ABSTRACT

Title of Dissertation: **MOLECULAR DISSECTION OF *BORRELIA BURGDORFERI* BB0323 PROTEIN COMPLEX SUPPORTING MICROBIAL BIOLOGY, INFECTIVITY, AND AS A NOVEL THERAPEUTIC TARGET**

Sandhya Bista**,** Doctor of Philosophy, 2023

Dissertation directed by: Professor Dr. Utpal Pal,
Department of Veterinary Medicine

Lyme disease (LD), also known as Lyme borreliosis, is the most common vector-borne disease in the United States, caused by the gram-negative bacteria of the *Borrelia burgdorferi* sensu *lato* group. This atypical bacterial group features distinct genomic and antigenic elements, does not possess any classical toxins, and the pathogenesis of LD is primarily due to the immune activity of the host. These multi-organotrophic spirochetes can elicit severe clinical complications in susceptible hosts, including neuroborreliosis, carditis, and arthritis. If diagnosed early, the disease can be treated with a conventional antibiotic regimen; however, persistent, or relapsing symptoms later develop in a subset

of patients. Six months to a year after the antibiotic treatment, up to 20% of the patients can experience various subjective symptoms pertaining to pain, cognitive dysfunction, or other neurological complications, collectively termed Post Treatment Lyme Disease Syndrome (PTLDS). The diagnosis, etiology, and treatment of PTLDS remain currently unknown. To better understand microbial pathogenesis, we have characterized a select set of structurally unique spirochete gene products that act as novel virulence determinants and support microbial infection in mammals. The current study focused on the BB0323 protein of *B. burgdorferi*, a unique and multifunctional virulence determinant undergoing a complex post-translational maturation process. The maturation, stability, and functions of BB0323 require multifaceted protein-protein interaction (PPI) events involving specific *B. burgdorferi* proteins, such as a protease-chaperone called BbHtrA, and a membrane-associated protein of unknown function annotated as BB0238. In our current study, we have further dissected the biological significances of the protein-protein interaction complex (PPI), either involving BbHtrA: BB0323 and BB0323:BB0238. The latter PPI event was more thoroughly investigated for its role in spirochete biology and infection and as a novel target for therapeutic intervention against *B. burgdorferi* infection. We identified a cleavage site where BB0323 full-length protein cleaves into N and C termini by BbHtrA. Subsequently, we have introduced point mutations in the recombinant BB0323 (at the cleavage site for BbHtrA- NL residues replaced with AA), as well as generated an isogenic *B. burgdorferi* isolates ($Bb^{bb0323NL}$) with the point mutations in native BB0323. Further analyses show that the cleavage site mutated BB0323 protein could not be processed by the recombinant BbHtrA. Notably, despite the inability of BbHtrA to process BB0323 *in vitro*, within $Bb^{bb0323NL}$, BB0323

could indeed be processed to some degree, which yields a basal level of mature N-terminal protein. Notably, in these *B. burgdorferi* cells, at least two other BB0323 polypeptides of lower molecular weight (less than 27 kDa of mature N-term BB0323) were also produced, possibly due to the action of BbHtrA on non-specific sites. However, the *Bb*^{bb0323NL} mutants were non-infectious in the murine host, demonstrating the importance of precise cleavage of BB0323 full-length protein and optimal production of N-terminal, which needed to form a complex with another PPI partner, BB0238. Overall, these results further underscored the event of BbHtrA and BB0323 interaction for processing the latter protein as an essential prerequisite for spirochete infection in mammals. Our previous studies have shown that BB0323 N-terminal and BB0238 interact and post-translationally stabilize each other. We used an interaction-deficient borrelial mutant, replacing the BB0323 interaction motif in BB238 (termed as *bb0238* Delta Interaction Motif, or *bb0238*Δ*IM*), which despite showing no growth defects *in vitro* or other abnormalities, is unable to infect mammalian host. We, therefore, explored the possibility of using the BB0323:BB0238 complex as a novel therapeutic target to combat *B. burgdorferi* infection in mammals. We first examined whether *bb0238*Δ*IM* mutants (without interaction motifs) can persist in mice for a long term or could be acquired by naïve ticks. The results show that, unlike the wild type or another *B. burgdorferi* mutant, The *bb0238*Δ*IM* could not establish the infection in mice and, as a result, could not be acquired by the ticks, suggesting blockade of BB0323:BB0238 interaction by small molecules could be a novel therapeutic approach to combat incidence of LD. An AlphaLisa assay platform was developed in our lab to monitor BB0323-BB0238 PPI on a high-throughput basis using 384-well microtiter plates, which was then

miniaturized to 1536 well at the National Center for Advancing Translational Sciences (NCATS) in a collaborative effort. An AlphaLisa quantitative HTS later screened several small molecule libraries available at NCATS, which were further filtered by counter assays, and a selected set of 84 compounds was tested in a secondary, cell-based assay for cell-permeable compounds that impair BB0323-BB0238 interaction with spirochete cells. A *B. burgdorferi* cell-based assay comprising a dot-blot assay and regrowth assay was developed to examine the PPI inhibitory activities of the molecules inside the cells. We finally selected one of the compounds, Lomibuvir, for the *in vivo* studies and demonstrated its PPI inhibitory activity in an *in vitro* experiment. A pharmacokinetic study in mice showed an increase in the level of the compound in plasma and liver over 21 days. Additional *in vivo* efficacy studies of Lomibuvir to reduce *B. burgdorferi* infection in mice were performed using vehicle and ceftriaxone as negative and positive controls, respectively. The results showed that the bacterial load in the skin and heart of the mice was significantly lower in the Lomibuvir-treated group, as compared to the vehicle-treated animals; however, the effect was not as dramatically effective as the antibiotic (ceftriaxone) treatment groups. While future medicinal chemistry approaches could be adopted to further enhance the impact of Lomibuvir as an anti-*B. burgdorferi* agent, to the best of knowledge, is the first proof-of-concept study that highlights the utility of targeting borrelial PPI events as a possible therapeutic target of Lyme disease.

MOLECULAR DISSECTION OF *BORRELIA BURGDORFERI* BB0323 PROTEIN COMPLEX SUPPORTING MICROBIAL BIOLOGY, INFECTIVITY, AND AS A NOVEL THERAPEUTIC TARGET

by

Sandhya Bista

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2023

Advisory Committee:
Professor Dr. Utpal Pal, Chair
Professor Dr. Zhengguo Xiao, Dean's Rep
Professor Dr. Daniel Nelson
Associate Professor Dr. George Belov
Special member Dr. Bolormaa Baljinnyam

Acknowledgments

I want to express my heartfelt gratitude to all those who have helped me complete this dissertation. First and foremost, I am deeply grateful to my advisor Dr. Utpal Pal for the valuable insight, support, guidance, and encouragement. Your passion for science has been a tremendous source of inspiration for me to pursue a research career in the future. Your constructive critiques and feedback helped me refine my techniques and produce finer results.

I would also like to thank my advisory committee members, Dr. Zhengguo Xiao (Dean's Rep), Dr. Daniel Nelson, Dr. George Belov, and Dr. Bolormaa Baljinnyam, for their time and valuable feedback, which helped to shape this research.

I am incredibly grateful to Dr. Xiuli Yang and Dr. Meghna Thakur for providing guidance. Their unwavering patience and helpful suggestions helped me overcome the moments of self-doubt and uncertainty.

I feel incredibly fortunate to get an opportunity to work with Dr. Quentin Bernard. I am also very grateful to our postdoctoral research scientist Dr. Vipin Singh Rana, for being the source of knowledge. Thank you for walking me through the protocols and experiments and clearing my doubts emerging from my scientific curiosities.

To Dr. Shraboni Dutta and Dr. Shelby D. Foor, amazing colleagues I was blessed with and who were always there with my highs and lows. Your words of encouragement and emotional support helped me stay motivated through the toughest times.

I would also like to extend my gratitude to all the past and present lab members whose support has helped in my project and provided valuable views in the lab meetings to shape my research. Additionally, I would like to thank Dr. Chrysoula Kitsou and Kathryn Nassar for always being ready to support me.

I thank the Department of Veterinary Medicine, faculties, post-doc fellows, graduate students, and staff for providing a conducive environment.

I would like to thank and dedicate this work to my parents, Shyam Sundar Bista and Nirmala Bista, for being constant sources of love, encouragement, and support. No words can be enough for what you have done for me to get here. Thank you for providing a nurturing home and instilling me and my siblings with good morals. Love, support, and motivation from my sister Nirjala and my brothers Nirdosh and Sandesh made this accomplishment a shared success.

Finally, I am grateful to my husband, Dr. Ravi Chetree. Thank you for the countless hours you spent listening to me and for your sacrifices to put our family first. Your untiring dedication to our family has been a great source of inspiration. Thank you for constantly motivating me by being there when needed and making me feel the abysmal years of hours pass by smoothly. I look forward to celebrating this achievement with you and our darling daughter, Sranvi.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADME	Absorption, Distribution, Metabolism and Excretion
BSK	Barbour Stoenner Kelly medium
BSA	Bovine serum albumin
CD	Circular dichroism
cDNA	Complementary DNA
^{o}C	Degrees Celsius
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EM	Erythema migrans
GST	Glutathione S-transferase
g	Gravitational force
HIS	6x Histidine Tag
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilo base pair
kDa	kilo Dalton
LB	Luria Bertani
µl	Microliter
µM	Micromolar
ml	Milliliter
mM	Millimolar
nM	Nanomolar

ORF	Open reading frame
PBS	Phosphate Buffered Saline
PBS-T	PBS+ Tween-20
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
mRNA	Messenger RNA
SDS-PAGE	Sodium dodecyl Sulphate- Polyacrylamide Gel Electrophoresis

Chapter 1: Introduction to Lyme Disease

1.1 History, geographical distribution, and prevalence of Lyme Disease

Borrelia burgdorferi is a gram-negative bacterium and falls under the family *Spirochaetaceae* (Johnson et al., 1984), which causes Lyme disease (LD), also known as Lyme borreliosis in humans and many domesticated animals. Besides *B. burgdorferi,* there are many other genospecies, such as *B. afezelii, B. garinii,* and *B. bavariensis* also elicit LD in humans and are prevalent in many parts of the globe. *B. burgdorferi* is transmitted by an arthropod vector - hard ticks belonging to several species of *Ixodes* genus that transmit LD (Mead, 2015; Steere et al., 2016). The zoonotic disease is now reported in over 80 countries across most continents but is particularly widespread in many North American and Eurasian continents. It was first recognized in Old Lyme, Connecticut, as "Lyme arthritis" and was characterized as recurring oligoarticular arthritis (Steere et al., 1977). It was later diagnosed as tick-borne Spirochetosis by Willy Burgdorfer et al. in 1982 (Barbour & Benach, 2019) and was isolated in 1983 in a fortified Kelly medium, i.e., Barbour-Stoenner-Kelly (BSK) medium (Benach et al., 1983). However, evidence suggests that LD has been around for much longer. There are records of cases with symptoms resembling LD in 19th-century Europe (Burgdorfer, 1993). In addition, the discovery of the LD bacterium in 5300 years old mummy and ticks from 15 million years ago presents it as an ancient ailment (Keller et al., 2012; Nuwer, 2014).

LD is primarily found in North America, Europe, and Asia. In the United States, it is mostly reported in the northeastern and upper mid-western regions (Adams et al., 2014; Kugeler et al., 2015). The number of cases of LD correlates positively with the availability of the vectors and reservoir hosts in the region. The vector population responsible for the transmission comprises *I. scapularis* in the eastern and *I. pacificus* in the western US (Nieto et al., 2018). *I. ricinus* and *I. persulcatus* are responsible for transmission in Europe and Asia, respectively (Burgdorfer et al., 1982; Mannelli et al., 2012; Tilly et al., 2008). This notifiable tick-borne disease is maintained in the environment by a wide range of vertebrate hosts, such as small white-footed mice (*Peromyscus Leucopus*) (in the eastern U.S), deer, and birds (Donahue et al., 1987; Magnarelli et al., 1992; Mannelli et al., 2012; Parise et al., 2020; Wilson et al., 1985).

The Centers for Disease Control and Prevention (CDC) reports almost half a million cases, even though only 30,000 cases are reported yearly. Most recent estimates from the CDC suggest that there are ~476,000 new cases annually in the U.S. alone, where the medical costs associated with managing Lyme disease and its sequelae approach $1.3 billion annually (Adrion et al., 2015). Most patients are clustered toward the northeastern, mid-Atlantic, and mid-Western states, especially in Maine, Massachusetts, Connecticut, Wisconsin, New Hampshire, and Pennsylvania. (Kugeler et al., 2021; Schwartz et al., 2017). Climate change, deer and rodent populations, their abundance or rehabilitation, and reforestation are implicated in the increasing incidence of LD (Levi et al., 2012; Dumic & Severnini, 2018; McPherson et al., 2017; Schwartz et al., 2017; Dumic & Severnini, 2018;). Selective host choice of the ticks in southern states, such as lizards, which are less efficient reservoirs compared to rodents, differences

in temperature, seasonal phenomena between the hemispheres, and the tendency of southern ticks to remain underneath the leaves instead of top to retain moisture are a few factors that could result in a low incidence of the disease in the zone (Ginsberg et al., 2017, 2021; Ogden & Tsao, 2009; Ostfeld & Keesing, 2000).

1.2 Transmission

The enzootic life cycle of *B. burgdorferi* consists of ticks as vectors, small rodents as reservoir hosts, and humans and dogs as incidental hosts. The ticks of *Ixodes sps.* have four stages of the lifecycle - eggs, larvae, nymph, and adults, and generally have a life span of two years (Dumic & Severnini, 2018b). Each active stage- larva, nymph, or adult, requires one blood feeding for further development and survival. After the egg hatches, the six-legged larvae quest for small mammals (Kocan et al., 2015). The feeding generally takes 4-5 days, and the larva drops off molts to nymph in about one month. The eight-legged nymph, after maturation, quests for a mammalian host and, upon finding one, attaches to other mammals and feeds for 4-5 days. After engorgement, the nymph falls off from the host, undergoes intermolt development, and later molts into either male or female adults. Both larvae and nymphs can acquire *B. burgdorferi* if they feed on infected animals. The pathogen survives in ticks through the transtadial development of the vector, and thus all subsequent stages of the tick will remain infected throughout the lifespan; thus, the transmission of *B. burgdorferi* in ticks is only transtadial and not transovarial. (Steere et al., 2004). In the eastern U.S., larval and nymphal stages of *I. scapularis* feed on white-footed mice, i.e., *Peromyscus leucopus,* which is also a primary

reservoir host of the bacteria. Nymphs, which can be infected with *B. burgdorferi,* are a significant vector for human and canine transmission. The adult ticks feed and mate on white-tailed deer and other larger mammals. After engorgement, female ticks fall off from the host, and after several weeks of ovarian development, lay thousands of eggs Nadelman & Wormser, 1998), following which the female dies.

When a naïve ticks feed on an infected mammalian reservoir host, the vector inserts its mouthpart into the host dermis to acquire a blood meal. Exposure to tick salivary proteins attracts the bacteria in the dermal feeding region and is ingested by ticks (Hodzic et al., 2001; Murfin et al., 2019). The acquired pathogen colonizes the tick gut until the next feeding, when the pathogen disseminates to the hemocoel and finally invades the salivary gland (de Silva & Fikrig, 1995; Kurokawa et al., 2020; Zung et al., 1988; Dunham-Ems et al., 2009). Once transmitted to the host dermis, the pathogen replicates locally and subsequently spreads to other dermal locations and disseminates to other distant organs via blood. Thus, the spirochete has adapted to adjust to highly variable environmental conditions from disparate tissues of vertebrate hosts and in the arthropod. The change in tissue environments, including temperature, pH, nutrition, and immune conditions, can initiate a global change in its transcriptome that ultimately dictate its survival strategy. In its life cycle, the pathogen is adapted to persist in extreme stress conditions, such as in metabolically quiescent conditions of unfed ticks' lumen, which is devoid of nutrients that are stored intracellularly within the gut epithelium (Kung et al., 2013).

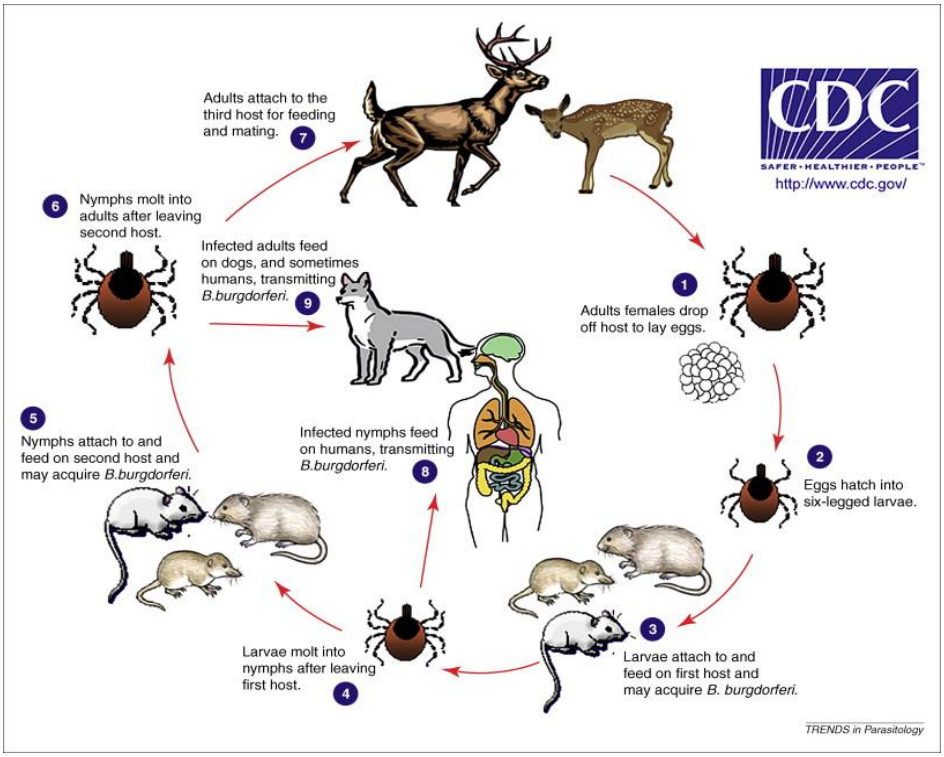


Figure 1. Life cycle of *B. burgdorferi* (Little et al., 2010).

1.3 General features of the causative agent Borrelia burgdorferi

B. burgdorferi is a complex microbe that is perpetually maintained between vector and host. It is a highly motile pathogen of 0.2 - 0.5µm diameter and 10 - 30µm length with a spiral shape and is microaerophilic (Goldstein et al., 1994; Johnson et al., 1984). Without a stain, the gram-negative bacterium is only visible by a specialized microscope, differential interference contrast (DIC) microscope, also known as the Nomarski microscope. The helical structure separates it from other eubacteria (Barbour & Hayes, 1986). *B. burgdorferi* can be differentiated from other pathogenic spirochaetes, such as *Leptospira* and *Treponema*, based on the number of periplasmic flagella,

presence or absence of hooks, cell coil wavelength, and pole shape (Wang et al., 2014). The varying section of the 16S rRNA gene is compared for species differentiation (Cyr et al., 2005; Lee et al., 2010). More than 40 species of *Borrelia* have been identified, out of which 20 belong to the LD group, Lyme *Borrelia* (LB) B. *burgdorferi sensu lato,* and the other 20 belong to the relapsing fever (RF) (Margos et al., 2011; Stanek & Reiter, 2011). In the *B. burgdorferi sensu lato* group, five species are the most frequent cause of the disease; *B. burgdorferi sensu stricto* in North America and Europe, and *B. garinii*, *B. afzelli*, *B. baverensis* in Eurasia and *B. speilmanii* in Europe (Casjens et al., 2011; Mannelli et al., 2012b; Marques et al., 2021; Wang et al., 2014). Diseases like RF and southern tick-associated rash illness (STARI) are caused by different factions of *Borrelia*, such as *B. hermsii*, B. *miyamotoi, and B. lonestari* (Herman-Giddens, 2012; Krause et al., 2015) although precise etiology of STARI remains as a subject of contention. Recent reclassification has divided the *Borrelia* genus between Relapsing fever and Lyme disease groups to *Borrelia* and *Borreliella*, respectively (Adeolu & Gupta, 2014; Barbour et al., 2017; Margos et al., 2017), although traditional classification is still being used in the mainstream literature.

B. burgdorferi features an atypical genome encompassing a liner chromosome and 14-21 various sizes (5 – 56 Kb) linear or circular extrachromosomal DNA elements called plasmids. The chromosome (~0.9 Mb) lacks several factors required for classical biosynthetic and metabolic pathways, rather features a series of membrane transporters encoding a unique proteome that exploits the host for its maintenance, contributing to the pathogenesis of LD (Fraser et al., 1997). *B. burgdorferi* is an obligate parasite that requires a vector and a host for survival but can also be grown in a lab in highly

sophisticated and serum-enriched media called BSK-II. The growth of spirochetes in vitro is optimal at 34 °C, 1.5% CO_2, with a regeneration time of 12 - 18 hours in the laboratory conditions.

The spirochete cell has a unique organization with an outer membrane (OM), periplasmic space featuring bundles of endoflagella, peptidoglycan layer (PG), inner membrane (IM), and enclosed cytoplasmic contents (Johnson et al., 1984; Kudryashev et al., 2009). The OM functions as a barrier from the environment and has several porins or receptors for the uptake of nutrients (Cullen et al., 2004; Nikaido, 2003). The membrane is distinct from other gram-negative bacteria as it lacks classical lipopolysaccharide (LPS) and phosphatidylethanolamine but contains low molecular weight glycolipids, which have immunogenic properties that the patient's serum can recognize (Belisle et al., 1994; Hossain et al., 2001; Stübs et al., 2009; Takayama et al., 1987). The membrane contains a low density of transmembrane-spanning proteins, making it more fragile during centrifugations and more susceptible to detergents than other gram-negative bacteria (Radolf et al., 1994; Walker et al., 1991). In contrast, OM contains many surface-exposed lipoproteins (Brandt et al., 1990). The *Borrelia* genome encodes at least 127 lipoproteins which are 7.8% of total open reading frames (ORFs), two-thirds of which are thought to be localized on the surface (Setubal et al., 2006a). Of 39 periplasmic proteins, 31 are speculated to be attached to the IM, and only eight are anchored to the OM on the periplasmic side. The lipoproteins act as significant components for immunogenicity and function as ligands to several host receptors (Brandt et al., 1990; Dowdell et al., 2017).

OM lipid rafts are microdomains highly studied for eukaryotes and have cholesterol and sphingolipids. Some prokaryotes like *Borrelia* and *Bacillus* also have the domain. The microdomain in *Borrelia* lacks sphingolipids. The major proteins present in the rafts are outer surface proteins A and B (OspA and OspB) although possibly there are additional proteins that are likely to be present (LaRocca et al., 2013).

The peptidoglycan (PG) layer in *B. burgdorferi* is situated between IM and OM and comprises N-acetyl Glucosamine (GlcNAc) and N-Acetyl Muramic Acid (MurNAc) cross-linked by a short peptide. The PG structure is closely located with the IM, and the composition differs from other bacteria at the third position of the peptide stem. *B. burgdorferi* contains L-ornithine in the third position, whereas most gram-negatives have Diaminopimelic acid (Beck et al., 1990; Vollmer et al., 2008). Therefore, the PG layer is atypical in *B. burgdorferi.* It is multilayered at the poles, unlike other gram negatives, which are single layered (Jutras et al., 2019). Flagella functions as the cytoskeletal feature instead of the rod-like PG, which pushes through the filaments and is elastic (Charon et al., 2012; Jutras et al., 2016). Enzymes required for PG recycling are absent, and forty-five percent of the muropeptides are shed outside the cell in each generation. The released PG has immunomodulatory properties and is implicated in the development of Lyme arthritis, including ones in humans (Jutras et al., 2019).

The flagella are responsible for the flat wave structure and are situated between the PG layer and periplasmic space. About 7 - 11 flagellin fibers on both subpolar ends are attached to the protoplasmic cylinder by motor and hooks. The fibers from both ends

overlap in the middle and are responsible for the corkscrew fashion movement (Jutras et al., 2016; Motaleb et al., 2000). Deletion of an essential flagellar gene shows that the periplasmic flagella (PF) are not only responsible for motility but also for holding the spiral architecture and cellular integrity during external stress (Kumar et al., 2017; Motaleb et al., 2000).

Periplasmic space is a versatile environment where mechanisms like protein maturation, folding, and degradation occur. Periplasm houses several candidates with diverse functions in protein secretion, oxidation, lipoprotein translocation, metal transport, osmoregulation, proteolysis, protein folding, cell division, and flagellar rotation (Miller & Salama, 2018). Recent articles show that in the case of *E. coli*, the periplasm's size correlates to the bacteria's environmental sensing (Asmar et al., 2017). The periplasmic space of *Borrelia* is not well understood, but several studies show that it houses multiple essential proteins of unknown structure or functions, including BB0323, BB0238, and BB0104 (BbHtrA), which also interact and confer essential functions in spirochete biology and infectivity – elucidation of their biological significance constitute the focus of our current study (Kariu et al., 2015; Östberg et al., 2004; Ye et al., 2016a; Zhang et al., 2009).

Metabolism in B. burgdorferi

B. burgdorferi has limited de-novo biosynthetic capabilities. The relatively small-sized genome of *Borrelia* houses a limited number of genes, leading to the restricted biosynthetic and metabolic capacity, making it an obligate parasite, unable to survive outside the hosts except for in highly enriched media. It requires to uptake of nutrients

from its environment for energy generation, metabolism, and survival. The borrelial genome encodes a large repertoire of transporter proteins, many of which have a broader binding affinity range. The phosphoenolpyruvate phospho-transferase system (PEP: PTS) uptakes carbohydrates such as glucose, maltose, fructose, and chitobiose. An ATP-binding cassette system uptakes ribose, which cannot be used for energy production but for nucleic acid synthesis (Lackum & Stevenson, 2005).

Carbohydrate

B. burgdorferi encodes all the genes required for glycolytic but none of the tricarboxylic acid cycle (TCA) or oxidative phosphorylation pathway, which leads to relatively less ATP production and a slower growth rate (Barbour, 1988; Fraser et al., 1997). It can survive in various environmental conditions by utilizing alternate carbon sources, such as maltose, fructose, mannose, N-acetylglucosamine (NAG), glycerol, and chitobiose instead of glucose (Lackum & Stevenson, 2005). In the case of ticks, NAG, a primary component of chitin, can be converted to fructose 6- phosphate and enter glycolysis (Fraser et al., 1997).

Lipid

B. burgdorferi speculated avoiding fatty acids as an energy source (Fraser et al., 1997; Johnson, 1977). It lacks the genes responsible for fatty acid synthesis, chain elongation, and cholesterol synthesis (Fraser et al., 1997). It acquires fatty acids and cholesterol from the environment and synthesizes glycolipids, phospholipids, lipoproteins, membrane lipids, etc., through many membrane transporters (Boylan &

Gherardini, 2008; Crowley et al., 2013). Notably, *B. burgdorferi* is known to influence the host cells to alter their lipid metabolism and increase the level of phosphatidylcholine in serum, which the bacteria could access (Fitzgerald et al., 2020).

Metal

Iron is an essential cofactor of many enzymes used for various functions in transcription, translation, oxidative phosphorylation, DNA metabolism, carbon metabolism, and gene regulation (Kronstadt & Caza, 2013). Most microbes need iron as an essential co-factor for their survival, and thus, hosts generally sequester iron availability as a well-known anti-bacterial defense strategy. Surprisingly, *B. burgdorferi* does not encode essential iron-containing proteins and, thus, can bypass the need for iron for its survival in the host or pathogenesis. In fact, the spirochetes could grow normally in the presence of iron chelators (Posey & Gherardini, 2000). On the other hand, *Borrelia* houses a gene BB0219, *Borrelia* metal transporter protein A (BmtA), a gene identified for manganese (Mn) transfer. Mutation of the gene resulted in decreased Mn uptake by the cells resulting in delayed growth and the inability of the bacteria to withstand oxidative stress (Ouyang et al., 2009). Mn, along with Zinc (Zn), regulates the expression of RpoS, a principal regulator of *B. burgdorferi* genes. Adding external manganese could repress RpoS and OspC production even at a higher temperature, revealing that Mn could be a part of the environmental sensor, supporting the spirochete infectious cycle in ticks and mammals. In contrast to Mn, an abundance of Zn could reverse the effect and increase the production of RpoS and OspC, displaying the regulatory impact of Zinc (Troxell et al., 2013).

Protein

B. burgdorferi genome encodes for relatively large numbers of membrane transporters (Fraser et al., 1997) and has the most significant percentage of phosphotransferase systems (Saier & Paulsen, 2000). The ATP binding cassette (ABC) and oligopeptide permeases (opps) help *Borrelia* in the uptake of peptides from the environment (Groshong et al., 2017). It has five homologs of oppA- oligopeptide binding fraction (Wang et al., 2002); two oppB and oppC- cytoplasmic membrane domain; one oppD- nucleotide domain; and one oppF- ATP dependent transport of peptide (Groshong et al., 2017).

Lipoprotein translocation in B. burgdorferi

Generally, in *E. coli,* lipoproteins are peripherally attached to the periplasmic face of IM or OM. The proteins reach the destination in three significant steps: Step 1 is the transportation of preprolipoprotein through IM by a Sec-dependent or TAT-mediated pathway to the outer face of the membrane (Hou & Brüser, 2011; Kudva et al., 2013; Nakayama et al., 2012). Typically, the N terminals of the prolipoproteins contain a signal peptide sequence that contains a "lipobox" with a conserved amino acid- cysteine at +1 position, whose sulfhydryl group is lapidated in Step 2, where a transfer of a diacylglycerol group to the prolipoprotein by the action of Lgt enzyme occurs. Sequentially, the prolipoprotein is acted by signal peptidase II (Lsp) to cleave off the signal peptide sequence in periplasmic space, turning it into an apolipoprotein (Nakayama et al., 2012; Sankaran & Wu, 1994). Finally, in Step 3**,** an enzyme Lnt adds

an acyl group resulting in a mature lipoprotein. Posttranslational modification by adding lipids helps the proteins to anchor to the hydrophobic zones (Babu et al., 2006).

The bacterial signal peptide generally consists of a positively charged N-terminal region, a hydrophobic-hinge region, and a lipobox at the C-terminal of the signal peptide sequence (Babu et al., 2006). The lipobox consists of (LVI)(ASTVI)(GAS)(C) with invariable cysteine at +1 position for lipid modification sequence (Babu et al., 2006; Braun & Wu, 1994). It is a molecular signature for predicting lipoproteins *in silico* (Setubal et al., 2006). With more sequences of bacterial proteins analyzed, the sequences in the canonical lipobox have become outdated, with only residue at the +1-position conserved (Zückert, 2014), although there are likely to be exceptions to the rule.

The mature lipoprotein, after removal of the signal peptide, is either associated with the cytoplasmic membrane, or is translocated to the periplasm, or get associated with the inner face or outer face of OM. Generally, the presence of the Lol avoidance signals or +2 sorting makes the protein unrecognizable to the Lol translocation machinery; therefore, the proteins stay in the cytoplasmic membrane; otherwise, they are transferred by either Type 2 secretion system (T2SS) or type 5 (T5SS) (Tokuda & Matsuyama, 2004; Zückert, 2014).

The components of the Sec translocase system, the enzymes required for lipid modification, and signal peptide cleavage are detected in *B. burgdorferi*. In comparison to *E coli, B. burgdorferi* encodes for Sec machinery (except SecB) β-barrel-assembly-machinery (BAM) complex, and partial Lol pathway (lacks LolB) and lacks components of T2SS (Casjens et al., 2011; Fraser et al., 1997). Also, the lipoproteins in *B. Burgdorferi* lack the signals for T5SS (Oomen et al., 2004).

Notably, *B. burgdorferi* does not follow the +2 sorting rule; in fact, the localization is dependent on the disordered tether sequences present in N-terminus; mutation of the peptide led to mislocalization of outer surface lipoproteins on the periplasmic leaflet (Kumru et al., 2010, 2011; Schulze et al., 2010; Schulze & Zückert, 2006). Furthermore, *B. burgdorferi* features a bundle of endoflagella located within the periplasm. Understandably, the conventional methods of protein transport do not work here, and various translocation steps are yet to be elucidated, and it is suggested that the trafficking to OM is likely to be assisted by the action of chaperones such as sixteen kilodalton protein (skp), survival protein A (SurA), DegP, and other holdase and flippase enzymes (Zückert, 2014).

1.4 Genome

B. burgdorferi, B31 strain is one of the first fully-sequenced genomes in bacteria (Fraser et al., 1997). All the *Borrelia* species have one linear chromosome of ~ 0.9MB and a variation of 7-21 plasmids ranging from 5 to 84 kbp; the B31 strain has 21- 12 linear and 9 circular plasmids. The chromosome primarily has housekeeping genes with fewer intergenic regions. It has low GC content, i.e., 28.6%, and houses 803 genes that encode proteins (Barbour, 1988; Plasterk et al., 1985; Casjens et al., 2002). The origin of replication is predicted at the center of the chromosome (Casjens et al., 2011; Picardeau et al., 1999). The genes and proteins are named after the locus tags; the genes are numbered from the 5’ region in ascending order; for example, genes are annotated as *bb0323* and the proteins as BB0323. The gene encoded by a plasmid contains a letter

instead of the plasmid name; for example., *bba52* encodes protein BBA52, and "A" represents plasmid lp54 (Casjens et al., 2011). The small and compact chromosome includes genes for conserved or housekeeping cellular functions, such as cell wall metabolism, DNA, RNA, protein synthesis, and glycolysis, etc. However, the *B. burgdorferi* genome lacks genes required for most biosynthetic pathways, including amino acid synthesis, nucleotide synthesis, respiration, and lipid synthesis are lacking. Therefore, the spirochete depends on its host (or cultural media when grown in vitro) for these essentials (Fraser et al., 1997).

The plasmids of LD agents mainly encode proteins of unknown functions, including many surface lipoproteins required for microbial virulence or host-pathogen interaction (Fraser et al., 1997; Sadziene et al., 1993). Plasmids vary in stability; some could be lost during passages during *in vitro* cultures (Byram et al., 2004; Grimm et al., 2005). Linear plasmids are coded as "lp" and circular plasmids as "cp," and their kb sizes are used for their designation, such as lp54 is a linear plasmid of 54 kb (S. R. Casjens et al., 2011; Picardeau et al., 1999; I. Schwartz et al., 2021). Plasmids encode a variety of genes that are essential for different stages of infection and survival in specific environments. Most proteins are lipidated in their N-terminal and do not have homologous counterparts outside the *Borrelia* genus **(**Dowdell et al., 2017). Elucidation of their function has made them exceptional diagnostic and vaccine development targets (Dowdell et al., 2017; Fraser et al., 1997; Kenedy et al., 2012). Cp26 is the only plasmid that is stably maintained in various species of *B. burgdorferi* sensu lato and is vital for spirochetal survival in vivo as well as growth *in vitro.* This plasmid encodes for outer surface protein OspC, which is essential for the spirochete migration through ticks

(Fingerle et al., 2007; Pal et al., 2004) as well as the establishment of early infection in mammals (Byram et al., 2004; Fraser et al., 1997; Grimm et al., 2005; I. Schwartz et al., 2021; Tilly et al., 2016). Plasmid cp26, lp54, and lp17 are present in all natural strains. Lp17 houses BBD18, a regulator of alternative sigma factor Rpos (Hayes et al., 2014; I. Schwartz et al., 2021). Lp54 comprises various genes essential for *B. burgdorferi* transmission from ticks and infection in the host. Proteins such as OspA, OspB, Decorin binding proteins A and B (DbpA and DbpB), and BBA57 are encoded by the plasmid (Bernard et al., 2019; Blevins et al., 2008; Caimano et al., 2019; Kenedy et al., 2012; Pal et al., 2004; Tilly et al., 2016). Lp28-1 contains the variable primary protein-like sequence Expressed, the *vlsE* gene, which is essential for antigenic variation inside the host (Norris, 2014). The lp36 plasmid encodes various genes, such as *bbk13, bbk32,* and *bbk46,* necessary for host association or immune evasion (I. Schwartz et al., 2021). Plasmids lp56 and lp25 are involved in the restriction modification system, the removal of which increases the transformation efficiency of exogenous DNA in the spirochete (Lawrenz et al., 2002; Rego et al., 2011). These represent only a few examples of many spirochetes genes that are encoded in specific plasmids and support *B. burgdorferi* biology and infection.

Overall, the *Borrelia* genome is small and highly segmented, harboring clusters of paralogous genes. The chromosome mainly carries the housekeeping genes with known functions, whereas proteins encoded by plasmids are responsible for enigmatic molecular function yet are essential for spirochete infection contributing to host-pathogen interaction and microbial immune evasion. Advanced technologies and high throughput

methods are broadening our knowledge of the structure and functions of encoded proteins.

1.5 Gene expression and regulation in in B. burgdorferi

LD is an enzootic disease that encompasses the acquisition of the spirochete by a naïve *Ixodes* tick vector, persistence within the tick, and then transmission back to a vertebrate host. The spirochete persists in extremely distinct environmental conditions in terms of temperature, pH, level of oxygen and carbon dioxide, transient metals, nutrients, osmolarity, and host factors (Bontemps-Gallo et al., 2016; Boyle et al., 2019; Brooks et al., 2003; Carroll et al., 2000; Hyde et al., 2007; Seshu et al., 2004). Spirochete harbors two major gene regulatory systems to adapt successfully in varied tissue environments in ticks and mammals, which are, 1) BosR/Rrp2/RpoS/RpoN pathway, herein referred as RpoS/RpoN alternative sigma factor cascade, 2) the two-component system (TCS) comprising Hk1/Rrp1, and RelBbu and DksA mediated stringent response (Samuels et al., 2022). Transcription of the *Borrelia* gene is carried out by the RNA polymerase, structurally similar to the other bacterial holoenzyme, but requires manganese instead of magnesium for the activity (Browning & Busby, 2016). The enzymatic activity of RNA polymerase is highest at 37ºC and pH 7.5 (Boyle et al., 2019). In contrast to *E. coli*, which has seven sigma factors, *Borrelia* harbors genes for three sigma factors, *rpoD*, *rpoN*, and *rpoS* (Burgess & Anthony, 2001; Caimano et al., 2019) *RpoD* is a primary sigma factor that initiates transcription in the logarithmic phase of the cell cycle (Burtnick et al., 2007; Hübner et al., 2001; Samuels et al., 2022). *RpoS/RpoN*, alternative sigma factor cascade activates the genes required during the transmission and infection

establishment in a host and represses those which are essential for the survival of spirochetes in ticks with help from *rrp2*- a transcription activator (Arnold et al., 2018; Fisher et al., 2005; Grove et al., 2017; Hübner et al., 2001). In addition, a transcription regulator, *BosR*- *Borrelia* oxidative stress regulator - a metalloregulator, acts both transcriptionally and post-transcriptionally in response to pH and carbon dioxide (Saputra et al., 2020). A transcription repressor of RpoS, *Borrelia* Host Adaptation Regulator (BadR), activates in the environment with a low carbohydrate environment mimicking unfed ticks (CMiller et al., 2013) RpoN recognizes the promoter sequence of RpoS, which is different from the promoter, recognized by RpoD (Studholme & Buck, 2000). RpoS generally activates stress responses in other bacteria (Hengge-Aronis, 2002). Still, in the case of *B. burgdorferi*, the activation is in response to the enzootic phase when the genes responsible for disease transmission are expressed instead of genes of the general stress response, and the genes required for survival inside ticks, regulated by RpoD are repressed (Caimano et al., 2007, 2019; Schwartz et al., 2021). Overall, in the RpoN/RpoS cascade, after activation of RpoS, several lipoproteins, such as DbpA, OspC, and BBk32, are upregulated (Caimano et al., 2007). OspC is essential in establishing infection but not for persistence (Tilly et al., 2008). It facilitates the invasion of the spirochete into salivary glands following feeding (Pal et al., 2004, 2022; Tilly et al., 2016). BBk32 is essential in the adhesion of spirochete in the extracellular matrix (ECM), and DbpA is crucial in binding host ligands such as decorin, heparin, and dermatan sulfate (Caine & Coburn, 2016). *B. burgdorferi* spends most of its time in the gut of unfed ticks until the start of the next feeding in the subsequent stage. Once *B. burgdorferi* are acquired in ticks from an infected host, and after the spirochete reaches the gut, the change in temperature and pH

initiates the changes in the outer surface proteins of *Borrelia*. Here, OspC is downregulated, and OspA- one of the significant antigens, is upregulated. OspA interacts with a tick receptor for *B. burgdorferi* called TROSPA, facilitating spirochete colonization of the tick gut (Pal et al., 2004). OspA has an affinity for plasminogen and helps in its conversion to plasmin- which down-regulates the production of complement-related proteins (Fuchs et al., 1994). The OspA also protects the spirochete from antibody-mediated actions (Kung et al., 2013; Kurokawa et al., 2020; Pal et al., 2004). Additionally, RpoS represses the expression of vector phase proteins in vertebrates (Grove et al., 2017).

The two-component signal transduction system (TCS) Hk1/Rrp1 and its secondary messenger c-di-GMP, as the second regulatory mechanism, are potentially activated by exogenous amino acids and their derivatives (Caimano et al., 2015). The TCS mainly functions in the tick phase of the cycle and the BSK-II medium. It upregulates the utilization of glycerol, expression of chitobiose, and protection from vector complement factors, among others (Caimano et al., 2011; Samuels et al., 2021).

In unfed ticks, alarmones guanosine pentaphosphate and guanosine tetraphosphate, known as ppGpp, are the major regulating factors in response to stringent factors (Caimano et al., 2016). The levels of ppGpp are altered by the enzyme Rel_{Bbu} (Drecktrah et al., 2015; Samuels et al., 2021). The alarmones bind to the RNA polymerase and alter the transcription of related genes (Gaca et al., 2015; Gourse et al., 2018; Liu et al., 2013). The activity of ppGpp is usually coordinated with the response of DnaK suppressor protein (DksA) with a distinct regulon. RelBbu facilitates glycerol uptake by enhancing glycerol kinase (gk) and glycerol uptake facilitator (glf). It also

upregulates the expression of BBK32 and DbpA/B, which is potentially important in spirochete survival in nutrient-deficient locations in vertebrate hosts, such as joints (Drecktrah et al., 2015). In addition to gk and glf, DksA regulates other tick-specific proteins globally, including OspA and Lp6.6 (Boyle et al., 2019). Overall, these signal transduction systems' independent contributions, their interplay, coordination, and other factors, such as small regulatory RNAs, chaperones, and 6s RNA, allow *B. burgdorferi* to survive and persist in dramatically distinct environmental conditions.

1.6 Immune response against B. burgdorferi

B. burgdorferi activates both innate and adaptive immunity in hosts, although without antibiotic treatments, neither response can fully eradicate the spirochete from an infected host (Sidman et al., 1992; Wang et al., 2005). Upon entry inside the host dermis via tick bite, innate immunity is activated and helps to suppress the spirochete population. Langerhans cells (LC) are one of the first cells to respond, followed by macrophages/dendritic cells (DC) and neutrophils (which can occur within 4 hours) (Nestle et al., 2009; Xu et al., 2007). However, spirochete motility and chemotaxis can facilitate the evasion of host cellular immune responses. For example, the spirochetes are estimated to move 300 times faster than the macrophages and 40 times faster than the neutrophils (Malawista & de Boisfleury Chevance, 2008; Park et al., 2018). Therefore, most bacteria disseminate to other tissues even though these sentinels capture some spirochete cells (Lazarus et al., 2006).

Amongst other innate immune responses, TLR-2 receptors are most relevant in LD pathogenesis, activated by the presence of lipoproteins (Hirschfeld et al., 2000). In the presence of diacylated lipoproteins and peptidoglycans, the TLR2 heterodimerizes with TLR6 and, to bind with triacylated lipoproteins- TLR2 heterodimerizes with TLR1 (Alexopoulou et al., 2002; Bockenstedt et al., 2020), which lead to an anti-bacterial response.

B. burgdorferi is an extracellular organism and inhibits complement activation to facilitate its survival in a host (Bockenstedt et al., 2020). A collection of proteins called complement regulator-acquiring surface proteins (CRASP) are produced by *B. burgdorferi and* are known for the inactivation of complement activity in the host. Five CRASP proteins are identified in *B.burgdorferi* (CRASP 1- 5) (Kraiczy et al., 2001, 2003, 2004; McDowell et al., 2003). The CRASP proteins (CRASP 1 and CRASP 2) bind with H factors in serum; thus, by inhibiting complement protein, that results in C3b accumulation. This, in turn, decreases the phagocytosis and membrane attack complex (Bockenstedt et al., 2020; Lin et al., 2020). Additional spirochete proteins, such as erpP, erpC, erpA, OspE, OspF, and ELp, also bind to the factor H leading to the inhibition of the complement cascade (Alitalo et al., 2002; Dulipati et al., 2020; Hellwage et al., 2001; Kraiczy et al., 2004). The outer surface protein OspC binds to a C4b, an opsonizing agent that also forms a complex with C2a to make C3 convertase, an enzyme required to convert C3 to C3a and C3b (Caine et al., 2017; Dulipati et al., 2020). In addition, another lipoprotein, BBK32, binds to the C1 complement complex and prevents the classical complement pathway (Garcia et al., 2016).

Adaptive immunity against *B. burgdorferi* comprises both T and B cell responses. Unconventionally, lipids and glycolipids such as glycolipid diacyl glycerol (BbGL-II) are presented to the invariant Natural Killer T cells (iNKT) (Kinjo et al., 2006). The activated iNKT cells produce INFγ (Dustin, 2008; Olson et al., 2010). In addition, studies have also shown γδ T cells make INFγ in response to *B. burgdorferi* infection (Bockenstedt et al., 2003) and the action of αβ T cells in response to spirochetal protein exposure leading to the production of TNFα and INFγ. The activation and differentiation of TH-1 cells upon infection by *Borrelia* lead to a high level of IL-14 and IFN-γ. On the contrary, IL-10 production might offer the host protective effects without proper pathogen clearance (Bockenstedt et al., 2020). The T-cell response is also implicated in developing autoimmunity in chronic Lyme disease. However, CD8+ cells have shown protective action against the condition (Singh & Girschick, 2004).

B cell activity is critical during LD resolution as humoral immune responses constitute the primary host defense against *B. burgdorferi*; in fact, early B cell response is second to antibiotic therapy in clearing the infection rapidly (Blum et al., 2018). The antibodies produced against the spirochetes are bactericidal by killing in a direct or complement-dependent manner (LaRocca & Benach, 2008, pp. 64-88) and assisting in the opsonization of the microbe (Modolell et al., 1994). However, the seroconversion takes longer time; with only 50% of the patients possessing antibodies at the time of presentation of initial skin lesion (erythema migrans); in mice, the seroconversion may take up to 14 days from the time of pathogen exposure (Aguero-Rosenfeld et al., 2005; Steere et al., 2008; Tunev et al., 2011; Wormser et al., 2008). The up and down-regulation of OM proteins and antigenic variations of proteins such as VlsE results in

different populations of spirochetes such that they are unrecognizable by the previous generation of antibodies (Norris, 2006, 2014). Altogether, B cell response is robust in chronic LD, and the antibodies produced are specific; the protection is incomplete, and re-infection can occur (Bockenstedt et al., 2020; Elsner et al., 2015).

