 2874-2883.

Bunikis, J., Noppa, L., and Bergström, S. (1995). Molecular analysis of a 66-kDa protein associated with the outer membrane of Lyme disease *Borrelia*. FEMS Microbiol Lett *131*, 139-145. https://doi.org/10.1111/j.1574-6968.1995.tb07768.x

Burman, N., Bergström, S., Restrepo, B.I., and Barbour, A.G. (1990). The variable antigens Vmp7 and Vmp21 of the relapsing fever bacterium *Borrelia hermsii* are structurally analogous to the VSG proteins of the African trypanosome. Mol Microbiol *4*, 1715-1726. https://doi.org/10.1111/j.1365-2958.1990.tb00549.x

Busby, W.H., Jr., Quackenbush, G.E., Humm, J., Youngblood, W.W., and Kizer, J.S. (1987). An enzyme(s) that converts glutaminyl-peptides into pyroglutamyl-peptides. Presence in pituitary, brain, adrenal medulla, and lymphocytes. J Biol Chem *262*, 8532-8536.

Byram, R., Stewart, P.E., and Rosa, P. (2004). The essential nature of the ubiquitous 26-kilobase circular replicon of *Borrelia burgdorferi*. J Bacteriol *186*, 3561-3569. https://doi.org/10.1128/JB.186.11.3561-3569.2004

Caccamo, P.D., and Brun, Y.V. (2018). The Molecular Basis of Noncanonical Bacterial Morphology. Trends Microbiol *26*, 191-208. https://doi.org/10.1016/j.tim.2017.09.012

Caimano, M.J., Bourell, K.W., Bannister, T.D., Cox, D.L., and Radolf, J.D. (1999). The *Treponema denticola* major sheath protein is predominantly periplasmic and has only limited surface exposure. Infect Immun *67*, 4072-4083. https://doi.org/10.1128/IAI.67.8.4072-4083.1999

Caimano, M.J., Eggers, C.H., Gonzalez, C.A., and Radolf, J.D. (2005). Alternate sigma factor RpoS is required for the in vivo-specific repression of *Borrelia burgdorferi* plasmid lp54-borne ospA and lp6.6 genes. J Bacteriol *187*, 7845-7852. https://doi.org/10.1128/JB.187.22.7845-7852.2005

Caimano, M.J., Groshong, A.M., Belperron, A., Mao, J., Hawley, K.L., Luthra, A., Graham, D.E., Earnhart, C.G., Marconi, R.T., Bockenstedt, L.K*., et al.* (2019). The RpoS Gatekeeper in *Borrelia burgdorferi*: An Invariant Regulatory Scheme That Promotes Spirochete Persistence in Reservoir Hosts and Niche Diversity. Front Microbiol *10*, 1923. https://doi.org/10.3389/fmicb.2019.01923

Caimano, M.J., Iyer, R., Eggers, C.H., Gonzalez, C., Morton, E.A., Gilbert, M.A., Schwartz, I., and Radolf, J.D. (2007). Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals provides insight into RpoS function during the enzootic cycle. Mol Microbiol *65*, 1193-1217. https://doi.org/10.1111/j.1365-2958.2007.05860.x

Caine, J.A., and Coburn, J. (2016). Multifunctional and Redundant Roles of *Borrelia burgdorferi* Outer Surface Proteins in Tissue Adhesion, Colonization, and Complement Evasion. Front Immunol *7*, 442. https://doi.org/10.3389/fimmu.2016.00442

Caine, J.A., Lin, Y.P., Kessler, J.R., Sato, H., Leong, J.M., and Coburn, J. (2017). *Borrelia burgdorferi* outer surface protein C (OspC) binds complement component C4b and confers bloodstream survival. Cell Microbiol *19*. https://doi.org/10.1111/cmi.12786

Carrasco, S.E., Troxell, B., Yang, Y., Brandt, S.L., Li, H., Sandusky, G.E., Condon, K.W., Serezani, C.H., and Yang, X.F. (2015). Outer surface protein OspC is an antiphagocytic factor that protects *Borrelia burgdorferi* from phagocytosis by macrophages. Infect Immun *83*, 4848-4860. https://doi.org/10.1128/IAI.01215-15

Carter, C.J., Bergström, S., Norris, S.J., and Barbour, A.G. (1994). A family of surface-exposed proteins of 20 kilodaltons in the genus *Borrelia*. Infect Immun *62*, 2792-2799. https://doi.org/10.1128/IAI.62.7.2792-2799.1994

Casjens, S., Palmer, N., van Vugt, R., Huang, W.M., Stevenson, B., Rosa, P., Lathigra, R., Sutton, G., Peterson, J., Dodson, R.J*., et al.* (2000). A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. Mol Microbiol *35*, 490-516. https://doi.org/10.1046/j.1365-2958.2000.01698.x

Cava, F., and de Pedro, M.A. (2014). Peptidoglycan plasticity in bacteria: emerging variability of the murein sacculus and their associated biological functions. Curr Opin Microbiol *18*, 46-53. https://doi.org/10.1016/j.mib.2014.01.004

Chan, J.M., and Dillard, J.P. (2016). *Neisseria gonorrhoeae* Crippled Its Peptidoglycan Fragment Permease To Facilitate Toxic Peptidoglycan Monomer Release. J Bacteriol *198*, 3029-3040. https://doi.org/10.1128/JB.00437-16

Chan, J.M., and Dillard, J.P. (2017). Attention Seeker: Production, Modification, and Release of Inflammatory Peptidoglycan Fragments in *Neisseria* Species. J Bacteriol *199*. https://doi.org/10.1128/JB.00354-17

Charon, N.W., Cockburn, A., Li, C., Liu, J., Miller, K.A., Miller, M.R., Motaleb, M.A., and Wolgemuth, C.W. (2012). The unique paradigm of spirochete motility and chemotaxis. Annual Reviews in Microbiology *66*, 349-370. https://doi.org/10.1146/annurev-micro-092611-150145

Charon, N.W., Goldstein, S.F., Marko, M., Hsieh, C., Gebhardt, L.L., Motaleb, M.A., Wolgemuth, C.W., Limberger, R.J., and Rowe, N. (2009). The flat-ribbon configuration of the periplasmic flagella of *Borrelia burgdorferi* and its relationship to motility and morphology. J Bacteriol *191*, 600-607. https://doi.org/10.1128/JB.01288-08

Chen, S., Kumru, O.S., and Zückert, W.R. (2011). Determination of *Borrelia* surface lipoprotein anchor topology by surface proteolysis. J Bacteriol *193*, 6379-6383. https://doi.org/10.1128/JB.05849-11

Chen, S., and Zückert, W.R. (2011). Probing the *Borrelia burgdorferi* surface lipoprotein secretion pathway using a conditionally folding protein domain. J Bacteriol *193*, 6724-6732. https://doi.org/10.1128/JB.06042-11

Chiba, S., Akiyama, Y., and Ito, K. (2002). Membrane protein degradation by FtsH can be initiated from either end. J Bacteriol *184*, 4775-4782. https://doi.org/10.1128/jb.184.17.4775-4782.2002

Cho, H., McManus, H.R., Dove, S.L., and Bernhardt, T.G. (2011). Nucleoid occlusion factor SlmA is a DNA-activated FtsZ polymerization antagonist. Proc Natl Acad Sci U S A *108*, 3773-3778. https://doi.org/10.1073/pnas.1018674108

Chu, C.Y., Stewart, P.E., Bestor, A., Hansen, B., Lin, T., Gao, L., Norris, S.J., and Rosa, P.A. (2016). Function of the *Borrelia burgdorferi* FtsH Homolog Is Essential for Viability both In Vitro and In Vivo and Independent of HflK/C. mBio *7*, e00404-00416. https://doi.org/10.1128/mBio.00404-16

Clausen, T., Kaiser, M., Huber, R., and Ehrmann, M. (2011). HTRA proteases: regulated proteolysis in protein quality control. Nat Rev Mol Cell Biol *12*, 152-162. https://doi.org/10.1038/nrm3065

Cloud-Hansen, K.A., Hackett, K.T., Garcia, D.L., and Dillard, J.P. (2008). *Neisseria gonorrhoeae* uses two lytic transglycosylases to produce cytotoxic peptidoglycan monomers. J Bacteriol *190*, 5989-5994. https://doi.org/10.1128/JB.00506-08

Cluss, R.G., Silverman, D.A., and Stafford, T.R. (2004). Extracellular secretion of the *Borrelia burgdorferi* Oms28 porin and Bgp, a glycosaminoglycan binding protein. Infect Immun *72*, 6279-6286. https://doi.org/10.1128/IAI.72.11.6279-6286.2004

Coburn, J., Chege, W., Magoun, L., Bodary, S.C., and Leong, J.M. (1999). Characterization of a candidate *Borrelia burgdorferi* beta3-chain integrin ligand identified using a phage display library. Mol Microbiol *34*, 926-940. https://doi.org/10.1046/j.1365-2958.1999.01654.x

Coburn, J., and Cugini, C. (2003). Targeted mutation of the outer membrane protein P66 disrupts attachment of the Lyme disease agent, *Borrelia burgdorferi*, to integrin alphavbeta3. Proc Natl Acad Sci U S A *100*, 7301-7306. https://doi.org/10.1073/pnas.1131117100

Coburn, J., Fischer, J.R., and Leong, J.M. (2005). Solving a sticky problem: new genetic approaches to host cell adhesion by the Lyme disease spirochete. Mol Microbiol *57*, 1182-1195. https://doi.org/10.1111/j.1365-2958.2005.04759.x

Coleman, J.L., Crowley, J.T., Toledo, A.M., and Benach, J.L. (2013). The HtrA protease of *Borrelia burgdorferi* degrades outer membrane protein BmpD and chemotaxis phosphatase CheX. Mol Microbiol *88*, 619-633. https://doi.org/10.1111/mmi.12213

Coleman, J.L., Toledo, A., and Benach, J.L. (2016). *Borrelia burgdorferi* HtrA: evidence for twofold proteolysis of outer membrane protein p66. Mol Microbiol *99*, 135-150. https://doi.org/10.1111/mmi.13221

Cordes, F.S., Roversi, P., Kraiczy, P., Simon, M.M., Brade, V., Jahraus, O., Wallis, R., Skerka, C., Zipfel, P.F., Wallich, R.*, et al.* (2005). A novel fold for the factor H-binding protein BbCRASP-1 of *Borrelia burgdorferi*. Nat Struct Mol Biol *12*, 276-277. https://doi.org/10.1038/nsmb902

Coutte, L., Botkin, D.J., Gao, L., and Norris, S.J. (2009). Detailed analysis of sequence changes occurring during vlsE antigenic variation in the mouse model of *Borrelia burgdorferi* infection. PLoS Pathog *5*, e1000293. https://doi.org/10.1371/journal.ppat.1000293

Coutte, L., Willery, E., Antoine, R., Drobecq, H., Locht, C., and Jacob-Dubuisson, F. (2003). Surface anchoring of bacterial subtilisin important for maturation function. Mol Microbiol *49*, 529-539. https://doi.org/10.1046/j.1365-2958.2003.03573.x

Crowley, J.T., Toledo, A.M., LaRocca, T.J., Coleman, J.L., London, E., and Benach, J.L. (2013). Lipid exchange between *Borrelia burgdorferi* and host cells. PLoS Pathog *9*, e1003109. https://doi.org/10.1371/journal.ppat.1003109

Cullen, P.A., Haake, D.A., and Adler, B. (2004). Outer membrane proteins of pathogenic spirochetes. FEMS Microbiol Rev *28*, 291-318. https://doi.org/10.1016/j.femsre.2003.10.004

Das, S., Shraga, D., Gannon, C., Lam, T.T., Feng, S., Brunet, L.R., Telford, S.R., Barthold, S.W., Flavell, R.A., and Fikrig, E. (1996). Characterization of a 30-kDa *Borrelia burgdorferi* substrate-binding protein homologue. Res Microbiol *147*, 739-751. https://doi.org/10.1016/s0923-2508(97)85121-2

de Silva, A.M., Telford, S.R., 3rd, Brunet, L.R., Barthold, S.W., and Fikrig, E. (1996). *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. J Exp Med *183*, 271-275. https://doi.org/10.1084/jem.183.1.271

Defoe, G., and Coburn, J. (2001). Delineation of *Borrelia burgdorferi* p66 sequences required for integrin alpha(IIb)beta(3) recognition. Infect Immun *69*, 3455-3459. https://doi.org/10.1128/IAI.69.5.3455-3459.2001

Demchick, P., and Koch, A.L. (1996). The permeability of the wall fabric of *Escherichia coli* and *Bacillus subtilis*. J Bacteriol *178*, 768-773. https://doi.org/10.1128/jb.178.3.768-773.1996

Deres, K., Schild, H., Wiesmuller, K.H., Jung, G., and Rammensee, H.G. (1989). In vivo priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine. Nature *342*, 561-564. https://doi.org/10.1038/342561a0

Desmarais, S.M., De Pedro, M.A., Cava, F., and Huang, K.C. (2013). Peptidoglycan at its peaks: how chromatographic analyses can reveal bacterial cell wall structure and assembly. Mol Microbiol *89*, 1-13. https://doi.org/10.1111/mmi.12266

Dombrowski, C., Kan, W., Motaleb, M.A., Charon, N.W., Goldstein, R.E., and Wolgemuth, C.W. (2009). The elastic basis for the shape of *Borrelia burgdorferi*. Biophys J *96*, 4409-4417. https://doi.org/10.1016/j.bpj.2009.02.066

