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# Characterization Of Infectious Isolates Of Borrelia Burgdorferi In North Dakota, Bb0399 And Bbb28 In B. Burgdorferi, And Borrelia Miyamotoi In Vitro And In Vivo

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# CHARACTERIZATION OF INFECTIOUS ISOLATES OF *BORRELIA BURGDORFERI* IN NORTH DAKOTA, BB0399 AND BBB28 IN *B. BURGDORFERI*, AND *BORRELIA MIYAMOTOI IN VITRO AND IN VIVO*

by

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Masters of Science, California State University, Chico, 2011

A Dissertation
Submitted to the Graduate Faculty

of the

University of North Dakota

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy in Microbiology and Immunology

Grand Forks, North Dakota
May
2018

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This dissertation, submitted by Brandee Lynn Stone in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Catherine Brissette, Chairperson

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Sergei Nechaev, PhD,
Member at Large

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

Grant McGimpsey, PhD
Dean of the Graduate School

May 1, 2018
Date

PERMISSION

Title: CHARACTERIZATION OF INFECTIOUS ISOLATES OF *BORRELIA BURGDORFERI* IN NORTH DAKOTA, BB0399 AND BBB28 IN *B. BURGDORFERI*, AND *BORRELIA MIYAMOTOI IN VITRO AND IN VIVO*

Department: Microbiology and Immunology

Degree: Doctor of Philosophy

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Brandee Lynn Stone
May, 2018

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## ACKNOWLEGEMENTS

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**ABSTRACT**

The *Borrelia burgdorferi sensu lato* (*s.l.*) complex contains species carried by hard-shell ticks (*Ixodes* spp.) causing Lyme disease and related non-pathogenic species. Relapsing fever *Borrelia* includes both tick-borne (soft-shell, *Ornithodoros* spp.) and louse-borne species knowns to cause relapsing fever. A subgroup includes relapsing fever spirochetes carried by hard-shell ticks, including *B. miyamotoi*, an emerging pathogen. Despite bordering high-risk counties in Minnesota, little attention has been given to Lyme disease, *B. burgdorferi, I. scapularis,* or reservoirs in eastern North Dakota. Reports of *B. burgdorferi* and *I. scapularis* in North Dakota, however, prompted a more detailed examination. Through trapping *Peromyscus* and *Myodes*, five *B. burgdorferi* populations were obtained. We confirmed the presence of established, unique (nonclonal), and infectious *B. burgdorferi* populations in eastern North Dakota. Species of the *B. burgdorferi s.l.* complex possess two highly conserved hypothetical genes, *bb0399* and *bbb28*, containing one of the most common protein motifs, ankyrin-repeat domains. The goal was to identify the function(s) of *bb0399* and *bbb28*. Our hypothesis was BB0399 is an essential DNA binding protein and BBB28 is regulated by the *Borrelia* oxidative stress response regulator, BosR in response to unknown stimuli. Exposing *B. burgdorferi* to *tert*-butyl hydroperoxide increased transcription of *bbb28* but not *bb0399*. Several

attempts to express recombinant BB0399 and BBB28 failed and the functionsof *bb0399* and *bbb28* remain unknown. *B. miyamotoi* is an emerging pathogen vectored by the same *Ixodes* spp. carrying and transmitting *B. burgdorferi*. *B. miyamotoi* binds human factor H *in vitro*. C57BL/6J Rag1$^{-/-}$ mice infected with a Japanese strain of *B. miyamotoi*, FR64b, developed a chronic infection, while both 2-4 and 6-8 week-old wild-type C3H/HeN groups cleared *B. miyamotoi*. *B. miyamotoi* FR64b, normally vectored by *I. persulcatus*, was acquired by North American *I. scapularis* and maintained *B. miyamotoi* throughout the molting process from larvae to nymph, suggesting unlike other relapsing fever *Borrelia*, *B. miyamotoi* is not vector specific.

# CHAPTER 1

# AN INTRODUCTION TO BORRELIA BURGDORFERI AND BORRELIA MIYAMOTOI

An array of viral, bacterial, and parasitic pathogens cause tick-borne diseases. Tick-borne pathogens are transmitted through the bite of an infected hard- or soft-shell tick belonging to four genera (*Ixodes, Dermacentor, Amblyomma,* and *Ornithodoros*) (1–3). This group of diseases include Lyme disease (*Borrelia burgdorferi sensu lato*), tick-borne relapsing fever (*Borrelia*), anaplasmosis (*Anaplasma phagocytophilum*), rickettsiosis (*Rickettsia*), babesiosis (*Babesia*), Powassan virus (Flavivirus), tick-borne encephalitis virus (Flavivirus), Colorado tick fever (Coltivirus), and Heartland virus (Phlebovirus) to name a few.

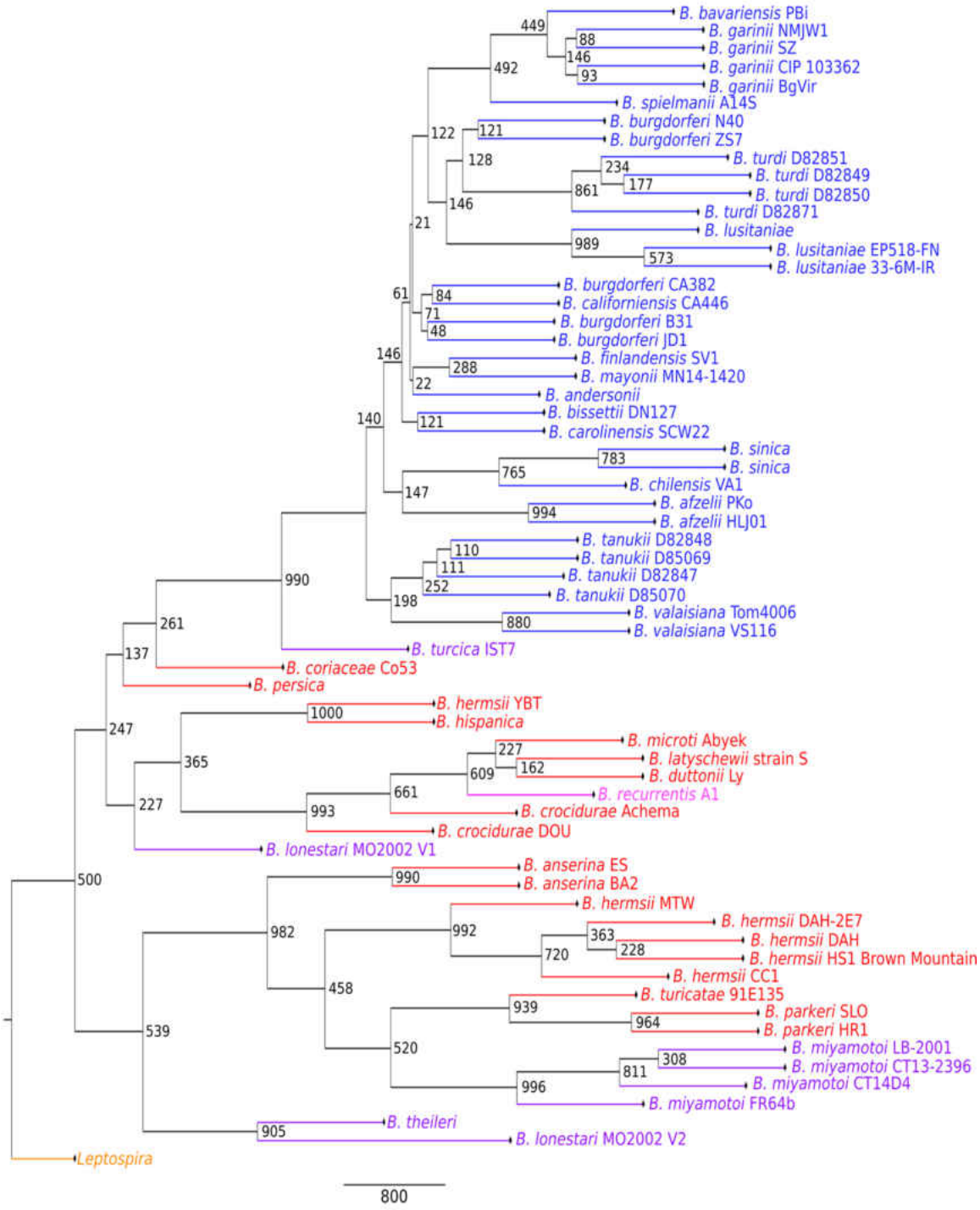
## Borrelia

Species of the *B. burgdorferi sensu lato* (*s.l.*) complex are primarily carried by *Ixodes* spp. (Ixodidae; hard shell). The *s.l.* complex includes nine *Borrelia* spp. causing Lyme disease and approximately 11 non-pathogenic but genetically similar *Borrelia* spp. (Table 1.1) (4–16). *Borrelia* spp. that cause tick-borne relapsing fever (TBRF) are primarily carried by *Ornithodoros* spp. (Argasidae; soft shell) (Table 1.1). Relapsing fever *Borrelia* are genetically distinct from *B. burgdorferi s.l.* species but are genetically similar to each other (Fig. 1.1).

Table 1. 1. *Borrelia* spp. confirmed to cause human disease and associated vectors. NA – North America, Eu – Europe, As – Asia, Af – Africa.

| Disease | Causative agent(s) | Vector(s) |
|---|---|---|
| Lyme disease | *B. burgdorferi sensu stricto* (*s.s.*) (NA, Eu)<br>*B. mayonii* (NA)<br>*B. bissettii* (NA, Eu)<br>*B. lusitaniae* (Eu)<br>*B. valaisiana* (Eu)<br>*B. afzelii* (Eu, As)<br>*B. garinii* (Eu, As)<br>*B. spielmanii* (Eu, As)<br>*B. bavariensis* (Eu, As; formerly *B. garinii* OspA serotype 4) | *I. scapularis* (NA)<br>*I. pacificus* (NA)<br>*I. ricinus* (Eu, As)<br>*I. persulcatus* (Eu, As) |
| Tick-borne relapsing fever | *B. hermsii* (NA)<br>*B. turicatae* (NA)<br>*B. parkeri* (NA)<br>*B. duttonii* (Af)<br>*B. crocidurae* (Af)<br>*B. hispanica* (Eu, Af)<br>*B. latyshevi* (As)<br>*B. persica* (As, Af) | *O. hermsi* (NA)<br>*O. turicata* (NA)<br>*O. parkeri* (NA)<br>*O. moubata* (Af)<br>*O. erraticus; O. sonrai* (Af)<br>*O. erraticus* (Eu, Af)<br>*O. tartakovski* (As)<br>*O. tholozani* (As, Af) |
| Hard tick-borne relapsing fever | *B. miyamotoi* (NA, Eu, As) | *I. scapularis*<br>*I. pacificus*<br>*I. ricinus*<br>*I. persulcatus* |
| Louse-borne relapsing fever | *B. recurrentis* (Af) | *P. humanus humanus* (Af) |

Figure 1. 1. Maximum likelihood phylogenetic analysis of *Borrelia* flagellin. *B. burgdorferi s.l.* species form a distinct, monophyletic clade; relapsing fever *Borrelia* do not form a monophyletic clade, however, they are separate from Lyme *Borrelia* species. Values at nodes represent bootstrap values from 1000 replicates. Species of the *B. burgdorferi s.l.* complex (Lyme) are shown in blue, relapsing fever *Borrelia* vectored or carried by soft-shell ticks (*Ornithodoros*, *Argas*) are red, relapsing fever *Borrelia* vectored by lice (*Pediculus*) is pink, relapsing fever *Borrelia* vectored or carried by hard-shell ticks (*Ixodes*, *Amblyomma*, *Hyalomma*, *Rhipichephalus*) are purple, and an outgroup (*Leptospira*) is orange.


B. bavariensis PBi
B. garinii NMJW1
B. garinii SZ
B. garinii CIP 103362
B. garinii BgVir
B. spielmanii A14S
B. burgdorferi N40
B. burgdorferi ZS7
B. turdi D82851
B. turdi D82849
B. turdi D82850
B. turdi D82871
B. lusitaniae
B. lusitaniae EP518-FN
B. lusitaniae 33-6M-IR
B. burgdorferi CA382
B. californiensis CA446
B. burgdorferi B31
B. burgdorferi JD1
B. finlandensis SV1
B. mayonii MN14-1420
B. andersonii
B. bissettii DN127
B. carolinensis SCW22
B. sinica
B. sinica
B. chilensis VA1
B. afzelii PKo
B. afzelii HLJ01
B. tanukii D82848
B. tanukii D85069
B. tanukii D82847
B. tanukii D85070
B. valaisiana Tom4006
B. valaisiana VS116
B. turcica IST7
B. coriaceae Co53
B. persica
B. hermsii YBT
B. hispanica
B. microti Abyek
B. latyschewii strain S
B. duttonii Ly
B. recurrentis A1
B. crocidurae Achema
B. crocidurae DOU
B. lonestari MO2002 V1
B. anserina ES
B. anserina BA2
B. hermsii MTW
B. hermsii DAH-2E7
B. hermsii DAH
B. hermsii HS1 Brown Mountain
B. hermsii CC1
B. turicatae 91E135
B. parkeri SLO
B. parkeri HR1
B. miyamotoi LB-2001
B. miyamotoi CT13-2396
B. miyamotoi CT14D4
B. miyamotoi FR64b
B. theileri
B. lonestari MO2002 V2
Leptospira
800

The general classification for *Borrelia* currently holds as several Lyme disease *Borrelia* are associated with hard-shell ticks and relapsing fever *Borrelia* are associated with soft-shell ticks. However, five exceptions have been documented. *B. recurrentis* is a louse-borne relapsing fever spirochete presently endemic predominantly to sub-Saharan Africa. *B. theileri* is the causative agent of bovine borreliosis and is transmitted by *Rhipicephalus microplus*, a hard-shell tick that parasitizes livestock (17). *B. lonestari* and *B. turcica* are genetically similar to relapsing fever borreliae and carried by the hard-shell ticks *Amblyomma americanum* and *Hyalomma aegyptium*, respectively (18, 19). The status of *B. lonestari* and *B. turcica* as an animal or human pathogens is unknown. Finally, *B. miyamotoi* is a relapsing fever spirochete vectored by the same *Ixodes* spp. transmitting species of the *B. burgdorferi s.l.* complex (20, 21).

### *The Unusual Nature of B. burgdorferi S.L. Genetics*

All known *Borrelia* spp. have unusual genomes compared to traditional bacterial genomes, which consist of a circular chromosome. Species of the *B. burgdorferi s.l.* complex have a highly fragmented genome comprised of a linear chromosome (ca. 910 kilobases [kb]) and an array of linear and circular plasmids (size from ca. 5 to 54 kb) (22). Twelve linear and nine circular plasmids have been characterized from the *B. burgdorferi s.s.* B31 type strain, though the number and arrangement of plasmids vary by *B. burgdorferi s.l.* strain (23, 24).

The chromosome mainly consists of essential genes (e.g. metabolism, motility, replication) and appears to be stable (22, 25). Some essential genes are found on plasmids thereby making those plasmids essential. The presence of essential plasmids is in stark contrast to traditional bacterial genomes where plasmids are usually unessential or extra-chromosomal genetic elements. However, it should be noted the definition of "essential plasmid" requires consideration of the environment and *B. burgdorferi* exists in several environment types: *in vivo* in ticks (fed and unfed), *in vivo* in numerous reservoirs and hosts, and *in vitro* in laboratory cultures. A plasmid required for survival in a mouse may not be required for survival in a tick. Prolonged *in vitro* passage results in strains losing plasmids (likely due to a lack of selective pressure), which are required for establishing or maintaining an infection in mice and/or ticks resulting in non-infectious isolates (26, 27). The loss of plasmids, however, is not universal as some isolates do retain plasmids and, thus, infectivity (28).

The genome, in terms of open reading frames (ORFs), is significantly reduced compared to free-living bacteria but is on par with genome sizes of other obligate parasitic bacteria such as *Rickettsia* and *Chlamydia*. *B. burgdorferi* B31 currently has 1427 putative or known ORFs and 136 pseudogenes (22, 29). Of these 1427 ORFs, 588 (41.2%) are predicted "hypothetical proteins," or proteins meeting all the requirements to be functional but lacking empirical data to demonstrate functionality. Very few hypothetical proteins reside on the chromosome (26.4%) as many of these are essential genes that have been assigned a function either through

identification of conserved domains and/or empirically. By comparison, 50% of ORFs on 16 of the 21 plasmids found in *B. burgdorferi* B31 are hypothetical proteins. Function and functionality of many of these will require experimental data as only approximately 7.9% of predicted ORFs on *B. burgdorferi*'s plasmids share homology to proteins from any genera outside of *Borrelia* (30).

### *Relapsing Fever Borrelia Genetics is also Unusual*

Relapsing fever species also share similar genetic traits though much less is known about relapsing fever *Borrelia* as sequence data for most species is still incomplete. As a result, much of what we know about relapsing fever *Borrelia* comes from work with *B. hermsii*. Like species of the *B. burgdorferi s.l.* complex, relapsing fever *Borrelia* have a linear chromosome (ca. 923 kb) and several circular plasmids homologous to the *B. burgdorferi s.l.* cp32 family (31, 32). Unlike *B. burgdorferi s.l.* spp., relapsing fever *Borrelia* also have a linear megaplasmid (ca. 183 kb). *B. hermsii* HS1 (type strain) also has two circular plasmids (ca. 6.5 and 28.8 kb) and seven linear plasmids (size from ca. 27 to 58 kb) (32).

Of the 1272 ORFs, 36.6% are predicted hypothetical proteins. However, the vast majority of these hypothetical proteins are found on the plasmids (ranges from linear plasmid B58 with 43.8% ORFs predicted as hypothetical proteins to the megaplasmid with 87.9% hypothetical protein ORFs). As with *B. burgdorferi* B31, the chromosome of *B. hermsii* HS1 has the fewest hypothetical proteins (19.9%). Given the number of hypothetical proteins predicted on *B. hermsii* plasmids, we presume a similarly small

percentage of predicted ORFs in *B. hermsii* lack homology to proteins from genera outside *Borrelia* and will require empirical testing to determine the function and functionality of many of these proteins.

## Vectors and Associated *Borrelia* spp.

There are distinct morphological differences between *Ixodes* and *Ornithodoros* ticks. *Ixodes* possess a hard plate on the back (dorsal scutum). *Ornithodoros*, on the other hand, have leathery but soft skin. *Ixodes* have mouthparts visible from dorsal and ventral perspectives, while *Ornithodoros* mouthparts are only visible when observing the ventral side. In addition to the gross anatomical differences, there are also behavioral differences between *Ixodes* and *Ornithodoros* that may contribute to differences observed between *B. burgdorferi s.l.* spp. and relapsing fever *Borrelia*.

### *Life Cycle of Ixodes and Enzootic Cycle of B. burgdorferi s.l.*

*Ixodes* have a two-year, four-stage life cycle (Fig. 1.2). During year 1, eggs are laid in spring and hatch to larvae in summer. Larvae will feed on their first host, typically a small to medium mammal, rodent, or bird, some of which are competent reservoirs for *B. burgdorferi s.l.* spp. Indeed, the larval bloodmeal is a crucial point in the enzootic cycle of *B. burgdorferi s.l.*, which is not transovarially maintained in ticks. That is, an infected adult cannot pass *B. burgdorferi s.l.* to the eggs. *Ixodes* can only acquire *B. burgdorferi s.l.* by feeding on an infected reservoir. As a result, Lyme disease is not considered be obtained through the bite of a larval *Ixodes*. After feeding, replete *Ixodes* will detach and find refuge in a suitable habitat for the fall and winter months. During year 2, larvae will molt to nymphs, emerge in the

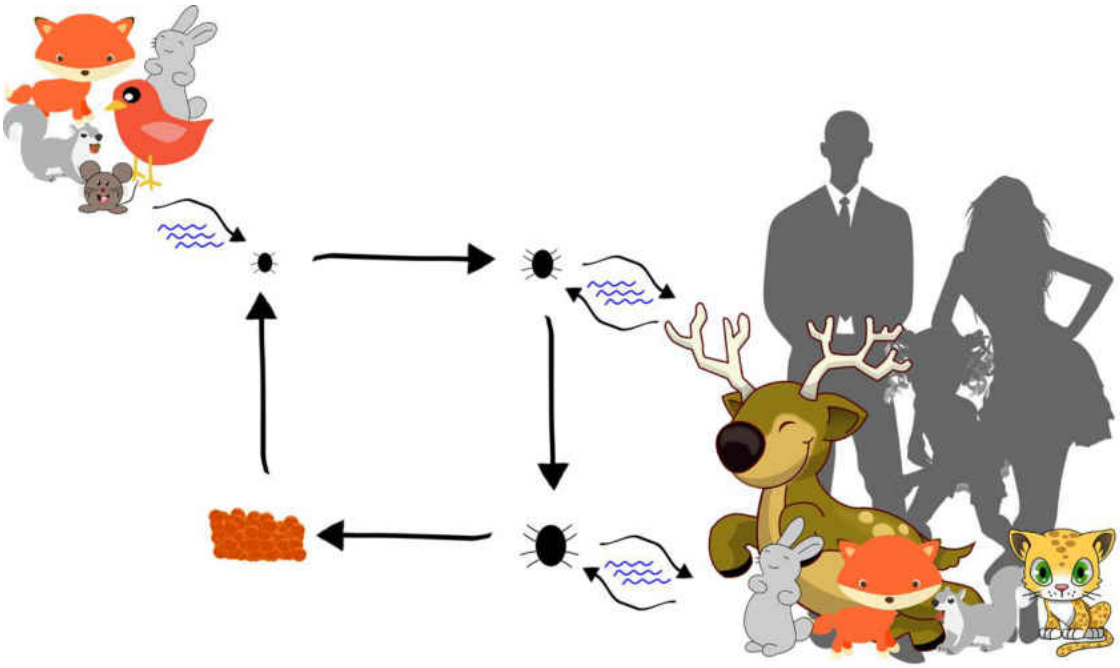


Figure 1. 2. Life cycle of *Ixodes* spp. and enzootic cycle of *B. burgdorferi s.l.* Larvae (six legs) feed on small- to medium-sized animals, providing the first opportunity for *Ixodes* to acquire *B. burgdorferi s.l.* After feeding, larvae molt into nymphs (eight legs) and feed on medium- to large-sized animals. A final bloodmeal is consumed by adult females and pairs mate on a medium- to large-sized animals. Females will drop from the host to lay eggs.

spring, and seek its next bloodmeal. Nymphs will usually feed on small to medium mammals, rodents, and birds. After detaching, nymphs will molt and emerge as adults during the fall of year 2. Adults will find a medium to large mammal, in particular, *Odocoileus virginianus* (white-tailed deer), a common host for adults. Females, but not males, will take a bloodmeal and both will mate. Males die shortly after mating, while females will drop from the host, find suitable habitat, and lay eggs.

Each life stage feeds once but *Ixodes* are considered generalist feeders. Even though there are preferences for hosts (e.g. larvae tend to feed on *Peromyscus* or white-footed mice more often than other hosts), as a whole, *Ixodes* spp. feed on a variety of animals (33–45). Humans and domestic animals can become hosts for all stages of *Ixodes* but are considered dead-end hosts for *B. burgdorferi s.l.* as neither can sustain an enzootic cycle. Species of the *B. burgdorferi s.l.* complex are not maintained transovarially, a subject of debate for years. In retrospect, early reports of *B. burgdorferi* being transovarially transmitted (46) were likely due to the presence of *B. miyamotoi*, a relapsing fever spirochete transmitted by *Ixodes* spp. discovered in 1995 (20). To become infected with *B. burgdorferi s.l.*, ticks must feed on an infected animal and any stage can acquire *B. burgdorferi s.l.* Larvae cannot transmit Lyme disease, thus the largest threat for humans and domestic animals to acquire *B. burgdorferi s.l.* from ticks come from unfed nymphs and adult females. Adult males can be infected, however, since adult males do not typically partake in a bloodmeal, they rarely transmit *B. burgdorferi s.l*. (47).

It is important to note the general perception of *Ixodes*, including host preference by different life stages and even active periods, is based on the most studied and largest endemic region for Lyme disease in North America, Northeastern US. However, as we understand more about Lyme disease (i.e. *Ixodes*, *B. burgdorferi s.l.*, reservoirs, and hosts) in the upper Midwest, southern US, California, Canada, Europe, and Asia, it is clear we need to view Lyme disease as a regional disease, especially in terms of the maintenance of

*B. burgdorferi s.l.*, the chance to acquire Lyme disease, and public health strategies for controlling Lyme disease.

*P. leucopus* (white-footed mouse) is a primary reservoir for *B. burgdorferi s.s.* in the northeastern US and host for larval *I. scapularis* (48, 49, 49–52). In the upper Midwest, *P. leucopus* and *P. maniculatus* (deer mouse) are both major reservoirs and hosts. However, in California, the list of *Ixodes* hosts is long and several species are competent reservoirs for *B. burgdorferi s.l.*, including western grey squirrels (*Sciurus griseus*), California kangaroo rats (*Dipodomys californicus*), and dusky-footed wood rats (*Neotoma fuscipes*) (53–55). These species, not the *Peromyscus* spp. present, represent the primary reservoirs for *B. burgdorferi s.l.* in California. The primary hosts for larval and nymphal *I. pacificus*, however, are lizards, particularly the western fence lizard (*Sceloporus occidentalis*) and the southern alligator lizard (*Elgaria multicarinata*) (53). Both lizards are refractory hosts for *B. burgdorferi s.l.* Likewise, in Europe and Asia, there does not appear to be a single, predominant reservoir as a diverse population of rodents, small and medium mammals, and birds serve as adequate *B. burgdorferi s.l.* reservoirs and *Ixodes* hosts (56).

### *Life Cycle of Ornithodoros and Enzootic Cycle of Tick-Borne Relapsing Fever (TBRF) Borrelia*

Unlike *Ixodes, Ornithodoros* have a multi-year life cycle that is not dictated by climate (Fig. 1.3). Eggs hatch into larvae, which feed on their first host. *Ornithodoros* spp. are considered specialized feeders. Collectively, *Ornithodoros* parasitize myriad mammals, rodents, and birds. Each species, however, has preferred hosts and each individual *Ornithodoros* population

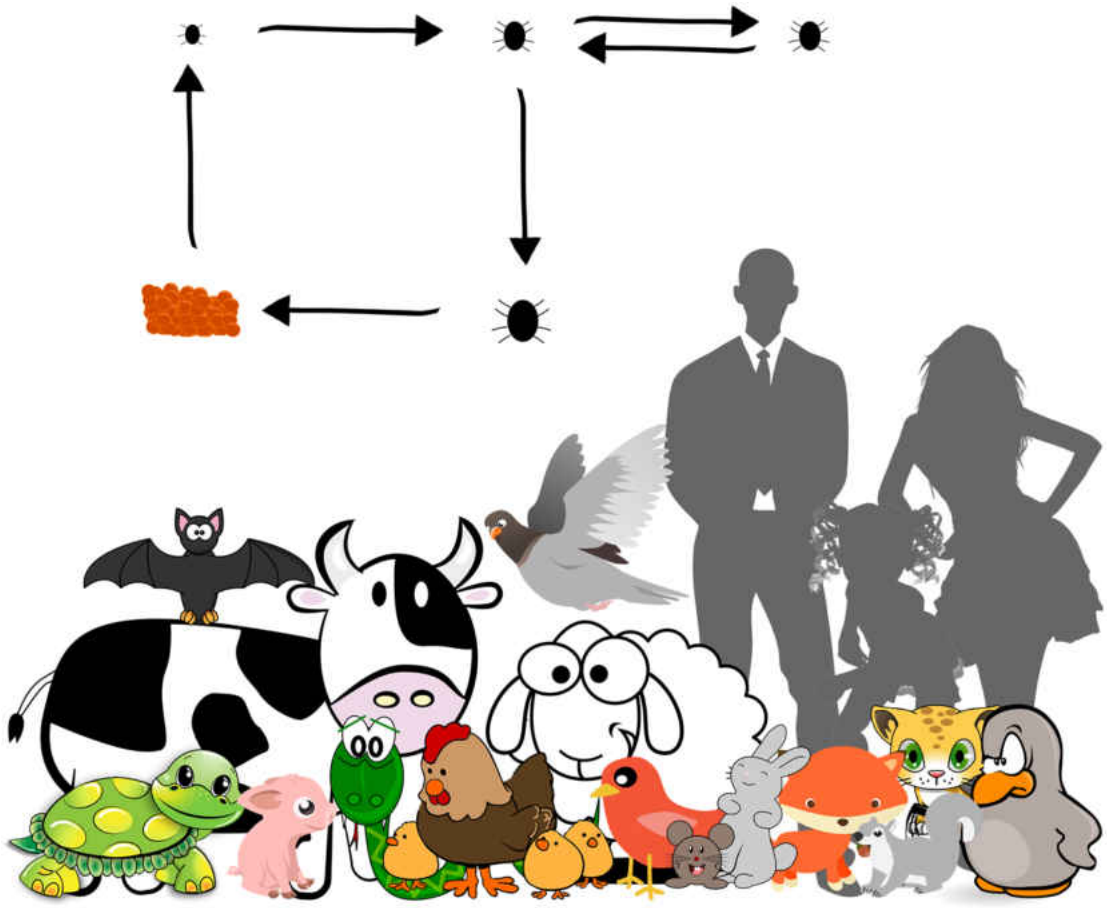


Figure 1. 3. Life cycle of *Ornithodoros* spp. With the exception of *Ornithodoros* eggs, each stage generally feeds upon the same host species and is capable of acquiring and transmitting relapsing fever *Borrelia*.

will be limited primarily to one host. After feeding, larvae detach and molt to nymphs. *Ornithodoros* spp. have multiple nymphal stages. Each nymphal stage will feed, detach, and molt to a new nymphal stage. After approximately seven nymphal stages, *Ornithodoros* will molt to an adult. In stark contrast to *Ixodes*, adult *Ornithodoros* can feed, without molting, several times. Eventually, adults will mate and lay eggs.

Tick-borne relapsing fever (TBRF) *Borrelia* carried by *Ornithodoros* are transovarially maintained. Thus, a tick can acquire *Borrelia* during any

feeding (if the host is infected) or from an infected adult female. As with *Ixodes*, humans and domestic animals can become incidental hosts for *Ornithodoros* and dead-end hosts for relapsing fever *Borrelia*.

### *Habitats and Behavior of Ixodes and Ornithodoros*

The geographic distribution of *Ixodes* spp. is governed by the distribution of hosts and limited by temperature and humidity, with ticks preferring environments with warm, humid summers and mild winters (57). While off-host *I. scapularis* are highly susceptible to desiccation and low temperature in laboratory experiments, established populations have been found in regions that experience frigid and dry winters (38, 58–60). This suggests that microclimates are invaluable for *I. scapularis* survival. Deciduous and mixed forests provide leaf litter that maintains high relative humidity and are regarded as the classic microhabitat for ticks. However, several temperate biomes, including coniferous forests, grasslands, and pastures also maintain microclimates that sustain *Ixodes* spp. (61–64) some urban, peri-urban, and recreational environments support or are capable of supporting *Ixodes* spp. and host populations (65–67).

*Ixodes* find hosts by questing. Ticks will crawl up vegetation, extend their front legs, and wait for something, hopefully, a tasty bloodmeal and not a researcher's collection cloth, to brush against the vegetation. The tick will attach and begin feeding. Desiccation is not a concern during feeding as ticks absorb water from the bloodmeal. If a suitable bloodmeal is not obtained and desiccation is a concern, *Ixodes* will crawl back to their humid habitat. *Ixodes* are long-term, slow feeders, remaining attached to a host for approximately

three to five days. Long attachment times, as well as the open environments *Ixodes* inhabit, facilitates dispersal of a population, especially if *Ixodes* attaches to a migrating mammal or bird.

*Ornithodoros*, in comparison, are found in more contained environments such as animal dens, burrows, and nests. As a result, *Ornithodoros* do not need to "hunt" for a bloodmeal and desiccation is less of a concern. *Ornithodoros* are short-term, quick feeders, able to attach, feed, and detach from a host in minutes to hours. Not surprisingly, dispersal of *Ornithodoros* populations is quite limited.

***Borrelia spp. Suitably Adapted to the Differences in Vector***

*B. burgdorferi s.l.* are found in the tick midgut. When an infected *Ixodes* feeds, *Borrelia* must sense a bloodmeal is occurring and begin migrating from the midgut to the salivary glands and into the feeding pit while shifting gene expression from tick-phase genes to mammalian-phase genes. This process can take time for the slow-replicating microbe and works quite well with *Ixodes*' long attachment time.

A feeding nymph is the last chance *B. burgdorferi s.l.* has to ensure survival of its genetic material since *B. burgdorferi s.l.* is not transovarially maintained, *Ixodes* feed once per life stage, adult males do not feed, and adult female *Ixodes* typically feed on deer, which are not competent reservoirs for *B. burgdorferi s.l.* (68). TBRF *Borrelia*, on the other hand, are found in the salivary glands of infected *Ornithodoros*, which allows for rapid transmission from tick to host (69). With nymph and adult *Ornithodoros*

capable of multiple feedings per life stage, TBRF *Borrelia* have ample opportunities for transmission to and from a host.

An important and intriguing, but not yet understood difference between *B. burgdorferi s.l.* and relapsing fever *Borrelia* involves vector specificity, exclusivity, and complementarity. Vector specificity occurs when serologically distinct bacterial strains have closely related vectors; vector exclusivity occurs when one vector species carries one bacterial strain; and vector complementarity occurs when one vector species carries multiple bacterial strains (70, 71). An unspoken assumption is that species of the *B. burgdorferi s.l.* complex are not vector specific or exclusive. That is, any species of the *B. burgdorferi s.l.* complex is assumed to be able to establish an infection in any *Ixodes* spp. While no experiments have shown exclusivity, numerous surveys have cataloged the species of the *B. burgdorferi s.l.* complex found in species of *Ixodes*. From these surveys and basic knowledge of evolution, it appears some *B. burgdorferi s.l.* species are exclusive. For example, *B. bavariensis* NT29, an Asian strain, is only found in *I. persulcatus*, an Asian species, while European *B. bavariensis* strains are only found in the European *I. ricinus* (72–74). Whether *B. bavariensis* demonstrates exclusivity or simple geographic isolation remains unknown. An experiment attempting to infect *I. ricinus* with an Asian strain of *B. bavariensis* is required. Other species of the *B. burgdorferi s.l.* complex appear to be highly promiscuous. *B. burgdorferi s.s.* has been found in *I. scapularis, I. pacificus*, *I. ricinus*, and *I. persulcatus*. There may be strain restrictions, such that *B. burgdorferi s.s.* B31 may not infect any species of *Ixodes* except *I. scapularis*. Still, the

degree of specificity or exclusivity that may be present with *B. burgdorferi s.l.* species pales in comparison to the exclusivity observed with relapsing fever *Borrelia*. To date, all relapsing fever *Borrelia* identified are exclusive to their respective vector, despite some significant overlap in geographic distribution for some *Ornithodoros* spp. (75). That is, *B. hermsii* cannot establish an infection in *O. turicata*; *B. hermsii* can only infect *O. hermsi*. The mechanism by which this exclusivity occurs is still not understood.

## Tick-Borne Spirochete Diseases

### *Lyme Disease*

Diseases caused by *Borrelia burgdorferi s.l.*, especially Lyme disease, are relatively new diseases to be described. The first recorded account of erythema migrans (EM), an inconsistent but distinguishing symptom of Lyme disease, was made in Europe in 1909 by Dr. Arvid Afzelius (76). While unconfirmed at the time, Dr. Afzelius suspected a tick bite (*I. ricinus*) was involved in the development of EM. Lyme disease went unrecognized in the US until an outbreak of juvenile rheumatoid arthritis occurred in Connecticut in 1977 (77). By 1982, Dr. Willy Burgdorfer had isolated spirochetes from *I. scapularis* (*I. dammini*) and shown antibodies to *B. burgdorferi* reacted strongly with sera from Lyme disease patients (4).

Lyme disease is the top tick-borne disease reported to the Centers for Disease Control and Prevention (CDC), with 38,069 confirmed and probable cases reported in 2015. However, in 2012, the CDC released estimates indicating the number of people infected with Lyme disease is underreported

by about 10% and the actual number of cases in the US is closer to 300,000 (78).

Symptoms of Lyme disease in humans vary by species and shows a distinct geographical pattern, though some symptoms are universal (Table 1.2) (79, 80). In North America, where the primary etiological agent is *B. burgdorferi s.s.*, early stage Lyme disease (3-30 days post-infection) is characterized by influenza-like symptoms (e.g. mild fever, malaise, myalgia/arthralgia) and an associated, though not guaranteed, bullseye rash (erythema migrans). Arthritis is a key symptom of late stage Lyme disease (after 30 days post-infection). Lyme neuroborreliosis and carditis are also late stage Lyme symptoms in the US, though the prevalence is much lower compared to arthritis. In Europe, however, late-stage symptoms are more diverse because there are more pathogenic *Borrelia* spp. present. *B. burgdorferi s.s.* is found in Europe but is not a predominant strain. Thus, Lyme arthritis does occur but at significantly lower rates compared to North America. Acrodermatitis chronica atrophicans is a common late stage symptom caused by *B. afzelii*. Neurological symptoms (Lyme neuroborreliosis) characterized by numbness, Bell's palsy, neck stiffness, declining memory, and sleep disorders are also common late-stage symptoms associated with *B. bavariensis* infection. Infection with *B. burgdorferi s.l.* is not self-resolving and does require treatment, usually a course of ceftriaxone or doxycycline (81).