Immune response in ticks:

The *Ixodes* tick is a highly evolved hematophagous vector. The tick's adaptation to avoid the host immunity during acquisition of a blood meal benefits the *B. burgdorferi* in transmission (Bonnet & Boulanger, 2017; Frischknecht, 2007). During feeding, the tick spits a pool of saliva in the feeding pit of the dermis, which contains several pharmacologically active substances to prevent host immunity and wound healing and cause vasodilation (Ribeiro et al., 1985, 2006; Bernard et al., 2017), which provides adequate time and an appropriate environment for the pathogen from an infected host to be acquired by the ticks (Nuttall, 2019). Here, within a tick environment, OspC is downregulated, and OspA is upregulated. An *in vitro* study showed that a salivary protein, Salp15, binds to OspC, shielding the bacteria from the antibody-mediated effects (Ramamoorthi et al., 2005).

In ticks, *B. burgdorferi* spends most of its time in the lumen of the gut (until the start of the next feeding). After the spirochete reaches the gut, the biotic and abiotic factors, such as the change in temperature, pH, etc., initiate a change in the outer surface proteins of *B. burgdorferi*. For example, OspC, which is a temperature and pH-regulated gene, is downregulated, whereas OspA transcripts are increased. The antigen has an affinity for plasminogen and helps in conversion to plasmin- which, in turn, down-

regulates the production of complement-related proteins and is also found to protect the spirochetes from antibody-mediated actions (Kurokawa et al., 2020).

Ticks feature major innate immune pathways, such as Toll, IMD, and JAK/STAT pathways; these pathways are not thoroughly studied yet, although initial studies suggest their critical roles in the control of tick-borne bacterial pathogens, including *B. burgdorferi* (Kitsou & Pal, 2018; Rana et al., 2022; Shaw et al., 2017; Smith et al., 2016). Duox and peroxidase genes mediate the formation of the Dityrosine Network (DTN) in the ticks, forming a barrier on the gut epithelium, down-regulating immune activation, and favoring the survival of the bacteria (Rana et al., 2023; Smith & Pal, 2014). Complement inactivating factors in tick saliva, such as Salp20, also favors for protection of the pathogen (Kurokawa et al., 2020). *B. burgdorferi* also protects itself from free radical nitrogen inside the tick environment due to the presence of nucleotide excision repair gene *uvrB* (Bourret et al., 2016).

1.7 Pathogenesis and clinical manifestation

Pathogenesis

The bacteria do not contain any toxins that could directly harm or destroy the host tissues. The inflammatory responses incited upon entry and colonization of *B. burgdorferi* in susceptible hosts can cause severe pathological outcome (Steere et al., 2016). Upon an infected tick bite, the spirochetes transmit through saliva to the hosts. A percentage of spirochetes is cleared by the host immune cells, such as by langerhans cells, neutrophils, and macrophages (Xu et al., 2007). The bacteria can disseminate through lymphatic ducts, dermis, and blood. Following the initial entry within the host

dermis, the spirochetes can take 7-10 days to reach and colonize the other internal organs. Despite a strong anti-bacterial response, the immune system cannot completely clear the infection (Bolz et al., 2004; Troy et al., 2013; Wooten et al., 2002). The spirochete can adapt to the new environment, evade the immune responses, multiply, and persist in the tissues. The inflammatory reactions damage the residing tissues and display the related symptoms (Coburn et al., 2022).

Clinical manifestations

Transmission of the spirochetes by the ticks to hosts can result in a multi-system illness that can be clinically classified in three stages of LD. Febrile symptoms, such as fever, chills, fatigue, and flu-like symptoms, characterize the early localized stage (Stage 1). 70-80% of the cases present with a typical rash featuring bull's eye rash, called erythema migrans (Steere, 2001). Motile and spreading bacteria can attract immune cells, causing the characteristic skin lesion to clear in the center of the tick bite (Radolf and Samuels, 2021; Nadelman, 2015; Wormser et al., 2006). Almost 20% of these patients could have a secondary rash (Wormser, 2005). In addition, the patients may display various symptoms, including swollen lymph nodes, pain in joints and muscles, conjunctivitis, cough, and sore throat (Steere et al., 1987), amongst other symptoms. If not treated at this phase, LD could progress to the subsequent phase, the early dissemination stage (Stage 2). Spirochetes invading the central and peripheral nervous system could elicit complications of Lyme neuroborreliosis, characterized by severe headaches, facial palsy, neck stiffness, and cognitive disorders (Steere et al., 1987; Radolf et al., 2022). Around 1% of the cases have cardiac involvement featuring

atrioventricular shunt and acute myopericarditis, resulting in palpitations, shortness of breath, and, sometimes, sudden death (Forrester & Mead, 2014; Marx et al., 2020). The patients could continue to experience musculoskeletal discomfort in this stage. If not addressed timely, the disease could progress to a late stage (Stage 3) - chronic Lyme disease ensues several months to years after infections. It is generally associated with the musculoskeletal and nervous systems, including Lyme arthritis (LA), which is a severe outcome of LD affecting especially large joints like the knee resulting in swelling and effusions (Arvikar & Steere, 2015; Radolf et al., 2022). Appropriate antibiotic therapy is often successful in resolving Lyme arthritis, but some patients could experience post-treatment, antibiotic-refractory Lyme arthritis (Radolf- et al., 2022; Steere & Angelis, 2006). Several speculations on the etiology of persistence have been made. Jutras et al. have shown that peptidoglycan debris in the synovial fluid can initiate chronic inflammation (Jutras et al., 2019). In addition, excessive production of Th1-type cytokines, the inability of T cells to downregulate the response of TH1, and imbalance in synovial tissue repairment are linked with dysregulated inflammation (Lochhead, Arvikar, et al., 2019; Lochhead, Ordoñez, et al., 2019; Vudattu et al., 2013). Despite appropriate antibiotic treatment, usually 6 months to a year after antibiotic therapy, about 10-20% of the patients continue to have symptoms such as fatigue, cognitive disorders, and fibromyalgia, which does not respond to further antibiotic therapy; these conditions are termed as Post-treatment Lyme disease syndrome (PTLDS) (Berende et al., 2016). The syndrome's etiology, diagnosis, and treatment are still unknown and are further discussed in the subsequent sections.

1.8 Diagnosis, prevention, and treatment

Diagnosis

Diagnosis of LD is often complicated as its symptoms vary and resemble many other febrile diseases. The history of tick bites on a subject residing in an endemic area is a critical aspect of diagnosis. The development of a skin rash (EM) is the most noticeable first sign of LD, but its appearance and size are not universal and vary greatly amongst affected individuals. LD symptoms in general are vague, so a differential diagnosis is a must. The identification comprises two major strategies: a direct method that detects *B. burgdorferi* and an indirect way that detects the antibody produced against the bacteria. The direct methods include culture of organs and fluids (cerebrospinal fluid, synovial fluid), PCR assays, xenodiagnoses, and histopathology (Marques, 2008). However, these methods have limited usage for detecting LD in humans. The CDC recommends a two-tiered system for LD clinical diagnosis. It comprises an Enzyme immunoassay (EIA) against *B. burgdorferi* as the first step. The second step is performed in case the EIA is positive. Immunoblotting or Western blotting (WB) is performed against the whole cell antigen and developed either against Immunoglobulin M or G (IgM or IgG) depending upon the stage of the disease (Branda & Steere, 2021; Marques, 2015).

The diagnosis of LD lacks effectiveness in the early stages of the disease, resulting in subsequent delays in treatment. And again, the current diagnosis modalities do not discriminate between active or inactive forms, and the antibody persists for an extended period (Marques, 2015). This leads to confusion in the accurate diagnosis of the

disease and delays the treatment, which might result in more serious episodes of the disease. Thus, developing a better diagnostic tool remains a top priority in LD research efforts. Recently, in 2019, FDA approved a modified two-tiered system (MTT), which includes a second EIA in place of a western blot. It is expected to be more sensitive and streamlined than the conventional system (Mead et al., 2019).

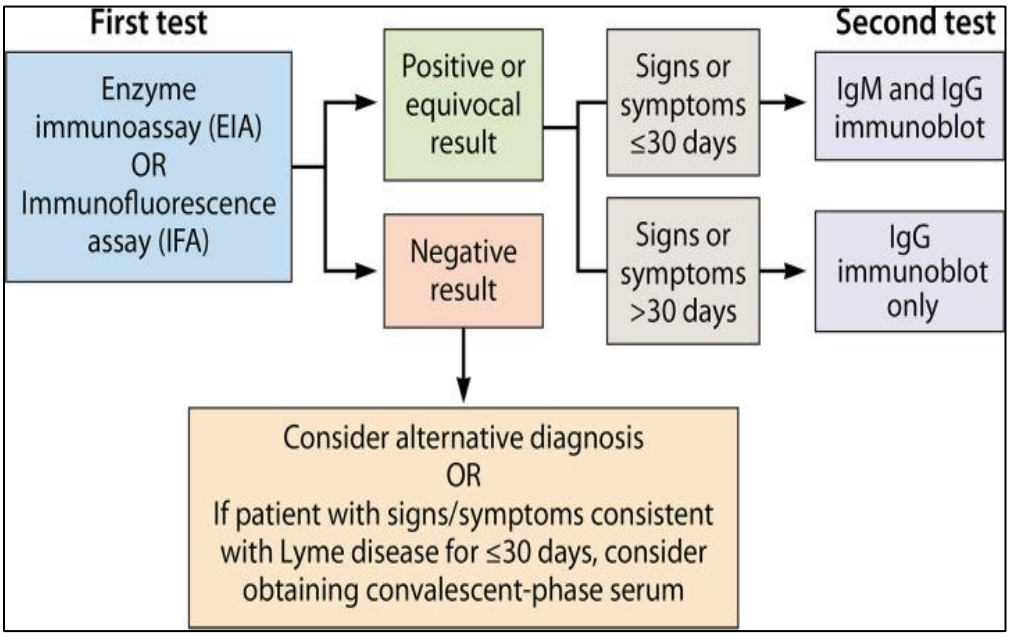


Figure 2. Conventional two-tiered serologic testing protocol for diagnosing Lyme Borreliosis (Branda & Steere, 2021).

Prevention of LD

The vaccine is one of the primary defenses in preventing infectious diseases, but we currently lack one for LD in humans. Intensive research throughout the late 1990s to

early 2000, vaccines were developed using outer surface protein A (OspA) as an immunogen. Three shots of the vaccine in humans provided 76% efficacy. The antibody produced in the host against OspA blocked the transmission of *Borrelia* from feeding ticks (Fikrig et al., 1992; Steere et al., 1998)), thus the vaccine was characterized as a "transmission-blocking vaccine" (A. M. de Silva et al., 1996). LYMerix, developed by SmithKline Beecham (SKB, now GlaxoSmithKline, GSK), was approved by FDA in 1998. Despite initial enthusiasm, due to immunological side effects perceived by the public, sales of dropped substantially, and the vaccine was subsequently retracted from the market. (Nigrovic & Thompson, 2007). However, subsequent Vaccine Adverse Event Reporting System (VAERS), which is a U.S. national vaccine safety surveillance program co-sponsored by FDA, did not find major adverse effects associated with the vaccine administration. There are ongoing studies on identifying other proteins such as OspB, OspC, DbpA, BbK32, and the next generation of OspA-based vaccine candidates (Gomes-Solecki et al., 2020).

In addition, other ecological factors to consider for preventing LD are the reduction of vector and reservoir hosts and the reduction of pathogen prevalence in them. Avoiding tick bites, such as wearing protective clothing and tick repellents outdoors in endemic areas, is highly recommended in case of tick bites. (CDC, 2021).

Treatment

Early diagnosis and timely treatment of LD are highly warranted to prevent more serious conditions. Guidelines published by the Infectious Disease Society of America

suggest treatment involving antibiotics (CDC, 2022; Lantos et al., 2020). Single-dose doxycycline as a prophylactic agent is only recommended when the tick is recognized as *Ixodes sps* and attached for more than 36 hours in an endemic area. For the early stage of the disease, in the case of EM, doxycycline (10 days) and amoxicillin or cefuroxime (14 days) is usually recommended. Seven days regimen of azithromycin is indicated in cases where doxycycline or Beta-lactam antibiotics cannot be used. In the cases of neurological and cardiac involvement, an initial intravenous infusion of ceftriaxone or cefotaxime followed by oral antibiotics such as doxycycline is indicated. Lyme arthritis requires more prolonged treatment of up to 28 days. Antibiotic therapy of more than eight weeks is not deemed valid, and in case of persistent symptoms, secondary treatment strategies, including corticosteroids, are expected to provide aid (Lantos et al., 2020).

1.9 Novel Therapeutic target against Lyme disease

As mentioned above, most clinical cases of LD are adequately cured by antibiotics. However, as discussed earlier, up to 20% of people may experience persistent symptoms such as pain, fatigue, and mental unclarity, which can initiate six months to a year after completion of the treatment known as PTLDS (CDC, 2022). The exact reason for the development of this condition is unknown; however, many speculations have led to the general belief that it might be due to the persistence of a population of spirochetes in the hosts, which might occur due to the stress exerted by the antibiotics during the treatment (Embers et al., 2012; Lewis, 2008, 2010; Preac Mursic et al., 1990). As presented by CDC, *B. burgdorferi*-induced “autoimmune” response or persistent but

difficult-to-detect infection could also be a cause of PTLDS, while others believe that conditions are unrelated to the *B. burgdorferi* infection. Additionally, some studies suggested an association between immune dysregulation and PTLDS (Wong et al., 2022). Immune mediators like CCL19 and IL-23 were found to be elevated in the case of cohorts with confirmed LD in acute cases and were likely to increase later in patients with PTLDS symptoms; Notably, these patients were negative for *B. burgdorferi* culture, and the antibody response decreased over time, confirming, absence of active infection (Aucott et al., 2016; Strle et al., 2014; Wong et al., 2022). An *in vitro* study has showed an increment in proinflammatory cytokines associated with microglial cells in exposure to dead bacteria instead of live ones (Greenmyer et al., 2018). Additionally, Positron emission tomography (PET**)** scanning of the brains of patients with PTLDS showed microglial activation that could be linked with cognitive symptoms (Coughlin et al., 2018). However, these results are preliminary and hence remain inconclusive. Some studies provide evidence of the elevation of inflammatory markers, such as C-reactive peptides or immune response against autoantigens, Endothelial growth factors (EGF), that could be linked with PTLDS (Drouin et al., 2013; Uhde et al., 2016). However, the improvement of symptoms in 40% of the placebo groups of patients with PTLDS-without immunity-related treatments shows that it is unlikely to be autoimmunity mediated (Klempner et al., 2001; Wong et al., 2022). Overall, there is inadequate data to confirm that PTLDS is due to autoimmunity.

Generally, the immune system is significantly influenced by its relationship with microbiota. The symbiotic relationship between the host and its microflora helps to modulate and train immunity, the imbalance of which has been linked to developing

immune diseases (Belkaid & Hand, 2014; Morrissette et al., 2020). Gut microbiome studies done with a group of people with PTLDS showed a significant increase in the population of *Enterobacteriaceae* and *Blautia species* (Morrissette et al., 2020). These species of bacteria are associated with multiple immune-related ailments such as IBD, Multiple Sclerosis, and Alzheimer's disease (Chen et al., 2016; Vogt et al., 2017; Zeng et al., 2017). Notably, the study also showed depletion of *Bacteroides*, which are associated with maintaining a healthy immune system (Arpaia et al., 2013; Furusawa et al., 2013; Morrissette et al., 2020). One of the probable reasons for the dysbiosis of the microbiome is the extensive use of antibiotics in the conventional treatment of LD, which can give rise to the post-treatment appearance of symptoms (Morrissette et al., 2020). Therefore, it is imperative to investigate novel therapeutic targets for the treatment.

Recent investigations on potential antibiotics for LD have presented two main targets, Azocillin and Hygromycin A (Leimer et al., 2021; Pothineni et al., 2020). Azocillin is an antibiotic that can kill the persister cells produced by doxycycline *in vitro* and clear the infection *in vivo*; however, it has broad-spectrum activity and cannot be used for patients with sensitivity against β-lactam antibiotics (Pothineni et al., 2020). Although a promising target, Hygromycin A also might have a non-targeted effect on commensal spirochetes (Leimer et al., 2021). Overall, the exact etiology for the development of PTLDS is not confirmed yet, and the unavailability of proper diagnostic tools and an array of non-objective symptoms further aggravates the situation. The above-presented studies suggest the syndrome might be due to extensive antibiotic usage, which could result in a disbalance in host microflora and immune dysregulation or the induction of antibiotic-tolerant cells that can persist, although the precise etiology of

PTLDS remains unknown. Given that the current antibiotic treatment of LD is not complete, the development of novel therapeutic strategies for comprehensive interventions against *B. burgdorferi* remains an important future goal.

Review of BB0323 Complex as potential targets for LD Treatment

Borrelia comprises several protein complexes essential for cellular metabolism, transmission, persistence, or infection (Yang et al., 2011).These interactions support the spirochete biology and infection throughout the enzootic life cycle (Bernard et al., 2019). A deeper understanding of such events is highly warranted not only for a deeper understanding of *B. burgdorferi* biology but also for identifying novel therapeutic agents and vaccine targets against the disease. One such protein complex involved the BB0323 protein. BB0323 is a membrane-associated immunogenic lipoprotein expressed throughout the tick-host phase and is essential for infection in murine hosts (Zhang et al., 2009). The protein is conserved in the sensu lato complex and has a vital function in outer membrane organization and stability as well as supporting spirochete infection; the knockout mutant cannot segregate the OM and presents with huge blebs (Stewart et al., 2004; Zhang et al., 2009). The protein at the C-terminal region features a known motif called the Lysin Motif domain (LysM), which is a peptidoglycan binding motif (NCBI, 2022). The LysM is present in all life forms except archaea (Mesnage et al., 2014; Steen et al., 2005). This domain is known to bind the polysaccharide portion of peptidoglycan (PG) in bacteria and chitin in fungi, mainly to the GlcNac polymer (Mesnage et al., 2014). The domain usually contains 44-65 amino acids and is generally present in enzymes responsible for cell division and, in some cases, host-pathogen interaction (Buist

et al., 2008; Mesnage et al., 2014; Zhang et al., 2009). However, the BB0323 protein has no homology to the other bacterial proteins with defined functions, but previous studies show that it interacts with the peptidoglycan (Zhang et al., 2009). Similar to PG, ticks possess abundant chitin, which is also a GlcNAc polymer. Thus, the possibility of whether the BB0323 LysM domain also interacts with the tick chitin when the spirochete resides inside the vector has yet to be explored.

The putative lipoprotein BB0323 comprises 377 amino acids with signal peptides in the first 22 amino acids and has an expected molecular weight of 41 kDa. However, the mature protein corresponds to a 27 kDa N-terminal and 12 kDa C-terminal containing the LysM domain (Kariu et al., 2013). The protein undergoes a series of post-translational processing by at least two proteases BB0104 (High-temperature requirement protease A, BbHtrA) and BB0359 (Carboxy terminal processing protease A, CtpA) (Kariu et al., 2013; Östberg et al., 2004) Furthermore, the two polypeptides of BB0323 interact with each other. The N-terminal of the protein is essential for cell division, and C-terminal is indispensable for infection in murine hosts but is expendable in cell division (Kariu et al., 2013).

Gene knockout studies have suggested that the function of BB0323 is analogous to the Tol-PAL complex of *E. coli* (Bernadac et al., 1998). However, it has no homology to Tol or PAL or related proteins (Kariu et al., 2013; Zhang et al., 2009). The Tol-PAL complex is a well-conserved protein complex in gram-negative bacteria and is composed of at least five core proteins, namely, TolA, TolQ, TolR- inner membrane; TolB, a periplasmic and PAL- peptidoglycan associated lipoprotein- linked with the outer

membrane. TolA, Q, and R interact in the inner membrane, and TolB and PAL form complexes near the outer membrane. In addition to its association with the peptidoglycan layer, PAL interacts with TolAs to connect the two membranes (Lazzaroni et al., 1999; Lloubès et al., 2001). The Tol-PAL knockout mutants show defects in cell division – especially in outer membrane constriction and peptidoglycan splitting procedure, hence forming cell chains (Bernadac, Gavioli, Lazzaroni, Raina, & Lloubès, 1998; Kowata et al., 2016; Szczepaniak et al., 2020; Yakhnina & Bernhardt, 2020). The mutation also increases the cell sensitivity to antibiotics and detergents (Bernadac, Gavioli, Lazzaroni, Raina, & Lloubès, 1998; Szczepaniak et al., 2020; Yakhnina & Bernhardt, 2020). Mutation of another abundant lipoprotein, Murein peptide/lpp, in *E. coli* forms a bleb in the outer membrane (Asmar & Collet, 2018; Cascales et al., 2002). Lpp tethers the OM with the PG layer in gram-negative bacteria (Gerding et al., 2007). However, this globular protein has no structural similarity to BB0323 (Brangulis et al., 2019).

Crystal structure of the N-terminal domain of BB0323 (22-202 amino acids) revealed a rod-like structure of a member of the spectrin family similar to EzrA protein, a member of cell membrane divisome complex in bacteria (Brangulis et al., 2019). Cell division in bacteria is controlled by the tubulin homolog FtsZ. Polymerization of this protein in the mid-cell region forms a Z-ring. The Z-ring with actin homolog FtsA recruits other essential proteins to form a divisome complex (Gamba et al., 2009; Osawa et al., 2008; Szwedziak et al., 2014). EzrA, an inhibitor of the polymerization of FtsZ, one of the first proteins to be recruited in the mid-cell area, helps correctly assemble the Z-ring. Deletion of Ezra results in delayed cell division and improperly shaped and irregular-sized cells (Jorge et al., 2011; Steele et al., 2011). BB0323 is a periplasmic

protein containing two spectrin repeats in contrast to 5-100 repeats of cytosolic EzrA. The spectrin repeats have cytoskeletal properties and act as linkers between the cell structures. The distance between the peptidoglycan layer and OM in *B. burdorferi* cells is 11-12 nm approximately, and in the crystallized structure, the N-terminal of BB0323 (BB0323 $_{22\text{-}202}$, not including the first 41 amino acids) is 10.5 nm long, which shows that the N-terminal domain can reach out to PG and OM layer, and possibly act as a linker between cell components and may have a cytoskeletal property (Brangulis et al., 2019). However, the C-terminal domain containing the Lysm domain is essential for interaction with the PG layer. C - terminal mutation shows no issue with cell growth and fission, whereas N-terminal does not associate with the PG layer (Kariu et al., 2013). Interestingly, m-cherry fusion at the C-terminal of BB0323 shows its accumulation in the central cell area initially, in the $1/4^{th}$ and $3/4^{th}$ positions- as the cell grows, representing the future cell division sites, and at the poles after cell division (Takacs et al., 2018); this area fascinatingly coincides with a region of active peptidoglycan synthesis which has a higher concentration of PG and may act as an anchor for the protein (Jutras et al., 2016).

BB0238

Previous attempts of coimmunoprecipitation (Co-IP) and LC-MS/MS approach disclosed multiple interacting partners of BB0323, mainly a HtrA homolog and BB0238 (Kariu et al., 2013). BB0238 is a 27 kDa protein, annotated by NCBI database as a hypothetical protein containing one tetracopeptide repeat (TPR) domain at N-terminal (Groshong et al., 2014; Kariu et al., 2015; NCBI, 2022). The conserved protein is present

throughout the enzootic cycle and follows the expression pattern of BB0323. Notably, it exists in periplasmic space and interacts with the N-terminal of BB0323. The levels of both terminals of BB0323 are diminished in spirochetes deficient in BB0238. However, the N-terminal of B0323 is produced enough to support OM fission, but the infection in mice is impaired. Conversely, the level of BB0238 completely diminished in isolates devoid of BB0323, although mRNA levels remain unchanged. Thus, BB0323 and BB0238 mutually stabilize each other post-translationally. BB0238 interacts directly with BB0323 N-terminal, with its epitope mapped between 120-130 amino acids. The TPR domain, which is generally involved in protein interactions, is not involved in this case. Deletion or alanine substitutions of the 11 amino acids in BB0238 encompassing the BB0323 binding site lower the level of the latter protein over time and render the spirochete non-infectious in mice (Kariu et al., 2015; Thakur et al., 2017). The PPI between BB0323 and BB0238 is vital for the transmission and persistence of LD. In following chapters, we have utilized the knowledge to discover small molecules that can be further explored as novel therapeutics against the disease.

BbHtrA

High temperature requirement A (HtrA), a serine protease, also known as Deg-Degradation of periplasmic protein, is a highly conserved protein found in all domains of life, including bacteria and humans (Clausen et al., 2002; Song et al., 2022; Spiess et al., 1999). The heat shock-induced protein is mostly envelope associated. HtrA mainly functions as a protease and/or chaperone and is a critical factor for homeostasis and

quality control of other proteins in ATP independent manner (Clausen et al., 2011; Harkness et al., 2021). HtrA family members comprise a serine protease domain and at least one C terminal-associated PDZ domain involved in PPI. It stays as a trimeric unit fundamentally and oligomerizes as a hexamers-resting stage, 12 mers, and 24 mers, forming a funnel/cage-like structure, with protease domain at the top and PDZ domains extending out to bind substrate and translocate them to the protease zone (Song et al., 2022). The height of the cage is maintained to exclude the entry of well-folded protein (Clausen et al., 2002). *E coli* contains three homologs of HtrA, namely, Deg P, Deg Q, and Deg S (Clausen et al., 2002; Hansen & Hilgenfeld, 2013; Kim et al., 2011). Deg P is responsible for deleting misfolded periplasmic proteins and guides the unfolded protein to the membrane (Sawa et al., 2011). It comprises an N-terminal protease domain and two C-terminal PDZ domains (Song et al., 2022). Deg Q, a homolog, has high sequential and functional similarity to Deg P. Both proteins consist of two PDZ domains with protease and chaperone functions (Clausen et al., 2002; Waller & Sauer, 1996; Wrase et al., 2011); however, the LA loop- essential in maintaining hexameric structure, is significantly shortened in Deg Q (Song et al., 2022). Deg S protein is attached to the membrane by N-terminal and has one PDZ domain. Upon accumulation of unfolded proteins, Deg S activates, resulting in a cascade of events ensuing in the upregulation of quality control proteins, such as Deg P (Chaba et al., 2011; Hasenbein et al., 2010). The function of DegP switches from a chaperone -refolding proteins at a lower temperature to proteolytic machinery at a high temperature, possibly due to the presence of more substrates during increased temperature. Binding of a substrate by PDZ domain, due to surface exposed hydrophobic region of a substrate, or due to recognition of conserved C-

terminal domain of activator results in a structural and conformational change of HtrA, converting into a multimeric structure with an active catalytic triad (Clausen et al., 2002; Jones et al., 2002; Song et al., 2022).

The proteolytic activity of HtrA results from a catalytic triad composed of amino acids Serine, Aspartate, and Histidine (Ser-Asp-His) (Clausen et al., 2002; Song et al., 2022). Ser is a principal actor, which attacks the peptide bond of the substrate, where Histidine acts as a proton acceptor at the beginning and a donor at the end of the enzymatic action. Asp stabilizes the His and holds it in correct confirmation. Upon activation, Ser attacks the peptide bond of the substrate, releasing H^+; His accepts the proton and is stabilized by Asp. Ser forms a covalent bond with the substrate. This tetrahedral structure is stabilized by an "Oxyanion hole" made from an amide group of Ser. His transfers a proton to the amine leaving group, yielding the acyl-enzyme, which is, in turn, attacked by water with His assistance, releasing the substrate's C-terminal fragment. His now donates the proton to Ser, as it leaves the N-terminal of the substrate resulting in peptide bond hydrolysis (Hedstrom, 2002).

B. burgdorferi genome encodes a single HtrA homolog, BB0104, designated Deg Q family serine endoprotease by NCBI database, named BbHtrA (Coleman et al., 2013; Kariu et al., 2013; Russell & Johnson, 2013). BbHtrA can form a trimer without substrate, has caseinolytic activity in increased temperature conditions (Coleman et al., 2013), and maintains both proteolytic and chaperone activity *in vitro* (Ronzetti et al., 2022). In addition, the protease is present in both soluble and membrane fractions, is secreted out, and degrades extracellular matrix (ECM) fibronectin and aggrecan,

promoting tissue invasion (Coleman et al., 2013; Russell & Johnson, 2013). Among others, BbHtrA is responsible for the processing of a periplasmic protein-BB0323, bacterial membrane protein D-BMPD, an outer membrane protein-LMP1, a chemotaxis protein-Chex, and a porin- P66, thus has a diverse function in OM stability, host-pathogen interaction, immune evasion, chemotaxis, and motility, consequently, has a deleterious effect upon gene deletion (Coleman et al., 2013; Russell & Johnson, 2013; Ye et al., 2016; Zhuang et al., 2018). Spirochetes devoid of BbHtrA show defects in growth and morphology at 37ºC, forming huge blebs and significantly reducing the ability to infect hosts. Notably, it also results in a diminished level of mature N-terminal of BB0323 with elevated full-length protein in higher temperatures (Ye et al., 2016). The interaction and proteolytic processing of BB0323 by the action of BbHtrA is deemed a crucial event. In the following chapter, we have characterized the importance of BbHtrA-mediated precise proteolysis of BB0323 for its optimal function and infection in murine.

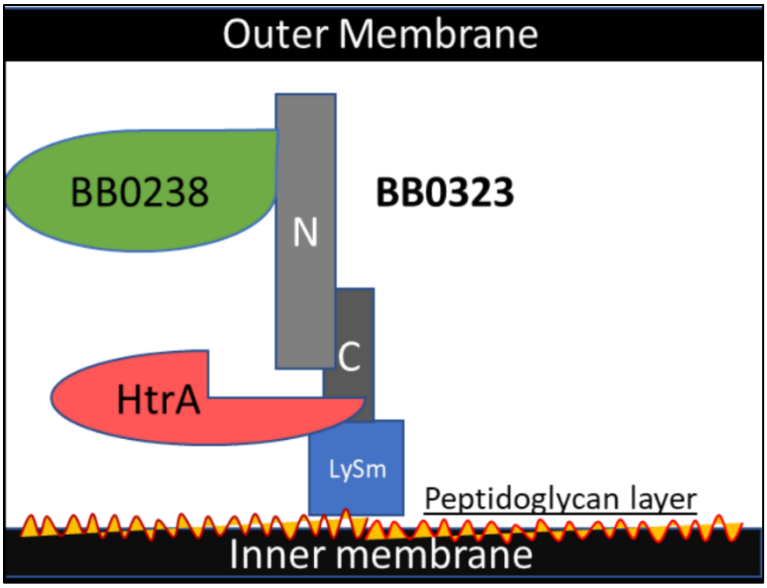


Figure 3. A model displaying BbHtrA mediated processing of BB0323 and its interaction with BB0238 in periplasm of *B. burgdorferi*. The BB0323 protein cleaves into N and C termini by the action of BbHtrA. Both terminals interact with each other. The LysM domain of C-terminal interacts with the PG layer. BB0238 interacts with the N-terminal.

2.0 Research objectives

The spirochetes deficient of BB0323 (in null mutants) cannot divide normally and are non-infectious to murine hosts (Zhang et al., 2009). Full-length BB0323 processing is impaired in BbHtrA deletion mutants, displaying the importance of BbHtrA in the physiology of BB0323 (Ye et al., 2016). However, the exact cleavage site remains undetected. In this study, we focus on elucidating the position of cleavage of the N and C terminus of BB0323 by the action of BbHtrA and explore the biological significance of such proteolytic cleavage. We intend to expand our knowledge on the significance of this cleavage in the growth of *B. burgdorferi* and its ability to sustain in the tick-vertebrate enzootic cycle and maintain the infection. Elucidation of the importance of this phenomenon will help provide opportunities for exploring novel therapeutic targets.

The PPI between BB0323 and BB0238 was studied to divulge that this interaction is critical to the physiology of both proteins and their post-translational stability (Kariu et al., 2015; Thakur et al., 2017). Identifying small molecules that inhibit this interaction will render spirochete non-infectious. We speculate that blocking PPI, essential for infection, is a novel mode of therapeutic strategy to combat *B. burgdorferi*. Unlike

conventional antibiotics that target housekeeping functions and thus can generate a stress-induce response in microbes (Mitosch et al., 2017), the PPI blocking approach targets borrelial virulence and help bypass the side effects of existing therapeutics. It is also possible that these alternate (PPI blocking) approaches will lead to a complete cure and prevent the emergence of PTLDS in the host if used as an antimicrobial. While other functions of BB0238 are being further explored, we are intrigued to identify the inhibitor of the interaction with BB0323, which is essential for infection. Here, in collaboration with National Center for Advancing Translational Science (NCATS), NIH, our lab has established a high throughput small molecule screen assay based on Amplified Luminescent Proximity Homogenous Assay (Alpha) screen technology for the identification of PPI inhibitors of BB0323 and BB0238. The compounds identified by the assay-utilizing recombinant proteins as specific and potent inhibitors are further tested for the effect in native proteins inside the *B. burgdorferi* cells. The secondary, cell-based assay selected the compounds with confirmed activity in cells for further studies.

The *in vitro* assay explained above is inadequate to evaluate the small compounds' effects as therapeutic agents against *B. burgdorferi*, especially in an animal model. Thus, suitable candidates with proper Minimum Inhibitory Concentration (MIC) were identified and were further used for *in vivo* testing in a tick-borne murine model of LD. These compounds selected from a series of prior experiments, including primary alpha assays, TrueHits assays, and cell-based secondary assays, short-listed candidates, are further used in infected mice to reduce the number of spirochetes in tissues. Several preliminary experiments are performed to standardize variables and establish a protocol for animal

testing. Factors such as the injection time, route, and dosage of the compounds are considered.

Pharmacokinetics, a study of the biochemical and biophysical effect of the body on drugs, is an essential tool to determine the drug profile after administration (Yamashita & Hashida, 2013). Distribution of the compounds in the organs is crucial to prevent the colonization of pathogens in the tissues; therefore, it is essential to measure the concentration of the drugs inside the concerned tissues of mice at various time points. Here, this study presents the pharmacokinetic analysis of the selected compound in mice tissues and its analysis by LC-MS/MS.

In conclusion, we herein detailed our efforts to elucidate the biological significance of a protein complex in *B. burgdorferi* essential for infection and presented proof-of-concept evidence that targeting PPI using small molecule therapeutics could be a novel approach to combat LD.

Chapter 2: Materials and methods

2.1 B. Burgdorferi, mice and tick

The spirochetes of the B31-A3 background were used in the study. Stocks of *B. burgdorferi* were kept in cryovials at -80°C freezer in a 70-80% sterile glycerol for long-term storage. The isolates were grown at either 33°C or 37°C in BSK-H media. The *bb0323* deleted mutants were previously produced in the lab; the details of the generation of the mutant are described in a published paper (Zhang et al., 2009). The egg mass of *I. scapularis* ticks was obtained from a tick-rearing facility at Oklahoma State University. The larvae feed on naïve or infected mice placed in a tick-feeding cage with a wire mesh and water at the bottom. Vaseline was applied on the walls of cages, and the lids contained filters; the cages were also placed on sticky papers so that no ticks could escape the area. The fed larvae were collected from the water around three days after tick placement. The collected ticks were incubated in an environmental chamber at 25°C for molting, and subsequent stages were maintained in the lab (Daniel E. Sonenshine, 2013). C3H/HEN mice of four to six weeks of age were purchased from Charles River, and all the experiments on the animals were performed according to guidelines of the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC) of the University of Maryland College park.

2.2 RNA isolation, cDNA synthesis, and quantitative PCR

Total RNA was isolated from ticks and murine tissues using TRIzol Reagent (Invitrogen) and treated with DNase I (NEB). The tissues stored at -80°C were homogenized by mortar and pestle, cooled with liquid nitrogen, and resuspended with TRIzol. 200µl of chloroform was added to every 1 ml of TRIzol reagent for phase separation. The tubes were mixed evenly and centrifuged at 12,000g for 20 minutes at 4°C. The top aqueous phase containing RNA was separated and added with 500µl of isopropanol for 1 hour/overnight at -20°C for precipitation. The tubes were centrifuged again at the abovementioned conditions, the supernatants were discarded, and the RNA pellets were washed with 70% ethanol. The samples were centrifuged at 7500g for 5 minutes at 4°C. The RNA pellets were air-dried for 10 minutes and resuspended with RNase-free- ultra-pure water containing DNase I and DNase buffer. After incubating at 37°C and 75 °C for 10 minutes each, the samples were stored at -80 °C until further use. cDNA was transcribed from RNA using the SuperScript Vilo cDNA Synthesis Kit. The primers used in quantitative PCR (qPCR) are listed in **Table 1.** The primers were designed to amplify PCR products between 75-200 bp and Tm of 55-60°C using OligoPerfect Primer Designer (ThermoFischer Scientific). The cDNA was analyzed by qPCR using Bio-Rad thermocycler using SYBR select master mix (ThermoFischer Scientific). *flaB* cDNA copies were measured and normalized to tick or mouse *β-actin* levels to quantify spirochetes.

2.3 Site-directed mutagenesis

Bacterial protein expression vector pGEX-6P-1 and pET28-b were used to express BB0323 full length and BB0323-terminus spanning 22-242 amino acids (BB0323-N) as previously described (Kariu et al., 2013, 2015). The plasmids were double Alanine substitution mutagenesis templates at $N236^{th}$ and $L237^{th}$ positions. The mutagenic primer for double amino acids substitution was designed by mismatching at the center of primers. The primers contained at least ten nucleotides of plasmid at the 3' end. The forward and reverse primers listed in **Table 1**, had overlapped and proceeded in opposite directions (https://www.neb.com/faqs/2013/01/28/how-do-i-design-primers-to-use-with-the-q5-site-directed-mutagenesis-kit). The template DNA was mixed with Q5 Host Start Master Mix and primers and transferred to a thermocycler for Polymerase Chain Reaction (PCR). The PCR product was incubated at room temperature for 5 minutes with *Kinase, Ligase,* and *DpnI (KLD)* enzyme mix. This step phosphorylates-ligates the new product, and the *DpnI* enzyme degrades the template DNA https://www.neb.com/products/m0554-kld-enzyme-mix#Product%20Information. The KLD mix was transformed into NEB competent cells, and the clones grown in selective plates were isolated and sequenced for the desired mutation. The clones, grown in Luria-Bertani (LB) broth, were mixed with 70-80% sterile glycerol, and stored in a -80°C freezer.

2.4 Generation of recombinant proteins

2.4.1 For cleavage mutation studies

Plasmids from correct clones were isolated from DH5α cells using PureLink Quick Plasmid Miniprep Kit (Invitrogen). The plasmids were transformed into BL21 *E. coli* competent cells, plated in agar plates with ampicillin for pGEX-6p1 and Kanamycin for pET-28-b vector, and incubated at 37°C overnight. The clones were grown overnight in 5ml LB media containing appropriate antibiotics. The culture was used as a starter for 500ml LB in the morning and placed in a shaker at 37°C until OD_{600} reached 0.5. The recombinant protein was induced at 25°C by adding 0.25-0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3-4 hours. The cell pellets were centrifuged and stored at -80°C until further use. Purification of the recombinant proteins that are either fused to Glutathione S-transferase (GST) or without any fusion after removal of the GST tag was performed according to the manufacturer's instructions (GE Healthcare). The pellets were resuspended in 20ml PBS with 1% TritonX-100 added with 1mg/ml lysozyme. The suspension was incubated for 30 minutes in ice, vortexed every 10 minutes, and then sonicated for 5-10 minutes. The lysate was centrifuged at 12,000g for 15 minutes at four °C. The supernatants were incubated in Glutathione Sepharose 4B beads containing a pre-equilibrated column for 1 hour at four °C. The unbound protein in the mixture was drained and washed with 1XPBS 7-10 times. After adequately washing, the beads were incubated with a digestion buffer containing 50mM Tris-Hydrochloric acid (HCl) pH 7.5, 150mM Sodium Chloride (NaCl), 1mM Ethylenediaminetetraaceticacid (EDTA), 1mM Dithiothreitol (DTT) with precision protease, overnight at 4 °C. The proteins were

collected the following day at 2ml for five elutions. The genes were also cloned into plasmid pET-2-8b. The proteins were expressed in *E. coli* cells and purified with 6x His tag using HisPur Cobalt (Thermo Fisher Scientific). The pellets were mixed in a resuspension buffer containing 20mM sodium phosphate, 300mM sodium chloride, and 5mM imidazole pH 7.4 with 1mg/ml lysozyme. After sonication, the soluble protein supernatant was collected and incubated with equilibrated beads in a column for 1 hour at 4°C. As mentioned, the beads were washed with the sodium phosphate buffer with 15 mM imidazole. The proteins were isolated by increasing the imidazole concentration to 150mM. The collected proteins were subjected to appropriate buffer exchange and checked for purity in 12% SDS page gel.

2.4.2 For therapeutic studies

Recombinant proteins GST-BB0238, His-BB0238, GST-BB0323, and His-BB0323 were obtained from previous studies (Thakur et al., 2017; Kariu et al. 2015, 2013). All proteins were expressed in *E. coli* BL21 with either pET28a, pGEX-6P-1, or pET303 expression vectors. Standard expressions were performed with one mM IPTG at 25°C for 4 hours. PET28a and pET303 HIS-Tag proteins were isolated using the manufacturer's instructions using Nickel Beads from Pro Bond. PGEX-6P-1 GST fused proteins were isolated following the manufacturer's instructions (GE Healthcare). Elution with tags was done using imidazole and reduced glutathione as per the manufacturer's protocol. Dialysis of proteins to remove imidazole or reduced glutathione was performed

in dialysis membrane chambers (Sigma) over 24 hours. Proteins were concentrated via Amicon Ultra Centrifugal Filter units.

2.5 Generation of antisera

Mice were injected subcutaneously with at least 10 µg of BB0323 proteins in PBS mixed with complete Freund's adjuvant (CFA) 1:1 (SIGMA). The cloning and isolation of proteins used for antibody production in this study are described elsewhere (Kariu et al., 2013, 2015). The animals were boosted after ten days with the protein and incomplete Freund's adjuvant. On the 21st day, the mice were re-boosted, and sera were collected on the 28th-31st day. The anti-BB0323 murine antisera was used to detect recombinant full-length and N-terminus BB0323, processed N-terminus in BB0323 in *B. burgdorferi* extracts and used in Far Western assays. The antisera could not detect the C-terminus, so we obtained an aliquot of antibody against the terminus raised in chicken from Dr. Philip Stewart at NIH.

2.6 Protease cleavage assay and N-terminal sequencing

In vitro, protease cleavage assay of full-length BB0323 by BbHtrA has been described (Kariu et al., 2013). Briefly, 5ug of recombinant full-length wild type or mutated BB0323 ($BB0323^{NL}$) was incubated with 500 nanograms (ng) of recombinant wild-type BbHtrA in 20 µl buffer (50 mM Tris-HCl pH 7.0, 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol) at 37°C. The reactions were added with sample buffer at the indicated times. Digests were analyzed by western blotting using BB0323 or BbHtrA

antisera, western and far-western blotting (for detection of cleaved C-terminus) using N-terminal BB0323.

Edman degradation technique was used for N-terminal sequencing (Besant & Attwood, 2010). The digested products were transferred to a nylon membrane and stained with amido black. The membrane was cut in size corresponding to C-terminus and was modified with phenyl isothiocyanate (PITC). The terminal amino acid is cleaved off as phenylthiohydantoin (PTH) derivative, thus leaving a new α-amino group and the following amino acid available subsequent PITC reaction. The protein sequence was thereby analyzed for its amino acid compositions.

2.7 Triton X-114 phase partitioning assay

Triton X-114 is a nonionic detergent helpful in separating proteins in different phases. The detergent separates into aqueous and detergent phases above 20°C (Sigma). The assay in this study is applied to check if the mutation of BB0323 protein at $N236^{th}$ and $L237^{th}$ would alter the localization. The biphasic extraction was performed as the protocol described elsewhere (Zhang et al., 2009). The spirochetes were grown until the late log phase and were centrifuged at 8000g for 15 minutes at 4°C. The pellets were washed with ice-cold 1X Phosphate-Buffered Saline (PBS) (pH 7.4) four times to remove the remnants of the BSK buffer. The spirochetes are then resuspended in 800 µl PBS. The mixture was sonicated and extracted with 2% Triton X-114. The mix was centrifuged to obtain aqueous and detergent phage and precipitated separately using ice-cold acetone. Centrifugation at 13,000g for 15 minutes at 4°C pelleted down the proteins, resuspended

them in PBS, and used for SDS PAGE separation and western blot analysis. The location of NL mutated BB0323 was determined using FlaB and OspC as controls.

2.8 Western blotting and Far-Western blotting

The western blotting and far-western blotting analyses were performed, as described (Kariu et al., 2013, 2015; Zhang et al., 2009). The spirochetes growing up to the late log phase were centrifuged and washed four times with ice-cold 1XPBS. The cell pellets were resuspended in 1X laemmli buffer with β-mercaptoethanol (BME) and boiled at 95°C for 10 minutes. The lysates were separated in 12-15% SDS PAGE gel in 100v for 2 hours and transferred in a 0.45µM polyvinylidene (PVDF) membrane 20V for 20 minutes. The membrane was incubated in Ponceau S staining buffer to visualize protein bands and confirm equal loading. The membrane was blocked with 3% skim milk in PBST (0.05% Tween 20) for 1 hour at room temperature. For detection of protein levels by western blot, the membrane was incubated to the related antibody (1:3000) overnight and then with the horseradish peroxidase (HRP) labeled secondary antibody (1:10,000) in PBST for 1 hour. The protein levels were visualized using an Enhanced Chemiluminescent HRP substrate (Thermos Fischer). To detect the C-terminus, the lysates were separated in 15% SDS PAGE gel for far-Western blotting and transferred to a membrane. The blot was incubated with recombinant BB0323-N and detected with an antibody against BB0323-N. To check the proper loading between the wells, the membranes were stripped and blotted against FlaB. ImageJ software measured The protein bands and analyzed them by GraphPad Prism 5.

2.9 Growth analysis of spirochetes

The growth of wild-type, *bb0323* deletion mutant, and *bb0323*^{NL} spirochetes was assessed *in vitro*. Spirochetes were grown at 34°C in BSK medium to early log phase and passaged (10^5 spirochetes/ml) into fresh medium. The spirochetes were cultivated in triplicates at 34°C and 37°C and counted every 24 hours with a Petroff-Hausser cell counter under a dark-field microscope as described.

2.10 Labeling of peptidoglycan in B. burgdorferi

B. burgdorferi Wild type (*Bb*^{WT}), *bb0323* deletion mutant (*Bb*$^{bb0323-}$), or mutant harboring cleavage site mutated *bb0323* (*Bb*^{bb0323NL}) complemented spirochetes were used in the study. The cultures were grown at 37C in BSK medium until the mid-log stage. Fluorescent blue D-amino acid for labeling of peptidoglycans, HADA - 3-[[(7-Hydroxy-2-oxo-2*H*-1-benzopyran-3-yl) carbonyl] amino]-D-alanine hydrochloride was used for the labeling purpose (Jutras et al., 2016). All three strains were incubated with 0.2mM HADA re-suspended in 1% DMSO (Final concentration). The Borrelia treated with only DMSO was used as a control. The samples were spotted on 1% agarose (weight/volume)/PBS pads and imaged in a fluorescence microscope at room temperature. Observer Z1 Motorized Epifluorescence Microscope was used for imaging. The exposure time for epifluorescence imaging ranged from 50ms to 2s, and phase contrast was made at 150ms. The pictures were analyzed using ImageJ software.