Dong, C., Beis, K., Nesper, J., Brunkan-Lamontagne, A.L., Clarke, B.R., Whitfield, C., and Naismith, J.H. (2006). Wza, the translocon for *E. coli* capsular polysaccharides, defines a new class of membrane protein. Nature *444*, 226-229. https://doi.org/10.1038/nature05267

Dowdell, A.S., Murphy, M.D., Azodi, C., Swanson, S.K., Florens, L., Chen, S., and Zückert, W.R. (2017). Comprehensive Spatial Analysis of the *Borrelia burgdorferi* Lipoproteome Reveals a Compartmentalization Bias toward the Bacterial Surface. J Bacteriol *199*. https://doi.org/10.1128/JB.00658-16

Dresser, A.R., Hardy, P.O., and Chaconas, G. (2009). Investigation of the genes involved in antigenic switching at the vlsE locus in *Borrelia burgdorferi*: an essential role for the RuvAB branch migrase. PLoS Pathog *5*, e1000680. https://doi.org/10.1371/journal.ppat.1000680

Du, S., and Lutkenhaus, J. (2019). At the Heart of Bacterial Cytokinesis: The Z Ring. Trends Microbiol *27*, 781-791. https://doi.org/10.1016/j.tim.2019.04.011

Dunham-Ems, S.M., Caimano, M.J., Eggers, C.H., and Radolf, J.D. (2012). *Borrelia burgdorferi* requires the alternative sigma factor RpoS for dissemination within the vector during tick-to-mammal transmission. PLoS Pathog *8*, e1002532. https://doi.org/10.1371/journal.ppat.1002532

Dunn, J.P., Kenedy, M.R., Iqbal, H., and Akins, D.R. (2015). Characterization of the beta-barrel assembly machine accessory lipoproteins from *Borrelia burgdorferi*. BMC Microbiol *15*, 70. https://doi.org/10.1186/s12866-015-0411-y

Dworkin, J. (2014). The medium is the message: interspecies and interkingdom signaling by peptidoglycan and related bacterial glycans. Annual Reviews in Microbiology *68*, 137-154. https://doi.org/10.1146/annurev-micro-091213-112844

Egan, A.J.F., Errington, J., and Vollmer, W. (2020). Regulation of peptidoglycan synthesis and remodelling. Nat Rev Microbiol *18*, 446-460. https://doi.org/10.1038/s41579-020-0366-3

Eicken, C., Sharma, V., Klabunde, T., Lawrenz, M.B., Hardham, J.M., Norris, S.J., and Sacchettini, J.C. (2002). Crystal structure of Lyme disease variable surface antigen VlsE of *Borrelia burgdorferi*. J Biol Chem *277*, 21691-21696. https://doi.org/10.1074/jbc.M201547200

Eicken, C., Sharma, V., Klabunde, T., Owens, R.T., Pikas, D.S., Hook, M., and Sacchettini, J.C. (2001). Crystal structure of Lyme disease antigen outer surface protein C from *Borrelia burgdorferi*. J Biol Chem *276*, 10010-10015. https://doi.org/10.1074/jbc.M010062200

Eiffert, H., Lotter, H., Jarecki-Khan, K., and Thomssen, R. (1991). Identification of an immunoreactive non-proteinaleous component in *Borrelia burgdorferi*. Med Microbiol Immunol *180*, 229-237. https://doi.org/10.1007/BF00202557

Erdile, L.F., Brandt, M.A., Warakomski, D.J., Westrack, G.J., Sadziene, A., Barbour, A.G., and Mays, J.P. (1993). Role of attached lipid in immunogenicity of *Borrelia burgdorferi* OspA. Infect Immun *61*, 81-90. https://doi.org/10.1128/IAI.61.1.81-90.1993

Errington, J., Daniel, R.A., and Scheffers, D.J. (2003). Cytokinesis in bacteria. Microbiol Mol Biol Rev *67*, 52-65, table of contents. https://doi.org/10.1128/mmbr.67.1.52-65.2003

Ferenci, T., Schwentorat, M., Ullrich, S., and Vilmart, J. (1980). Lambda receptor in the outer membrane of *Escherichia coli* as a binding protein for maltodextrins and starch polysaccharides. J Bacteriol *142*, 521-526. https://doi.org/10.1128/JB.142.2.521-526.1980

Fingerle, V., Hauser, U., Liegl, G., Petko, B., Preac-Mursic, V., and Wilske, B. (1995). Expression of outer surface proteins A and C of *Borrelia burgdorferi* in Ixodes ricinus. J Clin Microbiol *33*, 1867-1869. https://doi.org/10.1128/JCM.33.7.1867-1869.1995

Fingerle, V., Liegl, G., Munderloh, U., and Wilske, B. (1998). Expression of outer surface proteins A and C of *Borrelia burgdorferi* in Ixodes ricinus ticks removed from humans. Med Microbiol Immunol *187*, 121-126. https://doi.org/10.1007/s004300050083

Finlay, B.B., and Falkow, S. (1989). Common themes in microbial pathogenicity. Microbiol Rev *53*, 210-230.

Fischer, J.R., LeBlanc, K.T., and Leong, J.M. (2006). Fibronectin binding protein BBK32 of the Lyme disease spirochete promotes bacterial attachment to glycosaminoglycans. Infect Immun *74*, 435-441. https://doi.org/10.1128/IAI.74.1.435-441.2006

Fischer, J.R., Parveen, N., Magoun, L., and Leong, J.M. (2003). Decorin-binding proteins A and B confer distinct mammalian cell type-specific attachment by *Borrelia burgdorferi*, the Lyme disease spirochete. Proc Natl Acad Sci U S A *100*, 7307-7312. https://doi.org/10.1073/pnas.1231043100

Flak, T.A., and Goldman, W.E. (1999). Signalling and cellular specificity of airway nitric oxide production in pertussis. Cell Microbiol *1*, 51-60. https://doi.org/10.1046/j.1462-5822.1999.00004.x

Fleurie, A., Lesterlin, C., Manuse, S., Zhao, C., Cluzel, C., Lavergne, J.P., Franz-Wachtel, M., Macek, B., Combet, C., Kuru, E.*, et al.* (2014). MapZ marks the division sites and positions FtsZ rings in *Streptococcus pneumoniae*. Nature *516*, 259-262. https://doi.org/10.1038/nature13966

Francetic, O., and Pugsley, A.P. (2005). Towards the identification of type II secretion signals in a nonacylated variant of pullulanase from *Klebsiella oxytoca*. J Bacteriol *187*, 7045-7055. https://doi.org/10.1128/JB.187.20.7045-7055.2005

Fraser, C.M., Casjens, S., Huang, W.M., Sutton, G.G., Clayton, R., Lathigra, R., White, O., Ketchum, K.A., Dodson, R., Hickey, E.K.*, et al.* (1997). Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature *390*, 580-586. https://doi.org/10.1038/37551

Fraser, C.M., Norris, S.J., Weinstock, G.M., White, O., Sutton, G.G., Dodson, R., Gwinn, M., Hickey, E.K., Clayton, R., Ketchum, K.A.*, et al.* (1998). Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. Science *281*, 375-388. https://doi.org/10.1126/science.281.5375.375

Froger, A., Rolland, J.P., Bron, P., Lagree, V., Caherec, F.L., Deschamps, S., Hubert, J.F., Pellerin, I., Thomas, D., and Delamarche, C. (2001). Functional characterization of a microbial aquaglyceroporin. Microbiology (Reading) *147*, 1129-1135. https://doi.org/10.1099/00221287-147-5-1129

Fukuda, A., Matsuyama, S., Hara, T., Nakayama, J., Nagasawa, H., and Tokuda, H. (2002). Aminoacylation of the N-terminal cysteine is essential for Lol-dependent release of lipoproteins from membranes but does not depend on lipoprotein sorting signals. J Biol Chem *277*, 43512-43518.

Gan, L., Chen, S., and Jensen, G.J. (2008). Molecular organization of Gram-negative peptidoglycan. Proc Natl Acad Sci U S A *105*, 18953-18957. https://doi.org/10.1073/pnas.0808035105

Garcia, B.L., Zhi, H., Wager, B., Hook, M., and Skare, J.T. (2016). *Borrelia burgdorferi* BBK32 Inhibits the Classical Pathway by Blocking Activation of the C1 Complement Complex. PLoS Pathog *12*, e1005404. https://doi.org/10.1371/journal.ppat.1005404

Garcia, D.L., and Dillard, J.P. (2008). Mutations in ampG or ampD affect peptidoglycan fragment release from *Neisseria gonorrhoeae*. J Bacteriol *190*, 3799-3807. https://doi.org/10.1128/JB.01194-07

Garcia Monco, J.C., Wheeler, C.M., Benach, J.L., Furie, R.A., Lukehart, S.A., Stanek, G., and Steere, A.C. (1993). Reactivity of neuroborreliosis patients (Lyme disease) to cardiolipin and gangliosides. J Neurol Sci *117*, 206-214. https://doi.org/10.1016/0022-510x(93)90175-x

Garcia-Heredia, A., Pohane, A.A., Melzer, E.S., Carr, C.R., Fiolek, T.J., Rundell, S.R., Lim, H.C., Wagner, J.C., Morita, Y.S., Swarts, B.M.*, et al.* (2018). Peptidoglycan precursor synthesis along the sidewall of pole-growing mycobacteria. Elife *7*. https://doi.org/10.7554/eLife.37243

Garcia-Monco, J.C., Seidman, R.J., and Benach, J.L. (1995). Experimental immunization with *Borrelia burgdorferi* induces development of antibodies to gangliosides. Infect Immun *63*, 4130-4137. https://doi.org/10.1128/IAI.63.10.4130-4137.1995

Geiger, O., Lopez-Lara, I.M., and Sohlenkamp, C. (2013). Phosphatidylcholine biosynthesis and function in bacteria. Biochim Biophys Acta *1831*, 503-513. https://doi.org/10.1016/j.bbalip.2012.08.009

Gherardini, F.C. (2013). *Borrelia burgdorferi* HtrA may promote dissemination and irritation. Mol Microbiol *90*, 209-213. https://doi.org/10.1111/mmi.12390

Gilmore, R.D., Jr., and Piesman, J. (2000). Inhibition of *Borrelia burgdorferi* migration from the midgut to the salivary glands following feeding by ticks on OspC-immunized mice. Infect Immun *68*, 411-414. https://doi.org/10.1128/iai.68.1.411-414.2000

Goldman, W.E., Klapper, D.G., and Baseman, J.B. (1982). Detection, isolation, and analysis of a released *Bordetella pertussis* product toxic to cultured tracheal cells. Infect Immun *36*, 782-794. https://doi.org/10.1128/IAI.36.2.782-794.1982

Goldstein, S.F., Charon, N.W., and Kreiling, J.A. (1994). *Borrelia burgdorferi* swims with a planar waveform similar to that of eukaryotic flagella. Proceedings of the National Academy of Sciences of the United States of America *91*, 3433-3437.

Gominet, M., Slamti, L., Gilois, N., Rose, M., and Lereclus, D. (2001). Oligopeptide permease is required for expression of the *Bacillus thuringiensis* plcR regulon and for virulence. Mol Microbiol *40*, 963-975. https://doi.org/10.1046/j.1365-2958.2001.02440.x

Goodell, E.W., and Higgins, C.F. (1987). Uptake of cell wall peptides by *Salmonella typhimurium* and *Escherichia coli*. J Bacteriol *169*, 3861-3865. https://doi.org/10.1128/jb.169.8.3861-3865.1987

Gorrell, R.J., Guan, J., Xin, Y., Tafreshi, M.A., Hutton, M.L., McGuckin, M.A., Ferrero, R.L., and Kwok, T. (2013). A novel NOD1- and CagA-independent pathway of interleukin-8 induction mediated by the *Helicobacter pylori* type IV secretion system. Cell Microbiol *15*, 554-570. https://doi.org/10.1111/cmi.12055

Grabowicz, M., and Silhavy, T.J. (2017). Redefining the essential trafficking pathway for outer membrane lipoproteins. Proc Natl Acad Sci U S A *114*, 4769-4774. https://doi.org/10.1073/pnas.1702248114

Greene, N.P., Hinchliffe, P., Crow, A., Ababou, A., Hughes, C., and Koronakis, V. (2013). Structure of an atypical periplasmic adaptor from a multidrug efflux pump of the spirochete *Borrelia burgdorferi*. FEBS Lett *587*, 2984-2988. https://doi.org/10.1016/j.febslet.2013.06.056

Grimm, D., Tilly, K., Byram, R., Stewart, P.E., Krum, J.G., Bueschel, D.M., Schwan, T.G., Policastro, P.F., Elias, A.F., and Rosa, P.A. (2004). Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. Proc Natl Acad Sci U S A *101*, 3142-3147. https://doi.org/10.1073/pnas.0306845101

Groshong, A.M., Dey, A., Bezsonova, I., Caimano, M.J., and Radolf, J.D. (2017). Peptide uptake is essential for *Borrelia burgdorferi* viability and involves structural and regulatory complexity of its oligopeptide transporter. mBio *8*. https://doi.org/10.1128/mBio.02047-17

Guo, B.P., Brown, E.L., Dorward, D.W., Rosenberg, L.C., and Hook, M. (1998). Decorin-binding adhesins from *Borrelia burgdorferi*. Mol Microbiol *30*, 711-723. https://doi.org/10.1046/j.1365-2958.1998.01103.x