Table 1. 2. Clinical symptoms of human *Borrelia* diseases.

| Disease | Clinical symptom(s) |
|---|---|
| Lyme disease | Symptom onset after exposure: early stage generally 3-30 days<br>–Influenza-like (e.g. mild fever, malaise, myalgia/arthralgia; *B. burgdorferi s.s.*)<br>–Erythema migrans (*B. burgdorferi s.s.*, *B. afzelii*)<br>–Symptom onset after exposure: late stage generally > 30 days<br>–Arthritis<br>–Acrodermatitis chronica atrophicans (*B. afzelii*)<br>–Neurological (Lyme neuroborreliosis, e.g. numbness, Bell's palsy, stiffness of neck, declining memory, sleep disorders; *B. burgdorferi s.s.*, *B. bavariensis*) |
| Tick-borne relapsing fever | Symptom onset: ca. 7 days<br>–Influenza-like<br>–Recurring high fever<br>–Headache<br>–Myalgia<br>–Arthritis<br><br>Approximately 3-10 febrile episodes (relapses) occur; mortality rates are variable but generally less than 5% |
| Hard tick-borne relapsing fever/*Borrelia miyamotoi* disease | Symptom onset after exposure: ca. 15 days (82)<br>–Influenza-like<br>–Most common:<br>–Fever<br>–Malaise<br>–Headache<br>–Chills<br>–Arthritis/arthralgia<br>–Meningoencephalitis (immunocompromised patients)<br><br>Rare (less than 10% of patients):<br>–Rash/Erythema migrans<br>–Gastrointestinal (e.g. vomiting, nausea, diarrhea)<br>–Cardiac/respiratory (shortness of breath)<br>–Neurological (e.g. dizziness, confusion)<br>–Stiffness of neck |
| Louse-borne relapsing fever | Symptom onset after exposure: ca. 4-8 days<br>–Recurring high fever<br>–Malaise<br>–Headache<br>–Chills<br>–Meningism<br>–Myalgia<br>–Nausea<br>–Vomiting<br><br>Approximately 3-5 relapses occur; mortality rate varies greatly (30-70% without treatment during outbreaks) |

### *Tick-Borne Relapsing Fever*

Unlike Lyme disease, TBRF is not nationally reportable. TBRF is, however, reportable in 11 endemic states (California, Washington, Colorado, Idaho, Nevada, Oregon, Arizona, Texas, New Mexico, Montana, Utah, Wyoming) and one bordering state (North Dakota, note no TBRF cases have been reported in North Dakota). Approximately 70% of TBRF cases are reported in California, Washington, and Colorado. Data collected from these 12 states between 1990 and 2011 indicate 504 cases of TBRF have been reported (83).

There is less diversity in the symptoms of TBRF in humans. Influenza-like symptoms appear approximately seven days post-infection (Table 1.2). The defining characteristic of TBRF is recurring spirochetemia events leading to febrile episodes. TBRF can be fatal (mortality rate is generally less than 5%) but is usually self-resolving after approximately three to ten recurring febrile episodes. Louse-borne relapsing fever (*B. recurrentis*) has similar symptoms though approximately three to five febrile episodes occur and mortality can be much higher (30-70% without treatment during outbreaks).

### *Hard Tick-Borne Relapsing Fever or Borrelia miyamotoi Disease*

Hard tick-borne relapsing fever (HTBRF) or *Borrelia miyamotoi* disease (BMD) is an emerging disease first described in 2011 (82). Numerous cases have been documented for Lyme disease and TBRF allowing for a defined set of typical symptoms. Much of the data currently available for *B. miyamotoi* infections come from retrospective serological analyses of banked patient samples, which provide valuable epidemiological information but can lack the

detailed patient history or clinical aspects required to sufficiently define a disease. Two large studies in Russia (82) and the US (84) suggest HTBRF is a disease similar to TBRF.

The Russian patients described by Platonov et al (82) reported tick bites, developed moderate or severe disease, and were hospitalized as a precautionary measure against more severe tick-borne diseases, particularly viral tick-bone encephalitis. Forty-six patients were classified as having a confirmed *B. miyamotoi* infection with no detected current *B. burgdorferi s.l.* coinfection by PCR though all patient sera reacted with whole cell lysates of *B. burgdorferi s.s., B. afzelii,* and *B. garinii*. The most common symptoms were fever, headache, and malaise or fatigue (Table 1.3). Five patients reported recurrent fever with an average duration of 3.4 days, and 9 days between relapses, a similar timeline for TBRF. All patients were successfully treated with ceftriaxone or doxycycline.

Table 1. 3. Comparison of *B. miyamotoi* symptoms reported from US (84) and Russian (82) patients

| Symptom | US (*n* = 51) | Russia (*n* = 46) |
|---|---|---|
| Fever, chills | 96% | 96%, 35% [a] |
| Headache | 96% [b] | 89% |
| Myalgia | 84% | 59% |
| Arthralgia | 76% | 28% |
| Malaise/fatigue | 82% | 98% |
| Rash/EM [c] | 8% | 9% |
| Gastrointestinal [d] | 6% | 30% (nausea)<br>7% (vomiting) |
| Respiratory [e] | 6% | na [f] |
| Neurological (dizziness, confusion, vertigo) | 8% | na |
| Stiff neck | na | 2% |

[a] Fever and chills were reported in separate categories
[b] Authors noted in most patients the headaches were severe
[c] US patients were described as having a rash. Russian patients were noted for having a single erythema migrans
[d] For US patients, GI symptoms included nausea, abdominal pain, diarrhea, anorexia. For Russian patients, GI symptoms included nausea and vomiting
[e] Labored breathing or shortness of breath
[f] Not reported

In the US, 97 of 11.515 patient samples submitted by clinical laboratories for tick-borne disease analysis were PCR-positive for *B. miyamotoi* (84). Patients with known or suspected *B. burgdorferi s.s.* coinfection or a history of Lyme disease were omitted from further analysis. Fever, headache, and malaise were commonly reported among US patients with two patients reporting recurrent fever (Table 1.2). The duration of febrile episodes and the time between relapses were not reported. Spirochetemia was noted in US patients but either not reported or documented in Russian patients. Strikingly, a rash or single erythema migrans of unknown origin, a symptom associated with *B. burgdorferi s.l.* infections, was reported in 8 and 9% of US and Russian patients, respectively.

In addition, some symptoms were different between US and Russia patients, which suggest clinical manifestations vary by *B. miyamotoi* strain, similar to that seen with *B. burgdorferi s.l.* strains (Table 1.2) (80). Arthralgia was more common in US (76%) compared to Russian patients (28%), and leukopenia, thrombocytopenia, and elevated liver enzymes were found in some US patients but none of the Russian patients. These differences may be explained by genetic differences between American and Asian type *B. miyamotoi*. Genetic analyses of *B. miyamotoi* isolates reveal heterogeneity between, and a high degree of homology among, strains from the US (American types; *I. scapularis, I. pacificus*), Europe (European type; *I. ricinus*), and Asia (Asian type; *I. persulcatus*) (85, 86).

Detailed case reports are currently available for nine patients in the US, Europe, and Japan. For immunocompetent patients, symptoms were similar to those observed in the aforementioned studies (e.g. fever, headache, malaise) (87–91). One US patient did not seek treatment, providing additional evidence that *B. miyamotoi* can result in recurrent fever and be self-resolving, similar to other relapsing fever infections (92, 93). This patient experienced two episodes of fever separated by three weeks, significantly longer than in other *B. miyamotoi* or relapsing fever patients, with each episode lasting 4-5 days, on par with *B. miyamotoi* or relapsing fever patients.

The pathology of *B. miyamotoi* infection is dramatically different in immunocompromised patients, specifically, those treated for non-Hodgkin's lymphoma (NHL) treated with rituximab. Two patients treated with rituximab

for NHL, one from the US (94) and one from the Netherlands (95), with reported recent tick bites developed meningoencephalitis. Motile spirochetes were detected in cerebral spinal fluid in both cases. Interestingly, *glpQ* (glyphosphodiesterase; among *Borrelia*, *glpQ* is unique to relapsing fever *Borrelia*) was amplified and sequenced from both patient's samples yet no anti-GlpQ antibodies were detected in the blood or cerebral spinal fluid of the European patient. IgM against *B. burgdorferi* was negative for both patients. Neither patient reported any of the commonly associated symptoms of a *B. miyamotoi* infection (e.g. fever, headache, myalgia, malaise). Instead, both patients exhibited neurological symptoms (cognitive processing defects, disturbed gait). A third patient from Germany, also with treated with rituximab for NHL developed Lyme neuroborreliosis-like symptoms (dizziness, vomiting, and headache) (96).

### *General Immune Responses and How Borrelia Deal with Them*

To establish long-term infection in rodents and mammals (late or disseminated phase of Lyme disease in humans), *B. burgdorferi s.l.* migrates to various sites, such as joints, skin, heart, and bladder (97–105). These sites offer much protection from cells of the immune system, however, *Borrelia* has not unlocked the mysteries of teleportation. As a result, *B. burgdorferi s.l.* must migrate to these sites from the inoculation site (tick bite) and will encounter immune cells producing complement and a humoral immune response. *Ixodes* saliva contains adaptive and innate immunomodulatory and anti-complement proteins that offer some protection for *B. burgdorferi s.l.* (106–113). *B. burgdorferi s.l.* possess a handful of anti-

complement proteins with the best understood being factor H binding proteins (114–117). The ability to bind and neutralize complement through binding factor H is vital for *B. burgdorferi s.l.* to establish infection (114).

Relapsing fever *Borrelia* migrate from the inoculation site to the blood where they remain until cleared by the immune system. *Ornithodoros* salivary gland extracts also possess proteins that inhibit the host immune response (118–121). Despite possessing factor H binding proteins, complement is not an effective method for relapsing fever *Borrelia* to establish infection, meaning complement is not the primary mechanism to clear relapsing fever *Borrelia* (122, 123). Instead, the primary mechanism for clearance is an humoral response.

The humoral response is important for controlling and preventing both *B. burgdorferi s.l.* and relapsing fever *Borrelia* infections (124–131). Indeed, these responses form the basis of an intense research effort for effective Lyme vaccines. Fortunately for *Borrelia*, they are quite adept at evading the host humoral response primarily through variation of surface-exposed proteins. Lyme disease *Borrelia* possess outer surface proteins (Osps) and variable membrane protein-like (Vls) proteins, while relapsing fever *Borrelia* possess variable membrane proteins (Vmps; include variable large and variable small proteins) (132–136).

### *Osps and VlsE of Lyme Borrelia*

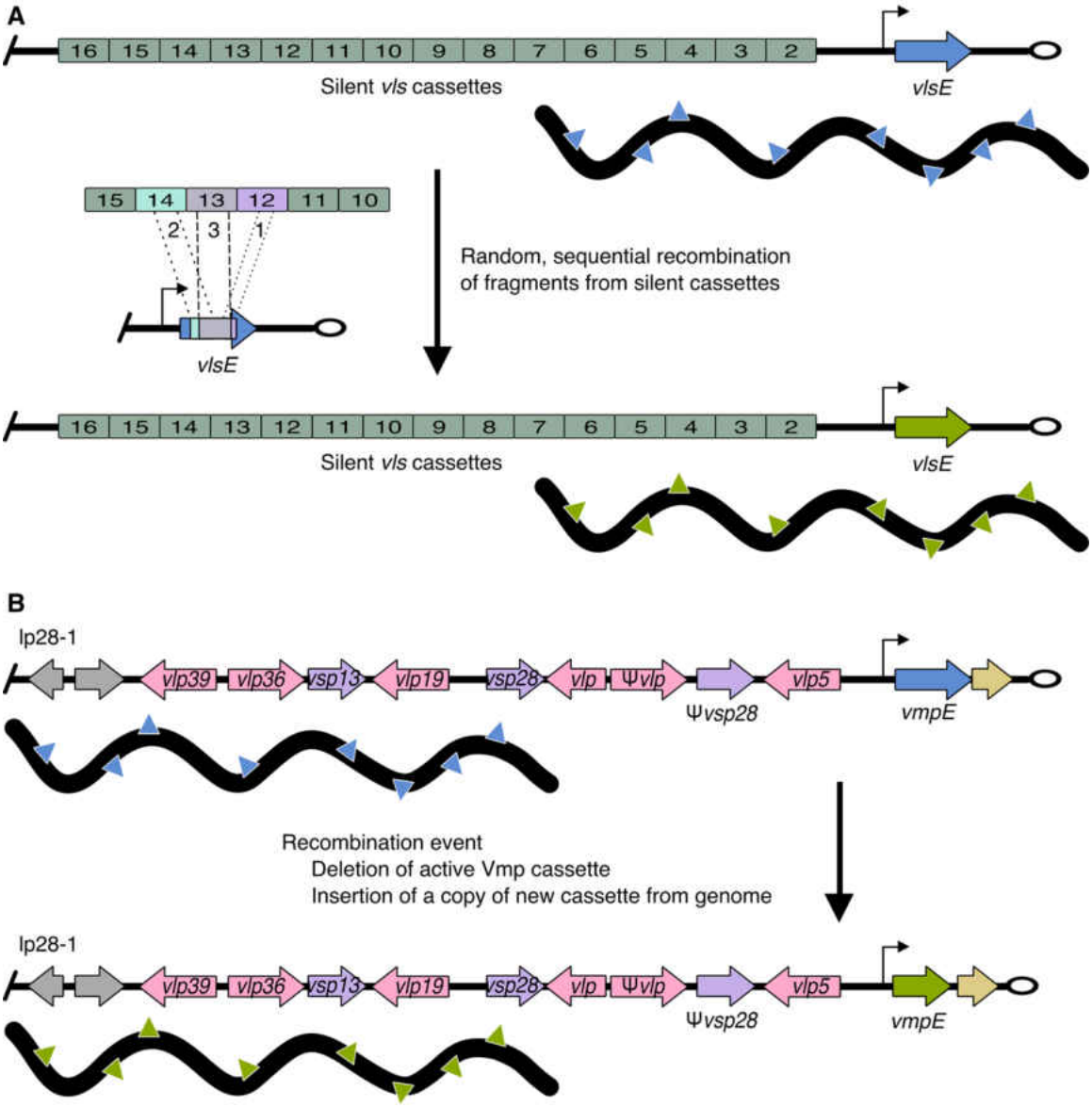
The outer surface proteins (Osps), particularly OspC, are one of the most studied groups of *Borrelia* proteins (117, 137, 138). ErpA (OspE) and OspF are factor H-binding proteins. OspA, a protein predominantly involved

in uptake and survival in tick, is immunogenic and able to block antibody binding to another surface-exposed protein, P66 (139, 140). OspC has diverse roles, many of which are essential for transmission from *Ixodes* and establishing infection in mammals (141–148). These studies were key in demonstrating that *ospC* is upregulated during the early stages of infection, downregulated after infection has been established, and deleting or overexpressing *ospC* results in spirochetes that are quickly cleared from a host.

A handful of immune evasion functions have been identified for OspC. OspC protects *Borrelia* by binding Salp15, an *Ixodes* salivary protein. OspC also prevents phagocytosis by macrophages (148). In addition, several OspC types have been identified and correlated with a strains ability to establish infection in hosts and reservoirs (98, 99, 149–152). However, as each Osp is present as a single-copy locus, genetic variation is seen at the population level. That is, outside of random mutation or horizontal gene transfer events, a single spirochete cannot produce different OspC types *in situ*.

In contrast, the Vls (Vmp-like sequences) system can change the expressed surface antigen *in situ* (Fig. 1.4). Antigenic recombination of VlsE is important in maintaining infection in mammals and helps Lyme *Borrelia* evade the humoral immune response (153–166). The Vls system is composed of approximately 16 *vls* cassettes (the exact number varies by strain) and one expression locus, *vlsE*. All of the identified *vls* cassettes are located on the same plasmid (lp28-1) in close proximity to but in the opposite direction of *vlsE*. Expression at *vlsE* occurs through the random

Figure 1. 4. Antigenic variation of Lyme borreliae VlsE and relapsing fever borreliae Vmp systems. (A) VlsE. The expression locus (vlsE) is located near the telomere of linear plasmid (lp) 28-1 (blue or green arrow, promoter is indicated by a black arrow). Silent *vls* cassettes are located upstream and in the opposite orientation of *vlsE*. Antigenic variation occurs through the random and sequential insertion of silent cassette fragments (labeled 1, 2, and 3). (B) *vlp* (pink arrows) and *vsp* (purple arrows) cassettes are located throughout the genome on lp28-1, 28-2, 28-3, 28-4, and 32-1. The expression locus (blue or green arrow, promoter is indicated by a black arrow) is found on lp28-1 near the telomere (open oval). Changing the expressed Vmp cassette is achieved through deletion of the current cassette (blue arrow) followed by insertion of a copy of a new cassette (green arrow via recombination events) resulting in a change in the expressed Vmp on the surface of the bacterium (denoted by blue or green triangles, respectively). Grey arrows indicate non-Vmp ORFs; tan arrows indicate downstream homology sequences (DHS, sequences found throughout the genome and required for mapping recombination events at the Vmp expression locus).


A
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2
Silent vls cassettes
vlsE
15 14 13 12 11 10
2 3 1
vlsE
Random, sequential recombination
of fragments from silent cassettes
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2
Silent vls cassettes
vlsE
B
lp28-1
vlp39 vlp36 vsp13 vlp19 vsp28 vlp Ψvlp Ψvsp28 vlp5 vmpE
Recombination event
Deletion of active Vmp cassette
Insertion of a copy of new cassette from genome
lp28-1
vlp39 vlp36 vsp13 vlp19 vsp28 vlp Ψvlp Ψvsp28 vlp5 vmpE

recombination of segments of multiple *vls* cassettes rather than recombination of an entire, single *vls* cassette. Thus, recombination events result in thousands of unique VlsE variants, all approximately 36-kD.

### *Vmps of Relapsing Fever Borrelia*

Vmps, a system similar to Vls, are one of the best characterized immune evasion mechanisms (132, 167–170). *B. hermsii* has approximately 60 unique and promoterless *vmp* cassettes (i.e. silent cassettes) scattered throughout its genome and one promoter-driven *vmp* expression locus (Fig. 1.4). A single *vmp* cassette is expressed when the entire cassette is moved to the expression locus.

The majority of spirochetes are cleared from the host through specific anti-Vmp IgM antibodies raised against the predominantly expressed Vmps, which results in a significant decrease in spirochete load (from approximately $10^5$-$10^7$ spirochetes/mL to < $10^4$ spirochetes/mL). The remaining spirochetes consist of a small population expressing different cassettes. Since the host has not raised a strong antibody response to these non-dominantly expressed Vmps, this minority population of spirochetes can proliferate to high concentrations until an antibody response is mounted and the majority of spirochetes are once again cleared. This cycle of *vmp* conversion, peaking spirochete loads, and antibody-mediated clearing repeats a minimum of two times resulting in the characteristic symptoms of a relapsing fever illness.

### *Mechanisms of Immune Evasion by B. miyamotoi*

Given the genetic similarity of *B. miyamotoi* to relapsing fever spirochetes, it is likely *B. miyamotoi* utilizes some homologous mechanisms

to evade host immune responses. While *B. miyamotoi* is resistant to complement *in vitro* (171, 172), complement inactivation is not required for relapsing fever spirochetes to establish infection. OspE homologues have been identified in *B. miyamotoi* FR64b (isolated from the blood of *A. argenteus*) however, McDowell et al. were unable to demonstrate binding of factor H (115). We observed factor H binding using whole-cell *B. miyamotoi* FR64b lysates and Röttgerding et al. (173) showed *B. miyamotoi* HT31 expresses a functional FH-binding protein. CbiA, which binds FH *in vitro*. While the role of FH-binding remains unknown, it appears, as is the case for relapsing fever spirochetes, inactivation of complement may not be required to resolve spirochetemia during infection with *B. miyamotoi* (126, 174).

Instead, *B. miyamotoi* likely utilizes a Vmp system (175, 176), which relies on antigenic variation of Vmp. C3H/HeN mice infected with *B. miyamotoi* LB-2001 produced anti-Vsp1 IgM and IgG antibodies that were effective in clearing the initial spirochetemic peak of *B. miyamotoi* from SCID mice (176). Despite this clearing, a second spirochetemic relapse occurred. Analyses of the secondary *B. miyamotoi* population revealed expression of *vlpC2*, not *vsp1*, as would be expected in the case of antigenic variation. They also noted *vlpC2* was present in the initial *B. miyamotoi* population in a much lower prevalence compared to *vsp1*.

## Problem

*B. burgdorferi s.l.* has had a significant impact on human health of both immunocompetent and immunocompromised individuals and Lyme disease is expected, at a minimum, to maintain its status as a public health

threat. The effect *B. miyamotoi* has had and will have remains unknown, though there is a clear impact on immunocompromised individuals that is cause for concern.

Despite both *B. burgdorferi s.l.* and *B. miyamotoi* being demonstrated human pathogens, several essential questions remain unanswered. Both possess uncharacterized genes, which prevent a comprehensive understanding of the physiology, pathogenesis, prevention, and treatment of Lyme disease and HTBRF. While we have a decent understanding of the enzootic maintenance of *B. burgdorferi s.s.* in the northeastern US, we are still filling in large holes in the enzootic maintenance of *B. burgdorferi s.l.* in other areas of North America. There are more unknowns regarding the enzootic maintenance of *B. miyamotoi*.

During my graduate career, I attempted to address some basic questions regarding *B. burgdorferi s.s.* and *B. miyamotoi*. Specifically, 1. The enzootic maintenance of *B. burgdorferi* in North Dakota, 2. The physiological role of proteins in the survival and pathogenesis of *B. burgdorferi*, and 3. The enzootic maintenance of *B. miyamotoi*.

# CHAPTER 2

# THE WESTERN PROGRESSION OF LYME DISEASE: INFECTIOUS AND NON-CLONAL *BORRELIA BURGDORFERI SENSU LATO* POPULATIONS IN GRAND FORKS COUNTY, NORTH DAKOTA

## Introduction

Eastern North Dakota borders some Minnesota counties (i.e., Kittson, Marshall, Polk, and Norman) where the risk of contracting the tick-borne diseases Lyme disease or human granulocytic anaplasmosis is moderate to high based on confirmed human cases (177, 178), the abundance of *Borrelia burgdorferi*-positive *Ixodes scapularis* (179), and the density of nymphal *I. scapularis* (177). Despite this close geographical proximity, there has been a paucity of studies on the migration of *I. scapularis* or *B. burgdorferi* into North Dakota (38). Eastern North Dakota is classified as a transition zone for Lyme disease based on studies investigating the expansion of *I. scapularis* and *B. burgdorferi* in the Midwest (179). However, in 2011, the North Dakota Department of Health reported results of a 2010 survey showing established *I. scapularis* populations in six eastern North Dakota counties, including Grand Forks county (180). In addition, the pathogens *Anaplasma phagocytophilum, Babesia* sp., and *B. burgdorferi* were detected in *I. scapularis* via PCR (180).

Surveillance of *I. scapularis* has shown an increase in its geographic distribution (181–183), which has been accompanied by a concomitant

increase in the distribution of confirmed Lyme disease cases (184). The primary causative agents of Lyme disease differ between the US and Europe with *B. burgdorferi sensu stricto* being the primary agent in the US and *B. afzelli* and *B. garinii* being the primary agents in Europe (185, 186). While it is generally accepted that *B. burgdorferi s.l.* is the sole cause of Lyme disease in the US, there is increasing evidence that other members of the *B. burgdorferi sensu lato* complex, a group consisting of approximately 20 closely related species, could also cause Lyme disease in the US (4–16). For example, *B. bissettii*, has been identified in DNA isolated from human sera samples in California residents by sequence analysis of *p66*; however, it has not been clearly associated with Lyme disease in the US because many of the samples that were positive for *B. bissettii* were also positive for *B. burgdorferi* (187). It has been demonstrated, however, to be associated with Lyme disease in Europe (185, 188) and has been shown to be infectious and pathogenic in a mouse model (189). *B. americana*, found in *I. pacificus* and *I. minor*, is predominantly found in California and South Carolina and has not yet been associated with Lyme disease in humans (185), but antibodies to *B. americana* have reportedly been detected in blood from Lyme disease patients (190). These data underscore the need to correctly identify newly isolated *Borrelia* species in order to assess their potential contribution to human disease.

Several schemes have emerged to classify presumptive Lyme disease *Borrelia*. One is based on outer surface protein C (OspC), a protein expressed only during transmission from vector to host (141, 144) that is required for

*B. burgdorferi* to infect mammals (143, 146). While *ospC* is highly polymorphic, many groups or types have been described (98, 149). OspC types are commonly used to determine a strains ability to cause disseminated infections in humans (98, 191, 192). OspC groups A-U have been identified (98, 149) with A-O, T, and U found in North America (98, 99, 191). Groups A, B, I, K, and N have been found most commonly associated with disseminated infections in humans (98, 152, 193). Groups C, D, E, F, G, H, and M have also been found capable of causing disseminated infection in humans, although the occurrence of infection with these types is much lower (151, 193). *B. burgdorferi* small mammal reservoirs have been found to carry specific OspC types (150). For example, it has previously been shown *Peromyscus leucopus* (white-footed mouse) tested positive for groups A, B, D, F, G, I, and K, while *Tamias striatus* (eastern chipmunk) tested positive for groups A, D, F, G, I, K, T, and U (150).

Antigenic outer surface proteins (e.g., OspC) are highly variable and there are indications they are subject to horizontal gene transfer (194–196), which make them less than ideal candidates for evaluating evolutionary history and geographical relationships of *B. burgdorferi* strains. Multilocus sequence typing (MLST) schemes have been developed that have proven to be the most reliable method for determining the history and relationship within many bacterial genera and species, including *B. burgdorferi s.l.* strains (197, 198). MLST is based on the analysis of housekeeping genes, which are under strong pressure to minimize large-scale mutation events, such as those events seen in outer surface proteins. The *B. burgdorferi* MLST scheme

utilizes eight housekeeping genes: *clpA, clpX, nifS, pepX, pyrG, rplB, recG,* and *uvrA* (198). Each gene is assigned an allele number based on the sequence identity to previously submitted alleles. The profile produced from all eight loci corresponds to a sequence type (ST), which can be used to compare strains to determine evolutionary history and relationships.

With the discovery of *I. scapularis* and *B. burgdorferi* in eastern North Dakota, we sought to characterize previously unidentified spirochete cultures obtained from five hearts of *Peromyscus* spp. (deer mice) and *Myodes gapperi* (Southern red-backed vole) trapped in the Turtle and Forest River areas of eastern North Dakota. All five small mammals harbored unique or non-clonal populations of spirochetes determined to be *B. burgdorferi s.l*. The presence of non-clonal populations is significant as it indicates the *B. burgdorferi* populations present in eastern North Dakota are not the result of a recent or single migration event. Two of the five small mammals were carrying at least two different OspC types. OspC typing showed the infectious *B. burgdorferi* types A, B, E, F, and I are disproportionately represented in our samples. However, one isolate typed to the non-infectious group L and one typed to none of the previously described groups. *B. burgdorferi* M3 (*ospC* group B) was obtained from *M. gapperi* at Forest River and predicted to be a highly infectious isolate. Using this isolate, we determined *B. burgdorferi* M3 is infectious in laboratory mice via both artificial and natural routes of exposure, culturable from mouse tissues, and survives *I. scapularis* molting. These data confirm *B. burgdorferi* is present in eastern North Dakota and is infectious and transmissible in a laboratory model.

## Materials and Methods

### *Animal Care and Use*

Infection experiments were performed per a University of North Dakota Institutional Animal Care and Use (UND IACUC) approved protocol #1101-2 at the University of North Dakota Center for Biomedical Research. Four to six week-old BALB/c mice (Harlan; Madison, WI) were cared for in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines (Animal Welfare Assurance A3917-01) and the National Research Council of the National Academics *Guide for the Care and Use of Laboratory Animals* (8th edition). Wild rodents were collected, euthanized, and necropsied in the field as described in the UND IACUC approved protocol #1304-3. All efforts were made to minimize animal suffering.

### *Sample Collection and Culturing Spirochetes*

Live trapping of rodents was conducted during June and July 2012 in the two largest forested areas within an otherwise agricultural landscape in Grand Forks County: i.e., Turtle River State Park (47.94°N, -97.50°W; ca. 254 hectares) and Forest River Biological Station and Wildlife Management Area (48.17°N, -97.66°W; ca 349 hectares). Sherman live traps (H.B. Sherman Traps; Tallahassee, Florida) were baited with peanut butter and oatmeal, supplied with cotton bedding, set in the evening, and recovered in the morning. Captured mammals were identified as *M. gapperi* and *Peromyscus* spp. based on morphological characteristics. This method makes it difficult to identify *Peromyscus* species in the field; thus, *Peromyscus* were

identified only to the genus level. However, all of the *Peromyscus* in this study were trapped deep in deciduous forests, a preferred habitat for *P. leucopus*, while *P. maniculatus* prefer more open terrain and/or coniferous forest, strongly suggesting the captured *Peromyscus* were *P. leucopus* (Robert Seabloom, personal communication; (199). *Peromyscus* spp. and *M. gapperi* were euthanized with isoflurane, necropsied, and hearts immediately inoculated into modified Barbour-Stoenner-Kelly (BSK-II) medium containing 6% rabbit serum and 50 µg/mL rifampin (200). Surgical tools were sterilized with 95-99% ethanol prior to each necropsy to prevent possible cross-contamination between animals. Three days later, uncontaminated cultures were blind passed into modified BSK-II with 6% rabbit serum without rifampin, incubated for three additional days, then examined for spirochetes via dark field microscopy.

### *Amplification and Sequencing of ospA, ospC, flaB, 16S, 16S-ile tRNA IGS, and p66*

*ospA, ospC, p66,* and the 16S-*ile* tRNA IGS were obtained using amplification conditions previously described (201), except the annealing temperature was adjusted to 48°C (see Table 2.1 for primers used). 16S and *flaB* were amplified using the following conditions: 1 cycle 94°C for 5 min, 40 cycles of 94°C for 30 sec, 50°C for 30 sec, 68°C for 1 min, 1 cycle 72°C for 5 min. Sequencing was performed at Davis Sequencing (Davis, California). Chromatograms were visually inspected then the forward (coding) and reverse (template) strand sequences were aligned to obtain a double-stranded consensus sequence. 16S sequences were queried using the Ribosomal Database Project's Sequence Match (Seqmatch) program (202)

Table 2. 1. Primer sequences used in this study and the predicted amplicon sizes. Primers obtained from other sources are indicated after primer name.

| **Primer name** | **Sequence (5'-3')** | **Predicted amplicon size in nucleotides[a]** |
|---|---|---|
| 16S For | GGT CAA GAC TGA CGC TGA GTC A | 136 nt |
| 16S Rev | GGC GGC ACA CTT AAC ACG TTA G | |
| *flaB* For | GGG TCT CAA GCG TCT TGG | 139 nt |
| flaB Rev | GAA CCG GTG CAG CCT GAG | |
| ospA For | ATG AAA AAA TAT TTA TTG GGA ATA GG | 829 nt |
| ospA Rev | ATT CTC CTT ATT TTA AAG CG | |
| ospC For (200) | ATG AAA AAG AAT ACA TTA AGT GC | 638 nt |
| ospC Rev | CTT AAT TAA GGT TTT TTT GG | |
| p66 For | GAT TTT TCT ATA TTT GGA CAC AT | 755 nt |
| p66 Rev | TGT AAA TCT TAT TAG TTT TTC AAG | |
| 16S IGS For | AGT GCG GCT GGA TCA CCT CC | 950 nt |
| ileT IGS Rev (200) | GTC TGA TAA ACC TGA GGT CGG A | |
| nid1 For | CCA GCC ACA GAA TAC CAT CC | 153 nt |
| *nid1* Rev | GGA CAT ACT CTG CTG CCA TC | |
| *recA* For | GTG GAT CTA TTG TAT TAG ATG AGG CT | 171 nt |
| *recA* Rev | GCC AAA GTT CTG CAA CAT TAA CAC CT | |
| *I. scap* 16S For | CGG TCT GAA CTC AGA TCA AG | 300 nt |
| *I. scap* 16S Rev | GGG ACA AGA AGA CCC TAT C | |
| MLST primers for amplification and sequencing from borrelia.mlst.net/misc/info.asp | | |
| *clpA* For | AAA GAT AGA TTT CTT CCA GAC | 982 nt |
| *clpA* Rev | GAA TTT CAT CTA TTA AAA GCT TTC | |
| *clpX* For | GCT GCA GAG ATG AAT GTG CC | 884 nt |
| *clpX* Rev | GAT TGA TTT CAT ATA ACT CTT TTG | |
| *nifS* For | ATG GAT TTC AAA CAA ATA AAA AG | 1049 nt |
| *nifS* Rev | GAT ATT ATT GAA TTT CTT TTA AG | |
| *pepX* For | ACA GAG ACT TAA GCT TAG CAG | 811 nt |
| *pepX* Rev | GTT CCA ATG TCA ATA GTT TC | |
| *pyrG* For | GAT TGC AAG TTC TGA GAA TA | 801 nt |
| *pyrG* Rec | CAA ACA TTA CGA GCA AAT TC | |
| *recG* For | CCC TTG TTG CCT TGC TTT C | 805 nt |
| recG Rev | GAA AGT CCA AAA CGC TCA G | |
| rplB For | TGG GTA TTA AGA CTT ATA AGC | 760 nt |
| rplB Rev | GCT GTC CCC AAG GAG ACA | |
| uvrA For | GAA ATT TTA AAG GAA ATT AAA AGT AG | 911 nt |
| uvrA Rev | CAA GGA ACA AAA ACA TCT GG | |

[a] Predicted amplicon size was determined using the following respective accession versions: *B. burgdorferi* B31 AE000783.1 (chromosome), AE000790.2 (lp54), AE000792.1 (cp26); Mouse: NC_000079.6 (nid1).

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and the Nucleotide Collection (nr/nt) BLAST database (blast.ncbi.nlm.nih.gov) to obtain genus and species identifications. *flaB, ospA, ospC, p66,* and the IGS region nucleotide sequences were queried using BLAST.

### *NCBI Nuccore and BLAST Database Searches*

Sequences were obtained by searching the NCBI nuccore database (www.ncbi.nlm.nih.gov/nuccore) using the following terms: *Borrelia* + *p66*; *Borrelia* + *ospC*; *Borrelia* + "outer surface protein C;" *Borrelia* + *ospA*; *Borrelia* + "outer surface protein A;" *Borrelia* + "intergenic spacer region;" *Borrelia* + IGS; *Borrelia* + 16S + 23S + IGS; *Borrelia* + 16S + 23S + "intergenic spacer region." A search was performed in the non-redundant protein sequence (nr) BLAST database using the complete *B. burgdorferi* B31 protein sequences for p66 (chromosome accession: NC_001318.1), OspC (cp26 accession: NC_001903.1), and OspA (lp54 accession: NC_001857.2).

### *Alignments and Phylogeny*

Sequences for *ospA, ospC, p66,* and the IGS regions from eastern North Dakota isolates were aligned along with BLAST and NCBI database sequences in ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/). The shaded alignment was generated using BoxShade (ExPASy, www.ch.embnet.org/software/BOX_form.html). Duplicate sequences (identified as the same species and found to be 100% identical) were represented in the analyses by a single sequence. Sequences obtained from BLAST were included in analyses if the query coverage was greater than 90%. Obtained sequences were manually trimmed to conserved regions

aligning to the shortest sequence obtained for the eastern North Dakota isolates. Trimmed sequences meeting the above criteria were then used in phylogenetic analyses. For OspA and OspC, a subset of sequences from each clade was chosen to create representative phylogenetic trees. Final phylogenetic analyses for OspA, OspC, and p66 were performed using the PHYLIP programs SeqBoot, Proml, and Consense (Version 3.695, http://evolution.genetics.washington.edu/phylip/). Briefly, sequence files were put into SeqBoot and analyzed with1000 bootstrap replicates. The SeqBoot output file was analyzed in Proml using the Jones-Taylor-Thornton model with multiple data sets, slower analysis, a random number seed of 9, data sets jumbled 5 times, and an outgroup root when appropriate. The resulting file was input into Consense to obtain a single consensus tree using the majority rule (extended) consensus type and treated as rooted when appropriate. DNA trees were created using Dnaml in PHYLIP. Trees were visualized using FigTree (Version 1.4, http://tree.bio.edu.ac.uk/software/figtree) and labeled using Adobe Illustrator CS3 (San Jose, California).

### *OspC Typing*

To sequence *ospC* from mixed populations, PCR products for *ospC* from samples M6, M7, and M9 were gel purified and cloned into *E. coli* using the pCR2.1 TOPO TA Cloning Kit per the manufacturer's instructions (Life Technologies; Carlsbad, California). Plasmids were purified using the QIAGEN Plasmid Mini Prep kit (Valencia, California) and sequenced at Davis Sequencing. OspC amino acid sequences for previously typed isolates

(98, 99) were obtained from NCBI. Nucleotide sequences from the eastern North Dakota isolates were translated (ExPASy; web.expasy.org/translate/) and aligned with the previously typed isolates in ClustalW2. A Percent Identity Matrix (PIM) was obtained. An OspC type group was assigned to each eastern North Dakota isolate if the sequence did not diverge more than 2% from a particular group (99).