2.11 Genetic manipulation of B. burgdorferi

A *bb0323* DNA was inserted in a plasmid pXLF14301 to complement a *bb0323* deletion mutant spirochete previously generated in the lab (Zhang et al., 2009). Plasmid *pXLF14301* is a suicide vector with streptomycin as a selective marker. The vector is targeted for homologous recombination at position *bb0445* and *bb0446* via Allelic exchange. To create mutants with amino acid NL replaced with AA at 236th and 237th amino acid positions. The *pXLF14301* vector carrying *bb0323* complementary DNA was used as a template for substitution double point mutation. The mutagenesis was performed according to the manufacturer's protocol, Q5 Site-Directed Mutagenesis kit (NEB), as described above. The primers used for the purpose are listed in Table 1. The plasmid was sequenced to check the intended mutation. The positive clone was transformed into chemically competent *E. coli* cells to generate enough plasmids. PureLinkTM HiPure Plasmid Maxiprep kit (Invitrogen) obtained a highly concentrated and sterile aliquot of plasmids from 500ml LB culture. Two 50ml tubes of *bb0323* deletion mutant kanamycin borrelia at the mid-log phase were centrifuged, washed with 1XPBS, and resuspended in an electroporation solution (EPS). The mix was electroporated and incubated at 37°C overnight without antibiotics. The culture was plated in 96 well plates with fresh BSK, streptomycin, and kanamycin (Kariu et al., 2013; Samuels et al., 1994; Zhang et al., 2009) out of two clones that showed improper processing of BB0323, one clone designated as $Bb^{bb0323NL}$ was chosen for further studies.

2.12 Infection studies using mice and ticks

2.12.1 Infection by subcutaneous injection

To assess the persistence of spirochete in murine hosts, groups of C3H/HeN mice (three animals/group) were injected intradermally with an equal number (10^5 cells/per mouse) of Wild type (Bb^{WT}), *bb0323* deletion mutant ($Bb^{bb0323-}$) or mutant harboring cleavage site mutated *bb0323* ($Bb^{bb0323NL}$) complemented spirochetes (Zhang et al., 2009). The mice were intradermally injected with the spirochetes. After two weeks of infection, sera were used to assess immunological response against whole cell lysates from wild-type *Borrelia*. Samples of skin, heart, bladder, and joints were isolated 14 days after infection. The level of the spirochetes was measured by analysis of levels of *flaB* transcripts normalized against mouse *β-actin* gene using qRT-PCR. The mice tissues were also cultured in BSK media at 33°C and examined under dark field microscopy for the presence of Borrelia (Kariu et al., 2013; Zhang et al., 2009).

2.12.2 *bb0238ΔIM* animal infection studies

To assess the effect of disruption of BB0323 and BB0238 in long-term infection, mice (3 mice/ group) were injected with 10^5 cells intradermally with Wild type (WT), *bba57* deletion mutant or *bb0238ΔIM*-complemented spirochetes. The procedure of *bba57* deletion and *bb0238ΔIM*-complemention is detailed elsewhere (Thakur et al., 2017; Yang et al., 2013). Immune response against the cell extracts of *B. burgdorferi* (WT) was assessed with serum collected two weeks after the injection to confirm the establishment of infection, at least by positive controls. Eight weeks after injection, the mice were infested with naïve nymph (7 ticks/mouse). Ticks were collected after

complete repletion and were subjected to qRT-PCR analysis to compare the levels of acquired spirochetes. Tick *β actin* levels normalized levels of *flaB* transcripts. Tissues of skin, heart, bladder, and joints were collected on the ninth week and processed to compare levels of spirochete *flaB* transcripts normalized against mice *β actin*. Some parts of the tissues were cultured in BSK media to assess the presence of viable spirochete.

2.12.3 Infection by transmission via ticks

To perform transmission studies by ticks, naïve nymphal ticks were microinjected with spirochetes resuspended in PBS. The ticks were incubated overnight at 25°C and were placed in anesthetized mice, five ticks/mouse, and three mice/group. The animals were placed in tick-feeding cages provided with feed and water. The fully repleted ticks were collected three to five days after the placement. The ticks were immediately stored at -80°C for the future. The ticks were analyzed for spirochete burden using qPCR. The mice tissues were collected after twelve days of tick feeding and analyzed as detailed above. Tissues were also cultured in BSK to observe the presence of spirochetes (Kariu et al., 2013; Zhang et al., 2009).

2.13 Circular dichroism

Circular dichroism (CD) was performed on the proteins after dialysis into 10 mM KPO4 buffer at pH 7.6. The concentrations of the proteins were determined using A280 and specific extinction coefficients. BB0323 had extinction coefficients of 65,320 M^{-1} cm^{-1}, while BB0323 ^{NL}-N and BB0323-N had extinction coefficients of 27,390 M^{-1} cm^{-1}.

CD spectra were collected using a Chirascan CD spectrometer from Applied Photophysics LTD, with a 0.1-mm demountable quartz cell. The spectra were recorded in the wavelength 188 to 280 nm at 25°C, with 1-nm steps and 3-s averaging per data point. Multiple scans showed consistent results. The CD data was converted to mean residue molar ellipticity (degrees per square centimeter per decimole) after subtracting the baseline signal from the buffer. To estimate the secondary structure content, the CD data were analyzed in CDNN deconvolution program, provided by Gerald Bohn in 1977 at the Institut für Biotechnologie, Martin- Luther Universität Halle-Wittenberg, distributed with Applied Photophysics software.

2.14 Microscale thermophoresis (MST)

Microscale thermophoresis assay was performed at NCATS, NIH, using a Monolith NT instrument. The interaction affinity of BB0238 with either BB0323-N or $BB0323^{NL}$-N was measured. 10nm of HST-tagged proteins BB0323 were mixed with NanoTemper Red-tris-NTA dye diluted in PBS-T buffer and incubated with 12 points 1:1 serially diluted GST-BB0238. The mixtures were analyzed at 10% LED and 40% MST power. MO analyzed data from three biological replicates— affinity analysis software-presented by GraphPad Prism 9.

Target engagement of the drug by MST was performed to identify the binding partner of the small molecule compounds. The proteins HST-BB0323 N-terminal and HST-BB0238 were labeled with NanoTemper Red-tris-NTA dye. The methodology for preparing the proteins is detailed elsewhere (Kariu et al., 2013, 2015). Proteins at concentrations of 3nM were mixed with compounds at 1:1 dilution series of 12 points.

After incubation, the mixture was loaded in capillaries and placed in the MST instrument. Data recording and analysis were done as mentioned above.

2.15 Biolayer interferometry (BLI)

The Biolayer interferometry assay was performed at IBBR. Briefly, His tagged BB0323FL, BB0323-N, and BB0323^{NL}-N were diluted in a series to final concentrations of 5.5 nM to 0.7 µM. The Anti-Penta-HIS biosensors containing penta-HIS-antibody were equilibrated in Kinetics Buffer (KB). The results were analyzed with a global fit and a 2:1 heterogeneous ligand-binding model.

2.16 B. burgdorferi cellular assay

The wild type *B. burgdorferi* (strain B31, isolate A3) was grown to the mid-log phase. 100µl of 1x10^6 cells/well were seeded in each well. 2 µl of drugs (Stock - 10mM)/well, to make the final drug concentration 200 µM, were added to the cells. The culture was incubated at 37°C for 16-18 hours. 10mm DMSO (2µl/well) was used as a control. After incubation, 1 µl of culture was added to fresh 200 µl of BSK to observe the re-growth of *B. burgdorferi* after 4-6 days. The rest of the culture was pelleted, and the cells were treated with 20 µl PBS with 1% TritonX100 and vortexed while incubating in ice for 30 minutes. A dot blot assay was performed with 1 µl of the cell lysate. Anti-BB0323 antibody was used as a method of detection of the protein. The assay was performed thrice with at least three technical replicates each.

2.17 Dot blot assay

1 µl of the compound-treated cell lysate was dotted in pre-wetted (1X PBS) and semi-dried nitrocellulose membrane of 0.45µM pore size. The dots were allowed to dry for 10 minutes. The membrane was blocked with 3% milk in Phosphate-buffered saline pH 7.5 with 0.05% Tween 20 (PBST), followed by incubation with antibody against BB0323 N terminal protein overnight (The procedure for the production of antibody against BB0323 N terminal protein described elsewhere) and HRP conjugated anti-mice antibody. Immunoblot was developed using enhanced chemiluminescence. The densities of the blots were further analyzed by ImageJ 2.1.0 software.

2.18 Validation of the cellular assay with a dose-response kinetics

The cellular assay explained above was used for the dose-response effect on PPI inhibition. Serially diluted drugs, 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.125 µM, 1.56 µM, 0.78 µM, 0.39 µM, 0.19 µM, 0.097 µM and 0 µM were used for the purpose. The compounds were diluted in DMSO, and 2 µL of the compounds were added to 100µl of culture and incubated at 37°C for 18 hours. As stated above, the cells were subjected to dot blot and regrowth analysis. The assay was performed at least three times with at least three technical repeats.

2.19 Validation of the efficacy of the compounds in mice model of LD

The mice were infested with spirochete-infected nymphal ticks. Five mice per group were used for the experiment. Treatment with the compounds, ceftriaxone, or control started after the final tick fall (4-5 days). The treatment was continued for 21 days, once or twice daily, depending on the dose regime. The effect of the treatment was checked by tick acquisition, serology, tissue culture, and qRT-PCR.

2.20 Enzyme-linked immunosorbent assay (ELISA)

96-well plates (Nunc) were coated with 500ng of Borrelia B31 lysates overnight at 4°C and blocked with 1%BSA in PBS for 1 hour at room temperature. The wells were incubated with the Borrelia-infected serum at 1:3000 concentration and incubated for 1 hour at 37°C, followed with HRP-conjugated goat anti-mouse IgG, 1:10,000, and was incubated for 1 hour at RT. The signal was developed with TMB peroxidase substrate reagents, stopped with 0.1N Hydrochloric acid, and read at 450nm. The assay was done in triplicates at least two times.

2.21 Formulation of compounds

Compound NCGC00687633/VCH-222/Lomibuvir

5mg compound in a clear vial was added with 50 uL of NMP and sonicated/vortexed to get a clear solution. 550 uL PEG300 was added to the mix and

sonicated to mix well. Furthermore, 400 uL of water was added and mixed well using sonication for a clear solution.

2.22 Pharmacokinetics study

A total of 18 mice 3/data points were selected for the study. The animals were injected with 10^5 cells of *B. burgdorferi* B31, resuspended in 1X PBS, subcutaneously. The treatment regimen started immediately.

The animals were injected with 15 mg/kg of small molecules/day for 21 days. The injections were done intraperitoneally. The optimal dose was decided based on previous animal studies of the compound. The route of administration and dose rate was standardized through all mice to warrant consistent results.

The samples were collected on days 0,3,6,9,15,21. Three mice were sacrificed at each data point. The tissues were collected 24 hours after the last injections. Blood was drawn by the submandibular vein and cardiac puncture (non-survival). The whole blood was placed in a Vacutainer tube containing Heparin. The contents were mixed thoroughly, placed in ice, and centrifuged. The collected plasma and other organs were stored at -80°C until further analysis.

Tissues were analyzed at ChemoGenics Biopharma, NC. The samples were homogenized and analyzed by LC-MS/MS method to determine compound concentrations in mice tissues. Sciex API 5500 (MDS Sciex, Ontario, Canada) Agilent 1260 with thermostat and CTC PAL autosampler were used for the procedure.

Plasma preparation- 45 uL of plasma was precipitated with 3X acetonitrile, vortexed, and centrifuged.

45 uL of plasma were taken in centrifuge tubes, then spiked with the standard solutions and vortexed. 70 uL of the supernatant was transferred into vial inserts and injected into the LC/MS/MS system.

Other tissue samples- Sterile PBS were added to the tissues, four times their weight, and subjected to homogenize with an Omni Bead Ruptor instrument. The 50 µl tissue samples were kept in a 96-well plate, where 150 µl of acetonitrile was added. The plate was sonicated for 30 seconds and centrifuged. The supernatant was collected on a clean plate. Five microliters of the samples were injected into the LC-MS/MS system at 50 °C. Data analysis: Analyst® software (Version 1.7.1) was used to capture the data and further analysis.

2.23 Small molecule library

The small molecules were synthesized and obtained from NCATS, NIH. One of the studies was also performed with the compound purchased commercially (MedChem Express).

2.24 Statistical analysis

All the results are presented in mean ± standard error of the mean (SEM). GraphPad Prism software, version 4.0-9.0, was used to measure the significant difference and which was evaluated using student's t-test or ANOVA.

Table 1. Oligonucleotide primers used in this study

Sequence (5'→ 3')	**Purpose**
CAGTCACTTAGCCGCTTTAAATACTAATAAAGACACTTATC	Forward primers for N236A/L237A site-directed mutagenesis
TTTCTTTCTTTAATGAATGCTCTAC	Reverse primers for N236A/L237A site-directed mutagenesis
TTGCTGATCAAGCTCAATATAACCA	Forward primer *B. burgdorferi flaB* qRT-PCR
TTGAGACCCTGAAAGTGATGC	Reverse primer *B. burgdorferi flaB* qRT-PCR
AGAGGGAAATCGTGCGTGAC	Forward primer mouse *β-actin* qRT-PCR
CAATAGTGATGACCTGGCCGT	Reverse primer mouse *β-actin* qRT-PCR
GGTATCGTGCTCGACTC	Forward primer tick *β-actin* qRT-PCR
ATCAGGTAGTCGGTCAGG	Reverse primer tick *β-actin* qRT-PCR

Chapter 3: Post-translational processing of BB0323 and its biological significance

Abstract

BbHtrA mediated cleavage of BB0323 is previously characterized *in vitro.* However, the biological significance, particularly the relationship between proteolysis and its effect on the physiology and pathogenicity of the spirochete remained unknown. To elucidate the significance of the phenomena, we have identified the BbHtrA-mediated N- and C-terminal cleavage site of BB0323 through N-terminal Edman sequencing of the C-terminus produced *in vitro* by the action of the protease. The cleavage site was identified as Asparagine, N (position 236) and Leucine, L (position 237). A mutated BB0323 recombinant protein with the altered cleavage site - N-236 and L-237 (NL) to Alanine-Alanine (AA) was generated , which became resistant to proteolysis by BbHtrA *in vitro*. Surprisingly, unlike BB0323 null mutants that display fission defects, severe OM blebbing and growth impairment, spirochetes carrying mutated BB0323 protein display normal growth and morphology. In fact, the mutant *Borrelia* produced N-terminus BB0323 protein, albeit in a lower level, but with at least two lower aberrant bands. This could be due to the nonspecific activity of BbHtrA in other NL sites present in the sequence of BB0323 in the absence of the conventional location. Notably, despite a certain degree of BB0323 processing and normal growth and morphology, the cleavage site mutated spirochetes still could not infect mice. The results demonstrate that precise cleavage of BB0323 into N and C termini at N236 and L237 amino acid positions is

essential for the maintenance of infectivity in the murine host, and prevention of this cleavage can be appropriate for the establishment of novel anti-*Borrelial* targets.

3.1 Introduction

As detailed in Chapter 1, the vaccine for LD is currently unavailable for humans, and although effective antibiotic treatment is available, in about 20% cases later complications of PTLDS develops. Usually, the efficacy and better outcome of antibiotic treatment depend on timely diagnosis and prompt treatment initiation (Mead, 2015). However, a precise and reliable diagnostic for LD, especially one that can detect early diagnosis is lacking. This warrants the discovery of novel strategies and microbial targets for prevention, diagnosis, and treatment. Several studies have elucidated the bacteria's biology, physiology, and pathogenicity, but the mechanism is vastly unknown. Several proteins are studied for their structure and function during the establishment of spirochete infection, although in most cases their precise molecular functions remain enigmatic (Radolf et al., 2012). One such gene product is BB0323, an immunogenic protein increased in the tick-mouse phase, which is indispensable for *B. burgdorferi* infection. The protein primarily exists as a subsurface protein in spirochetes and is consistently expressed in the skin, heart, and joints during the mammalian infection. The expression is more in early-stage cells than in the late-stage ones grown *in vitro.* BB0323-deficient spirochetes could not be transmitted by the ticks, and the mice were not infected even via injections (Zhang et al., 2009).

A GST-pulldown assay displayed that GST-BB0323 coupled with Glutathione

Sepharose could pull down BbHtrA. Notably, in the spirochetes, BbHtrA is also present in the periplasmic compartment as BB0323, even though the exact subcellular location where BB0323-BbHtrA interacts remains unknown. Incubation of full-length recombinant BB0323 (rBB0323FL) with recombinant BbHtrA (rBbHtrA) at 37°C results in a gradual increment of N-terminus at 35kDa molecular weight (MW) region; in addition, C-terminus fragment corresponding to 15KDa also increased over the time. In contrast, incubation with BbHtrA devoid of catalytical activity, in which a serine present at the 226th amino acid position is mutated to alanine (rBbHtrA S226A), did not result in discernible lower fragments of BB0323 (Kariu et al., 2013).

Herein, the current study underscores the importance of precise BbHtrA-mediated processing of BB0323 protein during maturation and its importance in forming an optimal complex with BB0238, underscoring the PPI function in spirochete infectivity. The results displayed by the following experiments also present the gene products as vital therapeutic targets to thwart LD.

3.2 Results

3.2.1 Proteolytic cleavage of recombinant BB0323

For the identification of the cleavage site in BB0323 protein by the proteolytic effect of BbHtrA, we performed an *in vitro* digestion assay. The full-length BB0323 recombinant protein was incubated with BbHtrA at 37°C. The digested product was separated in 15% SDS PAGE gel, transferred to a nylon membrane, and stained with an

amido black stain. A prominent band between 11 and 17 kDa molecular weight, corresponding to the size of the C-terminal **(Figure 4A)**, was excised, and processed for the N- terminal sequencing by the Edman degradation method. Sequencing shows that the first six amino acids matched BB0323 residues LLNTNK, covering sites 237 – 242 **(Figure 4B-C)**, indicating that the C-terminus originated from the 237th residue of BB0323. These results show that the probable cleavage site in BB0323 by the action of BbHtrA are asparagine 236 (N236) and leucine 237 (L237) amino acids.

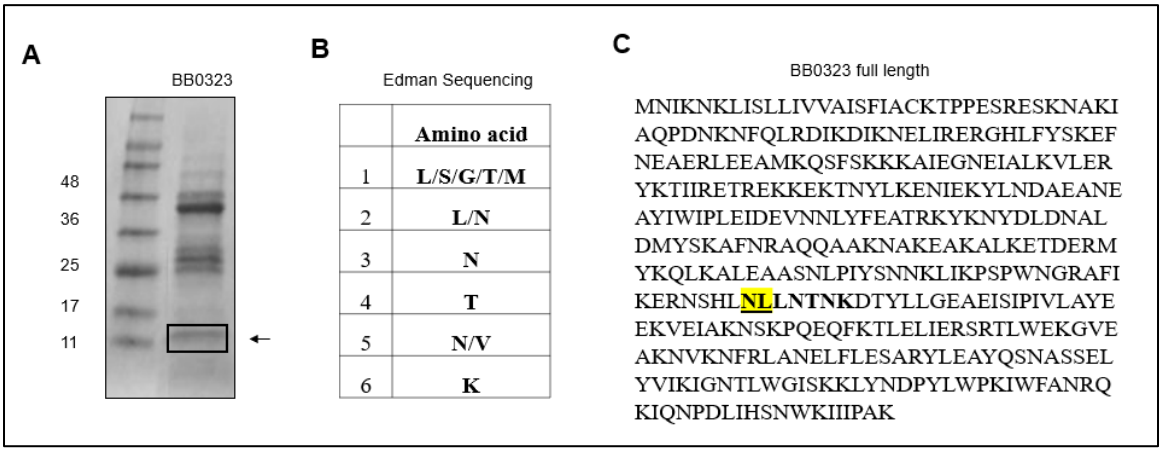


Figure 4: Identification and localization of BbHtrA mediated cleavage site in BB0323 protein. (A) Digestion of full-length BB0323 by BbHtrA. Full-length BB0323 was incubated with BbHtrA in a digestion buffer at 37 °C for 2.5 h. The digested product was boiled, separated in 15% SDS PAGE gel, and transferred to a nylon membrane. After incubation with Amido black, a prominent band between 11 and 17 kDa corresponding to the size of the C-terminal (indicated by an arrow and a box) was excised and processed for N-terminal sequencing. (B) N-terminal sequencing of C-terminal. The N-terminal sequencing of the C-terminal by the Edman degradation method. The sequence of the first six probable amino acids of the C-terminal. (C) Localization of the amino acids in full-length BB0323 protein sequence. The amino acid residues composing the cleavage site are underlined and highlighted (Yellow).

3.2.2 Mutation of the cleavage site affects BB0323 proteolysis

In silico analysis of BB0323 protein using I-Tasser on high-performance computing systems available at the National Institutes of Health (NIH), based on the available crystallized section of the N-terminal, predicted the surface availability of the

N236 and L237 residues **(Figure 5 A- B).** The figure shows probable surface exposure of the cleavage site **(Figure 5B).** Site-directed mutagenesis was performed to confirm further the cleavage site involving amino acids. Alanine substituted the amino acids at positions 236 and 237. The mutated protein was named BB0323^{NL} **(Figure 5A).** Alanine being polar, hydrophobic, aliphatic, and possessing a similar isoelectric point (PI) as that of asparagine and Leucine, is expected to produce the least untargeted effects (Gray et al., 2017; Lefevre, 1997).To examine whether the mutation of the residues affected the cleavage of BB0323 by proteolytic effects of BbHtrA, the BB0323^{NL} protein was subjected to a digestion assay as stated in the method section and described elsewhere (Kariu et al., 2013). The recombinant wild-type BB0323 and BB0323^{NL} were incubated with recombinant BbHtrA protein in a digestion buffer. The samples were collected at various time points, separated in a 15% SDS PAGE gel, and transferred to a nitrocellulose membrane. In the case of the wild-type BB0323 panel, the band of N-terminal detected with antibody produced against BB0323 N-term was observed at around 35 kDa MW, with increased intensity over time, along with decreasing levels of the full-length protein (**Figure 5C, right panel).** As expected, we also observed elevated levels of C-terminal, detected by far-western blotting with BB0323-N term protein and antibody produced against it (Toru et al., 2013). In contrast, the incubation of BbHtrA with BB0323^{NL} at 37°C in the digestion buffer could not produce N and C termini **(Figure 5C, left panel).**

Notably, an aberrant polypeptide slightly lower than 35 kDa was present in mutant *B. burgdorferi* cell **(Figure 5C, left panel, arrow).** To confirm that the unusual peptide, which co-purifies with the mutated full-length protein, is not the N-terminus

protein produced by the action of BbHtrA; here, we have used a mutated version of BbHtrA (does not have protease activity-HtrA SA) to incubate with BB0323^{NL} protein **(Figure 5D).** The recombinant BB0323^{NL} was incubated with recombinant BbHtrA S226A(SA), and the cleavage was tested at various time points. As expected, we observed that irrespective of the presence of HtrA SA, the aberrant peptides were present at all the time points, as shown in the previous figure. The density of full length did not decrease over time, and there was no appearance of C-terminus protein **(Figure 5D, middle panel),** confirming that the aberrant peptide was not produced due to the action of BbHtrA.

Overall, the digestion assays showed that the mutation of BB0323 protein at the 236th and 237th position to double Alanine prevented the protein's cleavage by the protease's action. This is the position for the primary division of BB0323 into mature polypeptides.

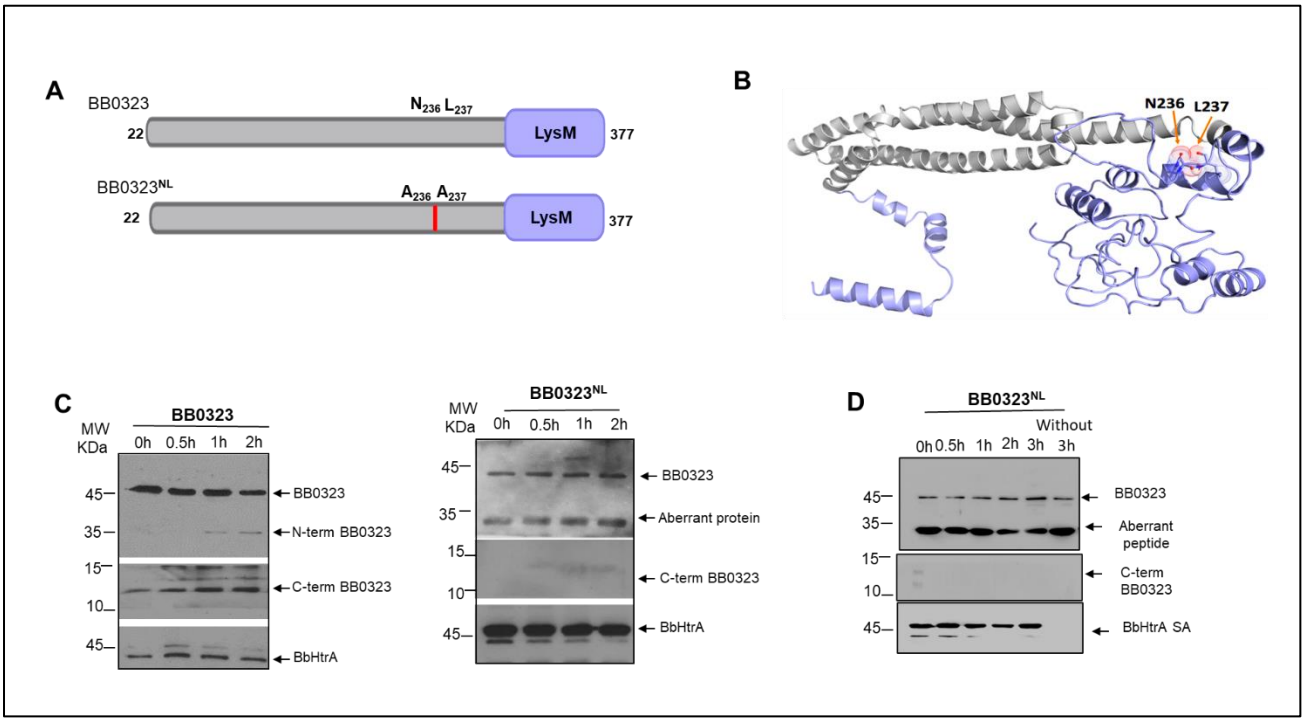


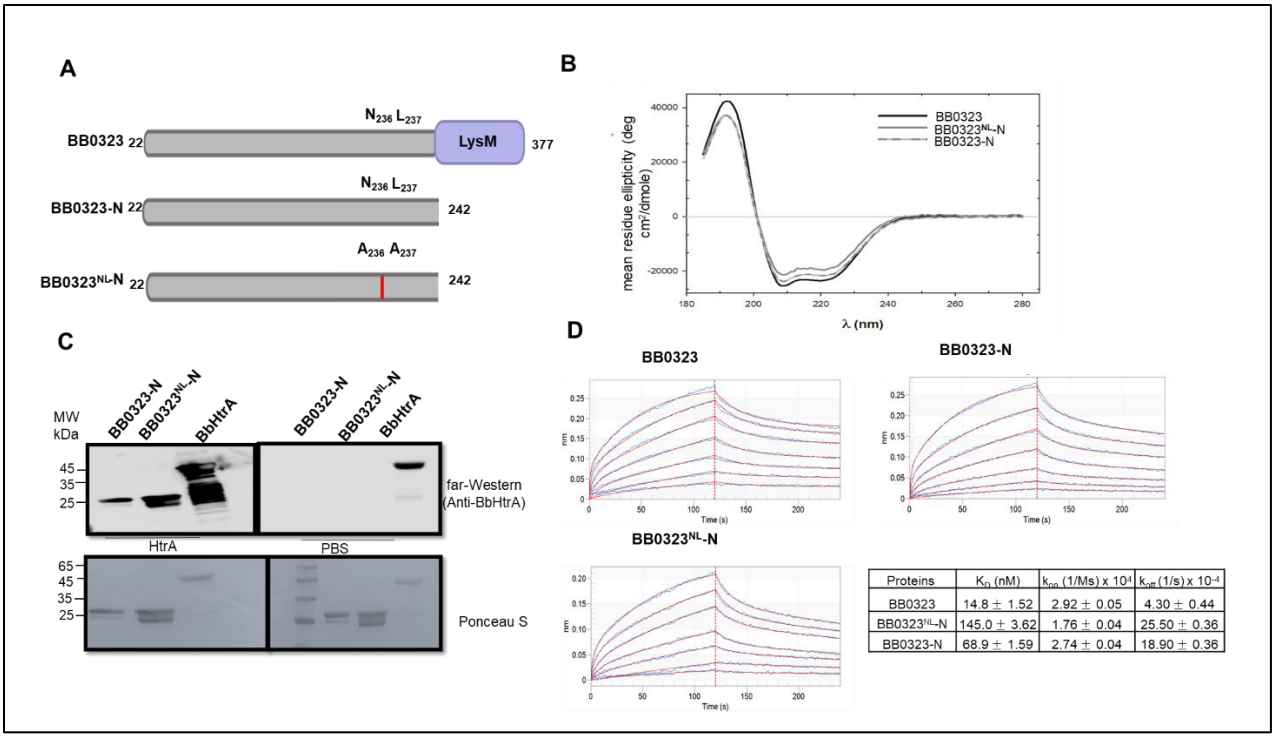
Figure 5. Site-directed mutagenesis of BB0323 and confirmation of the cleavage site. (A) Representation of wild-type and cleavage site mutated BB0323 full-length proteins without signal peptide sequences. Replacement of asparagine (N) at 236th and Leucine (L) at 237th position by double Alanine (AA), shown in the lower panel (red band). (B) The BB0323 model predicted by I-TASSER highlights the position of the BbHtrA-mediated cleavage site, N236 and L237. Additionally, the model showcases homology-guided five N-terminal alpha-helices. (C) The proteolytic processing of BB0323 is hindered by cleavage site mutation. The incubation of full-length BB0323 and BB0323^{NL} at 37°C was carried out for the specified time points. The samples were separated in 15% SDS PAGE gel and were immunoblotted using specific antibodies. BB0323 full-length, N-terminal (arrow, upper panel), and C-terminal (arrow, middle panel) were detected. The amount of protease used in the assay was determined using an anti-BbHtrA antibody (arrow, bottom panel). In the digestion of BB0323^{NL}, there was no detectable presence of N or C-termini, except for the abnormally reactive polypeptide. (D) Digestion of Full-length BB0323^{NL} with HtrA SA. BB0323^{NL} protein was incubated with BbHtrA SA protein at 37°C over indicated periods. SDS PAGE separated the samples, transferred them to a nitrocellulose membrane, and probed with either BB0323 N-term or C-term BB0323 or BbHtrA antibody. The aberrant peptides were present at all the time points, the density of full-length BB0323 does not decrease over time, and there is no appearance of C-terminus protein.

3.2.3 Characterization of mutated BB0323

To assess if the mutation in the protein altered the binding affinity of BB0323 and BbHtrA. We performed a far-western assay. The full-length BB0323 protein is difficult to purify, so we did further *in vitro* analysis using N-terminal protein containing 22-242 residues, BB0323-N; NL to AA mutated version at 236th and 237th position of this protein is termed as BB0323^{NL}-N. To determine if the mutation changed the secondary structure of the protein BB0323^{NL}-N, we employed Far-UV circular dichroism (CD), as detailed in the previous chapter for the methods section. We found that the α-helicity and β-turn of full-length BB0323 protein and 2% and 11%, for BB0323-N and 12% and BB0323^{NL}-N was 62% and 13%, respectively. The results confirmed that the secondary structure of BB0323^{NL}-N protein was not altered and was comparable to BB0323-N **(Figure 6A-B)**.

We further performed the far-western assay, which displayed that the mutated protein still interacted with the BbHtrA protein. Recombinant proteins BB0323-N, BB0323^{NL}-N, and BbHtrA were separated in SDS page gel and transferred to a nitrocellulose membrane. The first set **(Figure 6C)** was incubated with BbHtrA protein, and the second set was with PBS only. After washing the membrane with PBST, a western blot was performed with an anti-BbHtrA antibody. The production details of anti-BbHtrA antibodies are described elsewhere (Ye et al., 2016a). The PBS panel was performed to confirm that BbHtrA antibodies do not nonspecifically probe BB0323-N and BB0323NL-N proteins incubated with only PBS instead of BbHtrA and probed by antibody against BbHtrA. The assay showed that the interaction between the proteins was specific.

To determine the difference in affinity between the wild-type truncated BB0323-N and mutated BB0323^{NL}-N proteins with BbHtrA, we used Bio Layer Interferometry (BLI). The results showed a highly tight affinity of Full-length protein (K_D~15 nM), while the truncated protein BB0323-N had (K_D~70 nM). The K_D value of the BbHtrA interaction with the cleavage site mutated protein BB0323^{NL}-N was substantially higher than the wild-type proteins, i.e., 145 nM, displaying a significant decrease in the binding affinity **(Figure 6D).**



Proteins	K_D (nM)	k_{on} (1/Ms) x 10^{-4}	k_{off} (1/s) x 10^{-4}
BB0323	14.8 ± 1.52	2.92 ± 0.05	4.30 ± 0.44
BB0323NL-N	145.0 ± 3.62	1.76 ± 0.04	25.50 ± 0.36
BB0323-N	68.9 ± 1.59	2.74 ± 0.04	18.90 ± 0.36

Figure 6. Compared to BB0323 with native cleavage sites, the mutated BB0323 N-term exhibits a lower binding affinity to BbHtrA. (A) A diagram representing the BB0323- N term (22-242 amino acids) and the full-length proteins. The native cleavage site of BB0323 is shown in both full-length and truncated N-terminal proteins. The bottom panel presents the mutation of the cleavage site BB0323-N designated as BB0323^{NL}-N. The double Alanine (AA) mutation residues at N236th and L237th positions are indicated (red band). (B) The mutation in cleavage sites does not impact the secondary structure of the BB0323 protein. The circular dichroism technique was employed to analyze the protein structures. The BB0323-N and BB0323^{NL}-N proteins contained comparable percentages of α and β helices. (C) Far-Western analysis of BB0323-BbHtrA interaction. The recombinant N-terminal BB0323 protein carrying the native BbHtrA cleavage site (BB0323-N) and its mutated version BB0323^{NL}-N, were separated by SDS-PAGE, and transferred to a nitrocellulose membrane, incubated with recombinant BbHtrA (left panel) or PBS(Right panel), and probed with anti-BbHtrA antibodies. (D) Depiction of BB0323-BbHtrA interaction using Biolayer Interferometry (BLI) sensorgrams. The BLI findings indicated that the BB0323^{NL}-N had weaker interaction with BbHtrA than the proteins with native cleavage sites. The blue lines show the experimental data, and the smooth red lines indicate the fitted model for each protein and ligand concentration. The chosen model was selected for the best fit for all data rather than the individual ones. The red dotted lines represent the breakpoint between the 120-second association and 120-second dissociation, as detailed Materials and Methods section. The table summarizes the kinetic and thermodynamic interaction parameters between BB0323 proteins and BbHtrA.

3.2.4 Proteolytic cleavage of native BB0323 in B. burgdorferi and impact on spirochete fission or growth in vitro

To investigate the significance of cleavage in BB0323 mediated by BbHtrA, a cleavage site mutant *B. burgdorferi* was produced. Site-directed mutagenesis in the *pxlf14301* plasmid containing the *bb0323* gene was performed and was electroporated in the *bb0323* deleted spirochete and was denoted $Bb^{bb0323NL}$ **(Figure 7A)**. The mutant is expected to produce $BB0323^{NL}$ instead of the wild-type protein. The plasmids analysis displayed that the $Bb^{bb0323NL}$ isolates retained all the essential plasmids **(Figure 7B)**. In addition, $Bb^{bb0323NL}$ showed no defects in growth at both 35°C and 37°C **(Figure 7C)**. The spirochetes under a dark field microscope had no discernible clumping effect or fission defects as the *bb0323* deletion mutants (Fig. not shown).

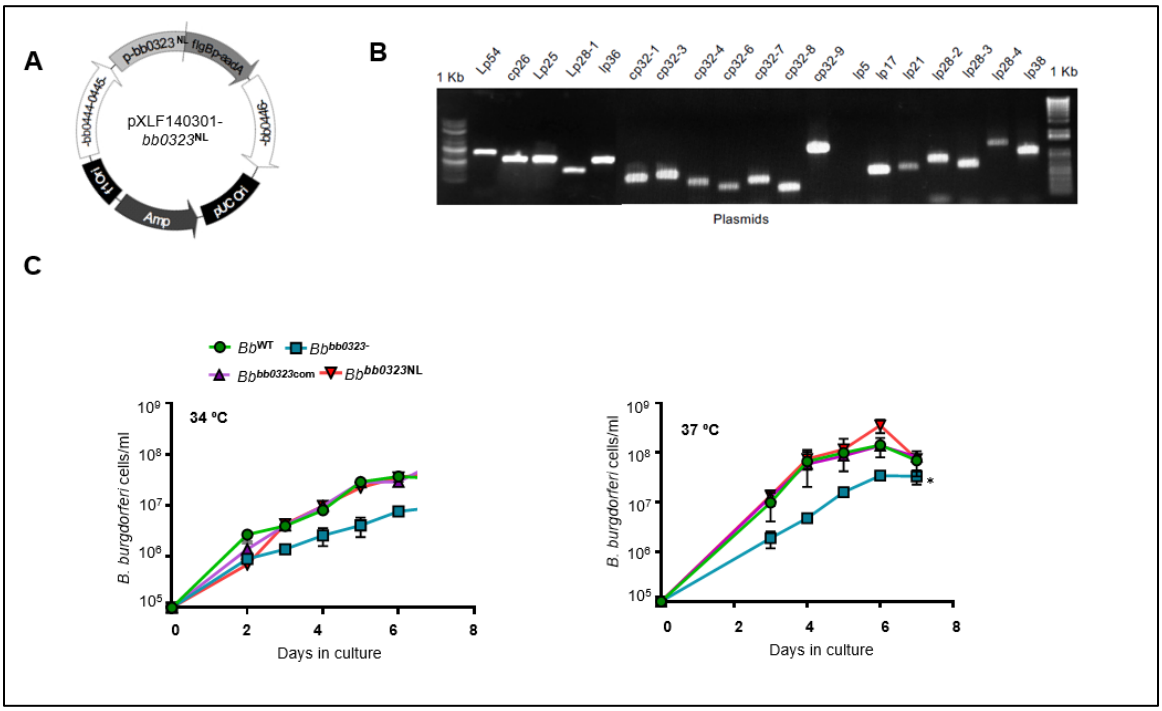


Figure 7. Mutation of the cleavage site in BB0323 does not impact Borrelial cell fission. (A) The vector construct, *pXLF14301-bb0323*^{NL}, used to introduce mutation, is illustrated in the diagram. This construct was created by introducing double-point mutations in the *bb0323* gene and was electroporated inside *bb0323* deleted spirochetes resulting in the Borrelia that produces bb0323^{NL} protein, designated as *Bb*^{bb0323NL} isolates. (B) Plasmid analysis of *Bb*^{bb0323NL} mutants. The *Bb*^{bb0323}NL possesses all but lp5, a non-essential plasmid. Specific primer sets were used to conduct polymerase chain reaction (PCR) on the genomic DNA samples taken from the mutant isolates. (C)The *Bb*^{bb0323NL} mutants exhibit normal growth patterns *in vitro* compared to wild-type spirochetes. Borrelia cells diluted to 10^5/ml were incubated in BSK-H media at 34°C and 37°C. The cultures were enumerated once every 24 hours using a Petroff-Hausser cell counter under a dark field microscope. Compared to other isolates, the *bb0323* deletion mutant (*Bb*$^{bb0323-}$) exhibited significant growth abnormalities.

3.2.5 Mutating the cleavage site in BB0323 in B. burgdorferi generated abnormal polypeptides

Unexpectedly, the cleavage site mutants produced the mature 27-kDa N-terminal and at least two abnormally processed polypeptides of slightly lower molecular weights. The anti-BB0323 antibody probed the lower polypeptides and was present in cells grown at 25°C, 34°C, and 37°C **(Figure 8A)**. The exact reason for the presence of the lower two bands is unknown; however, there are two other pairs of Asparagine and Lysine at 147-148 (Blue) and 207-208 (Red) that could be nonspecifically cleaved **(Figure 8B)**. The results show that producing a mature band at 27 kDa size by the cleavage site mutant was enough to support average cell growth and morphology, irrespective of the lower two polypeptides. Additionally, the mutation did not alter the localization and membrane association of BB0323 and BB0238 (an interacting partner). The phase partitioning assay by Triton X-114 displayed that *Bb*^{bb0323NL} was present in the detergent as the wild-type **(Figure 8C).**

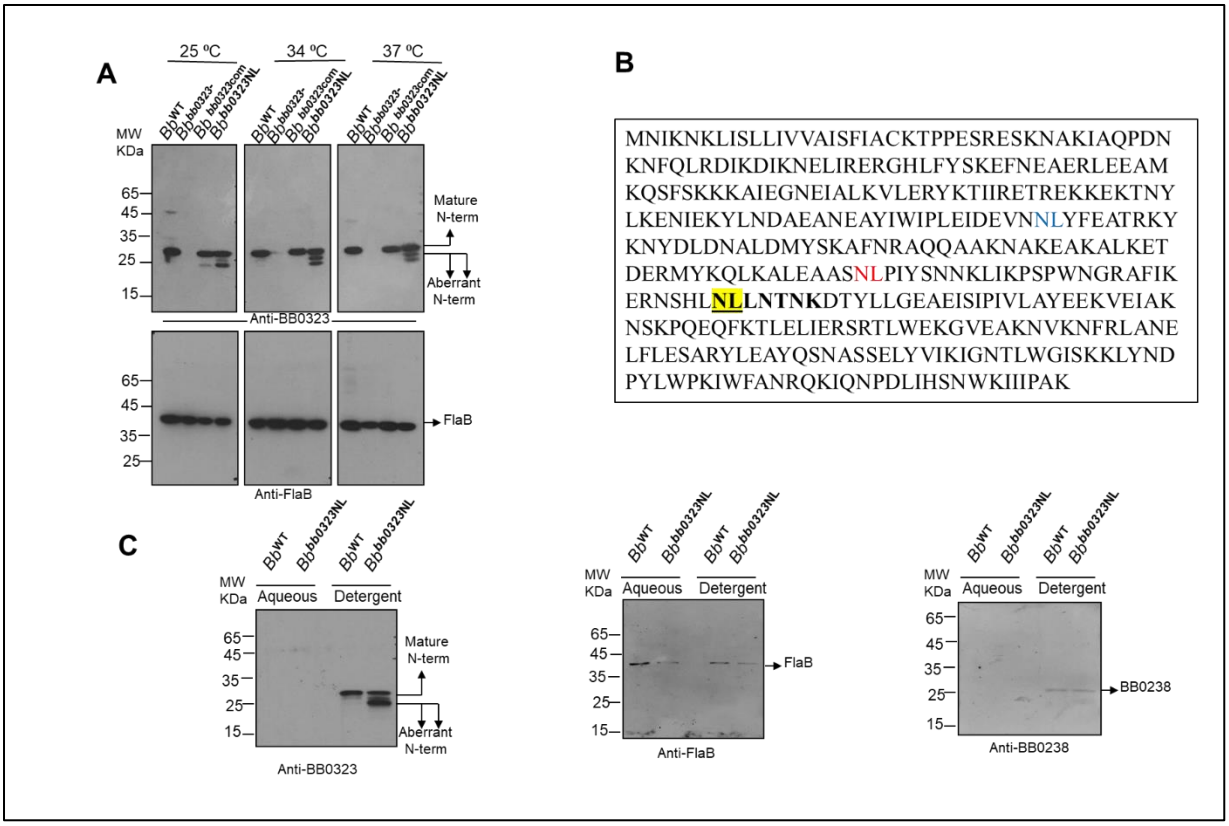


Figure 8. Mutation of the cleavage site in BB0323 results in unusual processing of the N-terminal domain without impact on localization. (A) Production of N-terminal polypeptides in *B. burgdorferi* isolates. Temperatures ranging from 25°C to 37°C were used to grow wild-type (*Bb*^{WT}), bb0323^{-} mutant (*Bb*$^{bb0323-}$), bb0323 complement (*Bb*^{bb0323com}), and cleavage site mutant (*Bb*^{bb0323NL}). After growth, the spirochetes were centrifuged and lysed, and the resulting lysates were subjected to SDS PAGE separation and transferred onto a nitrocellulose membrane. The blot was probed with antiserum targeting BB0323 N-term (upper part), and even loading was confirmed with FlaB (lower part). The image reveals the presence of mature N-term at 27kDa MW(arrow upwards) and the polypeptides with slightly lower MW (arrow downwards). (B) The amino acids in Full length BB0323 sequence that make up the primary cleavage site at N236th and L237th positions (underlined and highlighted yellow), and other probable sites where the cleavage can occur after mutation in the primary site, NL (147-148) (Blue), NL (207-208) (red). (C) Localization of BB0323 N-term proteins in detergent soluble fractions. Phase partitioning assay of the spirochetes using Triton X-114 separates the membrane-bound and soluble proteins into aqueous and detergent phases. The separated portions were boiled at 95°C in Laemmli buffer, subjected to the SDS PAGE separation, and transferred onto a nitrocellulose membrane. Antibodies against BB0323 N-term, BB0238, and FlaB were used to check the localization of the proteins.

3.2.6 A precise BB0323 proteolytic cleavage in B. burgdorferi is required to support microbial infectivity in mammals

To assess the effect of cleavage site mutation of BB0323 in the infectivity of Borrelia, mice were subcutaneously injected with 10^5 spirochetes of either wild-type, *bb0323* deletion mutant or *Bb*^{bb0323NL} isolates. After fourteen days of injection, the animals were euthanized, and several organs were collected. The antibody response generated against *Borrelia* was detected by immunoblot analysis, where wild-type lysates were separated in 12% SDS PAGE gel and probed with the sera collected from the mice. The blot displayed that comparable to *bb0323* deletion mutants, the cleavage site mutants *Bb*^{bb0323NL} did not produce any antibodies against the bacterial proteins. In contrast, the antibody production in mice infected with wild-type was robust **(Figure 9A).** In addition, the tissues cultured from the mutant groups did not have any detectable spirochetes in contrast to the wild-type group **(Figure 9B).** Mice tissues such as skin, heart, and bladder were tested for spirochetes; qPCR analysis was performed to check the *flaB* load per β-actin of mice tissues. The levels of *Borrelial* burden in both the mutant groups were mostly indiscernible, unlike the wild-type infected groups **(Figure 9C).** Overall, the results indicate that the same cleavage of BB0323 at the N236th and L237th positions is crucial to maintain the infectivity of the Borrelia in the murine hosts.