Guo, B.P., Norris, S.J., Rosenberg, L.C., and Hook, M. (1995). Adherence of *Borrelia burgdorferi* to the proteoglycan decorin. Infect Immun *63*, 3467-3472. https://doi.org/10.1128/IAI.63.9.3467-3472.1995

Haake, D.A. (2000). Spirochaetal lipoproteins and pathogenesis. Microbiology (Reading) *146 (Pt 7)*, 1491-1504. https://doi.org/10.1099/00221287-146-7-1491

Haas, R., and Meyer, T.F. (1986). The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: evidence for gene conversion. Cell *44*, 107-115. https://doi.org/10.1016/0092-8674(86)90489-7

Hagan, C.L., Silhavy, T.J., and Kahne, D. (2011). β-Barrel membrane protein assembly by the Bam complex. Annu Rev Biochem *80*, 189-210. https://doi.org/10.1146/annurev-biochem-061408-144611

Hagblom, P., Segal, E., Billyard, E., and So, M. (1985). Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. Nature *315*, 156-158. https://doi.org/10.1038/315156a0

Hagman, K.E., Lahdenne, P., Popova, T.G., Porcella, S.F., Akins, D.R., Radolf, J.D., and Norgard, M.V. (1998). Decorin-binding protein of *Borrelia burgdorferi* is encoded within a two-gene operon and is protective in the murine model of Lyme borreliosis. Infect Immun *66*, 2674-2683. https://doi.org/10.1128/IAI.66.6.2674-2683.1998

Hallstrom, T., Siegel, C., Morgelin, M., Kraiczy, P., Skerka, C., and Zipfel, P.F. (2013). CspA from *Borrelia burgdorferi* inhibits the terminal complement pathway. mBio *4*. https://doi.org/10.1128/mBio.00481-13

Hammerschmidt, C., Koenigs, A., Siegel, C., Hallstrom, T., Skerka, C., Wallich, R., Zipfel, P.F., and Kraiczy, P. (2014). Versatile roles of CspA orthologs in complement inactivation of serum-resistant Lyme disease spirochetes. Infect Immun *82*, 380-392. https://doi.org/10.1128/IAI.01094-13

Hancock, R.E., and Benz, R. (1986). Demonstration and chemical modification of a specific phosphate binding site in the phosphate-starvation-inducible outer membrane porin protein P of *Pseudomonas aeruginosa*. Biochim Biophys Acta *860*, 699-707. https://doi.org/10.1016/0005-2736(86)90569-9

Hankamer, B., Morris, E., Nield, J., Gerle, C., and Barber, J. (2001). Three-dimensional structure of the photosystem II core dimer of higher plants determined by electron microscopy. J Struct Biol *135*, 262-269. https://doi.org/10.1006/jsbi.2001.4405

Hanson, M.S., Cassatt, D.R., Guo, B.P., Patel, N.K., McCarthy, M.P., Dorward, D.W., and Hook, M. (1998). Active and passive immunity against *Borrelia burgdorferi* decorin binding protein A (DbpA) protects against infection. Infect Immun *66*, 2143-2153. https://doi.org/10.1128/IAI.66.5.2143-2153.1998

Hara, T., Matsuyama, S., and Tokuda, H. (2003). Mechanism underlying the inner membrane retention of *Escherichia coli* lipoproteins caused by Lol avoidance signals. J Biol Chem *278*, 40408-40414. https://doi.org/10.1074/jbc.M307836200

Harada, K., Ando, M., Fujimura-Ito, T., Kasahara-Imamura, M., Sako, Y., Uchida, A., Ishida, Y., Kadota, H., Ohnishi, T., and Moseley, B.E. (2003). Mechanism of thermotolerance induction by split-dose hyperthermia in *Deinococcus radiodurans* DNA repair deficient mutants. Int J Mol Med *12*, 741-747.

Harman, M.W., Hamby, A.E., Boltyanskiy, R., Belperron, A.A., Bockenstedt, L.K., Kress, H., Dufresne, E.R., and Wolgemuth, C.W. (2017). Vancomycin reduces cell wall stiffness and slows swim speed of the Lyme disease bacterium. Biophys J *112*, 746-754. https://doi.org/10.1016/j.bpj.2016.12.039

Hayashi, S., and Wu, H.C. (1990). Lipoproteins in bacteria. J Bioenerg Biomembr *22*, 451-471. https://doi.org/10.1007/BF00763177

Hayashi, Y., Tsurumizu, R., Tsukahara, J., Takeda, K., Narita, S., Mori, M., Miki, K., and Tokuda, H. (2014). Roles of the protruding loop of factor B essential for the localization of lipoproteins (LolB) in the anchoring of bacterial triacylated proteins to the outer membrane. J Biol Chem *289*, 10530-10539. https://doi.org/10.1074/jbc.M113.539270

Heiss, L.N., Lancaster, J.R., Jr., Corbett, J.A., and Goldman, W.E. (1994). Epithelial autotoxicity of nitric oxide: role in the respiratory cytopathology of pertussis. Proc Natl Acad Sci U S A *91*, 267-270. https://doi.org/10.1073/pnas.91.1.267

Heiss, L.N., Moser, S.A., Unanue, E.R., and Goldman, W.E. (1993). Interleukin-1 is linked to the respiratory epithelial cytopathology of pertussis. Infect Immun *61*, 3123-3128. https://doi.org/10.1128/IAI.61.8.3123-3128.1993

Hoffmann, P., Heinle, S., Schade, U.F., Loppnow, H., Ulmer, A.J., Flad, H.D., Jung, G., and Bessler, W.G. (1988). Stimulation of human and murine adherent cells by bacterial lipoprotein and synthetic lipopeptide analogues. Immunobiology *177*, 158-170. https://doi.org/10.1016/S0171-2985(88)80036-6

Holtje, J.V. (1998). Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. Microbiol Mol Biol Rev *62*, 181-203.

Hooda, Y., Lai, C.C., Judd, A., Buckwalter, C.M., Shin, H.E., Gray-Owen, S.D., and Moraes, T.F. (2016). Slam is an outer membrane protein that is required for the surface display of lipidated virulence factors in *Neisseria*. Nat Microbiol *1*, 16009. https://doi.org/10.1038/nmicrobiol.2016.9

Hossain, H., Wellensiek, H.J., Geyer, R., and Lochnit, G. (2001). Structural analysis of glycolipids from *Borrelia burgdorferi*. Biochimie *83*, 683-692. https://doi.org/10.1016/s0300-9084(01)01296-2

Howe, T.R., LaQuier, F.W., and Barbour, A.G. (1986). Organization of genes encoding two outer membrane proteins of the Lyme disease agent *Borrelia burgdorferi* within a single transcriptional unit. Infect Immun *54*, 207-212.

Hsu, Y.P., Rittichier, J., Kuru, E., Yablonowski, J., Pasciak, E., Tekkam, S., Hall, E., Murphy, B., Lee, T.K., Garner, E.C*., et al.* (2017). Full color palette of fluorescent d-amino acids for in situ labeling of bacterial cell walls. Chem Sci *8*, 6313-6321. https://doi.org/10.1039/c7sc01800b

Huang, Z., Toledo, A.M., Benach, J.L., and London, E. (2016). Ordered Membrane Domain-Forming Properties of the Lipids of *Borrelia burgdorferi*. Biophys J *111*, 2666-2675. https://doi.org/10.1016/j.bpj.2016.11.012

Iqbal, H., Kenedy, M.R., Lybecker, M., and Akins, D.R. (2016). The TamB ortholog of *Borrelia burgdorferi* interacts with the beta-barrel assembly machine (BAM) complex protein BamA. Mol Microbiol *102*, 757-774. https://doi.org/10.1111/mmi.13492

Irving, A.T., Mimuro, H., Kufer, T.A., Lo, C., Wheeler, R., Turner, L.J., Thomas, B.J., Malosse, C., Gantier, M.P., Casillas, L.N*., et al.* (2014). The immune receptor NOD1 and kinase RIP2 interact with bacterial peptidoglycan on early endosomes to promote autophagy and inflammatory signaling. Cell Host Microbe *15*, 623-635. https://doi.org/10.1016/j.chom.2014.04.001

Ito, H., Ura, A., Oyamada, Y., Yoshida, H., Yamagishi, J., Narita, S., Matsuyama, S., and Tokuda, H. (2007). A new screening method to identify inhibitors of the Lol (localization of lipoproteins) system, a novel antibacterial target. Microbiol Immunol *51*, 263-270. https://doi.org/10.1111/j.1348-0421.2007.tb03906.x

Ivleva, N.B., Shestakov, S.V., and Pakrasi, H.B. (2000). The carboxyl-terminal extension of the precursor D1 protein of photosystem II is required for optimal photosynthetic performance of the cyanobacterium *Synechocystis sp.* PCC 6803. Plant Physiol *124*, 1403-1412. https://doi.org/10.1104/pp.124.3.1403

Iyer, R., Caimano, M.J., Luthra, A., Axline, D., Jr., Corona, A., Iacobas, D.A., Radolf, J.D., and Schwartz, I. (2015). Stage-specific global alterations in the transcriptomes of Lyme disease spirochetes during tick feeding and following mammalian host adaptation. Mol Microbiol *95*, 509-538. https://doi.org/10.1111/mmi.12882

Jager, S.N., Porta, E.O.J., and Labadie, G.R. (2019). Convenient synthesis of the immunogenic glycolipid BbGL1. Steroids *141*, 41-45. https://doi.org/10.1016/j.steroids.2018.11.012

Jauris-Heipke, S., Liegl, G., Preac-Mursic, V., Rossler, D., Schwab, E., Soutschek, E., Will, G., and Wilske, B. (1995). Molecular analysis of genes encoding outer surface protein C (OspC) of *Borrelia burgdorferi* sensu lato: relationship to ospA genotype and evidence of

lateral gene exchange of ospC. J Clin Microbiol *33*, 1860-1866. https://doi.org/10.1128/JCM.33.7.1860-1866.1995

Johnson, R.C., Schmid, G.P., Hyde, F.W., Steigerwalt, A.G., and Brenner, D.J. (1984). *Borrelia burgdorferi* sp. nov.: Ethiologic agent og Lyme disease. International Journal of SysteaticBacteriology, 496-497.

Jones, J.D., Bourell, K.W., Norgard, M.V., and Radolf, J.D. (1995). Membrane topology of *Borrelia burgdorferi* and *Treponema pallidum* lipoproteins. Infect Immun *63*, 2424-2434. https://doi.org/10.1128/IAI.63.7.2424-2434.1995

Jones, K.L., Seward, R.J., Ben-Menachem, G., Glickstein, L.J., Costello, C.E., and Steere, A.C. (2009). Strong IgG antibody responses to *Borrelia burgdorferi* glycolipids in patients with Lyme arthritis, a late manifestation of the infection. Clin Immunol *132*, 93-102. https://doi.org/10.1016/j.clim.2009.03.510

Jutras, B.L., Lochhead, R.B., Kloos, Z.A., Biboy, J., Strle, K., Booth, C.J., Govers, S.K., Gray, J., Schumann, P., Vollmer, W.*, et al.* (2019). *Borrelia burgdorferi* peptidoglycan is a persistent antigen in patients with Lyme arthritis. Proc Natl Acad Sci U S A *116*, 13498-13507. https://doi.org/10.1073/pnas.1904170116

Jutras, B.L., Scott, M., Parry, B., Biboy, J., Gray, J., Vollmer, W., and Jacobs-Wagner, C. (2016). Lyme disease and relapsing fever *Borrelia* elongate through zones of peptidoglycan synthesis that mark division sites of daughter cells. Proc Natl Acad Sci U S A *113*, 9162-9170. https://doi.org/10.1073/pnas.1610805113

Kamio, Y., and Nikaido, H. (1976). Outer membrane of *Salmonella typhimurium*: accessibility of phospholipid head groups to phospholipase c and cyanogen bromide activated dextran in the external medium. Biochemistry *15*, 2561-2570. https://doi.org/10.1021/bi00657a012

Kariu, T., Sharma, K., Singh, P., Smith, A.A., Backstedt, B., Buyuktanir, O., and Pal, U. (2015). BB0323 and novel virulence determinant BB0238: *Borrelia burgdorferi* proteins that interact with and stabilize each other and are critical for infectivity. Journal of Infectious Diseases *211*, 462-471. https://doi.org/10.1093/infdis/jiu460

Kariu, T., Yang, X., Marks, C.B., Zhang, X., and Pal, U. (2013). Proteolysis of BB0323 results in two polypeptides that impact physiologic and infectious phenotypes in *Borrelia burgdorferi*. Mol Microbiol *88*, 510-522. https://doi.org/10.1111/mmi.12202

Karnauchov, I., Herrmann, R.G., Pakrasi, H.B., and Klosgen, R.B. (1997). Transport of CtpA protein from the cyanobacterium *Synechocystis* 6803 across the thylakoid membrane in chloroplasts. Eur J Biochem *249*, 497-504. https://doi.org/10.1111/j.1432-1033.1997.t01-1-00497.x

Katona, L.I., Beck, G., and Habicht, G.S. (1992). Purification and immunological characterization of a major low-molecular-weight lipoprotein from *Borrelia burgdorferi*. Infect Immun *60*, 4995-5003.