### *Multilocus Sequence Typing (MLST)*

MLST was performed for each eastern North Dakota sample (198). Primer sequences for the eight housekeeping genes used (*clpA, clpX, nifS, pepX, pyrG, recG, rplB,* and *uvrA*) were obtained from the Imperial College London's *B. burgdorferi* MLST website (http://borrelia.mlst.net). For amplification and sequencing, the outer forward and outer reverse primers for each gene were used. For each gene, a 50 µL reaction was set up using the HotStarTaq *Plus* Master Mix (QIAGEN) per the manufacturer's instructions. Primers were added to a final concentration of 1 µM and 1 µL of purified genomic DNA was added. Previously described amplification conditions were used (198) with the following modifications: 1. The initial denaturing step was decreased to 5 min per the HotStarTaq *Plus* Master Mix instructions and 2. The annealing temperature for *recG* was decreased to 48°C. Sequencing was performed at Eton BioSciences, Inc (San Diego, CA). Chromatograms were inspected for double-peaks, which indicated a mixed population. Chromatograms indicating mixed populations were omitted from further analyses. Single locus queries were performed for each sequence to obtain an allele number. An allelic profile query was performed with the

available loci for each eastern North Dakota sample using the *B. burgdorferi* MLST website. When data for eight loci were available, the query type chosen was "Exact or nearest match." When less loci were available, the query type chosen was n-1, where n = number of available loci.

### *Infectivity of B. burgdorferi M3*

Six female 4-6 week old BALB/c mice were each subcutaneously injected with $10^6$ spirochetes/mL. Two weeks post-injection, infection was preliminarily determined by assaying pre- and post-infection sera by enzyme-linked immunosorbent assay (ELISA). Larval *I. scapularis* were allowed to feed on infected mice as previously described (203). Briefly, approximately 200-300 uninfected larval *I. scapularis* (Oklahoma State University-Stillwater) were placed on infected BALB/c mice, allowed to attach, and feed. Four to seven days after attachment, engorged larval *I. scapularis* dropped off, were collected, and placed in a humidified chamber until they molted to nymphs. Mice were euthanized twenty-four hours after all *I. scapularis* detached (i.e., day 8). One tibiotarsal joint, ear pinnae, and the heart were collected for qPCR analysis. The second tibiotarsal joint and ear pinnae were cultured in BSK-II medium with 6% rabbit serum and 50 µg/mL rifampin, blind passed, and examined by dark field microscopy as described above. After molting, approximately 15 infected nymphal *I. scapularis* were placed on 6 naïve female 4-6 week old BALB/c mice. Engorged *I. scapularis,* mouse tissues, and cultures were treated as described above.

### *Enzyme-Linked Immunosorbent Assay (ELISA)*

Anti-*B. burgdorferi* IgG in sera from inoculated mice was detected as previously described (203). Briefly, 96-well plates were coated with a 10 µg/mL *B. burgdorferi* lysate in a carbonate coating buffer and incubated O/N at 4°C. All washes were performed with PBS-Tween. Serum samples were diluted 1:100 in PBS. Anti-mouse IgG was diluted 1:5000 in PBS. Each serum sample was analyzed in triplicate.

### *DNA Extraction*

DNA from bacterial cultures was extracted with a 25:24:1 phenol-chloroform-isoamyl alcohol extraction. DNA was further purified with two consecutive ethanol precipitations. Total (mouse and spirochete) DNA for use in qPCR was extracted from tibiotarsal joints using a phenol-chloroform-isoamyl alcohol protocol and further purified with the QIAGEN DNeasy Blood and Tissue Kit per manufacturer's specifications. Total DNA from hearts and ear pinnae was extracted using a modified DNeasy Blood and Tissue Kit protocol. Briefly, minced tissues were suspended in Buffer ATL with proteinase K and incubated O/N at 56°C. Samples were further purified per manufacturer's specifications and as previously described (203).

### *Quantitative PCR (qPCR)*

Primers used are listed in Table 2.1. Reactions were performed using Bio-Rad iQ SYBR Green Supermix (Hercules, California). Mouse DNA was detected using primers for nidogen (*nid1*) and quantified against 500, 50, 5, 0.5, 0.05, and 0.005 ng mouse DNA standards. Amplification conditions: 95°C for 3 min, 40 cycles of 95°C for 30, 49°C for 1 min, 1 cycle of 95°C for

1 min, 50°C for 1 min, 1 cycle 49°C for 1 min, 49-95°C Δ0.5°C for 10 sec each step. *B. burgdorferi* DNA was detected using primers for *recA* and quantified against six *B. burgdorferi* DNA standards ranging in concentration from $10^{-6}$ to $10^{-1}$ copy number. Amplification conditions: 95°C for 3 min, 40 cycles of 95°C for 30 sec, 50°C for 1 min, 1 cycle of 95°C for 1 min, 50°C for 1 min, 1 cycle 50°C for 1 min, 50-95°C Δ0.5°C for 10 sec each step. Each sample and a no-template control were run in triplicate.

***Detection of B. burgdorferi DNA in Nymphal I. scapularis***

Total DNA was extracted from *I. scapularis* allowed to molt to nymphs after feeding as larvae using a modified QIAGEN DNeasy Blood and Tissue Kit protocol. Ten molted *I. scapularis* per mouse were homogenized in Buffer ATL (600 µL) with proteinase K (20 µL) and incubated overnight at 56°C. Buffer AL was added (200 µL); tubes were vortexed and incubated at 70°C for 10 min. Wheat germ transfer RNA, Type V (1 µL of 10 mg/mL; R-7876, Sigma-Aldrich; St. Louis, Missouri) was added and tubes vortexed. Ethanol (230 µL, 95%) was added, tubes were vortexed, transferred to DNeasy spin columns, and centrifuged for 1 min at 8000 rpm. Buffer AW1 (500 µL) was added and centrifuged for 1 min at 8000 rom. Buffer AW2 (500 µL) was then added and centrifuged at 14000 rpm for 3 min. DNA was eluted from the spin column with 100 µL nuclease-free water twice. PCR was performed using primers for *I. scapularis* 16S, *B. burgdorferi* 16S, and *B. burgdorferi flaB* (Table 2.1) with the following amplification conditions: initial denaturation at 94°C for 3 min; 40 cycles of 94°C for 30 sec, 50°C for 30 sec, 65°C for 30

sec; final elongation at 65°C for 5 min. Reactions were run on a 2.5% NuSieve gel.

### *GenBank Accession Numbers*

Sequences obtained in this study have been deposited with accession numbers KM676013-KM676070.

## Results

### *Sequence and Phylogeny Confirm Spirochetes are B. burgdorferi and Represent Non-Clonal Populations*

To confirm the eastern North Dakota samples were *B. burgdorferi s.l.*, we sequenced 136 and 139 nucleotides of the 16S rDNA and *flaB*, respectively. The 16S rDNA sequences were queried against the Ribosomal Database Project. The sequences for each of the five samples returned hits to various *Borrelia* species (data not shown). A BLAST search of the *flaB* sequences obtained from all five samples showed 100% sequence identity matches to *B. burgdorferi* (data not shown). These data confirmed the spirochetes were members of the *B. burgdorferi s.l.* group.

To determine whether the samples represented multiple *B. burgdorferi* populations or a single population, sequencing and phylogenetic analyses were performed for *ospA, ospC, p66,* and the 16S-*ile* tRNA IGS. For comparison, various *B. burgdorferi s.l.* and *B. hermsii* sequences were obtained from the NCBI and BLAST databases. A BLAST search using the 16S-*ile* tRNA DNA sequence from *B. burgdorferi* B31 returned results for *B. burgdorferi s.l.* (data not shown). There were no differences in the sequences of *ospA* and *p66* for the five samples. Protein maximum likelihood analyses of

OspA (Fig. 2.1) and p66 (Fig. 2.2) grouped the eastern North Dakota samples with *B. burgdorferi s.l.*

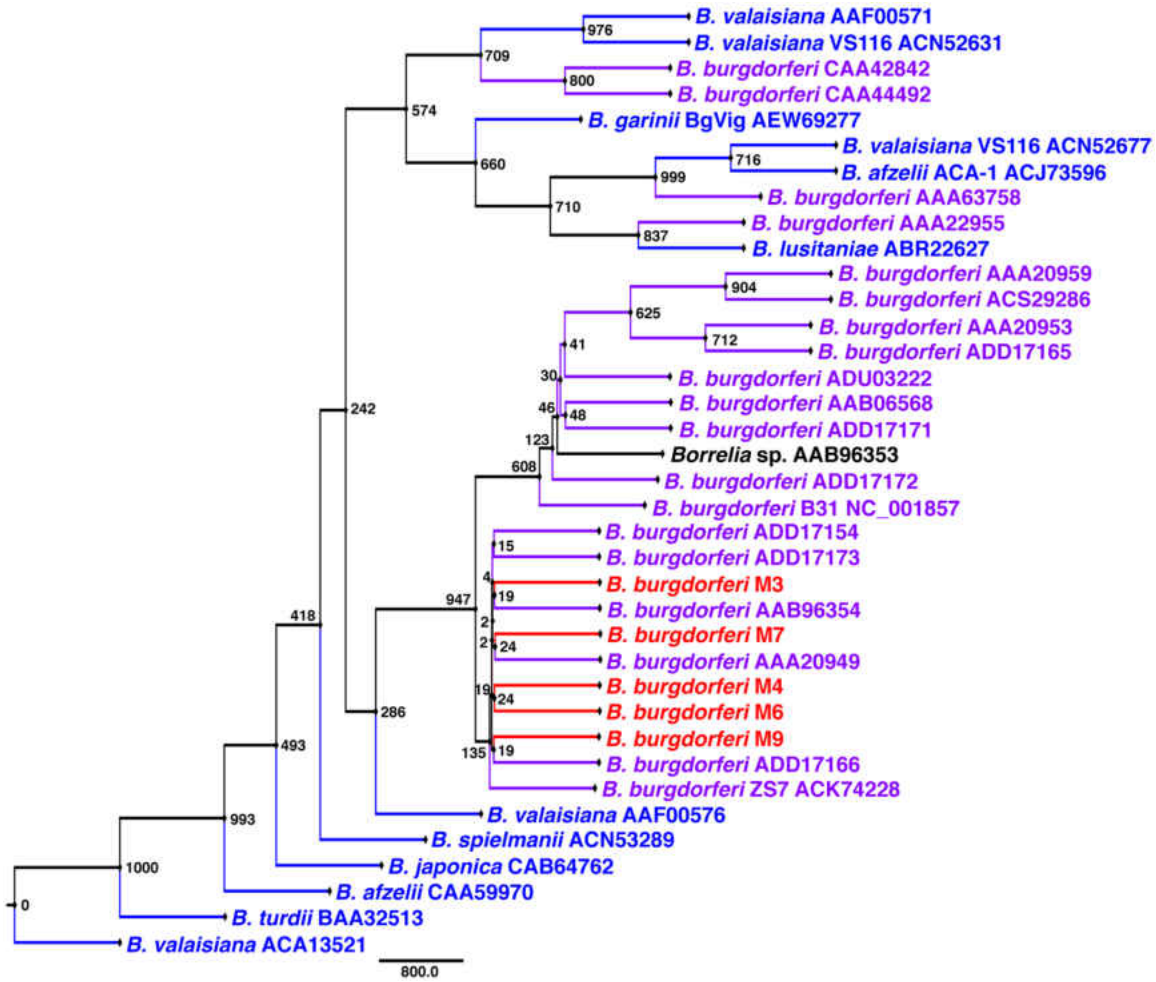



Figure 2. 1. Unrooted protein maximum likelihood analysis of OspA shows the eastern North Dakota isolates group with North American *B. burgdorferi*. Sequences included, approximately, residues 41-236. Node values represent bootstrap values from 1000 replicates. Red: eastern North Dakota isolates; purple: North America Lyme disease-associated *Borrelia*; blue: Eurasia Lyme disease-associated *Borrelia*; black: unknown species.

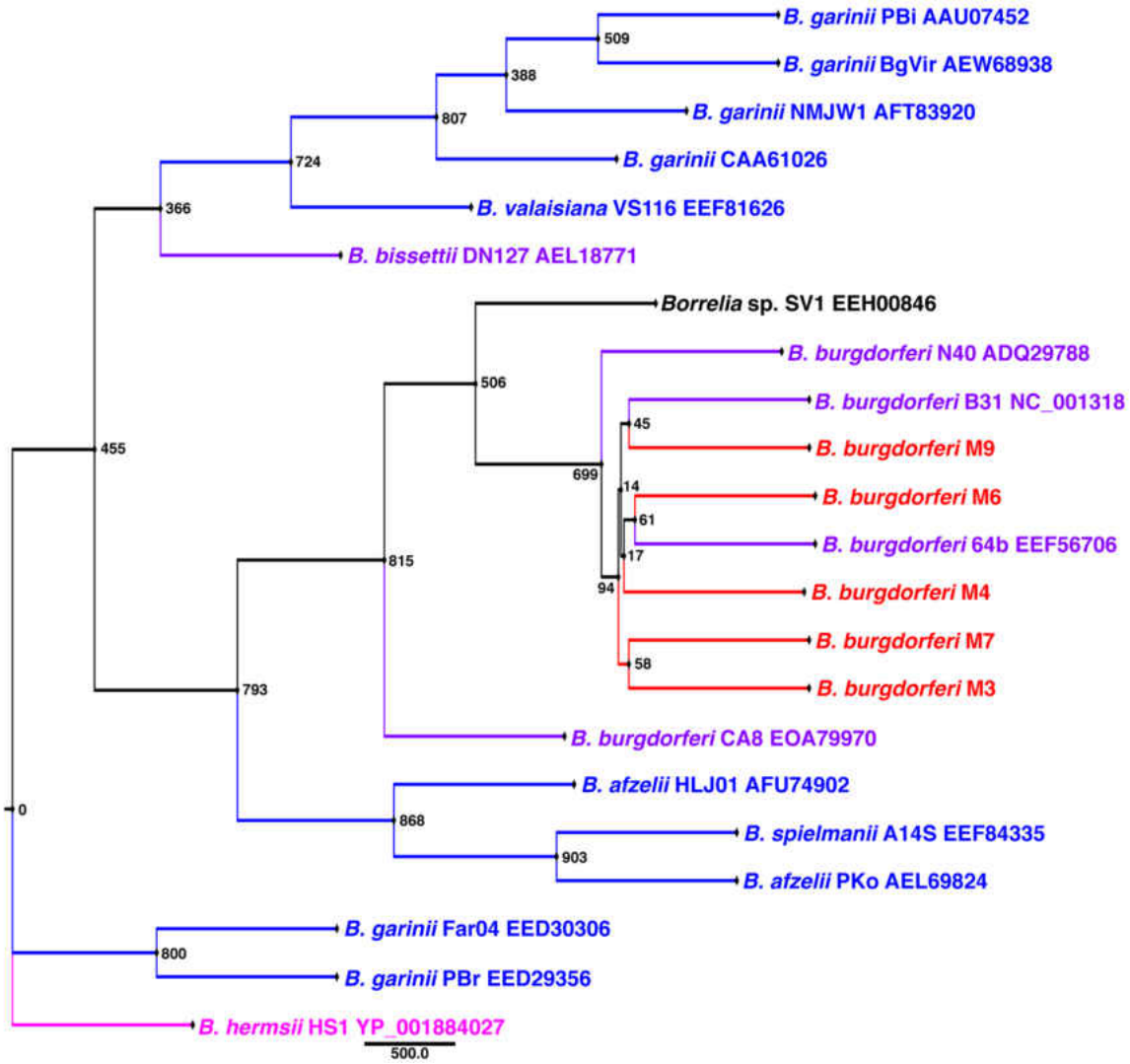
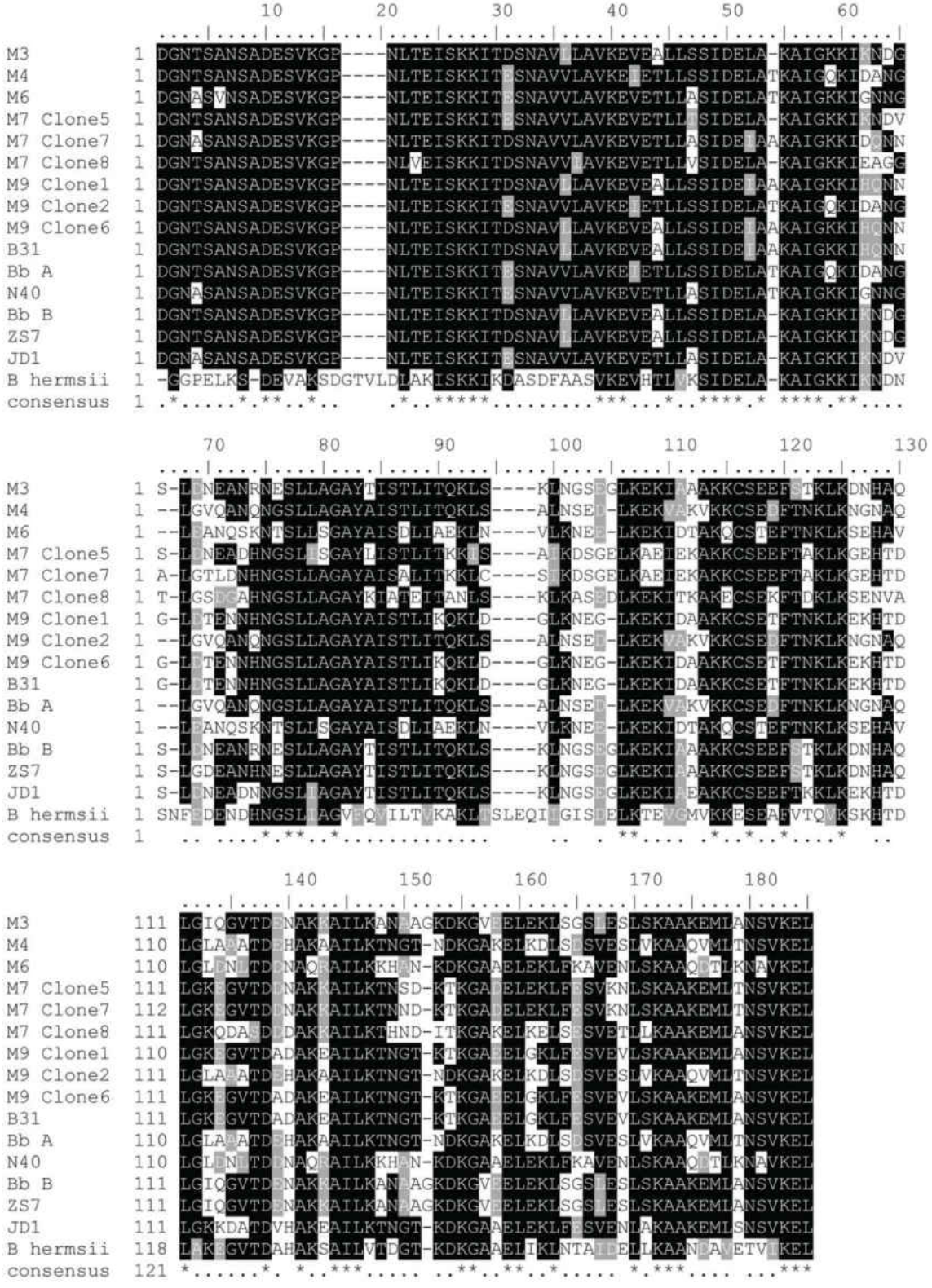



Figure 2. 2. Rooted protein maximum likelihood analysis of p66 shows the eastern North Dakota isolates group with North American *B. burgdorferi*. Sequences included, approximately, residues 364-548. Node values represent bootstrap values from 1000 replicates. Red: eastern North Dakota isolates; purple: North America Lyme disease-associated *Borrelia*; blue: Eurasia Lyme disease-associated *Borrelia*; black: unknown species; pink: Relapsing fever outgroup.

Unlike OspA and p66, OspC showed variation among the eastern North Dakota samples, sharing between 66 and 100% identity (Figs. 2.3 and 2.4). In two of the small mammals sampled (M7 and M9), multiple *B. burgdorferi* strains were detected. With the exception of *B. burgdorferi* M7 Clone 7, which grouped with a clade consisting of both North American and Eurasian *Borrelia*, the eastern North Dakota populations grouped most closely with *B. burgdorferi s.l.* (Fig. 2.4). *B. burgdorferi* M9 Clones 1 and 6 were 100% identical across the region used for OspC analyses, but showed variation outside of this region, particularly at four residues immediately downstream. Further sequencing would be required to determine whether Clones 1 and 6 are indeed different strains. OspC typing revealed a diverse group of spirochetes (Table 2.2). Interestingly, one of the isolates identified, *B. burgdorferi* M7 Clone 8, belongs to one of the rarer groups (L) to be identified in reservoir animals (204). One isolate, *B. burgdorferi* M7 Clone 7, did not group with OspC groups A-U. This is not surprising given it did not clearly group with North American *B. burgdorferi s.l.* Taken together, these data confirm non-clonal, invasive populations of *B. burgdorferi* are present in eastern North Dakota.

Figure 2. 3. Alignment of OspC suggests the eastern North Dakota isolates are genetically distinct strains of *B. burgdorferi.* Samples M7 and M9 contain a mixture of clones. Sequences included, approximately, residues 25-198. *B. burgdorferi* B31 (AE000792.1), N40 (DQ437463.1), *B. burgdorferi* A (a non-type strain, ABQ42987.1), B (a non-type strain, ABK41066.1), ZS7 (AF500204.1), and JD1 (DQ437462.1), as well as Vsp3, an OspC ortholog found in *B. hermsii* (relapsing fever, AAA22967.1) were included for comparisons. Alignment was performed in ClustalW2 and shading was performed using ExPASy's BoxShade.



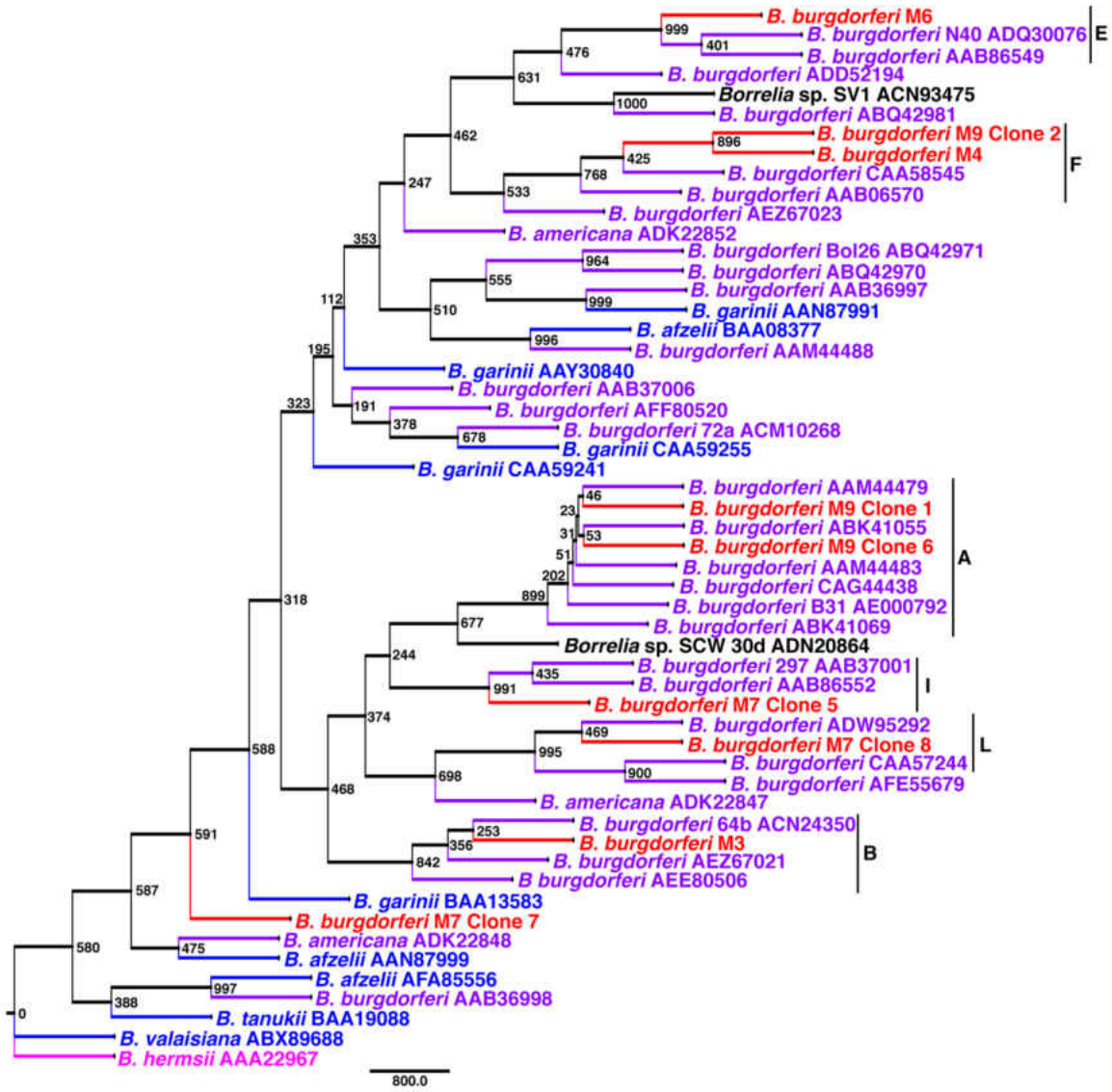



Figure 2. 4. Rooted protein maximum likelihood analysis of OspC shows the eastern North Dakota isolates group with North American *B. burgdorferi*. OspC groups are indicated by a vertical black line and single letter code. Sequences included, approximately, residues 25-198. Node values represent bootstrap values from 1000 replicates Red: eastern North Dakota isolates; purple: North America Lyme disease-associated *Borrelia*; blue: Eurasia Lyme disease-associated *Borrelia*; black: unknown species; pink: Relapsing fever outgroup.

Table 2. 2. OspC group for each eastern North Dakota isolate using Wang et al. (99) and Seinost et al. (98) groupings.

| Eastern ND Isolate | OspC group |
|---|---|
| *B. burgdorferi* M9 Clones 1 and 6 | A |
| *B. burgdorferi* M3 | B |
| *B. burgdorferi* M6 | E |
| *B. burgdorferi* M4 and *B. burgdorferi* M9 Clone 2 | F |
| *B. burgdorferi* M7 Clone 5 | I |
| *B. burgdorferi* M7 Clone 8 | L |
| *B. burgdorferi* M7 Clone 7 | None |

### *The Eastern North Dakota Populations are Most Closely Related to B. burgdorferi Found in the Upper Midwest*

MLST analysis was performed to determine the regional source of the eastern North Dakota populations. Loci were omitted from further analysis if the chromatograms suggested a mixed population for that locus (i.e., double peak at a single nucleotide location) (Tables 2.3 and 2.4). Sequence for all eight loci could be obtained only for one sample (*B. burgdorferi* M9), thus single locus analyses were performed for the remaining samples. The database profiles that most closely matched the incomplete eastern North Dakota sample profiles were obtained (Tables 2.3 and 2.4). *B. burgdorferi* M3 most closely matched ST30 strains for all loci except *nifS*, which was unavailable. The available loci information for *B. burgdorferi* M4 matched ST56 strains. *B. burgdorferi* M6 matched at five loci to ST31 and ST229 but had a different *recG* loci than ST31 or ST229. *B. burgdorferi* M7 differed at *clpA* from ST225 strains. *B. burgdorferi* M9 matched at all eight loci to ST56 strains. Each database strain that was the closest match to the eastern North Dakota samples was initially identified in the Upper Midwest (Illinois, Wisconsin, Minnesota, and Manitoba, Canada) (Fig. 2.5, Table 2.4). These data suggest the eastern North Dakota samples are most closely related to

Upper Midwest strains and thus, the Upper Midwest is the most probable source of the eastern North Dakota samples.

Table 2. 3. Allele scores for *clpA, clpX, nifS, pepX, pyrG, recG, rplB,* and *uvrA* and closest matching database STs.

| Eastern ND Isolate | *clpA* | *clpX* | *nifS* | *pepX* | *pyrG* | *recG* | *rplB* | *uvrA* | ST |
|---|---|---|---|---|---|---|---|---|---|
| *B. burgdorferi* M3 | 19 | 1 | - [a] | 1 | 2 | 1 | 1 | 10 | 30 |
| *B. burgdorferi* M4 | 24 | - | - | 18 | - | 19 | 1 | - | 56, 231 |
| *B. burgdorferi* M6 | 20 | 4 | - | 3 | 3 | 1 | 3 | - | 31, 229 |
| *B. burgdorferi* M7 | 18 | 2 | - | - | 2 | 8 | 1 | - | 225 |
| *B. burgdorferi* M9 | 24 | 14 | 4 | 18 | 11 | 19 | 1 | 12 | 56, 231 |

[a] Chromatogram indicated a mixed population

Table 2. 4. Allelic profiles and sequence types (ST) for the eastern North Dakota samples and the most closely matching multilocus sequence typing database strains. Alleles that differ from the respective eastern North Dakota allele are highlighted.

| Sample/Strain | ST | *clpA* | *clpX* | *nifS* | *pepX* | *pyrG* | *recG* | *rplB* | *uvrA* |
|---|---|---|---|---|---|---|---|---|---|
| *B. burgdorferi* M3 | | 19 | 1 | -[a] | 1 | 2 | 1 | 1 | 10 |
| 51405[b] | 30 | 19 | 1 | 5 | 1 | 2 | 1 | 1 | 10 |
| MC132 | 30 | 19 | 1 | 5 | 1 | 2 | 1 | 1 | 10 |
| MC108 | 30 | 19 | 1 | 5 | 1 | 2 | 1 | 1 | 10 |
| *B. burgdorferi* M4 | | 24 | - | - | 18 | - | 19 | 1 | - |
| 1469205 | 56 | 24 | 14 | 4 | 18 | 11 | 19 | 1 | 12 |
| MC73 | 56 | 24 | 14 | 4 | 18 | 11 | 19 | 1 | 12 |
| MC78 | 56 | 24 | 14 | 4 | 18 | 11 | 19 | 1 | 12 |
| Mid761 | 56 | 24 | 14 | 4 | 18 | 11 | 19 | 1 | 12 |
| MC150 | 231 | 24 | 14 | 4 | 18 | 11 | 90 | 1 | 12 |
| *B. burgdorferi* M6 | | 20 | 4 | - | 3 | 3 | 1 | 3 | - |
| 48102 | 31 | 20 | 4 | 3 | 3 | 3 | 3 | 3 | 3 |
| 50302 | 31 | 20 | 4 | 3 | 3 | 3 | 3 | 3 | 3 |
| Mid471 | 31 | 20 | 4 | 3 | 3 | 3 | 3 | 3 | 3 |
| MC101 | 229 | 20 | 4 | 3 | 3 | 3 | 18 | 3 | 3 |
| MC92 | 229 | 20 | 4 | 3 | 3 | 3 | 18 | 3 | 3 |
| *B. burgdorferi* M7 | | 18 | 2 | - | - | 2 | 8 | 1 | - |
| MC110 | 225 | 8 | 2 | 5 | 93 | 2 | 8 | 1 | 84 |
| MC120 | 225 | 8 | 2 | 5 | 93 | 2 | 8 | 1 | 84 |
| BP-2 | 225 | 8 | 2 | 5 | 93 | 2 | 8 | 1 | 84 |
| *B. burgdorferi* M9 | | 24 | 14 | 4 | 18 | 11 | 19 | 1 | 12 |
| 1469205 | 56 | 24 | 14 | 4 | 18 | 11 | 19 | 1 | 12 |
| MC73 | 56 | 24 | 14 | 4 | 18 | 11 | 19 | 1 | 12 |
| MC78 | 56 | 24 | 14 | 4 | 18 | 11 | 19 | 1 | 12 |
| Mid761 | 56 | 24 | 14 | 4 | 18 | 11 | 19 | 1 | 12 |
| MC150 | 231 | 24 | 14 | 4 | 18 | 11 | 90 | 1 | 12 |

[a] Chromatogram indicated a mixed population

[b] Database strain identifications

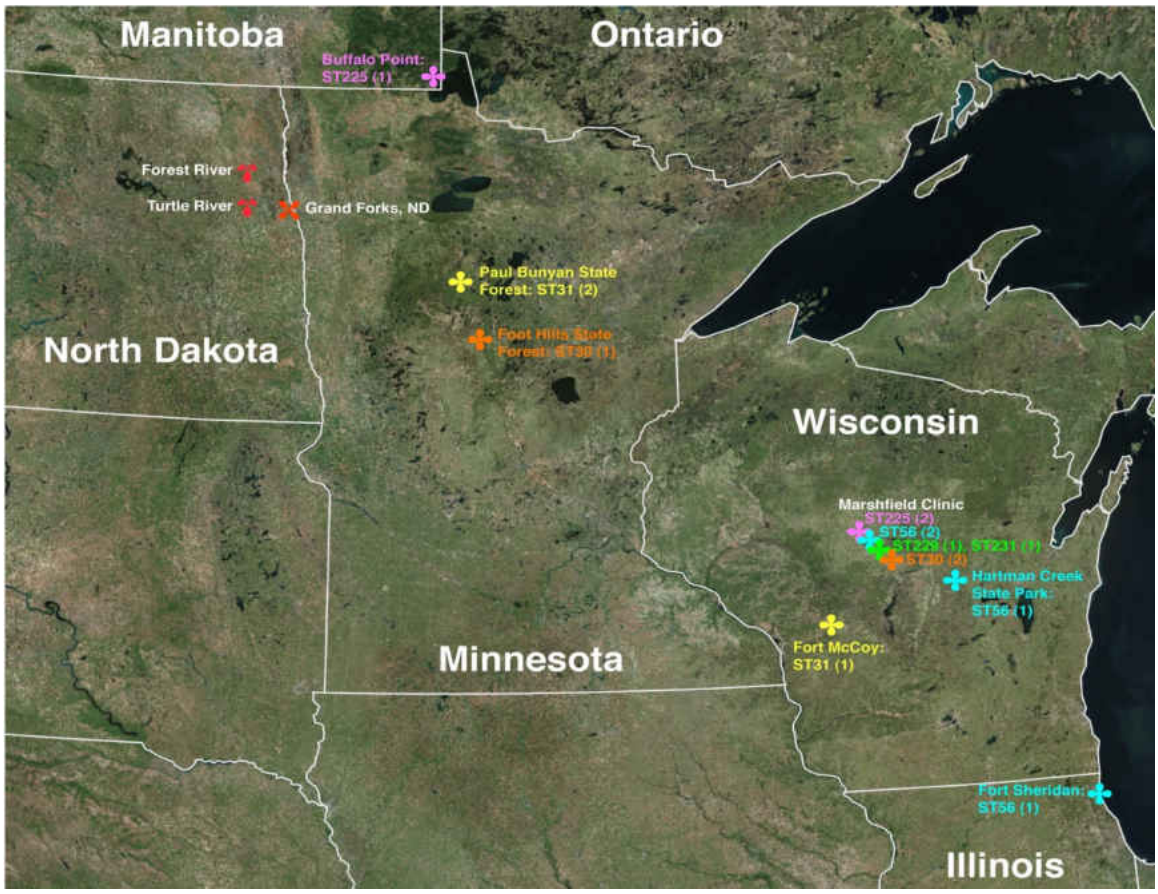



Figure 2. 5. Location of database STs from Table 2.4. Strains from Marshfield Clinic, WI were obtained from unknown locations in Wisconsin. All were identified from human erythema migran samples. The remaining strains were reported to be isolated from the identified location. Pink icons are ST225, yellow ST31, orange ST30, green ST229 and ST231, cyan ST56. Numbers in parentheses indicate the number of unique strains comprising each ST. Forest River and Turtle River were sites of sample collection in this study. Satellite images obtained from NOAA via Google Earth.

### *Needle-Injected B. burgdorferi M3 Infects and Survives in BALB/c Mice and is Acquired by Larval I. scapularis During Feeding*

To determine whether infectious and transmissible populations were present in eastern North Dakota, an infection and transmission study was performed using *B. burgdorferi* M3. M3 was the only sample obtained from *M. gapperi*, whose reservoir status is unknown. Further, the OspC data suggested M3 was a clonal population belonging to the infectious B group. An ELISA of pre- and post-infection sera from mice subcutaneously injected with $10^6$ *B. burgdorferi* M3/mL showed increased anti-*Borrelia* antibodies 2-weeks post-injection (Table 2.5). Tibiotarsal joints and ear pinnae were culture-positive, except for one mouse where culture data were unavailable due to contamination. *B. burgdorferi flaB* was detected in molted nymphs fed on five of the six needle-injected mice (Table 2.5). Neither *B. burgdorferi* 16S nor *flaB* could be positively identified in molted nymphs fed on mouse 6. *B. burgdorferi recA* was detected in one of three hearts, six of six tibiotarsal joints, and three of six ear pinnae (Table 2.6). The mouse nidogen gene, *nid1*, was detected in all tissues (Table 2.6). These data demonstrate *B. burgdorferi* M3 is infectious to mice, able to disseminate to multiple tissues, and capable of being acquired by *I. scapularis* during a blood meal.