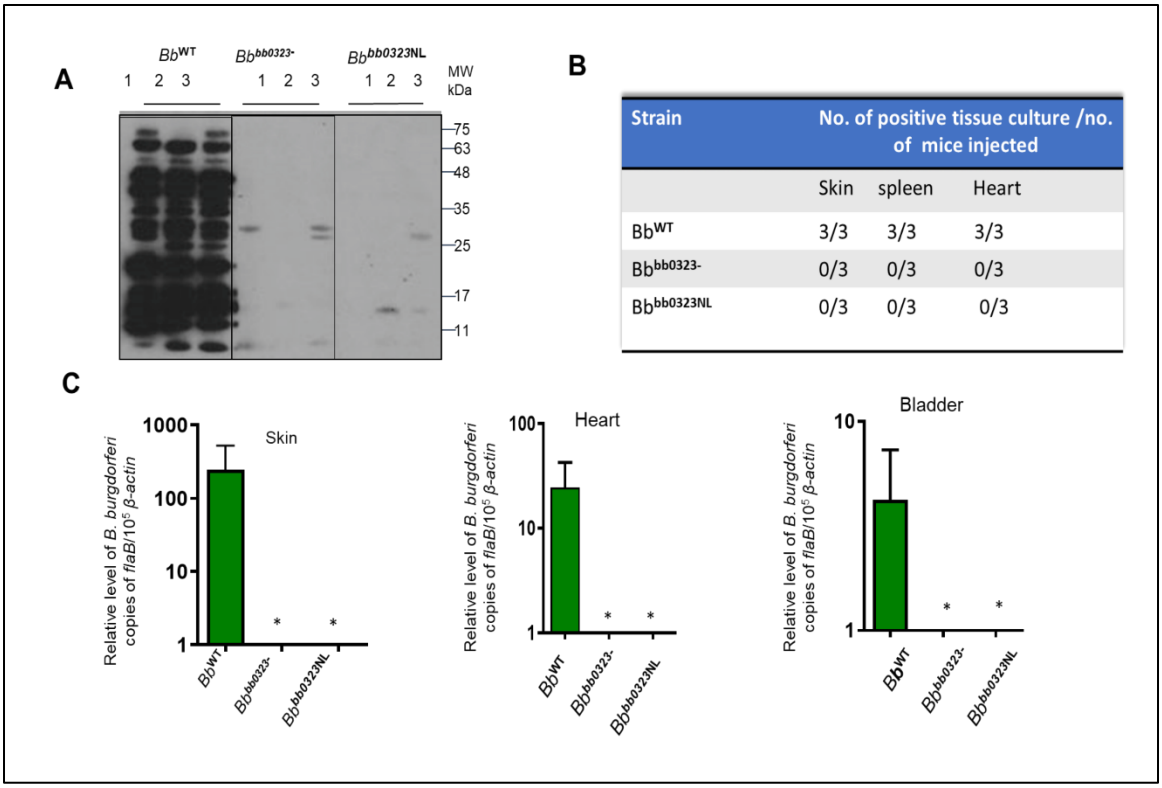


Figure 9. Cleavage of BB0323 by the action of BbHtrA is a crucial factor for spirochete infectivity in mice. (A) Examination of serological response of infected mice against the spirochetes. Mice were subcutaneously injected with 10^5 cells of wild-type (Bb^{WT}), *bb0323* deletion mutants ($Bb^{bb0323-}$), and cleavage site mutants ($Bb^{bb0323NL}$). Sera were collected fourteen days after the inoculation and immunoblot was performed against the wild-type *Borrelia* lysates. (B) Analysis of cultured tissues collected from the *Borrelia*-infected mice. The organs (Skin, spleen, and heart) collected from mice fourteen days after the injection of spirochetes were incubated in BSK-H media at 34°C and checked for the presence of *Borrelia* under a dark field microscope at weeks two, three, and four. (C) The levels of spirochetes in the mice's skin, heart, and bladder were evaluated using qPCR to measure the copies of *flaB.* The data was normalized against mouse *β-actin* to ensure an accurate comparison. In all mice tissues, there was a significant decrease in pathogen load when infected with $Bb^{bb0323-}$ and $Bb^{bb0323NL}$ compared to those infected with BbWT (*, Borrelia not detected). The data presented show the means ± SEM of four qPCR analyses. Two separate experiments were performed.

3.2.7 A precise BB0323 proteolytic cleavage in B. burgdorferi is required to support the microbial transmission

To confirm if ticks can transfer the cleavage site mutant *Bb*^{bb0323NL} to mice, naïve nymphal ticks were microinjected with either wild-type, *bb0323* deletion mutant or *Bb*^{bb0323NL} spirochetes. After overnight incubations, the ticks were allowed to parasitize the non-infected mice (5 ticks/mice, 3 mice/ group). After their repletion and detachment, the ticks were collected and tested for their spirochete load by qPCR analysis, which showed that ticks in all groups contained comparable levels of spirochete cells **(Figure 10A)**. The animals were euthanized 12 days after the last tick fell. Immunoblots were performed, and the blot was consistent with the infection studies; the mice infested with the mutant microinjected ticks did not produce antibodies against the *Borrelia* lysates **(Figure 10B).** The organ culture incubated in BSK-H media showed that the samples from both mutants did not grow any spirochetes **(Figure 10C).** Accordingly, the qPCR analysis of the mice tissues showed that the *bb0323* deletion mutants and the *Bb*^{bb0323NL} mutants could not establish comparable infection levels in the mice tissues **(Figure 10D).** Altogether, the results show that the precise BbHtrA-mediated cleavage in BB0323 is essential for transmitting *Borrelia* from ticks and its establishment in the mice tissues. However, it is not vital for survival inside the ticks.

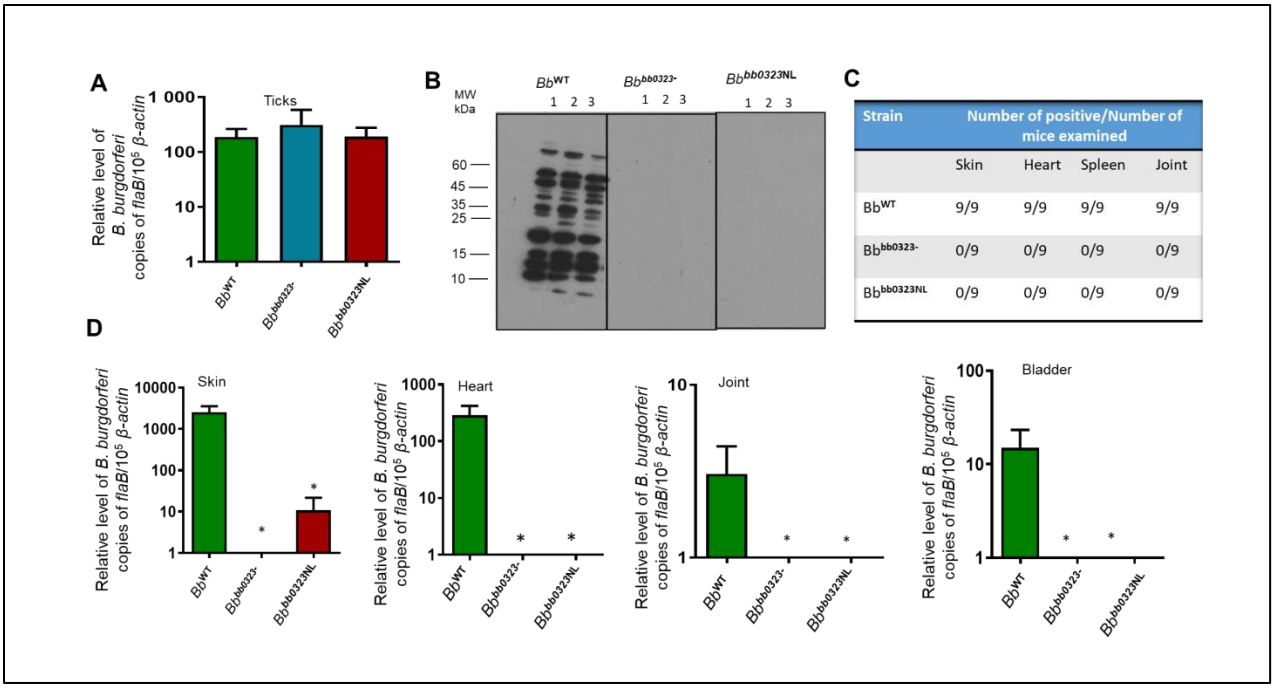


Figure 10. Effect of cleavage site mutation in transmitting *Borrelia* by ticks to naïve mice. The spirochetes of *Bb*^{Wt}, *Bb*$^{bb0323-}$, and *Bb*^{bb0323NL} groups were microinjected in naïve nymphal ticks. After overnight incubation, the ticks were allowed to parasitize on naïve mice. Two weeks after tick repletion, samples of blood, skin, heart, spleen, bladder, and joints were collected. (A) Level of spirochetes in the ticks. The ticks used for the spirochete transfer were collected and processed further by qPCR analyses. The results displayed that those ticks microinjected with *Bb*$^{bb0323-}$and *Bb*^{bb0323NL} retained comparable Borrelia burden to ticks containing the *Bb*^{Wt} group after completion of feeding. (B) The collected sera were used for immunoblotting against the wild-type *Borrelia* lysates separated by SDS PAGE and transferred to a nitrocellulose membrane. The immune response produced by the mice infested by the ticks with mutants *Bb*$^{bb0323-}$and *Bb*^{bb0323NL} was indiscernible compared to *the Bb*^{Wt} group. (C) Culture analysis of the organs collected from the mice. The organs collected from mice infested with the ticks microinjected with *Bb*^{Wt}, *Bb*$^{bb0323-}$, and *Bb*^{bb0323NL} groups were incubated in BSK-H media at 34°C. The spirochetes were visualized under dark field microscopy after two, three, and four weeks. (D) Determination of Borrelia burden in the mice tissues. The organs, skin, heart, joint, and bladder, collected two weeks after the ticks fall, were processed for qPCR analysis. Levels of *flaB* mRNA were normalized by mice *β-actin.* The results show a significant reduction in spirochete burden in the animals infected with *Bb*$^{bb0323-}$and *Bb*^{bb0323NL} compared to the *Bb*^{Wt} group (*, $P < 0.05$). Overall, The results exhibit that the ticks cannot transfer the cleavage site mutant sufficiently to establish the infection in murine hosts. The data presents the average of four qPCR analyses obtained from two animal experiments.

3.3 Discussion

BB0323 is a membrane-associated lipoprotein that goes through a series of proteolytic events to produce mature 27 kDa N and 12 kDa C-terminal polypeptides from 42kDa full-length protein (Kariu et al., 2013). These products support various aspects of biology and infection of *B. burgdorferi*. The full-length protein is processed by at least two proteases, primarily BbHtra, followed by CtpA (Östberg et al., 2004; Ye et al., 2016a). The whole phenomenon of the maturation of BB0323 still needs to be completed. Still, this study demonstrates that BbHtrA-mediated protein cleavage is crucial and a potential therapeutic target to thwart Lyme disease. Point mutation of BB0323 protein at positions Asparagine, N236 and Leucine, L237 to double Alanine (A236 and A 237) made it resistant to the BbHtrA mediated cleavage *in vitro* and also non-infectious in the murine model.

Previous studies have shown the role of BbHtrA in processing various gene products of Borrelia, such as Lmp1, BmpD, cheX, and p66, modulating the functions of the proteins (Coleman et al., 2013a; Russell et al., 2016). Additionally, some *in vitro* studies have demonstrated that BbHtrA degrades some parts of the extracellular matrix (ECM), such as fibronectin, and also stimulates the production of cytokines (Russell & Johnson, 2013). The present study has given additional evidence that the protease of the HtrA family plays a critical role in controlling the processing of microbial pathogenic determinants. Gene deletion studies of BbHtrA results in defects in fission, and the spirochetes cannot infect the murine host; additionally, there are pleiotropic effects on the multiple aspects of *Borrelia* cells such that it is difficult to figure out its one-directional effect on BB0323. However, deleting the HtrA gene disrupted the production of mature

N-terminal protein (Ye et al., 2016a). It is interesting to note that BbHtrA or BB0238 deleted mutants, similar to *Bb*^{bb0323NL}- cleavage site mutants, could still produce basal amount of mature BB0323 N-terminal that was sufficient for OM division. Therefore, blocking BB0323 interaction and proteolytic processing, such as in the cleavage site mutant, although didn't impose growth or morphological abnormalities, still rendered spirochete non-infectious, which create new possibilities for therapeutic drugs development.

Our research indicates that BB0323 has a multifaceted role in the biology and pathogenicity of the spirochete. The production of N-terminal BB0323 and its function depend on interaction with BbHtrA as well as, as shown before, with another spirochete protein BB0238 (Kariu et al., 2015). The cleavage of BB0323 at a specific site by the action of BbHtrA is essential in producing optimal levels of N-terminal to support the infection in murine hosts.

Interestingly, as we have shown here in case of LD agents, a controlled proteolysis of transcription factors and sigma factors of *Vibrio cholerae* helps the bacteria adapt to environmental changes (Pennetzdorfer et al., 2019). Similarly, mutation studies of a cleavage site of cytosolic protein RIN4 of a plant, *Arabidopsis thaliana*, which is acted and cleaved by a putative serine protease of *Pseudomonas syringae,* AvrRpt2, shows the importance of molecular characterization of protease substrate interaction, not only within the bacteria but also in host-pathogen interface (Chisholm et al., 2005). Overall, we conclude that BB0323 is a *Borrelial* protein that participate in multiple PPI events essential for spirochetal biology and infectivity and, subjected to regulated proteolysis. The complete process of post-translational processing of the spirochetal

proteins is still unclear, and a better understanding of the maturation of various pathogenic determinants, such as BB0323, BB0238, and BbHtrA, remain as a subject of future investigation. However, the actual phenomena by which the cleavage site mutant was non-infectious is enigmatic and is further explored in the next chapter. Overall, these findings will increase our understanding of *Borrelial* biology and bring us a step closer to identifying new targets for new treatments of LD. Another probable aspect would be identifying the area at which BbHtrA interacts with BB0323 protein; specific disruption of this complex, such as via small molecule inhibitors, would also provide an exciting target of potential new therapeutics.

Chapter 4: BB0323:BB0238 interaction and their stoichiometry: a key mechanism to infection establishment

Abstract

The study in the previous chapter demonstrates the inability of the cleavage site mutants of BB0323 to establish infection in murine hosts. We make efforts to understand the molecular mechanism of why cleavage site mutants became non-infectious in mice. We have previously shown that N-terminal and C-terminal BB0323 proteins can interact. Herein, we found that the level of mature N-terminal BB0323 protein was significantly lower in $Bb^{bb0323NL}$ isolates. However, C-terminal BB0323 was produced compared to the wild-type, and the interaction between N- and C- terminal BB0323 was undisturbed. Additionally, The immunogenic profile of the cleavage site mutant was altered. Notably, in spirochete cells, the N-terminal BB0323 produced in low amounts could only recruit less BB0238 protein. Altogether, these results suggest, the failure of the cleavage (NL site) mutants to infect mice is likely due to less abundance of mature N-terminus and its availability to form BB0323:BB0238 complex. Overall, these studies highlight the significance of the precise processing of a critical virulence determinant and underscore the importance of studying essential PPI events and their stoichiometry in LD pathogenesis.

4.1 Introduction

The protein-protein interaction (PPI) is a fundamental process in many organisms, including pathogenic bacteria, and thus its optimal levels is likely required to perform vital cellular functions such as gene regulation and signal transduction in cells (Feng et al., 2015; Snider et al., 2015). The PPI can involve between the same protein (homomers) or different protein molecules (heteromers) (Marsh & Teichmann, 2015). The stoichiometry of a PPI refers to the number and arrangements of subunits of protein molecules in a complex, which can affect the complex's binding affinity, specificity, and functionality (Taggart et al., 2020). Therefore, it is essential to study the stoichiometry balance/dosage balance, as their disturbance via small molecules could be a novel strategy for development of antimicrobials.

In eukaryotic cells, fine-tuning optimal protein levels is achieved by various mechanisms, including regulations at the transcriptional levels by controlling the transcription, mRNA production rates, and removal of excess production post-translationally (Taggart et al., 2021). The levels of protein synthesis are either coregulated by the same regulon or the relative rates are fine-tuned after transcription (Sampson et al., 1988; Sastry et al., 2019; Schmid & Roth, 1987). After translation, for protein homeostasis, excess proteins and the subunits which fail to dimerize (unstable orphan proteins) are degraded and removed (Juszkiewicz & Hegde, 2018; Taggart et al., 2021).

Studies on extensive process protein level maintenance are ongoing in bacteria. One example of the requirement for tight regulation of protein complexes is FtsZ regulation in the cell divisome machinery complex of *E coli*. Production of optimal levels

of FtsZ in the complex is essential. A lower level of FtsZ resulted in filamentous forms of cells being unable to divide, whereas mini nucleoid-free cells are formed if the production of FtsZ and FtsA is above the threshold (Dai & Lutkenhaus, 1992; Ortiz et al., 2016).

Additionally, different domains of the FliF protein of *Salmonella enterica* in the flagella basal body could form 34-mers and 23-mers in the assembly of the MS ring (Kawamoto et al., 2021). It demonstrates the ability of the same protein to oligomerize in different ratios according to the functional requirement to control the flagellar machinery (S. Johnson et al., 2021; Kawamoto et al., 2021; Ortiz et al., 2016).

Levels of proteins in cells are widely controlled by oxidative stress, nutritional availability, pH, and the environment of the host or vector, among others (B. Chen et al., 2011; Wolff et al., 2014). The type of PPI stoichiometry within the complexes made by the identical protein subunits can vary depending on the biological context. Abruption of this relationship pattern can cause structural and functional changes and produce unstable and unfunctional aggregates (Taggart et al., 2020, 2021).

The previous chapter showed that the mutation of the BbHtrA-mediated cleavage site in BB0323 renders the protein being resistant to proteolytic cleavage *in vitro*. The mature N-terminal was produced at a basal level, which could support the normal fission of the spirochete. However, the mutated spirochetes were not able to infect mice. Herein, we continued our efforts to understand biological significance of such BB0323 processing, as the cleavage site mutants lost infectivity despite retaining the production of mature BB0323 proteins. First, we examined the mutants' immunogenic profile *in vivo.* Next, we addressed whether BB0323 distribution in spirochete cells was altered. As

BB0323 supports bacterial fission and binds peptidoglycan, we particularly address possible altered localization of the peptidoglycan layer due to deletion or mutation in BB0323 protein. Finally, we assessed the BB0323:BB0238 complex, particularly whether suboptimal level of production of BB0323 N-terminal influence the complex stoichiometry.

4.2 Results

4.2.1 Cleavage site mutants display distinct immunogenic profiles

Although the cleavage site mutant retained its mature N-terminal, it could not infect the murine host. Here, we aimed to investigate any alteration in the spirochetes' immunogenic profile. The lysates of Bb^{Wt}, $Bb^{bb0323-}$, and $Bb^{bb0323NL}$ *groups* grown at 37°C were transferred to a nitrocellulose membrane, and an immunoblot was performed using an anti-outer membrane antibody. The results displayed observable increments in at least three bands of proteins **(Figure 11A).** In addition, a western blot was performed using the serum collected from wild-type infected mice to probe the proteins of Bb^{Wt}, $Bb^{bb0323-}$, and $Bb^{bb0323NL}$ isolates. The blot displayed a change in the mutants' immunogenic protein profile **(Figure 11 B).** Overall, the results demonstrated that the mutation of the BbHtrA-mediated cleavage site in BB0323 led to a change in the immunogenic profile of the spirochetes.

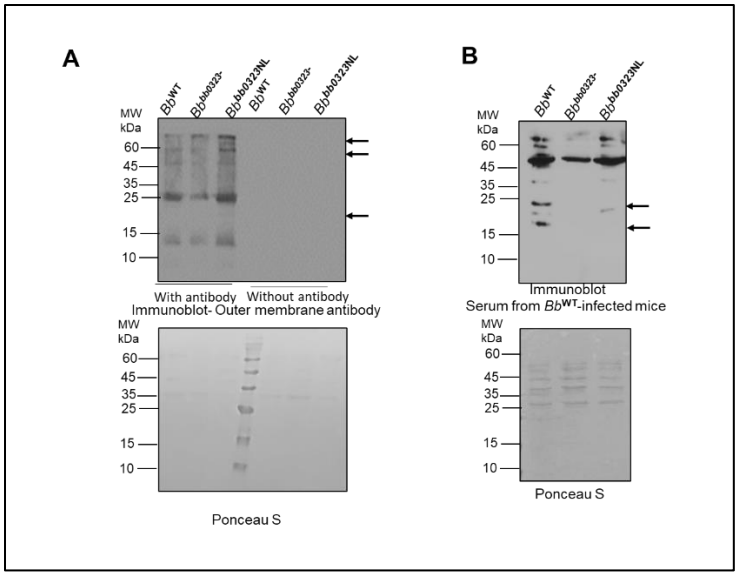


Figure 11. Immunogenic profile of cleavage site mutant. (A) The cleavage mutant displays an altered outer membrane protein profile *in vitro*. Borrelia Lysate grown at 37°C to check the difference in protein expression between Bb^{Wt}, $Bb^{bb0323-}$, and $Bb^{bb0323NL}$groups (arrow). Immunoblots display the difference in the levels of outer membrane proteins in the cleavage mutant compared to the wild-type. In the upper panel (left), the blot displays Bb^{WT}, $Bb^{bb0323-}$, and $Bb^{bb0323NL}$ lysates, probed with *anti-Borrelia* outer membrane (OM) antibodies. The upper panel (right), serving as a negative control, was incubated with PBS instead of an anti-OM antibody. The lower panel shows the blot stained with Ponceau S. as a loading control. (B) The immune profile of the cleavage mutant is modified *in vivo*. Compared to the wild-type, the *bb0323* mutants exhibit alteration in the immunogenic proteins. The cells of Bb^{WT}, $Bb^{bb0323-}$, and $Bb^{bb0323NL}$ spirochetes were lysed and separated in SDS PAGE and transferred to a nitrocellulose membrane. The blot was probed with the serum samples obtained from the mice infected with the wild-type *Borrelia* (upper panel). The distinct bands are highlighted with arrows.

4.2.2 Unaltered peptidoglycan layer organization in Cleavage site mutants

Previous studies have shown that the BB0323 protein interacts with the peptidoglycan layer (PG), which has a role in the pathogenesis of LD (Kariu et al., 2013); here, we checked if the mutation in BB0323 proteins altered the localization of the active zones of PG layer. Localization of PG with the fluorescent D-alanine analog 7-hydroxycoumarin-amino-D-alanine (HADA) demonstrated the cellular distribution of PG similar to the wild-type **(Figure 12).** The results obtained from fluorescence microscopy

displayed that despite the mutations in BB0323 proteins, the localization of PG was comparable to the wild-type spirochetes.

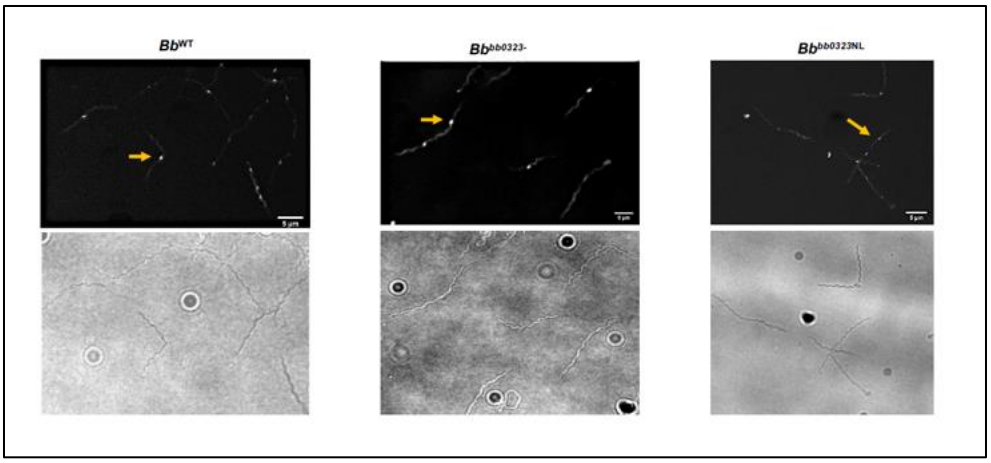


Figure 12. Labeling of the peptidoglycans in the spirochetes. The upper panels display the HADA-labelled spirochetes. The spirochetes from Bb^{Wt}, $Bb^{bb0323-}$, and $Bb^{bb0323NL}$groups grew to mid-log phase and were incubated with o.2 mM HADA and were incubated at 37°C for two hours. The cells were centrifuged, resuspended in PBS, and spotted in a slide with 1% agarose. A fluorescent microscope was used to record the labeling in the cells. The lower panel shows pictures of the spirochetes of different groups by phase contrast microscopy.

4.2.3 Cleavage site mutant produces lower levels of mature N-terminal BB0323

To understand the potential mechanisms behind the inability of the cleavage site mutants to establish the infection in murine, we performed a densitometric analysis of the mature BB0323 N-terminal protein produced by the $Bb^{bb0323NL}$ isolates compared with the wild-types. Western blot probed with anti-BB0323 -N terminal antibody showed that the mature N-term polypeptide, 27kDA molecular weight, was significantly lower in $Bb^{bb0323NL}$ mutants **(Figure 13A)**. It was previously established that Borrelia requires the C-terminal to maintain infectivity (Kariu et al., 2013). We performed western blotting

(using an anti-C-terminal antibody) and far-western blotting (incubation with BB0323-N term protein). Here, we confirmed that the level of the C-terminal of BB0323 is unaltered in *Bb*^{bb0323NL} mutants **(Figure 13B)**, and the interaction between N and C- termini of the cleavage site mutant was comparable to the wild-type isolates **(Figure 13C)**.

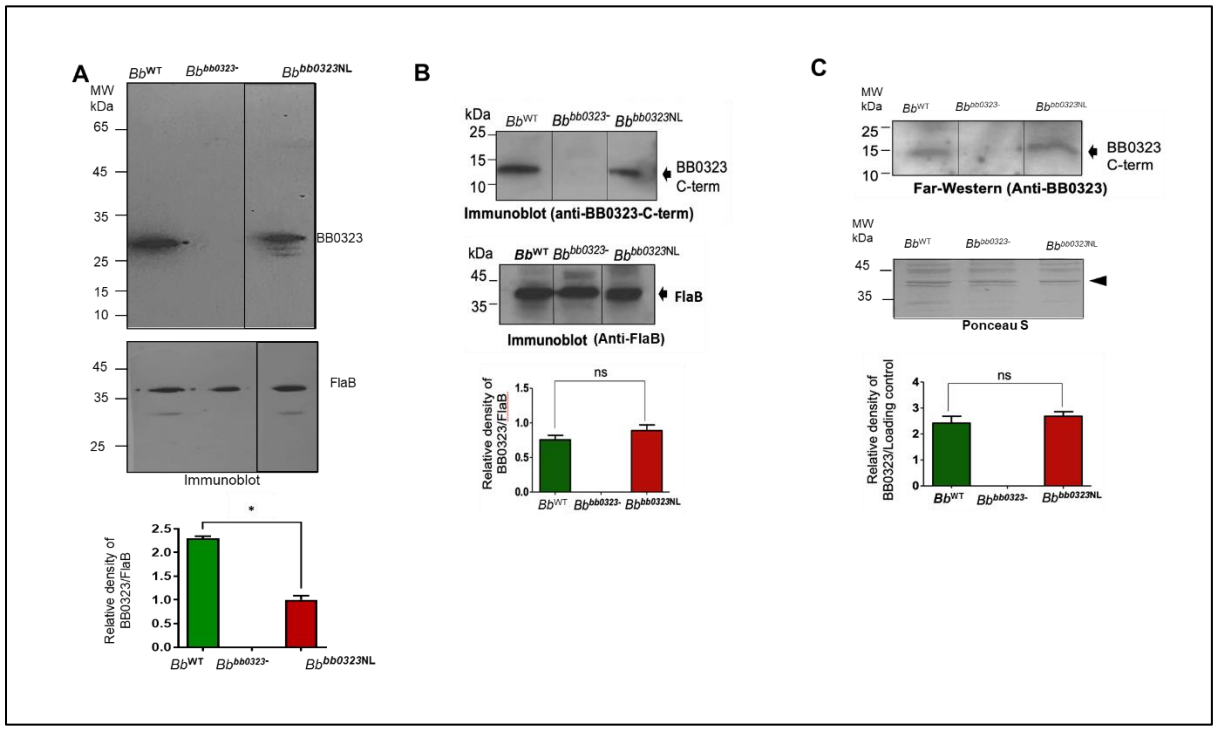


Figure 13. The cleavage site mutants produced reduced quantities of mature BB0323-N terminal proteins. (A) Densitometric analysis displayed that the level of the mature N-terminal polypeptide produced by *Bb*^{bb0323NL} was significantly lower than *Bb*^{Wt} spirochetes. After separating the lysates produced from *Bb*^{Wt}, *Bb*$^{bb0323-}$, and *Bb*^{bb0323NL} groups using SDS PAGE, the gel was transferred to a nitrocellulose membrane and incubated with anti-BB0323 N-term antibody (Upper panel). The level of FlaB was demonstrated using an anti-FlaB antibody as a loading control (Middle panel). Using ImageJ software, the densities of the proteins were measured and normalized against the levels of FlaB. The results presented by GraphPad Prism 9 showed that the mature N-terminal protein is significantly reduced in the cleavage site mutant compared to the wild-type (*, $P < 0.05$). (B) The amount of BB0323 C-terminal fragment produced in *Bb*^{bb0323NL} is similar to *Bb*^{Wt} isolates. *Bb*^{Wt}, *Bb*$^{bb0323-}$, and *Bbbb0323NL groups* were separated through 15% SDS PAGE and transferred to a nitrocellulose membrane. The blot was probed with an anti-BBo323 C-terminus antibody (upper panel, arrow). Anti-FlaB antibody was used to probe FlaB as a loading control (Middle panel, arrow). The densitometry analysis using ImageJ displayed the comparable level of C-terminal in both *Bb*^{bb0323NL} and *Bb*^{Wt} isolates (Lower panel). (C) Interaction between BB0323 C terminal

and rBB0323 N-terminal remains unaffected. The spirochetes were lysed, separated as described above, and transferred to a nitrocellulose membrane. The blot was incubated with recombinant BB0323-N terminal protein in PBST. The bound N-terminal protein was probed with a ti-BB0323 N-terminal antibody (Upper panel). The level loading in the blot is displayed by Ponceau S stain (Middle panel, the row represents the band selected for normalization). The densitometric analysis displaying a similar level of biding in *Bb*^{wt} and *Bb*^{bb0323NL} spirochetes was done by ImageJ (Lower panel).

4.2.4 Optimal levels of BB0323:BB0238 interaction complex are required to support microbial infectivity

A previous study with BB0323:BB0238 interaction has demonstrated that spirochete isolates producing mutant BB0238 protein unable to interact with BB0323, cannot infect the mice, and the interaction between the proteins is essential for the post-translational stability (Kariu et al., 2015; Thakur et al., 2017). However, the BB0238 level in *Bb*^{bb0323NL} mutants was comparable to the wild-type **(Figure 14A)**. Far-western analysis showed that BB0238 only interacts with the 27 kDa polypeptide of BB0323 but not with the lower two bands **(Figure 14B)**. Notably, the interaction complex in *Bb*^{bb0323NL} mutants was significantly lower than the wild-type isolates. Additionally, MST (**Figure 14C)** analyses with recombinant BB0238 with BB0323 N-term and BB0323^{NL}-N proteins demonstrated cleavage site mutation in the BB0323 did not alter the binding affinity and specificity to the BB0238 protein. Altogether, these findings underline the importance of precise cleavage of BB0323 at positions N236th and L237th and maintaining an optimal level of mature N-terminal polypeptides to preserve the proportional makeup of BB0323:BB0238 complex, an imbalance of which can render the spirochete non-infectious.

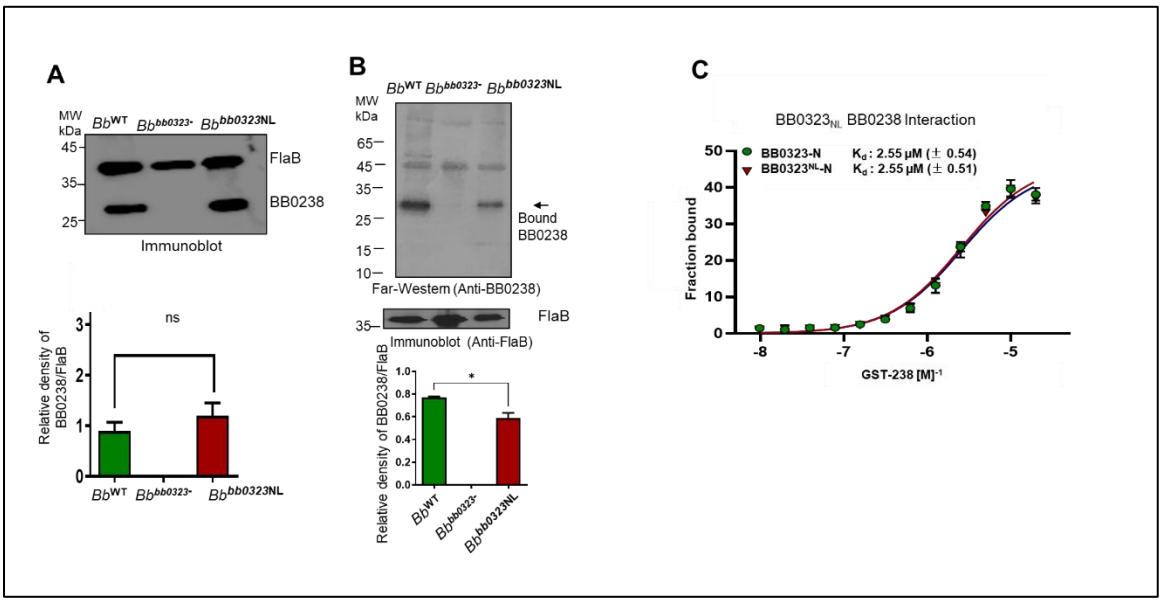


Figure 14. The diminished N-terminal BB0323 produced by Bbbb0323NL recruits lower BB0238 protein to bind in the BB0323:BB0238 complex. (A) The mutation in the cleavage site of BB0323 did not alter the BB0238 levels. The lysates of *Bb*^{wt}, *Bb*$^{bb0323-}$ and *Bb*^{bb0323NL} isolates were run in the SDS PAGE and transferred to the nitrocellulose membrane. The level of BB0238 protein was probed by using an anti-BB0238 antibody. The level of FlaB protein was used as a loading control (Upper panel). The densities of the bands analyzed by ImageJ software and presented by GraphPad prism (Lower panel) show similar levels of BB0238 in *Bb*^{bb0323NL} as in *Bb*^{wt} isolates. (B) Recombinant BB0238 interacts only with mature N-terminal BB0323 and not with the lower aberrant polypeptides. The lysates of *Bb*^{Wt}, *Bb*$^{bb0323-}$, and *Bb*^{bb0323NL} spirochetes were separated by SDS PAGE and transferred to a nitrocellulose membrane. Next, the blot was incubated with recombinant BB0238 protein and probed with an anti-BB0238 antibody. The mature N-term BB0323 could bind the BB0238 but not the lower polypeptides (Upper panel); FlaB was probed using an anti-flaB antibody and used as a loading control (Middle panel). Densitometric analysis of the blots (Lower panel) demonstrates a significantly lower level of BB0238 recruited for the interaction by N-terminal BB0323 in *Bb*^{bb0323NL} isolates (*, P< 0.05). (C) The cleavage site mutation in the BB0323 protein did not alter the binding affinity with BB0238. Microscale Thermophoresis Assay measured the binding kinetics of recombinant BB0323-N and BB0323NL-N with BB0238; the results showed that kD values between both groups were similar.

4.3 Discussion

It has been previously established that interaction between BB0323 and BB0238 is essential to maintain *Borrelial* infectivity (Thakur et al., 2017). Size exclusion chromatography and crosslinking assays showed that these proteins interact in a ratio of 1:1 or 2:2 (Thakur et al., 2017). The study above was performed by altering the cleavage site, which BB0323 protein displays that the resulting mutant has altered its immunogenic profile *in vivo.* The NL site mutant spirochetes produced decreased amounts of mature N-terminal, but the level of C-terminal was maintained. Interestingly, despite the level differences between the two termini, their interaction in the cleavage site mutant was still comparable to the wild-type.

The diminished level of N-terminal could not recruit a similar amount of BB0238, even though the affinity between the recombinant proteins was unaltered, displayed by an MST assay. It suggests that the decrement in the level of one of the interacting partners could disrupt the stoichiometry of the complex and have perturbed the function of the animal infection.

Several studies addressed the functions of the ratio balance of protein complexes (Dai & Lutkenhaus, 1992; Feucht et al., 2001; Jensen et al., 2005). One of the relevant examples is the protein subunits FtsZ and FtsA involved in forming the Z-ring divisome complex in *E. coli*. The ratio of FtsZ and FtsA inside *E. coli* and *B. subtilis* is 5:1 (Feucht et al., 2001; Jensen et al., 2005). Maintenance of this ratio is essential as overexpression of either of these proteins hindered cell division and produced filamentous cells. The flaw could be corrected by simultaneous over-expression of the other protein (Dai & Lutkenhaus, 1992).

This study showed the decreased formation of the BB0323:BB0238 complex, displaying that the proper ratio of these two proteins might be essential to maintain the infectivity in mice. The result is consistent with the infectivity of the spirochetes with either of the genes deleted (Kariu et al., 2013, 2015; Zhang et al., 2009). However, the mechanism behind the altered immune response is still unclear. The questions regarding the increased production of Outer membrane proteins *in vitro* and diminished response against some proteins *in vivo* hint at the role of either the N-terminal BB0323 protein or the complex in immune response inside the host. Similarly, the study that demonstrates the significance of the interacting complex and the feature of its stoichiometry is yet to be done.

Overall, our studies underscore the biological significance of BB0323 complexes, including one involving BB0238. While additional research is needed to fully unravel the mechanistic details of how these PPI or BB0323 complexes support spirochete biology and infectivity, our results clearly shown blocking the BB0323-BB0238 interaction renders spirochetes non-transmissible through ticks and non-infectious in a mammalian host. There, as a proof-of-concept, we have explored the possibility of the BB0323:BB0238 complex as a target for new therapeutic development against LD, as detailed in the following chapters of this dissertation.

Chapter 5: Assessment of BB0323:BB0238 complex as a novel therapeutic target against LD

Abstract

Identification of novel and more efficient therapeutic agents against LD is not only essential to develop a complete treatment strategy but also to limit the current usage of antibiotics. This effort can prevent the occurrence of PTLDS, associated with the current use of antibiotic treatment. In this study, we explored the prospect of blocking BB0323: BB0238 PPI as a new drug target against the persistence of *B. burgdorferi* in mouse hosts. Herein, we assessed whether blocking BB0323-BB0238 PPI prevents long-term persistence of *B. burgdorferi* in murine hosts. We show that, unlike wild type or other *B. burgdorferi* mutants, *bb0238*Δ*IM* mutants (that are impaired in BB0323-BB0238 PPI despite producing the proteins), are unable to persist in murine hosts in long term. While ticks fed on various mouse groups ingested wild type or control mutant spirochetes from respective infected animals, *bb0238*Δ*IM* mutants could not be acquired in ticks. An AlphaLisa assay was developed for a quantitative high-throughput qHTS screening of various small molecule libraries (with over 8000 small molecules) using resources and expertise at NCATS. The short-listed HITs molecules were further confirmed using a secondary *B. burgdorferi* cell-based assay, as detailed in the next chapter.

5.1 Introduction

Antibiotic discovery is one of the most significant achievements of humans. However, over the years, excessive usage of antibiotics has resulted in developing resistance, such as Methicillin-resistant Staphylococcus aureus (MRSA) or other Multidrug resistant (MDR) bacteria (Belete, 2019; Czaplewski et al., 2016; Michael et al., 2014). The emerging situation warranted need for new interventions and provides a clear opportunity to search for compounds that treat the infections without exerting evolutionary pressure on the organism (Gwynn et al., 2010). Novel molecular targets specific to the bacteria, such as anti-virulence agents - targeting virulence factors of a bacteria, such as adherence, invasion, LPS, and toxins, instead of killing causes less stress in the bacteria (Belete, 2019).

Identification of a valid target is the first step in drug development. Targets refer to the biomolecules such as proteins, DNA, RNA, or peptides where the drugs bind and provide therapeutic efficacy (Santos et al., 2017). Databases are crucial in drug discovery; Drug banks, Therapeutic target databases, ChEMBL databases, and comparative toxic genomic databases are a few examples of these resources (Shangguan, 2021). The records comprise the molecules' structure, function, and potential interaction pattern, making it easier to find the related targets. Several biological assays are performed to identify and analyze biological functions. Functional genomic or genetic assays such as RNA interference, Gene knockout, Gene transfection, DNA microarray, and RNA sequencing allow the functional analyses of the molecule and its relevance as a drug target (Hughes et al., 2011; Schenone et al., 2013). Machine-based methods can be utilized to simulate and predict the binding of the target to a drug *in silico*, reducing time

and cost in the drug discovery process. The method also involves sequential and structural analysis of the target to a pre-established receptor and predicts a druggable target (Dara et al., 2022; Duch et al., 2007; Hughes et al., 2011). The analyses could be done either based on the structure, such as structure-based drug discovery or ligand, such as ligand-based drug discovery (Shaker et al., 2021; Vamathevan et al., 2019). Additionally, approaches to drug discovery include a conventional approach or bottom-up strategy, which involves screening several thousand molecules against specific assays which reflect the function of the disease target, and a rational approach or deconvolution method, which consists in finding out the structure of the target first and designing a drug to fit the space. (Rick NG, 2016; Shangguan, 2021).

Once a target has been identified, validating its effects and functions is crucial. The early-stage validation comprises *in vitro* or cell-based assays and *in vivo* experiments, usually in animal model to confirm a target's biological functions upon a ligand's activity (Rick NG, 2016). For example, mutation of the interacting interface of a protein of a bacteria might predict the results expected from using a molecule to prevent this PPI. Using an animal model that mimics the human model of the disease is of utmost importance (Embers et al., 2013; Herrmann, 1995; Vandamme, 2014).

In a conventional method of drug discovery, following target identification and validation, it is essential to develop assays that can reflect the functions (Gwynn et al., 2010). Early-phase assay development incorporates a potential drug's enzymatic or physiological effect on the target (Hughes et al., 2011; Rick NG, 2016). HTS-compatible Assays such as ELISA, fluorescence polarization, fluorescent resonance energy transfer, and bead-based assays such as AlphaLisa platforms are utilized. The acronym "Alpha"

stands for amplified luminescent proximity homogeneous assay. Orthogonal assays that use different principles and modalities than primary assays should be applied to confirm the results (Arkin MR et al., 2012).

Sequentially, the assay is miniaturized for a qHTS or ultra qHTS and used to screen small molecule or drug libraries. Additionally, Fragment-based screening, focused screening, structure-aided drug design, Virtual screening/Docking, and Physiological assays could be used for screening purposes (Boppana et al., 2009; Hughes et al., 2011; McInnes, 2007).

The HTS assays significantly reduce time and cost in compound screening. Using robotics, liquids are dispensed in nanoliters to Picolitres volumes (Rick NG, 2016). Several thousand molecules are screened using different libraries, using 1536 microwell plates. A hit is a molecule that produces the expected result in the assay when tested multiple times (Fox et al., 2006; Hughes et al., 2011). The series of HIT molecules should be further screened for the structures and activities. The compounds should display competitive behavior in dose-response assays. Tissue/cell-based assays should be additionally performed. Furthermore, several *in vitro* assays are performed to investigate the compounds' Absorption, Distribution, Metabolism, and Excretion (ADME) properties (Arkin MR et al., 2012; Fox et al., 2006; Hughes et al., 2011). The compounds which display the expected biological activity are selected as Leads (Hughes et al., 2011; Rick NG, 2016). Lead optimization involves modifying the compounds to retain the desired property and remove other flaws (Hughes et al., 2011).

As mentioned in the previous chapters, previous work from our lab has confirmed that the N-terminal of BB0323 forms a complex with another periplasmic protein BB0238. This interaction is vital for the infectivity of *B. burgdorferi* in mice and its transmission from ticks. These two proteins also depend on maintaining their mutual post-translational stability *in vitro* (Kariu et al., 2015; Thakur et al., 2017). Furthermore, disturbances in the stoichiometry also make the spirochete non-infectious (Previous chapter).

BB0323 and BB0238 both are conserved proteins in the *B. burgdorferi sensu lato complex* and do not have any homology to other bacteria (Kariu et al., 2013, 2015; Zhang et al., 2009). So, identifying these two proteins as targets for developing novel therapeutics against LD that are likely to have no or minor effects on other bacteria population is highly imperative. A previous study has shown that the interaction can be saturated or inhibited (Thakur et al., 2017), which indicates that the complex is an appropriate candidate. This chapter confirms that the BB0323:BB0238 complex is required for long-term infection establishment in mice and is a druggable target. Furthermore, a description of the development and usage of the Alphalisa assay is provided. The assay was used to screen over 8000 small molecules. Overall, this chapter shows the target validation and utilization of Alpha assay to screen four different compound libraries. The qHTS facilitated the discovery of the HIT series. Finally, the PPI activity of these compounds is confirmed and tested in upcoming chapters.

5.2 Results

5.2.1 Impaired BB0323 and BB0238 interaction diminishes the ability of *B. burgdorferi* to persist in mice

Previous studies have shown that the interaction of BB0323 and BB0238 is vital for the virulence and persistence of spirochete in the enzootic cycle for the early stages of infection (Thakur et al., 2017). Here, we sought to assess whether this interaction is also essential for long-term persistence. We used the *bb0238*Δ*IM* mutant that produces BB0238ΔIM protein devoid of interaction motifs; hence the association of BB0238 and BB0323 is disrupted. The mutant protein lacks 11 amino acids, i.e., 120-130 amino acid residues in BB0238. The generation of the isolates, and initial infection results are detailed elsewhere (Kariu et al., 2015; Thakur et al., 2017). To examine whether *bb0238*Δ*IM* mutants have defects in long-term survival in mice, we used *bba57*$^{-}$ mutants as an appropriate control. BBA57 is an outer membrane protein essential in early but dispensable for late infection in murine. The levels of *bba57*$^{-}$ mutants in the mice are significantly lowered in the first and second week, but it becomes comparable to the wild-type from the third week onwards. Generation of the *bba57*$^{-}$ mutant is detailed in a previously published paper from our lab (Yang et al., 2013).

To assess the effects of disruption of BB0323 and BB0238 in long-term infection, *in vivo* experiments with murine hosts (3 mice/ group), injected with 10^5 cells subcutaneously with wild-type (WT), *bba57*$^{-}$ deletion mutant or *bb0238*Δ*IM*-complemented spirochetes were performed. We sought to investigate whether the *bb0238*Δ*IM* isolates could persist in the mice for long time, when compared to wild type

or *bba57*$^{-}$. After nine weeks of injection, immunological responses against *B. burgdorferi* lysate were observable with serum from mice infected with wild-type and *bba57*$^{-}$mutant but not in mice infected with *bb0238ΔIM* isolates **(Figure 15A).** No viable spirochetes were recovered from *bb0238ΔIM* mutant infected tissues, whereas wild-type and *bba57*$^{-}$ mutants were easily observable **(Figure 15B).** Unlike the wild-type and *bba57*$^{-}$mutant, *bb0238ΔIM* mutant burdens in the tested murine tissues were mostly indiscernible **(Figure 15C).** The results clearly demonstrated that the association between the two proteins was essential in maintaining long-term *B. burgdorferi* infection in murine hosts.