Kenedy, M.R., Luthra, A., Anand, A., Dunn, J.P., Radolf, J.D., and Akins, D.R. (2014). Structural modeling and physicochemical characterization provide evidence that P66 forms a beta-barrel in the *Borrelia burgdorferi* outer membrane. J Bacteriol *196*, 859-872. https://doi.org/10.1128/JB.01236-13

Kenedy, M.R., Scott, E.J., 2nd, Shrestha, B., Anand, A., Iqbal, H., Radolf, J.D., Dyer, D.W., and Akins, D.R. (2016). Consensus computational network analysis for identifying candidate outer membrane proteins from *Borrelia* spirochetes. BMC Microbiol *16*, 141. https://doi.org/10.1186/s12866-016-0762-z

Kenedy, M.R., Vuppala, S.R., Siegel, C., Kraiczy, P., and Akins, D.R. (2009). CspA-mediated binding of human factor H inhibits complement deposition and confers serum resistance in *Borrelia burgdorferi*. Infect Immun *77*, 2773-2782. https://doi.org/10.1128/IAI.00318-09

Khandke, K.M., Fairwell, T., Chait, B.T., and Manjula, B.N. (1989). Influence of ions on cyclization of the amino terminal glutamine residues of tryptic peptides of streptococcal PepM49 protein. Resolution of cyclized peptides by HPLC and characterization by mass spectrometry. Int J Pept Protein Res *34*, 118-123. https://doi.org/10.1111/j.1399-3011.1989.tb01499.x

Kihara, A., Akiyama, Y., and Ito, K. (1995). FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. Proc Natl Acad Sci U S A *92*, 4532-4536. https://doi.org/10.1073/pnas.92.10.4532

Kihara, A., Akiyama, Y., and Ito, K. (1997). Host regulation of lysogenic decision in bacteriophage lambda: transmembrane modulation of FtsH (HflB), the cII degrading protease, by HflKC (HflA). Proc Natl Acad Sci U S A *94*, 5544-5549. https://doi.org/10.1073/pnas.94.11.5544

Kim, S., Malinverni, J.C., Sliz, P., Silhavy, T.J., Harrison, S.C., and Kahne, D. (2007). Structure and function of an essential component of the outer membrane protein assembly machine. Science *317*, 961-964. https://doi.org/10.1126/science.1143993

Koebnik, R., Locher, K.P., and Van Gelder, P. (2000). Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol Microbiol *37*, 239-253. https://doi.org/10.1046/j.1365-2958.2000.01983.x

Konovalova, A., Kahne, D.E., and Silhavy, T.J. (2017). Outer membrane biogenesis. Annual Reviews in Microbiology *71*, 539-556. https://doi.org/10.1146/annurev-micro-090816-093754

Koomey, M. (1997). Bacterial pathogenesis: a variation on variation in Lyme disease. Curr Biol *7*, R538-540. https://doi.org/10.1016/s0960-9822(06)00275-2

Kornacki, J.A., and Oliver, D.B. (1998). Lyme disease-causing *Borrelia* species encode multiple lipoproteins homologous to peptide-binding proteins of ABC-type transporters. Infect Immun *66*, 4115-4122.

Koronakis, V., Sharff, A., Koronakis, E., Luisi, B., and Hughes, C. (2000). Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature *405*, 914-919. https://doi.org/10.1038/35016007

Kraiczy, P., Skerka, C., Kirschfink, M., Zipfel, P.F., and Brade, V. (2001). Mechanism of complement resistance of pathogenic *Borrelia burgdorferi* isolates. Int Immunopharmacol *1*, 393-401. https://doi.org/10.1016/s1567-5769(00)00041-2

Kudryashev, M., Cyrklaff, M., Baumeister, W., Simon, M.M., Wallich, R., and Frischknecht, F. (2009). Comparative cryo-electron tomography of pathogenic Lyme disease spirochetes. Mol Microbiol *71*, 1415-1434. https://doi.org/10.1111/j.1365-2958.2009.06613.x

Kumar, D., Ristow, L.C., Shi, M., Mukherjee, P., Caine, J.A., Lee, W.Y., Kubes, P., Coburn, J., and Chaconas, G. (2015). Intravital Imaging of Vascular Transmigration by the Lyme Spirochete: Requirement for the integrin binding residues of the *B. burgdorferi* P66 protein. PLoS Pathog *11*, e1005333. https://doi.org/10.1371/journal.ppat.1005333

Kumaran, D., Eswaramoorthy, S., Luft, B.J., Koide, S., Dunn, J.J., Lawson, C.L., and Swaminathan, S. (2001). Crystal structure of outer surface protein C (OspC) from the Lyme disease spirochete, *Borrelia burgdorferi*. EMBO J *20*, 971-978. https://doi.org/10.1093/emboj/20.5.971

Kumru, O.S., Bunikis, I., Sorokina, I., Bergström, S., and Zückert, W.R. (2011a). Specificity and role of the *Borrelia burgdorferi* CtpA protease in outer membrane protein processing. J Bacteriol *193*, 5759-5765. https://doi.org/10.1128/JB.05622-11

Kumru, O.S., Schulze, R.J., Rodnin, M.V., Ladokhin, A.S., and Zückert, W.R. (2011b). Surface localization determinants of *Borrelia* OspC/Vsp family lipoproteins. J Bacteriol *193*, 2814-2825. https://doi.org/10.1128/JB.00015-11

Kumru, O.S., Schulze, R.J., Slusser, J.G., and Zückert, W.R. (2010). Development and validation of a FACS-based lipoprotein localization screen in the Lyme disease spirochete *Borrelia burgdorferi*. BMC Microbiol *10*, 277. https://doi.org/10.1186/1471-2180-10-277

Kuru, E., Hughes, H.V., Brown, P.J., Hall, E., Tekkam, S., Cava, F., de Pedro, M.A., Brun, Y.V., and VanNieuwenhze, M.S. (2012). *In situ*

probing of newly synthesized peptidoglycan in live bacteria with fluorescent D-amino acids. Angew Chem Int Ed Engl *51*, 12519-12523. https://doi.org/10.1002/anie.201206749

Kuru, E., Radkov, A., Meng, X., Egan, A., Alvarez, L., Dowson, A., Booher, G., Breukink, E., Roper, D.I., Cava, F*., et al.* (2019). Mechanisms of incorporation for D-Amino acid probes that target peptidoglycan biosynthesis. ACS Chem Biol *14*, 2745-2756. https://doi.org/10.1021/acschembio.9b00664

Lagal, V., Portnoi, D., Faure, G., Postic, D., and Baranton, G. (2006). *Borrelia burgdorferi* sensu stricto invasiveness is correlated with OspC-plasminogen affinity. Microbes Infect *8*, 645-652. https://doi.org/10.1016/j.micinf.2005.08.017

Lagal, V., Postic, D., Ruzic-Sabljic, E., and Baranton, G. (2003). Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC* gene and its association with invasiveness. J Clin Microbiol *41*, 5059-5065. https://doi.org/10.1128/jcm.41.11.5059-5065.2003

Lahdenne, P., Porcella, S., Hagman, K., Akins, D., Popova, T., Cox, D., Katona, L., Radolf, J., and Norgard, M. (1997). Molecular characterization of a 6.6-kilodalton *Borrelia burgdorferi* outer membrane-associated lipoprotein (lp6.6) which appears to be downregulated during mammalian infection. Infect Immun *65*, 412-421.

LaRocca, T.J., Crowley, J.T., Cusack, B.J., Pathak, P., Benach, J., London, E., Garcia-Monco, J.C., and Benach, J.L. (2010). Cholesterol lipids of *Borrelia burgdorferi* form lipid rafts and are required for the bactericidal activity of a complement-independent antibody. Cell Host Microbe *8*, 331-342. https://doi.org/10.1016/j.chom.2010.09.001

LaRocca, T.J., Holthausen, D.J., Hsieh, C., Renken, C., Mannella, C.A., and Benach, J.L. (2009). The bactericidal effect of a complement-independent antibody is osmolytic and specific to *Borrelia*. Proc Natl Acad Sci U S A *106*, 10752-10757. https://doi.org/10.1073/pnas.0901858106

LaRocca, T.J., Pathak, P., Chiantia, S., Toledo, A., Silvius, J.R., Benach, J.L., and London, E. (2013). Proving lipid rafts exist: membrane domains in the prokaryote *Borrelia burgdorferi* have the same properties as eukaryotic lipid rafts. PLoS Pathog *9*, e1003353. https://doi.org/10.1371/journal.ppat.1003353

Lawson, C.L., Yung, B.H., Barbour, A.G., and Zückert, W.R. (2006). Crystal structure of neurotropism-associated variable surface protein 1 (Vsp1) of *Borrelia turicatae*. J Bacteriol *188*, 4522-4530. https://doi.org/10.1128/JB.00028-06

Lazazzera, B.A. (2001). The intracellular function of extracellular signaling peptides. Peptides *22*, 1519-1527. https://doi.org/10.1016/s0196-9781(01)00488-0

Lenhart, T.R., and Akins, D.R. (2010). *Borrelia burgdorferi* locus BB0795 encodes a BamA orthologue required for growth and efficient localization of outer membrane proteins. Mol Microbiol *75*, 692-709. https://doi.org/10.1111/j.1365-2958.2009.07015.x

Lenhart, T.R., Kenedy, M.R., Yang, X., Pal, U., and Akins, D.R. (2012). BB0324 and BB0028 are constituents of the *Borrelia burgdorferi* beta-barrel assembly machine (BAM) complex. BMC Microbiol *12*, 60. https://doi.org/10.1186/1471-2180-12-60

Lenz, J.D., Hackett, K.T., and Dillard, J.P. (2017). A Single Dual-Function Enzyme Controls the Production of Inflammatory NOD Agonist Peptidoglycan Fragments by *Neisseria gonorrhoeae*. mBio *8*. https://doi.org/10.1128/mBio.01464-17

Leonard, B.A., Podbielski, A., Hedberg, P.J., and Dunny, G.M. (1996). *Enterococcus faecalis* pheromone binding protein, PrgZ, recruits a chromosomal oligopeptide permease system to import sex pheromone cCF10 for induction of conjugation. Proc Natl Acad Sci U S A *93*, 260-264. https://doi.org/10.1073/pnas.93.1.260

Lescot, M., Audic, S., Robert, C., Nguyen, T.T., Blanc, G., Cutler, S.J., Wincker, P., Couloux, A., Claverie, J.M., Raoult, D*., et al.* (2008). The genome of *Borrelia recurrentis*, the agent of deadly louse-borne relapsing fever, is a degraded subset of tick-borne *Borrelia duttonii*. PLoS Genet *4*, e1000185. https://doi.org/10.1371/journal.pgen.1000185

Levin, P.A., Kurtser, I.G., and Grossman, A.D. (1999). Identification and characterization of a negative regulator of FtsZ ring formation in *Bacillus subtilis*. Proc Natl Acad Sci U S A *96*, 9642-9647. https://doi.org/10.1073/pnas.96.17.9642

Lewenza, S., Mhlanga, M.M., and Pugsley, A.P. (2008). Novel inner membrane retention signals in *Pseudomonas aeruginosa* lipoproteins. J Bacteriol *190*, 6119-6125. https://doi.org/10.1128/JB.00603-08

Lewenza, S., Vidal-Ingigliardi, D., and Pugsley, A.P. (2006). Direct visualization of red fluorescent lipoproteins indicates conservation of the membrane sorting rules in the family *Enterobacteriaceae*. J Bacteriol *188*, 3516-3524. https://doi.org/10.1128/JB.188.10.3516-3524.2006

Lex, A., Wiesmuller, K.H., Jung, G., and Bessler, W.G. (1986). A synthetic analogue of *Escherichia coli* lipoprotein, tripalmitoyl pentapeptide, constitutes a potent immune adjuvant. J Immunol *137*, 2676-2681.

Li, H., Dunn, J.J., Luft, B.J., and Lawson, C.L. (1997). Crystal structure of Lyme disease antigen outer surface protein A complexed with an Fab. Proc Natl Acad Sci U S A *94*, 3584-3589. https://doi.org/10.1073/pnas.94.8.3584

Li, H., and Lawson, C.L. (1995). Crystallization and preliminary X-ray analysis of *Borrelia burgdorferi* outer surface protein A (OspA) complexed with a murine monoclonal antibody Fab fragment. J Struct Biol *115*, 335-337. https://doi.org/10.1006/jsbi.1995.1058

Li, X.Z., and Nikaido, H. (2004). Efflux-mediated drug resistance in bacteria. Drugs *64*, 159-204. https://doi.org/10.2165/00003495-200464020-00004

Lilly, A.A., Crane, J.M., and Randall, L.L. (2009). Export chaperone SecB uses one surface of interaction for diverse unfolded polypeptide ligands. Protein Sci *18*, 1860-1868. https://doi.org/10.1002/pro.197

Lin, B., Short, S.A., Eskildsen, M., Klempner, M.S., and Hu, L.T. (2001). Functional testing of putative oligopeptide permease (Opp) proteins of *Borrelia burgdorferi*: a complementation model in *opp(-) Escherichia coli*. Biochim Biophys Acta *1499*, 222-231. https://doi.org/10.1016/s0167-4889(00)00121-x

Lin, T., Gao, L., Edmondson, D.G., Jacobs, M.B., Philipp, M.T., and Norris, S.J. (2009). Central role of the Holliday junction helicase RuvAB in vlsE recombination and infectivity of *Borrelia burgdorferi*. PLoS Pathog *5*, e1000679. https://doi.org/10.1371/journal.ppat.1000679

Lin, T., Gao, L., Zhang, C., Odeh, E., Jacobs, M.B., Coutte, L., Chaconas, G., Philipp, M.T., and Norris, S.J. (2012). Analysis of an ordered, comprehensive STM mutant library in infectious *Borrelia burgdorferi*: insights into the genes required for mouse infectivity. PLoS One *7*, e47532. https://doi.org/10.1371/journal.pone.0047532