Table 2. 5. Summary of ELISA and culture results from mice either needle-injected (N) with $10^6$ cells/mL *B. burgdorferi* M3 or tick-infected (T), as well as PCR detection of *B. burgdorferi flaB* from nymphs reported as *B. burgdorferi flaB* positive/nymph 16S positive.

| ELISA | | Culture | | | | PCR |
|---|---|---|---|---|---|---|
| Needle-injected (N) | Tick-infected (T) | Tibiotarsal joint | | Ear pinnae | | Bb/Nymphs |
| | | N | T | N | T | |
| 6/6 | 6/6 | 5/6[a] | 6/6 | 6/6 | 6/6 | 5[b]/6 |

[a] One culture contaminated after initial inoculation
[b] PCR with *B. burgdorferi* 16S and *fla* were not clearly positive or negative for one mouse

Table 2. 6. Summary of qPCR for needle-injected and tick-infected mice reported as *B. burgdorferi recA* positive/mouse *nid1* positive.

| Needle-injected | | | Tick-infected | | |
|---|---|---|---|---|---|
| Heart | Tibiotarsal joint | Ear pinnae | Heart | Tibiotarsal joint | Ear pinnae |
| 1/3[a] | 6/6 | 3/6 | 6/6 | 6/6 | 6/6 |

[a] Neither *recA* nor *nid1* were detectable in three mice

## *B. burgdorferi* M3 Survives the *I. scapularis* Larval Molt and is Subsequently Transmitted to Naïve BALB/c mice During a Blood Meal

To determine whether *B. burgdorferi* M3 was transtadially maintained during the *I. scapularis* larval molt and capable of transmission to naïve mice, *I. scapularis* nymphs were fed on naïve BALB/c mice. Infection was confirmed by ELISA, which showed an increase in absorbance post-feeding (Table 2.5). Tibiotarsal joints and ear pinnae were all culture-positive. *nid1* and *recA* were detected in all six hearts, tibiotarsal joints, and ear pinnae. These data show *B. burgdorferi* M3 is transtadially maintained in *I. scapularis* and capable of dissemination in naïve hosts.

## Discussion

Studies on the prevalence and spread of *B. burgdorferi*, *I. scapularis*, and Lyme disease in the Upper Midwest typically focus on Minnesota and Wisconsin (183, 205, 206). The vast majority of Lyme disease cases, and thus *B. burgdorferi* and *I. scapularis*, reported in the Upper Midwest are found in Minnesota and Wisconsin. However, there are numerous factors that demanded a detailed investigation of *B. burgdorferi* in eastern North Dakota (e.g., the close proximity to high-risk Minnesota counties with a history of Lyme disease, *B. burgdorferi*, and *I. scapularis*; the presence of known small mammal reservoirs; and recent studies (37, 180) identifying stable *I. scapularis* populations and *B. burgdorferi* in North Dakota further confirming the expansion of *I. scapularis* described in other US regions and Canada (41, 179, 181, 182, 207)]).

The data presented here demonstrate the spirochetes isolated in eastern North Dakota from *Peromyscus* spp. and *M. gapperi* hearts are members of the *B. burgdorferi s.l.* complex. While OspA and p66 are identical among all of the eastern North Dakota populations, OspC typing shows they are distinctly non-clonal populations. The M9 population consisted of at least two OspC types, A and F; M7 consisted of at least two types, I and L (Table 2.2, Fig. 2.4). *B. burgdorferi* M7 Clone 7 does not appear to belong to any of the previously defined OspC groups. In phylogenetic analyses, Clone 7 also does not appear to group clearly with either North American *B. burgdorferi s.l.* or Eurasian Lyme disease *Borrelia* (Fig. 2.4). A BLAST analysis with the sequence from Clone 7 returns results for *B. burgdorferi s.l.* OspC but with a

maximum identity score of 87% (data not shown). It is clear Clone 7 is a member of the *B. burgdorferi s.l.* complex*,* but its OspC type and infectivity remain unknown. Despite obtaining sequence data for a single clone, the M6 population may consist of multiple OspC types due to difficulties obtaining *ospC* sequence prior to cloning *ospC*. Since less than five *ospC* clones were obtained and sequenced from three of the five eastern North Dakota samples, it is not possible to determine the proportion of OspC types in each sample. It is clear at least three of the most common OspC types known to cause disseminated infection in humans (A, B, and I) (98, 99) are present in eastern North Dakota (Table 2.2, Fig. 2.4). A more comprehensive survey is required to determine the presence and distribution of OspC types.

The MLST data, though limited, suggest the eastern North Dakota populations are derived from the Upper Midwest populations and not a recent transplant from another region. Specifically, the eastern North Dakota populations appear to be most closely related to strains found in Minnesota, Wisconsin, Illinois, and southeastern Manitoba on the Minnesota-Canada border. A number of the housekeeping genes appeared to be identical in the populations that the OspC data showed were mixed populations. Likewise, a number of housekeeping genes appeared to indicate mixed populations in a single sample when the OspC data suggested populations in each sample were clonal. This was surprising because the *ospC* gene is highly polymorphic, while the housekeeping genes are generally more conserved. Viewing the OspC and MLST data together suggest that in addition to small-scale random mutation events, large-scale mutation events also occurred

with OspC. The sequence analyses, taken as a whole, suggest a regional population structure larger and more complex than was captured by the five samples partially characterized here.

The results of the infection study show *B. burgdorferi* M3 is infectious through both an artificial and natural route of infection. *B. burgdorferi* M3 is capable of disseminating from the site of inoculation to the heart, tibiotarsal joint, and ear pinnae indicating it is highly infectious (101). The ability to disseminate is not surprising since *B. burgdorferi* M3 belongs to the *ospC* group B, a group associated with disseminated disease in humans (98, 152, 193).

In the US, Lyme disease remains a significant public health issue. From 2001 to 2011, the number of confirmed cases reported to the CDC averaged 24,000 making it the most reported tick-borne disease in the US (CDC). In 2013, the CDC released revised yearly estimates based on continuing studies including analyzing data from tests conducted by seven participating commercial laboratories in 2008 (78). Based on these analyses, the estimated number of individuals infected with *B. burgdorferi* in the US was revised to approximately 288,000 per year, about ten times more than the average yearly number of reported and confirmed Lyme disease cases. North Dakota is not the Lyme disease hotspot Minnesota is, but Lyme disease is poised to be a significant public health issue in North Dakota. The number of Lyme disease cases reported yearly in North Dakota is low (205 reported cases between 1996 and 2015, North Dakota Department of Health), compared to the number of cases reported in neighboring Minnesota (16,439

confirmed cases between 1996 and 2015, Minnesota Department of Health). However, just as the national cases are underestimated, there are a number of factors that make a reasonable argument for cases in North Dakota being underestimated: the classification of eastern North Dakota as a transition zone (179), the conventional opinion that *B. burgdorferi* and *I. scapularis* are not found in North Dakota, the evolving criteria for reporting and confirming Lyme disease, the increasing number of Lyme disease cases in North Dakota, and the rural nature of North Dakota.

To develop comprehensive, informed public health policies in both the US and Canada, it is imperative to understand whether *I. scapularis*, and subsequently *B. burgdorferi*, are expanding outside of the previously identified geographical regions. While changes in habitat, and the reasons for those changes, are outside the scope of this study, it is clear that *B. burgdorferi* and *I. scapularis* have migrated westward in the Upper Midwest. This information is relevant to North Dakota residents, visitors, and medical professionals who should be aware of the risk of contracting Lyme disease in eastern North Dakota. This information is also important beyond the borders of North Dakota as it provides additional data on the ever-evolving state of Lyme disease.

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# CHAPTER 3

# *IN VITRO* AND *IN VIVO* CHARACTERIZATION OF TWO ANKYRIN PROTEINS, BB0399 AND BBB28, IN *BORRELIA BURGDORFERI*

## Introduction

Lyme disease, an infection with *Borrelia burgdorferi* spread through the bite of an infected *Ixodes scapularis*, is the leading tick-borne disease in the US (4, 77). Each year 20,000-30,000 cases are reported to the CDC, but new estimates increase the suspected cases ten-fold to 240,000-444,000 per year (78). Despite the high incidence of Lyme disease, we still do not fully understand mechanisms *B. burgdorferi* utilize to cause disease and survive in reservoirs and vectors (208–210). One potential mechanism involves ankyrin-repeat (Ank) proteins, which have recently been identified in prokaryotes with several identified as virulence factors (211–251). Ank domains commonly facilitate protein-protein interactions in eukaryotes. In bacteria, three general functional classes of Ank proteins have been identified: 1. DNA-binding (252), 2. Oxidative stress response (248), and 3. Mimicking host Ank proteins (216, 238, 253, 254).

Based on BLAST searches and previous studies, our hypothesis was *bb0399* belonged to the first functional class and *bbb28* belonged to the second functional class.

## Ankyrin Proteins

The ankyrin-repeat domain (Ank) is one of the most common protein motifs and has been identified in the genomes of bacteria, archaea, eukaryotes, and viruses. The general structure of an ankyrin-repeat domain consists of a 33-residue repeat forming a series of alpha helices that stack upon each other forming a spring-like structure. A single domain can possess between 2 and 34 repeats, with prokaryotic Ank proteins having an average of four to six repeats for free-living/facultative host-associated and obligate intracellular bacteria, respectively (242).

Eukaryotic Ank proteins exclusively mediate protein-protein interactions (212) in diverse cellular processes, e.g. cell cycle regulation, with functions such as transcription factors (246), toxins (widow spider neurotoxins, (255)), and nucleases (RNase L, (256)). While less is known regarding the role of bacterial Ank proteins, a handful have been characterized to some degree and it is increasingly clear that Ank domains are important virulence factors and play important roles in the survival of bacteria (211, 216, 218, 238, 240, 248, 249, 252–254, 257, 258). One theme that has emerged is the role of Ank proteins in mediating some aspect of oxidative stress, with the two best characterized examples being AnkA from *Anaplasma phagocytophilum* and AnkB from *Pseudomonas aeruginosa*.

### *AnkA*

DNA-binding Ank proteins have been identified in *Anaplasma* (259) and *Ehrlichia* (244). One of the better characterized is AnkA, a secreted, soluble protein from *A. phagocytophilum*, a Gram-negative, obligate

intracellular pathogen living in neutrophils. Once inside a neutrophil, *A. phagocytophilum* secrets AnkA via a type IV secretion system. AnkA translocates to the host nucleus where it: 1. Binds the host phosphatase, SHP-1, to interfere with host cell signaling (223, 257) and 2. Directly binds host DNA, specifically the cytochrome b-245 beta chain gene (*CYBB*) promoter, to decrease *CYBB* transcription (211, 252). Cytochrome b-245 is part of the oxidase system in phagocytic cells and is involved in the formation of superoxide radicals.

### *AnkB*

*Pseudomonas aeruginosa* is a Gram-negative, opportunistic pathogen. AnkB is an integral cytoplasmic membrane protein, with the Ank domain on the periplasmic side (248). *ankB* is cotranscribed in a small operon with *katB*, a catalase only induced in response to $H_2O_2$ (248, 260). Deletion of *ankB* decreases transcription of *katB* and makes cells more sensitive to $H_2O_2$ (248). While the mechanism or function of AnkB is unknown, two hypotheses were proposed (248): 1. AnkB binds to and stabilizes or anchors KatB allowing KatB to take an optimal conformation to bind and degrade $H_2O_2$, or 2. AnkB provides structural support for the cytoplasmic membrane to mitigate an increase in turgor pressure from oxygen accumulated during the degradation of $H_2O_2$.

## Oxidative Stress

Oxygen is toxic, even to obligate aerobic bacteria (261). An inevitable byproduct of aerobic cellular metabolism, especially oxidative phosphorylation, is the production of reactive oxygen species (ROS), such as

superoxide ($O_2 \cdot^-$) and hydrogen peroxide ($H_2O_2$) (262). If not fully detoxified, superoxide or intermediates can cause irreparable damage eventually leading to cell death. Some microaerophilic bacteria, which require low concentrations of atmospheric oxygen, will use oxidative phosphorylation as a major or sole source of ATP production. As a result, microaerophiles can have a full collection of ROS-neutralizing enzymes. *Helicobacter pylori* and some *Campylobacter* species have a superoxide dismutase (SOD) and catalase (263–267); these microaerophilic species are able to fully detoxify ROS. *B. burgdorferi* has no catalase and only a single manganese-dependent SOD identified (22, 268). In fact, *B. burgdorferi* has no apparent sources of endogenous ROS, e.g. oxidative phosphorylation, except for a putative flavoenzyme (*bb0812*) that might be a source of endogenous superoxide radicals (22, 268).

### *Oxidative Stress and B. burgdorferi*

Whereas all other known pathogens have mechanisms to obtain iron from hosts (e.g. siderophores) and have myriad iron-dependent enzymes, *B. burgdorferi* has no siderophores, iron-dependent enzymes, or iron transport mechanisms (268). Coupled with an intracellular iron concentration of less than 10 atoms (269), *B. burgdorferi* is a pathogen that does not require iron. The outer membrane lipid profile of *B. burgdorferi* is also unique among pathogens. In addition to lipoproteins commonly found in the outer membrane of most bacteria, e.g. phosphatidylethanolamine and phosphatidylglycerol, *B. burgdorferi* also incorporates a large percentage of eukaryotic-derived cholesterol and cholesterol-glycolipids and thus, have

more polyunsaturated fatty acids (36% of the total fatty acids when grown *in vitro*) compared to other bacteria (270). Because *B. burgdorferi* obtains membrane lipids from its environment, the lipid profile can be highly variable and reflective of the environment (271, 272). Polyunsaturated fatty acids are more susceptible to lipid peroxidation, a potentially lethal reaction whereby ROS create lipid peroxyl radicals and can eventually decrease membrane fluidity.

This unique physiology precludes *B. burgdorferi* from the obvious and well-characterized bacterial targets of ROS. *B. burgdorferi* is not susceptible to DNA damage resulting from exposure to ROS (270). Hydroxyl radicals from the breakdown of $H_2O_2$ can lead to protein carbonylation. While carbonylation can be catalyzed by any metal, there is no evidence that protein carbonylation occurs in *B. burgdorferi* as a result of ROS. The main targets for ROS damage are the polyunsaturated fatty acids found in *B. burgdorferi*'s membranes (270).

With no apparent source of endogenous ROS, *B. burgdorferi* likely encounters ROS from exogenous sources, *I. scapularis,* and mammalian hosts. Much remains unexplained regarding dissolved oxygen concentrations and ROS production in *I. scapularis*. The dissolved oxygen content in the midgut and salivary glands of *I. scapularis* have not been determined and so whether or to what extent *B. burgdorferi* encounters ROS in *I. scapularis* is unknown (273). We do know *I. scapularis* releases a large number of proteins during feeding to minimize host immune responses (107), including proteins that aid *B. burgdorferi* in various ways, particularly with ROS. Two

salivary proteins directly inhibit neutrophils from producing ROS (274) and Salp25D, a salivary peroxidase directly protects *B. burgdorferi* from mammalian host oxidative damage during host-to-vector transmission (275, 276).

*B. burgdorferi* will encounter rapidly evolving oxygen concentrations as it disseminates throughout a host. In rats, there is a concentration gradient of dissolved oxygen with a four-fold higher concentration in arterial blood than skin (273, 277, 278). Based on this, one hypothesis is that *B. burgdorferi* will encounter much lower concentrations of ROS in the host's dermal layer until it encounters neutrophils. In addition to the neutrophil's intracellular oxidative burst, which releases ROS within a phagolysosome to kill pathogens, neutrophils can release ROS into the extracellular space (276, 279, 280). Oxygen and ROS concentrations will likely vary by host organ and the extent of oxygenation in areas where *B. burgdorferi* reside, e.g. connective tissue at the base of the heart, is unknown. Still, *B. burgdorferi* will have to survive some exposure to oxygen and ROS.

Studies on the effects of oxidative stress, particularly from hydrogen peroxide ($H_2O_2$) on *B. burgdorferi* have produced conflicting results with some groups showing *B. burgdorferi* is resistant to $H_2O_2$ (281) and others showing *B. burgdorferi* is susceptible (282, 283). Recently, Troxell *et al*. (282) confirmed wild-type *B. burgdorferi* is resistant to oxidative stress and demonstrated the discrepancies in previous studies may be due to the presence of pyruvate in *B. burgdorferi* culture medium. Pyruvate is a potent

scavenger of $H_2O_2$ and added in excess of physiological concentrations in *B. burgdorferi* culture medium.

## *B. burgdorferi* Ankyrin Proteins

*B. burgdorferi* possesses two genes encoding proteins with Ank domains, *bb0399* and *bbb28*. These genes are two of the 588 hypothetical proteins in the *B. burgdorferi* genome. Both *bb0399* and *bbb28* are actively transcribed (284), however, the function(s) of *bb0399* and *bbb28* remain unknown.

## *B. burgdorferi bbb28*

*B. burgdorferi* type strain B31 possesses a gene, *bbb28*, with a putative Ank domain consisting of two Ank repeats (Fig. 3.1). *bbb28* is located on circular plasmid 26 (cp26), a plasmid essential for *B. burgdorferi* survival (285), and is highly conserved among *B. burgdorferi* strains (89-100% sequence identity). A few studies have identified *bbb28* or BBB28 but those that have suggest *bbb28* is functional, transcribed, and translated. Yang *et al.* (286) found BBB28 co-precipitated with the inner membrane protein, La7. Caimano *et al.* (287) found *bbb28* decreased by approximately half when the response regulator, *rrp1*, was knocked out. Rrp1, part of the Hk1/Rrp1 two-component system, is vital for *B. burgdorferi* survival in *I. scapularis* (288–290). Ojaimi *et al.* (284) found *bbb28* was one of 50 highly transcribed genes and transcription was high independent of temperature (35°C used to mimic a mammalian infection and 23°C used to mimic *I. scapularis* colonization) in wild-type (wt) *B. burgdorferi* type strain B31. Ojaimi *et al*. (291) also compared global transcription in a wild-type, virulent

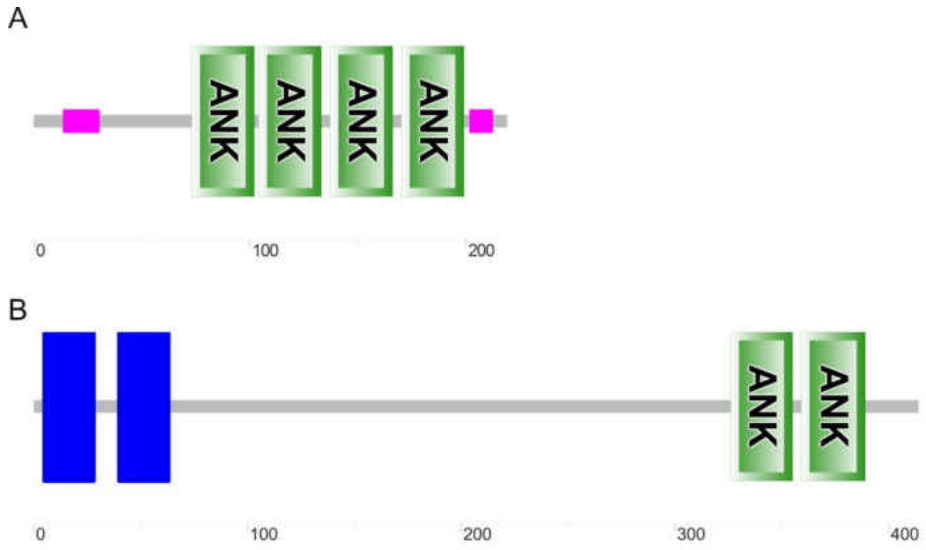



Figure 3. 1. Predicted features of BB0399 and BBB28 using European Molecular Biology Laboratory's Simple Modular Architecture Research Tool database (EMBL SMART). A. BB0399 has four Ank repeats and four regions of low complexity (pink boxes at aa 14-29 and aa 202-211; two regions are not shown at aa 106-114 and 184-197). B. BBB28 has two Ank repeats and two predicted transmembrane domains. (EMBL SMART found at http://smart.embl-heidelberg.de/)

*B. burgdorferi* B31 isolate (BL206) and a high-passage, attenuated isolate (B356; lacks lp25, cp32-6, and cp32-8). *bbb28* transcription was higher in the B356 isolate. RNA-seq analysis also found *bbb28* was transcribed in wild-type *B. burgdorferi* B31 grown under normal conditions (292).

Hyde *et al* (293) found *bbb28* transcription was downregulated in a mutant B31 strain, JS167, that was as resistant to oxidative stress as the wild-type strain, MSK5 (294). This difference in phenotype between mutant and wild-type was attributed to the activity of a single protein, the *Borrelia* oxidative stress regulator (BosR). In wild-type *B. burgdorferi*, BosR activates genes involved in an oxidative stress response, e.g. superoxide dismutase

(*sodA*) and neutrophil activating protein A (*napA*; homologous with *Escherichia coli*'s Dps, an iron-dependent DNA-binding protein that functions to protect DNA during starvation and oxidative stress). *B. burgdorferi* B31 strain CHP100 was found to be hyper-sensitive to *tert*-butyl hydroperoxide (*t*-BHP, damages outer membrane). The hyper-sensitive phenotype was due to a single point mutation in *bosR* resulting in an arginine to lysine substitution (*bosRR39K*). BosRR39K does not bind the promoter of *napA* but does bind the promoter and repress *sodA*. Inactivation of *bosRR39K* by transposon mutagenesis resulted in strain JS167 and restoration of the wild-type phenotype, ROS resistant. Thus, the model proposed is that BosR activates transcription of oxidative stress response genes, BosRR39K represses transcription of those genes, and inactivation of *bosRR39K* derepresses those genes. Thus, we can infer *bbb28* is activated in a wild-type strain, repressed by BosRR39K, and derepressed by the inactivation of *bosRR39K*.

Finally, signature-tagged mutagenesis (STM) of *B. burgdorferi* was able to produce *bbb28*::kan$^R$ mutants that were used to infect mice (105). Culture and PCR of five tissues were utilized to screen tissues for *B. burgdorferi* at two and four weeks post-infection. The overall trend was one of defective dissemination and/or survival of *bbb28*::kan$^R$ mutants outside of the inoculation site.

### *B. burgdorferi bb0399*

Less is known about *bb0399*. Expression of BB0399 is different in mid-exponential phase compared to stationary phase. A RelA/SpoT homolog

($Rel_{Bbu}$) involved in *B. burgdorferi* persistence in ticks upregulates *bb0399* expression during stationary phase, however, the increase in expression is small compared to other $Rel_{Bbu}$-regulated genes ($log_2$ fold change = 1.96) (295). Another study, also found *bb0399* transcription was also different during stationary phase but found *bb0399* was decreased in stationary phase ($log_2$ fold change = -1.3) (292)Expression in wild-type *B. burgdorferi* B31 is minimal and unaffected by temperature (284). Expression of *bb0399* becomes undetectable when *rrp1* is knocked out (287). Unlike with BBB28, there are no data suggesting *bb0399* transcripts are translated. STM was unable to produce a *bb0399*::$kan^R$ mutant suggesting *bb0399* is an essential gene (105).

## Objective

The goal of this work was to identify the function(s) of *bbb28* and *bb0399*. Our initial hypothesis focused on a BBB28 belonging to the oxidative stress class of ankyrin proteins. Our hypothesis stated BBB28 had a direct role in an oxidative stress response with an additional, or perhaps related, role in host dissemination. Our oxidative stress hypothesis shifted to BBB28 not having a direct role in an oxidative stress response, rather, being regulated by BosR in response to unknown stimuli. Our hypothesis for BB0399 was that BB0399 is an essential gene and may have DNA-binding properties, similar to AnkA of *A. phagocytophilum*.

## Materials and Methods

### *Cloning*

Full-length *bb0399*, full-length *bbb28*, and truncated *bbb28* (nt 187-1245, *bbb28*Δ186 encoding protein BBB28Δ62) were cloned into five vectors (Table 3.1). After ligating the target gene into the vector backbone, *E. coli* Top10 was transformed with each plasmid except pMAL-p5X and pMAL-c5X, which were transformed into NEB Express Competent *E. coli* (New England BioLabs, Inc). Cultures were grown overnight in LB with the appropriate antibiotic for selection. Up to 50 colonies per transformation were screened by PCR for the presence of the correct insert using universal T7 or M13 primers as appropriate. Plasmid was then extracted from up to five PCR-positive colonies using Qiagen's Midi Plasmid Purification kit per the manufacturer's instructions. Plasmids were sent for sequencing to either Davis Sequencing or Eton Bio. Both strands were sequenced using either universal T7 or M13 primers, as appropriate. Each sequence file was visually inspected for sequence quality. A consensus sequence was obtained by aligning the coding and non-coding strand files. Consensus sequence for each insert was aligned to the appropriate sequence from *B. burgdorferi* B31. A cloned insert had to match 100% to *B. burgdorferi* B31 before proceeding to the transformation of an expression strain.

To transform an expression strain of *E. coli*, 50 ng of purified plasmid was added to 50 μL of chemically competent *E. coli*. Five expression strains were used: BL21(DE3) (Novagen), Rosetta pLysS (Novagen), Tuner (Novagen), Tuner pLysS (Novagen), NEB Express Competent *E. coli*.

Table 3. 1. Cloning vectors, primers, and *E. coli* strains used in this study. Restriction sites in primers, if present, are underlined.

| Gene | Plasmid Name | Cloning Primers | *E. coli* Strain(s) Used | Vector Backbone |
|---|---|---|---|---|
| *bb0399* | pBLS2 | F: CGTCTAAAATTAAACACTTTATTAAAGG<br>R: ATATCCAAGCATCTGATGGG | Top10 | pCR2.1 (Thermo Fisher Scientific) |
| | pBLS4 | F: CACCATGCTTTTACTRTTATTGCAAACAATAATG<br>R: GGCTATTTTACTAAAATTCG | BL21(DE3)<br>Rosetta pLysS<br>Tuner<br>Tuner pLysS | pET200 (Thermo Fisher Scientific) |
| | pBLS10 | F: PO4-atgaaaaaagaattcattatgctttactgttattgcaaaca<br>R (SbfI): GAGAGAGACCTGCAGGCTATTTTACTAAAATTCG | NEB Express | pMAL-c5X (New England BioLabs, Inc) |
| | pBLS9 | F: PO4-ATGAAAAAAGAATTCATTATGCTTTTACTGTTATTGCAAACA<br>R (SbfI): GAGAGAGACCTGCAGGCTATTTTACTAAAATTCG | NEB Express | pMAL-p5X (New England BioLabs, Inc) |
| | pBLS15 | F (BamHI): CTCTCTGGATCCATGAAAAAAGAATTCATTATGCTTTTACTG<br>R (SalI): CTCTCTGTCGACCTATTTTACTAAAATTCGAACTATTTCTTTGTT | Tuner<br>Tuner pLysS | pGEX-6P-1 (GE Healthcare Life Sciences) |
| *bbb28* | pBLS1 | F: TATAATTTAAAAATAAACTTTAAAAGGATG<br>R: GCAATGGAATTAATCATCAATTAGC | Top10 | pCR2.1 |
| | pBLS3 | F: CACCATGAGTTATTATGTGCTAAGCAAAATATT<br>R: GTTAAATACCGATTAAATATTTATAGATTTCACTAG | BL21(DE3)<br>Rosetta pLysS<br>Tuner<br>Tuner pLysS | pET200 |
| | pBLS8 | F: $PO_4$-ATGAGTTATTATGTGCTAAGCAAAATATTTCTATATTCTGGG | NEB Express | pMAL-c5X |

| | | | | |
|---|---|---|---|---|
| | | R (SbfI): GAGAGAGACCTGCAGGTTA AATACCGATTAAATATTT | | |
| | pBLS7 | F: $PO_4$-ATGAGTTATTATGTGCTAAG CAAAATATTTCTATATTCTG GG<br>R (SbfI): GAGAGAGACCTGCAGGTTA AATACCGATTAAATATTT | NEB Express | pMAL-p5X |
| | pBLS14 | F (BamHI): CTCTCTGGATCCatgagttatt atgtgctaagcaaaatattt<br>R (SalI): CTCTCTGTCGACTTAAATAC CGATTAAATATTTATAGATT TC | Tuner<br>Tuner pLysS | pGEX-6P-1 |
| *bbb28Δ186* | pBLS6 | F: CACCttcaccgaaaagcaattatt agaagattttaatattttcgaa<br>R: GTTAAATACCGATTAAATAT TTATAGATTTCACTAG | BL21(DE3)<br>Rosetta pLysS<br>Tuner<br>Tuner pLysS | pET200 |
| | pBLS12 | F: PO4-ATGAGTTATTATGTGCTAAG CAAAATATTTCTATATTCTG GG<br>R (SbfI): GAGAGAGACCTGCAGGTTA AATACCGATTAAATATTT | NEB Express | pMAL-c5X |
| | pBLS11 | F: PO4-ATGAGTTATTATGTGCTAAG CAAAATATTTCTATATTCTG GG<br>R (SbfI): GAGAGAGACCTGCAGGTTA AATACCGATTAAATATTT | NEB Express | pMAL-p5X |
| | pBLS13 | F (BamHI): CTCTCTGGATCCTTCACCGA AAAGCAATTATTAGAAGAT<br>R (SalI): CTCTCTGTCGACTTAAATAC CGATTAAATATTTATAGATT TC | Tuner<br>Tuner pLysS | pGEX-6P-1 |

### *Expression of Recombinant BB0399 and BBB28*

Several protocols were used to attempt expression of recombinant BB0399 and BBB28.

For the traditional induction, one colony was inoculated into 50 mL Super Broth (SB) + 100 μL 50 μg/mL kanamycin (Kan) and grown overnight at 37°C. Five mL of the starter culture was subcultured into 500 mL SB + 2.5 mL Kan and grown with shaking until $OD_{600}$ was 0.4-0.6. One-hundred μL was removed from each flask, pelleted, and stored at -20°C (pre-induction sample). Isopropyl ß-D-1 thiogalactopyranoside (IPTG) was added to each flask to a final concentration of 0.5 mM. One-hundred μL of induced culture was removed 1 and 4 hours post-induction. After 4 hours, the remaining induced culture was pelleted (6000 x *g*, 10 min) and stored at -20°C. The same protocol was used for cultures grown and induced at 30 and 20°C. In addition, all three protocols were attempted with 0.1 and 1 mM IPTG.

Two auto-induction protocols were used. The first attempt at auto-induction was performed using the Dual Media Set (EB + OB) (Zymo Research) at 37, 30, and 20°C. Protocol II, which is designed for induction of toxic proteins and insoluble proteins, was used. For each temperature, a freshly streaked colony was inoculated into 5 mL EB medium and grown at the appropriate temperature for 8 hours (starter culture). Fifteen mL of pre-warmed EB medium was added with 0.25 mM IPTG to the starter culture and incubated for 16 hours.

The second attempt at auto-induction was performed using Studier's methods at 37, 30, and 20°C (296). Briefly, strains were cultured in ZYM-

5052 (rich, auto-induction medium), MDA-5052 (enriched, fully defined auto-inducing medium), and MD-5052 (minimal, auto-inducing medium) at 37 and 20°C without or with 0.1 mM IPTG for 16 hours.

Drunken induction was also attempted using a protocol was obtained from Dr. Suba Nookala. Briefly, an overnight culture was grown with the appropriate antibiotic. The overnight culture was subcultured (1:100 dilution) into fresh LB with the appropriate antibiotic to an $OD_{600}$ of 1.4-1.8. Cultures were chilled to 8-10°C in an ice-water bath. One mL of culture was removed, pelleted, resuspended in SDS-PAGE sample buffer, and stored at -20°C. IPTG was added to a final concentration of 100 µM and 2% (v/v) ethanol was added. Cultures were induced overnight at 20°C. After induction, the culture was pelleted and resuspended in binding buffer (5 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl, pH 8), 0.1% Triton X-100, 100 µL DNase (10000 U/mL stock), 1 mg/mL lysozyme, 1 mM MgCl2, and 5 mM ß-mercaptoethanol. Samples were homogenized by pipetting for 2-3 minutes. One mL of 50X phenylmethane sulfonyl fluoride (PMSF) was added. The lysate was sonicated on ice and centrifuged at 8000 rpm, 4°°C, 30 min. Supernatants were removed (soluble protein fraction) and both supernatants and pellets were saved and analyzed by SDS-PAGE.

All whole cell lysates (pre- and post-induction) and purified protein fractions were analyzed via SDS-PAGE.

### *Growth Curves*

pBLS4 (*bb0399* in pET200) and pBLS3 (*bbb28* in pET200) in Tuner, Tuner pLysS, and Rosetta pLysS and pBLS9 (*bb0399* in pMAL-p5X) and

pBLS7 (*bbb28* in pMAL-p5X) in NEB Express were grown in LB, LB + 1% glucose, LB + 1% glycerol, SB, ZYM-5052, MDA-5052, MD-5052, and MDG (non-inducing medium) at 20 and 37°C for 140 and 68 hours, respectively, using a Bioscreen C Analyzer (Growth Curves USA). Plates were shaken continuously and $OD_{600}$ was read every hour.

### *Purification of Recombinant Proteins*

Soluble (pET200, pGEX-6P-1 constructs), insoluble (pET200, pGEX-6P-1 constructs), inclusion body (pGEX-6P-1 constructs), cytoplasmic (pMAL-c5X constructs), and periplasmic (pMAL-p5X constructs) fraction was extracted when appropriate per manufacturer's instructions.

Briefly, soluble and insoluble fractions from pET200 constructs were isolated using B-PER II (Bacterial Protein Extraction Reagent; Pierce). Soluble protein fractions were purified with the MagneHis Protein Purification System (Promega) per manufacturer's instructions. Protein fractions where recombinant BB0339, BBB28, or BBB28Δ62 would have been were they expressed in *E. coli* transformed with pMAL-c5X or pMAL-p5X were purified using the New England BioLab's cytoplasmic or periplasmic (osmotic shock) isolation protocol, respectively. Inclusion bodies were purified Protein fractions from *E. coli* transformed with pGEX-6P-1 were purified by resuspending pellets in 1/20 volume PBS. Cells were sonicated on ice to lyse (8 cycles at 40% amp, 20 sec on, 30 sec off). Triton x-100 was added to a final concentration of 1% then incubated for 30 min at RT on an orbital shaker. Samples were centrifuged 5 min, 4°C, 10000 x *g*. Supernatant was removed and both supernatant and pellet were saved.

Inclusion bodies were purified by one of two methods. The first method used the MagneHis Protein Purification System protocol per manufacturer's instructions. The second method is as follows: 100 mL cultures were pelleted and resuspended in 4 mL 20 mM Tris-HCl, pH 8. Cell suspensions were sonicated on ice (4 cycles at 30% amp, 10 sec on, 20 sec off) and centrifuged (20 min, 4°C, 15000 x *g*). Pellets were resuspended in 6 mL ice-cold purification buffer (2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton X-100), sonicated, and centrifuged until the supernatant was clear. The pellet was resuspended in 5 mL solubilization buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine-HCl, 1 mM ß-mercaptoethanol, pH 8) and incubated 60 min, RT, on an orbital shaker. Samples were centrifuged 30 min, 4°C, 15000 x *g*. Prior to analyzing samples by SDS-PAGE, proteins resuspended in guanidine were precipitated and resuspended in urea using a modified protocol (297). Briefly, 200 μL of guanidine extract was added to 1 mL of RT 95% ethanol, incubated overnight at -80°C, then centrifuged at 14000 x *g*, 30 min, 4°C. Supernatant was removed and the pellet was resuspended in 250 μL of -20°C 70% ethanol, vortexed, and centrifuged 14000 x *g*, 10 min, RT. Supernatant and residual ethanol was removed and the pellet was air dried for 15 min. Pellets were resuspended in 50-100 μL 8 M urea. Samples were then mixed with SDS-PAGE loading buffer and incubated at RT for 30 minutes before loading on a gel.

### *Dot Blots*

pGEX dot blots were performed using pBLS14 and pBLS15 in Rosetta pLysS, BL21, Tuner, and Tuner pLysS induced at 20 and 37°C as described

above for the traditional induction and Studier's ZYM-5052 autoinduction medium. After induction, soluble fraction was isolated as described above for pGEX-6P-1 constructs. PVDF membranes were dipped in methanol until transparent then equilibrated in 1X TBS without Tween-20 for 10 minutes. Whatman filter paper was dipped in 1X TBS and placed on a clean weigh boat. The PVDF membrane was placed on the filter paper and quickly dotted with 1 and 2 μL of each supernatant. The membrane was removed from the filter paper and allowed to dry completely. The dried membrane was wet in methanol, briefly washed in 1X TBS, then blocked for 1 h at RT in 5% blocking buffer (1X TBS-T + 5% non-fat dried milk). The membrane was incubated with primary antibody (1:1000 mouse anti-GST, Pierce) for 2 h at RT. The membrane was washed eight times with 1X TBS-T then incubated with secondary antibody (1:10000 mouse anti-IgG, Pierce) for 1 h at RT. The membrane was immediately developed with the Pierce SuperSignal West Pico Chemiluminescent kit per manufacturer's instructions.

Inclusion bodies were also analyzed via dot blot. The same protocol as described above was used with pET200 constructs, 1:2500 rabbit anti-His primary antibody (Thermo Fisher), and 1:10000 rabbit anti-IgG ECL secondary (GE Healthcare).

### *Production of Polyclonal Anti-BBB28 Antibodies*

Using the full-length BBB28 sequence, putative antigenic regions were determined using EMBOSS explorer antigenic tool and the Universidad Complutense Madrid antigenic peptide prediction tool (298). A 411 bp region of the putative exposed region was cloned into a pET200/D-TOPO vector

(Invitrogen) and expressed in *Escherichia coli* λDE3 BL21 Star (Invitrogen). Recombinant protein was purified using the MagneHis Protein Purification System (Promega) per manufacturer's instructions and used to produce polyclonal anti-BBB28 antibodies from 4- to 6-week-old C3H/HeN mice. Purified protein was dialyzed into 1X PBS treated for animal injection. A pre-immune cheek bleed was obtained for each mouse and analyzed via an ELISA using whole-cell *B. burgdorferi* lysates and purified BBB28 peptide. Purified peptide was suspended 1:1 in Alhydrogel adjuvant 2% (InvivoGen) per manufacturer's instructions. Each mouse received 50 µL of the peptide and adjuvant mixture intraperitoneally on day 1, 14, and 28. On day 28, blood was collected via submandibular bleed and analyzed via ELISA to confirm production of antibodies against *B. burgdorferi* whole cell lysate. Mice were then sacrificed, blood was collected, and serum was separated and stored at -20°C.