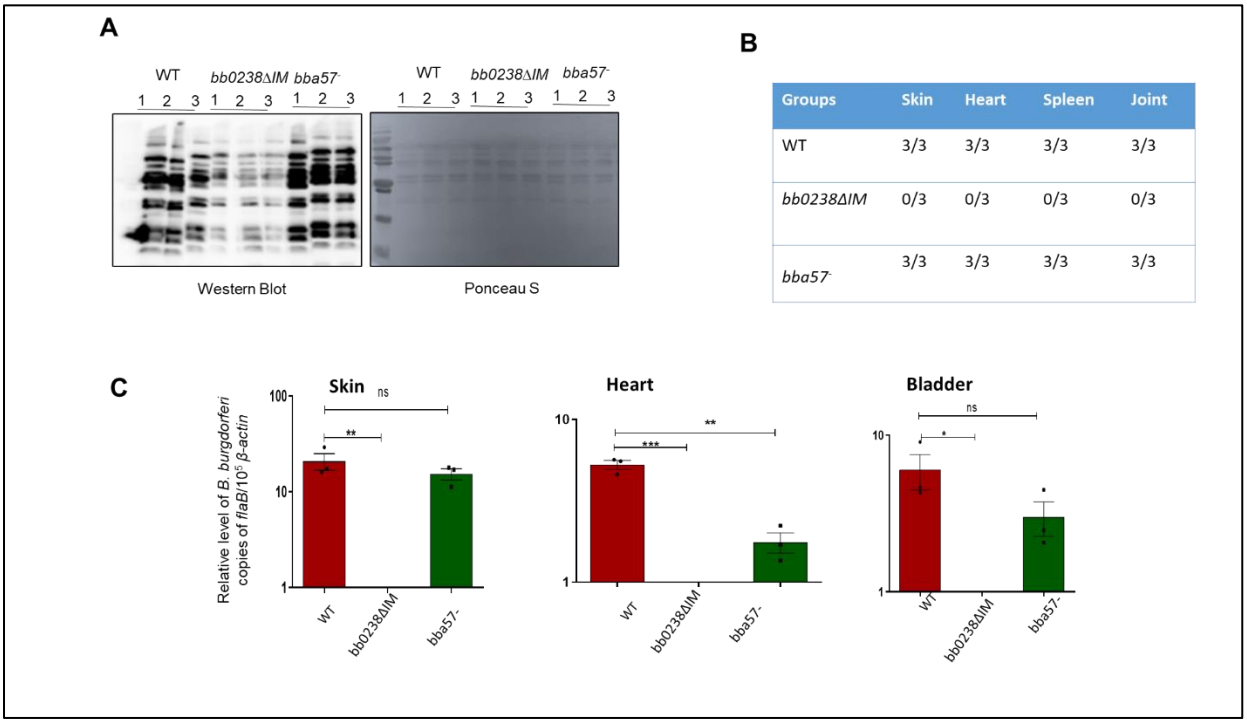


Figure 15: Impaired BB0323 and BB0238 interaction diminishes spirochete ability to persist in mice for the long term. A: Serological responses in infected murine. Mice were infected with equal numbers of wild-type, *bb0238ΔIM,* or *bba57*$^{-}$ mutant spirochetes via subcutaneous inoculation. Immunoblot analysis was performed against *B. burgdorferi* cell lysates with sera collected nine weeks after injection. B: Infected skin, heart, joints, and spleen. The organ cultures in BSK were incubated for two weeks at 34ºC and

observed under dark field microscopy for the presence or absence of the spirochetes. **C**: Assessments of spirochete level in mice skin, heart, joints, and bladder. The differences in the level of spirochetes were evaluated by measuring *flaB* transcripts levels using qPCR, and levels of mouse *β-actin* were used to normalize between groups. *Borrelia* burden was significantly reduced in all mice tissues infected with *bb0238ΔIM*, compared to wild type and *bba57*$^{-}$ mutant (*$P < 0.05$, **, $P< 0.01$,***, $P<0.001$). The results were derived from two animal experiments. Bars represent the mean ± SEM of four qPCR analyses from two biological repeats.

5.2.2 BB0323 and BB0238 interaction is necessary for acquiring the spirochetes by naïve ticks

Treatment of infected mice with antibiotics like doxycycline or ceftriaxone clear *B. burgdorferi* in the animal. However, spirochetes can persist in low levels where *B. burgdorferi* could not be detectable in an antibiotic treated hosts. Surprisingly, ticks fed on these mice apparently cleared of *B. burgdorferi*, can acquire the spirochetes – a mode of microbial detection called xenodiagnoses (Bockenstedt & Radolf, 2014; Marques et al., 2014). It has been shown in some studies that the animals which have *B. burgdorferi*-negative tissue culture are still able to transfer it to a naïve tick (Bockenstedt & Radolf, 2014; Marques et al., 2014). Thus, we next sought to examine whether the ticks could acquire the *bb0238ΔIM* mutant upon ingesting blood from the spirochete-injected mice.

Naïve nymphal ticks (7 ticks/mice) were allowed to parasitize the mice eight weeks after the *Borrelia* injection. The qRT-PCR analysis showed that the repleted ticks could not acquire spirochetes in *bb0238ΔIM* mutant infected mice in contrast to the other two groups, such as either wild type or *bba57*$^{-}$ mutants that were readily present in fed ticks . These results suggest that the interaction of BB0323 and BB0238 is essential for

the persistence of *B. burgdorferi* for a longer term in murine hosts and also for the acquisition by the ticks **(Figure 16).**

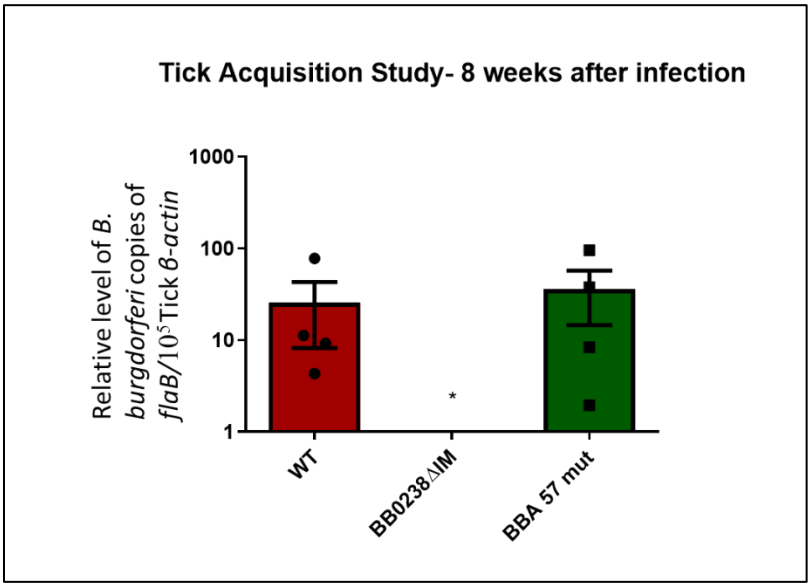


Figure 16. **Acquisition of spirochetes by ticks from *Borrelia-injected* hosts**. After eight weeks of Borrelia injection, ticks were allowed to parasitize the mice. CDNA from fully repleted ticks were collected and assessed for spirochetes using qRT-PCR. The spirochete level was significantly reduced in ticks fed on mice injected with *bb0238ΔIM* compared to wild type and *bba57*$^{-}$ mutant. (*$P < 0.05$).

5.2.3 Confirmation of the BB0323:BB0238 complex as a target of therapeutic development

In order to examine the inhibitory action of a potential small molecules against LD by targeting BB0323-BB0238 PPI, we have used a bead-based qHTS compatible assay called AlphaLisa, in collaboration with NCATS/NIH. The Amplified Luminescent Proximity Homogeneous Assay (ALPHA) assay consists of two latex-based beads coated with hydrogels (PerkinElmer, 2023). The donor bead, as the name suggests, upon illumination at 680nm, can convert ambient oxygen to excited singlet oxygen molecules.

The bead is coated with a photosensitizing molecule, phthalocyanine, which causes the conversion. This singlet oxygen can move to a distance of up to 200nm and can transfer the energy to Acceptor beads. On the other hand, these beads are coated with Thioxene derivative, which causes illumination at 615nm upon receiving the energy. The donor and acceptor beads contained Glutathione and Nickle, respectively, to bind with the proteins with appropriate tags (Arkin MR et al., 2012; PerkinElmer, 2023). The recombinant proteins HST-BB0323 N-terminal and GST-BB0238 were used for the PPI interaction examination **(Figure 17)**. The assay was initially developed in our lab using 96 well followed by 384 well plates using a volume of 100 and 340 µl, respectively. Finally, it was miniaturized at NCATS lab to a 6 µl reaction to use in the qHTS compatible 1536 well plates. The assay was highly specific to the interaction of BB0323 and BB0238, as evaluated by a series of competitive inhibition assays, where non-tagged BB0238 protein could inhibit the signal output in a dose-dependent manner, and assays with non-interacting proteins such as a non-specific tick protein did not have any signal output (figure not shown). The experiments, such as development, optimization, and miniaturization of Alpha assay, were performed at UMD by our previous lab members (Unpublished).

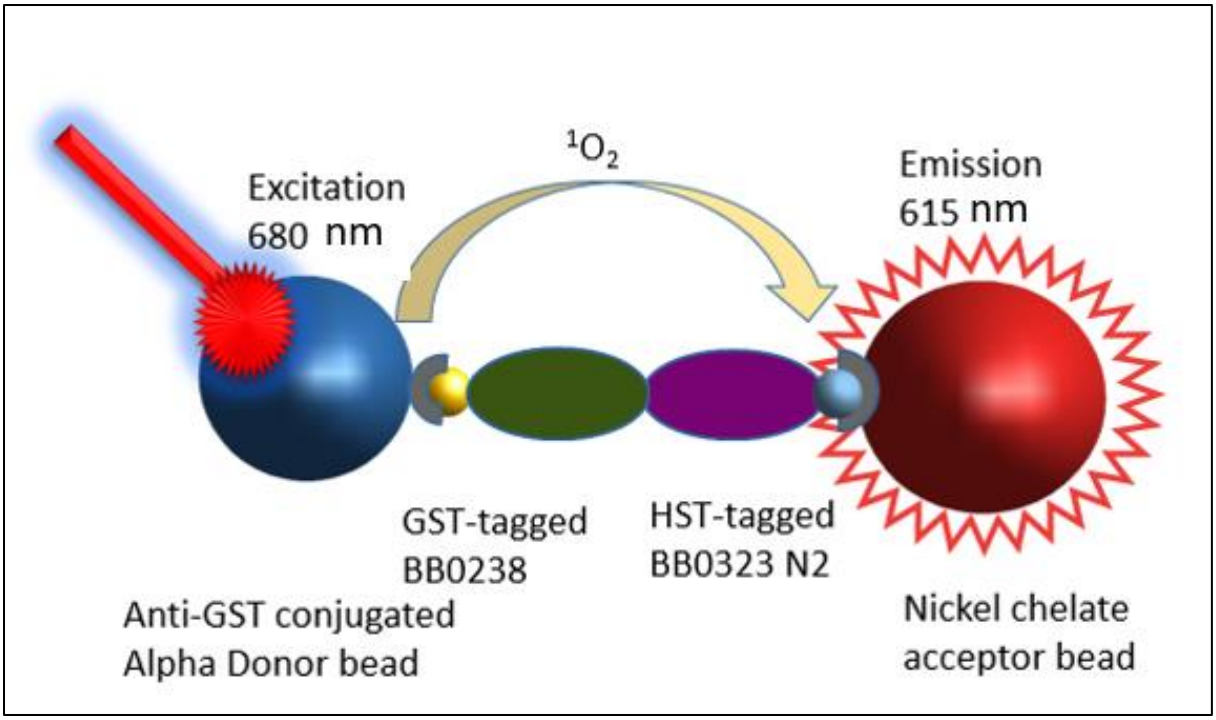


Figure 17. A Schematic representation of the Alphalisa assay. Alpha assay with GST-BBO238 protein binding to anti-GST conjugated beads as a donor bead and HST-tagged BB0323 N2 protein attached with a Nickle chelate acceptor bead. The donor and acceptor beads are kept close, within a 250nm diameter distance, by the interaction between BB0238 and BB0323-N2 proteins. Upon excitation at 680nm, a singlet oxygen molecule is transferred to the ambient acceptor bead, thus emitting light at 615 nm.

As mentioned earlier, the assay was developed in our lab and further standardization and utilization to screen the molecules were conducted at NCATS. The PPI assay was miniaturized and optimized to a 1536-well-plate format to enable automated high-throughput screening via robotics platforms. Subsequently, qHTS used the final and optimized Alphalisa BB0323/BB0238 PPI inhibition assay. A total of 8,988 compounds from several approved drug libraries from NCATS, which included investigational drugs with known mechanisms of action, and natural products that were screened. The qHTS assays often non-specific inhibitors; in most cases they bind non-competitively and have a nonspecific affinity. It could be due to colloidal aggregation,

some may contain privileged structure for binding, and others might alter fluorometric or calorimetric aspects of the assay, altering the final readout (Jadhav et al., 2010; McGovern et al., 2002). Accordingly, compounds with interfering and nonspecific properties were removed from the list after applying filters (Jadhav et al., 2010; McGovern et al., 2002, 2003). Two hundred sixty-nine compounds were selected for follow-up experiments based on the potency (half maximum inhibitory concentration (IC_{50}) below 30 μM) and efficacy (≥ 60%). The compounds were retested in dilution series using AlphaLisa PPI inhibition assay, and 163 compounds were further selected with confirmed inhibitory activity. Furthermore, the primary Hits were subjected to TruHits assay (PerkinElmer) that screens out false positives such as oxygen and color quenchers, among others. Also, another counter screen which comprised a His-tagged GST protein in an identical assay format, screened out the compounds which could act by inhibiting the binding of tags to the beads (data not shown). Finally, 84 compounds were selected for further orthogonal cell-based assessment.

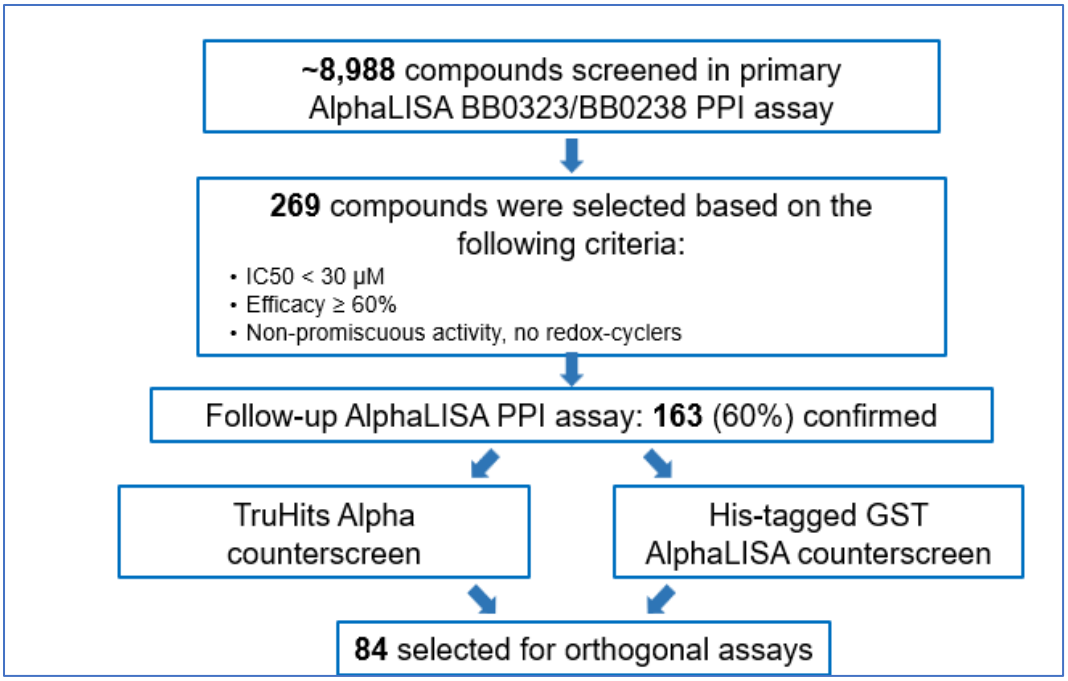


Figure 18. **A chart displaying the drug development process from screening small molecules by qHTS. (**Obtained from NCATS by courtesy of Dr. Bolormaa Baljinnyam). Discovery flow chart for small molecule inhibitors of BB0323: BB0238 PPI. The chart shows the key stages of drug screening, including primary screening- performed by HTS-AlphaLisa assay, selection of 160 compounds based on their IC50, and Efficacy. The next stage comprised of counter screening with TruHits assay and HST-tagged GST counter screen. 84 compounds were selected from around 8988 small molecules.

5.3 Discussion

This chapter presents the initial stage of the drug discovery process against LD by evaluating the BB0323:BB0238 PPI complex as a potential therapeutic target. As confirmed by the serology, qPCR, and organ cultures in BSK media, the impairment in the interaction of the BB0323:BB0238 complex blocked the ability of the spirochete to persist in mice for an extended period. Additionally, the xenodiagnoses experiment displayed that the ticks feeding on the mice injected with the mutants could not acquire

the *Borrelia*, indicating impairment in disseminating the disease, as ticks are the sole transmission mode.

The PPI inhibitor screening used the AlphaLisa assay with HST-BB0323 and GST-BB0238 proteins. This bead-based assay was initially developed 96 well plates and optimized for 384 well plates at our Lab. Furthermore, the miniaturization and screening of the libraries were performed at NCATS/NIH. The qHTS assay facilitated the screening of the molecules with predefined activities and natural products. After applications of several screening techniques, filters, secondary assays, and counter screens, 84 compounds were selected as HIT series. Further testing of these compounds using secondary assays is essential for confirming the inhibitory effect of these compounds and selecting Lead molecules.

The potential of BB0323: BB0238 complex as a drug target is also supported by previous studies in our lab. In short-term studies, the *bb0238*Δ*IM* isolates could not establish the infection in murine models (Thakur et al., 2017). The same study additionally used a luciferase-based assay and demonstrated that BB0323: BB0238 binding was saturable and could be inhibited by anti-BB0323 antibody or the recombinant partner proteins.

In conclusion, validating BB0323 and BB0238 PPI complex as potential therapeutic target provides a new avenue in the drug discovery process against LD. The use of the AlphaLisa platform expedited the process of screening several thousand molecules. The small molecules which could prevent the interaction identified by the assay were processed through several filters, controls, and counter assays, which presented 84 promising compounds which are further studied in the following chapters.

Chapter 6: Development of a cell-based assay to screen small molecule inhibitors against BB0323:BB0238 complex

Abstract

The drug discovery process requires the usage of appropriate assays, including cell-based assays that can select for cell permeable compounds, for example, and provide essential functional readouts of the expected effects of compounds on disease targets. We have performed a primary screen and identified a cohort of small molecule inhibitors that inhibit interactions between recombinant forms of BB0323 and BB0238. Our goal is to further short list the compounds with cell permeable properties, and that can inhibit interactions between native BB0323 and BB0238 within a spirochete cell. In this study, we have developed a *Borrelia* cell-based assay using a dot blot assay format coupled with a regrowth assay, that identifies the desired shortlisted hit molecules, and we opted to use the AmershamTM Protran Premium NC membrane (Hybond). Pre-charging the membrane with 1XPBS and drying before pipetting the dots made the dot sizes more consistent. A reliable and reproducible dot blot assay was established by changing and optimizing several parameters, that included optimal processing of *B. burgdorferi* cell lysates, membrane type used for blotting the lysates, and development of a semi-HTS screening using the dot blot assay, using anti-BB0323N antibodies. The assay principle was based on the fact that the prevention of interaction of BB0323 and BB0238, herein with selected cell permeable inhibiters, would result in the degradation of the proteins

over time. The optimized assay subsequently analyzed the 84 compounds selected from the primary Alphalisa screen. Two compounds were selected based on their PPI inhibitory activity and least impact on the growth of *B. burgdorferi* cells. Finally, one of the small molecules with the molecular formula $C_{25}H_{35}NO_4S$, Lomibuvir, was further analyzed by a dose-response assay based on the dot blots and the IC_{50} value. Therefore, in the current chapter, we detailed our efforts to successfully develop a cell based assay, which can be a reliable tool for identifying cell permeable inhibitors that can impact protein stability, likely by impairing selected PPI.

6.1 Introduction

Drug development against a disease or an infection is a rigorous, time-consuming, and highly costly enterprise (Hughes et al., 2011). The process involves several essential steps: target identification, target validation, hit discovery, leads selection, and optimization, and if applicable medicinal chemistry to enhance efficacy. The effort entails identifying an aspect of a disease that could be targeted to prevent or treat an infection. The selected hits from the screening outcome are further confirmed by biological assays predictive of the principle of the disease targets. It is necessary to ensure the bioactivity of the compounds, the molecules that can enter the cellular architecture and reach the target are valid candidates for further development as potential therapeutics.

Herein, we describe our efforts to develop a *B. burgdorferi* cell-based assay to screen the molecules with PPI inhibitory effects in two native proteins: BB0323 and

BB0238. The first part of the assay was checking the functional impact of the compounds on the protein stability, as inhibition of BB0323:BB0238 interacting reduces their stability. The second part was to ensure whether those compounds did not have toxic impact on the *B. burgdorferi* cells. We thus optimized a dot-blot assay to measure functional outcome of protein stability and used a regrowth assay to examine any cytotoxic effects of the compounds.

Dot-blot (DB) is a highly dynamic, adaptable, and cost-effective technique to study the interaction of immune complexes (Rupprecht et al., 2010). Here, the antigens bind to a nitrocellulose membrane which can be detected by applying antibodies against it, and work in similar principles of ELISA. The DB is a highly sensitive technique and can be used conveniently for large sample sizes (Helbing et al., 2022; Rupprecht et al., 2010; H. O. Silva et al., 2021; Tsukazaki et al., 1998). The assay removes the need for gel electrophoresis as done in Western blotting.

Herein, we have adapted the dot blot assay for the phenotypic readout of the activity of the small molecules on PPI of BB0323 and BB0238 inside the *B. burgdorferi* cells. It effectively and reproducibly detects the target cellular proteins, in this case, the degradation of BB0323 protein level as it can not to associate with BB0238.
As detailed in the previous chapter, we used a qHTS system to examine several thousands of small molecules and selected 84 compounds that prevented the interaction between recombinant BB0323 and BB0238 proteins. To determine which of these compounds can enter spirochete cells and prevent the PPI in native BB0323 and BB0238, we have developed and standardized a spirochete cell-based dot-blot assay to further select HIT compounds. Furthermore, the assay is also used for the dose-dependent

confirmation of the action and IC_{50} of a lead compound, Lomibuvir. Sequentially, we selected the compound for *in vivo* studies in an animal model of LD.

6.2 Results

6.2.1 Standardization of dot blot assay

We sought to develop a dot blot assay in order to identify cell permeable compounds and their effect on inhibiting the BB0323:BB0238 complex, by measuring the decrease in the level of BB0323 protein. Our published protein turnover assay show that, in spirochete cells, BB0323 is unstable in the absence of BB0238 (Kariu et al., 2015). The result was also similar in the presence of BB0238 protein devoid of interaction motifs, so it cannot interact with BB0323 (Bernard et al., 2019; Thakur et al., 2017). Here, we utilized the principle and developed a *Borrelia* cell-based assay to measure the effects of small molecules on the interaction of BB0323: BB0238 and their post-translational stability. We initially used a basic protocol comprising lysis of wild-type Borrelia cells as described below, standardized, and optimized the parameters to use the assay for drug response studies.

Basic protocol

The cultures were grown until the mid-log phase. Wild-type, 10^6 spirochetes in 100µl of cultures were centrifuged at 12,000g for 5 minutes at 4°C. The experiment was performed in triplicates, and at least three biological repeats were performed. The cell pellets were resuspended with 1XPBS with 1% triton X-100. The mix was vortexed for

15 seconds three times in 10 minutes. The sample was centrifuged for 10 minutes at 12,000g at 4°C, and the supernatant was used for the assay. 1 µl and 2 µl samples were dotted on nitrocellulose membranes. The samples were air-dried overnight. The following morning the blots were blocked with 5% skimmed milk in PBST and processed for immunoblot by anti-BB0323 antiserum. The results from the assay were not ideal, and several discrepancies were noted. We further standardized the assay as follows:

6.2.1.1 Standardization of the dot blot assay

As BB0323 and BB0238 are abundant in *B. burgdorferi* periplasm (Kariu et al., 2013, 2015; Zhang et al., 2009), we initially used the soluble fraction of *B. burgdorferi* cells for the dot-blot assay. However, during the standardization process of initial blotting of soluble protein fraction in the NC membrane, we realized there was an inconsistency in the amounts of antigens bound by the membrane, even between the samples extracted in the same experiment. We sought to explore if the inconsistency is due to the variable dislodgement of the cell lysates from the NC membrane at any processing stage. Hence the loading sample on NC membrane had a varied amount of protein, as visualized by Ponceau S Staining. Therefore, we compared the consistency in the amount of antigen that can be used for the loading with different cell fractions, after solubilizing the cellular proteins **(Figure 19A).** The samples containing cell supernatant were extracted after centrifugation and separation of the lysates, and as expected, variable protein levels were detected in supernatant and pellet fractions**.** However, the whole cell lysates displayed fairly even and consistent loading profiles throughout the blot, as shown by the Ponceau S staining, which were further used for the rest of the assays.

Additionally, we optimized the level of detergent to lyse the cells. Here, we used Triton X-100 in 1XPBS for its effective cell (membrane) lysis and non-ionic properties. We inspected the effect of varying concentrations of Triton X-100 in the cell lysis and the level of protein binding with the nitrocellulose membrane **(Figure 19B)**. And to determine the optimal amount of sample to load onto the nitrocellulose membrane in the dot-blot assay, wild-type *B. burgdorferi* lysates were applied in 1 uL and 2 uL volumes. The membrane was probed with an anti-BB0323 antibody (1:3000) followed by an anti-mouse HRP-labelled secondary antibody (1:10,000)**.** The result shows a sequential increase in intensity with the increase in the blotted volume **(Figure 19C)**. However, the lower volume of 1 uL still had significant detectable output signal.

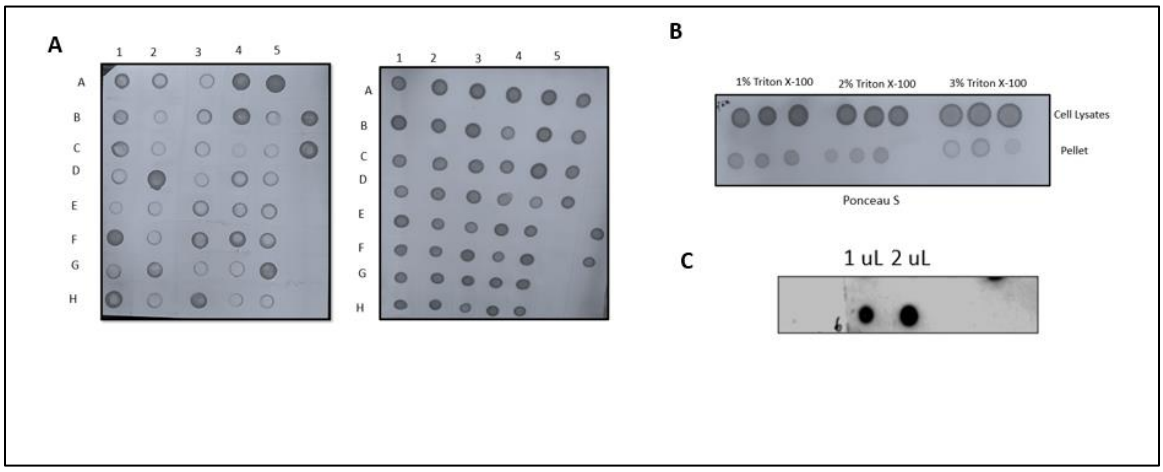


Figure 19. Standardization of dot-blot. (A) Inconsistencies in dot blot loading from different cell fractions. The figure displays Ponceau S staining of one of the first experiments performed. The wild-type *B. burgdorferi* lysates grown at 10^7 and seeded at 10^6 cells were centrifuged and lysed by 1XPBS Triton X-100. The lysates were centrifuged at 12,000g for 10 minutes, and (left) The supernatant was used for the dots. (right) The whole cell lysate was used for the dots. (B) Ponceau S staining of the *B. burgdorferi* samples extracted by different concentrations of Triton X-100. The nitrocellulose membrane with the antigen dots was incubated with Ponceau S for several

minutes and washed with water to remove excess dye. The resulting image shows the presence of protein dots corresponding to the samples, with varying concentrations of Triton X-100, i.e., 1%, 2%, or 3%. (C) Comparison of *B. burgdorferi* lysate signal intensity with different sample volumes on a dot blot. A representative image shows the difference between 1 uL and 2 uL samples applied on a nitrocellulose membrane, and the blot was developed using an anti-BB0323 N-terminal antibody (1:3000) and an anti-mice secondary HRP-labelled antibody (1:10,000). The signal intensity in the blot increased with the sample volumes.

Table 2. Optimized Dot-blot protocol for protein detection in *B. burgdorferi* lysates.

S.N	Steps
1.	Culture wild type *Borrelia burgdorferi* (A3) to mid-log phase. Adjust *B. burgdorferi* concentration to 1x 10^7/ml.
2	Seed 100µl of *B. burgdorferi* to 0.6ml Eppendorf tube (1x10^6 cells/well)
3.	Add 2 µl of drugs (Stock - 10mM)/well to make the final drug concentration 200 µM
4.	Incubate at 37°C for 16-18 hours. Note that control well (without drug addiction) contain a similar number of *B. burgdorferi* with DMSO (2µl/well)
5.	After incubation, add 1 µl of culture to fresh 200 µl of BSK to observe the re-growth of *B. burgdorferi*
6.	Centrifuge the rest of the culture to pellet the cells. Spin at 8000g for 15 minutes. Discard supernatant and collect *B. burgdorferi* pellet.
7.	Add 20 µl PBS with 1% TritonX100 to the cell pellet. Vortex to mix and then incubate on ice for 30 minutes. Mix the tube every 10 minutes with a vortex.
8.	Collect the supernatant, add 20µl PBS, and mix. The solution is now ready for dot-blot. Storing the mix at -20°C reduces the consistency of the results.
9.	Pre-wet the nitrocellulose membrane with 1XPBS for 5 minutes and air dry for 10 minutes.
10.	Dot 1µl of the lysate to the membrane and air dry at room temperature (RT) for 10 minutes.
11.	Block the membrane using 5% milk in PBST for 1 hour at RT.
12.	Add primary antibody (anti-BB0323-N terminal Antibody) at 1:3000 dilution and incubate Overnight at 4°C. Wash three times with PBS-T (PBS with 0.05% Tween-20)
13.	Add secondary antibody (HRP labeled goat anti-mouse IgG) at a dilution (1:100,000 or as appropriate) and incubate for 1 hour at RT.
14.	Develop the dot-blot result using standard chemiluminescence procedures.

15.	The density of each is scanned, and relative density is calculated via normalized against control s (*B. burgdorferi* treated only with DMSO), using ImageJ software.

6.2.2 Utilization of the cell-based assay to screen the small molecules obtained from AlphaLisa assay

Once we optimized the dot-blot assay, we tested the short-listed 84 compounds that were selected form the primary qHTS of 8988 small molecules. We would like to test the selected compounds' ability to enter borrelial cell and inhibit the native interaction of BB0323 and BB0238. The compounds were evaluated for their potential inhibitory effect by dot-blot and re-growth assays. In the first part of the assay, the wild-type *B. burgdorferi* were treated with selected compounds at 200 µM final concentration for 12-16 hours at 37ºC. DMSO-treated cells were taken as controls. Then, 1µl of the treated cells was added to 200 µl of fresh BSK media for the re-growth. A dot blot showed the effect of compounds on BB0323 N-terminal protein **(Figure 20A, left panel)**; in addition, the densitometric analysis of the blot, analyzed by ImageJ software, is displayed as a heat map (Green to Red) **(Figure 20A, Right panel).** Furthermore, the results of the regrowth assay six days after the initiation prompted the selection of the compounds with a minor effect (Purple to Yellow) on the fitness of the spirochetes **(Figure 20C).** The green circle in the left panel represents the compound selection based on low density and the least effect on cell growth as represented by densitometric and regrowth analyses. Two small molecules displaying the proper attributes were selected.

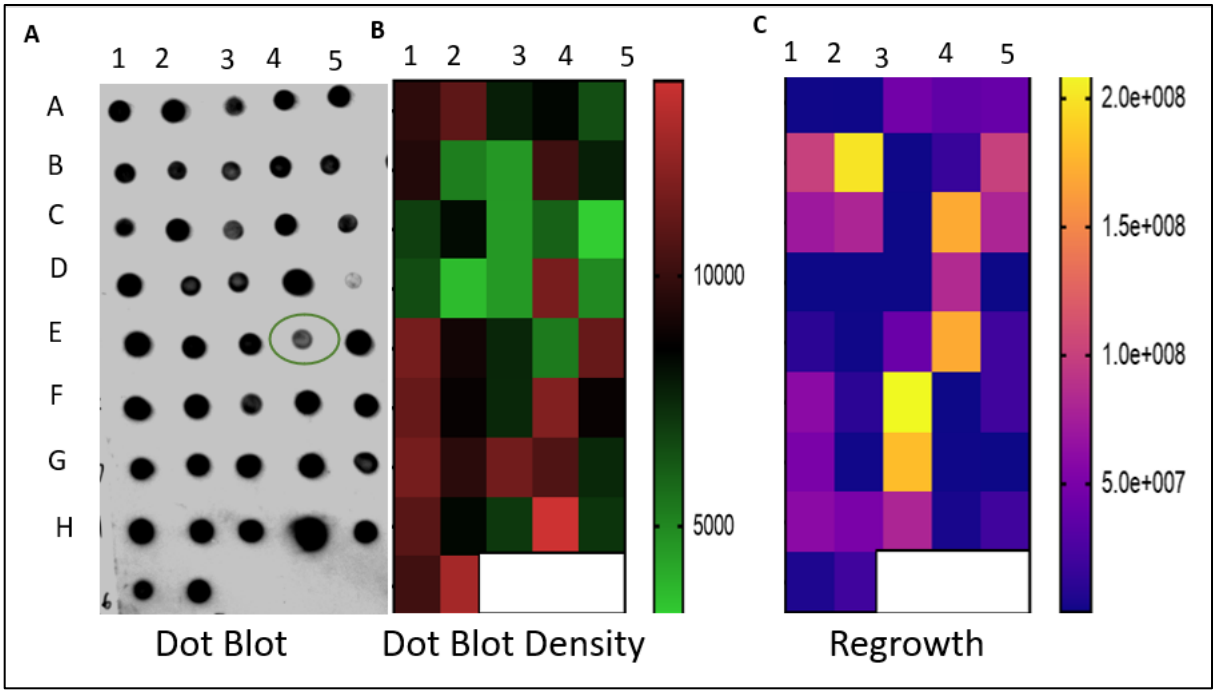


Figure 20. A representative Dot blot assay and a regrowth assay for selecting Lead molecules from the Hits series. (A) A dot blot assay was performed against the compounds obtained from the Alphalisa assay. The green circle represents a dot produced by a representative compound selected for further studies. (B) Densitometric analysis of the dot blot analyzed by ImageJ**.** (C) Regrowth assay results were obtained secondary to the incubation of Borrelia cells with compounds. The samples were displayed by GraphPad Prism 9.

6.2.3 The PPI inhibitory effect of the selected small molecule-Lomibuvir

The selected compound Lomibuvir was further tested for dose-response inhibitory effect using the cell-based assay as described above. The compound was serially diluted (2-fold) with concentrations ranging from 0.39 µM to 200 µM incubated with Borrelia cells overnight. The processed cell lysates were immobilized on the nitrocellulose membrane and incubated with Ponceau S stain to visualize the even loading of the lysate taken as a control **(Figure 21A right panel).** The blot was incubated with BB0323 N-terminal antibody, and the image was developed using chemiluminescent **(Figure 21A left panel)**. The dot blot showed dose response to BB0323 N-terminal protein detection.

We observed reduced signal intensity with increased concentration compared to wild-type and DMSO controls. The signal intensity of the dot plotted as loading control displayed that the compound dose response inversely correlates to BB0323 protein detection **(Figure 21B).** This indicates increasing in compound concentration correlates with a reduction of BB0323 target protein expression with IC_{50} of 1.5 µM **(Figure 21B).** As shown in the regrowth assay **(Figure 21C),** in which the cultures were counted six days after the re-growth assay initiation, the compound concentration beyond 6.2 µM affected the regrowth of *B. burgdorferi.* This level was higher than the IC50 of the PPI inhibitor effect; the lower concentrations did not affect cell growth. All the parameters confirm the ability of the small molecule to inhibit endogenous BB0323 and BB0238 interaction in a dose-dependent manner. This further confirmed that Lomibuvir has an inhibitory effect against BB0323:BB0238 interaction to suppress the expression of BB0323.

Additionally, the effect of higher concentrations on the re-growth of the spirochetes suggests that the compound might have an off-target impact on the spirochete cells in addition to the PPI inhibition.

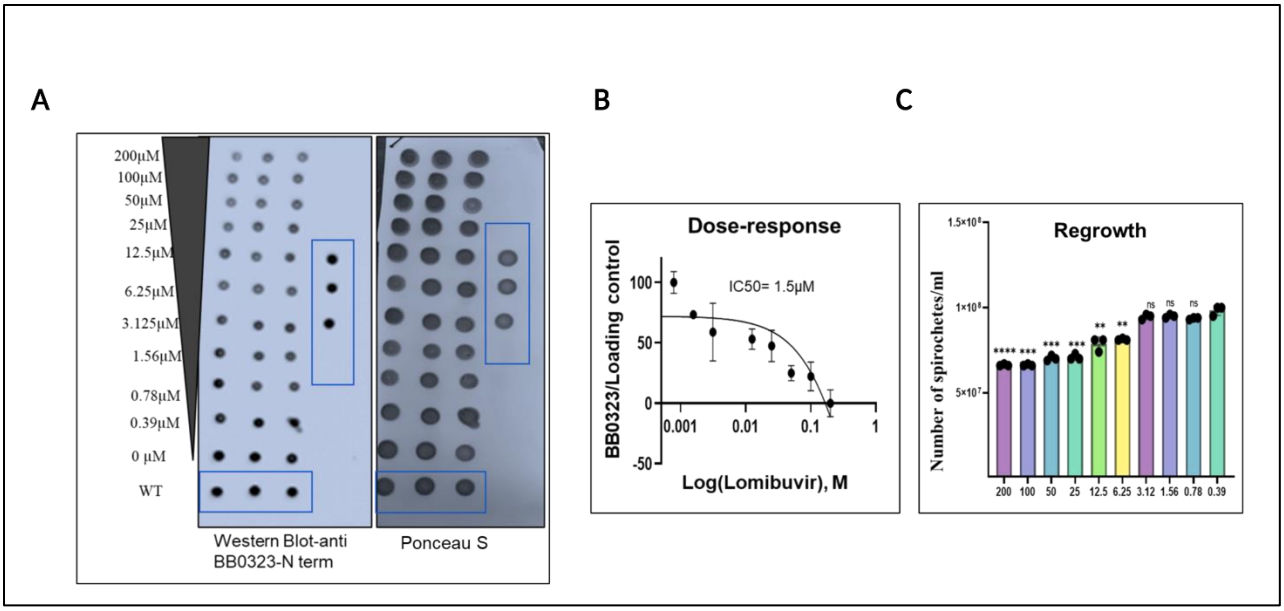


Figure 21. Assessment of the inhibitory effect of Lomibuvir. (A) Dot blot representing dose response of endogenous inhibition of BB0323 and BB0238 interaction in *Borrelia* culture by selected inhibitor- Lomibuvir. Immunoblot developed using anti-BB0323 N term antibody (left panel), Ponceau S stain showing even loading (Right panel). Samples from wild-type (untreated) spirochetes are presented inside the box. (B) A Dose-response curve. Analyses of dot blot by ImageJ software and presentation by GraphPad Prism 9. (C) Re-growth assay. Wild-type cells, pre-incubated with different concentrations of Lomibuvir, were used for the assay. 1 µL of the treated cells was added to the 200 µL of fresh BSK media and was incubated at 34 °C for 4-6 days. The cells were counted under a dark field microscope using a Petroff-hausser counting chamber. The result is representative of three repeats.

6.2.4 Effect of Lomibuvir on other proteins

We studied the inhibitory effects of Lomibuvir in BB0238- the other partner of the BB0323 complex. The spirochetal cell-based inhibition assay, comprised of dot blot analysis and densitometric analysis, was performed as discussed above. We also checked the level of another borrelial protein that is responsive to environmental changes and display varied expression in borrelial strains (Pal, et al., 2004). The dot-blot analysis was normalized based on a *B. burgdorferi* house-keeping protein, FlaB that was also unaltered

in the dot blot. Anti-BB0238, anti-OspC and anti-FlaB antibodies were used accordingly for the detection. The results show that the level of BB0238 protein was significantly lower in the group treated with Lomibuvir than those treated with DMSO **(Figure 22A).** However, the level of OspC was unaltered in both groups **(Figure 22B).** The results suggest that Lomibuvir exerts its specific effect on the BB0323: BB0238 complex and possibly does not affect other proteins in the cell.

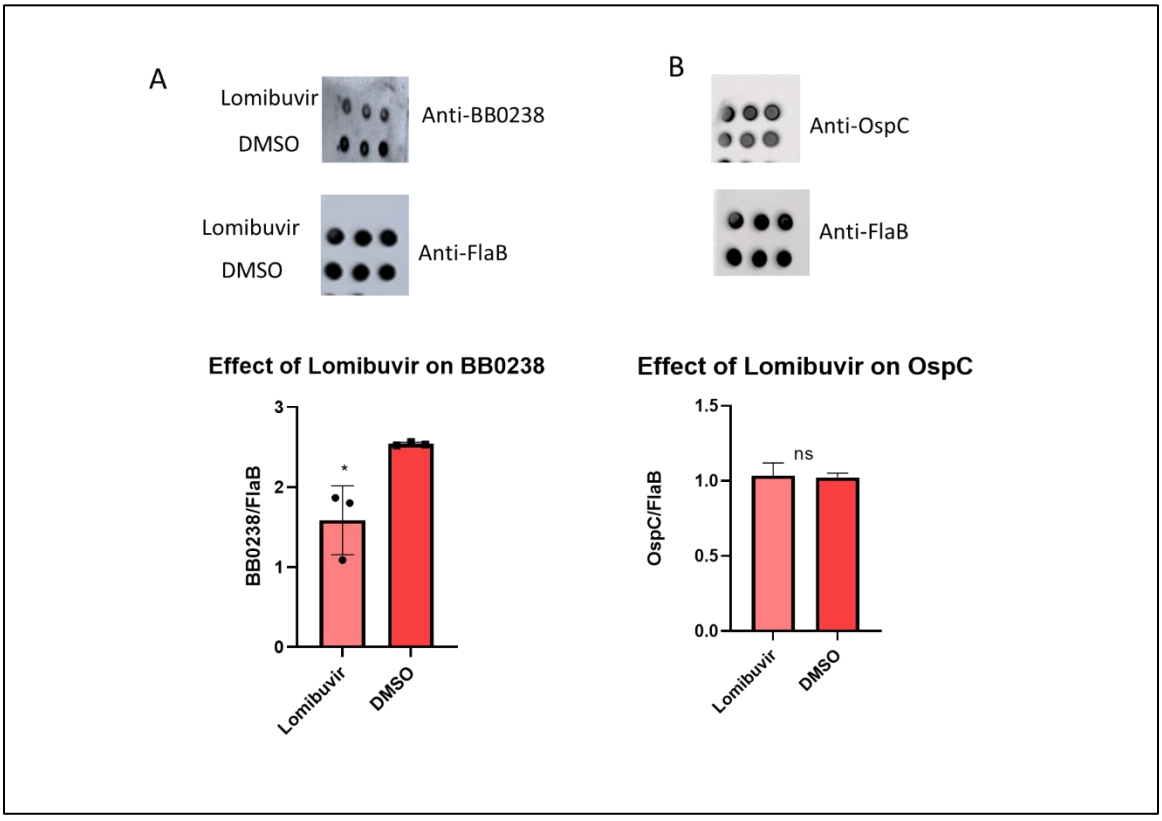


Figure 22. Specificity of the inhibitory effect of Lomibuvir on BB0323: BB0238 complex. (A)A dot blot image showing the effect of Lomibuvir on BB0238 protein. The *B. burgdorferi* cells treated with Lomibuvir/DMSO were centrifuged and lysed. The lysate was applied to a nitrocellulose membrane. The upper panel shows a dot blot developed against anti-BB0238, the middle panel is a dot blot developed against FlaB, and the lower panel shows the densitometric analysis of the above two blots by ImageJ. The results are presented by GraphPad analysis. (*P<0.05). (B) A dot blot image showing the effect of Lomibuvir on the OspC level. The upper panel shows a dot blot developed against an anti-OspC antibody, the middle panel is a dot blot developed against FlaB, and

the lower panel displays the densitometric analysis of the above two blots. The figure depicts that the small molecule had no observable effect on the OspC protein level.

6.3 Discussion

The success of a drug discovery process depends on the reliability and repeatability of the screening assay used in it. Herein, a relatively simple dot blot assay was optimized to screen 84 small molecules obtained from the AlphaLisa screening. The blot was developed using anti-BB0323 antibodies, and it measured the difference in BB0323-N-terminal protein level after using a PPI inhibitor molecule (Kariu et al., 2015). Adding a regrowth assay allowed us to select for the compounds with non-intended cytotoxic effects. The dot blot assays, like ELISA, can accommodate more than 96 samples. It is affordable and requires no sophisticated reaction equipment or reagents.

One of the compounds, Lomibuvir, was selected to validate the activity in the assay described above based on its PPI effect with minimal impact on the fitness of the spirochetes. The consistent effect of the compound on BB0238 bolsters the results of its impact on the inhibition of BB0323:BB0238 interactions. Notably, the level of OspC was unaffected by the use of Lomibuvir, suggesting the compound possibly does not influence expression of other non-target proteins. Altogether, the results provide strong evidence for the specificity of the compound on the BB0323:BB0238 complex. Furthermore, additional studies are warranted to validate the compound's effectiveness *in vivo*.

Chapter 7: Evaluation of selected small molecule inhibitors in animal model of Lyme borreliosis

Abstract

We have tested a small molecule, Lomibuvir, for its effectiveness on inhibiting interaction of BB0323 and BB0238 *in vivo*, which in turn impact spirochete infectivity, thus affording host protection against *B. burgdorferi* infection in a tick-borne murine model of LD. The results indicated that the compounds could lower the infection significantly when administered in C3H mice immediately after the tick-borne infection with *B. burgdorferi*. The study aimed to confirm the safety and efficacy of the small molecules using intraperitoneal injections for 21 days, with ceftriaxone as a positive control and the vehicle that molecule was solubilized as a negative control. The initial study showed a lowered infection, particularly in the skin of animals receiving the compound. A pharmacokinetic study of Lomibuvir showed a constant rise in the concentration of the compound in plasma and the liver. However, it was not the case in other organs. A dose of 5,15, or 20 mg/kg for 21 days did not display toxicity in the animals. The lowered infection level in the skin showed the efficacy of Lomibuvir in preventing infection. However, we recognize that the probability of the off-target effect of the compound cannot be discounted, and further studies are to be performed to confirm the PPI inhibition activity in the other borrelial species. It is also necessary to optimize the molecule properties and treatment strategies to enhance the therapeutic efficacy.