Lin, T., Troy, E.B., Hu, L.T., Gao, L., and Norris, S.J. (2014). Transposon mutagenesis as an approach to improved understanding of *Borrelia* pathogenesis and biology. Front Cell Infect Microbiol *4*, 63. https://doi.org/10.3389/fcimb.2014.00063

Lin, Y.P., Chen, Q., Ritchie, J.A., Dufour, N.P., Fischer, J.R., Coburn, J., and Leong, J.M. (2015). Glycosaminoglycan binding by *Borrelia burgdorferi* adhesin BBK32 specifically and uniquely promotes joint colonization. Cell Microbiol *17*, 860-875. https://doi.org/10.1111/cmi.12407

Lin, Y.P., Frye, A.M., Nowak, T.A., and Kraiczy, P. (2020a). New Insights Into CRASP-Mediated Complement Evasion in the Lyme Disease Enzootic Cycle. Front Cell Infect Microbiol *10*, 1. https://doi.org/10.3389/fcimb.2020.00001

Lin, Y.P., Tan, X., Caine, J.A., Castellanos, M., Chaconas, G., Coburn, J., and Leong, J.M. (2020b). Strain-specific joint invasion and colonization by Lyme disease spirochetes is promoted by outer

surface protein C. PLoS Pathog *16*, e1008516. https://doi.org/10.1371/journal.ppat.1008516

Liu, G., Topping, T.B., and Randall, L.L. (1989). Physiological role during export for the retardation of folding by the leader peptide of maltose-binding protein. Proc Natl Acad Sci U S A *86*, 9213-9217. https://doi.org/10.1073/pnas.86.23.9213

Livermore, B.P., Bey, R.F., and Johnson, R.C. (1978). Lipid metabolism of *Borrelia hermsii*. Infect Immun *20*, 215-220. https://doi.org/10.1128/IAI.20.1.215-220.1978

Livey, I., Gibbs, C.P., Schuster, R., and Dorner, F. (1995). Evidence for lateral transfer and recombination in OspC variation in Lyme disease *Borrelia*. Mol Microbiol *18*, 257-269. https://doi.org/10.1111/j.1365-2958.1995.mmi_18020257.x

London, E. (2002). Insights into lipid raft structure and formation from experiments in model membranes. Curr Opin Struct Biol *12*, 480-486. https://doi.org/10.1016/s0959-440x(02)00351-2

Lone, A.G., and Bankhead, T. (2020). The *Borrelia burgdorferi* VlsE Lipoprotein Prevents Antibody Binding to an Arthritis-Related Surface Antigen. Cell Rep *30*, 3663-3670 e3665. https://doi.org/10.1016/j.celrep.2020.02.081

Lopez, D., and Kolter, R. (2010). Functional microdomains in bacterial membranes. Genes Dev *24*, 1893-1902. https://doi.org/10.1101/gad.1945010

Malinverni, J.C., Werner, J., Kim, S., Sklar, J.G., Kahne, D., Misra, R., and Silhavy, T.J. (2006). YfiO stabilizes the YaeT complex and is essential for outer membrane protein assembly in *Escherichia coli*. Mol Microbiol *61*, 151-164. https://doi.org/10.1111/j.1365-2958.2006.05211.x

Masuda, K., Matsuyama, S., and Tokuda, H. (2002). Elucidation of the function of lipoprotein-sorting signals that determine membrane localization. Proc Natl Acad Sci U S A *99*, 7390-7395.

Matsuyama, S., Tajima, T., and Tokuda, H. (1995). A novel periplasmic carrier protein involved in the sorting and transport of *Escherichia coli* lipoproteins destined for the outer membrane. EMBO J *14*, 3365-3372.

Matsuyama, S., Yokota, N., and Tokuda, H. (1997). A novel outer membrane lipoprotein, LolB (HemM), involved in the LolA (p20)-dependent localization of lipoproteins to the outer membrane of *Escherichia coli*. EMBO J *16*, 6947-6955. https://doi.org/10.1093/emboj/16.23.6947

Medrano, M.S., Ding, Y., Wang, X.G., Lu, P., Coburn, J., and Hu, L.T. (2007). Regulators of expression of the oligopeptide permease A proteins of *Borrelia burgdorferi*. J Bacteriol *189*, 2653-2659. https://doi.org/10.1128/JB.01760-06

Medzhitov, R., and Janeway, C., Jr. (2000). Innate immune recognition: mechanisms and pathways. Immunol Rev *173*, 89-97. https://doi.org/10.1034/j.1600-065x.2000.917309.x

Melly, M.A., McGee, Z.A., and Rosenthal, R.S. (1984). Ability of monomeric peptidoglycan fragments from *Neisseria gonorrhoeae* to damage human fallopian-tube mucosa. Journal of Infectious Diseases *149*, 378-386. https://doi.org/10.1093/infdis/149.3.378

Mitchell, S.J., and Minnick, M.F. (1997). A carboxy-terminal processing protease gene is located immediately upstream of the invasion-associated locus from *Bartonella bacilliformis*. Microbiology (Reading) *143 (Pt 4)*, 1221-1233. https://doi.org/10.1099/00221287-143-4-1221

Miyamoto, A., Matsuyama, S., and Tokuda, H. (2001). Mutant of LolA, a lipoprotein-specific molecular chaperone of *Escherichia coli*, defective in the transfer of lipoproteins to LolB. Biochem Biophys Res Commun *287*, 1125-1128. https://doi.org/10.1006/bbrc.2001.5705

Mizutani, M., Mukaiyama, K., Xiao, J., Mori, M., Satou, R., Narita, S., Okuda, S., and Tokuda, H. (2013). Functional differentiation of structurally similar membrane subunits of the ABC transporter LolCDE complex. FEBS Lett *587*, 23-29. https://doi.org/10.1016/j.febslet.2012.11.009

Moffett, S., Brown, D.A., and Linder, M.E. (2000). Lipid-dependent targeting of G proteins into rafts. J Biol Chem *275*, 2191-2198. https://doi.org/10.1074/jbc.275.3.2191

Monnet, V. (2003). Bacterial oligopeptide-binding proteins. Cell Mol Life Sci *60*, 2100-2114. https://doi.org/10.1007/s00018-003-3054-3

Moore, D.A., Whatley, Z.N., Joshi, C.P., Osawa, M., and Erickson, H.P. (2017). Probing for Binding Regions of the FtsZ Protein Surface through Site-Directed Insertions: Discovery of Fully Functional FtsZ-Fluorescent Proteins. J Bacteriol *199*. https://doi.org/10.1128/JB.00553-16

Motaleb, M.A., Corum, L., Bono, J.L., Elias, A.F., Rosa, P., Samuels, D.S., and Charon, N.W. (2000). *Borrelia burgdorferi* periplasmic flagella have both skeletal and motility functions. Proc Natl Acad Sci U S A *97*, 10899-10904. https://doi.org/10.1073/pnas.200221797

Narita, S., and Tokuda, H. (2011). Overexpression of LolCDE allows deletion of the *Escherichia coli* gene encoding apolipoprotein N-acyltransferase. J Bacteriol *193*, 4832-4840. https://doi.org/10.1128/JB.05013-11

Nascimento, A.L., Ko, A.I., Martins, E.A., Monteiro-Vitorello, C.B., Ho, P.L., Haake, D.A., Verjovski-Almeida, S., Hartskeerl, R.A., Marques, M.V., Oliveira, M.C*., et al.* (2004). Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. J Bacteriol *186*, 2164-2172. https://doi.org/10.1128/jb.186.7.2164-2172.2004

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev *67*, 593-656. https://doi.org/10.1128/mmbr.67.4.593-656.2003

Nilsson, C.L., Cooper, H.J., Hakansson, K., Marshall, A.G., Östberg, Y., Lavrinovicha, M., and Bergström, S. (2002). Characterization of the P13 membrane protein of *Borrelia burgdorferi* by mass spectrometry. J Am Soc Mass Spectrom *13*, 295-299. https://doi.org/10.1016/S1044-0305(01)00365-8

Nixon, P.J., Trost, J.T., and Diner, B.A. (1992). Role of the carboxy terminus of polypeptide D1 in the assembly of a functional water-oxidizing manganese cluster in photosystem II of the cyanobacterium *Synechocystis* sp. PCC 6803: assembly requires a free carboxyl group at C-terminal position 344. Biochemistry *31*, 10859-10871. https://doi.org/10.1021/bi00159a029

Noppa, L., Östberg, Y., Lavrinovicha, M., and Bergström, S. (2001). P13, an integral membrane protein of *Borrelia burgdorferi*, is C-terminally processed and contains surface-exposed domains. Infect Immun *69*, 3323-3334. https://doi.org/10.1128/IAI.69.5.3323-3334.2001

Norman, M.U., Moriarty, T.J., Dresser, A.R., Millen, B., Kubes, P., and Chaconas, G. (2008). Molecular mechanisms involved in vascular interactions of the Lyme disease pathogen in a living host. PLoS Pathog *4*, e1000169. https://doi.org/10.1371/journal.ppat.1000169

Oguchi, Y., Takeda, K., Watanabe, S., Yokota, N., Miki, K., and Tokuda, H. (2008). Opening and closing of the hydrophobic cavity of LolA coupled to lipoprotein binding and release. J Biol Chem *283*, 25414-25420. https://doi.org/10.1074/jbc.M804736200

Ohnishi, J., Piesman, J., and de Silva, A.M. (2001). Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks. Proc Natl Acad Sci U S A *98*, 670-675. https://doi.org/10.1073/pnas.98.2.670

Ojaimi, C., Brooks, C., Casjens, S., Rosa, P., Elias, A., Barbour, A., Jasinskas, A., Benach, J., Katona, L., Radolf, J*., et al.* (2003). Profiling of temperature-induced changes in *Borrelia burgdorferi* gene expression by using whole genome arrays. Infect Immun *71*, 1689-1705. https://doi.org/10.1128/iai.71.4.1689-1705.2003

Ojaimi, C., Mulay, V., Liveris, D., Iyer, R., and Schwartz, I. (2005). Comparative transcriptional profiling of *Borrelia burgdorferi* clinical isolates differing in capacities for hematogenous dissemination. Infect Immun *73*, 6791-6802. https://doi.org/10.1128/IAI.73.10.6791-6802.2005

Okuda, S., and Tokuda, H. (2009). Model of mouth-to-mouth transfer of bacterial lipoproteins through inner membrane LolC, periplasmic LolA,

and outer membrane LolB. Proc Natl Acad Sci U S A *106*, 5877-5882. https://doi.org/10.1073/pnas.0900896106

Okuda, S., and Tokuda, H. (2011). Lipoprotein sorting in bacteria. Annual Reviews in Microbiology *65*, 239-259. https://doi.org/10.1146/annurev-micro-090110-102859

Okuda, S., Watanabe, S., and Tokuda, H. (2008). A short helix in the C-terminal region of LolA is important for the specific membrane localization of lipoproteins. FEBS Lett *582*, 2247-2251. https://doi.org/10.1016/j.febslet.2008.05.022

Onufryk, C., Crouch, M.L., Fang, F.C., and Gross, C.A. (2005). Characterization of six lipoproteins in the sigmaE regulon. J Bacteriol *187*, 4552-4561. https://doi.org/10.1128/JB.187.13.4552-4561.2005

Östberg, Y., Berg, S., Comstedt, P., Wieslander, A., and Bergström, S. (2007). Functional analysis of a lipid galactosyltransferase synthesizing the major envelope lipid in the Lyme disease spirochete *Borrelia burgdorferi*. FEMS Microbiol Lett *272*, 22-29. https://doi.org/10.1111/j.1574-6968.2007.00728.x

Östberg, Y., Carroll, J.A., Pinne, M., Krum, J.G., Rosa, P., and Bergström, S. (2004). Pleiotropic effects of inactivating a carboxyl-terminal protease, CtpA, in *Borrelia burgdorferi*. J Bacteriol *186*, 2074-2084. https://doi.org/10.1128/jb.186.7.2074-2084.2004

Östberg, Y., Pinne, M., Benz, R., Rosa, P., and Bergström, S. (2002). Elimination of channel-forming activity by insertional inactivation of the p13 gene in *Borrelia burgdorferi*. J Bacteriol *184*, 6811-6819. https://doi.org/10.1128/jb.184.24.6811-6819.2002

Pal, U., de Silva, A., Montgomery, R., Fish, D., Anguita, J., Anderson, J., Lobet, Y., and Fikrig, E. (2000). Attachment of *Borrelia burgdorferi* within Ixodes scapularis mediated by outer surface protein A. J Clin Invest *106*, 561-569.