## *Oxidative Stress*

### *Determining Effects of Preparing Culture for Oxidative Stress Exposure*

*B. burgdorferi* 5A18NP1, an infectious isolate of the B31 type strain (299), was cultured in BSK-II medium with 6% rabbit serum (complete BSK-II) at 34°C + 5% $CO_2$ to a cell density of 5 x $10^7$ cells/mL. Cells were centrifuged, washed, and resuspended in modified BSK-II (no rabbit serum or sodium pyruvate) as previously described (282) with one modification. Resuspended cultures were incubated at 34°C + 5% $CO_2$ for 30 minutes in modified BSK-II prior to ROS exposure. A 10 mL aliquot was removed immediately prior to transfer, immediately after transfer, after a 30-minute

incubation in modified BSK-II, and then 5, 10, 20, 30, and 60 minutes post-incubation.

RNA was isolated using Trizol and isopropanol precipitation. cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) and used in quantitative PCR (qPCR). Transcripts for *bosR* and *napA* were normalized to *flaB* and compared to expression at: 1. Pre-transfer (+pyruvate) baseline, 2. Immediately post-transfer (-pyruvate, 0 min), or 3. 30 minutes post-transfer (-pyruvate, 30 min).

***Oxidative Stress***

*B. burgdorferi* 5A18NP1 was cultured in BSK-II with 6% rabbit serum at 34°C + 5% $CO_2$ to a density of 5 x $10^7$ cells/mL. Cells were centrifuged, washed, and resuspended as described above in modified BSK-II. After a 30 minute acclimation period, a 10 mL aliquot was removed (time 0). One mM $H_2O_2$ or *t*-BHP were added to each culture. Ten mL aliquots were removed 5, 10, 20, 30, and 60 minutes post-treatment. Cultures were maintained in an ice-water bath until centrifuged. Pellets were washed three times with 1X PBS and stored at -80°C until RNA extraction.

***Quantitative Real-Time PCR***

RNA was isolated using Trizol and isopropanol precipitation. cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) and used in quantitative PCR (qPCR). qPCR was performed for *flaB, bosR, napA, sodA, bb0399,* and *bbb28* (primers in Table 3.2) using SYBR Green Master Mix (Bio-Rad) and the following protocol: 1 cycle at 95°C for 3 min, 40 cycles at 95°C for 30 sec and 56°C for 1 min. Melt curves were 1 cycle at 95°C for 1

min, 1 cycle at 56°C, 10 seconds at 56-95°C with 0.5°C temperature change per cycle. Transcripts were normalized to *flaB*.

Table 3. 2. qRT-PCR primers used in this study**.**

| Amplicon target | Primer sequence |
|---|---|
| *flaB* | F: GGGTCTCAAGCGTCTTGG<br>R: GAACCGGTGCAGCCTGAG |
| *bosR* | F: AGCTTGGCTTCCACAATAGC<br>R: TTTGTTTCCCAGTTTTCTCCA |
| *napA* | F: GAAAGCATTGTTTGCAGTCT<br>R: AAAACAATCGCAATTTTCAA |
| sodA | F: AGAACTTTAAGGCCAGGAAA<br>R: CAATACTAACCATGCCCAAC |
| bb0399 | F: TGCACTAAATCTTGGAGCAGAA<br>R: GCTCCGCTTTCTTTTAAAAATTC |
| bbb28 | F: TTCCAACGGCAATCCAATA<br>R: GCGCCTTTTTCGATAAGTGA |

## Results and Discussion

### *In silico Analysis of BB0399 and BBB28*

BB0399 is a small protein of 219 amino acids (Fig. 3.1A, Fig. 3.2A). It has four predicted Ank repeats and four predicted regions of low complexity. The presence of low complexity regions near the C- and N-termini suggest BB0399 may be a promiscuous protein with numerous binding partners (300). BBB28 is 414 amino acids with two predicted Ank repeats (residues 326-355 and 359-389) and two transmembrane domains residues 5-27 and 40-62) (Fig. 3.1B, Fig. 3.2B). Interestingly, the transmembrane domains predicted for BBB28 in *B. burgdorferi* B31 are not predicted in *B. bavariensis* PBi, a related Lyme disease *Borrelia*. In *B. bavariensis* PBi, the N-terminal region is annotated as a low complexity region.

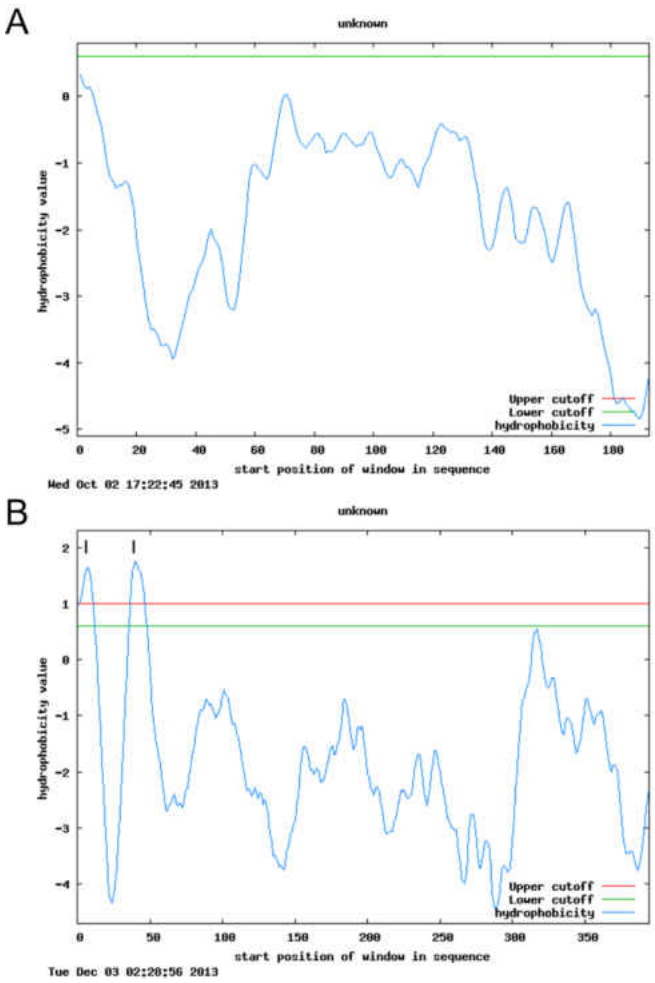



Figure 3. 2. Hydrophobicity plots by TopPred for BB0399 and BBB28. A. Hydrophobicity plot for BB0399 predicts a soluble protein. B. Plot predicts two transmembrane domains, indicated by the two hydrophobic regions near the N-terminal.

Hydrophobicity plots (TopPred Version 1.10, (301)) suggest BB0399 is a soluble protein with the Ank domain (residues 73-199) representing the least hydrophilic region (Fig. 3.2A). TopPred also predicted two transmembrane domains in BBB28 (Fig. 3.2B). Again, there is a sharp increase in hydrophobicity within the Ank domain (residues 326-389) compared to the rest of the protein (excluding the transmembrane domains).

Protein BLAST (Blastp, NCBI) searches of BB0399 and BBB28 from *B. burgdorferi* B31 produced no significant matches when *Borrelia* spp. were excluded, except the conserved Ank domain structure. The Ank domain aligned with a variety of organisms from bacteria *(*e.g. *Wolbachia*, *Bacillus, Lentisphaerae*) to insects (e.g. *Culex* spp., *Vollenhovia* spp., *Athalia* spp.) to trees and grasses (e.g. *Prunus* spp., *Zea* spp.) to mollusks (*Mizuhopecten* spp.) (Fig. 3.3, 3.4; Tables 1 and 2 in Appendix).

Phyre2 is a tool that predicts protein structure through alignments and domain homology to create 3D models (302). The most likely models for BB0399 and BBB28 were produced using an intensive search (Fig. 3.5). For BB0399, 97% of the residues were modeled at greater than 90% confidence and for BBB28, 71% of the residues were modeled at greater than 90% confidence.

### *Cloning and Recombinant Protein Expression*

Numerous inductions for recombinant BB0399 and BBB28 failed regardless of induction conditions or expression vector used. After one failed induction, plasmid was extracted from cultures and sequenced. These results revealed a stop codon resulting in premature termination of both proteins. Since both *bb0399* and *bbb28* were sequenced prior to expression and no stop codons were present, this suggests *bb0399* and *bbb28* are toxic to *E. coli* and were mutated to prevent expression. The presence of an antibiotic gene on each expression plasmid and culturing under selective pressure prevented *E. coli* from removing the plasmid containing *bb0399* or *bbb28*.

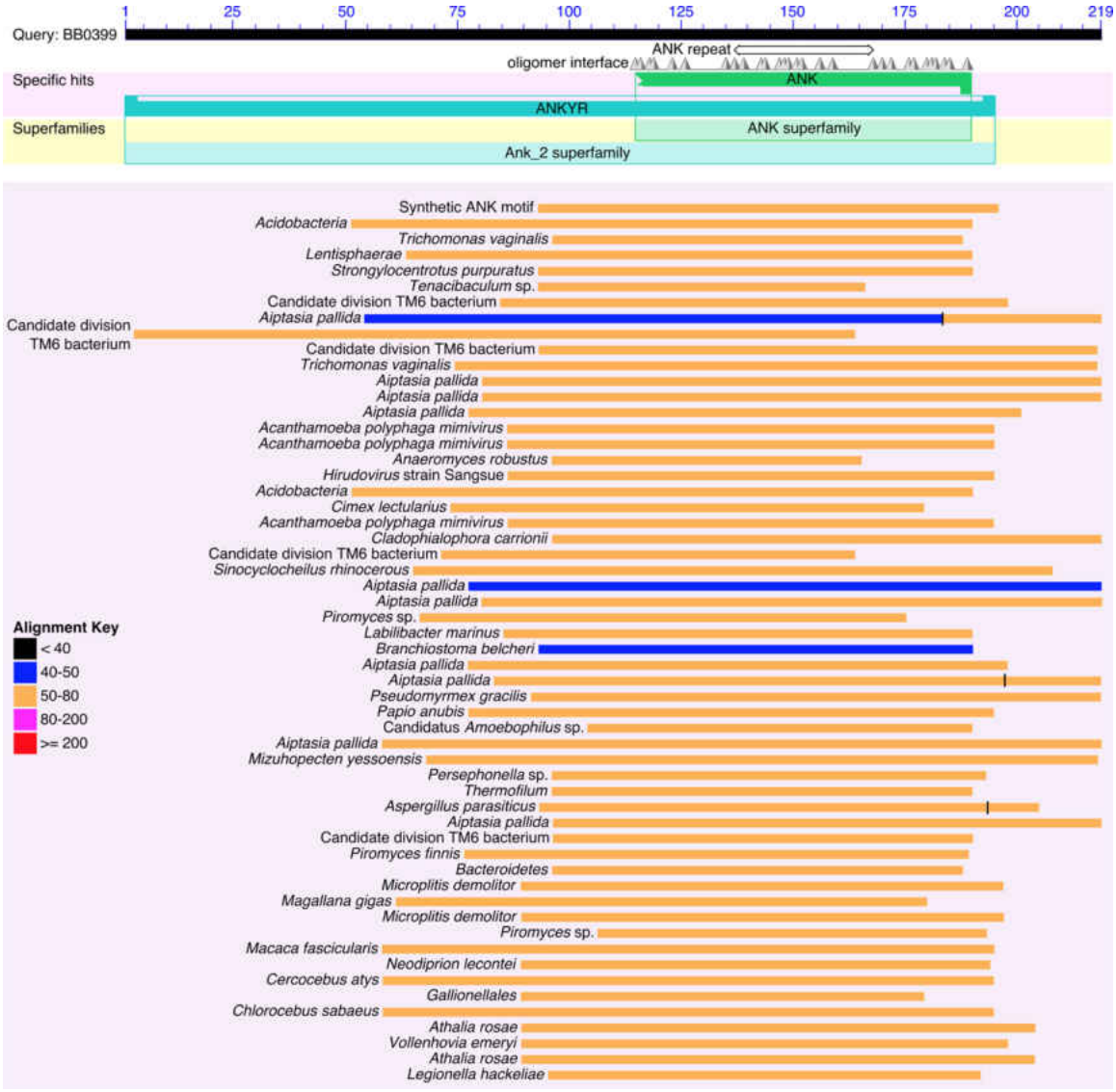



Figure 3. 3. Alignment for BB0399 adapted from NCBI protein BLAST results.


Figure 3. 4. Alignment for BBB28 adapted from NCBI protein BLAST results.

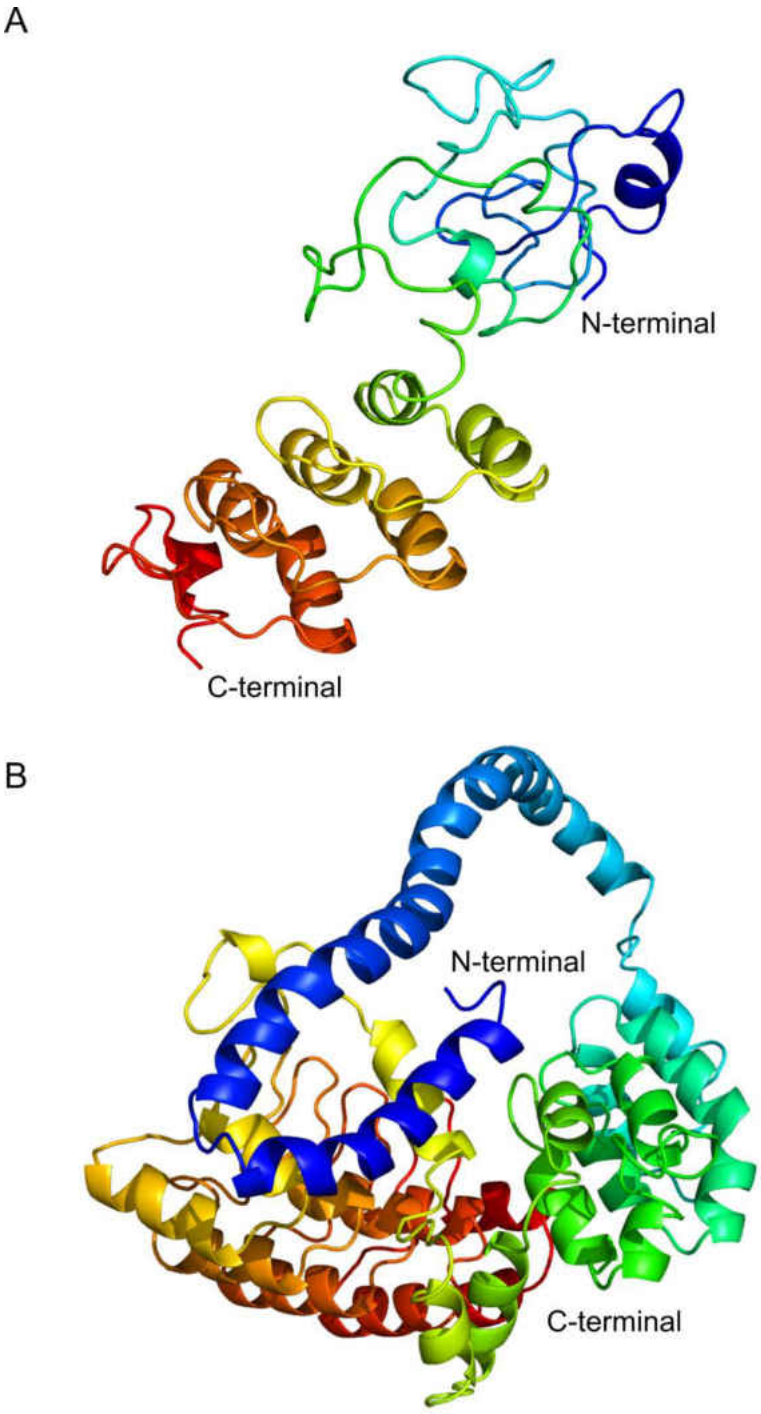



Figure 3. 5. Predicted structures of BB0399 (A) and BBB28 (B) by Phyre2. Each structure is colored from the N-terminal (blue) to the C-terminal (red). A. Ank repeats show the most secondary structure (green to red helix-turn-helix motifs near the C-terminal). B. Putative transmembrane domains are shown in blue at the N-terminal. Ank repeats are in light green to red.

A handful of induction attempts produced promising SDS-PAGE, dot blot, and Western results but were unable to be confirmed by additional methods. For example, a dot blot for the glutathione *S*-transferase (GST) tag found on recombinant protein expressed from pGEX vectors and 6X-Histidine (His) tag found on recombinant protein expressed from pET vectors showed some strains produced GST or His after induction but not before induction (Fig. 3.6). The negative dots did have detectable protein when stained with either Coomassie blue or Ponceau S. These results confirmed inducing expression of recombinant BB0399 or BBB28 was possible with some strains. An obvious drawback of dot blots is the inability to determine whether antibodies are detecting the purification tag alone or tagged recombinant protein. Western blots with the same samples were not positive, even for the tag alone.

The goal for producing recombinant protein was to produce antibodies against BB0399 and BBB28 for use in *in vivo* experiments. In a final attempt to obtain antibodies to BB0399 and BBB28, a non-coding, highly antigenic peptide was cloned into a pET200 TOPO vector (Invitrogen) (Fig. 3.7). The peptides chosen covered several putative antigenic regions and at least part of the peptide was expected to be available for antibody binding under native folding conditions. There was a 50% success rate as only the *bbb28* peptide was successfully cloned and expressed. ELISA results from mice injected with *bbb28* peptide showed antibodies were produced that recognized whole *B. burgdorferi* lysate.

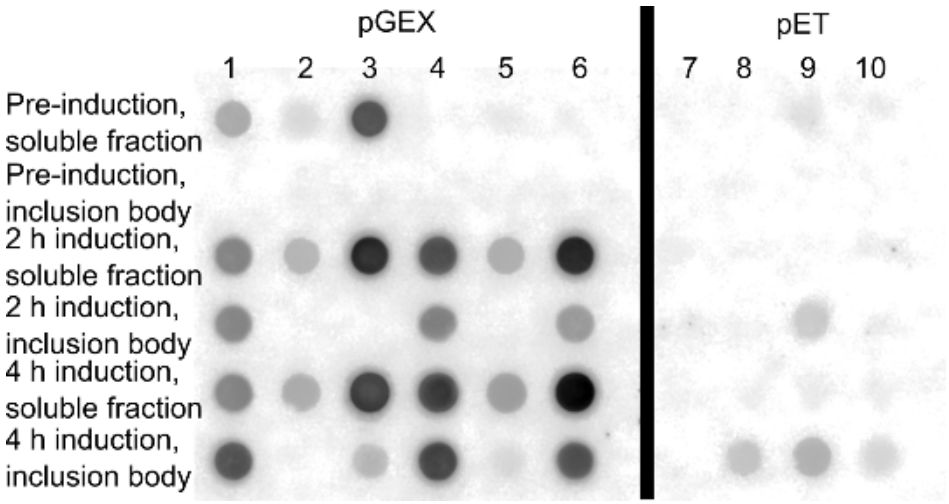



Figure 3. 6. Dot blot for GST (pGEX) and His (pET) in soluble and inclusion body fractions isolated from Tuner and Tuner pLysS *E. coli* strains. Three *E. coli* strains transformed with pGEX constructs (columns 1-3) had detectable GST in the soluble fraction throughout the experiment, while the other three strains (columns 4-6) showed detectable GST after induction. Detection of His was weak throughout the experiment. Column 1 – *E. coli* Tuner *bb0399* in pGEX, 2 – Tuner pLysS *bb0399*, 3 – Tuner *bbb28*, 4 – Tuner pLysS *bbb28*, 5 – Tuner with truncated *bbb28* (*bbb28*Δ186), 6 – Tuner pLysS *bbb28*Δ186, 7 – Tuner *bb0399* in pET200, 8 – Tuner pLysS *bb0399*, 9 – Tuner *bbb28*, 10 – Tuner pLysS *bbb28*.

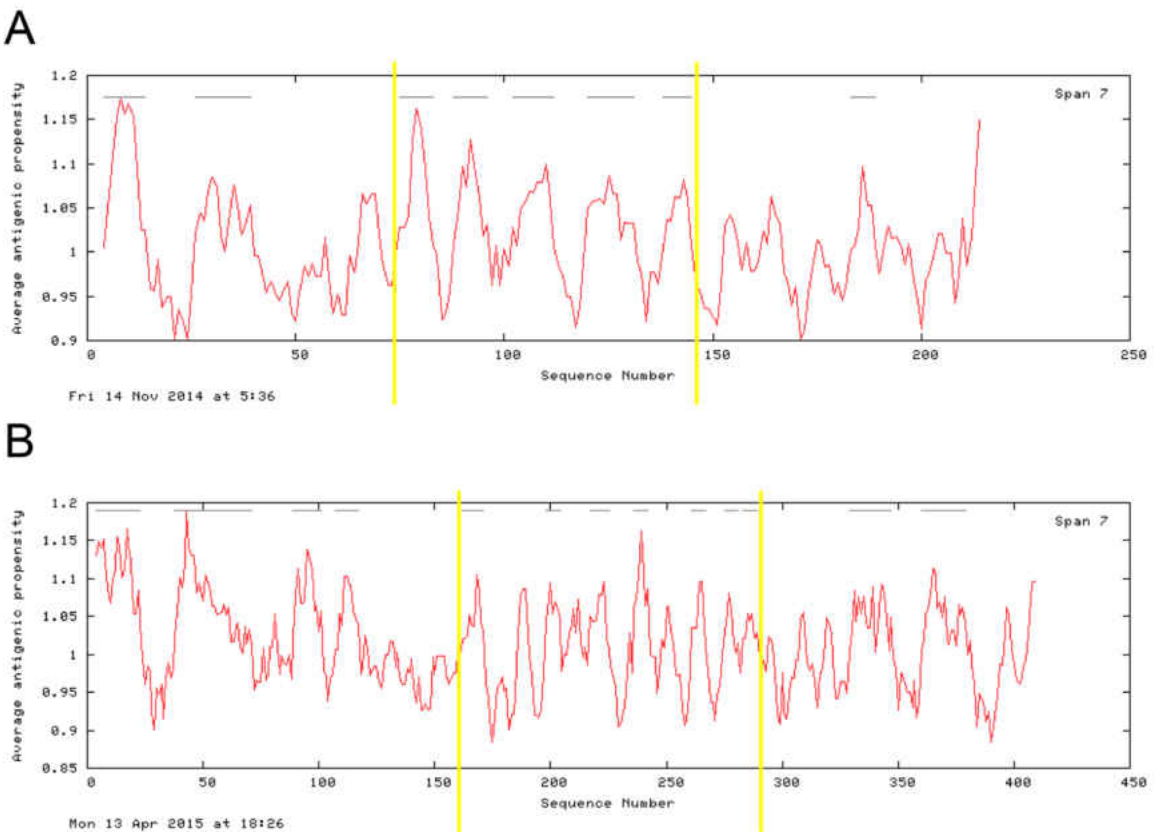
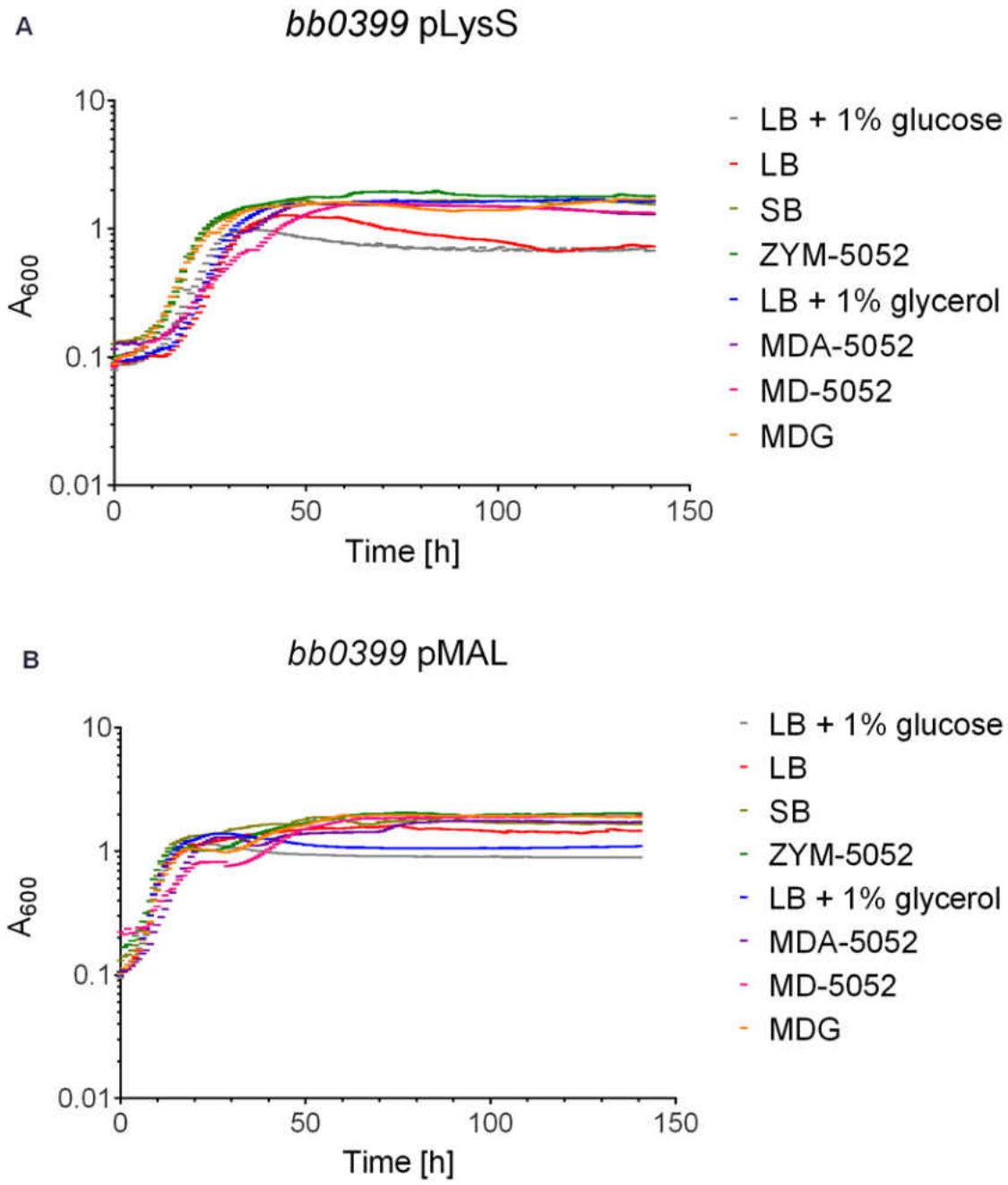



Figure 3. 7. Predicted antigenic peptides. (A) BB0399 peptides predicted to antigenic are shown as horizontal grey bars at the top of the plot. A region covering five putative antigenic peptides, marked by yellow vertical lines, was cloned into a pET200 TOPO vector. (B) BBB28 peptides predicted to be antigenic. A region covering seven putative antigenic peptides, marked by yellow vertical lines, was cloned into a pET200 TOPO vector.

### *Growth Curves*

With evidence that both BB0399 and BBB28 are toxic to *E. coli* and after several failed attempts to express recombinant protein, auto-induction medium was used. Smaller yields of toxic proteins can be obtained under certain conditions, such as inducing at lower temperatures or at higher cell density. During auto-induction, starter cultures are grown in non-inducing media containing a *lac* repressor, such as excess glucose. Cultures are then subcultured into auto-inducing media, which also contains an appropriate *lac* repressor as well as an additional carbon source. As the culture expands, glucose depletes, cells begin using the second carbon source, such as lactose, *lac* is no longer repressed, and recombinant protein is expressed. Growth curves were performed to determine the best non-inducing medium, auto-inducing medium, as well as the optimal length for induction.

All strains grew faster at 37°C (Fig. 3.9) compared to 20°C (Fig. 3.8). MDG was found to be the best non-inducing medium. Strains grew well in all auto-inducing media but for induction at 20°C, 16-24 hours was not enough time for cultures to reach stationary phase. Cultures required at least 40 hours to reach stationary phase at 20°C. As a result, prior cultures were harvested too early. Unfortunately, longer induction times in auto-inducing media failed.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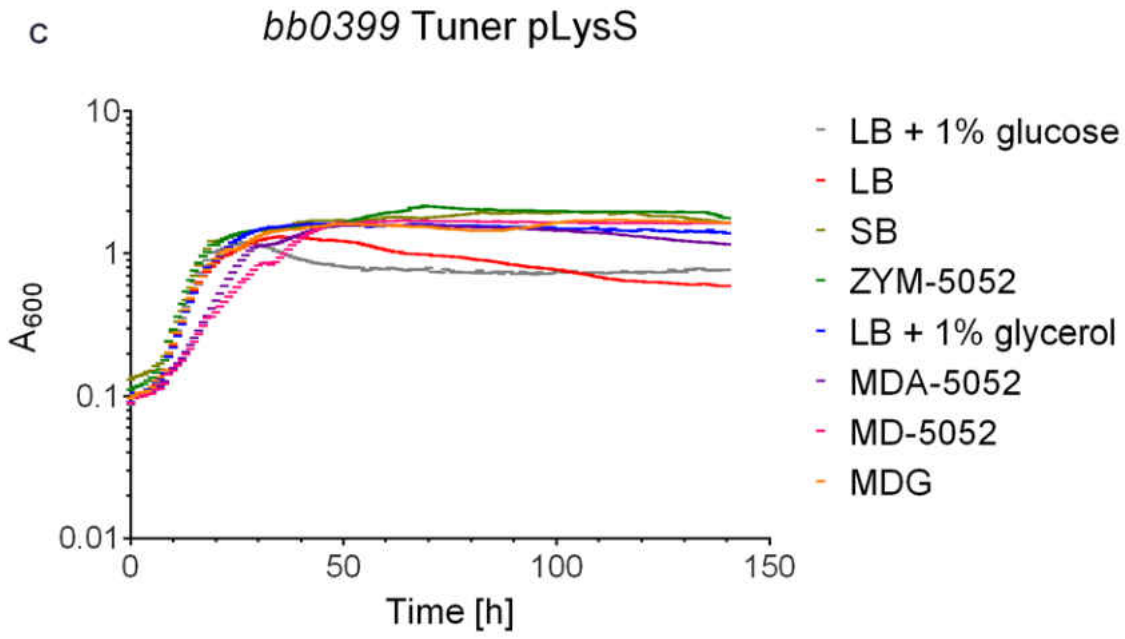
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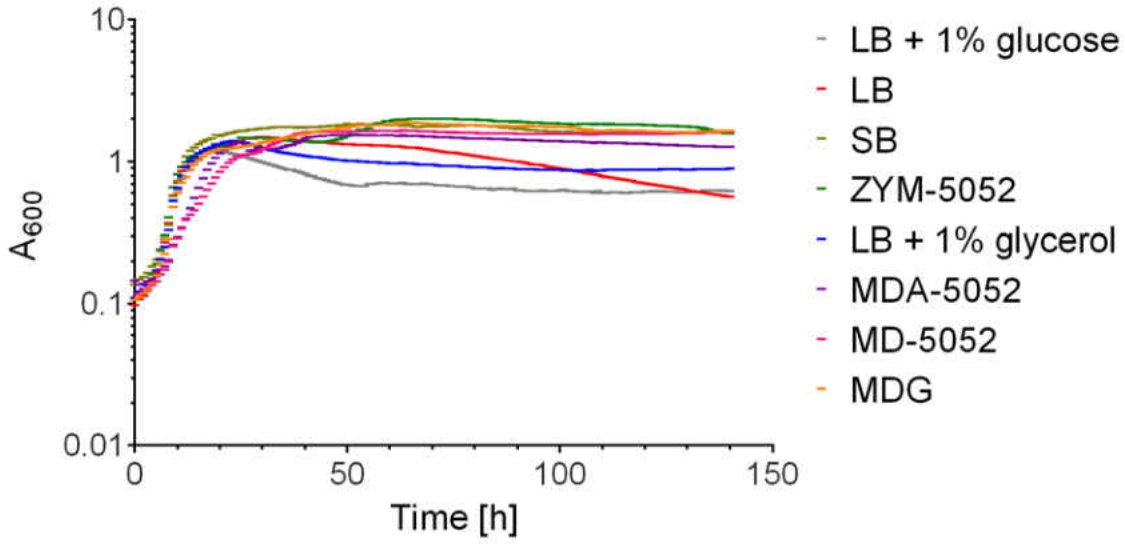
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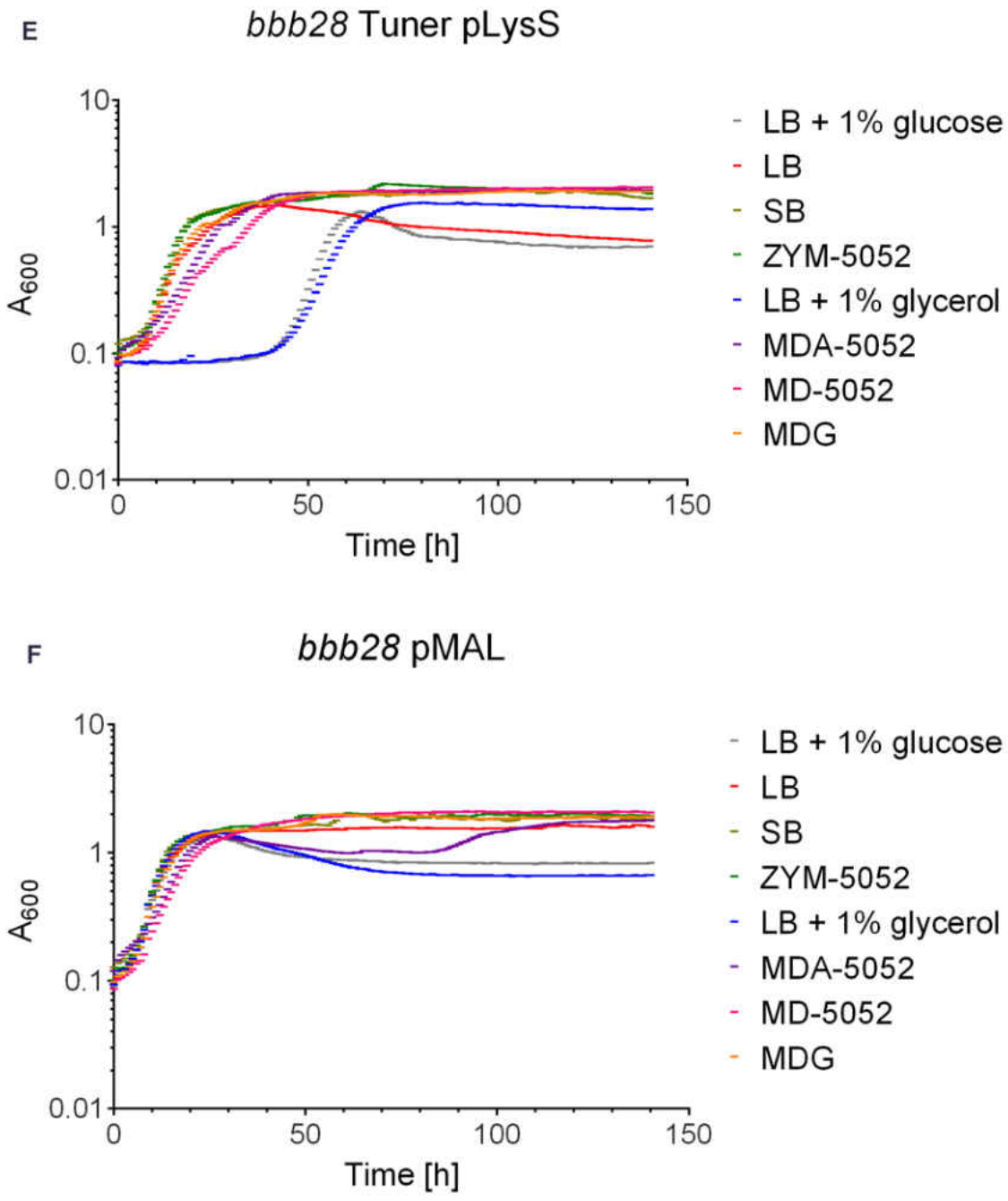
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ZYM-5052
LB + 1% glycerol
MDA-5052
MD-5052
MDG
F
bbb28 pMAL
A600
10
1
0.1
0.01
0
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LB
SB
ZYM-5052
LB + 1% glycerol
MDA-5052
MD-5052
MDG