7.1 Introduction

The interfaces of biomolecular interactions, including PPIs are highly diverse, yet emerge as a viable target for anti-microbial therapeutic strategies, and the specific compounds can be discovered that inhibit the essential interactions (Chène, 2006). Small molecules, peptides, and antibodies are commonly used as PPI modulators. Out of three, small molecules have better cell permeability, metabolic stability, pharmacokinetics, and cost-effectivity (Lu et al., 2020; Ran & Gestwicki, 2018). Over the few years, interest in small molecules has increased. Unlike enzyme-substrate interactions, PPI interfaces are uniquely specific making it a difficult target (Akhter & Hussain, 2020; Choi et al., 2021; Rupprecht et al., 2010). The compounds might bind directly to the interaction site (Orthosteric site) or Allosteric sites, causing a conformational change for the effect (Ma & Nussinov, 2014; Nussinov & Tsai, 2012).

Using small molecules as new therapeutics has produced promising results in cancer and autoimmune diseases (Dhanak et al., 2017; Huck et al., 2018; G. Liu et al., 2022; Wu et al., 2022; Zhong et al., 2021). However, very few studies have been conducted to explore these exciting therapeutic spaces targeting bacterial infections (Carro, 2018). In this dissertation, we have explored a proof-of-principle study, whether PPI could be a valid target as anti-*B. burgdorferi* therapeutic strategy. We utilized our knowledge of virulence determinant factors of LD pathogens to identify small molecules to intervene in the infection. The proteins involved in the interaction, BB0323 and BB0238 are unique to the bacteria, so the molecules would be specific to it and have the least off-target effect. The *in vivo* study involving infected mice displays a promising

effect on bacterial clearance and illustrates the proof-of-concept that the BB0323: BB0238 complex is a potential molecular target for developing a novel therapeutic strategy to combat LD.

7.2 Results

7.2.1 Pharmacokinetic study

Pharmacokinetics studies are essential to study concentration of the compound in various post-injection time and at the target site (Yamashita & Hashida, 2013). The principles were mainly focused on quantitative analysis, including drug absorption, distribution, and excretion. The present study evaluated Lomibuvir for pharmacokinetics properties using a murine model after successfully screening for its pharmacological activities against *B. burgdorferi*. A total of 18 C3H mice were used to characterize the pharmacokinetics of the selected drug, and the mice were administered 15mg/kg consecutively for 21 days. The above study revealed that Lomibuvir at 15mg/kg was safe as a therapeutic dosage without any detectable toxic effects. In general, it is crucial to investigate the toxicological profile of any chemical substance to develop potential drug discovery in the pharmaceutical industry. Apart from that, the selection of drug delivery routes is another essential aspect of the animal model in pharmacokinetic studies to maximize the biodistribution of the test drug at the target site (Kushwaha et al., 2014). In this study, Lomibuvir at 15mg/kg was administered Intraperitoneally (IP). The

bioavailability of the test drug was evaluated by using LCMS/MS technique at different time points.

Three mice were euthanized sequentially at 0, 3, 6, 9, 15, and 21 days. The blood was drawn by cardiac puncture, and the other target organs, Liver, Skin, Heart, Bladder, and Joints, were collected. The blood and tissue samples were processed for LCMS/MS according to the method described in Sample Preparation. The processed samples were serially diluted in standard solution and 5µl injected into C18 5µm column. The LCMS/MS system was operated with an appropriate mobile phase with a 1.0min/ml flow rate.

The results of the Pharmacokinetics are summarized in (**Figure 23A-B)**. The mean drug concentrations released during the first 3 to 21 days significantly increase in blood plasma. Peak drug concentrations in blood were observed at 3 to 21 days. Apart from that, the liver is the main target organ in the general screening system which can trigger the safety dose evaluation in the early phase of drug discovery, primarily through pharmacokinetic studies (Cho & Yoon, 2015). In this study, the liver's drug concentrations decreased after the initial dose at 3 to 6 days **(Figure 23B).** This is probably due to the initial amount of the chemical compounds, which the active liver enzymes may convert to inactive metabolites to protect from drug-induced liver injury. Therefore, this may influence the biodistribution and elimination of the drug; hence, the bioavailability of Lomibuvir declined in the liver for the first three time points. However, the concentration of the compounds significantly increased from 15 to 21 days **(Figure 23A-B).**

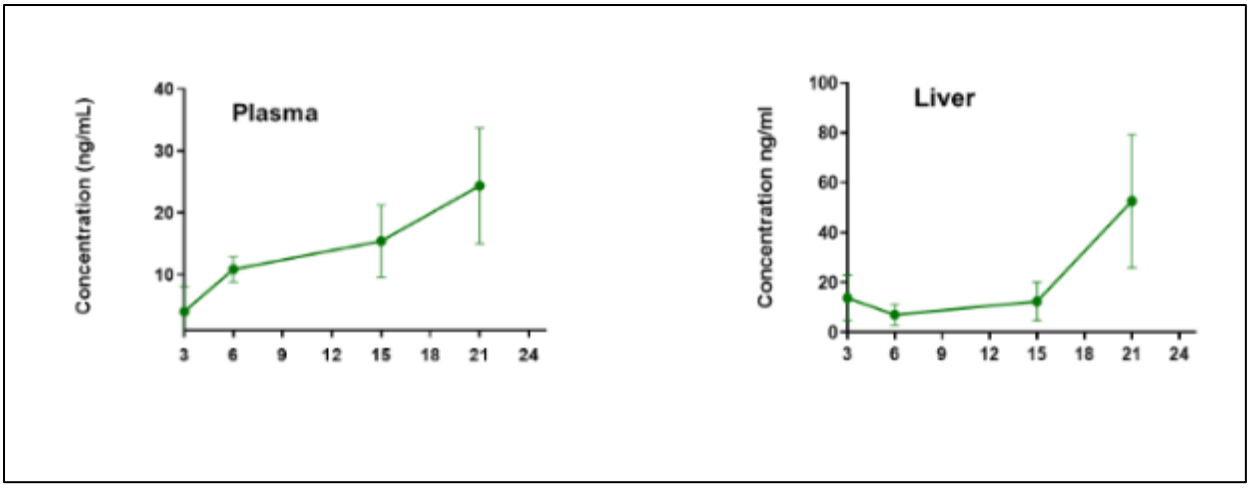


Figure 23. Pharmacokinetics study showing drug release profile. (A) Pharmacokinetic effect on plasma. The figure shows the pK effect of Lomibuvir on plasma over 21 days, the drugs were injected each day, and the sample was collected 24 hours after the last injection. (B) Pharmacokinetic effect of Lomibuvir on Liver. The data displays the concentration of the compound on each data point.

7.2.2 Target Engagement of Lomibuvir by Microscale Thermophoresis (MST)

Target engagement is an essential aspect in a drug discovery process to identify the molecular partner. In this study, we have used MST assay to identify the binding partner of Lomibuvir in the target BB0323: BB0238 complex. In the MST assay, the changes in the fluorescence in the HST-tagged protein with increasing compound concentration were measured. The results showed that the compound was bound to BB0238 protein with high affinity **(Figure 24A).** The binding affinity was further calculated by the dissociation constant (kd). The kd value for BB0238:Lomibuvir was 7.61 μM (± 0.67). In contrast, the compound didn't show valid affinity towards the HST-BB0323 protein **(Figure 24B)**. Additionally, the compound displayed no binding

properties toward the poly-histidine (HST) peptide **(Figure 24C)**. Overall, the results show a successful binding affinity of Lomibuvir towards BB0238.

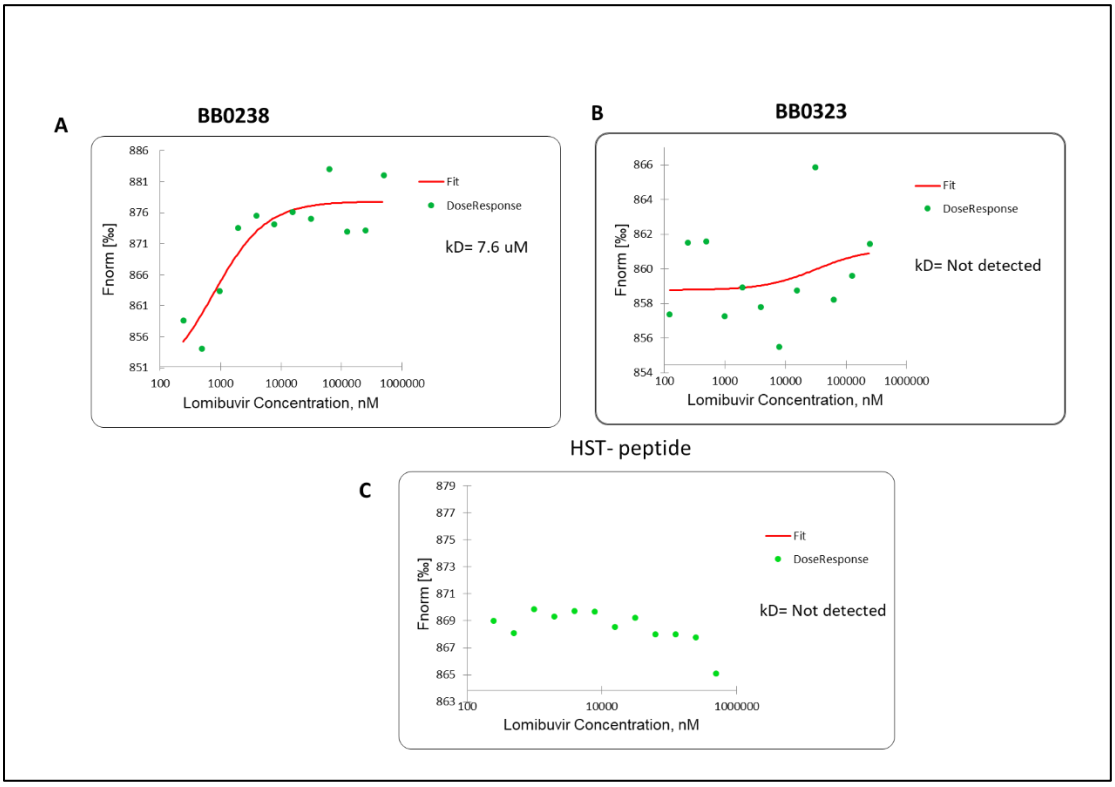
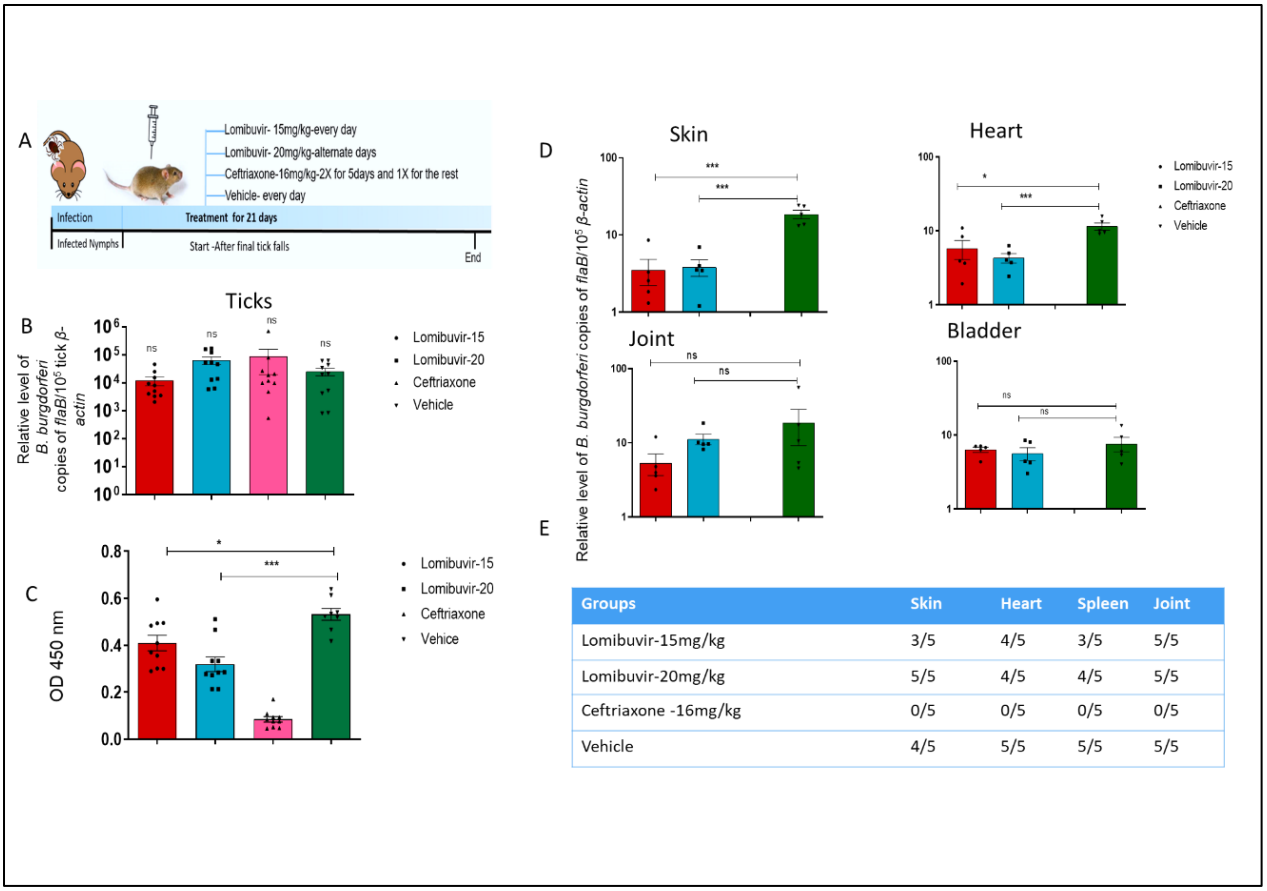


Figure 24. Target engagement of Lomibuvir with HST-BB0238, HST-BB0323, and HST peptide. The graphs display the binding affinity of Lomibuvir with the (A) BB0238, (B) BB0323, and (C) HST peptide, as measured by MST. The Y-axis shows normalized fluorescence percentage values (Fnorm %), and X-axis shows the concentration of Lomibuvir in the nano-molar unit.

7.2.3 Efficacy of Lomibuvir against *B. burgdorferi* in a tick-transmitted murine model of LD

We finally assessed the efficacy of Lomibuvir against *B. burgdorferi* infection using a reliable animal model of LD. A schematic representation of the disease model and treatment regimen is shown **(Figure 25A)**. To assess the efficacy of Lomibuvir in the treatment of *B. burgdorferi*, naïve nymphal ticks that infected with wild-type *B.*

burgdorferi using a microinjection, were used. Seven ticks on each mouse (5 animals/group) were allowed to feed on four separate groups of C3H mice (two separate doses of Lomibuvir, positive control (ceftriaxone) and negative control (vehicle). The spirochete burden in fully fed ticks was analyzed using qRT-PCR analysis of copies of the *flaB* gene and normalized against tick *β-actin* **(Figure 25B)**. After tick feeding, the animals were administered either of the two separate doses of the compound, antibiotic (positive control) or vehicle (negative control): 15mg/kg of Lomibuvir/day, 20 mg/kg of Lomibuvir alternate days, and 16 mg/kg ceftriaxone/day or with vehicle. The injections were performed Intraperitoneally (IP) for 21 days. Serological analysis (ELISA with *B. burgdorferi* lysate against antiserum from infected mice), suggested the infection was significantly lower in compound-treated groups than in the vehicle injected mice **(Figure 25C).** The bacterial levels in skin and heart tissues were also significantly reduced in both compounds treated groups compared to vehicles. In contrast, bladder and joint tissues did not differ significantly despite the lowering trend in joints **(Figure 25D)** and spirochetes were recovered from mice tissues treated with Lomibuvir **(Figure 25E)**. As expected, no spirochete burden was observed in ceftriaxone-treated mice Figure **(Figure 25D-E)**. Overall, the results show that Lomibuvir could lower the *B. burgdorferi* burden in the mice, at least in a tissue specific manner.



Groups	Skin	Heart	Spleen	Joint
Lomibuvir-15mg/kg	3/5	4/5	3/5	5/5
Lomibuvir-20mg/kg	5/5	4/5	4/5	5/5
Ceftriaxone -16mg/kg	0/5	0/5	0/5	0/5
Vehicle	4/5	5/5	5/5	5/5

Figure 25. In-vivo validation of the efficacy of Lomibuvir on *B. burgdorferi* clearance. (A) A schematic model of Borrelia transmission and treatment with Lomibuvir in mice. (B) Transmission of *B. burgdorferi* to mice by infected ticks. Naïve nymphal ticks were microinjected with equal numbers of wild-type spirochetes. The fully fed ticks were collected and assessed for *B. burgdorferi* burden by analyzing levels of *flaB* gene normalized against tick *β-actin* by qPCR. (C) Serological response in mice treated with Lomibuvir. Mice were infected with wild-type Borrelia by ticks microinjected with the spirochetes. Treatment was started immediately after repletion of ticks, either with Lomibuvir 15 mg/kg/day, Lomibuvir 20 mg/kg/alternate day, Ceftriaxone 16mg/kg/day, or Vehicle. Sera were collected after 21 days of injections to screen for differences with ELISA. (D) *B. burgdorferi* burden levels in murine skin, heart, joints, and bladder were assessed by using qPCR. The spirochete burden was significantly reduced in both Lomibuvir-treated groups in the skin and heart but not in joints and bladder. Borrelia burden was not detected in Ceftriaxone treated group. (*,P<0.05, **,P<0.01,***,P<0.001). (E) Culture analysis of treated mice tissues. The collected murine tissues were incubated at 37ºC in BSK media for 2-4 weeks. The samples were observed by dark field microscopy. The figure is representative of two animal experiments.

7.3 Discussion

Our results show that the compound, Lomibuvir, which has shown promising results *in vitro* as an effective PPI inhibitor of the BB0323: BB0238 protein complex, thus, we wanted to evaluate the compound's efficacy *in vivo* as well as its safety and pharmacokinetic properties. The ADME (Absorption, distribution, metabolism, and excretion) properties of Lomibuvir are unknown in mice. Thus, we performed a pilot pharmacokinetic study to determine the compound's ADME properties and checked the level of compounds in plasma and liver tissues at different treatment points, and measured the concentration by LC-MS/MS. The levels were lower than the available data on rats administered with the drug orally i.e., 30% bioavailability (Chauret et al., 2009). It might be due to the fact that we had different routes of administration and delayed timing of tissue collection.

Next, we conducted an *in vivo* efficacy study of Lomibuvir, including ceftriaxone and vehicle as a positive and negative control, respectively. The ceftriaxone antibiotic is widely used in LD treatment and in several *in vivo* studies (Wormser & Schwartz, 2009). Herein, we observed a consistent decrease in bacterial load compared to the vehicle-treated animals. Additionally, effects were also observed in the heart. However, the load was significantly higher than the ceftriaxone-treated group in all tissues. The optimization of the compound and the use of combinational therapy could improve the results. Furthermore, drug trials in other species should also be performed. Target engagement studies done by MST showed that the compound binds with BB0238 protein with the affinity of micromolar concentrations.

Lomibuvir is a known RNA polymerase inhibitor of the Hepatitis C virus (HCV) (Fenaux et al., 2013; PubChem, 2023). The polymerase enzyme is present in all life forms and is essential for gene expression and cellular functioning although there are species-specific divergences. In eukaryotes, RNA polymerase exists in three forms: Pol I, II, and III; each enzyme transcribes different types of RNA (Hsieh & Borger, 2022). In the case of viruses, depending on the type of its genetic material, various types of polymerase enzymes are present; namely, RNA-dependent RNA polymerase (RdRp) found in RNA viruses, DNA-dependent RNA polymerase found in DNA viruses, and Reverse transcriptase found in Retrovirus (Choi, 2012). Notably, in *B. burgdorferi*, a single RNA polymerase transcribes all kinds of RNAs (Boyle et al., 2020; Sutherland & Murakami, 2018). Further studies on the probability of Lomibuvir acting on *B. burgdorferi* RNA polymerase should be conducted more in order to confirm its activity as a sole PPI inhibitor. Nevertheless, an RNA polymerase inhibition treatment, as antimicrobial strategy, is not available yet for LD and should be explored further to identify novel treatment targets. Still, to the best of our knowledge, our study represents a handful of earlier studies targeting PPI as an anti-microbial therapeutic strategy against all human pathogens and constitutes the first study in the field of *B. burgdorferi* infection. As we have others have identified additional PPI events in *B. burgdorferi*, targeting these essential events could be exploited as novel anti-virulence therapeutic strategies to combat LD where a more effective and complete antibiotic therapy is warranted.

Chapter 8: Conclusions and Future Directions

LD is complex zoonotic infection with almost half a million new estimated cases yearly in the United States. It is caused by an atypical and highly-evolved gram-negative bacterium, *B. burgdorferi*, which has unique characteristics such as the absence of classical lipopolysaccharides or any toxins, contains endoflagella, and a structurally distinct peptidoglycan layer. On the other hand, *B. burgdorferi* features a segmented genome with a linear chromosome and up to 21 extra-chromosomal and self-replicating genetic elements called plasmids. The bacteria produce many outer surface lipoproteins that display little to no homology to known proteins yet are involved in the bacteria's entrance, adherence, colonization, and transmission. The spirochete genome encodes for minimal biosynthetic capacities making the bacteria an obligatory parasite. It is transmitted by ticks of *Ixodes species*, and the prevalence of the disease is increasing geographically. The disease can affect multiple organs; the symptoms are shared with many other febrile diseases, and thus the diagnosis still remains problematic. The treatment is more straightforward if the infection is identified in an earlier phase, which is challenging due to the unavailability of appropriate diagnostic aids. Additionally, as most tick bites go unnoticed, patients can seek physicians' help mainly in the later, more serious stages of infection. The disease often progresses to the chronic phases where extensive usage of antibiotics like doxycycline is required, including intravenous injections or even hospitalizations. Around 20% of the cases develop a vague set of symptoms after six months to a year after an apparently successful antibiotic treatment, collectively called as Post-treatment Lyme disease Syndrome (PTLDS) (CDC, 2022). The etiology of the PTLDS condition is not known yet, but theories suggest persistent

bacteria, immune dysregulation, and the alteration of the microbiome might be the contributing factors although a precise reason remains elusive. Some studies suggest that these conditions arise due to the effect of antibiotic therapy, again convincing evidence is lacking. Therefore, it is highly imperative to identify novel strategies for treatment against LD.

In this dissertation research, we have targeted a borrelial protein complex essential for spirochete infectivity, and explored whether it can serve as a molecular target for novel therapeutics discovery against LD. The first part of the complex involves PPI between BbHtrA and BB0323, which is essential for processing full-length BB0323 in smaller mature N and C terminal polypeptides. In lower temperatures, the spirochete can produce the basal level of the N-terminal, but in higher temperatures, the proteolytic processing by BbHtrA is required. Herein, in the first half, we investigated the necessity of the proteolytic activity of the protease on BB0323. At first, we identified the cleavage site of N and C termini and produced a protein with alteration in the cleavage site. This mutated protein could not be processed by HtrA *in vitro*, confirmed the identity of the cleavage site. We next produced a mutant of *B.* burgdorferi, which carried BB0323 with its cleavage site mutated for BbHtrA, instead of the wild type copy of BB0323. The cleavage mutant in the *B. burgdorferi* still produced BB0323 yet in a basal level along with two other aberrant polypeptides of lower molecular weight. This could be because there are two more areas with the same amino acid sequence in the protein where BbHtrA might act in the absence of the primary site. Although the cleavage site mutant did not affect the growth and fission of spirochetes, it was non-infectious in the murine host and had a unique immune profile, highlighting the biological significance of the BB0323

processing by BbHtrA. Additionally, as BB0323 N- and C-terminal fragments form heteromer, we checked the level of the C-terminal protein and its binding affinity to the N-terminal, which was unaltered. Again, we tested the level of BB0238, which was also unaffected, and binding was comparable to the wild-type. Still, due to a lower level of mature N-terminal, less BB0238 was recruited, leading to fewer BB0323:BB0238 complexes. Previous studies show that BB0323:BB0238 complex is essential in maintaining infection *in vivo*; here, in this study, we identified that the optimal stoichiometric balance of the complex is crucial to drive *B. burgdorferi* infection in a mammalian host.

We next explored the BB0323: BB0238 complex as a target for the novel drug discovery. In this study, we tested the prospect of BB0323: BB0238 complex as a drug target against the long-term persistence of *B. burgdorferi* in mice hosts. We show that the mutant with altered PPI motifs for BB0323: BB0238, called *bb0238ΔIM* mutants, which produces BB0323 and BB0238, but these proteins failed to interact, are unable to establish long-term infection in murine hosts.

We next developed a qHTS compatible test in our lab, called AlphaLisa and in collaboration with NCATS, screened nearly 9000 small molecule compounds. Amongst 84 compounds that inhibited BB0323: BB0238 interaction, we further short listed to a single molecule Lomibuvir, based on a *B. burgdorferi* cell-based secondary assay we developed to confirm the cell permeability and activity of the compounds on native protein within spirochete cells.

The preliminary assessment of the molecule, Lomibuvir treatment in the mouse infection model of LD showed a promising result, as the bacterial load in the skin of the treated mice had a decreasing trend. The safety of the molecules at the given dose was also confirmed by closely observing the health and well-being of the treated mice. A pharmacokinetic study showed an increase in compound concentration in plasma and liver over the period of 21 days. The compound's efficacy was further confirmed by *in vivo* experiments, including ceftriaxone treatment as a positive and vehicle treatment as a negative control.

Herein we detailed our efforts to test a core hypothesis - whether a PPI event essential for establishment of *B. burgdorferi* infection in a mammalian host – could be a viable target for small molecule therapeutics against LD. Taken together, these results, although promising as a first proof-of-concept study, could not produce a sterilizing clearance against the infection. Thus, it might also be necessary to optimize the molecule, via medicinal chemistry, to increase its efficacy and pharmacokinetic properties. The prospect of using the small molecule as a combination therapy to lower the antibiotic dose should also be explored. Additionally, as Lomibuvir is a known RdRp inhibitor of the Hepatitis C Virus, a study of the probable effect on RNA polymerase activity of *B. burgdorferi* is highly warranted. In addition to Lomibuvir, other compounds identified by the AlphaLisa assay and confirmed by the spirochete cell-based assay could also be studied in greater detail for their efficacy against *B. burgdorferi*. As our study represents a first-hand and pioneering study of utilizing PPI as viable anti-microbial therapeutic target, additional molecular targets, such as additional virulence determinants of LD

pathogens, including PPI events essential for infection should be explored for development of novel drug targets and comprehensive treatment strategies.

Contributions Statement

Some of the data provided in this dissertation, produced through collaborative efforts, are detailed here. The circular dichroism and biolayer interferometry assays were performed at Dr. Daniel Nelson's lab, IBBR. The high throughput screening of small molecules in the AlphaLisa platform and microscale thermophoresis were performed at NCATS, NIH. The processing of tissue samples for pharmacokinetic studies was done at ChemoGenics BioPharma, NC.

Additionally, the mutation of *B. burgdorferi* to obtain *Bb*^{bb0323NL} *isolates* and the production of recombinant proteins -BB0323^{NL} and BB0323^{NL}-N were performed by our previous lab member, Dr. Meghna Thakur. The initial optimization of the AlphaLisa assay was performed by Dr. Meghna Thakur and Dr. Shelby Foor in our lab. We appreciate and acknowledge everyone involved in carrying out these studies and recognize their contributions and scientific input.

Appendices

List of publications

1. Meghna Thakur*, **Sandhya Bista***, Shelby D. Foor, Shraboni Dutta, Xiuli Yang, Michael Ronzettia, Vipin S. Rana, Chrysoula Kitsou, Sara B. Linden, Amanda S. Altieri, Bolormaa Baljinnyam, Daniel C. Nelson, Anton Simeonov, Utpal Pal. Controlled Proteolysis of an Essential Virulence Determinant Dictates Infectivity of Lyme Disease Pathogens. Infection and Immunity. Vol - 90(5):e0005922 (2022). (Selected as American Society of Microbiology Spotlight Article.) *Meghna Thakur and Sandhya Bista contributed equally to this article.

2. Juraj Koči, **Sandhya Bista,** Payal Chirania, XiuliYang, Chrysoula Kitsou, Vipin Singh Rana, Ozlem Buyuktanir, Daniel E. Sonenshine, Utpal Pal. Antibodies against EGF-like domains in *Ixodes scapularis* BM86 orthologs impact tick feeding and survival of *Borrelia burgdorferi*. Scientific Reports. Vol -11, Article number: 6095 (2021).

3. Chrysoula Kitsou, Shelby D. Foor, Shraboni Dutta**, Sandhya Bista**, Utpal Pal. Tick gut barriers impacting tick–microbe interactions and pathogen persistence. Molecular Microbiology. Vol - 116(5):1241-1248, (2021).

4. **Sandhya Bista**, Preeti Singh, Quentin Bernard, Xiuli Yang, Thomas Hart, Yi-Pin Lin,2 Chrysoula Kitsou, Vipin Singh Rana, Fuming Zhang, Robert J. Linhardt, Kai Zhnag, Darrin R. Akins, Lucy Hritzo, Yuri Kim, Dennis J. Grab, J. Stephen Dumler, and Utpal Pal. University of Maryland., A Novel Laminin-Binding Protein Mediates Microbial-Endothelial Cell Interactions and Facilitates Dissemination of Lyme Disease Pathogens. The Journal of Infectious Diseases. Vol - 221, 1438–1447, (2020).

Bibliography

Adams, D. A., Jajosky, R. A., Ajani, U., Kriseman, J., Sharp, P., Onwen, D. H., Schley, A. W., Anderson, W. J., Grigoryan, A., Aranas, A. E., Wodajo, M. S., Abellera, J. P., & Centers for Disease Control and Prevention (CDC). (2014). Summary of notifiable diseases--United States, 2012. *MMWR. Morbidity and Mortality Weekly Report*, *61*(53), 1–121.

Adeolu, M., & Gupta, R. S. (2014). A phylogenomic and molecular marker based proposal for the division of the genus *Borrelia* into two genera: the emended genus *Borrelia* containing only the members of the relapsing fever *Borrelia*, and the genus *Borreliella* gen. nov. containing the members of the Lyme disease *Borrelia* (*Borrelia burgdorferi* sensu lato complex). *Antonie van Leeuwenhoek*, *105*(6), 1049–1072. https://doi.org/10.1007/s10482-014-0164-x

Adrion, E. R., Aucott, J., Lemke, K. W., & Weiner, J. P. (2015). Health care costs, utilization and patterns of care following Lyme disease. *PloS One*, *10*(2), e0116767. https://doi.org/10.1371/journal.pone.0116767

Aguero-Rosenfeld, M. E., Wang, G., Schwartz, I., & Wormser, G. P. (2005). Diagnosis of Lyme Borreliosis. *Clinical Microbiology Reviews*, *18*(3), 484–509. https://doi.org/10.1128/CMR.18.3.484-509.2005

Akhter, Y., & Hussain, R. (2020). *Protein-protein complexes as targets for drug discovery against infectious diseases* (pp. 237–251). https://doi.org/10.1016/bs.apcsb.2019.11.012

Alexopoulou, L., Thomas, V., Schnare, M., Lobet, Y., Anguita, J., Schoen, R. T., Medzhitov, R., Fikrig, E., & Flavell, R. A. (2002). Hyporesponsiveness to vaccination with *Borrelia burgdorferi* OspA in humans and in TLR1- and TLR2-deficient mice. *Nature Medicine*, *8*(8), 878–884. https://doi.org/10.1038/nm732

Alitalo, A., Meri, T., Lankinen, H., Seppälä, I., Lahdenne, P., Hefty, P. S., Akins, D., & 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. *The Journal of Immunology*, *169*(7), 3847–3853. https://doi.org/10.4049/jimmunol.169.7.3847

Arkin MR, Glicksman MA, & Fu H. (2012). Inhibition of Protein-Protein Interactions: Non-Cellular Assay Formats. In Markossian S, Grossman A, & Brimacombe K et al. (Eds.), *Assay Guidance Manual*. Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Arnold, W. K., Savage, C. R., Lethbridge, K. G., Smith, T. C., Brissette, C. A., Seshu, J., & Stevenson, B. (2018). Transcriptomic insights on the virulence-controlling CsrA, BadR, RpoN, and RpoS regulatory networks in the Lyme disease spirochete. *PLOS ONE*, *13*(8), e0203286. https://doi.org/10.1371/journal.pone.0203286

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P., Liu, H., Cross, J. R., Pfeffer, K., Coffer, P. J., & Rudensky, A. Y. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature*, *504*(7480), 451–455. https://doi.org/10.1038/nature12726

Arvikar, S. L., & Steere, A. C. (2015). Diagnosis and Treatment of Lyme Arthritis. *Infectious Disease Clinics of North America*, *29*(2), 269–280. https://doi.org/10.1016/j.idc.2015.02.004

Asmar, A. T., & Collet, J.-F. (2018). Lpp, the Braun lipoprotein, turns 50—major achievements and remaining issues. *FEMS Microbiology Letters*, *365*(18). https://doi.org/10.1093/femsle/fny199

Asmar, A. T., Ferreira, J. L., Cohen, E. J., Cho, S.-H., Beeby, M., Hughes, K. T., & Collet, J.-F. (2017). Communication across the bacterial cell envelope depends on the size of the periplasm. *PLOS Biology*, *15*(12), e2004303. https://doi.org/10.1371/journal.pbio.2004303

Aucott, J. N., Soloski, M. J., Rebman, A. W., Crowder, L. A., Lahey, L. J., Wagner, C. A., Robinson, W. H., & Bechtold, K. T. (2016). CCL19 as a Chemokine Risk Factor for Posttreatment Lyme Disease Syndrome: a Prospective Clinical Cohort Study. *Clinical and Vaccine Immunology*, *23*(9), 757–766. https://doi.org/10.1128/CVI.00071-16

Babu, M. M., Priya, M. L., Selvan, A. T., Madera, M., Gough, J., Aravind, L., & Sankaran, K. (2006). A Database of Bacterial Lipoproteins (DOLOP) with Functional Assignments to Predicted Lipoproteins. *Journal of Bacteriology*, *188*(8), 2761–2773. https://doi.org/10.1128/JB.188.8.2761-2773.2006

Barbour, A. G. (1988). Plasmid analysis of *Borrelia burgdorferi*, the Lyme disease agent. *Journal of Clinical Microbiology*, *26*(3), 475–478. https://doi.org/10.1128/jcm.26.3.475-478.1988

Barbour, A. G., Adeolu, M., & Gupta, R. S. (2017). Division of the genus *Borrelia* into two genera (corresponding to Lyme disease and relapsing fever groups) reflects their genetic and phenotypic distinctiveness and will lead to a better understanding of these two groups of microbes (Margos et al. (2016) There is inadequate evidence to support the division of the genus *Borrelia.* Int. J. Syst. Evol. Microbiol. doi: 10.1099/ijsem.0.001717). *International Journal of Systematic and Evolutionary Microbiology*, *67*(6), 2058–2067. https://doi.org/10.1099/ijsem.0.001815

Barbour, A. G., & Benach, J. L. (2019). Discovery of the Lyme Disease Agent. *MBio*, *10*(5). https://doi.org/10.1128/mBio.02166-19

Barbour, A. G., & Hayes, S. F. (1986). Biology of *Borrelia* species. *Microbiological Reviews*, *50*(4), 381–400. https://doi.org/10.1128/mr.50.4.381-400.1986

Beck, G., Benach, J. L., & Habicht, G. S. (1990). Isolation, preliminary chemical characterization, and biological activity of *Borrelia burgdorferi* peptidoglycan. *Biochemical and Biophysical Research Communications*, *167*(1), 89–95. https://doi.org/10.1016/0006-291X(90)91734-A

Belete, T. M. (2019). Novel targets to develop new antibacterial agents and novel alternatives to antibacterial agents. *Human Microbiome Journal*, *11*, 100052. https://doi.org/10.1016/j.humic.2019.01.001

Belisle, J. T., Brandt, M. E., Radolf, J. D., & Norgard, M. v. (1994). Fatty acids of Treponema pallidum and *Borrelia burgdorferi* lipoproteins. *Journal of Bacteriology*, *176*(8), 2151–2157. https://doi.org/10.1128/jb.176.8.2151-2157.1994

Belkaid, Y., & Hand, T. W. (2014). Role of the Microbiota in Immunity and Inflammation. *Cell*, *157*(1), 121–141. https://doi.org/10.1016/j.cell.2014.03.011

Benach, J. L., Bosler, E. M., Hanrahan, J. P., Coleman, J. L., Habicht, G. S., Bast, T. F., Cameron, D. J., Ziegler, J. L., Barbour, A. G., Burgdorfer, W., Edelman, R., & Kaslow, R. A. (1983). Spirochetes Isolated from the Blood of Two Patients with Lyme Disease. *New England Journal of Medicine*, *308*(13), 740–742. https://doi.org/10.1056/NEJM198303313081302

Berende, A., ter Hofstede, H. J. M., Vos, F. J., van Middendorp, H., Vogelaar, M. L., Tromp, M., van den Hoogen, F. H., Donders, A. R. T., Evers, A. W. M., & Kullberg, B. J. (2016). Randomized Trial of Longer-Term Therapy for Symptoms Attributed to Lyme Disease. *New England Journal of Medicine*, *374*(13), 1209–1220. https://doi.org/10.1056/NEJMoa1505425

Bernadac, A., Gavioli, M., Lazzaroni, J.-C., Raina, S., Lloube`s, R., & Lloube`s, L. (1998). *Escherichia coli* tol-pal Mutants Form Outer Membrane Vesicles. In *JOURNAL OF BACTERIOLOGY* (Vol. 180, Issue 18).

Bernard, Q., Thakur, M., Smith, A. A., Kitsou, C., Yang, X., & Pal, U. (2019). *Borrelia burgdorferi* protein interactions critical for microbial persistence in mammals. *Cellular Microbiology*, *21*(2). https://doi.org/10.1111/cmi.12885

Besant, P. G., & Attwood, P. V. (2010). *Histidine Phosphorylation in Histones and in Other Mammalian Proteins* (pp. 403–426). https://doi.org/10.1016/S0076-6879(10)71021-1

Blevins, J. S., Hagman, K. E., & Norgard, M. V. (2008). Assessment of decorin-binding protein A to the infectivity of *Borrelia burgdorferi* in the murine models of needle and tick infection. *BMC Microbiology*, *8*(1), 82. https://doi.org/10.1186/1471-2180-8-82

Blum, L. K., Adamska, J. Z., Martin, D. S., Rebman, A. W., Elliott, S. E., Cao, R. R. L., Embers, M. E., Aucott, J. N., Soloski, M. J., & Robinson, W. H. (2018). Robust B Cell Responses Predict Rapid Resolution of Lyme Disease. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.01634

Bockenstedt, L. K., & Radolf, J. D. (2014). Editorial Commentary: Xenodiagnosis for Posttreatment Lyme Disease Syndrome: Resolving the Conundrum or Adding to It? *Clinical Infectious Diseases*, *58*(7), 946–948. https://doi.org/10.1093/cid/cit942

Bockenstedt, L. K., Shanafelt, M.-C., Belperron, A., Mao, J., & Barthold, S. W. (2003). Humoral Immunity Reflects Altered T Helper Cell Bias in *Borrelia burgdorferi* -Infected γδ T-Cell-Deficient Mice. *Infection and Immunity*, *71*(5), 2938–2940. https://doi.org/10.1128/IAI.71.5.2938-2940.2003

Bockenstedt, L. K., Wooten, R. M., & Baumgarth, N. (2020). Immune response to *Borrelia*: Lessons from lyme disease spirochetes. *Current Issues in Molecular Biology*, *42*, 145–190. https://doi.org/10.21775/cimb.042.145

Bolz, D. D., Sundsbak, R. S., Ma, Y., Akira, S., Kirschning, C. J., Zachary, J. F., Weis, J. H., & Weis, J. J. (2004). MyD88 Plays a Unique Role in Host Defense but Not Arthritis Development in Lyme Disease. *The Journal of Immunology*, *173*(3), 2003–2010. https://doi.org/10.4049/jimmunol.173.3.2003

Bonnet, S., & Boulanger, N. (2017). Ixodes Tick Saliva. In *Arthropod Vector: Controller of Disease Transmission, Volume 2* (pp. 231–248). Elsevier. https://doi.org/10.1016/B978-0-12-805360-7.00013-7

Bontemps-Gallo, S., Lawrence, K., & 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 Pathogens, 12*(8), e1005791. https://doi.org/10.1371/journal.ppat.1005791

Boppana, K., Dubey, P. K., Jagarlapudi, S. A. R. P., Vadivelan, S., & Rambabu, G. (2009). Knowledge based identification of MAO-B selective inhibitors using pharmacophore and structure based virtual screening models. *European Journal of Medicinal Chemistry*, *44*(9), 3584–3590. https://doi.org/10.1016/j.ejmech.2009.02.031

Bourret, T. J., Lawrence, K. A., Shaw, J. A., Lin, T., Norris, S. J., & Gherardini, F. C. (2016). The Nucleotide Excision Repair Pathway Protects *Borrelia burgdorferi* from Nitrosative Stress in Ixodes scapularis Ticks. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01397

Boylan, J. A., & Gherardini, F. C. (2008). Determining the Cellular Targets of Reactive Oxygen Species in *Borrelia burgdorferi*. In *Bacterial Pathogenesis* (pp. 213–221). Humana Press. https://doi.org/10.1007/978-1-60327-032-8_17

Boyle, W. K., Groshong, A. M., Drecktrah, D., Boylan, J. A., Gherardini, F. C., Blevins, J. S., Samuels, D. S., & Bourret, T. J. (2019). DksA Controls the Response of the Lyme Disease Spirochete *Borrelia burgdorferi* to Starvation. *Journal of Bacteriology*, *201*(4). https://doi.org/10.1128/JB.00582-18

Boyle, W. K., Hall, L. S., Armstrong, A. A., Dulebohn, D. P., Samuels, D. S., Gherardini, F. C., & Bourret, T. J. (2020). Establishment of an in vitro RNA polymerase transcription system: a new tool to study transcriptional activation in *Borrelia burgdorferi*. *Scientific Reports*, *10*(1), 8246. https://doi.org/10.1038/s41598-020-65104-y

Branda, J. A., & Steere, A. C. (2021). Laboratory Diagnosis of Lyme Borreliosis. *Clinical Microbiology Reviews*, *34*(2). https://doi.org/10.1128/CMR.00018-19

Brandt, M. E., Riley, B. S., Radolf, J. D., & Norgard, M. v. (1990). Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. *Infection and Immunity*, *58*(4), 983–991. https://doi.org/10.1128/iai.58.4.983-991.1990

Brangulis, K., Akopjana, I., Kazaks, A., & Tars, K. (2019). Crystal structure of the N-terminal domain of the major virulence factor BB0323 from the Lyme disease agent *Borrelia burgdorferi*. *Acta Crystallographica Section D: Structural Biology*, *75*, 825–830. https://doi.org/10.1107/S2059798319010751

Braun, V., & Wu, H. C. (1994). *Chapter 14 Lipoproteins, structure, function, biosynthesis and model for protein export* (pp. 319–341). https://doi.org/10.1016/S0167-7306(08)60417-2

Brooks, C. S., Hefty, P. S., Jolliff, S. E., & Akins, D. R. (2003). Global Analysis of *Borrelia burgdorferi* Genes Regulated by Mammalian Host-Specific Signals. *Infection and Immunity*, *71*(6), 3371–3383. https://doi.org/10.1128/IAI.71.6.3371-3383.2003

Browning, D. F., & Busby, S. J. W. (2016). Local and global regulation of transcription initiation in bacteria. *Nature Reviews Microbiology*, *14*(10), 638–650. https://doi.org/10.1038/nrmicro.2016.103

Buist, G., Steen, A., Kok, J., & Kuipers, O. P. (2008). LysM, a widely distributed protein motif for binding to (peptido)glycans. *Molecular Microbiology*, *68*(4), 838–847. https://doi.org/10.1111/j.1365-2958.2008.06211.x

Burgdorfer, W. (1993). The Historical Road to the Discovery of *Borrelia burgdorferi*. In *Aspects of Lyme Borreliosis* (pp. 21–28). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-77614-4_2

Burgdorfer, W., Barbour, A. G., Hayes, S. F., Benach, J. L., Grunwaldt, E., & Davis, J. P. (1982). Lyme Disease—a Tick-Borne Spirochetosis? *Science*, *216*(4552), 1317–1319. https://doi.org/10.1126/science.7043737

Burgess, R. R., & Anthony, L. (2001). How sigma docks to RNA polymerase and what sigma does. *Current Opinion in Microbiology*, *4*(2), 126–131. https://doi.org/10.1016/S1369-5274(00)00177-6

Burtnick, M. N., Downey, J. S., Brett, P. J., Boylan, J. A., Frye, J. G., Hoover, T. R., & Gherardini, F. C. (2007). Insights into the complex regulation of rpoS in *Borrelia burgdorferi*. *Molecular Microbiology*, *65*(2), 277–293. https://doi.org/10.1111/j.1365-2958.2007.05813.x

Byram, R., Stewart, P. E., & Rosa, P. (2004). The Essential Nature of the Ubiquitous 26-Kilobase Circular Replicon of *Borrelia burgdorferi*. *Journal of Bacteriology*, *186*(11), 3561–3569. https://doi.org/10.1128/JB.186.11.3561-3569.2004

Caimano, M. J., Drecktrah, D., Kung, F., & Samuels, D. S. (2016). Interaction of the Lyme disease spirochete with its tick vector. *Cellular Microbiology*, *18*(7), 919–927. https://doi.org/10.1111/cmi.12609

Caimano, M. J., Dunham-Ems, S., Allard, A. M., Cassera, M. B., Kenedy, M., & Radolf, J. D. (2015). Cyclic di-GMP Modulates Gene Expression in Lyme Disease Spirochetes at the Tick-Mammal Interface To Promote Spirochete Survival during the Blood Meal and Tick-to-Mammal Transmission. *Infection and Immunity*, *83*(8), 3043–3060. https://doi.org/10.1128/IAI.00315-15

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., Blevins, J. S., & Radolf, J. D. (2019). The RpoS Gatekeeper in *Borrelia burgdorferi*: An Invariant Regulatory Scheme That Promotes Spirochete Persistence in Reservoir Hosts and Niche Diversity. *Frontiers in Microbiology*, *10*. 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., & 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. *Molecular Microbiology*, *65*(5), 1193–1217. https://doi.org/10.1111/j.1365-2958.2007.05860.x

Caimano, M. J., Kenedy, M. R., Kairu, T., Desrosiers, D. C., Harman, M., Dunham-Ems, S., Akins, D. R., Pal, U., & Radolf, J. D. (2011). The hybrid histidine kinase Hk1 is part of a two-component system that is essential for survival of *Borrelia burgdorferi* in feeding Ixodes scapularis ticks. *Infection and Immunity*, *79*(8), 3117–3130. https://doi.org/10.1128/IAI.05136-11

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

Caine, J. A., Lin, Y.-P., Kessler, J. R., Sato, H., Leong, J. M., & Coburn, J. (2017). *Borrelia burgdorferi* outer surface protein C (OspC) binds complement component C4b and confers bloodstream survival. *Cellular Microbiology*, *19*(12), e12786. https://doi.org/10.1111/cmi.12786

Carro, L. (2018). Protein–protein interactions in bacteria: a promising and challenging avenue towards the discovery of new antibiotics. *Beilstein Journal of Organic Chemistry*, *14*, 2881–2896. https://doi.org/10.3762/bjoc.14.267

Carroll, J. A., Cordova, R. M., & Garon, C. F. (2000). Identification of 11 pH-Regulated Genes in *Borrelia burgdorferi* Localizing to Linear Plasmids. *Infection and Immunity*, *68*(12), 6677–6684. https://doi.org/10.1128/IAI.68.12.6677-6684.2000

Cascales, E., Bernadac, A., Gavioli, M., Lazzaroni, J.-C., & Lloubes, R. (2002). Pal Lipoprotein of *Escherichia coli* Plays a Major Role in Outer Membrane Integrity. *Journal of Bacteriology*, *184*(3), 754–759. https://doi.org/10.1128/JB.184.3.754-759.2002

Casjens, S., Palmer, N., Van Vugt, R., Mun Huang, W., Stevenson, B., Rosa, P., Lathigra, R., Sutton, G., Peterson, J., Dodson, R. J., Haft, D., Hickey, E., Gwinn, M., White, O., & M. Fraser, C. (2002). A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. *Molecular Microbiology*, *35*(3), 490–516. https://doi.org/10.1046/j.1365-2958.2000.01698.x

Casjens, S. R., Fraser-Liggett, C. M., Mongodin, E. F., Qiu, W.-G., Dunn, J. J., Luft, B. J., & Schutzer, S. E. (2011). Whole Genome Sequence of an Unusual *Borrelia burgdorferi* Sensu Lato Isolate. *Journal of Bacteriology*, *193*(6), 1489–1490. https://doi.org/10.1128/JB.01521-10

CDC. (2021). *Preventing Tick Bites*. Http://Www.Cdc.Gov/.