Pal, U., Li, X., Wang, T., Montgomery, R.R., Ramamoorthi, N., Desilva, A.M., Bao, F., Yang, X., Pypaert, M., Pradhan, D*., et al.* (2004a). TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. Cell *119*, 457-468. https://doi.org/10.1016/j.cell.2004.10.027

Pal, U., Yang, X., Chen, M., Bockenstedt, L.K., Anderson, J.F., Flavell, R.A., Norgard, M.V., and Fikrig, E. (2004b). OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands. J Clin Invest *113*, 220-230. https://doi.org/10.1172/JCI19894

Park, S., Liu, G., Topping, T.B., Cover, W.H., and Randall, L.L. (1988). Modulation of folding pathways of exported proteins by the leader sequence. Science *239*, 1033-1035. https://doi.org/10.1126/science.3278378

Parveen, N., Caimano, M., Radolf, J.D., and Leong, J.M. (2003). Adaptation of the Lyme disease spirochaete to the mammalian host environment results in enhanced glycosaminoglycan and host cell binding. Mol Microbiol *47*, 1433-1444. https://doi.org/10.1046/j.1365-2958.2003.03388.x

Parveen, N., and Leong, J.M. (2000). Identification of a candidate glycosaminoglycan-binding adhesin of the Lyme disease spirochete *Borrelia burgdorferi*. Mol Microbiol *35*, 1220-1234. https://doi.org/10.1046/j.1365-2958.2000.01792.x

Paulsen, I.T., Nguyen, L., Sliwinski, M.K., Rabus, R., and Saier, M.H., Jr. (2000). Microbial genome analyses: comparative transport capabilities in eighteen prokaryotes. J Mol Biol *301*, 75-100. https://doi.org/10.1006/jmbi.2000.3961

Payne, J.W., and Gilvarg, C. (1968). Size restriction on peptide utilization in *Escherichia coli*. J Biol Chem *243*, 6291-6299.

Perego, M., Higgins, C.F., Pearce, S.R., Gallagher, M.P., and Hoch, J.A. (1991). The oligopeptide transport system of *Bacillus subtilis* plays a role in the initiation of sporulation. Mol Microbiol *5*, 173-185. https://doi.org/10.1111/j.1365-2958.1991.tb01838.x

Peters, K., Schweizer, I., Beilharz, K., Stahlmann, C., Veening, J.W., Hakenbeck, R., and Denapaite, D. (2014). *Streptococcus pneumoniae* PBP2x mid-cell localization requires the C-terminal PASTA domains and is essential for cell shape maintenance. Mol Microbiol *92*, 733-755. https://doi.org/10.1111/mmi.12588

Pinho, M.G., Kjos, M., and Veening, J.W. (2013). How to get (a)round: mechanisms controlling growth and division of coccoid bacteria. Nat Rev Microbiol *11*, 601-614. https://doi.org/10.1038/nrmicro3088

Pinne, M., Denker, K., Nilsson, E., Benz, R., and Bergström, S. (2006). The BBA01 protein, a member of paralog family 48 from *Borrelia burgdorferi*, is potentially interchangeable with the channel-forming protein P13. J Bacteriol *188*, 4207-4217. https://doi.org/10.1128/JB.00302-06

Pinne, M., Östberg, Y., Comstedt, P., and Bergström, S. (2004). Molecular analysis of the channel-forming protein P13 and its paralogue family 48 from different Lyme disease *Borrelia* species. Microbiology (Reading) *150*, 549-559. https://doi.org/10.1099/mic.0.26728-0

Pinne, M., Thein, M., Denker, K., Benz, R., Coburn, J., and Bergström, S. (2007). Elimination of channel-forming activity by insertional inactivation of the p66 gene in *Borrelia burgdorferi*. FEMS Microbiol Lett *266*, 241-249. https://doi.org/10.1111/j.1574-6968.2006.00529.x

Popova, A.V., and Hincha, D.K. (2011). Thermotropic phase behavior and headgroup interactions of the nonbilayer lipids phosphatidylethanolamine and monogalactosyldiacylglycerol in the dry state. BMC Biophys *4*, 11. https://doi.org/10.1186/2046-1682-4-11

Pozsgay, V., Kubler-Kielb, J., Coxon, B., Marques, A., Robbins, J.B., and Schneerson, R. (2011). Synthesis and antigenicity of BBGL-2 glycolipids of *Borrelia burgdorferi*, the causative agent of Lyme disease. Carbohydr Res *346*, 1551-1563. https://doi.org/10.1016/j.carres.2011.04.045

Probert, W.S., Allsup, K.M., and LeFebvre, R.B. (1995). Identification and characterization of a surface-exposed, 66-kilodalton protein from *Borrelia burgdorferi*. Infect Immun *63*, 1933-1939. https://doi.org/10.1128/IAI.63.5.1933-1939.1995

Probert, W.S., and Johnson, B.J. (1998). Identification of a 47 kDa fibronectin-binding protein expressed by *Borrelia burgdorferi* isolate B31. Mol Microbiol *30*, 1003-1015. https://doi.org/10.1046/j.1365-2958.1998.01127.x

Promnares, K., Kumar, M., Shroder, D.Y., Zhang, X., Anderson, J.F., and Pal, U. (2009). *Borrelia burgdorferi* small lipoprotein Lp6.6 is a member of multiple protein complexes in the outer membrane and facilitates pathogen transmission from ticks to mice. Mol Microbiol *74*, 112-125. https://doi.org/10.1111/j.1365-2958.2009.06853.x

Pugsley, A.P. (1993). The complete general secretory pathway in gram-negative bacteria. Microbiol Rev *57*, 50-108.

Pugsley, A.P., Kornacker, M.G., and Ryter, A. (1990). Analysis of the subcellular location of pullulanase produced by *Escherichia coli* carrying the pulA gene from Klebsiella pneumoniae strain UNF5023. Mol Microbiol *4*, 59-72. https://doi.org/10.1111/j.1365-2958.1990.tb02015.x

Radolf, J.D., Arndt, L.L., Akins, D.R., Curetty, L.L., Levi, M.E., Shen, Y., Davis, L.S., and Norgard, M.V. (1995). *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocytes/macrophages. J Immunol *154*, 2866-2877.

Radolf, J.D., Bourell, K.W., Akins, D.R., Brusca, J.S., and Norgard, M.V. (1994). Analysis of *Borrelia burgdorferi* membrane architecture by freeze-fracture electron microscopy. J Bacteriol *176*, 21-31. https://doi.org/10.1128/jb.176.1.21-31.1994

Radolf, J.D., and Caimano, M.J. (2008). The long strange trip of *Borrelia burgdorferi* outer-surface protein C. Mol Microbiol *69*, 1-4. https://doi.org/10.1111/j.1365-2958.2008.06226.x

Ramamoorthi, N., Narasimhan, S., Pal, U., Bao, F., Yang, X.F., Fish, D., Anguita, J., Norgard, M.V., Kantor, F.S., Anderson, J.F., *et al.* (2005). The Lyme disease agent exploits a tick protein to infect the mammalian host. Nature *436*, 573-577. https://doi.org/10.1038/nature03812

Ramamoorthy, R., and Philipp, M.T. (1998). Differential expression of *Borrelia burgdorferi* proteins during growth in vitro. Infect Immun *66*, 5119-5124. https://doi.org/10.1128/IAI.66.11.5119-5124.1998

Randall, L.L., and Hardy, S.J. (2002). SecB, one small chaperone in the complex milieu of the cell. Cell Mol Life Sci *59*, 1617-1623. https://doi.org/10.1007/pl00012488

Remans, K., Pauwels, K., van Ulsen, P., Buts, L., Cornelis, P., Tommassen, J., Savvides, S.N., Decanniere, K., and Van Gelder, P. (2010). Hydrophobic surface patches on LolA of *Pseudomonas aeruginosa* are essential for lipoprotein binding. J Mol Biol *401*, 921-930. https://doi.org/10.1016/j.jmb.2010.06.067

Ristow, L.C., Bonde, M., Lin, Y.P., Sato, H., Curtis, M., Wesley, E., Hahn, B.L., Fang, J., Wilcox, D.A., Leong, J.M., *et al.* (2015). Integrin binding by *Borrelia burgdorferi* P66 facilitates dissemination but is not required for infectivity. Cell Microbiol *17*, 1021-1036. https://doi.org/10.1111/cmi.12418

Ristow, L.C., Miller, H.E., Padmore, L.J., Chettri, R., Salzman, N., Caimano, M.J., Rosa, P.A., and Coburn, J. (2012). The beta(3)-integrin ligand of *Borrelia burgdorferi* is critical for infection of mice but not ticks. Mol Microbiol *85*, 1105-1118. https://doi.org/10.1111/j.1365-2958.2012.08160.x

Rivera-Milla, E., Stuermer, C.A., and Malaga-Trillo, E. (2006). Ancient origin of reggie (flotillin), reggie-like, and other lipid-raft proteins: convergent evolution of the SPFH domain. Cell Mol Life Sci *63*, 343-357. https://doi.org/10.1007/s00018-005-5434-3

Rizzitello, A.E., Harper, J.R., and Silhavy, T.J. (2001). Genetic evidence for parallel pathways of chaperone activity in the periplasm of *Escherichia coli*. J Bacteriol *183*, 6794-6800. https://doi.org/10.1128/JB.183.23.6794-6800.2001

Robert, V., Volokhina, E.B., Senf, F., Bos, M.P., Van Gelder, P., and Tommassen, J. (2006). Assembly factor Omp85 recognizes its outer membrane protein substrates by a species-specific C-terminal motif. PLoS Biol *4*, e377. https://doi.org/10.1371/journal.pbio.0040377

Rogovskyy, A.S., Casselli, T., Tourand, Y., Jones, C.R., Owen, J.P., Mason, K.L., Scoles, G.A., and Bankhead, T. (2015). Evaluation of the Importance of VlsE Antigenic Variation for the Enzootic Cycle of *Borrelia burgdorferi*. PLoS One *10*, e0124268. https://doi.org/10.1371/journal.pone.0124268

Rudner, D.Z., LeDeaux, J.R., Ireton, K., and Grossman, A.D. (1991). The spo0K locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence. J Bacteriol *173*, 1388-1398. https://doi.org/10.1128/jb.173.4.1388-1398.1991

Russell, T.M., Delorey, M.J., and Johnson, B.J. (2013). *Borrelia burgdorferi* BbHtrA degrades host ECM proteins and stimulates release of inflammatory cytokines in vitro. Mol Microbiol *90*, 241-251. https://doi.org/10.1111/mmi.12377

Russell, T.M., and Johnson, B.J. (2013). Lyme disease spirochaetes possess an aggrecan-binding protease with aggrecanase activity. Mol Microbiol *90*, 228-240. https://doi.org/10.1111/mmi.12276

Sadziene, A., Thomas, D.D., and Barbour, A.G. (1995). *Borrelia burgdorferi* mutant lacking Osp: biological and immunological characterization. Infect Immun *63*, 1573-1580. https://doi.org/10.1128/IAI.63.4.1573-1580.1995

Saier, M.H., Jr., and Paulsen, I.T. (2000). Whole genome analyses of transporters in spirochetes: *Borrelia burgdorferi* and *Treponema pallidum*. J Mol Microbiol Biotechnol *2*, 393-399.

Salah Ud-Din, A.I.M., and Roujeinikova, A. (2017). Methyl-accepting chemotaxis proteins: a core sensing element in prokaryotes and archaea. Cell Mol Life Sci *74*, 3293-3303. https://doi.org/10.1007/s00018-017-2514-0

Samuelson, J.C., Chen, M., Jiang, F., Moller, I., Wiedmann, M., Kuhn, A., Phillips, G.J., and Dalbey, R.E. (2000). YidC mediates membrane protein insertion in bacteria. Nature *406*, 637-641. https://doi.org/10.1038/35020586

Sanchez-Pulido, L., Devos, D., Genevrois, S., Vicente, M., and Valencia, A. (2003). POTRA: a conserved domain in the FtsQ family and a class of beta-barrel outer membrane proteins. Trends Biochem Sci *28*, 523-526. https://doi.org/10.1016/j.tibs.2003.08.003

Sankaran, K., Gupta, S.D., and Wu, H.C. (1995). Modification of bacterial lipoproteins. Methods Enzymol *250*, 683-697. https://doi.org/10.1016/0076-6879(95)50105-3

Sauvonnet, N., and Pugsley, A.P. (1996). Identification of two regions of *Klebsiella oxytoca* pullulanase that together are capable of promoting beta-lactamase secretion by the general secretory pathway. Mol Microbiol *22*, 1-7. https://doi.org/10.1111/j.1365-2958.1996.tb02650.x

Schaub, R.E., Chan, Y.A., Lee, M., Hesek, D., Mobashery, S., and Dillard, J.P. (2016). Lytic transglycosylases LtgA and LtgD perform distinct roles in remodeling, recycling and releasing peptidoglycan in *Neisseria gonorrhoeae*. Mol Microbiol *102*, 865-881. https://doi.org/10.1111/mmi.13496

Schroder, N.W., Schombel, U., Heine, H., Gobel, U.B., Zahringer, U., and Schumann, R.R. (2003). Acylated cholesteryl galactoside as a novel immunogenic motif in *Borrelia burgdorferi* sensu stricto. J Biol Chem *278*, 33645-33653. https://doi.org/10.1074/jbc.M305799200

Schulze, R.J., Chen, S., Kumru, O.S., and Zückert, W.R. (2010). Translocation of *Borrelia burgdorferi* surface lipoprotein OspA through the outer membrane requires an unfolded conformation and can initiate at the C-terminus. Mol Microbiol *76*, 1266-1278. https://doi.org/10.1111/j.1365-2958.2010.07172.x

Schulze, R.J., and Zückert, W.R. (2006). *Borrelia burgdorferi* lipoproteins are secreted to the outer surface by default. Mol Microbiol *59*, 1473-1484. https://doi.org/10.1111/j.1365-2958.2006.05039.x

Schwan, T., Battisti, J., Porcella, S., Raffel, S., Schrumpf, M., Fischer, E., Carroll, J., Stewart, P., Rosa, P., and Somerville, G. (2003). Glycerol-3-phosphate acquisition in spirochetes: distribution and biological activity of glycerophosphodiester phosphodiesterase (GlpQ) among *Borrelia* species. J Bacteriol *185*, 1346-1356.