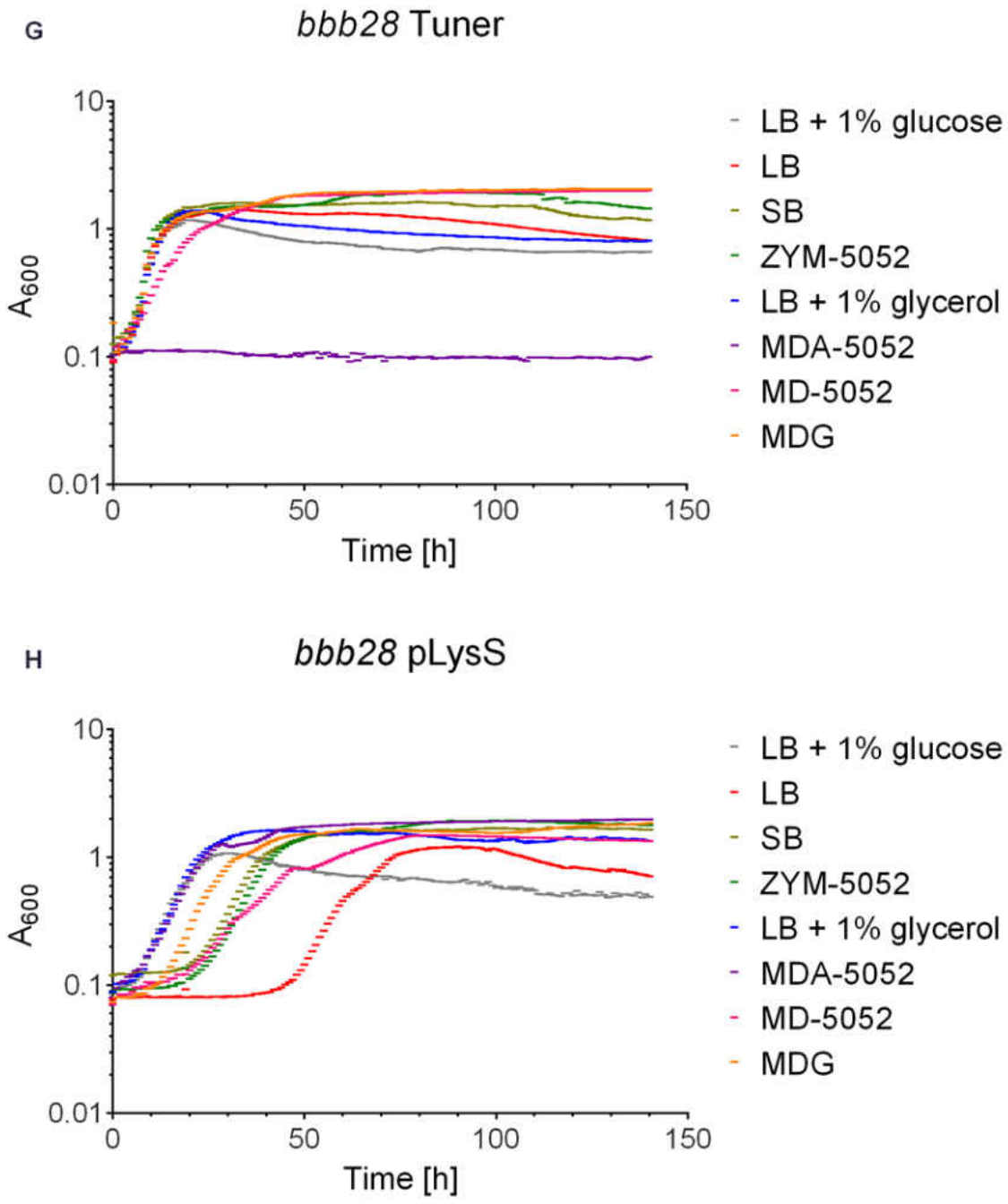
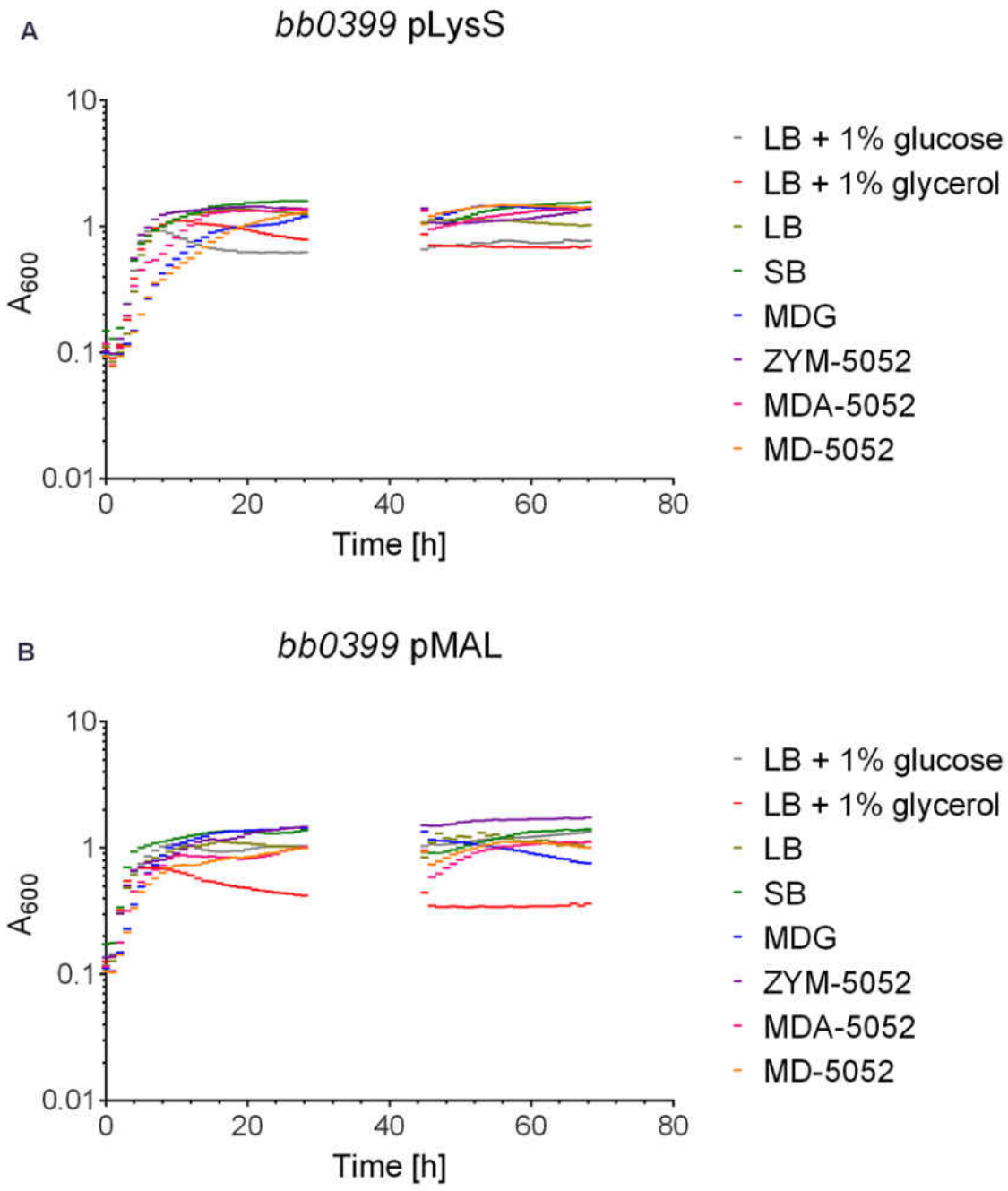



Figure 3. 8. Growth curves for *E. coli* transformed with one of eight plasmids and cultured in eight types of media at 20°C. For all strains, MDG medium was found to be the best non-inducing medium. For auto-induction, all strains grew well in all auto-inducing media but required much longer incubations than recommended (30-40 hours to reach stationary phase compared to the 16-24 hours recommended).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bb0399 pLysS
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MDG
ZYM-5052
MDA-5052
MD-5052
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bb0399 pMAL
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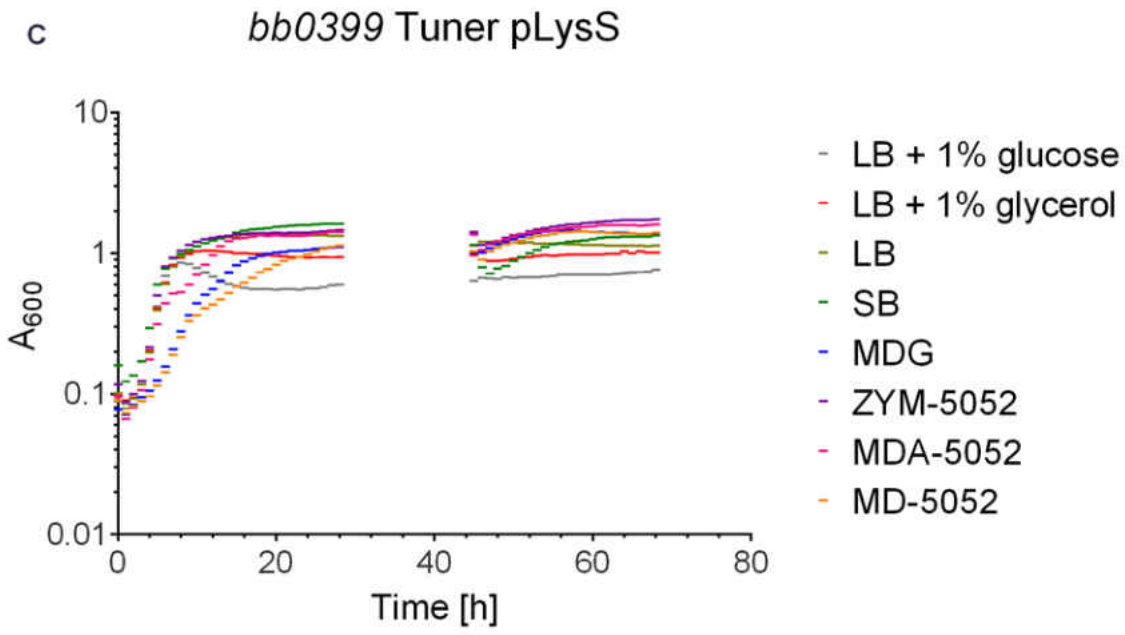
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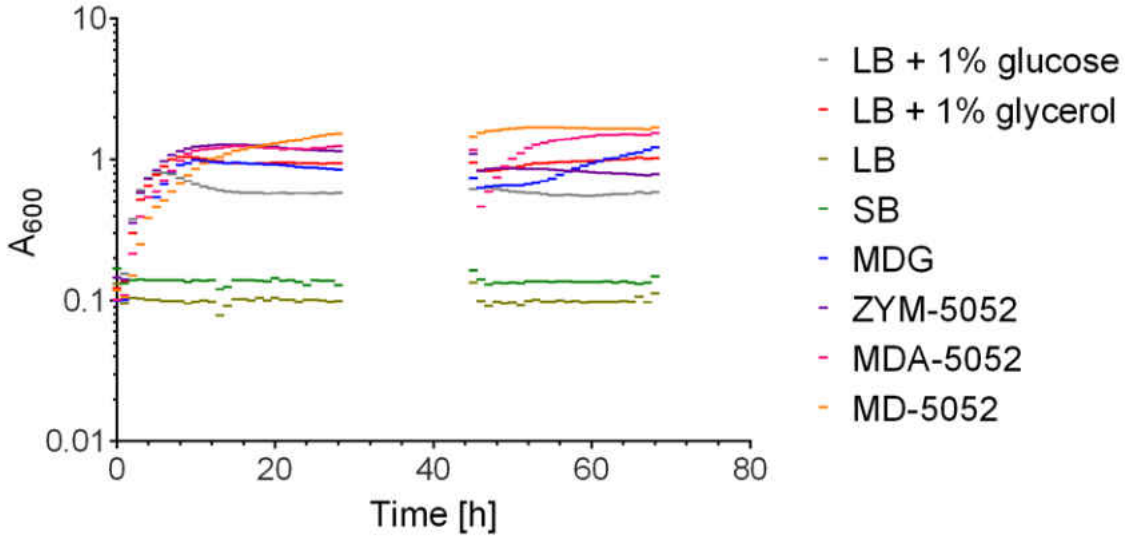
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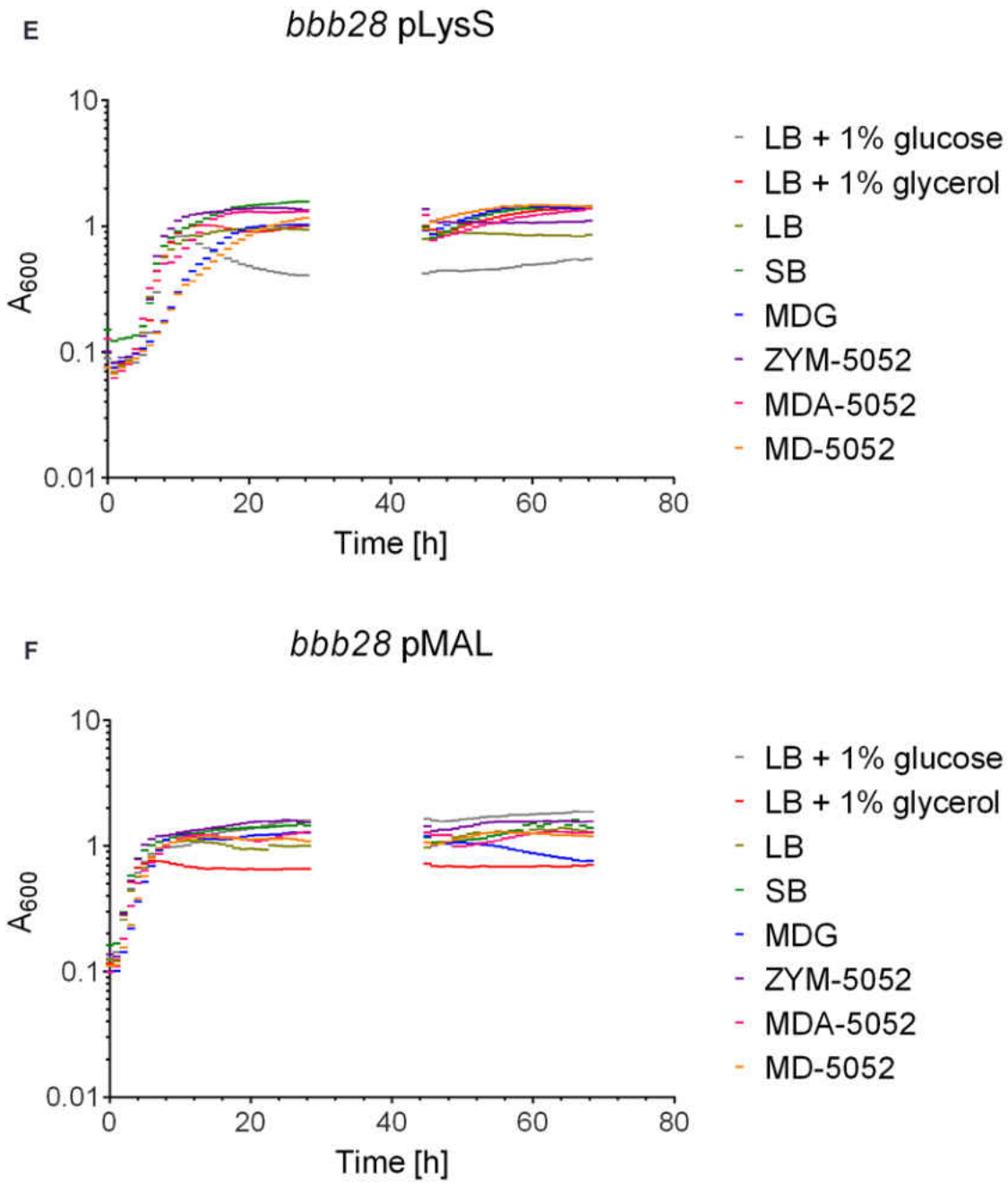
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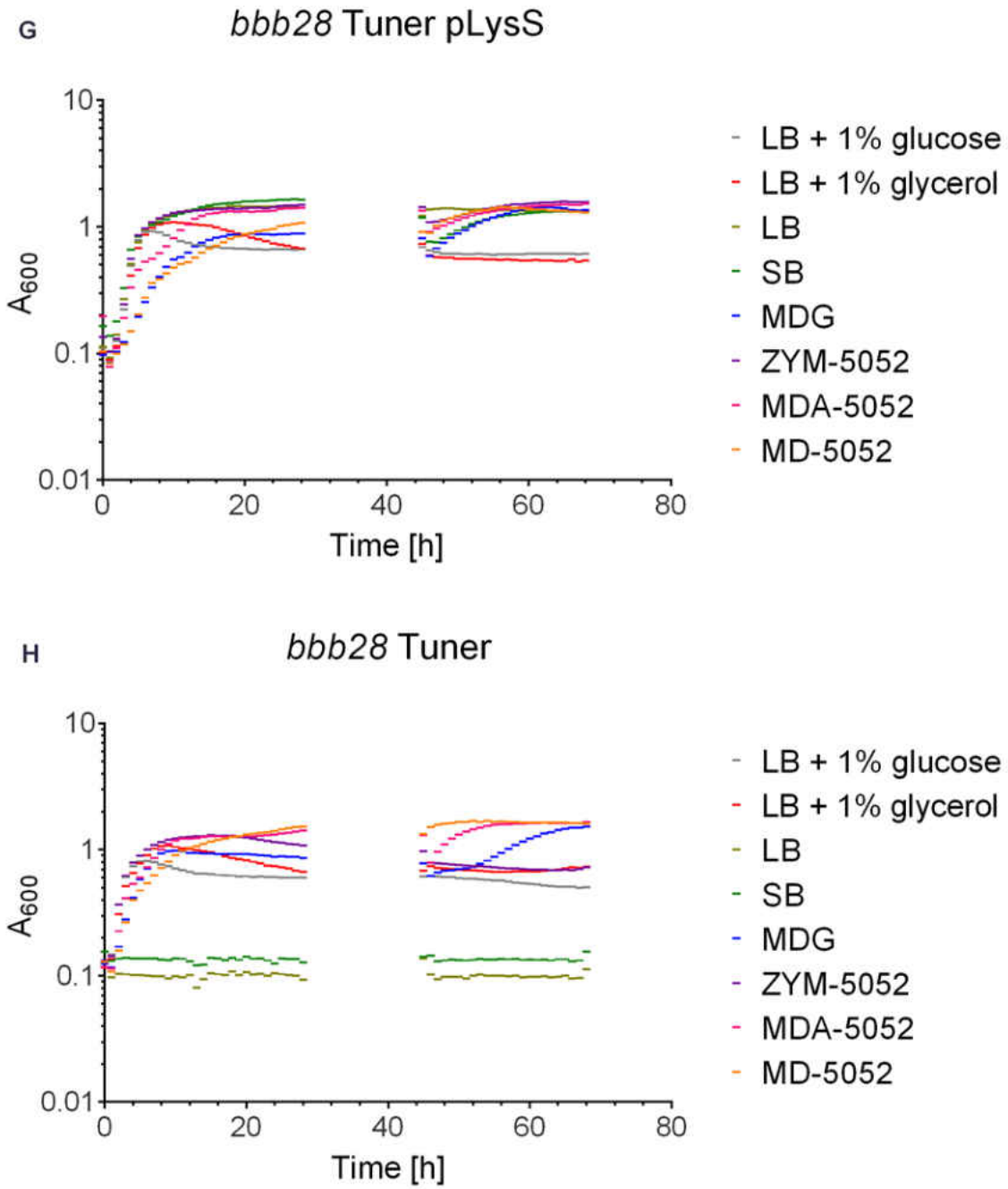


Figure 3. 9. Growth curves for *E. coli* transformed with one of eight plasmids and cultured in eight types of media at 37°C. For unknown reasons, no data points were recorded between 29 and 44 hours resulting in missing data points. In addition, two strains (bb0399 and *bbb28* Tuner) did not grow in LB or SB. These wells were likely not inoculated with the starter culture.

Troubleshooting failed recombinant protein expression can be a maddening experience as there are several factors (e.g. codon optimization, properties of the chosen protein, vectors, expression strains, and induction parameters) that can affect expression.

### *Codon Usage*

*Borrelia* tends to use rare codons much more frequently. To circumvent this problem, use of an *E. coli* strain capable of overproducing tRNAs for these rare codons, such as a Rosetta pLysS *E. coli* strain is usually recommended for routine expression of *Borrelia* proteins. Codon optimization (replacing rare codons with common codons) is also performed and recommended for genes encoded with several rare codons. Codon optimization was not performed as neither BB0399 nor BBB28 possess a high percentage of rare codons compared to other *B. burgdorferi* proteins. Expressing recombinant BB0399 and BBB28 using any *E. coli* transformed with pLysS was deemed sufficient.

### *Recombinant Protein Properties*

Transmembrane domains can also impede recombinant protein expression. Two approaches are available to express recombinant integral proteins: 1. Clone the protein without the transmembrane domains or 2. Express recombinant protein in an *E. coli* strain designed to express membrane proteins, such as the OverExpress C41(DE3) or C43(DE3) *E. coli* strains (Lucigen).

### *Vector and Expression E. coli Choice*

If you adore customization, cloning is for you. Every piece of a vector can be customized to fit your needs. The choice of purification tag can help increase solubility of recombinant proteins, making them easier to purify. There are also non-bacterial expression systems available, which could eliminate several problems associated with expressing bacterial proteins.

### *Induction Parameters*

If using an IPTG-inducible promoter, the presence of repressors in culture medium, temperature before and during induction, duration of induction, cell concentration at the time of induction, and IPTG concentration used can all affect expression.

## *Oxidative Stress*

### *Preparing Cultures for t-BHP Exposure Affects Gene Transcription*

Care must be taken when determining the effect of ROS on *B. burgdorferi*. In addition to the unusual physiology (e.g. little intracellular iron, no oxidative phosphorylation pathway), the medium used to culture *B. burgdorferi*, BSK-II, possesses several components capable of scavenging ROS. The one component found to be a potent scavenger of ROS is pyruvate, which is added in physiological excess in BSK-II (282). Initially, $H_2O_2$ or *t*-BHP were added immediately after transferring *B. burgdorferi* to pyruvate-free BSK-II. Results from these experiments and knowledge of bacterial stress responses suggested *B. burgdorferi* was experiencing stress unrelated to the presence of ROS. To test this hypothesis, expression of two control genes, *bosR* and *napA*, was evaluated under three culture conditions.

Expression of *bosR* and *napA* was found to significantly increase immediately after transfer to pyruvate-free medium (-pyruvate, 0 min; Fig. 3.10). A 30-minute incubation in pyruvate-free BSK-II returned expression of *bosR* and *napA* to levels seen prior to resuspension (-pyruvate, 30 min; Fig. 3.10). Based on these results, cultures were washed and resuspended in pyruvate-free BSK-II and incubated for 30 minutes prior to the addition of either $H_2O_2$ or *t*-BHP.

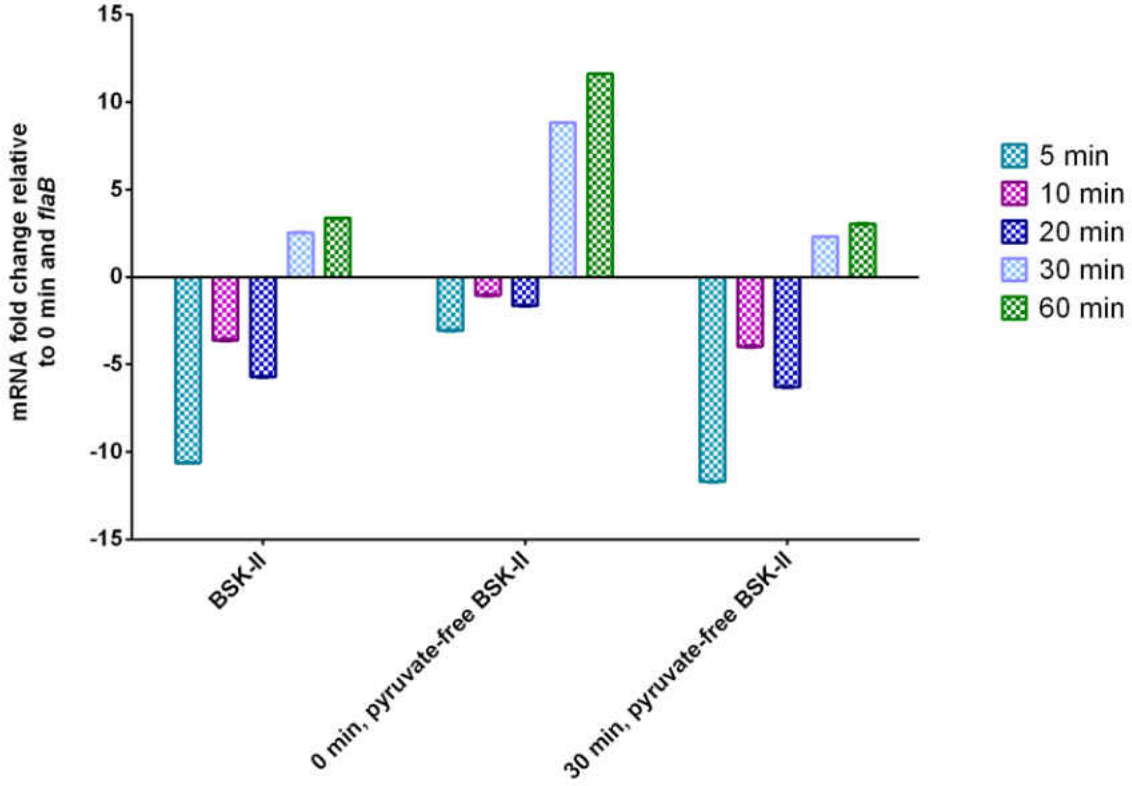


Figure 3. 10. Preparing *B. burgdorferi* for exposure to ROS appears to affect the expression of *bosR*. A similar trend was observed with *napA*.

### *Transcription of bbb28, but not bb0399, Appears to Increase in the Presence of t-BHP*

To determine the expression of *bb0399* and *bbb28* in response to *t*-BHP, *B. burgdorferi* was cultured in complete BSK-II, resuspended in pyruvate-free BSK-II, incubated for 30 minutes then treated with 0 or 1 mM *t*-BHP. Aliquots were removed immediately prior to ROS addition and then at 5, 10, 20, 30, and 60 minutes after addition of ROS. qRT-PCR data were unavailable for some time points and genes. However, expression of *bbb28* was highest 5 minutes after the addition of *t*-BHP and a time-dependent decrease in transcription was observed (Fig. 3.11).

Whether the changes in *bbb28* transcription observed after the addition of *t*-BHP are due to the presence of ROS is still unknown. Undoubtedly, centrifugation, resuspension, and placement into different medium affect gene transcription in *B. burgdorferi*. To better elucidate the effect of ROS on *bbb28* transcription, a promoter fusion or reporter strain is required. A reporter strain would allow direct observation of promoter activation in response to ROS.

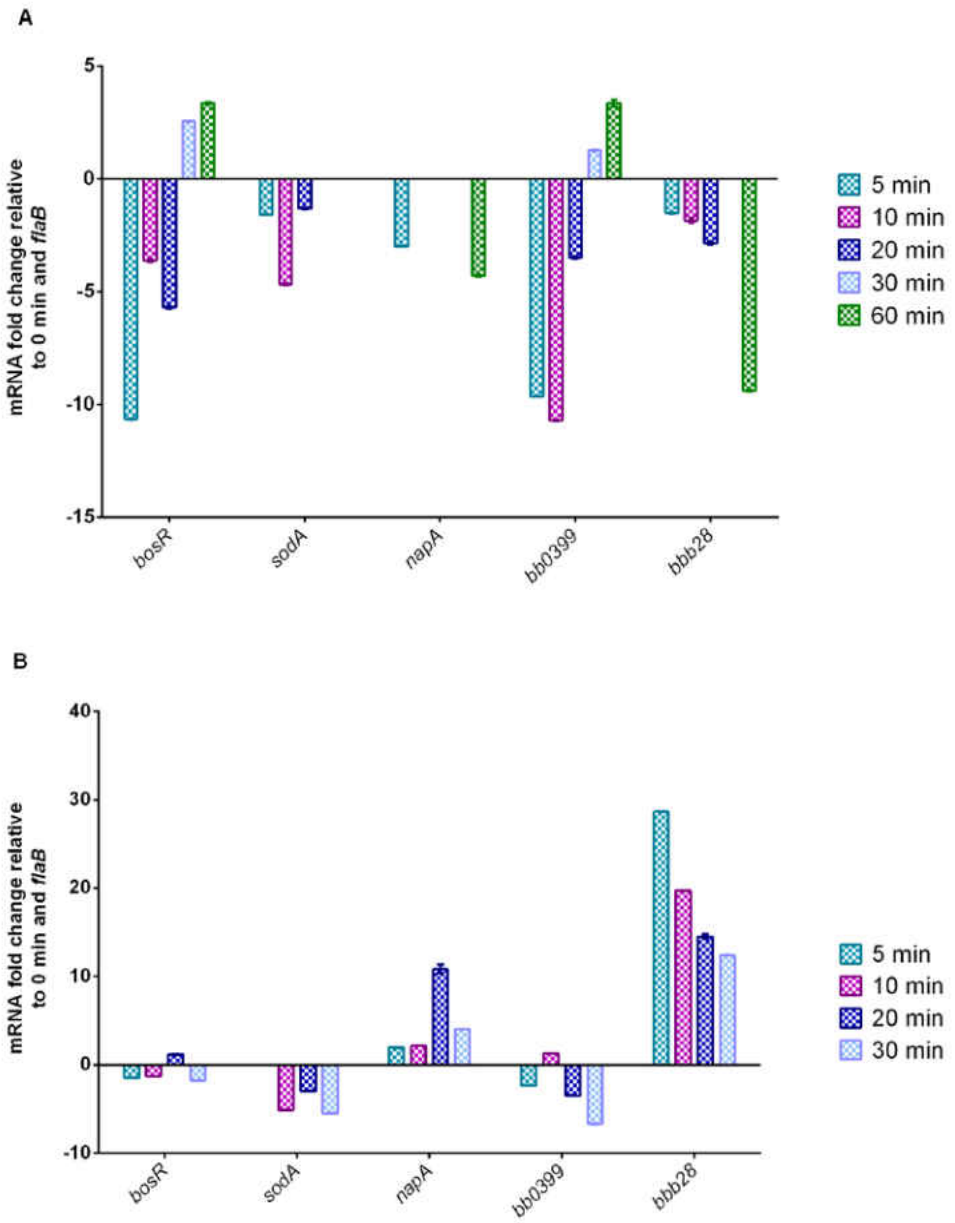


Figure 3. 11. Transcription of *bbb28* increases after exposure to 1 mM *t*-BHP. (A) Transcription of *bosR, sodA, napA, bb0399,* and *bbb28* in the absence of *t*-BHP. Transcription of *bosR* and *bb0399* increased, while bbb28 transcription decreased over time in the absence of *t*-BHP. qRT-PCR failed for *sodA* at 30 and 60 minutes, *napA* at 10, 20, and 30 minutes, and *bbb28* at 30 minutes. (B) Transcription in the presence of 1 mM *t*-BHP. A strong increase in *bbb28* transcription was observed 5 minutes after addition of 1 mM *t*-BHP. qRT-PCR failed for *sodA* at 5 minutes and all transcripts at 60 minutes.

## Conclusions

This project provided ample learning opportunities to learn new methods and troubleshoot methods. Unfortunately, I was unable to provide additional information regarding the function of BB0399 or BBB28. Both *bb0399* and *bbb28* are transcribed and others have shown at least BBB28 is translated (286). The placement of *bb0399* on the chromosome (22) as well as the inability to knockout *bb0399* (105) strongly suggests this is an essential gene. Similarly, the placement of *bbb28* on cp26, an essential plasmid for *B. burgdorferi*, suggests the function of BBB28 is important, though not essential for survival *in vitro* or in mice as mutants have been recovered (105). In addition, understanding the function of *bb0399* and *bbb28* would add to our understanding of *B. burgdorferi*, regardless of whether this information would move from the realm of basic science into translational research.

# CHAPTER 4

# *IN VITRO* CHARACTERIZATION OF *BORRELIA MIYAMOTOI* AND THE EFFECT OF AGE ON *BORRELIA MIYAMOTOI* INFECTION IN WILD-TYPE C3H/HEN MICE

## Introduction

*Borrelia miyamotoi* is a relapsing fever spirochete (20, 21, 85, 86, 175, 303–309) carried by the same *Ixodes* spp. that vector and carry species of the *Borrelia burgdorferi sensu lato* (*s.l.*) complex in North America, Europe, and Asia (20, 310). While first characterized in 1995 by Fukunaga et al (20), *B. miyamotoi* was likely first reported in 1987 when Lane and Burgdorfer (46) noted transovarial transmission of spirochetes in *I. pacificus* and attributed the spirochetes as *B. burgdorferi*.

In general, Lyme disease *Borrelia* are associated with hard-shell ticks and relapsing fever *Borrelia* are associated with soft-shell ticks. In addition to *B. miyamotoi*, there are currently four documented exceptions. *B. recurrentis* is a louse-borne relapsing fever spirochete presently endemic predominantly to sub-Saharan Africa. *B. theileri* is the causative agent of bovine borreliosis and is transmitted by *Rhipicephalus microplus*, a hard-shell tick that parasitizes livestock (17). *B. lonestari* and *B. turcica* are genetically similar to relapsing fever borreliae and carried by the hard-shell ticks *Amblyomma americanum* and *Hyalomma aegyptium*, respectively (18, 19). The status of *B. lonestari* and *B. turcica* as an animal or human pathogens is unknown.

*B. recurrentis* and *B. miyamotoi* are the only relapsing fever *Borrelia* transmitted by a vector other than *Ornithodoros* that are confirmed to cause human disease (82, 87–93, 311). Despite the different vectors, pathology of *B. miyamotoi* disease (BMD) or hard tick-borne relapsing fever (HTBRF) appears to be similar to tick-borne relapsing fever (TBRF) with immunocompetent patients reporting mild, recurrent but self-resolving febrile episodes (Fig. 4.1) (87–93). Due to the generally mild nature of *B. miyamotoi* infection, much of the attention given to *B. miyamotoi* has been from researchers intrigued by this unusual pathogen.

There are several questions surrounding *B. miyamotoi*. So far, all of these questions stem from our current knowledge of *B. burgdorferi* and the relapsing fever *Borrelia*. Without an identified natural reservoir for *B. miyamotoi*, we are limited in our ability to study the physiology of *B. miyamotoi* as well as transmission and maintenance in *Ixodes* in the laboratory. An initial hurdle to studying *B. miyamotoi* was the inability to culture *B. miyamotoi in vitro*. Despite initial reports of growth in Barbour-Stoenner-Kelly-II (BSK-II) medium (20, 21, 175, 200), more recent attempts to culture *B. miyamotoi* had been unsuccessful (310, 312). In 2014 and 2015, however, a major breakthrough was made when two *in vitro* culture systems were developed (172, 313) and subsequently modified for use in our laboratory (314). Additionally, to study transmission and maintenance in *Ixodes* requires an adequate animal model. Immunocompromised mice are able to maintain an infection (176, 315). However, an immunocompetent mouse model would allow for more accurate investigations of the acquisition

of *B. miyamotoi* by *Ixodes* as well as allow further study into the immunological responses by hosts.

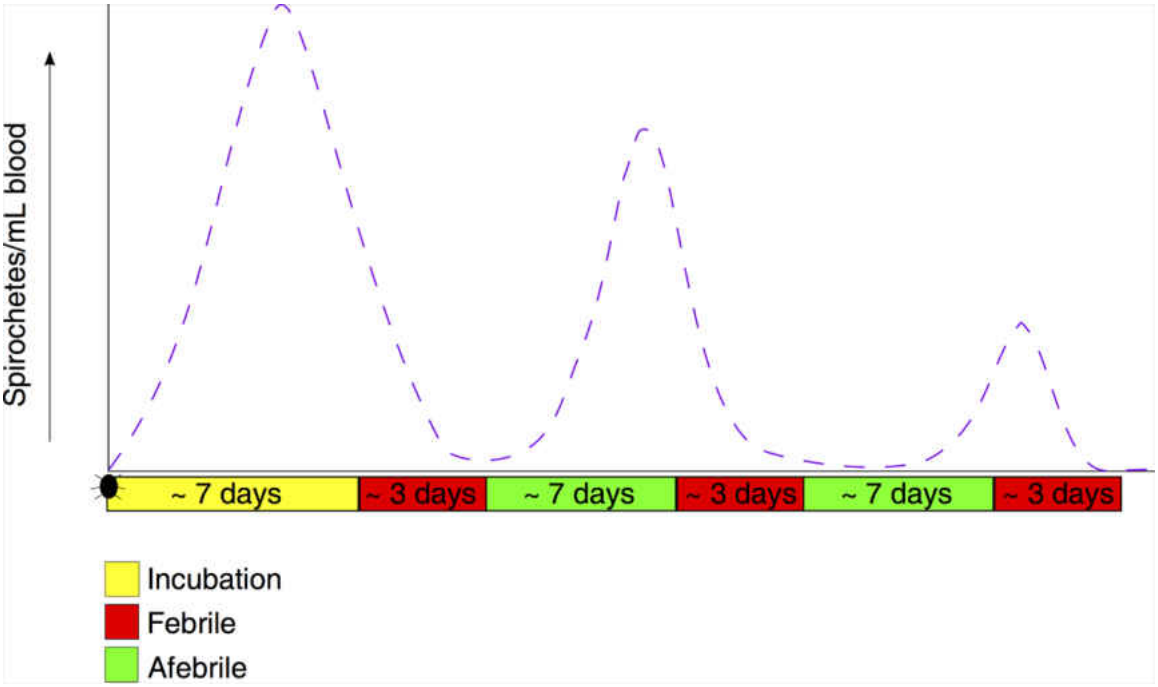


Figure 4. 1. Depiction of the general course of tick-borne relapsing fever. Approximately seven days after an infected tick bites, an increase is observed in the concentration of spirochetes in blood. Concomitant with a peak in spirochete concentration is the onset of the first febrile episode. As spirochetes are cleared from the blood, antigenic variation of variable membrane proteins (VMPs) allows a second population of spirochetes to expand. This leads to another, yet slightly less severe, peak in spirochetemia and febrile episode. This cycle of clearing and expansion occurs for an average of three to ten times.