CDC. (2022a). *Post-Treatment Lyme Disease Syndrome*.

CDC. (2022b). *Treatment of Lyme Disease*. https://www.cdc.gov/

Chaba, R., Alba, B. M., Guo, M. S., Sohn, J., Ahuja, N., Sauer, R. T., & Gross, C. A. (2011). Signal integration by DegS and RseB governs the σ^E-mediated envelope stress response in *Escherichia coli*. *Proceedings of the National Academy of Sciences*, *108*(5), 2106–2111. https://doi.org/10.1073/pnas.1019277108

Chauret, N., Chagnon-Labelle, C., Diallo, M., Laquerre, J., Laterreur, J., May, S., & Ste-Marie, L. (2009). *Preclinical Pharmacokinetic and ADME Characterization of VCH-222, a Novel Non-Nucleoside HCV NS5B Polymerase Inhibitor from Vertex/ViroChem*. https://www.natap.org/2009/EASL/EASL_65.htm

Chen, B., Retzlaff, M., Roos, T., & Frydman, J. (2011). Cellular strategies of protein quality control. *Cold Spring Harbor Perspectives in Biology*, *3*(8), a004374. https://doi.org/10.1101/cshperspect.a004374

Chen, J., Chia, N., Kalari, K. R., Yao, J. Z., Novotna, M., Paz Soldan, M. M., Luckey, D. H., Marietta, E. V., Jeraldo, P. R., Chen, X., Weinshenker, B. G., Rodriguez, M., Kantarci, O. H., Nelson, H., Murray, J. A., & Mangalam, A. K. (2016). Multiple

sclerosis patients have a distinct gut microbiota compared to healthy controls. *Scientific Reports*, *6*(1), 28484. https://doi.org/10.1038/srep28484

Chène, P. (2006). Drugs Targeting Protein–Protein Interactions. *ChemMedChem*, *1*(4), 400–411. https://doi.org/10.1002/cmdc.200600004

Chisholm, S. T., Dahlbeck, D., Krishnamurthy, N., Day, B., Sjolander, K., & Staskawicz, B. J. (2005). Molecular characterization of proteolytic cleavage sites of the *Pseudomonas syringae* effector AvrRpt2. *Proceedings of the National Academy of Sciences*, *102*(6), 2087–2092. https://doi.org/10.1073/pnas.0409468102

Cho, H.-J., & Yoon, I.-S. (2015). Pharmacokinetic Interactions of Herbs with Cytochrome P450 and P-Glycoprotein. *Evidence-Based Complementary and Alternative Medicine*, *2015*, 1–10. https://doi.org/10.1155/2015/736431

Choi, J., Yun, J. S., Song, H., Kim, N. H., Kim, H. S., & Yook, J. I. (2021). Exploring the chemical space of protein–protein interaction inhibitors through machine learning. *Scientific Reports*, *11*(1), 13369. https://doi.org/10.1038/s41598-021-92825-5

Choi, K. H. (2012). *Viral Polymerases* (pp. 267–304). https://doi.org/10.1007/978-1-4614-0980-9_12

Clausen, T., Kaiser, M., Huber, R., & Ehrmann, M. (2011). HTRA proteases: regulated proteolysis in protein quality control. *Nature Reviews Molecular Cell Biology*, *12*(3), 152–162. https://doi.org/10.1038/nrm3065

Clausen, T., Southan, C., & Ehrmann, M. (2002). Review The HtrA Family of Proteases: Implications for Protein Composition and Cell Fate HtrA represents the first well-studied protein quality control factor that acts in an ATP-independent manner. Furthermore, its recently described three-dimensional. In *Molecular Cell* (Vol. 10). http://www-sequence.stanford.edu/group/candida/.

Coburn, J., Garcia, B., Hu, L. T., Jewett, M. W., Kraiczy, P., Norris, S. J., & Skare, J. (2022). Lyme Disease Pathogenesis. *Current Issues in Molecular Biology*, 473–518. https://doi.org/10.21775/cimb.042.473

Coleman, J. L., Crowley, J. T., Toledo, A. M., & Benach, J. L. (2013). The HtrA protease of *Borrelia burgdorferi* degrades outer membrane protein BmpD and chemotaxis phosphatase CheX. *Molecular Microbiology*, *88*(3), 619–633. https://doi.org/10.1111/mmi.12213

Coughlin, J. M., Yang, T., Rebman, A. W., Bechtold, K. T., Du, Y., Mathews, W. B., Lesniak, W. G., Mihm, E. A., Frey, S. M., Marshall, E. S., Rosenthal, H. B., Reekie, T. A., Kassiou, M., Dannals, R. F., Soloski, M. J., Aucott, J. N., & Pomper, M. G. (2018). Imaging glial activation in patients with post-treatment Lyme disease symptoms: a pilot study using [11C]DPA-713 PET. *Journal of Neuroinflammation*, *15*(1), 346. https://doi.org/10.1186/s12974-018-1381-4

Cullen, P. A., Haake, D. A., & Adler, B. (2004). Outer membrane proteins of pathogenic spirochetes. *FEMS Microbiology Reviews*, *28*(3), 291–318. https://doi.org/10.1016/j.femsre.2003.10.004

Cyr, T. L., Jenkins, M. C., Hall, R. D., Masters, E. J., & McDonald, G. A. (2005). Improving the specificity of 16S rDNA-based polymerase chain reaction for detecting *Borrelia burgdorferi* sensu lato-causative agents of human Lyme disease. *Journal of Applied Microbiology*, *98*(4). https://doi.org/10.1111/j.1365-2672.2005.02539.x

Czaplewski, L., Bax, R., Clokie, M., Dawson, M., Fairhead, H., Fischetti, V. A., Foster, S., Gilmore, B. F., Hancock, R. E. W., Harper, D., Henderson, I. R., Hilpert, K., Jones, B. V, Kadioglu, A., Knowles, D., Ólafsdóttir, S., Payne, D., Projan, S., Shaunak, S., … Rex, J. H. (2016). Alternatives to antibiotics—a pipeline portfolio review. *The Lancet Infectious Diseases*, *16*(2), 239–251. https://doi.org/10.1016/S1473-3099(15)00466-1

Dai, K., & Lutkenhaus, J. (1992). The proper ratio of FtsZ to FtsA is required for cell division to occur in *Escherichia coli*. *Journal of Bacteriology*, *174*(19), 6145–6151. https://doi.org/10.1128/jb.174.19.6145-6151.1992

Dara, S., Dhamercherla, S., Jadav, S. S., Babu, C. M., & Ahsan, M. J. (2022). Machine Learning in Drug Discovery: A Review. *Artificial Intelligence Review*, *55*(3), 1947–1999. https://doi.org/10.1007/s10462-021-10058-4

de Silva, A. M., Telford, S. R., Brunet, L. R., Barthold, S. W., & Fikrig, E. (1996). *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. *The Journal of Experimental Medicine*, *183*(1), 271–275. https://doi.org/10.1084/jem.183.1.271

Dhanak, D., Edwards, J. P., Nguyen, A., & Tummino, P. J. (2017). Small-Molecule Targets in Immuno-Oncology. *Cell Chemical Biology*, *24*(9), 1148–1160. https://doi.org/10.1016/j.chembiol.2017.08.019

Donahue, J. G., Piesman, J., & Spielman, A. (1987). Reservoir Competence of White-Footed Mice for Lyme Disease Spirochetes. *The American Journal of Tropical Medicine and Hygiene*, *36*(1), 92–96. https://doi.org/10.4269/ajtmh.1987.36.92

Dowdell, A. S., Murphy, M. D., Azodi, C., Swanson, S. K., Florens, L., Chen, S., & Zückert, W. R. (2017). Comprehensive Spatial Analysis of the *Borrelia burgdorferi* Lipoproteome Reveals a Compartmentalization Bias toward the Bacterial Surface. *Journal of Bacteriology*, *199*(6). https://doi.org/10.1128/JB.00658-16

Drecktrah, D., Lybecker, M., Popitsch, N., Rescheneder, P., Hall, L. S., & Samuels, D. S. (2015). The *Borrelia burgdorferi* RelA/SpoT Homolog and Stringent Response Regulate Survival in the Tick Vector and Global Gene Expression during Starvation. *PLOS Pathogens*, *11*(9), e1005160. https://doi.org/10.1371/journal.ppat.1005160

Drouin, E. E., Seward, R. J., Strle, K., McHugh, G., Katchar, K., Londoño, D., Yao, C., Costello, C. E., & Steere, A. C. (2013). A novel human autoantigen, endothelial cell growth factor, is a target of T and B cell responses in patients with Lyme disease. *Arthritis & Rheumatism*, *65*(1), 186–196. https://doi.org/10.1002/art.37732

Duch, W., Swaminathan, K., & Meller, J. (2007). Artificial Intelligence Approaches for Rational Drug Design and Discovery. *Current Pharmaceutical Design*, *13*(14), 1497–1508. https://doi.org/10.2174/138161207780765954

Dulipati, V., Meri, S., & Panelius, J. (2020). Complement evasion strategies of *Borrelia burgdorferi* sensu lato. *FEBS Letters*, *594*(16), 2645–2656. https://doi.org/10.1002/1873-3468.13894

Dumic, I., & Severnini, E. (2018). "Ticking Bomb": The Impact of Climate Change on the Incidence of Lyme Disease. *Canadian Journal of Infectious Diseases and Medical Microbiology*, *2018*, 1–10. https://doi.org/10.1155/2018/5719081

Dunham-Ems, S. M., Caimano, M. J., Pal, U., Wolgemuth, C. W., Eggers, C. H., Balic, A., & Radolf, J. D. (2009). Live imaging reveals a biphasic mode of dissemination

of *Borrelia burgdorferi* within ticks. *Journal of Clinical Investigation*, *119*(12), 3652–3665. https://doi.org/10.1172/JCI39401

Dustin, M. L. (2008). T-cell activation through immunological synapses and kinapses. *Immunological Reviews*, *221*(1), 77–89. https://doi.org/10.1111/j.1600-065X.2008.00589.x

Elsner, R. A., Hastey, C. J., Olsen, K. J., & Baumgarth, N. (2015). Suppression of Long-Lived Humoral Immunity Following *Borrelia burgdorferi* Infection. *PLOS Pathogens*, *11*(7), e1004976. https://doi.org/10.1371/journal.ppat.1004976

Embers, M. E., Barthold, S. W., Borda, J. T., Bowers, L., Doyle, L., Hodzic, E., Jacobs, M. B., Hasenkampf, N. R., Martin, D. S., Narasimhan, S., Phillippi-Falkenstein, K. M., Purcell, J. E., Ratterree, M. S., & Philipp, M. T. (2012). Persistence of *Borrelia burgdorferi* in Rhesus Macaques following Antibiotic Treatment of Disseminated Infection. *PLoS ONE*, *7*(1), e29914. https://doi.org/10.1371/journal.pone.0029914

Embers, M. E., Grasperge, B. J., Jacobs, M. B., & Philipp, M. T. (2013). Feeding of ticks on animals for transmission and xenodiagnosis in Lyme disease research. *Journal of Visualized Experiments : JoVE*, *78*. https://doi.org/10.3791/50617

Fenaux, M., Eng, S., Leavitt, S. A., Lee, Y.-J., Mabery, E. M., Tian, Y., Byun, D., Canales, E., Clarke, M. O., Doerffler, E., Lazerwith, S. E., Lew, W., Liu, Q., Mertzman, M., Morganelli, P., Xu, L., Ye, H., Zhang, J., Matles, M., … Watkins, W. J. (2013). Preclinical Characterization of GS-9669, a Thumb Site II Inhibitor of the Hepatitis C Virus NS5B Polymerase. *Antimicrobial Agents and Chemotherapy*, *57*(2), 804–810. https://doi.org/10.1128/AAC.02052-12

Feng, S., Zhou, L., Huang, C., Xie, K., & Nice, E. C. (2015). Interactomics: toward protein function and regulation. *Expert Review of Proteomics*, *12*(1), 37–60. https://doi.org/10.1586/14789450.2015.1000870

Feucht, A., Lucet, I., Yudkin, M. D., & Errington, J. (2001). Cytological and biochemical characterization of the FtsA cell division protein of Bacillus subtilis. *Molecular Microbiology*, *40*(1), 115–125. https://doi.org/10.1046/j.1365-2958.2001.02356.x

Fikrig, E., Barthold, S. W., Kantor, F. S., & Flavell, R. A. (1992). Long-term protection of mice from Lyme disease by vaccination with OspA. *Infection and Immunity*, *60*(3), 773–777. https://doi.org/10.1128/iai.60.3.773-777.1992

Fingerle, V., Goettner, G., Gern, L., Wilske, B., & Schulte-Spechtel, U. (2007). Complementation of a *Borrelia* afzelii OspC mutant highlights the crucial role of OspC for dissemination of *Borrelia* afzelii in Ixodes ricinus. *International Journal of Medical Microbiology : IJMM*, *297*(2), 97–107. https://doi.org/10.1016/j.ijmm.2006.11.003

Fisher, M. A., Grimm, D., Henion, A. K., Elias, A. F., Stewart, P. E., Rosa, P. A., & Gherardini, F. C. (2005). *Borrelia burgdorferi* σ^{54} is required for mammalian infection and vector transmission but not for tick colonization. *Proceedings of the National Academy of Sciences*, *102*(14), 5162–5167. https://doi.org/10.1073/pnas.0408536102

Fitzgerald, B. L., Molins, C. R., Islam, M. N., Graham, B., Hove, P. R., Wormser, G. P., Hu, L., Ashton, L. V., & Belisle, J. T. (2020). Host Metabolic Response in Early Lyme Disease. *Journal of Proteome Research*, *19*(2), 610–623. https://doi.org/10.1021/acs.jproteome.9b00470

Forrester, J. D., & Mead, P. (2014). Third-Degree Heart Block Associated With Lyme Carditis: Review of Published Cases. *Clinical Infectious Diseases*, *59*(7), 996–1000. https://doi.org/10.1093/cid/ciu411

Fox, S., Farr-Jones, S., Sopchak, L., Boggs, A., Nicely, H. W., Khoury, R., & Biros, M. (2006). High-throughput screening: update on practices and success. *Journal of Biomolecular Screening*, *11*(7), 864–869. https://doi.org/10.1177/1087057106292473

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., Gwinn, M., Dougherty, B., Tomb, J.-F., Fleischmann, R. D., Richardson, D., Peterson, J., Kerlavage, A. R., Quackenbush, J., Salzberg, S., … Craig Venter, J. (1997). Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. In *NATURE* (Vol. 390). http://www.tigr.org/tdb/mdb/bbdb/bbdb.htm.

Frischknecht, F. (2007). The skin as interface in the transmission of arthropod-borne pathogens. *Cellular Microbiology*, *9*(7), 1630–1640. https://doi.org/10.1111/j.1462-5822.2007.00955.x

Fuchs, H., Wallich, R., Simon, M. M., & Kramer, M. D. (1994). The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. *Proceedings of the National Academy of Sciences*, *91*(26), 12594–12598. https://doi.org/10.1073/pnas.91.26.12594

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., Takahashi, M., Fukuda, N. N., Murakami, S., Miyauchi, E., Hino, S., Atarashi, K., Onawa, S., Fujimura, Y., Lockett, T., … Ohno, H. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*, *504*(7480), 446–450. https://doi.org/10.1038/nature12721

Gaca, A. O., Colomer-Winter, C., & Lemos, J. A. (2015). Many Means to a Common End: the Intricacies of (p)ppGpp Metabolism and Its Control of Bacterial Homeostasis. *Journal of Bacteriology*, *197*(7), 1146–1156. https://doi.org/10.1128/JB.02577-14

Gamba, P., Veening, J.-W., Saunders, N. J., Hamoen, L. W., & Daniel, R. A. (2009). Two-Step Assembly Dynamics of the *Bacillus subtilis* Divisome. *Journal of Bacteriology*, *191*(13), 4186–4194. https://doi.org/10.1128/JB.01758-08

Garcia, B. L., Zhi, H., Wager, B., Höök, M., & Skare, J. T. (2016). *Borrelia burgdorferi* BBK32 Inhibits the Classical Pathway by Blocking Activation of the C1 Complement Complex. *PLOS Pathogens*, *12*(1), e1005404. https://doi.org/10.1371/journal.ppat.1005404

Gerding, M. A., Ogata, Y., Pecora, N. D., Niki, H., & De Boer, P. A. J. (2007). The trans-envelope Tol-Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in E. coli. *Molecular Microbiology*, *63*(4), 1008–1025. https://doi.org/10.1111/j.1365-2958.2006.05571.x

Ginsberg, H. S., Albert, M., Acevedo, L., Dyer, M. C., Arsnoe, I. M., Tsao, J. I., Mather, T. N., & LeBrun, R. A. (2017). Environmental Factors Affecting Survival of Immature Ixodes scapularis and Implications for Geographical Distribution of Lyme Disease: The Climate/Behavior Hypothesis. *PLOS ONE*, *12*(1), e0168723. https://doi.org/10.1371/journal.pone.0168723

Ginsberg, H. S., Hickling, G. J., Burke, R. L., Ogden, N. H., Beati, L., LeBrun, R. A., Arsnoe, I. M., Gerhold, R., Han, S., Jackson, K., Maestas, L., Moody, T., Pang, G., Ross, B., Rulison, E. L., & Tsao, J. I. (2021). Why Lyme disease is common in the northern US, but rare in the south: The roles of host choice, host-seeking behavior, and tick density. *PLOS Biology*, *19*(1), e3001066. https://doi.org/10.1371/journal.pbio.3001066

Goldstein, S. F., Charon, N. W., & Kreiling, J. A. (1994). *Borrelia burgdorferi* swims with a planar waveform similar to that of eukaryotic flagella. *Proceedings of the National Academy of Sciences*, *91*(8), 3433–3437. https://doi.org/10.1073/pnas.91.8.3433

Gomes-Solecki, M., Arnaboldi, P. M., Backenson, P. B., Benach, J. L., Cooper, C. L., Dattwyler, R. J., Diuk-Wasser, M., Fikrig, E., Hovius, J. W., Laegreid, W., Lundberg, U., Marconi, R. T., Marques, A. R., Molloy, P., Narasimhan, S., Pal, U., Pedra, J. H. F., Plotkin, S., Rock, D. L., … Schutzer, S. E. (2020). Protective Immunity and New Vaccines for Lyme Disease. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *70*(8), 1768–1773. https://doi.org/10.1093/cid/ciz872

Gourse, R. L., Chen, A. Y., Gopalkrishnan, S., Sanchez-Vazquez, P., Myers, A., & Ross, W. (2018). Transcriptional Responses to ppGpp and DksA. *Annual Review of Microbiology*, *72*(1), 163–184. https://doi.org/10.1146/annurev-micro-090817-062444

Gray, V. E., Hause, R. J., & Fowler, D. M. (2017). Analysis of Large-Scale Mutagenesis Data To Assess the Impact of Single Amino Acid Substitutions. *Genetics*, *207*(1), 53–61. https://doi.org/10.1534/genetics.117.300064

Greenmyer, J. R., Gaultney, R. A., Brissette, C. A., & Watt, J. A. (2018). Primary Human Microglia Are Phagocytically Active and Respond to *Borrelia burgdorferi* With Upregulation of Chemokines and Cytokines. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.00811

Grimm, D., Tilly, K., Bueschel, D. M., Fisher, M. A., Policastro, P. F., Gherardini, F. C., Schwan, T. G., & Rosa, P. A. (2005). Defining Plasmids Required by *Borrelia burgdorferi* for Colonization of Tick Vector *Ixodes scapularis* (Acari: Ixodidae). *Journal of Medical Entomology*, *42*(4), 676–684. https://doi.org/10.1093/jmedent/42.4.676

Groshong, A. M., Dey, A., Bezsonova, I., Caimano, M. J., & Radolf, J. D. (2017). Peptide Uptake Is Essential for *Borrelia burgdorferi* Viability and Involves Structural and Regulatory Complexity of its Oligopeptide Transporter. *MBio*, *8*(6). https://doi.org/10.1128/mBio.02047-17

Groshong, A. M., Fortune, D. E., Moore, B. P., Spencer, H. J., Skinner, R. A., Bellamy, W. T., & Blevins, J. S. (2014). BB0238, a presumed tetratricopeptide repeat-containing protein, is required during *Borrelia burgdorferi* mammalian infection. *Infection and Immunity*, *82*(10), 4292–4306. https://doi.org/10.1128/IAI.01977-14

Grove, A. P., Liveris, D., Iyer, R., Petzke, M., Rudman, J., Caimano, M. J., Radolf, J. D., & Schwartz, I. (2017). Two Distinct Mechanisms Govern RpoS-Mediated Repression of Tick-Phase Genes during Mammalian Host Adaptation by *Borrelia burgdorferi* , the Lyme Disease Spirochete. *MBio*, *8*(4). https://doi.org/10.1128/mBio.01204-17

Gwynn, M. N., Portnoy, A., Rittenhouse, S. F., & Payne, D. J. (2010). Challenges of antibacterial discovery revisited. *Annals of the New York Academy of Sciences*, *1213*(1), 5–19. https://doi.org/10.1111/j.1749-6632.2010.05828.x

Hansen, G., & Hilgenfeld, R. (2013). Architecture and regulation of HtrA-family proteins involved in protein quality control and stress response. *Cellular and Molecular Life Sciences*, *70*(5), 761–775. https://doi.org/10.1007/s00018-012-1076-4

Harkness, R. W., Toyama, Y., Ripstein, Z. A., Zhao, H., Sever, A. I. M., Luan, Q., Brady, J. P., Clark, P. L., Schuck, P., & Kay, L. E. (2021). Competing stress-dependent oligomerization pathways regulate self-assembly of the periplasmic protease-chaperone DegP. *Proceedings of the National Academy of Sciences*, *118*(32). https://doi.org/10.1073/pnas.2109732118

Hasenbein, S., Meltzer, M., Hauske, P., Kaiser, M., Huber, R., Clausen, T., & Ehrmann, M. (2010). Conversion of a Regulatory into a Degradative Protease. *Journal of Molecular Biology*, *397*(4), 957–966. https://doi.org/10.1016/j.jmb.2010.02.027

Hayes, B. M., Dulebohn, D. P., Sarkar, A., Tilly, K., Bestor, A., Ambroggio, X., & Rosa, P. A. (2014). Regulatory Protein BBD18 of the Lyme Disease Spirochete: Essential Role During Tick Acquisition? *MBio*, *5*(2). https://doi.org/10.1128/mBio.01017-14

Hedstrom, L. (2002). Serine protease mechanism and specificity. *Chemical Reviews*, *102*(12), 4501–4523. https://doi.org/10.1021/cr000033x

Helbing, D. L., Böhm, L., Oraha, N., Stabenow, L. K., & Cui, Y. (2022). A Ponceau S Staining-Based Dot Blot Assay for Rapid Protein Quantification of Biological Samples. *Gels*, *8*(1), 43. https://doi.org/10.3390/gels8010043

Hellwage, J., Meri, T., Heikkilä, T., Alitalo, A., Panelius, J., Lahdenne, P., Ilkka Seppälä, J. T., & Meri, S. (2001). The Complement Regulator Factor H Binds to the Surface Protein OspE of *Borrelia burgdorferi*. *Journal of Biological Chemistry*, *276*(11), 8427–8435. https://doi.org/10.1074/jbc.M007994200

Hengge-Aronis, R. (2002). Signal Transduction and Regulatory Mechanisms Involved in Control of the σ^S (RpoS) Subunit of RNA Polymerase. *Microbiology and Molecular Biology Reviews*, *66*(3), 373–395. https://doi.org/10.1128/MMBR.66.3.373-395.2002

Herman-Giddens, M. E. (2012). Yale Lyme Disease Risk Maps Are Not Accurate for the South in 2012. *The American Journal of Tropical Medicine and Hygiene*, *86*(6), 1085–1085. https://doi.org/10.4269/ajtmh.2012.12-0149a

Herrmann, J. L. (1995). Animal models and Lyme disease. *Clinical Microbiology and Infection*, *1*(2), 72–73. https://doi.org/10.1111/j.1469-0691.1995.tb00448.x

Hirschfeld, M., Ma, Y., Weis, J. H., Vogel, S. N., & Weis, J. J. (2000). Cutting Edge: Repurification of Lipopolysaccharide Eliminates Signaling Through Both Human and Murine Toll-Like Receptor 2. *The Journal of Immunology*, *165*(2), 618–622. https://doi.org/10.4049/jimmunol.165.2.618

Hodzic, E., Borjesson, D. L., Feng, S., & Barthold, S. W. (2001). Acquisition Dynamics of *Borrelia burgdorferi* and the Agent of Human Granulocytic Ehrlichiosis at the Host-Vector Interface. *Vector-Borne and Zoonotic Diseases*, *1*(2), 149–158. https://doi.org/10.1089/153036601316977750

Hossain, H., Wellensiek, H.-J., Geyer, R., & Lochnit, G. (2001). Structural analysis of glycolipids from *Borrelia burgdorferi*. *Biochimie*, *83*(7), 683–692. https://doi.org/10.1016/S0300-9084(01)01296-2

Hou, B., & Brüser, T. (2011). The Tat-dependent protein translocation pathway. *BioMolecular Concepts*, *2*(6), 507–523. https://doi.org/10.1515/BMC.2011.040
Hsieh, M., & Borger, J. (2022). RNA Polymerase. In *J. Biochemistry*. StatPearls Publishing.
Hübner, A., Yang, X., Nolen, D. M., Popova, T. G., Cabello, F. C., & Norgard, M. V. (2001). Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN–RpoS regulatory pathway. *Proceedings of the National Academy of Sciences*, *98*(22), 12724–12729. https://doi.org/10.1073/pnas.231442498
Huck, B. R., Kötzner, L., & Urbahns, K. (2018). Small Molecules Drive Big Improvements in Immuno-Oncology Therapies. *Angewandte Chemie (International Ed. in English)*, *57*(16), 4412–4428. https://doi.org/10.1002/anie.201707816
Hughes, J. P., Rees, S., Kalindjian, S. B., & Philpott, K. L. (2011). Principles of early drug discovery. *British Journal of Pharmacology*, *162*(6), 1239–1249. https://doi.org/10.1111/j.1476-5381.2010.01127.x
Hyde, J. A., Trzeciakowski, J. P., & Skare, J. T. (2007). *Borrelia burgdorferi* Alters Its Gene Expression and Antigenic Profile in Response to CO $_2$ Levels. *Journal of Bacteriology*, *189*(2), 437–445. https://doi.org/10.1128/JB.01109-06
Jadhav, A., Ferreira, R. S., Klumpp, C., Mott, B. T., Austin, C. P., Inglese, J., Thomas, C. J., Maloney, D. J., Shoichet, B. K., & Simeonov, A. (2010). Quantitative Analyses of Aggregation, Autofluorescence, and Reactivity Artifacts in a Screen for Inhibitors of a Thiol Protease. *Journal of Medicinal Chemistry*, *53*(1), 37–51. https://doi.org/10.1021/jm901070c
Jensen, S. O., Thompson, L. S., & Harry, E. J. (2005). Cell division in Bacillus subtilis: FtsZ and FtsA association is Z-ring independent, and FtsA is required for efficient midcell Z-Ring assembly. *Journal of Bacteriology*, *187*(18), 6536–6544. https://doi.org/10.1128/JB.187.18.6536-6544.2005
Johnson, R. C. (1977). THE SPIROCHETES. *Annual Review of Microbiology*, *31*(1), 89–106. https://doi.org/10.1146/annurev.mi.31.100177.000513
Johnson, R. C., Schmid, G. P., Hyde, F. W., Steigerwalt, A. G., & Brenner, D. J. (1984). *Borrelia burgdorferi* sp. nov.: Etiologic Agent of Lyme Disease. *International Journal of Systematic Bacteriology*, *34*(4), 496–497. https://doi.org/10.1099/00207713-34-4-496
Johnson, S., Furlong, E. J., Deme, J. C., Nord, A. L., Caesar, J. J. E., Chevance, F. F. V., Berry, R. M., Hughes, K. T., & Lea, S. M. (2021). Molecular structure of the intact bacterial flagellar basal body. *Nature Microbiology*, *6*(6), 712–721. https://doi.org/10.1038/s41564-021-00895-y
Jorge, A. M., Hoiczyk, E., Gomes, J. P., & Pinho, M. G. (2011). EzrA Contributes to the Regulation of Cell Size in Staphylococcus aureus. *PLoS ONE*, *6*(11), e27542. https://doi.org/10.1371/journal.pone.0027542
Juszkiewicz, S., & Hegde, R. S. (2018). Quality Control of Orphaned Proteins. *Molecular Cell*, *71*(3), 443–457. https://doi.org/10.1016/j.molcel.2018.07.001
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., Bockenstedt, L. K., Steere, A. C., & Jacobs-Wagner, C. (2019). *Borrelia burgdorferi* peptidoglycan is a persistent antigen in patients with Lyme arthritis. *Proceedings of the National Academy of Sciences*, *116*(27), 13498–13507. https://doi.org/10.1073/pnas.1904170116

Jutras, B. L., Scott, M., Parry, B., Biboy, J., Gray, J., Vollmer, W., & Jacobs-Wagner, C. (2016). Lyme disease and relapsing fever *Borrelia* elongate through zones of peptidoglycan synthesis that mark division sites of daughter cells. *Proceedings of the National Academy of Sciences*, *113*(33), 9162–9170. https://doi.org/10.1073/pnas.1610805113

Kariu, T., Sharma, K., Singh, P., Smith, A. A., Backstedt, B., Buyuktanir, O., & 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*(3), 462–471. https://doi.org/10.1093/infdis/jiu460

Kariu, T., Yang, X., Marks, C. B., Zhang, X., & Pal, U. (2013). Proteolysis of BB0323 results in two polypeptides that impact physiologic and infectious phenotypes in *Borrelia burgdorferi*. *Molecular Microbiology*, *88*(3), 510–522. https://doi.org/10.1111/mmi.12202

Kawamoto, A., Miyata, T., Makino, F., Kinoshita, M., Minamino, T., Imada, K., Kato, T., & Namba, K. (2021). Native flagellar MS ring is formed by 34 subunits with 23-fold and 11-fold subsymmetries. *Nature Communications*, *12*(1), 4223. https://doi.org/10.1038/s41467-021-24507-9

Keller, A., Graefen, A., Ball, M., Matzas, M., Boisguerin, V., Maixner, F., Leidinger, P., Backes, C., Khairat, R., Forster, M., Stade, B., Franke, A., Mayer, J., Spangler, J., McLaughlin, S., Shah, M., Lee, C., Harkins, T. T., Sartori, A., … Zink, A. (2012). New insights into the Tyrolean Iceman's origin and phenotype as inferred by whole-genome sequencing. *Nature Communications*, *3*(1), 698. https://doi.org/10.1038/ncomms1701

Kenedy, M. R., Lenhart, T. R., & Akins, D. R. (2012). The role of *Borrelia burgdorferi* outer surface proteins. *FEMS Immunology & Medical Microbiology*, *66*(1), 1–19. https://doi.org/10.1111/j.1574-695X.2012.00980.x

Kim, S., Grant, R. A., & Sauer, R. T. (2011). Covalent Linkage of Distinct Substrate Degrons Controls Assembly and Disassembly of DegP Proteolytic Cages. *Cell*, *145*(1), 67–78. https://doi.org/10.1016/j.cell.2011.02.024

Kinjo, Y., Tupin, E., Wu, D., Fujio, M., Garcia-Navarro, R., Benhnia, M. R.-E.-I., Zajonc, D. M., Ben-Menachem, G., Ainge, G. D., Painter, G. F., Khurana, A., Hoebe, K., Behar, S. M., Beutler, B., Wilson, I. A., Tsuji, M., Sellati, T. J., Wong, C.-H., & Kronenberg, M. (2006). Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nature Immunology*, *7*(9), 978–986. https://doi.org/10.1038/ni1380

Kitsou, C., & Pal, U. (2018). Ixodes Immune Responses Against Lyme Disease Pathogens. *Frontiers in Cellular and Infection Microbiology*, *8*, 176. https://doi.org/10.3389/fcimb.2018.00176

Klempner, M. S., Hu, L. T., Evans, J., Schmid, C. H., Johnson, G. M., Trevino, R. P., Norton, D., Levy, L., Wall, D., McCall, J., Kosinski, M., & Weinstein, A. (2001). Two Controlled Trials of Antibiotic Treatment in Patients with Persistent Symptoms and a History of Lyme Disease. *New England Journal of Medicine*, *345*(2), 85–92. https://doi.org/10.1056/NEJM200107123450202

Kocan, K. M., De La Fuente, J., & Coburn, L. A. (2015). Insights into the development of Ixodes scapularis: A resource for research on a medically important tick species. *Parasites and Vectors*, *8*(1). https://doi.org/10.1186/s13071-015-1185-7

Kowata, H., Tochigi, S., Kusano, T., & Kojima, S. (2016). Quantitative measurement of the outer membrane permeability in *Escherichia coli* lpp and tol-pal mutants defines the significance of Tol-Pal function for maintaining drug resistance. *The Journal of Antibiotics*, *69*(12), 863–870. https://doi.org/10.1038/ja.2016.50

Kraiczy, P., Hartmann, K., Hellwage, J., Skerka, C., Kirschfink, M., Brade, V., Zipfel, P. F., Wallich, R., & Stevenson, B. (2004). Immunological characterization of the complement regulator factor H-binding CRASP and Erp proteins of *Borrelia burgdorferi*. *International Journal of Medical Microbiology Supplements*, *293*, 152–157. https://doi.org/10.1016/S1433-1128(04)80029-9

Kraiczy, P., Hellwage, J., Skerka, C., Kirschfink, M., Brade, V., Zipfel, P. F., & Wallich, R. (2003). Immune evasion of *Borrelia burgdorferi*: mapping of a complement-inhibitor factor H-binding site of BbCRASP-3, a novel member of the Erp protein family. *European Journal of Immunology*, *33*(3), 697–707. https://doi.org/10.1002/eji.200323571

Kraiczy, P., Skerka, C., Brade, V., & Zipfel, P. F. (2001). Further Characterization of Complement Regulator-Acquiring Surface Proteins of *Borrelia burgdorferi*. *Infection and Immunity*, *69*(12), 7800–7809. https://doi.org/10.1128/IAI.69.12.7800-7809.2001

Krause, P. J., Fish, D., Narasimhan, S., & Barbour, A. G. (2015). Borrelia miyamotoi infection in nature and in humans. *Clinical Microbiology and Infection*, *21*(7), 631–639. https://doi.org/10.1016/j.cmi.2015.02.006

Kronstad, J. W., & Caza, M. (2013). Shared and distinct mechanisms of iron acquisition by bacterial and fungal pathogens of humans. *Frontiers in Cellular and Infection Microbiology*, *3*(80). https://doi.org/10.3389/fcimb.2013.00080

Kudryashev, M., Cyrklaff, M., Baumeister, W., Simon, M. M., Wallich, R., & Frischknecht, F. (2009). Comparative cryo-electron tomography of pathogenic Lyme disease spirochetes. *Molecular Microbiology*, *71*(6), 1415–1434. https://doi.org/10.1111/j.1365-2958.2009.06613.x

Kudva, R., Denks, K., Kuhn, P., Vogt, A., Müller, M., & Koch, H.-G. (2013). Protein translocation across the inner membrane of Gram-negative bacteria: the Sec and Tat dependent protein transport pathways. *Research in Microbiology*, *164*(6), 505–534. https://doi.org/10.1016/j.resmic.2013.03.016

Kugeler, K. J., Farley, G. M., Forrester, J. D., & Mead, P. S. (2015). Geographic Distribution and Expansion of Human Lyme Disease, United States. *Emerging Infectious Diseases*, *21*(8), 1455–1457. https://doi.org/10.3201/eid2108.141878

Kugeler, K. J., Schwartz, A. M., Delorey, M. J., Mead, P. S., & Hinckley, A. F. (2021). Estimating the Frequency of Lyme Disease Diagnoses, United States, 2010–2018. *Emerging Infectious Diseases*, *27*(2), 616–619. https://doi.org/10.3201/eid2702.202731

Kumar, B., Miller, K., Charon, N. W., & Legleiter, J. (2017). Periplasmic flagella in *Borrelia burgdoferi* function to maintain cellular integrity upon external stress. *PLOS ONE*, *12*(9), e0184648. https://doi.org/10.1371/journal.pone.0184648

Kumru, O. S., Schulze, R. J., Rodnin, M. V., Ladokhin, A. S., & Zückert, W. R. (2011). Surface Localization Determinants of *Borrelia* OspC/Vsp Family Lipoproteins. *Journal of Bacteriology*, *193*(11), 2814–2825. https://doi.org/10.1128/JB.00015-11

Kumru, O. S., Schulze, R. J., Slusser, J. G., & Zückert, W. R. (2010). Development and validation of a FACS-based lipoprotein localization screen in the Lyme disease spirochete *Borrelia burgdorferi*. *BMC Microbiology*, *10*(1), 277. https://doi.org/10.1186/1471-2180-10-277

Kung, F., Anguita, J., & Pal, U. (2013). *Borrelia burgdorferi* and tick proteins supporting pathogen persistence in the vector. In *Future Microbiology* (Vol. 8, Issue 1, pp. 41–56). https://doi.org/10.2217/fmb.12.121

Kurokawa, C., Lynn, G. E., Pedra, J. H. F., Pal, U., Narasimhan, S., & Fikrig, E. (2020). Interactions between *Borrelia burgdorferi* and ticks. In *Nature Reviews Microbiology* (Vol. 18, Issue 10, pp. 587–600). Nature Research. https://doi.org/10.1038/s41579-020-0400-5

Kushwaha, H. N., Mohan, N. K., Sharma, A. K., & Singh, S. K. (2014). Pharmacokinetic study and bioavailability of a novel synthetic trioxane antimalarial compound 97/63 in rats. *Malaria Research and Treatment*, *2014*, 759392. https://doi.org/10.1155/2014/759392

Lackum, K., & Stevenson, B. (2005). Carbohydrate utilization by the Lyme borreliosis spirochete, *Borrelia burgdorferi*. *FEMS Microbiology Letters*, *243*(1), 173–179. https://doi.org/10.1016/j.femsle.2004.12.002

Lantos, P. M., Rumbaugh, J., Bockenstedt, L. K., Falck-Ytter, Y. T., Aguero-Rosenfeld, M. E., Auwaerter, P. G., Baldwin, K., Bannuru, R. R., Belani, K. K., Bowie, W. R., Branda, J. A., Clifford, D. B., DiMario, F. J., Halperin, J. J., Krause, P. J., Lavergne, V., Liang, M. H., Meissner, H. C., Nigrovic, L. E., … Zemel, L. S. (2020). Clinical practice guidelines by the Infectious Diseases Society of America, American Academy of Neurology, and American College of Rheumatology. *Neurology*. https://doi.org/10.1212/WNL.0000000000011151.

LaRocca, T. J., Pathak, P., Chiantia, S., Toledo, A., Silvius, J. R., Benach, J. L., & London, E. (2013). Proving Lipid Rafts Exist: Membrane Domains in the Prokaryote *Borrelia burgdorferi* Have the Same Properties as Eukaryotic Lipid Rafts. *PLoS Pathogens*, *9*(5), e1003353. https://doi.org/10.1371/journal.ppat.1003353

Lawrenz, M. B., Kawabata, H., Purser, J. E., & Norris, S. J. (2002). Decreased Electroporation Efficiency in *Borrelia burgdorferi* Containing Linear Plasmids lp25 and lp56: Impact on Transformation of Infectious *B. burgdorferi*. *Infection and Immunity*, *70*(9), 4798–4804. https://doi.org/10.1128/IAI.70.9.4798-4804.2002

Lazarus, J. J., Meadows, M. J., Lintner, R. E., & Wooten, R. M. (2006). IL-10 Deficiency Promotes Increased *Borrelia burgdorferi* Clearance Predominantly through Enhanced Innate Immune Responses. *The Journal of Immunology*, *177*(10), 7076–7085. https://doi.org/10.4049/jimmunol.177.10.7076

Lazzaroni, J. C., Germon, P., Ray, M.-C., & Vianney, A. (1999). The Tol proteins of *Escherichia coli* and their involvement in the uptake of biomolecules and outer membrane stability. *FEMS Microbiology Letters*, *177*(2), 191–197. https://doi.org/10.1111/j.1574-6968.1999.tb13731.x

Lee, S. H., Vigliotti, V. S., Vigliotti, J. S., Jones, W., & Pappu, S. (2010). Increased sensitivity and specificity of *Borrelia burgdorferi* 16S ribosomal DNA detection.