Schwan, T.G., and Piesman, J. (2000). Temporal changes in outer surface proteins A and C of the lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. J Clin Microbiol *38*, 382-388.

Schwan, T.G., Piesman, J., Golde, W.T., Dolan, M.C., and Rosa, P.A. (1995). Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. Proc Natl Acad Sci U S A *92*, 2909-2913. https://doi.org/10.1073/pnas.92.7.2909

Seinost, G., Dykhuizen, D.E., Dattwyler, R.J., Golde, W.T., Dunn, J.J., Wang, I.N., Wormser, G.P., Schriefer, M.E., and Luft, B.J. (1999). Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans. Infect Immun *67*, 3518-3524. https://doi.org/10.1128/IAI.67.7.3518-3524.1999

Seshadri, R., Myers, G.S., Tettelin, H., Eisen, J.A., Heidelberg, J.F., Dodson, R.J., Davidsen, T.M., DeBoy, R.T., Fouts, D.E., Haft, D.H., *et al.* (2004). Comparison of the genome of the oral pathogen *Treponema denticola* with other spirochete genomes. Proc Natl Acad Sci U S A *101*, 5646-5651. https://doi.org/10.1073/pnas.0307639101

Setubal, J.C., Reis, M., Matsunaga, J., and Haake, D.A. (2006). Lipoprotein computational prediction in spirochaetal genomes. Microbiology (Reading) *152*, 113-121. https://doi.org/10.1099/mic.0.28317-0

Seydel, A., Gounon, P., and Pugsley, A. (1999). Testing the '+2 rule' for lipoprotein sorting in the *Escherichia coli* cell envelope with a new genetic selection. Mol Microbiol *34*, 810-821.

Shang, E.S., Skare, J.T., Exner, M.M., Blanco, D.R., Kagan, B.L., Miller, J.N., and Lovett, M.A. (1998). Isolation and characterization of the outer membrane of *Borrelia hermsii*. Infect Immun *66*, 1082-1091. https://doi.org/10.1128/IAI.66.3.1082-1091.1998

Silva-Herzog, E., Ferracci, F., Jackson, M.W., Joseph, S.S., and Plano, G.V. (2008). Membrane localization and topology of the *Yersinia pestis* YscJ lipoprotein. Microbiology (Reading) *154*, 593-607. https://doi.org/10.1099/mic.0.2007/013045-0

Simons, K., and Ehehalt, R. (2002). Cholesterol, lipid rafts, and disease. J Clin Invest *110*, 597-603. https://doi.org/10.1172/JCI16390

Sinha, R.K., and Rosenthal, R.S. (1980). Release of soluble peptidoglycan from growing conococci: demonstration of anhydro-muramyl-containing fragments. Infect Immun *29*, 914-925.

Skare, J.T., Mirzabekov, T.A., Shang, E.S., Blanco, D.R., Erdjument-Bromage, H., Bunikis, J., Bergström, S., Tempst, P., Kagan, B.L., Miller, J.N.*, et al.* (1997). The Oms66 (p66) protein is a *Borrelia burgdorferi* porin. Infect Immun *65*, 3654-3661. https://doi.org/10.1128/IAI.65.9.3654-3661.1997

Skare, J.T., Shang, E.S., Foley, D.M., Blanco, D.R., Champion, C.I., Mirzabekov, T., Sokolov, Y., Kagan, B.L., Miller, J.N., and Lovett, M.A. (1995). Virulent strain associated outer membrane proteins of *Borrelia burgdorferi*. J Clin Invest *96*, 2380-2392. https://doi.org/10.1172/JCI118295

Sklar, J.G., Wu, T., Kahne, D., and Silhavy, T.J. (2007). Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. Genes Dev *21*, 2473-2484. https://doi.org/10.1101/gad.1581007

Slamti, L., de Pedro, M.A., Guichet, E., and Picardeau, M. (2011). Deciphering morphological determinants of the helix-shaped *Leptospira*. J Bacteriol *193*, 6266-6275. https://doi.org/10.1128/JB.05695-11

Sohlenkamp, C., and Geiger, O. (2016). Bacterial membrane lipids: diversity in structures and pathways. FEMS Microbiol Rev *40*, 133-159. https://doi.org/10.1093/femsre/fuv008

Sohlenkamp, C., Lopez-Lara, I.M., and Geiger, O. (2003). Biosynthesis of phosphatidylcholine in bacteria. Prog Lipid Res *42*, 115-162. https://doi.org/10.1016/s0163-7827(02)00050-4

Stevenson, B., El-Hage, N., Hines, M.A., Miller, J.C., and Babb, K. (2002). Differential binding of host complement inhibitor factor H by *Borrelia burgdorferi* Erp surface proteins: a possible mechanism underlying the expansive host range of Lyme disease spirochetes. Infect Immun *70*, 491-497. https://doi.org/10.1128/iai.70.2.491-497.2002

Stewart, P.E., Hoff, J., Fischer, E., Krum, J.G., and Rosa, P.A. (2004). Genome-wide transposon mutagenesis of *Borrelia burgdorferi* for identification of phenotypic mutants. Appl Environ Microbiol *70*, 5973-5979. https://doi.org/10.1128/AEM.70.10.5973-5979.2004

Strobl, S., Gomis-Ruth, F.X., Maskos, K., Frank, G., Huber, R., and Glockshuber, R. (1997). The alpha-amylase from the yellow meal worm: complete primary structure, crystallization and preliminary X-ray analysis. FEBS Lett *409*, 109-114. https://doi.org/10.1016/s0014-5793(97)00451-1

Strominger, J.L., Ito, E., and Threnn, R.H. (1960). Competitive inhibition of enzymatic reactions by oxamycin J Am Chem Soc *82*, 998-999.

Stubenrauch, C., Grinter, R., and Lithgow, T. (2016). The modular nature of the beta-barrel assembly machinery, illustrated in *Borrelia burgdorferi*. Mol Microbiol *102*, 753-756. https://doi.org/10.1111/mmi.13527

Stubs, G., Fingerle, V., Wilske, B., Gobel, U.B., Zahringer, U., Schumann, R.R., and Schroder, N.W. (2009). Acylated cholesteryl galactosides are specific antigens of *Borrelia* causing lyme disease and frequently induce antibodies in late stages of disease. J Biol Chem *284*, 13326-13334. https://doi.org/10.1074/jbc.M809575200

Stubs, G., Fingerle, V., Zahringer, U., Schumann, R.R., Rademann, J., and Schroder, N.W. (2011). Acylated cholesteryl galactosides are ubiquitous glycolipid antigens among *Borrelia burgdorferi* sensu lato. FEMS Immunol Med Microbiol *63*, 140-143. https://doi.org/10.1111/j.1574-695X.2011.00827.x

Tajima, T., Yokota, N., Matsuyama, S., and Tokuda, H. (1998). Genetic analyses of the in vivo function of LolA, a periplasmic chaperone involved in the outer membrane localization of *Escherichia coli* lipoproteins. FEBS Lett *439*, 51-54. https://doi.org/10.1016/s0014-5793(98)01334-9

Takayama, K., Rothenberg, R.J., and Barbour, A.G. (1987). Absence of lipopolysaccharide in the Lyme disease spirochete, *Borrelia burgdorferi*. Infect Immun *55*, 2311-2313. https://doi.org/10.1128/IAI.55.9.2311-2313.1987

Takeda, K., Miyatake, H., Yokota, N., Matsuyama, S., Tokuda, H., and Miki, K. (2003). Crystal structures of bacterial lipoprotein localization factors, LolA and LolB. EMBO J *22*, 3199-3209. https://doi.org/10.1093/emboj/cdg324

Tanaka, K., Matsuyama, S., and Tokuda, H. (2001). Deletion of *lolB*, encoding an outer membrane lipoprotein, is lethal for *Escherichia coli* and causes accumulation of lipoprotein localization intermediates in the periplasm. J Bacteriol *183*, 6538-6542.

Taniguchi, N., Matsuyama, S., and Tokuda, H. (2005). Mechanisms underlying energy-independent transfer of lipoproteins from LolA to LolB, which have similar unclosed beta-barrel structures. J Biol Chem *280*, 34481-34488.

Tao, K., Watanabe, S., Narita, S., and Tokuda, H. (2010). A periplasmic LolA derivative with a lethal disulfide bond activates the Cpx stress response system. J Bacteriol *192*, 5657-5662. https://doi.org/10.1128/JB.00821-10

Terada, M., Kuroda, T., Matsuyama, S., and Tokuda, H. (2001). Lipoprotein sorting signals evaluated as the LolA-dependent release of lipoproteins from the cytoplasmic membrane of *Escherichia coli*. J Biol Chem *276*, 47690-47694.

Thanbichler, M., and Shapiro, L. (2006). MipZ, a spatial regulator coordinating chromosome segregation with cell division in *Caulobacter*. Cell *126*, 147-162. https://doi.org/10.1016/j.cell.2006.05.038

Thein, M., Bonde, M., Bunikis, I., Denker, K., Sickmann, A., Bergström, S., and Benz, R. (2012). DipA, a pore-forming protein in the outer membrane of Lyme disease spirochetes exhibits specificity for the permeation of dicarboxylates. PLoS One *7*, e36523. https://doi.org/10.1371/journal.pone.0036523

Thein, M., Bunikis, I., Denker, K., Larsson, C., Cutler, S., Drancourt, M., Schwan, T.G., Mentele, R., Lottspeich, F., Bergström, S.*, et al.* (2008). Oms38 is the first identified pore-forming protein in the outer membrane of relapsing fever spirochetes. J Bacteriol *190*, 7035-7042. https://doi.org/10.1128/JB.00818-08

Tilly, K., Bestor, A., and Rosa, P.A. (2013). Lipoprotein succession in *Borrelia burgdorferi*: similar but distinct roles for OspC and VlsE at different stages of mammalian infection. Mol Microbiol *89*, 216-227. https://doi.org/10.1111/mmi.12271

Tilly, K., Bestor, A., and Rosa, P.A. (2016). Functional Equivalence of OspA and OspB, but Not OspC, in Tick Colonization by *Borrelia burgdorferi*. Infect Immun *84*, 1565-1573. https://doi.org/10.1128/IAI.00063-16

Tilly, K., Elias, A.F., Errett, J., Fischer, E., Iyer, R., Schwartz, I., Bono, J.L., and Rosa, P. (2001). Genetics and regulation of chitobiose utilization in *Borrelia burgdorferi*. J Bacteriol *183*, 5544-5553. https://doi.org/10.1128/JB.183.19.5544-5553.2001

Tilly, K., Grimm, D., Bueschel, D.M., Krum, J.G., and Rosa, P. (2004). Infectious cycle analysis of a *Borrelia burgdorferi* mutant defective in transport of chitobiose, a tick cuticle component. Vector Borne Zoonotic Dis *4*, 159-168. https://doi.org/10.1089/1530366041210738

Tilly, K., Krum, J.G., Bestor, A., Jewett, M.W., Grimm, D., Bueschel, D., Byram, R., Dorward, D., Vanraden, M.J., Stewart, P.*, et al.* (2006). *Borrelia burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian infection. Infect Immun *74*, 3554-3564. https://doi.org/10.1128/IAI.01950-05

Tokuda, H., and Matsuyama, S. (2004). Sorting of lipoproteins to the outer membrane in E. coli. Biochim Biophys Acta *1694*, IN1-9.

Toledo, A., Crowley, J.T., Coleman, J.L., LaRocca, T.J., Chiantia, S., London, E., and Benach, J.L. (2014). Selective association of outer surface lipoproteins with the lipid rafts of *Borrelia burgdorferi*. mBio *5*, e00899-00814. https://doi.org/10.1128/mBio.00899-14

Toledo, A., Huang, Z., Benach, J.L., and London, E. (2018a). Analysis of Lipids and Lipid Rafts in *Borrelia*. Methods Mol Biol *1690*, 69-82. https://doi.org/10.1007/978-1-4939-7383-5_6

Toledo, A., Huang, Z., Coleman, J.L., London, E., and Benach, J.L. (2018b). Lipid rafts can form in the inner and outer membranes of *Borrelia burgdorferi* and have different properties and associated proteins. Mol Microbiol *108*, 63-76. https://doi.org/10.1111/mmi.13914

Toledo, A., Perez, A., Coleman, J.L., and Benach, J.L. (2015). The lipid raft proteome of *Borrelia burgdorferi*. Proteomics *15*, 3662-3675. https://doi.org/10.1002/pmic.201500093

Treuner-Lange, A., Aguiluz, K., van der Does, C., Gomez-Santos, N., Harms, A., Schumacher, D., Lenz, P., Hoppert, M., Kahnt, J., Munoz-Dorado, J., *et al.* (2013). PomZ, a ParA-like protein, regulates Z-ring formation and cell division in *Myxococcus xanthus*. Mol Microbiol *87*, 235-253. https://doi.org/10.1111/mmi.12094

Tsui, H.T., Boersma, M.J., Vella, S.A., Kocaoglu, O., Kuru, E., Peceny, J.K., Carlson, E.E., VanNieuwenhze, M.S., Brun, Y.V., Shaw, S.L., *et al.* (2014). Pbp2x localizes separately from Pbp2b and other peptidoglycan synthesis proteins during later stages of cell division of *Streptococcus pneumoniae* D39. Mol Microbiol *94*, 21-40. https://doi.org/10.1111/mmi.12745