### *An Overview of the Complement System*

The complement system, composed of the classical, lectin, and alternative branches, is a crucial component of the immune system (Fig. 4.2). Components of complement continuously circulate in blood making complement one of the first lines of defense against pathogens. Complement initiates an immune response by: 1. Triggering phagocytosis through opsonization, 2. Mediating inflammation through the release of chemotactic peptides, and 3. Lysing cells via the membrane attack complex (MAC, also called the terminal complement complex or TCC) (Fig. 4.2) (316).

The classical pathway is generally mediated by non-specific antibodies, immunoglobulin G (IgG) or IgM, binding a bacterial antigen. Importantly, recent studies have shown *Borrelia*-specific IgM is produced by a subset of B cells during infection and play a crucial role in clearing *Borrelia* (125–127, 129–131, 317–319). The C1 complex, composed of C1q, C1r, and C1s, forms upon recognition of bound IgG or IgM. C1 cleaves C2 (C2a, C2b) and C4 (C4a, C4b). C4b covalently binds the target's cell surface and complexes with C2a to form C3 convertase, which cleaves C3 into C3a and C3b. C3b covalently binds the target cell surface (opsonization, facilitates phagocytosis of foreign cells and cellular debris), while C3a remains soluble to act as a mediator of inflammation. C5 convertase forms when C3b binds C3 convertase. Not surprisingly, C5 convertase cleaves C5 into C5a, a soluble inflammatory mediator, and C5b. C5b binds the target cell surface and C6 forming C5b6, which binds C7 (C5b-7) then C8. The C5b-8 complex binds C9 (C5b-9) and facilitates polymerization of several additional C9 proteins.

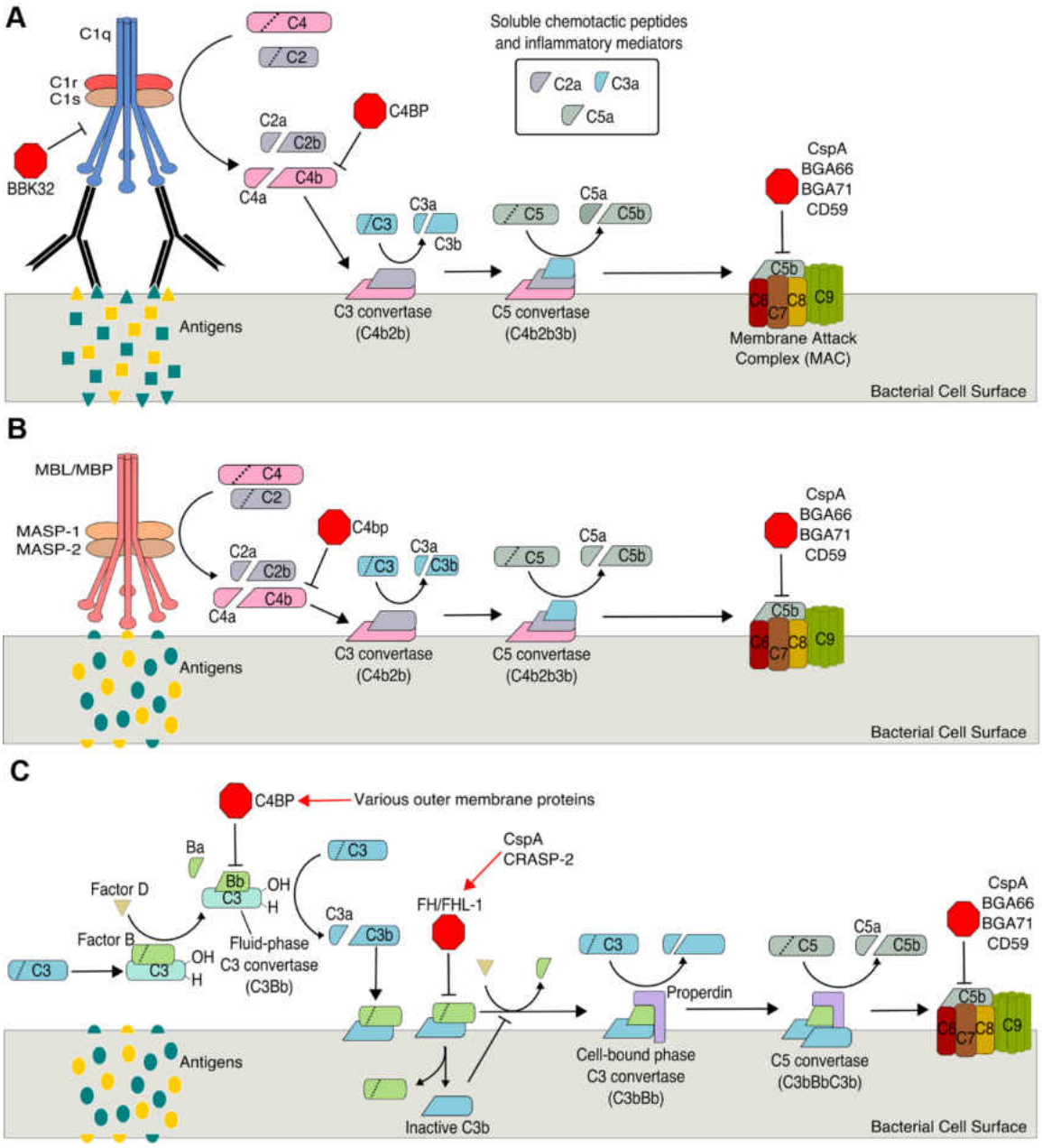


Figure 4. 2. Activation and regulation of complement pathways relevant to *Borrelia* spp. infection. Points of complement inhibition utilized by Borrelia spp. are indicated by red octagons. (A) Classical pathway. (B) Mannose-lectin pathway. (C) Alternative pathway. Red arrows indicate borrelial proteins that interact with a host regulatory protein.

These polymerized C9 proteins form the transmembrane pore of the MAC allowing an influx of extracellular fluid and subsequent lysis of the target cell.

The lectin pathway is very similar to the classical pathway, differing only in the initiation steps. The lectin pathway is typically initiated through mannose-binding lectins, a group of pattern recognition receptors (PRRs) on host cells, binding specific sets of carbohydrates on foreign cells (pathogen-associated molecular patterns, PAMPs). The lectin and classical pathways converge at the cleavage of C2 and C4 by different mechanisms. In the lectin pathway, C4 and C2 cleavage occurs through mannose-binding lectin-associated serine proteases (MASPs) (316).

Like the classical and lectin pathways, the alternative pathway forms a C3 convertase, C5 convertase, and results in the formation of the MAC. Unlike the classical and lectin pathways, the alternative pathway may not require antibody-antigen or PAMP-PRR interactions for activation. Rather, this pathway is initiated through hydrolysis of C3 to C3($H_2O$), which is thought to occur continuously at low levels. The pathway is propagated through interactions with bacterial antigens or a lack of host surface markers (e.g. sialic acid, glycosaminoglycans, sulfated polysaccharides) (316).

Factor B, after binding C3($H_2O$), is cleaved by factor D into Ba and Bb resulting in C3($H_2O$)Bb, the fluid-phase C3 convertase (cleaves C3 to C3a and C3b). C3b binds the bacterial cell surface where it complexes with additional factor B. Factor D again cleaves factor B, which results in the second, predominant and cell-bound C3 convertase (C3bBb). This cell-bound C3 convertase is stabilized by properdin (C3bBbP). Binding of additional C3b

to C3 convertase results in the formation of C5 convertase (C3bBbC3b), which cleaves C5 and initiates the formation of the MAC as described above.

### *Inhibition of the Mammalian Complement System by Borrelia and Ixodes*

Regulation of complement is critical for the survival of host cells (320, 321). Numerous mechanisms have evolved in hosts to prevent aberrant activation of complement on host cells including the use of complement regulatory factors and host cell surface components (e.g. sialic acid). Pathogens that inhibit host complement use mechanisms that are inextricably tied to host regulatory processes. *Borrelia* use several native proteins to inhibit complement (i.e. factor H-binding proteins or CRASPs, p43, BBK32, BGA66, BGA71, CD59-like protein) (322). The following sections focus on the complement regulators factor H (FH), factor H-like protein-1 (FHL-1), factor I (FI), C4-binding protein (C4bp), and CD59.

At least for Lyme borreliae, resistance to complement varies by strain and species (115, 323–327). Roughly 10% of *B. burgdorferi s.s.* are serum-resistant and 90% are intermediately resistant to serum; 75% of *B. afzelii* isolates are resistant, 25% are intermediate; 100% of *B. garinii* isolates are sensitive (specifically, OspA serotypes 3, 5, 6, 7); *B. bavariensis* (formerly *B. garinii* OspA serotype 4) is intermediately resistant. To the best of our knowledge, similar comparisons of multiple strains and species have not been published for relapsing fever *Borrelia*, though complement resistance is not universal among relapsing fever species among the strains observed. Resistance to complement is important for the transmission, survival, and dissemination of some *Borrelia* spp. in mammalian and rodent hosts and

reservoirs (328). Infectious strains of *Borrelia* are masters of complement evasion due to the native anti-complement proteins some possess and the ability all infectious strains possess to co-opt tick and host complement regulatory proteins.

### *Factor H, Factor H-Like Protein 1 (FHL-1), and Factor I*

FH is an ubiquitous 150-kDa soluble protein produced by diverse cell types throughout the human body (e.g. hepatic cells, fibroblasts, monocytes, endothelial cells) (329). FH consists of 20 short consensus repeats, while FHL-1 is a truncated variant of FH consisting of the FH N-terminal short consensus repeats 1 through 7. Both FH and FHL-1 are major direct regulators of the alternative complement pathway. In addition, FH and FHL-1 can directly regulate the classical and lectin pathways, though the regulatory roles in these pathways are minor compared to other classical and lectin regulatory mechanisms. Regulation is achieved through the recognition of self and non-self molecules via domains located on the C- and N-terminals, respectively (330–332). The C-terminal discriminates self from non-self through interactions with sialic acids, glycosaminoglycans, and sulfated polysaccharides, which are typically found only on host cells (333–337). FH binds self-molecules with high affinity to prevent activation of complement. FH regulates the classical and lectin pathways by acting as a co-factor for FI. In this capacity, FH facilitates the serine protease activity of FI in cleaving and inactivating C3b. The alternative pathway is regulated through FH targeting factor Bb, which prevents the formation of fluid-phase C3 convertase and promotes decay ("decay acceleration activity") of C3 and C5

convertases (338). For comprehensive reviews of FH and FHL-1 see references (329, 338, 339).

### *Factor H-Binding Proteins and CRASPs*

Interactions with FH are the best-studied mechanism for *Borrelia* complement inactivation and complement resistance is correlated with binding FH (114). *Borrelia* spp. bind FH and/or FHL-1 through various native proteins collectively termed factor H-binding proteins (FHBPs) or Complement Regulator-Acquiring Surface Proteins (CRASPs) (115–117). CRASPs can be grouped by their ability to bind only FH or both FH and FHL-1 as well as the species specificity of binding (that is, whether a FHBP can bind FH from only one or several host species) (115, 117): CRASP-1 (CspA) and CRASP-2 (CspZ) bind both FH and FHL-1, while CRASP-3 (ErpP), CRASP-4 (ErpC), and CRASP-5 (ErpA) bind only FH. CRASPs bind soluble FH and maintain it in an active conformation thereby allowing FH to inhibit completion of the complement response (i.e. MAC formation).

Several relapsing fever spirochetes bind FH *in vitro* (115, 340–345). Two FHBPs, FhbA and BhCRASP-1, have been identified in *B. hermsii* strains YOR and HS1, respectively (346, 347). However, binding FH is not as important for relapsing fever spirochetes to establish infection as it is for Lyme disease *Borrelia* (122, 123). Further supporting the non-essential nature of binding FH, Woodman et al. (122) found that despite FhbA being surface-exposed and strongly binding FH *in vitro*, only 16% of *B. hermsii* recovered from the blood of infected mice had detectable levels of bound FH.

### *C4b-Binding Protein*

C4b-binding protein (C4bp) has regulatory roles in all three pathways, though is the major regulator of the classical and lectin pathways. C4bp facilitates inactivation of C4b (classical, lectin) and fluid-phase C3b (alternative) by binding C4b, displacing C2a, and facilitating FI-mediated inactivation of C3 and C5 convertases (348).

Some Lyme and relapsing fever *Borrelia* spp. bind human and various animal C4bp (114, 342, 343, 349, 350). A comprehensive analysis identified outer surface proteins associated with C4bp including OspA, Vlps, Vmps, and several unidentified outer surface proteins (351). Other studies, however, have observed no binding of C4bp by *Borrelia* spp. (114, 344, 352). These contradictory data may be due to differences in experimental design including the use of different strains, growth medium, temperatures, growth phases, and the use of recombinant versus native human C4bp. A putative C4bp receptor, p43, has been identified in *B. burgdorferi s.l.* (349). The relapsing fever spirochetes *B. recurrentis* and *B. duttonii* produce CihC, a surface lipoprotein homologous in sequence and function to fibronectin-binding proteins of other relapsing fever spirochetes, which also binds C4bp (342).

### *FHBP, C4bp, and Borrelia Niche*

Resistance to complement is positively correlated to the infectivity of some *Borrelia* strains (327). With a higher resistance to complement, the more likely a bacterium can survive, disseminate, and proliferate. Co-opting tick proteins will protect spirochetes during the initial stages of transmission

and dissemination but sustained dissemination requires *Borrelia* to resist complement via its own native mechanisms.

This leads to the question of how complement sensitive strains can cause infection. An interesting hypothesis was developed regarding complement resistance and spirochete niche when a relationship was noted between binding of the complement inhibitors, C4bp and FH (79, 349, 350). Neurotropic strains (e.g. *B. bavariensis, B. garinii*, *B. turicatae*, *B. duttonii*, and to a lesser extent *B. hermsii*) do not have to be highly resistant to complement in immunoprivileged sites, such as the central nervous system. Finding neurotropic species strongly bind C4bp and very weakly bind FH and FHL-1, while species that are not neurotropic bind C4bp but preferentially bind FH and/or FHL-1 supports this hypothesis (349). Alitalo et al. (353) did find *B. garinii* strains isolated from neuroborreliosis patients not only express FHBPs not expressed by strains cultured *in vitro* for an extended time, but the FHBPs also bind FH. This implies complement-resistance, though this was not reported and one of the isolates (LU59) was later reported to be highly but not completely sensitive to complement (354). It is possible strong binding of FH is an artifact seen *in vitro*, similar to that observed with relapsing fever spirochetes (see section: Factor H binding proteins and CRASPs). Thus, binding FH is not required for neurotropic strains. Perhaps C4bp is sufficient to prevent complement activation during migration of neurotropic species from the site of inoculation to immunoprivileged sites. On the other hand, binding FH may be important for neurotropic strains to resist complement during migration and the incomplete sensitivity observed by

Sandholm et al. (350) may be due to *in vitro* culturing resulting in the population losing its ability to bind FH. It could also be that neither C4bp nor FHBPs play a role in complement-sensitive borreliae disseminating and a novel mechanism is utilized by complement-sensitive strains.

### *CD59-Like Protein*

Little information is available regarding the CD59-like protein of *B. burgdorferi*. Pausa et al. (355) demonstrated an increase in serum sensitivity and MAC formation in a serum-resistant *B. burgdorferi* isolate treated with anti-CD59 antibodies compared to the control treated *B. burgdorferi* and the serum-sensitive *B. garinii* isolate. In eukaryotic cells, CD59 is a surface-exposed membrane protein that prevents C9 polymerization and thus the formation of the MAC (19,20). Still, it is not clear *Borrelia* possesses a protein homologous to mammalian or rodent CD59. While human anti-CD59 antibodies bound a surface-exposed integral membrane protein (29 kDa), this protein has never been identified though several known borrelial proteins can and have been ruled out based on molecular weight (e.g. BGA66, BGA71, OspA, OspB, OspC) (322). Given the demonstrated complement-resistance conferred by this unknown borrelial protein, more attention should be given to identifying and clarifying the role this protein plays in complement resistance.

### *Complement Inhibition by Ixodes and Ornithodoros Salivary Proteins*

A large number of proteins with a vast array of functions have been identified in the saliva of feeding *Ixodes* spp. with more being identified and characterized (106, 107, 356–358). While the details and mechanisms for

some of these proteins remain to be elucidated, the beneficial nature of *Ixodes* salivary proteins to spirochete transmission and survival has been established (359–363, 274, 364, 365). *Ixodes* saliva contain adaptive and innate immunomodulatory and anti-complement proteins (106, 108–113). A recent study demonstrated changes in the salivary protein profile over the course of a feeding, which has implications for the efficacy of the host immune response at the feeding pit and for transmitting spirochetes (357). Currently, several members of the anti-complement family of proteins have been characterized from *I. scapularis*, *I. ricinus,* and *I. persulcatus* including Salp15, Salp20, Isac, Irac I, Irac II, and Ixac-B1, -2, -3, -4, -5.

Salp15 is able to inhibit both adaptive and innate immune responses (366, 367). Salp15 binds OspC, which both serum-resistant and serum-sensitive *B. burgdorferi s.l.* produce, to inhibit deposition of the MAC and block the recognition and binding of antibodies to OspC (361, 368–370). In addition, Salp15 expression increases when ticks are infected with *B. burgdorferi* (361). Interestingly, mice passively immunized with anti-Salp15 antibodies were protected from infection with *B. burgdorferi* (371).

Salp20 inhibits the alternative complement pathway through binding properdin, which prevents stabilization of C3 convertase and propagation of the alternative pathway (113, 372, 373). In addition, Salp20 enhances the activity of factor H to inhibit the alternative pathway (113). Incubating a serum-sensitive *B. garinii* strain with Salp20 protected the strain from complement activation and lysis (372). The mechanism(s) by which Salp20 confer(s) protection to *B. garinii* is unknown.

The Isac-like family of proteins includes Isac (*Ixodes scapularis* anticomplement), Irac I (*I. ricinus* anticomplement), Irac II, and Ixac-B1 through -5 (*I. ricinus* anticomplement). Proteins in this family are similar in function to Salp20 (110, 112, 374). Inhibition of the alternative complement pathway is achieved through targeting C3 convertase via interactions with properdin, as Salp20 does, and by preventing factor B from binding C3b or displacing factor B from C3 convertase.

*Ornithodoros* salivary gland extracts also possess proteins that inhibit the host immune response (118). To date, however, one complement inhibitor has been identified and characterized from one *Ornithodoros* spp. *O. moubata*, found in Africa, is the vector of the relapsing fever spirochete *B. duttonii* (119). *O. moubata* complement inhibitor (OmCI) is a lipocalin that binds to and prevents cleavage of C5 (120, 121). OmCI was found to be effective at inhibiting C5 cleavage in different mammalian and rodent hosts (121). It is unknown if OmCI protects *B. duttonii* or if homologous proteins are found in other *Ornithodoros* spp.

**Purpose**

In this study, we investigated the acquisition of *B. miyamotoi* by larval *I. scapularis* and found *B. miyamotoi* is efficiently acquired by uninfected larval *I. scapularis* and maintained through the molting process. Unlike other relapsing fever *Borrelia* (75), *B. miyamotoi* does not appear to be specific to an *Ixodes* spp. In addition, we found *B. miyamotoi* binds human factor H *in vitro* and a potential effect of mouse age on the duration of infection in immunocompetent C3H/HeN.

## Methods

### *B. miyamotoi*

An original Japanese strain of *B. miyamotoi*, FR64b, was kindly sent from Dr. Robert Gilmore (Centers for Disease Control, Fort Collins, CO) (chromosome sequence available under NCBI Reference Sequence ID NZ_CP004217.2).

### *Culture Conditions*

*B. miyamotoi* FR64b and mouse blood were cultured in MKP-F medium at 34°C with 5% $CO_2$ in capped Falcon tubes (8 or 16 mL), 1 mL deep-well 96-well plates sealed with silicone mat lids, or microcentrifuge tubes (1.5 or 2.0 mL) (314). Rifampicin (50 μg/mL) and phosphomycin (20 μg/mL) was added when appropriate. For growth curves, *B. miyamotoi* was cultured for four days in MKP-F medium at 34°C + 5% $CO_2$. Cultures were then diluted 1:100 into fresh MKP-F, incubated at 34°C + 5% $CO_2$. Total cells (i.e. motile and non-motile) were counted every 24 hours using a Petroff-Hausser Counting Chamber (Cat # 3900; Hausser Scientific, Horsham, PA). Cultures were diluted in 1X PBS when appropriate to facilitate accurate cell counts.

### *Serum Sensitivity*

Ten mL of $10^7$ *B. miyamotoi* were centrifuged at 6000 x *g* for 15 min at room-temperature. Pellets were washed once with 1X PBS, resuspended in 10 mL filter-sterilized normal human serum (NHS), and incubated at 34°C + 5% $CO_2$ for 27.5 hours. To verify spirochetes were still alive after transfer to human serum donated by Dr. Travis Alvine, 10 μL was viewed under

Darkfield. After incubation, the presence of motile spirochetes was determined by placing 10 μL on a slide and viewing 10 fields under Darkfield.

### *Factor H Binding*

1 x $10^6$ *B. miyamotoi* were harvested by centrifugation and resuspended in 100 μL veronal buffered saline supplemented with 1mM $Mg^{2+}$, 0.15mM $Ca^{2+}$, and 0.1% gelatin. Normal human serum (NHS) was incubated with 0.34 M EDTA to inhibit complement activation. The cell suspension was incubated with 1.5 mL NHS for 1 hour, washed with PBSA, and then proteins bound to the cells were eluted with 0.1 M glycine-HCL, pH 2. Cells were removed by centrifugation and the supernatant was analyzed by Western blotting with a mouse monoclonal anti-human Factor H antibody (Quidel). One μg recombinant human Factor H (aa 860-1231; ~65kDa on SDS-PAGE; R&D Systems) was used a positive control.

### *Mouse Infections*

Two to four week-old $Rag1^{-/-}$ C57BL/6J mice and 2-4 week-old and 6-8 week-old C3H/HeN were infected by intraperitoneal injection of $10^7$ spirochetes. Blood was collected from saphenous veins every 12 hours for two weeks. Mice were subcutaneously injected with sterile 0.9% saline as necessary. The collected blood was immediately added to 90 μL 0.11 M sodium citrate solution, pH 7.2. Spirochetes were cultured by adding 10 μL of diluted blood to 1 mL prewarmed MKP-F. The remaining blood solution was stored at -20°C for DNA isolation.

Four mice of strains 129S1, C57BL/6J, C57BL/10J, NOD/ShiLtJ, SJL, CBA/CaJ, DBA/1J, DBA/2J, A/J, BALB/cJ, CBA Jackson, and FVB/NJ (3-4

weeks old) were infected subcutaneously with $10^7$ *B. miyamotoi* FR64b. Blood was collected from saphenous veins every 24 hours for two weeks and cultured as described above. Four weeks after infection, mice were perfused with 1X PBS. Blood, ear pinnae, brain, thymus, heart, lungs, liver, pancreas, spleen, kidneys, bladder, and tibiotarsal joints were collected for PCR and culture.

To determine the minimum infectious dose, groups of three 4-6 week-old C3H/H3N mice were infected subcutaneously with the following doses of *B. miyamotoi* FR64b: $10^1$, $10^2$, $10^3$, $10^4$, or $10^5$ spirochetes. Blood was collected every 24 hours as described above.

### *Ixodes scapularis Feeding*

Uninfected larval *I. scapularis* were obtained from the Centers for Disease Control (Atlanta, GA). Uninfected larvae were fed to repletion on infected Rag1$^{-/-}$ C57BL/6J. Replete larvae were collected once per day. One to ten larvae per day were collected, placed in 70% ethanol, and stored at 4°C for DNA isolation and PCR. The remaining larvae were stored in vented conical tubes in a 95-99% humidified chamber at 23°C to molt. Ten larvae were collected once per week throughout the molting process. One week after molting, three flat nymphs were collected, placed in 70% ethanol, and stored at 4°C for DNA isolation and PCR.

### *DNA Isolation and PCR from Ixodes Larvae and Nymphs*

*I. scapularis* DNA was isolated by modifying a previous protocol (59). Ticks stored in 70% ethanol were dried at room temperature overnight. Ticks were crushed in 600 µL of buffer ATL (Qiagen). 20 µL proteinase K (Qiagen)

was added. Tubes were vortexed briefly and incubated overnight at 56°C. Tubes were briefly vortexed and incubated at 56°C for an additional 2 hours then centrifuged at maximum speed for 3 minutes at room temperature. Supernatants were transferred to a clean microcentrifuge tube. Ethanol precipitations were performed using 1/10 volume 3 M sodium acetate and 3 volumes 95% ethanol. Samples were incubated overnight at -20°C then centrifuged at maximum speed for 30 minutes at 4°C. Ethanol was decanted, and the pellet was resuspended in 1 mL nuclease-free water. If necessary, resuspension of the pellet and salt was facilitated by incubating the tube at 56°C. Samples were filtered through a Microcon DNA Fast Flow Centrifugal Filter Unit (Millipore). The filter membrane was washed once with 500 µL nuclease-free water. DNA was eluted twice with 100 µL nuclease-free water. Purified DNA was stored at 4°C overnight for use in PCR.

PCR was performed with primers for TROSPA (F: GTTGCTGTCCATGCTG; R: AAGTGTTCGTTTCCCTTT), *bipA* (F: AAATCCAGGAAATGTTGATG; R: GCCACCAGACTTAATAGCAC), and *oppA1* (F: ACTCAAATGAAGTAGAATTAGAAGAG; R: GTAAGCGTTTCTCTGTCAATAG) using Platinum PCR SuperMix High Fidelity (ThermoFisher). For each reaction, 200 nM of each primer and 1 µL of DNA was added to 45 µL of Platinum PCR SuperMix and the following PCR cycle was performed: 94°C for 2 min, 45 cycles of 94°C for 15 sec, 50°C for 30 sec, 68°C for 15 sec. Ten µL of each reaction was run on a 2% TBE gel and stained with ethidium bromide.

### *Rosetting Erythrocytes*

The ability of *B. miyamotoi* FR64b to rosette human erythrocytes was tested as previously described (375, 376). Briefly, *B. miyamotoi* FR64b and *B. burgdorferi* MI-16 were cultured to approximately $10^6$ spirochetes/mL. Red blood cells were separated and diluted to a final concentration of 5% in RPMI. The remaining whole blood was diluted 1:10 in 1X PBS. Four and six mL of *B. miyamotoi* and *B. burgdorferi* were centrifuged and resuspended in 500 µL diluted whole blood or RPMI. For one protocol, 5% erythrocytes and resuspended *Borrelia* were incubated separately for 15 minutes at 37°C. Erythrocytes and *Borrelia* were mixed at a 1:1 or 2:1 ratio then placed on a glass slide with a coverslip and sealed with clear nail polish. Slides were then incubated for 15 minutes at 23°C or 37°C. Slides were viewed under Darkfield immediately after incubations. For a second protocol, *Borrelia* were resuspended in diluted whole blood, placed on a glass slide, covered, and sealed with clear nail polish. Slides were incubated for 30 minutes at 23°C and 37°C then viewed under Darkfield.

## Results

### *Growth of B. miyamotoi*

*B. miyamotoi* was grown in modified MKP-F medium to assess the growth rate (Fig. 4.3). Generation time at 34°C + 5% $CO_2$ is approximately 16 hours, slightly longer than *B. burgdorferi* with a generation time of 8-12 hours. Maximum cell density peaked at approximately 5 x $10^7$ spirochetes/mL, again, slightly lower than *B. burgdorferi*, which reaches a maximum density of approximately $10^8$ spirochetes/mL. Growth curves were

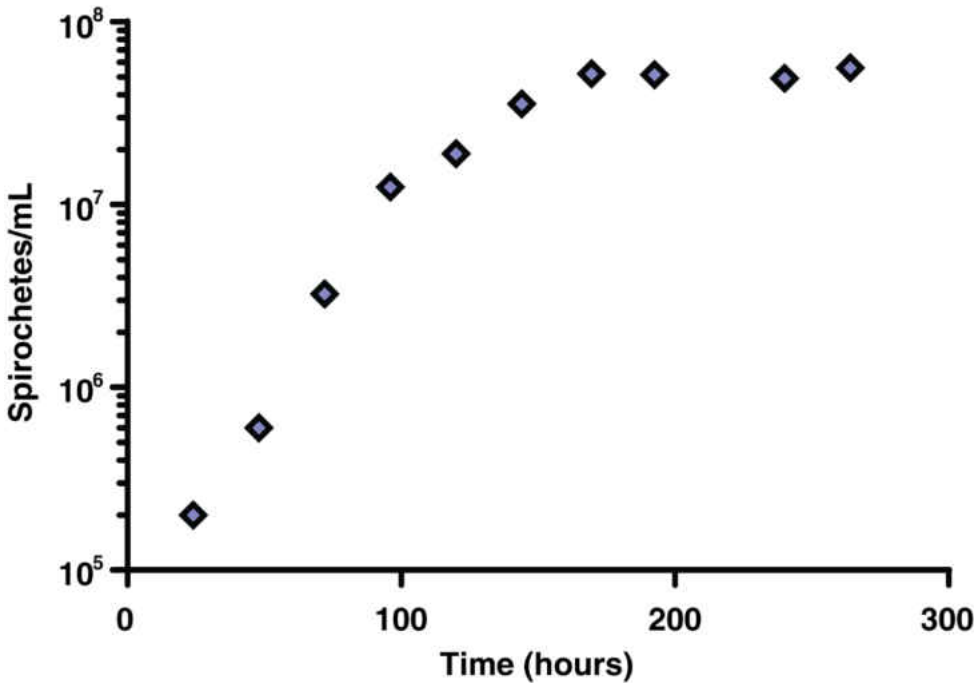


Figure 4. 3. Growth of *B. miyamotoi* in modified MKP-F medium. Spirochetes were counted every 24 hours. Generation time is approximately 16 hours and peak density is approximately 5 x $10^7$ cells/mL.

not performed at 23°C or 37°C, however, *B. miyamotoi* did grow at both temperatures.

### *Minimum Infectious Dose and the Effect of Mouse Strain on Infection*

None of the cultures from C3H/HeN mice infected with different concentrations of *B. miyamotoi* were positive. As we have successfully infected C3H/HeN mice with *B. miyamotoi*, negative cultures represent either mice were not sufficiently infected or there was a problem with the medium. One blood sample obtained at 24 hours for each of the 129S1, C57BL/6J, C57BL/10J, NOD/ShiLtJ, SJL, CBA/CaJ, DBA/1J, DBA/2J, A/J, BALB/cJ, CBA Jackson, and FVB/NJ mice was viewed via microscopy and showed viable spirochetes. Unfortunately, subsequent cultures were also negative.

### *B. miyamotoi Resists Human Serum and Binds Human Factor H*

*B. miyamotoi* was incubated with normal human serum (NHS). Immediately after resuspending spirochetes in NHS, no dead spirochetes were observed. This was important to ensure spirochetes were not killed during transfer and resuspension in NHS. Spirochetes were incubated for 27.5 hours, after which, ten random fields were observed with Darkfield microscopy. Motile spirochetes were observed in all fields showing *B. miyamotoi* is resistant to human serum.

Sequence alignments revealed a putative factor H binding protein homologous to factor H binding proteins of other relapsing fever spirochetes (*B. turicatae, B. parkeri,* and *B. hermsii*) (Fig. 4.4). *B. miyamotoi* was incubated with normal human serum to determine whether it binds human factor H. Proteins bound to the surface of *B. miyamotoi* were removed and analyzed via Western blot using anti-human factor H antibodies (Fig. 4.5). A band of approximately 65 kDa is visible in the eluate fraction, demonstrating human factor H was bound by *B. miyamotoi*.

### *Infection by Needle Inoculation is Detectable 12 Hours Post-Infection in Rag1$^{-/-}$ C57BL/6J and Immunocompetent C3H/HeN Mice*

Spirochetes were visible in wet whole blood mounts in all six mice 24 hours post-infection (hpi) (Fig. 4.6). However, all mice were positive by culture 12 hpi (Table 4.1). Rag1$^{-/-}$ mice became persistently infected, whereas the 3-4 week-old C3H/HeN mice were negative by culture 96 hpi. Blood was not routinely collected from Rag1$^{-/-}$ mice after 144 hpi. Even with saline injections, it became difficult to collect blood every 12 hours.

As a result, blood was collected from these mice at 252 and 336 hpi. The 6-8 week-old C3H/HeN mice were positive by culture 12 to 72 hpi. Both mice were negative by culture at 84 hpi but one mouse produced a positive culture 96 hpi, while the other mouse produced a positive culture 144 hpi.

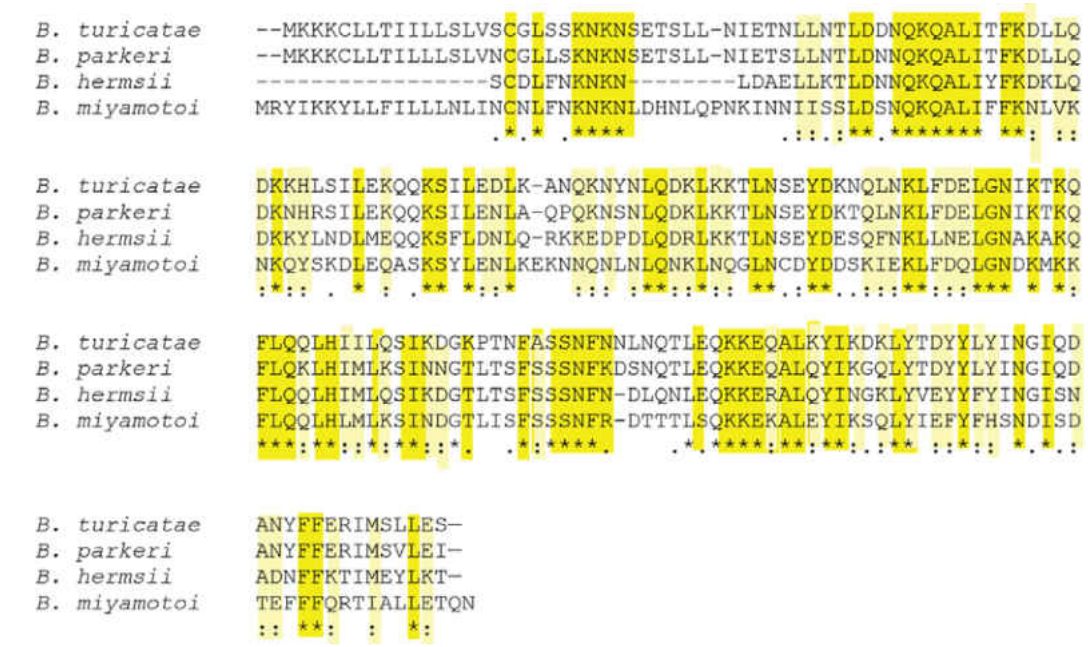


Figure 4. 4. Protein alignment shows homology between a putative factor H binding protein in *B. miyamotoi* and factor H binding proteins from *B. turicatae*, *B. parkeri*, and *B. hermsii*. Identical residues are highlighted in bright yellow and denoted with an asterisk. Residues strongly sharing similar biochemical properties are highlighted in light yellow and denoted with a colon. Residues weakly sharing similar biochemical properties are denoted with a period.

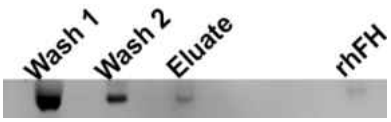


Figure 4. 5. *B. miyamotoi* binds human factor H. Recombinant human factor H (rhFH) is approximately 65 kDa. A band of similar size is present in the *B. miyamotoi* eluate fraction.

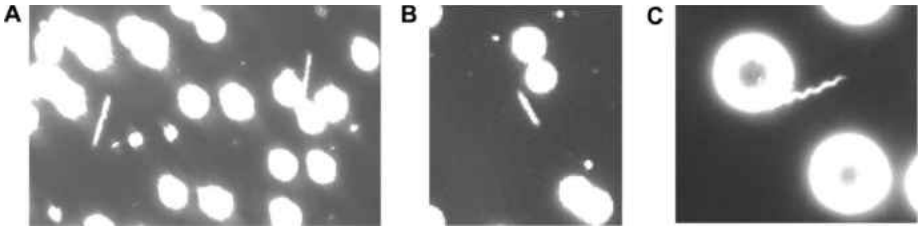


Figure 4. 6. Spirochetes were detected in whole blood mounts 24 hpi in all six mice. Three representative images are shown. (A) Rag1-/- C57BL/6J, (B) C3H/HeN, 2-4 week-old, (C) C3H/HeN, 6-8 week-old.