American Journal of Clinical Pathology, *133*(4). https://doi.org/10.1309/AJCPI72YAXRHYHEE

Lefevre, F. (1997). Alanine-stretch scanning mutagenesis: a simple and efficient method to probe protein structure and function. *Nucleic Acids Research*, *25*(2), 447–448. https://doi.org/10.1093/nar/25.2.447

Leimer, N., Wu, X., Imai, Y., Morrissette, M., Pitt, N., Favre-Godal, Q., Iinishi, A., Jain, S., Caboni, M., Leus, I. V., Bonifay, V., Niles, S., Bargabos, R., Ghiglieri, M., Corsetti, R., Krumpoch, M., Fox, G., Son, S., Klepacki, D., … Lewis, K. (2021). A selective antibiotic for Lyme disease. *Cell*, *184*(21), 5405-5418.e16. https://doi.org/10.1016/j.cell.2021.09.011

Levi, T., Kilpatrick, A. M., Mangel, M., & Wilmers, C. C. (2012). Deer, predators, and the emergence of Lyme disease. *Proceedings of the National Academy of Sciences*, *109*(27), 10942–10947. https://doi.org/10.1073/pnas.1204536109

Lewis, K. (2008). *Multidrug Tolerance of Biofilms and Persister Cells* (pp. 107–131). https://doi.org/10.1007/978-3-540-75418-3_6

Lewis, K. (2010). Persister Cells. *Annual Review of Microbiology*, *64*(1), 357–372. https://doi.org/10.1146/annurev.micro.112408.134306

Lin, Y.-P., Frye, A. M., Nowak, T. A., & Kraiczy, P. (2020). New Insights Into CRASP-Mediated Complement Evasion in the Lyme Disease Enzootic Cycle. *Frontiers in Cellular and Infection Microbiology*, *10*. https://doi.org/10.3389/fcimb.2020.00001

Little, S. E., Heise, S. R., Blagburn, B. L., Callister, S. M., & Mead, P. S. (2010). Lyme borreliosis in dogs and humans in the USA. *Trends in Parasitology*, *26*(4), 213–218. https://doi.org/10.1016/j.pt.2010.01.006

Liu, G., Chen, T., Zhang, X., Ma, X., & Shi, H. (2022). Small molecule inhibitors targeting the cancers. *MedComm*, *3*(4). https://doi.org/10.1002/mco2.181

Liu, X., Bushnell, D. A., & Kornberg, R. D. (2013). RNA polymerase II transcription: structure and mechanism. *Biochimica et Biophysica Acta*, *1829*(1), 2–8. https://doi.org/10.1016/j.bbagrm.2012.09.003

Lloubès, R., Cascales, E., Walburger, A., Bouveret, E., Lazdunski, C., Bernadac, A., & Journet, L. (2001). The Tol-Pal proteins of the *Escherichia coli* cell envelope: an energized system required for outer membrane integrity? *Research in Microbiology*, *152*(6), 523–529. https://doi.org/10.1016/S0923-2508(01)01226-8

Lochhead, R. B., Arvikar, S. L., Aversa, J. M., Sadreyev, R. I., Strle, K., & Steere, A. C. (2019). Robust interferon signature and suppressed tissue repair gene expression in synovial tissue from patients with postinfectious, *Borrelia burgdorferi* -induced Lyme arthritis. *Cellular Microbiology*, *21*(2). https://doi.org/10.1111/cmi.12954

Lochhead, R. B., Ordoñez, D., Arvikar, S. L., Aversa, J. M., Oh, L. S., Heyworth, B., Sadreyev, R., Steere, A. C., & Strle, K. (2019). Interferon-gamma production in Lyme arthritis synovial tissue promotes differentiation of fibroblast-like synoviocytes into immune effector cells. *Cellular Microbiology*, *21*(2). https://doi.org/10.1111/cmi.12992

Lu, Y., Deblais, L., Rajashekara, G., Miller, S. A., Helmy, Y. A., Zhang, H., Wu, P., Qiu, Y., & Xu, X. (2020). High-throughput screening reveals small molecule modulators inhibitory to *Acidovorax citrulli*. *Plant Pathology*, *69*(5), 818–826. https://doi.org/10.1111/ppa.13177

Radolf J.D., Strle K, Lemieux J.E., Strle F. Lyme Disease in Humans. Curr Issues Mol Biol. 2021;42:333-384. doi: 10.21775/cimb.042.333. Epub 2020 Dec 11. PMID: 33303701; PMCID: PMC7946767.Ma, B., & Nussinov, R. (2014). Druggable orthosteric and allosteric hot spots to target protein-protein interactions. *Current Pharmaceutical Design*, *20*(8), 1293–1301. https://doi.org/10.2174/13816128113199990073

Magnarelli, L. A., Oliver, J. H., Hutcheson, H. J., Boone, J. L., & Anderson, J. F. (1992). Antibodies to *Borrelia burgdorferi* in rodents in the eastern and southern United States. *Journal of Clinical Microbiology*, *30*(6), 1449–1452. https://doi.org/10.1128/jcm.30.6.1449-1452.1992

Malawista, S. E., & de Boisfleury Chevance, A. (2008). Clocking the Lyme Spirochete. *PLoS ONE*, *3*(2), e1633. https://doi.org/10.1371/journal.pone.0001633

Mannelli, A., Bertolotti, L., Gern, L., & Gray, J. (2012). Ecology of *Borrelia burgdorferi sensu lato* in Europe: transmission dynamics in multi-host systems, influence of molecular processes and effects of climate change. *FEMS Microbiology Reviews*, *36*(4), 837–861. https://doi.org/10.1111/j.1574-6976.2011.00312.x

Margos, G., Marosevic, D., Cutler, S., Derdakova, M., Diuk-Wasser, M., Emler, S., Fish, D., Gray, J., Hunfeldt, K.-P., Jaulhac, B., Kahl, O., Kovalev, S., Kraiczy, P., Lane, R. S., Lienhard, R., Lindgren, P. E., Ogden, N., Ornstein, K., Rupprecht, T., … Fingerle, V. (2017). There is inadequate evidence to support the division of the genus *Borrelia*. *International Journal of Systematic and Evolutionary Microbiology*, *67*(4), 1081–1084. https://doi.org/10.1099/ijsem.0.001717

Margos, G., Vollmer, S. A., Ogden, N. H., & Fish, D. (2011). Population genetics, taxonomy, phylogeny and evolution of *Borrelia burgdorferi* sensu lato. *Infection, Genetics and Evolution*, *11*(7), 1545–1563. https://doi.org/10.1016/j.meegid.2011.07.022

Marques, A.R. (2008). Chronic Lyme Disease: A Review. *Infectious Disease Clinics of North America*, *22*(2), 341–360. https://doi.org/10.1016/j.idc.2007.12.011

Marques, A. R. (2015). Laboratory Diagnosis of Lyme Disease. *Infectious Disease Clinics of North America*, *29*(2), 295–307. https://doi.org/10.1016/j.idc.2015.02.005

Marques, A. R., Strle, F., & Wormser, G. P. (2021). Comparison of Lyme Disease in the United States and Europe. *Emerging Infectious Diseases*, *27*(8), 2017–2024. https://doi.org/10.3201/eid2708.204763

Marques, A., Telford, S. R., Turk, S.-P., Chung, E., Williams, C., Dardick, K., Krause, P. J., Brandeburg, C., Crowder, C. D., Carolan, H. E., Eshoo, M. W., Shaw, P. A., & Hu, L. T. (2014). Xenodiagnosis to detect *Borrelia burgdorferi* infection: a first-in-human study. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *58*(7), 937–945. https://doi.org/10.1093/cid/cit939

Marsh, J. A., & Teichmann, S. A. (2015). Structure, Dynamics, Assembly, and Evolution of Protein Complexes. *Annual Review of Biochemistry*, *84*(1), 551–575. https://doi.org/10.1146/annurev-biochem-060614-034142

Marx, G. E., Leikauskas, J., Lindstrom, K., Mann, E., Reagan-Steiner, S., Matkovic, E., Read, J. S., Kelso, P., Kwit, N. A., Hinckley, A. F., Levine, M. A., & Brown, C. (2020). Fatal Lyme Carditis in New England: Two Case Reports. *Annals of Internal Medicine*, *172*(3), 222. https://doi.org/10.7326/L19-0483

McDowell, J. V., Wolfgang, J., Tran, E., Metts, M. S., Hamilton, D., & Marconi, R. T. (2003). Comprehensive Analysis of the Factor H Binding Capabilities of *Borrelia* Species Associated with Lyme Disease: Delineation of Two Distinct Classes of Factor H Binding Proteins. *Infection and Immunity*, *71*(6), 3597–3602. https://doi.org/10.1128/IAI.71.6.3597-3602.2003

McGovern, S. L., Caselli, E., Grigorieff, N., & Shoichet, B. K. (2002). A Common Mechanism Underlying Promiscuous Inhibitors from Virtual and High-Throughput Screening. *Journal of Medicinal Chemistry*, *45*(8), 1712–1722. https://doi.org/10.1021/jm010533y

McGovern, S. L., Helfand, B. T., Feng, B., & Shoichet, B. K. (2003). A Specific Mechanism of Nonspecific Inhibition. *Journal of Medicinal Chemistry*, *46*(20), 4265–4272. https://doi.org/10.1021/jm030266r

McInnes, C. (2007). Virtual screening strategies in drug discovery. *Current Opinion in Chemical Biology*, *11*(5), 494–502. https://doi.org/10.1016/j.cbpa.2007.08.033

McPherson, M., García-García, A., Cuesta-Valero, F. J., Beltrami, H., Hansen-Ketchum, P., MacDougall, D., & Ogden, N. H. (2017). Expansion of the Lyme Disease Vector *Ixodes Scapularis* in Canada Inferred from CMIP5 Climate Projections. *Environmental Health Perspectives*, *125*(5), 057008. https://doi.org/10.1289/EHP57

Mead, P., Petersen, J., & Hinckley, A. (2019). Updated CDC Recommendation for Serologic Diagnosis of Lyme Disease. *MMWR. Morbidity and Mortality Weekly Report*, *68*(32), 703. https://doi.org/10.15585/mmwr.mm6832a4

Mead, P. S. (2015). Epidemiology of Lyme Disease. *Infectious Disease Clinics of North America*, *29*(2), 187–210. https://doi.org/10.1016/j.idc.2015.02.010

Mesnage, S., Dellarole, M., Baxter, N. J., Rouget, J.-B., Dimitrov, J. D., Wang, N., Fujimoto, Y., Hounslow, A. M., Lacroix-Desmazes, S., Fukase, K., Foster, S. J., & Williamson, M. P. (2014). Molecular basis for bacterial peptidoglycan recognition by LysM domains. *Nature Communications*, *5*(1), 4269. https://doi.org/10.1038/ncomms5269

Michael, C. A., Dominey-Howes, D., & Labbate, M. (2014). The Antimicrobial Resistance Crisis: Causes, Consequences, and Management. *Frontiers in Public Health*, *2*. https://doi.org/10.3389/fpubh.2014.00145

Miller, C. L., Karna, S. L. R., & Seshu, J. (2013). *Borrelia* host adaptation Regulator (BadR) regulates *rpoS* to modulate host adaptation and virulence factors in *Borrelia burgdorferi*. *Molecular Microbiology*, *88*(1), 105–124. https://doi.org/10.1111/mmi.12171

Miller, S. I., & Salama, N. R. (2018). The gram-negative bacterial periplasm: Size matters. *PLOS Biology*, *16*(1), e2004935. https://doi.org/10.1371/journal.pbio.2004935

Mitosch, K., Rieckh, G., & Bollenbach, T. (2017). Noisy Response to Antibiotic Stress Predicts Subsequent Single-Cell Survival in an Acidic Environment. *Cell Systems*, *4*(4), 393-403.e5. https://doi.org/10.1016/j.cels.2017.03.001

Modolell, M., Schaible, U. E., Rittig, M., & Simon, M. M. (1994). Killing of *Borrelia burgdorferi* by macrophages is dependent on oxygen radicals and nitric oxide and can be enhanced by antibodies to outer surface proteins of the spirochete. *Immunology Letters*, *40*(2), 139–146. https://doi.org/10.1016/0165-2478(94)90185-6

Morrissette, M., Pitt, N., González, A., Strandwitz, P., Caboni, M., Rebman, A. W., Knight, R., D'Onofrio, A., Aucott, J. N., Soloski, M. J., & Lewis, K. (2020). A Distinct Microbiome Signature in Posttreatment Lyme Disease Patients. *MBio*, *11*(5). https://doi.org/10.1128/mBio.02310-20

Motaleb, M. A., Corum, L., Bono, J. L., Elias, A. F., Rosa, P., Samuels, D. S., & Charon, N. W. (2000). *Borrelia burgdorferi* periplasmic flagella have both skeletal and motility functions. *Proceedings of the National Academy of Sciences*, *97*(20), 10899–10904. https://doi.org/10.1073/pnas.200221797

Murfin, K. E., Kleinbard, R., Aydin, M., Salazar, S. A., & Fikrig, E. (2019). *Borrelia burgdorferi* chemotaxis toward tick protein Salp12 contributes to acquisition. *Ticks and Tick-Borne Diseases*, *10*(5), 1124–1134. https://doi.org/10.1016/j.ttbdis.2019.06.002

Nadelman, R. B. (2015). Erythema Migrans. *Infectious Disease Clinics of North America*, *29*(2), 211–239. https://doi.org/10.1016/j.idc.2015.02.001

Nadelman, R. B., & Wormser, G. P. (1998). Lyme borreliosis. *The Lancet*, *352*(9127), 557–565. https://doi.org/10.1016/S0140-6736(98)01146-5

Nakayama, H., Kurokawa, K., & Lee, B. L. (2012). Lipoproteins in bacteria: structures and biosynthetic pathways. *FEBS Journal*, *279*(23), 4247–4268. https://doi.org/10.1111/febs.12041

NCBI. (2022). *Borreliella burgdorferi B31, complete sequence*.

Nestle, F. O., Di Meglio, P., Qin, J.-Z., & Nickoloff, B. J. (2009). Skin immune sentinels in health and disease. *Nature Reviews Immunology*, *9*(10), 679–691. https://doi.org/10.1038/nri2622

Nieto, N. C., Porter, W. T., Wachara, J. C., Lowrey, T. J., Martin, L., Motyka, P. J., & Salkeld, D. J. (2018). Using citizen science to describe the prevalence and distribution of tick bite and exposure to tick-borne diseases in the United States. *PLOS ONE*, *13*(7), e0199644. https://doi.org/10.1371/journal.pone.0199644

Nigrovic, l. E., & thompson, k. M. (2007). The Lyme vaccine: a cautionary tale. *Epidemiology and Infection*, *135*(1), 1–8. https://doi.org/10.1017/S0950268806007096

Nikaido, H. (2003). Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiology and Molecular Biology Reviews*, *67*(4), 593–656. https://doi.org/10.1128/MMBR.67.4.593-656.2003

Norris, S. J. (2006). The dynamic proteome of Lyme disease *Borrelia*. In *Genome Biology* (Vol. 7, Issue 3). https://doi.org/10.1186/gb-2005-7-3-209

Norris, S. J. (2014). *vls* Antigenic Variation Systems of Lyme Disease *Borrelia* : Eluding Host Immunity through both Random, Segmental Gene Conversion and Framework Heterogeneity. *Microbiology Spectrum*, *2*(6). https://doi.org/10.1128/microbiolspec.MDNA3-0038-2014

Nussinov, R., & Tsai, C.-J. (2012). The different ways through which specificity works in orthosteric and allosteric drugs. *Current Pharmaceutical Design*, *18*(9), 1311–1316. https://doi.org/10.2174/138161212799436377

Nuttall, P. A. (2019). Tick saliva and its role in pathogen transmission. *Wiener Klinische Wochenschrift*. https://doi.org/10.1007/s00508-019-1500-y

Ogden, N. H., & Tsao, J. I. (2009). Biodiversity and Lyme disease: Dilution or amplification? *Epidemics*, *1*(3), 196–206. https://doi.org/10.1016/j.epidem.2009.06.002

Olson, J. A., Leveson-Gower, D. B., Gill, S., Baker, J., Beilhack, A., & Negrin, R. S. (2010). NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood*, *115*(21), 4293–4301. https://doi.org/10.1182/blood-2009-05-222190

Oomen, C. J., van Ulsen, P., Van Gelder, P., Feijen, M., Tommassen, J., & Gros, P. (2004). Structure of the translocator domain of a bacterial autotransporter. *The EMBO Journal*, *23*(6), 1257–1266. https://doi.org/10.1038/sj.emboj.7600148

Ortiz, C., Natale, P., Cueto, L., & Vicente, M. (2016). The keepers of the ring: regulators of FtsZ assembly. *FEMS Microbiology Reviews*, *40*(1), 57–67. https://doi.org/10.1093/femsre/fuv040

Osawa, M., Anderson, D. E., & Erickson, H. P. (2008). Reconstitution of Contractile FtsZ Rings in Liposomes. *Science*, *320*(5877), 792–794. https://doi.org/10.1126/science.1154520

Östberg, Y., Carroll, J. A., Pinne, M., Krum, J. G., Rosa, P., & Bergström, S. (2004). Pleiotropic Effects of Inactivating a Carboxyl-Terminal Protease, CtpA, in *Borrelia burgdorferi*. *Journal of Bacteriology*, *186*(7), 2074–2084. https://doi.org/10.1128/JB.186.7.2074-2084.2004

Ostfeld, R. S., & Keesing, F. (2000). Biodiversity and Disease Risk: the Case of Lyme Disease. In *Conservation Biology* (Vol. 14, Issue 3).

Ouyang, Z., He, M., Oman, T., Yang, X. F., & Norgard, M. V. (2009). A manganese transporter, BB0219 (BmtA), is required for virulence by the Lyme disease spirochete, *Borrelia burgdorferi*. *Proceedings of the National Academy of Sciences*, *106*(9), 3449–3454. https://doi.org/10.1073/pnas.0812999106

Pal, U., Kitsou, C., Drecktrah, D., Yaş, O. B., & Fikrig, E. (2022). Interactions Between Ticks and Lyme Disease Spirochetes. *Current Issues in Molecular Biology*, 113–144. https://doi.org/10.21775/cimb.042.113

Pal, U., Li, X., Wang, T., Montgomery, R. R., Ramamoorthi, N., deSilva, A. M., Bao, F., Yang, X., Pypaert, M., Pradhan, D., Kantor, F. S., Telford, S., Anderson, J. F., & Fikrig, E. (2004). TROSPA, an Ixodes scapularis Receptor for *Borrelia burgdorferi*. *Cell*, *119*(4), 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, & Fikrig, E. (2004). OspC facilitates *Borrelia burgdorferi* invasion of Ixodes scapularis salivary glands. *The Journal of Clinical Investigation*, *113*(2), 220–230. https://doi.org/10.1172/JCI19894

Parise, C. M., Breuner, N. E., Hojgaard, A., Osikowicz, L. M., Replogle, A. J., Eisen, R. J., & Eisen, L. (2020). Experimental Demonstration of Reservoir Competence of the White-Footed Mouse, Peromyscus leucopus (Rodentia: Cricetidae), for the Lyme Disease Spirochete, *Borrelia* mayonii (Spirochaetales: Spirochaetaceae). *Journal of Medical Entomology*, *57*(3), 927–932. https://doi.org/10.1093/jme/tjz242

Park, S. A., Choe, Y. H., Park, E., & Hyun, Y.-M. (2018). Real-time dynamics of neutrophil clustering in response to phototoxicity-induced cell death and tissue damage in mouse ear dermis. *Cell Adhesion & Migration*, 1–20. https://doi.org/10.1080/19336918.2018.1471322

Pennetzdorfer, N., Lembke, M., Pressler, K., Matson, J. S., Reidl, J., & Schild, S. (2019). Regulated Proteolysis in Vibrio cholerae Allowing Rapid Adaptation to Stress Conditions. *Frontiers in Cellular and Infection Microbiology*, *9*. https://doi.org/10.3389/fcimb.2019.00214

PerkinElmer. (2023). *AlphaLISA and AlphaScreen No-wash Assays*.

Picardeau, M., Lobry, J. R., & Hinnebusch, B. J. (1999). Physical mapping of an origin of bidirectional replication at the centre of the *Borrelia burgdorferi* linear chromosome. *Molecular Microbiology*, *32*(2), 437–445. https://doi.org/10.1046/j.1365-2958.1999.01368.x

Plasterk, R. H. A., Simon, M. I., & Barbour, A. G. (1985). Transposition of structural genes to an expression sequence on a linear plasmid causes antigenic variation in the bacterium *Borrelia* hermsii. *Nature*, *318*(6043), 257–263. https://doi.org/10.1038/318257a0

Posey, J. E., & Gherardini, F. C. (2000). Lack of a Role for Iron in the Lyme Disease Pathogen. *Science*, *288*(5471), 1651–1653. https://doi.org/10.1126/science.288.5471.1651

Pothineni, V. R., Potula, H.-H. S. K., Ambati, A., Mallajosyula, V. V. A., Sridharan, B., Inayathullah, M., Ahmed, M. S., & Rajadas, J. (2020). Azlocillin can be the potential drug candidate against drug-tolerant *Borrelia burgdorferi* sensu stricto JLB31. *Scientific Reports*, *10*(1), 3798. https://doi.org/10.1038/s41598-020-59600-4

Preac Mursic, V., Patsouris, E., Wilske, B., Reinhardt, S., Groß, B., & Mehraein, P. (1990). Persistence of *Borrelia burgdorferi* and histopathological alterations in experimentally infected animals. A comparison with histopathological findings in human Lyme disease. *Infection*, *18*(6), 332–341. https://doi.org/10.1007/BF01646399

PubChem, N. L. of M. (2023). *Lomibuvir*. Https://Pubchem.Ncbi.Nlm.Nih.Gov/Compound/Lomibuvir.

Radolf, J. D., Bourell, K. W., Akins, D. R., Brusca, J. S., & Norgard, M. v. (1994). Analysis of *Borrelia burgdorferi* membrane architecture by freeze-fracture electron microscopy. *Journal of Bacteriology*, *176*(1), 21–31. https://doi.org/10.1128/jb.176.1.21-31.1994

Radolf, J. D., Caimano, M. J., Stevenson, B., & Hu, L. T. (2012). Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. *Nature Reviews Microbiology*, *10*(2), 87–99. https://doi.org/10.1038/nrmicro2714

Radolf-, J. D., Strle, K., Lemieux, J. E., & Strle, F. (2022). Lyme Disease in Humans. *Current Issues in Molecular Biology*, 333–384. https://doi.org/10.21775/cimb.042.333

Ramamoorthi, N., Narasimhan, S., Pal, U., Bao, F., Yang, X. F., Fish, D., Anguita, J., Norgard, M. V., Kantor, F. S., Anderson, J. F., Koski, R. A., & Fikrig, E. (2005). The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature*, *436*(7050), 573–577. https://doi.org/10.1038/nature03812

Ran, X., & Gestwicki, J. E. (2018). Inhibitors of protein–protein interactions (PPIs): an analysis of scaffold choices and buried surface area. *Current Opinion in Chemical Biology*, *44*, 75–86. https://doi.org/10.1016/j.cbpa.2018.06.004

Rana, V. S., Kitsou, C., Dumler, J. S., & Pal, U. (2022). Immune evasion strategies of major tick-transmitted bacterial pathogens. In *Trends in Microbiology*. Elsevier Ltd. https://doi.org/10.1016/j.tim.2022.08.002

Rego, R. O. M., Bestor, A., & Rosa, P. A. (2011). Defining the Plasmid-Borne Restriction-Modification Systems of the Lyme Disease Spirochete *Borrelia burgdorferi*. *Journal of Bacteriology*, *193*(5), 1161–1171. https://doi.org/10.1128/JB.01176-10

Ribeiro, J. M. C., Alarcon-Chaidez, F., B. Francischetti, I. M., Mans, B. J., Mather, T. N., Valenzuela, J. G., & Wikel, S. K. (2006). An annotated catalog of salivary gland transcripts from Ixodes scapularis ticks. *Insect Biochemistry and Molecular Biology*, *36*(2), 111–129. https://doi.org/10.1016/j.ibmb.2005.11.005

Ribeiro, J. M., Makoul, G. T., Levine, J., Robinson, D. R., & Spielman, A. (1985). Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, Ixodes dammini. *Journal of Experimental Medicine*, *161*(2), 332–344. https://doi.org/10.1084/jem.161.2.332

Rick NG. (2016). *Drugs From Discovery to Approval* (Third). Wiley Blackwell.

Ronzetti, M., Baljinnyam, B., Jalal, I., Pal, U., & Simeonov, A. (2022). Application of biophysical methods for improved protein production and characterization: a case study on an HtrA -family bacterial protease . *Protein Science*. https://doi.org/10.1002/pro.4498

Rupprecht, K. R., Nair, R. K., Harwick, L. C., Grote, J., Beligere, G. S., Rege, S. D., Chen, Y.-Y., Lin, Z., & Fishpaugh, J. R. (2010). Development of a dot-blot assay for screening monoclonal antibodies to low-molecular-mass drugs. *Analytical Biochemistry*, *407*(2), 160–164. https://doi.org/10.1016/j.ab.2010.08.003

Russell, T. M., & Johnson, B. J. B. (2013). Lyme disease spirochaetes possess an aggrecan-binding protease with aggrecanase activity. *Molecular Microbiology*, n/a-n/a. https://doi.org/10.1111/mmi.12276

Russell, T. M., Tang, X., Goldstein, J. M., Bagarozzi, D., & Johnson, B. J. B. (2016). The salt-sensitive structure and zinc inhibition of *B orrelia burgdorferi* protease BbHtrA. *Molecular Microbiology*, *99*(3), 586–596. https://doi.org/10.1111/mmi.13251

Sadziene, A., Wilske, B., Ferdows, M. S., & Barbour, A. G. (1993). The cryptic ospC gene of *Borrelia burgdorferi* B31 is located on a circular plasmid. *Infection and Immunity*, *61*(5), 2192–2195. https://doi.org/10.1128/iai.61.5.2192-2195.1993

Saier, M. H., & Paulsen, I. T. (2000). Whole genome analyses of transporters in spirochetes: *Borrelia burgdorferi* and Treponema pallidum. *Journal of Molecular Microbiology and Biotechnology*, *2*(4), 393–399.

Sampson, L. L., Hendrix, R. W., Huang, W. M., & Casjens, S. R. (1988). Translation initiation controls the relative rates of expression of the bacteriophage lambda late genes. *Proceedings of the National Academy of Sciences*, *85*(15), 5439–5443. https://doi.org/10.1073/pnas.85.15.5439

Samuels, D. S., Lybecker, M. C., Yang, X. F., Ouyang, Z., Bourret, T. J., Boyle, W. K., Stevenson, B., Drecktrah, D., & Caimano, M. J. (2021). Gene regulation and transcriptomics. *Current Issues in Molecular Biology*, *42*, 223–266. https://doi.org/10.21775/cimb.042.223

Samuels, D. S., Lybecker, M. C., Yang, X. F., Ouyang, Z., Bourret, T. J., Boyle, W. K., Stevenson, B., Drecktrah, D., & Caimano, M. J. (2022). Gene Regulation and

Transcriptomics. *Current Issues in Molecular Biology*, 223–266. https://doi.org/10.21775/cimb.042.223

Samuels, D. S., Mach, K. E., & Garon, C. F. (1994). Genetic transformation of the Lyme disease agent *Borrelia burgdorferi* with coumarin-resistant gyrB. *Journal of Bacteriology*, *176*(19), 6045–6049. https://doi.org/10.1128/jb.176.19.6045-6049.1994

Sankaran, K., & Wu, H. C. (1994). Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol. *The Journal of Biological Chemistry*, *269*(31), 19701–19706.

Santos, R., Ursu, O., Gaulton, A., Bento, A. P., Donadi, R. S., Bologa, C. G., Karlsson, A., Al-Lazikani, B., Hersey, A., Oprea, T. I., & Overington, J. P. (2017). A comprehensive map of molecular drug targets. *Nature Reviews Drug Discovery*, *16*(1), 19–34. https://doi.org/10.1038/nrd.2016.230

Saputra, E. P., Trzeciakowski, J. P., & Hyde, J. A. (2020). *Borrelia burgdorferi* spatiotemporal regulation of transcriptional regulator bosR and decorin binding protein during murine infection. *Scientific Reports*, *10*(1), 12534. https://doi.org/10.1038/s41598-020-69212-7

Sastry, A. V., Gao, Y., Szubin, R., Hefner, Y., Xu, S., Kim, D., Choudhary, K. S., Yang, L., King, Z. A., & Palsson, B. O. (2019). The *Escherichia coli* transcriptome mostly consists of independently regulated modules. *Nature Communications*, *10*(1), 5536. https://doi.org/10.1038/s41467-019-13483-w

Sawa, J., Malet, H., Krojer, T., Canellas, F., Ehrmann, M., & Clausen, T. (2011). Molecular Adaptation of the DegQ Protease to Exert Protein Quality Control in the Bacterial Cell Envelope. *Journal of Biological Chemistry*, *286*(35), 30680–30690. https://doi.org/10.1074/jbc.M111.243832

Schenone, M., Dančík, V., Wagner, B. K., & Clemons, P. A. (2013). Target identification and mechanism of action in chemical biology and drug discovery. *Nature Chemical Biology*, *9*(4), 232–240. https://doi.org/10.1038/nchembio.1199

Schmid, M. B., & Roth, J. R. (1987). Gene location affects expression level in Salmonella typhimurium. *Journal of Bacteriology*, *169*(6), 2872–2875. https://doi.org/10.1128/jb.169.6.2872-2875.1987

Schulze, R. J., Chen, S., Kumru, O. S., & 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. *Molecular Microbiology*, *76*(5), 1266–1278. https://doi.org/10.1111/j.1365-2958.2010.07172.x

Schulze, R. J., & Zückert, W. R. (2006). *Borrelia burgdorferi* lipoproteins are secreted to the outer surface by default. *Molecular Microbiology*, *59*(5), 1473–1484. https://doi.org/10.1111/j.1365-2958.2006.05039.x

Schwartz, A. M., Hinckley, A. F., Mead, P. S., Hook, S. A., & Kugeler, K. J. (2017). Surveillance for Lyme Disease — United States, 2008–2015. *MMWR. Surveillance Summaries*, *66*(22), 1–12. https://doi.org/10.15585/mmwr.ss6622a1

Schwartz, I., Margos, G., Casjens, S. R., Qiu, W. G., & Eggers, C. H. (2021). Multipartite genome of lyme disease *Borrelia*: Structure, variation and prophages. *Current Issues in Molecular Biology*, *42*, 409–454. https://doi.org/10.21775/cimb.042.409

Seshu, J., Boylan, J. A., Gherardini, F. C., & Skare, J. T. (2004). Dissolved Oxygen Levels Alter Gene Expression and Antigen Profiles in *Borrelia burgdorferi*.

Infection and Immunity, *72*(3), 1580–1586. https://doi.org/10.1128/IAI.72.3.1580-1586.2004

Setubal, J. C., Reis, M., Matsunaga, J., & Haake, D. A. (2006). Lipoprotein computational prediction in spirochaetal genomes. *Microbiology*, *152*(1), 113–121. https://doi.org/10.1099/mic.0.28317-0

Shaker, B., Ahmad, S., Lee, J., Jung, C., & Na, D. (2021). In silico methods and tools for drug discovery. *Computers in Biology and Medicine*, *137*, 104851. https://doi.org/10.1016/j.compbiomed.2021.104851

Shangguan, Z. (2021). A Review of Target Identification Strategies for Drug Discovery: from Database to Machine-Based Methods. *Journal of Physics: Conference Series*, *1893*(1), 012013. https://doi.org/10.1088/1742-6596/1893/1/012013

Shaw, D. K., Wang, X., Brown, L. J., Chávez, A. S. O., Reif, K. E., Smith, A. A., Scott, A. J., McClure, E. E., Boradia, V. M., Hammond, H. L., Sundberg, E. J., Snyder, G. A., Liu, L., DePonte, K., Villar, M., Ueti, M. W., de la Fuente, J., Ernst, R. K., Pal, U., … Pedra, J. H. F. (2017). Infection-derived lipids elicit an immune deficiency circuit in arthropods. *Nature Communications*, *8*, 14401. https://doi.org/10.1038/ncomms14401

Sidman, C. L., Smith, A. L., & Barthold, S. W. (1992). Lyme Borreliosis in Genetically Resistant and Susceptible Mice with Severe Combined Immunodeficiency. *The American Journal of Tropical Medicine and Hygiene*, *47*(5), 605–613. https://doi.org/10.4269/ajtmh.1992.47.605

Silva, H. O., Souza, M. A. de, Tavares, T. C. F., Soares, P. M., & Lima, A. M. C. (2021). Development and standardization of the Dot Blot test for serological diagnosis of bovine leptospirosis. *Research, Society and Development*, *10*(6), e22710615091. https://doi.org/10.33448/rsd-v10i6.15091

Singh, S. K., & Girschick, H. J. (2004). Lyme borreliosis: from infection to autoimmunity. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *10*(7), 598–614. https://doi.org/10.1111/j.1469-0691.2004.00895.x

Smith, A. A., Navasa, N., Yang, X., Wilder, C. N., Buyuktanir, O., Marques, A., Anguita, J., & Pal, U. (2016). Cross-Species Interferon Signaling Boosts Microbicidal Activity within the Tick Vector. *Cell Host & Microbe*, *20*(1), 91–98. https://doi.org/10.1016/j.chom.2016.06.001

Smith, A. A., & Pal, U. (2014). Immunity-related genes in Ixodes scapularis--perspectives from genome information. *Frontiers in Cellular and Infection Microbiology*, *4*, 116. https://doi.org/10.3389/fcimb.2014.00116

Snider, J., Kotlyar, M., Saraon, P., Yao, Z., Jurisica, I., & Stagljar, I. (2015). Fundamentals of protein interaction network mapping. *Molecular Systems Biology*, *11*(12), 848. https://doi.org/10.15252/msb.20156351

Song, Y., Ke, Y., Kang, M., & Bao, R. (2022). Function, molecular mechanisms, and therapeutic potential of bacterial HtrA proteins: An evolving view. In *Computational and Structural Biotechnology Journal* (Vol. 20, pp. 40–49). Elsevier B.V. https://doi.org/10.1016/j.csbj.2021.12.004

Spiess, C., Beil, A., & Ehrmann, M. (1999). A Temperature-Dependent Switch from Chaperone to Protease in a Widely Conserved Heat Shock Protein. *Cell*, *97*(3), 339–347. https://doi.org/10.1016/S0092-8674(00)80743-6

Stanek, G., & Reiter, M. (2011). The expanding Lyme *Borrelia* complex—clinical significance of genomic species? *Clinical Microbiology and Infection*, *17*(4), 487–493. https://doi.org/10.1111/j.1469-0691.2011.03492.x

Steele, V. R., Bottomley, A. L., Garcia-Lara, J., Kasturiarachchi, J., & Foster, S. J. (2011). Multiple essential roles for EzrA in cell division of Staphylococcus aureus. *Molecular Microbiology*, *80*(2), 542–555. https://doi.org/10.1111/j.1365-2958.2011.07591.x

Steen, A., Buist, G., Horsburgh, G. J., Venema, G., Kuipers, O. P., Foster, S. J., & Kok, J. (2005). AcmA of Lactococcus lactis is an N-acetylglucosaminidase with an optimal number of LysM domains for proper functioning. *FEBS Journal*, *272*(11), 2854–2868. https://doi.org/10.1111/j.1742-4658.2005.04706.x

Steere, A. C. (1987). The Clinical Evolution of Lyme Arthritis. *Annals of Internal Medicine*, *107*(5), 725. https://doi.org/10.7326/0003-4819-107-5-725

Steere, A. C. (2001). Lyme Disease. *New England Journal of Medicine*, *345*(2), 115–125. https://doi.org/10.1056/NEJM200107123450207

Steere, A. C., & Angelis, S. M. (2006). Therapy for Lyme arthritis: Strategies for the treatment of antibiotic-refractory arthritis. *Arthritis & Rheumatism*, *54*(10), 3079–3086. https://doi.org/10.1002/art.22131

Steere, A. C., Coburn, J., & Glickstein, L. (2004). The emergence of Lyme disease. *Journal of Clinical Investigation*, *113*(8), 1093–1101. https://doi.org/10.1172/JCI21681

Steere, A. C., Malawista, S. E., Snydman, D. R., Shope, R. E., Andiman, W. A., Ross, M. R., & Steele, F. M. (1977). An epidemic of oligoarticular arthritis in children and adults in three connecticut communities. *Arthritis & Rheumatism*, *20*(1), 7–17. https://doi.org/10.1002/art.1780200102

Steere, A. C., McHugh, G., Damle, N., & Sikand, V. K. (2008). Prospective Study of Serologic Tests for Lyme Disease. *Clinical Infectious Diseases*, *47*(2), 188–195. https://doi.org/10.1086/589242

Steere, A. C., Sikand, V. K., Meurice, F., Parenti, D. L., Fikrig, E., Schoen, R. T., Nowakowski, J., Schmid, C. H., Laukamp, S., Buscarino, C., & Krause, D. S. (1998). Vaccination against Lyme Disease with Recombinant *Borrelia burgdorferi* Outer-Surface Lipoprotein A with Adjuvant. *New England Journal of Medicine*, *339*(4), 209–215. https://doi.org/10.1056/NEJM199807233390401

Steere, A. C., Strle, F., Wormser, G. P., Hu, L. T., Branda, J. A., Hovius, J. W. R., Li, X., & Mead, P. S. (2016). Lyme borreliosis. *Nature Reviews Disease Primers*, *2*. https://doi.org/10.1038/nrdp.2016.90

Stewart, P. E., Hoff, J., Fischer, E., Krum, J. G., & Rosa, P. A. (2004). Genome-wide transposon mutagenesis of *Borrelia burgdorferi* for identification of phenotypic mutants. *Applied and Environmental Microbiology*, *70*(10), 5973–5979. https://doi.org/10.1128/AEM.70.10.5973-5979.2004

Strle, K., Stupica, D., Drouin, E. E., Steere, A. C., & Strle, F. (2014). Elevated Levels of IL-23 in a Subset of Patients With Post-Lyme Disease Symptoms Following Erythema Migrans. *Clinical Infectious Diseases*, *58*(3), 372–380. https://doi.org/10.1093/cid/cit735

Stübs, G., Fingerle, V., Wilske, B., Göbel, U. B., Zähringer, U., Schumann, R. R., & Schröder, N. W. J. (2009). Acylated Cholesteryl Galactosides Are Specific Antigens

of *Borrelia* Causing Lyme Disease and Frequently Induce Antibodies in Late Stages of Disease. *Journal of Biological Chemistry*, *284*(20), 13326–13334. https://doi.org/10.1074/jbc.M809575200

Studholme, D. J., & Buck, M. (2000). The biology of enhancer-dependent transcriptional regulation in bacteria: insights from genome sequences. *FEMS Microbiology Letters*, *186*(1), 1–9. https://doi.org/10.1111/j.1574-6968.2000.tb09074.x

Sutherland, C., & Murakami, K. S. (2018). An Introduction to the Structure and Function of the Catalytic Core Enzyme of *Escherichia coli* RNA Polymerase. *EcoSal Plus*, *8*(1). https://doi.org/10.1128/ecosalplus.ESP-0004-2018

Szczepaniak, J., Press, C., & Kleanthous, C. (2020). The multifarious roles of Tol-Pal in Gram-negative bacteria. *FEMS Microbiology Reviews*, *44*(4), 490–506. https://doi.org/10.1093/femsre/fuaa018

Szwedziak, P., Wang, Q., Bharat, T. A. M., Tsim, M., & Löwe, J. (2014). Architecture of the ring formed by the tubulin homologue FtsZ in bacterial cell division. *ELife*, *3*. https://doi.org/10.7554/eLife.04601

Taggart, J. C., Lalanne, J.-B., & Li, G.-W. (2021). Quantitative Control for Stoichiometric Protein Synthesis. *Annual Review of Microbiology*, *75*(1), 243–267. https://doi.org/10.1146/annurev-micro-041921-012646

Taggart, J. C., Zauber, H., Selbach, M., Li, G.-W., & McShane, E. (2020). Keeping the Proportions of Protein Complex Components in Check. *Cell Systems*, *10*(2), 125–132. https://doi.org/10.1016/j.cels.2020.01.004

Takacs, C. N., Kloos, Z. A., Scott, M., Rosa, P. A., Jacobs-Wagner, C., & Stabb, E. V. (2018). *Fluorescent Proteins, Promoters, and Selectable Markers for Applications in the Lyme Disease Spirochete Borrelia burgdorferi*. https://doi.org/10.1128/AEM

Takayama, K., Rothenberg, R. J., & Barbour, A. G. (1987). Absence of lipopolysaccharide in the Lyme disease spirochete, *Borrelia burgdorferi*. *Infection and Immunity*, *55*(9), 2311–2313. https://doi.org/10.1128/iai.55.9.2311-2313.1987

Thakur, M., Sharma, K., Chao, K., Smith, A. A., Herzberg, O., & Pal, U. (2017). A protein-protein interaction dictates Borrelial infectivity. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-03279-7

Tilly, K., Bestor, A., & Rosa, P. A. (2016). Functional equivalence of OspA and OspB, but not OspC, in tick colonization by *Borrelia burgdorferi*. *Infection and Immunity*, *84*(5), 1565–1573. https://doi.org/10.1128/IAI.00063-16

Tilly, K., Rosa, P. A., & Stewart, P. E. (2008). Biology of Infection with *Borrelia burgdorferi*. *Infectious Disease Clinics of North America*, *22*(2), 217–234. https://doi.org/10.1016/j.idc.2007.12.013

Tokuda, H., & Matsuyama, S. (2004). Sorting of lipoproteins to the outer membrane in E. coli. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1693*(1), 5–13. https://doi.org/10.1016/j.bbamcr.2004.02.005

Troxell, B., Ye, M., Yang, Y., Carrasco, S. E., Lou, Y., & Yang, X. F. (2013). Manganese and Zinc Regulate Virulence Determinants in *Borrelia burgdorferi*. *Infection and Immunity*, *81*(8), 2743–2752. https://doi.org/10.1128/IAI.00507-13

Troy, E. B., Lin, T., Gao, L., Lazinski, D. W., Camilli, A., Norris, S. J., & Hu, L. T. (2013). Understanding Barriers to *Borrelia burgdorferi* Dissemination during Infection Using Massively Parallel Sequencing. *Infection and Immunity*, *81*(7), 2347–2357. https://doi.org/10.1128/IAI.00266-13

Tsukazaki, T., Yoshida, M., Namba, H., Yamada, M., Shimizu, N., & Nii, S. (1998). Development of a dot blot neutralizing assay for HHV-6 and HHV-7 using specific monoclonal antibodies. *Journal of Virological Methods*, *73*(2), 141–149. https://doi.org/10.1016/S0166-0934(98)00051-2

Tunev, S. S., Hastey, C. J., Hodzic, E., Feng, S., Barthold, S. W., & Baumgarth, N. (2011). Lymphoadenopathy during Lyme Borreliosis Is Caused by Spirochete Migration-Induced Specific B Cell Activation. *PLoS Pathogens*, *7*(5), e1002066. https://doi.org/10.1371/journal.ppat.1002066

Uhde, M., Ajamian, M., Li, X., Wormser, G. P., Marques, A., & Alaedini, A. (2016). Expression of C-Reactive Protein and Serum Amyloid A in Early to Late Manifestations of Lyme Disease. *Clinical Infectious Diseases*, *63*(11), 1399–1404. https://doi.org/10.1093/cid/ciw599

Vamathevan, J., Clark, D., Czodrowski, P., Dunham, I., Ferran, E., Lee, G., Li, B., Madabhushi, A., Shah, P., Spitzer, M., & Zhao, S. (2019). Applications of machine learning in drug discovery and development. *Nature Reviews Drug Discovery*, *18*(6), 463–477. https://doi.org/10.1038/s41573-019-0024-5

Vandamme, T. F. (2014). Use of rodents as models of human diseases. *Journal of Pharmacy & Bioallied Sciences*, *6*(1), 2–9. https://doi.org/10.4103/0975-7406.124301

Vogt, N. M., Kerby, R. L., Dill-McFarland, K. A., Harding, S. J., Merluzzi, A. P., Johnson, S. C., Carlsson, C. M., Asthana, S., Zetterberg, H., Blennow, K., Bendlin, B. B., & Rey, F. E. (2017). Gut microbiome alterations in Alzheimer's disease. *Scientific Reports*, *7*(1), 13537. https://doi.org/10.1038/s41598-017-13601-y

Vollmer, W., Blanot, D., & de Pedro, M. A. (2008). Peptidoglycan structure and architecture. *FEMS Microbiology Reviews*, *32*(2), 149–167. https://doi.org/10.1111/j.1574-6976.2007.00094.x

Vudattu, N. K., Strle, K., Steere, A. C., & Drouin, E. E. (2013). Dysregulation of CD4+CD25 ^{high} T Cells in the Synovial Fluid of Patients With Antibiotic-Refractory Lyme Arthritis. *Arthritis & Rheumatism*, *65*(6), 1643–1653. https://doi.org/10.1002/art.37910

Walker, E. M., Borenstein, L. A., Blanco, D. R., Miller, J. N., & Lovett, M. A. (1991). Analysis of outer membrane ultrastructure of pathogenic Treponema and *Borrelia species* by freeze-fracture electron microscopy. *Journal of Bacteriology*, *173*(17), 5585–5588. https://doi.org/10.1128/jb.173.17.5585-5588.1991

Waller, P. R., & Sauer, R. T. (1996). Characterization of degQ and degS, *Escherichia coli* genes encoding homologs of the DegP protease. *Journal of Bacteriology*, *178*(4), 1146–1153. https://doi.org/10.1128/jb.178.4.1146-1153.1996

Wang, G., Liveris, D., Mukherjee, P., Jungnick, S., Margos, G., & Schwartz, I. (2014). Molecular Typing of *Borrelia burgdorferi*. *Current Protocols in Microbiology*, *34*(1). https://doi.org/10.1002/9780471729259.mc12c05s34

Wang, X., Ma, Y., Weis, J. H., Zachary, J. F., Kirschning, C. J., & Weis, J. J. (2005). Relative Contributions of Innate and Acquired Host Responses to Bacterial Control and Arthritis Development in Lyme Disease. *Infection and Immunity*, *73*(1), 657–660. https://doi.org/10.1128/IAI.73.1.657-660.2005

Wang, X.-G., Lin, B., Kidder, J. M., Telford, S., & Hu, L. T. (2002). Effects of Environmental Changes on Expression of the Oligopeptide Permease (*opp*) Genes

of *Borrelia burgdorferi*. *Journal of Bacteriology*, *184*(22), 6198–6206. https://doi.org/10.1128/JB.184.22.6198-6206.2002
Wilson, M. L., Levine, J. F., & Spielman, A. (1985). Mice as Reservoirs of the Lyme Disease Spirochete. *The American Journal of Tropical Medicine and Hygiene*, *34*(2), 355–360. https://doi.org/10.4269/ajtmh.1985.34.355
Wolff, S., Weissman, J. S., & Dillin, A. (2014). Differential Scales of Protein Quality Control. *Cell*, *157*(1), 52–64. https://doi.org/10.1016/j.cell.2014.03.007
Wong, K. H., Shapiro, E. D., & Soffer, G. K. (2022). A Review of Post-treatment Lyme Disease Syndrome and Chronic Lyme Disease for the Practicing Immunologist. *Clinical Reviews in Allergy & Immunology*, *62*(1), 264–271. https://doi.org/10.1007/s12016-021-08906-w
Wooten, R. M., Ma, Y., Yoder, R. A., Brown, J. P., Weis, J. H., Zachary, J. F., Kirschning, C. J., & Weis, J. J. (2002). Toll-Like Receptor 2 Is Required for Innate, But Not Acquired, Host Defense to *Borrelia burgdorferi*. *The Journal of Immunology*, *168*(1), 348–355. https://doi.org/10.4049/jimmunol.168.1.348
Wormser, G. P. (2005). Prevention of Lyme borreliosis. *Wiener Klinische Wochenschrift*, *117*(11–12), 385–391. https://doi.org/10.1007/s00508-005-0362-7
Wormser, G. P., Brisson, D., Liveris, D., Hanincová, K., Sandigursky, S., Nowakowski, J., Nadelman, R. B., Ludin, S., & Schwartz, I. (2008). *Borrelia burgdorferi* Genotype Predicts the Capacity for Hematogenous Dissemination during Early Lyme Disease. *The Journal of Infectious Diseases*, *198*(9), 1358–1364. https://doi.org/10.1086/592279
Wormser, G. P., Dattwyler, R. J., Shapiro, E. D., Halperin, J. J., Steere, A. C., Klempner, M. S., Krause, P. J., Bakken, J. S., Strle, F., Stanek, G., Bockenstedt, L., Fish, D., Dumler, J. S., & Nadelman, R. B. (2006). The Clinical Assessment, Treatment, and Prevention of Lyme Disease, Human Granulocytic Anaplasmosis, and Babesiosis: Clinical Practice Guidelines by the Infectious Diseases Society of America. *Clinical Infectious Diseases*, *43*(9), 1089–1134. https://doi.org/10.1086/508667
Wormser, G. P., & Schwartz, I. (2009). Antibiotic Treatment of Animals Infected with *Borrelia burgdorferi*. *Clinical Microbiology Reviews*, *22*(3), 387–395. https://doi.org/10.1128/CMR.00004-09
Wrase, R., Scott, H., Hilgenfeld, R., & Hansen, G. (2011). The *Legionella* HtrA homologue DegQ is a self-compartmentizing protease that forms large 12-meric assemblies. *Proceedings of the National Academy of Sciences*, *108*(26), 10490–10495. https://doi.org/10.1073/pnas.1101084108
Wu, Y., Yang, Z., Cheng, K., Bi, H., & Chen, J. (2022). Small molecule-based immunomodulators for cancer therapy. *Acta Pharmaceutica Sinica B*, *12*(12), 4287–4308. https://doi.org/10.1016/j.apsb.2022.11.007
Xu, Q., Seemanapalli, S. V., Reif, K. E., Brown, C. R., & Liang, F. T. (2007). Increasing the Recruitment of Neutrophils to the Site of Infection Dramatically Attenuates *Borrelia burgdorferi* Infectivity. *The Journal of Immunology*, *178*(8), 5109–5115. https://doi.org/10.4049/jimmunol.178.8.5109
Yakhnina, A. A., & Bernhardt, T. G. (2020). The Tol-Pal system is required for peptidoglycan-cleaving enzymes to complete bacterial cell division. *Proceedings of the National Academy of Sciences*, *117*(12), 6777–6783. https://doi.org/10.1073/pnas.1919267117

Yamashita, F., & Hashida, M. (2013). Pharmacokinetic considerations for targeted drug delivery. *Advanced Drug Delivery Reviews*, *65*(1), 139–147. https://doi.org/10.1016/j.addr.2012.11.006

Yang, X., Promnares, K., Qin, J., He, M., Shroder, D. Y., Kariu, T., Wang, Y., & Pal, U. (2011). Characterization of multiprotein complexes of the *Borrelia burgdorferi* outer membrane vesicles. *Journal of Proteome Research*, *10*(10), 4556–4566. https://doi.org/10.1021/pr200395b

Yang, X., Qin, J., Promnares, K., Kariu, T., Anderson, J. F., & Pal, U. (2013). Novel Microbial Virulence Factor Triggers Murine Lyme Arthritis. *The Journal of Infectious Diseases*, *207*(6), 907–918. https://doi.org/10.1093/infdis/jis930

Ye, M., Sharma, K., Thakur, M., Smith, A. A., Buyuktanir, O., Xiang, X., Yang, X., Promnares, K., Lou, Y., Yang, X. F., & Pal, U. (2016). HtrA, a temperature- and stationary phase-activated protease involved in maturation of a key microbial virulence determinant, facilitates *Borrelia burgdorferi* infection in mammalian hosts. *Infection and Immunity*, *84*(8), 2372–2381. https://doi.org/10.1128/IAI.00360-16

Zeng, M. Y., Inohara, N., & Nuñez, G. (2017). Mechanisms of inflammation-driven bacterial dysbiosis in the gut. *Mucosal Immunology*, *10*(1), 18–26. https://doi.org/10.1038/mi.2016.75

Zhang, X., Yang, X., Kumar, M., & Pal, U. (2009). BB0323 function is essential for *Borrelia burgdorferi* virulence and persistence through tick-rodent transmission cycle. *Journal of Infectious Diseases*, *200*(8), 1318–1330. https://doi.org/10.1086/605846

Zhong, L., Li, Y., Xiong, L., Wang, W., Wu, M., Yuan, T., Yang, W., Tian, C., Miao, Z., Wang, T., & Yang, S. (2021). Small molecules in targeted cancer therapy: advances, challenges, and future perspectives. *Signal Transduction and Targeted Therapy*, *6*(1), 201. https://doi.org/10.1038/s41392-021-00572-w

Zhuang, X., Yang, X., Altieri, A. S., Nelson, D. C., & Pal, U. (2018). *Borrelia burgdorferi* surface-located Lmp1 protein processed into region-specific polypeptides that are critical for microbial persistence. *Cellular Microbiology*, *20*(9), e12855. https://doi.org/10.1111/cmi.12855

Zückert, W. R. (2014). Secretion of Bacterial Lipoproteins: Through the Cytoplasmic Membrane, the Periplasm and Beyond. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1843*(8), 1509–1516. https://doi.org/10.1016/j.bbamcr.2014.04.022