Tsukahara, J., Mukaiyama, K., Okuda, S., Narita, S., and Tokuda, H. (2009). Dissection of LolB function--lipoprotein binding, membrane targeting and incorporation of lipoproteins into lipid bilayers. FEBS J *276*, 4496-4504. https://doi.org/10.1111/j.1742-4658.2009.07156.x

Turner, R.D., Vollmer, W., and Foster, S.J. (2014). Different walls for rods and balls: the diversity of peptidoglycan. Mol Microbiol *91*, 862-874. https://doi.org/10.1111/mmi.12513

Twibanire, J.D., Omran, R.P., and Grindley, T.B. (2012). Facile synthesis of a library of Lyme disease glycolipid antigens. Org Lett *14*, 3909-3911. https://doi.org/10.1021/ol301697c

van Ulsen, P., van Alphen, L., ten Hove, J., Fransen, F., van der Ley, P., and Tommassen, J. (2003). A Neisserial autotransporter NalP modulating the processing of other autotransporters. Mol Microbiol *50*, 1017-1030. https://doi.org/10.1046/j.1365-2958.2003.03773.x

Verwer, R.W., Nanninga, N., Keck, W., and Schwarz, U. (1978). Arrangement of glycan chains in the sacculus of *Escherichia coli*. J Bacteriol *136*, 723-729. https://doi.org/10.1128/JB.136.2.723-729.1978

Viala, J., Chaput, C., Boneca, I.G., Cardona, A., Girardin, S.E., Moran, A.P., Athman, R., Memet, S., Huerre, M.R., Coyle, A.J., *et al.* (2004). Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori cag* pathogenicity island. Nat Immunol *5*, 1166-1174. https://doi.org/10.1038/ni1131

Vollmer, W. (2008). Structural variation in the glycan strands of bacterial peptidoglycan. FEMS Microbiol Rev *32*, 287-306. https://doi.org/10.1111/j.1574-6976.2007.00088.x

Vollmer, W., Blanot, D., and de Pedro, M.A. (2008). Peptidoglycan structure and architecture. FEMS Microbiol Rev *32*, 149-167. https://doi.org/10.1111/j.1574-6976.2007.00094.x

Vollmer, W., Massidda, O., and Tomasz, A. (2019). The Cell Wall of *Streptococcus pneumoniae*. Microbiol Spectr *7*. https://doi.org/10.1128/microbiolspec.GPP3-0018-2018

Vollmer, W., and Seligman, S.J. (2010). Architecture of peptidoglycan: more data and more models. Trends Microbiol *18*, 59-66. https://doi.org/10.1016/j.tim.2009.12.004

von Lackum, K., and Stevenson, B. (2005). Carbohydrate utilization by the Lyme borreliosis spirochete, *Borrelia burgdorferi*. FEMS Microbiol Lett *243*, 173-179. https://doi.org/10.1016/j.femsle.2004.12.002

Voulhoux, R., Bos, M.P., Geurtsen, J., Mols, M., and Tommassen, J. (2003). Role of a highly conserved bacterial protein in outer membrane protein assembly. Science *299*, 262-265. https://doi.org/10.1126/science.1078973

Walker, E.M., Borenstein, L.A., Blanco, D.R., Miller, J.N., and Lovett, M.A. (1991). Analysis of outer membrane ultrastructure of pathogenic *Treponema* and *Borrelia* species by freeze-fracture electron microscopy. J Bacteriol *173*, 5585-5588. https://doi.org/10.1128/jb.173.17.5585-5588.1991

Wang, G., van Dam, A.P., and Dankert, J. (1999). Evidence for frequent OspC gene transfer between *Borrelia valaisiana sp. nov.* and other Lyme disease spirochetes. FEMS Microbiol Lett *177*, 289-296. https://doi.org/10.1111/j.1574-6968.1999.tb13745.x

Wang, X.G., Kidder, J.M., Scagliotti, J.P., Klempner, M.S., Noring, R., and Hu, L.T. (2004a). Analysis of differences in the functional properties of the substrate binding proteins of the *Borrelia burgdorferi* oligopeptide permease (Opp) operon. J Bacteriol *186*, 51-60. https://doi.org/10.1128/jb.186.1.51-60.2004

Wang, X.G., Lin, B., Kidder, J.M., Telford, S., and Hu, L.T. (2002). Effects of environmental changes on expression of the oligopeptide permease (*opp*) genes of *Borrelia burgdorferi*. J Bacteriol *184*, 6198-6206. https://doi.org/10.1128/jb.184.22.6198-6206.2002

Wang, X.G., Scagliotti, J.P., and Hu, L.T. (2004b). Phospholipid synthesis in *Borrelia burgdorferi*: BB0249 and BB0721 encode functional phosphatidylcholine synthase and phosphatidyl-glycerolphosphate synthase proteins. Microbiology (Reading) *150*, 391-397. https://doi.org/10.1099/mic.0.26752-0

Watanabe, S., Oguchi, Y., Takeda, K., Miki, K., and Tokuda, H. (2008). Introduction of a lethal redox switch that controls the opening and closing of the hydrophobic cavity in LolA. J Biol Chem *283*, 25421-25427. https://doi.org/10.1074/jbc.M804737200

Wessel, M., Klusener, S., Godeke, J., Fritz, C., Hacker, S., and Narberhaus, F. (2006). Virulence of *Agrobacterium tumefaciens* requires phosphatidylcholine in the bacterial membrane. Mol Microbiol *62*, 906-915. https://doi.org/10.1111/j.1365-2958.2006.05425.x

Wheeler, C.M., Garcia Monco, J.C., Benach, J.L., Golightly, M.G., Habicht, G.S., and Steere, A.C. (1993). Nonprotein antigens of *Borrelia burgdorferi*. Journal of Infectious Diseases *167*, 665-674. https://doi.org/10.1093/infdis/167.3.665

Willemse, J., Borst, J.W., de Waal, E., Bisseling, T., and van Wezel, G.P. (2011). Positive control of cell division: FtsZ is recruited by SsgB during sporulation of *Streptomyces*. Genes Dev *25*, 89-99. https://doi.org/10.1101/gad.600211

Wilson, M.M., Anderson, D.E., and Bernstein, H.D. (2015). Analysis of the outer membrane proteome and secretome of *Bacteroides fragilis* reveals a multiplicity of secretion mechanisms. PLoS One *10*, e0117732. https://doi.org/10.1371/journal.pone.0117732

Wolgemuth, C.W., Charon, N.W., Goldstein, S.F., and Goldstein, R.E. (2006). The flagellar cytoskeleton of the spirochetes. J Mol Microbiol Biotechnol *11*, 221-227. https://doi.org/10.1159/000094056

Wu, L.J., and Errington, J. (2004). Coordination of cell division and chromosome segregation by a nucleoid occlusion protein in *Bacillus subtilis*. Cell *117*, 915-925. https://doi.org/10.1016/j.cell.2004.06.002

Wu, X., Sharma, B., Niles, S., O'Connor, K., Schilling, R., Matluck, N., D'Onofrio, A., Hu, L.T., and Lewis, K. (2018). Identifying Vancomycin as an Effective Antibiotic for Killing *Borrelia burgdorferi*. Antimicrob Agents Chemother *62*. https://doi.org/10.1128/AAC.01201-18

Xie, K., and Dalbey, R.E. (2008). Inserting proteins into the bacterial cytoplasmic membrane using the Sec and YidC translocases. Nat Rev Microbiol *6*, 234-244. https://doi.org/10.1038/nrmicro3595

Yakushi, T., Masuda, K., Narita, S., Matsuyama, S., and Tokuda, H. (2000). A new ABC transporter mediating the detachment of lipid-modified proteins from membranes. Nat Cell Biol *2*, 212-218. https://doi.org/10.1038/35008635

Yakushi, T., Yokota, N., Matsuyama, S., and Tokuda, H. (1998). LolA-dependent release of a lipid-modified protein from the inner membrane of *Escherichia coli* requires nucleoside triphosphate. J Biol Chem *273*, 32576-32581. https://doi.org/10.1074/jbc.273.49.32576

Yamaguchi, K., Yu, F., and Inouye, M. (1988). A single amino acid determinant of the membrane localization of lipoproteins in *E. coli*. Cell *53*, 423-432. https://doi.org/10.1016/0092-8674(88)90162-6

Yanagihara, Y., Kamisango, K., Yasuda, S., Kobayashi, S., Mifuchi, I., Azuma, I., Yamamura, Y., and Johnson, R.C. (1984). Chemical compositions of cell walls and polysaccharide fractions of spirochetes. Microbiol Immunol *28*, 535-544. https://doi.org/10.1111/j.1348-0421.1984.tb00706.x

Yang, X., Goldberg, M.S., Popova, T.G., Schoeler, G.B., Wikel, S.K., Hagman, K.E., and Norgard, M.V. (2000). Interdependence of environmental factors influencing reciprocal patterns of gene expression in virulent *Borrelia burgdorferi*. Mol Microbiol *37*, 1470-1479. https://doi.org/10.1046/j.1365-2958.2000.02104.x

Yang, X., Lyu, Z., Miguel, A., McQuillen, R., Huang, K.C., and Xiao, J. (2017). GTPase activity-coupled treadmilling of the bacterial tubulin FtsZ organizes septal cell wall synthesis. Science *355*, 744-747. https://doi.org/10.1126/science.aak9995

Yang, X.F., Pal, U., Alani, S.M., Fikrig, E., and Norgard, M.V. (2004). Essential role for OspA/B in the life cycle of the Lyme disease spirochete. J Exp Med *199*, 641-648. https://doi.org/10.1084/jem.20031960

Yasuda, M., Iguchi-Yokoyama, A., Matsuyama, S., Tokuda, H., and Narita, S. (2009). Membrane topology and functional importance of the periplasmic region of ABC transporter LolCDE. Biosci Biotechnol Biochem *73*, 2310-2316. https://doi.org/10.1271/bbb.90451

Yen, M.R., Peabody, C.R., Partovi, S.M., Zhai, Y., Tseng, Y.H., and Saier, M.H. (2002). Protein-translocating outer membrane porins of Gram-negative bacteria. Biochim Biophys Acta *1562*, 6-31. https://doi.org/10.1016/s0005-2736(02)00359-0

Yokota, N., Kuroda, T., Matsuyama, S., and Tokuda, H. (1999). Characterization of the LolA-LolB system as the general lipoprotein localization mechanism of *Escherichia coli*. J Biol Chem *274*, 30995-30999.

Young, K.D. (2006). The selective value of bacterial shape. Microbiol Mol Biol Rev *70*, 660-703. https://doi.org/10.1128/MMBR.00001-06

Zhang, J.R., Hardham, J.M., Barbour, A.G., and Norris, S.J. (1997). Antigenic variation in Lyme disease borreliae by promiscuous recombination of VMP-like sequence cassettes. Cell *89*, 275-285. https://doi.org/10.1016/s0092-8674(00)80206-8

Zhang, J.R., and Norris, S.J. (1998a). Genetic variation of the *Borrelia burgdorferi* gene *vlsE* involves cassette-specific, segmental gene conversion. Infect Immun *66*, 3698-3704. https://doi.org/10.1128/IAI.66.8.3698-3704.1998

Zhang, J.R., and Norris, S.J. (1998b). Kinetics and in vivo induction of genetic variation of vlsE in *Borrelia burgdorferi*. Infect Immun *66*, 3689-3697. https://doi.org/10.1128/IAI.66.8.3689-3697.1998

Zhang, K., Qin, Z., Chang, Y., Liu, J., Malkowski, M.G., Shipa, S., Li, L., Qiu, W., Zhang, J.R., and Li, C. (2019). Analysis of a flagellar filament cap mutant reveals that HtrA serine protease degrades unfolded flagellin protein in the periplasm of *Borrelia burgdorferi*. Mol Microbiol *111*, 1652-1670. https://doi.org/10.1111/mmi.14243

Zhang, Q.Y., DeRyckere, D., Lauer, P., and Koomey, M. (1992). Gene conversion in *Neisseria gonorrhoeae*: evidence for its role in pilus antigenic variation. Proc Natl Acad Sci U S A *89*, 5366-5370. https://doi.org/10.1073/pnas.89.12.5366

Zückert, W.R. (2013). A call to order at the spirochaetal host-pathogen interface. Mol Microbiol *89*, 207-211. https://doi.org/10.1111/mmi.12286

Zückert, W.R. (2014). Secretion of bacterial lipoproteins: through the cytoplasmic membrane, the periplasm and beyond. Biochim Biophys Acta *1843*, 1509-1516. https://doi.org/10.1016/j.bbamcr.2014.04.022

Zückert, W.R. (2019). Protein Secretion in Spirochetes. Microbiol Spectr *7*. https://doi.org/10.1128/microbiolspec.PSIB-0026-2019

Zückert, W.R., Kerentseva, T.A., Lawson, C.L., and Barbour, A.G. (2001). Structural conservation of neurotropism-associated VspA within the variable *Borrelia* Vsp-OspC lipoprotein family. J Biol Chem *276*, 457-463. https://doi.org/10.1074/jbc.M008449200

Zückert, W.R., Lloyd, J.E., Stewart, P.E., Rosa, P.A., and Barbour, A.G. (2004). Cross-species surface display of functional spirochetal lipoproteins by recombinant *Borrelia burgdorferi*. Infect Immun *72*, 1463-1469. https://doi.org/10.1128/iai.72.3.1463-1469.2004

Zückert, W.R., Meyer, J., and Barbour, A.G. (1999). Comparative analysis and immunological characterization of the *Borrelia* Bdr protein family. Infect Immun *67*, 3257-3266. https://doi.org/10.1128/IAI.67.7.3257-3266.1999