Table 4. 1. Presence of spirochetes determined from culture and/or microscopy.

| Time (h) | Rag1$^{-/-}$ C57BL/6J 2-4 wo | | WT C3H/HeN 2-4 wo | | WT C3H/HeN 6-8 wo | |
|---|---|---|---|---|---|---|
| 0 | -[a] | - | - | - | - | - |
| 12 | +[b] | + | + | + | + | + |
| 24 | + | + | + | + | + | + |
| 36 | + | + | + | + | + | + |
| 48 | + | + | + | + | C[c] | + |
| 60 | + | + | + | + | + | C |
| 72 | + | C | + | + | + | + |
| 84 | + | + | + | + | - | - |
| 96 | + | + | - | - | - | + |
| 108 | + | + | - | - | - | - |
| 120 | + | + | - | - | - | - |
| 132 | + | + | - | C | - | - |
| 144 | + | + | - | - | + | - |
| 156 | NC[d] | NC | - | - | - | - |
| 168 | NC | NC | - | - | - | - |
| 180 | NC | NC | C | C | - | C |
| 192 | NC | NC | - | - | - | - |
| 204 | NC | NC | - | - | - | - |
| 216 | NC | NC | - | - | - | - |
| 228 | NC | NC | - | - | - | - |
| 240 | NC | NC | - | - | - | - |
| 252 | + | + | - | - | - | - |
| 264 | NC | NC | - | - | - | - |
| 276 | NC | NC | - | - | - | - |
| 288 | NC | NC | - | - | - | - |
| 300 | NC | NC | - | - | - | - |
| 312 | NC | NC | - | - | - | - |
| 324 | NC | NC | - | - | - | - |
| 336 | + | + | - | - | - | - |

[a] No spirochetes detected by microscopy or culture
[b] Spirochetes observed by microscopy and/or culture
[c] Culture contaminated
[d] No blood collected

### *B. miyamotoi does not Appear to be Vector-Specific or Exclusive*

To determine whether *B. miyamotoi* is specific to an *Ixodes* spp., North American *I. scapularis* larvae were fed to repletion on infected Rag1$^{-/-}$ mice infected with a Japanese strain of *B. miyamotoi* (FR64b). *B. miyamotoi* FR64b was isolated from *Apodemus argenteus* (small Japanese field mouse) but is vectored by *I. persulcatus* (20). If *B. miyamotoi* is exclusive to its respective vector, we would not expect a Japanese strain to survive in North American *I. scapularis*. DNA extracted from *I. scapularis* larvae was positive for *bipA* immediately after feeding and in the weeks leading up to molting (Fig. 4.7). A faint PCR band is present for ticks collected from mouse 2 (M2) on the first day replete ticks began dropping (D1) (Fig. 4.7A). Importantly, *I. scapularis* were positive for *bipA* after molting to nymphs meaning *B. miyamotoi* is able to establish an infection in *I. scapularis* and survive the molting process (Fig. 4.7C). This suggests *B. miyamotoi* is not exclusive to one *Ixodes* spp.

### *Rosetting Erythrocytes*

To determine whether *B. miyamotoi* is capable of rosetting erythrocytes like some relapsing fever *Borrelia*, *B. miyamotoi* was incubated with whole blood and purified erythrocytes. No erythrocyte rosetting was observed under any conditions used (Fig. 4.8).

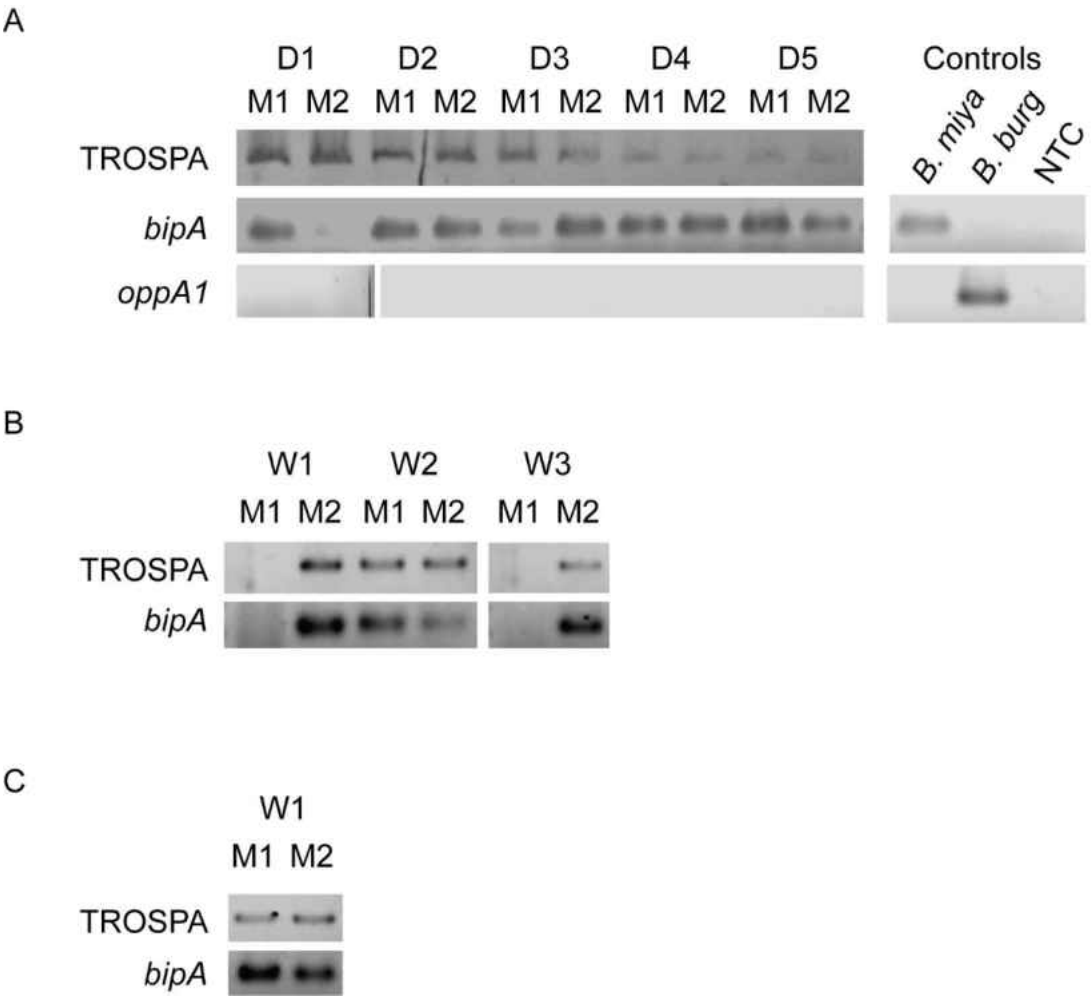


Figure 4. 7. *B. miyamotoi* DNA was detected in I. scapularis throughout and after the molting process. PCR was performed for the tick-specific TROSPA gene, the relapsing fever-specific *bipA* gene, and the Lyme *Borrelia*-specific *oppA1* gene. *bipA* was detected in each tick sample except M1 week 2 and 4. *oppA1* was not detected in any tick samples analyzed showing the ticks and mice are not infected with *B. burgdorferi*. D1: Day 1, first day replete ticks fell off each mouse; M1: mouse 1, M2: mouse 2; W1: week 1, one week after ticks were collected; *B. miya*: *B. miyamotoi* genomic DNA, *B. burg*: *B. burgdorferi* genomic DNA, NTC: no DNA template added.

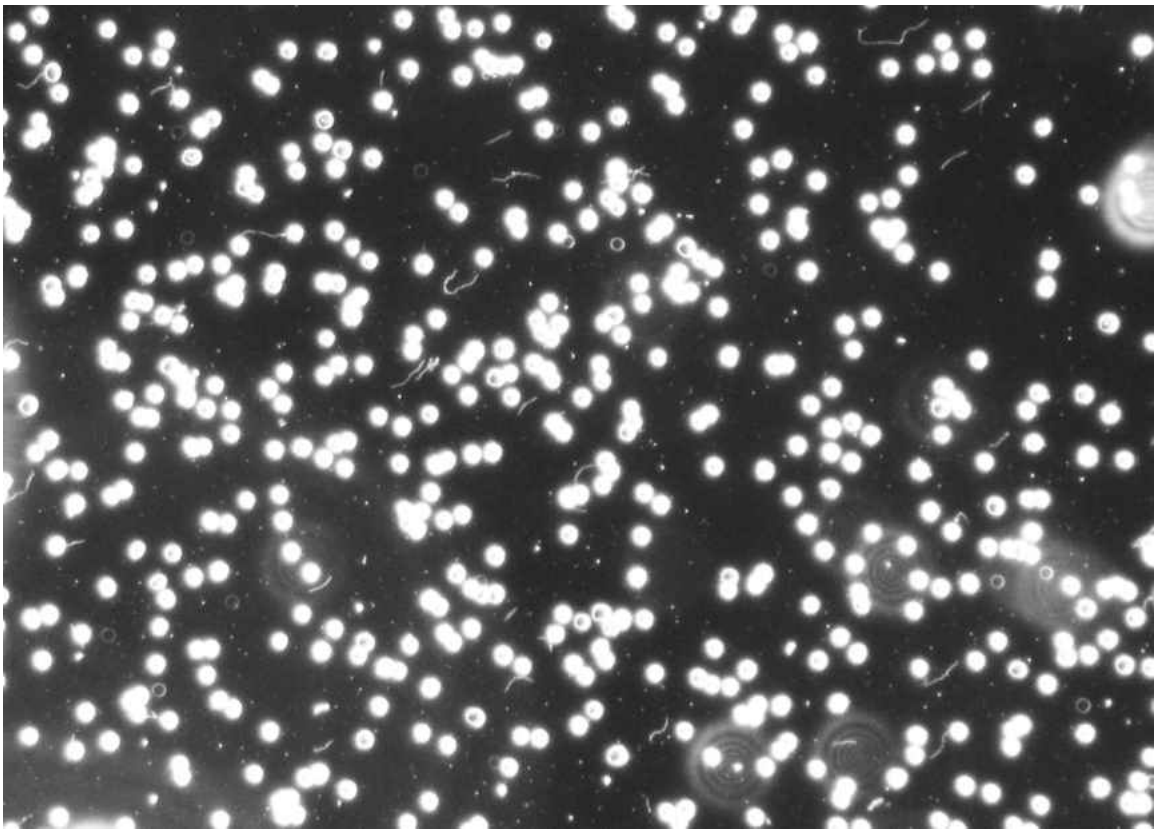

Figure 4. 8. *B. miyamotoi* does not appear to rosette erythrocytes. *B. miyamotoi* was incubated 1:1 with 5% purified erythrocytes at 23°C. Similar results were observed at 37°C, with whole blood, at 1:2 (*B. miyamotoi*:erythrocytes) ratios, and with *B. burgdorferi*.

## Discussion

We can develop effective treatment and prevention methods by understanding the mechanisms by which a pathogen causes disease. Modeling human infection in the laboratory is difficult. Various animals can model isolated aspects of disease but generally fall short of recapitulating a complete human disease. To gain a full understanding of a human pathogen requires patching together several pieces of information, such as identifying virulence factors. An additional method to assess a pathogen's ability to establish an infection is by determining serum susceptibility; a pathogen

susceptible to human sera will likely not be able to establish infection in humans (328, 377). *B. miyamotoi* has previously been shown to resist human serum (171, 172), however, complement resistance varies by strain and species with *B. burgdorferi s.l.* species (115, 323–327). Without a complete history for the strain and culture of *B. miyamotoi* used in our lab, we verified serum resistance for *B. miyamotoi* FR64b.

We showed *B. miyamotoi* FR64b possesses a putative FHBP and is capable of binding human factor H *in vitro* (Fig. 4.4, Fig. 4.5). Whether *B. miyamotoi* binds factor H *in vivo* and is a biologically relevant function remains to be determined. Species of the *B. burgdorferi s.l.* complex rely heavily on complement inactivation to establish an infection, while relapsing fever *Borrelia* do not (122, 123). Still, several relapsing fever *Borrelia* possess FHBPs (346, 347) and bind factor H *in vitro* (115, 122, 340–345). This begs the question if binding factor H is not biologically relevant for relapsing fever *Borrelia*, why maintain FHBPs? FHBPs are located on plasmids in *B. burgdorferi* and while significant rearrangement has occurred between plasmids across strains, the content has remained relatively unchanged (23). That is, *B. burgdorferi* moves genes around but has not removed or added genes. In addition, the FHBP found in relapsing fever *Borrelia*, FhbA, is also found on a plasmid, which is also stable (123, 346, 347). Species of the *B. burgdorferi s.l.* complex maintain numerous FHBPs but only one, FhbA and homologs, has been identified in relapsing fever *Borrelia* (123, 346). Perhaps, despite plasmid stability, relapsing fever *Borrelia* once had but has

since lost several FHBPs from their genomes and FhbA may be on the genomic chopping block.

### *Mechanisms of Immune Evasion by B. miyamotoi: Where We Are*

Given the genetic similarity of *B. miyamotoi* to relapsing fever spirochetes, it is likely *B. miyamotoi* utilizes some homologous mechanisms to evade host immune responses. While *B. miyamotoi* is resistant to complement *in vitro* (171, 172), complement inactivation is not required for relapsing fever spirochetes to establish infection. OspE homologues have been identified in *B. miyamotoi* FR64b (isolated from the blood of *A. argenteus*) however, McDowell et al. were unable to demonstrate FH-binding (115). This suggests, as is the case for relapsing fever spirochetes, inactivation of complement may not be required to resolve spirochetemia during infection with *B. miyamotoi* (126, 174).

Instead, it appears *B. miyamotoi* utilizes a Vmp system (175) and Wagemakers et al. (176) recently demonstrated antigenic variation of Vmps in *B. miyamotoi*. C3H/HeN mice infected with *B. miyamotoi* LB-2001 produced anti-Vsp1 IgM and IgG antibodies that were effective in clearing the initial spirochetemic peak of *B. miyamotoi* from SCID mice. Despite this clearing, a second spirochetemic relapse occurred. Analyses of the secondary *B. miyamotoi* population revealed expression of *vlpC2*, not *vsp1*, as would be expected in the case of antigenic variation. They also noted *vlpC2* was present in the initial *B. miyamotoi* population in a much lower prevalence compared to *vsp1*.

An additional mechanism some relapsing fever *Borrelia* use to evade host immune responses is by rosetting erythrocytes or coating the outer membrane in erythrocytes (375, 376, 378). Infection with species capable of rosetting erythrocytes, *B. duttonii* and *B. crociduriae*, is generally more severe than infection with species unable to rosette erythrocytes (378, 379). In addition to shielding *Borrelia* from an immune response, rosetting erythrocytes can result in microemboli and tissue damage (379, 379). The apparent inability of *B. miyamotoi* to rosette erythrocytes may, at least in part, explain the decreased severity of *B. miyamotoi* infection in immunocompetent patients.

### *Mechanisms of Immune Evasion by B. miyamotoi: Where We Need to Be*

Even though *B. miyamotoi* is genetically similar to relapsing fever spirochetes, it has evolved and exists in different vectors (*Ixodes* not *Ornithodoros*) with different enzootic cycles and different co-pathogens compared to relapsing fever spirochetes. We should not assume *B. miyamotoi* utilizes the same set of mechanisms as other relapsing fever spirochetes. *B. miyamotoi* may use a combination of relapsing fever and Lyme *Borrelia* mechanisms as well as completely novel mechanisms. There are many open questions regarding how *B. miyamotoi* evades host responses and establishes infection.

The role of IgM in clearing *B. miyamotoi* has not been demonstrated. As discussed above, IgM is key in clearing relapsing fever infections. During *B. hermsii* infections, IgM targets FhbA and other surface proteins (131). IgM likely is important in clearing *B. miyamotoi*. All immunocompromised patients

diagnosed with a *B. miyamotoi* infection developed meningoencephalitis. A shared factor with these patients has been treatment with rituximab, a monoclonal anti-CD20 antibody targeting IgM-producing CD20-positive B cells. Depletion of B cells may explain how *B. miyamotoi* is able to migrate to the CNS and causes meningoencephalitis in patients treated with rituximab. The presence of unknown complement inhibitors, however, could contribute to the complement-resistance of *B. miyamotoi* and may be useful in establishing infection (171, 172).

The effects of tick saliva on *B. miyamotoi* survival have not yet been studied. However, being vectored by *Ixodes*, *B. miyamotoi* likely takes advantage of the protective proteins in tick saliva. In addition, understanding interactions between host, vector, and pathogen will aid in the development of Lyme and relapsing fever prevention strategies and thus requires more attention.

### *Needle Infection*

Via intraperitoneal injection, Rag1$^{-/-}$ C57BL/6J mice became persistently infected, as expected. These mice lack mature B cells, which are the cells responsible for mounting a response to other relapsing fever *Borrelia* and provide an alternative immunodeficient mouse model to severe combined immunodeficiency (SCID) mice (125–127, 129–131, 317–319). Also, as expected, the C3H/HeN mice became infected. Wagemakers et al (176) had previously infected 6-8 week-old C3H/HeN mice and shown spirochetes were detected at 24 hpi. They also found three of eight mice had a brief relapse at 6 and 7 dpi. This lead us to wonder whether the age of the

mouse affected the duration and/or recurrence of spirochetemia since as with humans, mice show deterioration of the immune system as they age (380). Indeed, no recurrent spirochetemia was observed in young immunocompetent C3H/HeN mice (Table 4.1). A brief spirochetemic event was observed in older immunocompetent C3H/HeN mice (Table 4.1). It is possible the 6-8 week-old mice did not experience a relapse and the negative cultures observed were due to inadequate culturing. If this is true, the older mice were still infected longer than the 2-4 week-old mice. DNA isolation and PCR of blood during this potential clearing event would clarify these data. Unfortunately, DNA isolation failed.

***Limitations of Needle-Infection***

There are several inherent limitations to be considered. The use of needle infections as this is a less desirable method to replicate tick-borne infections. In addition to being an artificial method to introduce *Borrelia*, spirochete physiology and adaptation to a host can be negatively impacted. Tick saliva is known to contain factors that help *B. burgdorferi* in establishing infections in hosts (106–113, 274, 356–365). However, needle infections, if successful, represent the most efficient method for introducing *Borrelia* to a host for additional experiments. In addition, injection site can affect disease pathology in mice when infecting with *B. burgdorferi* (381). For example, subcutaneous injections of *B. burgdorferi* near the hind end of a mouse results in more severe joint pathology. Whether the pathology of relapsing fever is affected by injection site is unknown. Injection site may not affect *B. miyamotoi* disease progression considering *B. burgdorferi* establishes

persistent infections in tissues while relapsing fever *Borrelia* are mainly blood-borne pathogens. Still, it is important to keep in mind that hosts will not be exposed to *Borrelia* (Lyme or relapsing fever) by an intraperitoneal tick bite.

Multiple methods should be used to confirm infection results. A negative culture or blood smear does not mean an infection has cleared. One 2-4 week-old C3H/HeN mouse was negative by microscopy but positive by culture at 84 hpi.

### *Vector Specificity*

Tick-borne relapsing fever *Borrelia* demonstrate strong vector exclusivity. For example, *B. hermsii* cannot establish an infection in any *Ornithodoros* spp. besides *O. hermsi*. The reason for this specificity is unknown but does appear to be universal among the soft tick-borne *Borrelia*. Analyses have shown *B. miyamotoi* forms regional clades or group types (85, 86, 382). The exact groupings are still in flux as more researchers obtain field-caught ticks and sequence *B. miyamotoi*. It appears a slightly different strain of *B. miyamotoi* is carried by each species of *Ixodes* (vector specificity). For example, strains of *B. miyamotoi* found in *I. scapularis* is slightly different than strains found in *I. pacificus, I. ricinus,* or *I. persulcatus* but strains found in *I. scapularis* are highly similar to each other. However, dissimilarity in sequences should not automatically translate to vector specificity. It can be difficult to test vector specificity in the US as it can be difficult to obtain either European and Asian *Borrelia* or *Ixodes*. Through

sheer luck, we were able to investigate vector specificity using a Japanese strain of *B. miyamotoi* and North American *I. scapularis*.

PCR demonstrated *B. miyamotoi* DNA was present in *Ixodes* immediately after feeding and in the weeks leading up to the molt (Fig 4.2). Acquisition, however, is not sufficient to determine vector specificity. The presence of *Borrelia* DNA or ability to culture *Borrelia* from a tick before molting only demonstrates the spirochete was present in the host. For *Borrelia* to establish an infection in ticks it must survive the tick molting, a process during which the blood meal is digested and the tick undergoes a traumatic restructuring process (47, 383).

Detecting *B. miyamotoi* DNA is flat nymphs confirms the Rag1$^{-/-}$ mice were infected (Fig 4.1 and 4.2). It also demonstrates *B. miyamotoi* is acquired by feeding ticks and survives the tick molting suggesting *B. miyamotoi* can establish an infection.

## Concluding Remarks

Although *B. miyamotoi* is similar to other relapsing fever *Borrelia*, it has evolved, adapted, and exists in dramatically different vectors with different enzootic cycles and different co-pathogens. In addition, infection with *B. miyamotoi* in immunocompromised patients generally results in non-specific symptoms (e.g. headache, malaise), recurrent fever, and spirochetemia characteristic of relapsing fever. However, additional symptoms characteristic of relapsing fever have not been demonstrated, namely rapid symptom onset with a crisis event. This suggests *B. miyamotoi* infection is not synonymous with relapsing fever and is, rather, a relapsing

fever-like illness (90). The cause of this slightly different disease pathology is unknown but there is no doubt the different lifestyle of *B. miyamotoi* plays a role. Though the differences between *B. miyamotoi* and other relapsing fever spirochetes identified thus far are minor, we should exercise caution and not assume *B. miyamotoi* is like other relapsing fever *Borrelia.*

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# APPENDIX A

Appendix A

Table 1. Additional alignment information from blastp results of BB0399

| Organism | Additional Organism Information | NCBI Description | Query Coverage | Identity to Query | E value | GenBank Accession |
|---|---|---|---|---|---|---|
| None | Synthetic ANK motif | Chain A, 4ank: A Designed Ankyrin Repeat Protein With Four Identical Consensus Repeats | 47% | 29% | 4e-09 | 1N0R_A |
| *Acidobacteria* | Bacteria; Various terrestrial and aquatic environments | hypothetical protein CSA81_06935 [Acidobacteria bacterium] | 63% | 28% | 9e-09 | PIE02542.1 |
| *Trichomonas vaginalis* | Protozoa; Causative agent of STD trichomoniasis | hypothetical protein [Trichomonas vaginalis G3] | 42% | 35% | 1e-08 | XP_001326093.1 |
| *Lentisphaerae* | Bacteria; Marine, aquifer metagenome | hypothetical protein A2283_08585 [Lentisphaerae bacterium RIFOXYA12_ FULL_48_11] | 57% | 27% | 1e-08 | OGV66031.1 |
| *Strongylocentrotus purpuratus* | Animal; Purple sea urchin | PREDICTED: serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit B-like [Strongylocentrotus purpuratus] | 44% | 33% | 2e-08 | XP_011672323.1 |
| *Tenacibaculum* sp. | Bacteria; Fish pathogen | hypothetical protein [Tenacibaculum sp. 4G03] | 33% | 42% | 2e-08 | WP_099213746.1 |
| Unknown | Bacteria; Groundwater metagenome | hypothetical protein UR12_C0049G0006 [candidate division TM6 bacterium GW2011_ GWF2_30_66] | 52% | 33% | 2e-08 | KKP25019.1 |

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|---|---|---|---|---|---|---|
| *Aiptasia pallida* (*Exaiptasia pallida*) | Animal; Brown anemone, glass anemone, pale anemone | Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit B [Exaiptasia pallida] | 75% | 29% | 3e-08 | KXJ19934.1 |
| Unknown | Bacteria; Groundwater metagenome | hypothetical protein UR12_C0053G0001 [candidate division TM6 bacterium GW2011_GWF2_ 30_66] | 73% | 28% | 4e-08 | KKP24550.1 |
| Unknown | Bacteria; Aquifer metagenome | hypothetical protein A3F66_04490 [candidate division TM6 bacterium RIFCSPHIGHO2_12_FU LL_32_22] | 57% | 31% | 4e-08 | OGB84173.1 |
| *Trichomonas vaginalis* | Protozoa; Causative agent of STD trichomoniasis | ankyrin repeat protein [Trichomonas vaginalis G3] | 65% | 29% | 7e-08 | XP_001318422.1 |
| *Aiptasia pallida* (*Exaiptasia pallida*) | Animal; Brown anemone, glass anemone, pale anemone | uncharacterized protein LOC110238015 isoform X2 [Exaiptasia pallida] | 63% | 30% | 7e-08 | XP_020899308.1 |
| *Aiptasia pallida* (*Exaiptasia pallida*) | Animal; Brown anemone, glass anemone, pale anemone | uncharacterized protein LOC110238015 isoform X1 [Exaiptasia pallida] | 63% | 30% | 7e-08 | XP_020899305.1 |
| *Aiptasia pallida* (*Exaiptasia pallida*) | Animal; Brown anemone, glass anemone, pale anemone | putative ankyrin repeat protein MM-0045 [Exaiptasia pallida] | 56% | 32% | 9e-08 | KXJ20897.1 |
| *Acanthamoeba polyphaga mimivirus* | DNA virus; Amoeba hosts | putative ankyrin repeat protein [Acanthamoeba polyphaga mimivirus] | 49% | 32% | 9e-08 | YP_003986769.1 |

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| *Acanthamoeba polyphaga mimivirus* | DNA virus; Amoeba hosts | ankyrin repeat-containing protein [Acanthamoeba polyphaga mimivirus] | 49% | 31% | 1e-07 | AKI79037.1 |
| *Anaeromyces robustus* | Fungi; Anaerobic gut | ankyrin, partial [Anaeromyces robustus] | 31% | 42% | 1e-07 | ORX81127.1 |
| *Hirudovirus* strain Sangsue | DNA Mimiviridae virus; Isolated from *Hirudo medicinalis* leech (yep, a medical leech) | putative ankyrin repeat protein [Hirudovirus strain Sangsue] | 49% | 32% | 2e-07 | AHA45596.1 |
| *Acidobacteria* | Bacteria; Various terrestrial and aquatic environments | hypothetical protein CR997_04995 [Acidobacteria bacterium] | 63% | 27% | 2e-07 | PIE90600.1 |
| *Cimex lectularius* | Insect; Bed bug | PREDICTED: tankyrase-1-like [Cimex lectularius] | 48% | 33% | 2e-07 | XP_014249139.1 |
| *Acanthamoeba castellanii mamavirus* | DNA virus; Amoeba hosts | ankyrin repeat-containing protein [Acanthamoeba castellanii mamavirus] | 49% | 31% | 2e-07 | AEQ60456.1 |
| *Cladophialophora carrionii* | Fungi; Causative agent of chromoblastomycosis | hypothetical protein CLCR_07477 [Cladophialophora carrionii] | 56% | 26% | 2e-07 | OCT49800.1 |
| Unknown | Bacteria; Groundwater metagenome | hypothetical protein UR12_C0053G0002 [candidate division TM6 bacterium GW2011_GWF2_30_66] | 42% | 34% | 3e-07 | KKP24551.1 |
| *Sinocyclocheilus rhinocerous* | Fish; Horned Golden-line barbel | PREDICTED: ankyrin-1-like [Sinocyclocheilus rhinocerous] | 65% | 26% | 3e-07 | XP_016407777.1 |

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|---|---|---|---|---|---|---|
| *Aiptasia pallida* (*Exaiptasia pallida*) | Animal; Brown anemone, glass anemone, pale anemone | Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit B [Exaiptasia pallida] | 64% | 30% | 3e-07 | KXJ19941.1 |
| *Aiptasia pallida* (*Exaiptasia pallida*) | Animal; Brown anemone, glass anemone, pale anemone | uncharacterized protein LOC110249705 [Exaiptasia pallida] | 63% | 30% | 3e-07 | XP_020911949.1 |
| *Piromyces* sp. | Fungi; Anaerobic gut | hypothetical protein PIROE2DRAFT_25186 [Piromyces sp. E2] | 49% | 32% | 4e-07 | OUM66985.1 |
| *Labilibacter marinus* (*Saccharicrinis marinus*) | Bacteria; Marine sediment | hypothetical protein [Labilibacter marinus] | 47% | 26% | 5e-07 | WP_083631127.1 |
| *Branchiostoma belcheri* | Animal; Marine lancelet | PREDICTED: ankyrin repeat domain-containing protein 50-like [Branchiostoma belcheri] | 44% | 31% | 6e-07 | XP_019622329.1 |
| *Aiptasia pallida* (*Exaiptasia pallida*) | Animal; Brown anemone, glass anemone, pale anemone | serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit B-like [Exaiptasia pallida] | 55% | 31% | 7e-07 | XP_020894601.1 |
| *Aiptasia pallida* (*Exaiptasia pallida*) | Animal; Brown anemone, glass anemone, pale anemone | serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit B-like [Exaiptasia pallida] | 62% | 30% | 8e-07 | XP_020894695.1 |
| *Pseudomyrmex gracilis* | Insect; Elongate twig ant | ankyrin-1-like isoform X3 [Pseudomyrmex gracilis] | 58% | 28% | 8e-07 | XP_020279924.1 |

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|---|---|---|---|---|---|---|
| *Papio anubis* | Primate; Anubis baboon, Olive baboon | LOW QUALITY PROTEIN: ankyrin repeat domain-containing protein 7 [Papio anubis] | 53% | 28% | 8e-07 | XP_009202018.2 |
| Candidatus *Amoebophilus* sp. | Bacteria; Bioreactor metagenome | hypothetical protein BGO68_01365 [Candidatus Amoebophilus sp. 36-38] | 39% | 34% | 9e-07 | OJW67195.1 |
| *Aiptasia pallida* (*Exaiptasia pallida*) | Animal; Brown anemone, glass anemone, pale anemone | uncharacterized protein LOC110251065 [Exaiptasia pallida] | 73% | 28% | 9e-07 | XP_020913391.1 |
| *Mizuhopecten yessoensis* | Animal; Japanese scallop, Yesso scallop, ezo giant scallop | serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C-like [Mizuhopecten yessoensis] | 68% | 24% | 1e-06 | XP_021378548.1 |
| *Persephonella* sp. | Bacteria; Deep-sea hydrothermal vents | hypothetical protein [Persephonella sp. IF05-L8] | 44% | 29% | 1e-06 | WP_051654701.1 |
| *Thermofilum* | Archaea; Hot springs | MULTISPECIES: hypothetical protein [Thermofilum] | 42% | 34% | 1e-06 | WP_020962764.1 |
| *Aspergillus parasiticus* | Fungi; Pathogenic, mainly associated with improperly stored food grains and peanuts | hypothetical protein P875_00042660 [Aspergillus parasiticus SU-1] | 51% | 36% | 1e-06 | KJK61139.1 |
| *Aiptasia pallida* (*Exaiptasia pallida*) | Animal; Brown anemone, glass anemone, pale anemone | serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit A-like [Exaiptasia pallida] | 56% | 30% | 1e-06 | XP_020913952.1 |

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|---|---|---|---|---|---|---|
| Unknown | Bacteria; Groundwater metagenome | Ankyrin-3 [candidate division TM6 bacterium GW2011_GWF2_ 30_66] | 42% | 32% | 1e-06 | KKP25236.1 |
| *Piromyces finnis* | Fungi; Anaerobic gut | ankyrin [Piromyces finnis] | 51% | 35% | 1e-06 | ORX41735.1 |
| *Bacteroidetes* | Bacteria; Isolate from aquifer metagenome but genus found in various terrestrial and aquatic environments, animal gut | hypothetical protein A2W98_07475 [Bacteroidetes bacterium GWF2_33_38] | 42% | 32% | 2e-06 | OFY18134.1 |
| *Microplitis demolitor* | Insect; Parasitoid wasp useful as a biocontrol agent in agriculture | PREDICTED: GA-binding protein subunit beta-2 isoform X2 [Microplitis demolitor] | 49% | 26% | 2e-06 | XP_008549170.1 |
| *Magallana gigas* (*Crassostrea gigas*) | Animal; Pacific oyster, Japanese oyster, Miyagi oyster | PREDICTED: serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C [Crassostrea gigas] | 54% | 27% | 2e-06 | XP_011454531.1 |
| *Microplitis demolitor* | Insect; Parasitoid wasp useful as an biocontrol agent in agriculture | PREDICTED: GA-binding protein subunit beta-2 isoform X1 [Microplitis demolitor] | 49% | 26% | 2e-06 | XP_008549169.1 |
| *Piromyces* sp. | Fungi; Anaerobic gut | hypothetical protein PIROE2DRAFT_31296 [Piromyces sp. E2] | 39% | 36% | 2e-06 | OUM64010.1 |
| *Macaca fascicularis* | Primate; Crab-eating macaque | RecName: Full=Ankyrin repeat domain-containing protein 7 | 62% | 29% | 2e-06 | Q4R3S3.1 |
| *Neodiprion lecontei* | Insect; Redheaded pine sawfly, Leconte's sawfly | PREDICTED: ankycorbin [Neodiprion lecontei] | 47% | 24% | 2e-06 | XP_015516779.1 |

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| *Cercocebus atys* | Primate; Sooty mangabey | PREDICTED: ankyrin repeat domain-containing protein 7 [Cercocebus atys] | 62% | 29% | 2e-06 | XP_011943227.1 |
| *Gallionellales* | Bacteria; Groundwater metagenome | hypothetical protein COZ77_10025 [Gallionellales bacterium CG_4_8_14_3_um_ filter_54_18] | 41% | 30% | 2e-06 | PIX03766.1 |
| *Chlorocebus sabaeus* | Primate; Green monkey | PREDICTED: ankyrin repeat domain-containing protein 7 isoform X1 [Chlorocebus sabaeus] | 62% | 29% | 2e-06 | XP_007980895.1 |
| *Athalia rosae* | Insect; Turnip sawfly | GA-binding protein subunit beta-2 isoform X1 [Athalia rosae] | 52% | 23% | 2e-06 | XP_012266656.1 |
| *Vollenhovia emeryi* | Insect; Ant (if you made it this far, you deserve a bonus factoid: this genus has no worker caste) | PREDICTED: tankyrase-2 [Vollenhovia emeryi] | 49% | 24% | 2e-06 | XP_011881177.1 |
| *Athalia rosae* | Insect; Turnip sawfly | GA-binding protein subunit beta-2 isoform X3 [Athalia rosae] | 52% | 23% | 2e-06 | XP_020711614.1 |
| *Legionella hackeliae* | Bacteria; Isolated from patient with Legionnaires' disease, but unable to replicate in amoeba | hypothetical protein [Legionella hackeliae] | 44% | 36% | 2e-06 | WP_045105250.1 |

# APPENDIX B

Appendix B

Table 2. Additional alignment information from blastp results of BBB28

| Organism | Additional Organism Information | NCBI Description | Query Coverage | Identity to Query | E value | GenBank Accession |
|---|---|---|---|---|---|---|
| None | Synthetic ANK motif | Chain A, Crystal Structure Of Engineered Protein. Northeast Structural Genomics Consortium Target Or265 | 28% | 32% | 4e-06 | 4HQD_A |
| *Prunus mume* | Tree; Chinese plum, Japanese apricot | PREDICTED: ankyrin repeat domain-containing protein EMB506, chloroplastic [Prunus mume] | 20% | 38% | 9e-06 | XP_008229006.1 |
| *Prunus persica* | Tree; Peach tree | ankyrin repeat domain-containing protein EMB506, chloroplastic [Prunus persica] | 20% | 38% | 1e-05 | XP_007198840.2 |
| Ziziphus jujuba | Tree; Jujube red date, Chinese date, Korean date, Indian date | PREDICTED: ankyrin repeat domain-containing protein EMB506, chloroplastic [Ziziphus jujuba] | 35% | 31% | 3e-05 | XP_015895680.1 |
| *Armatimonadetes* | Bacteria; Groundwater metagenome | hypothetical protein AUJ92_05150 [Armatimonadetes bacterium CG2_30_59_28] | 33% | 26% | 1e-04 | OIO96820.1 |
| *Eutrema salsugineum* | Plant; Saltwater cress | hypothetical protein EUTSA_v10027842mg [Eutrema salsugineum] | 17% | 37% | 1e-04 | XP_006405540.1 |

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|---|---|---|---|---|---|---|
| None | Synthetic ANK motif | Chain A, Crystal Structure Of Engineered Protein. Northeast Structural Genomics Consortium Target Or267 | 28% | 30% | 2e-04 | 4HB5_A |
| *Piromyces finnis* | Fungi; Anaerobic gut | ankyrin [Piromyces finnis] | 34% | 29% | 2e-04 | ORX46187.1 |
| *Apostichopus japonicus* | Animal; Japanese spiky sea cucumber | hypothetical protein BSL78_25314 [Apostichopus japonicus] | 17% | 41% | 2e-04 | PIK37853.1 |
| Wolbachia endosymbiont of Diaphorina citri | Bacteria; Endosymbiont of insect Diaphorina citri, Asian citrus psyllid | ankyrin repeat domain-containing protein [Wolbachia endosymbiont of Diaphorina citri] | 21% | 38% | 2e-04 | WP_017532031.1 |
| *Brachypodium distachyon* | Grass; Purple false brome, stiff brome | PREDICTED: ankyrin repeat domain-containing protein EMB506, chloroplastic [Brachypodium distachyon] | 17% | 36% | 3e-04 | XP_003564031.1 |
| *Bacillus sp*. | Bacteria | ankyrin repeat domain-containing protein [Bacillus sp. FJAT-18017] | 18% | 35% | 3e-04 | WP_053598089.1 |
| *Wolbachia pipientis* | Bacteria; Arthropod endosymbiont important in host mating incompatibility | ankyrin repeat domain-containing protein [Wolbachia pipientis] | 21% | 38% | 3e-04 | WP_096676758.1 |

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| *Solanum tuberosum* | Plant; Potato, Irish potato | PREDICTED: ankyrin repeat domain-containing protein EMB506, chloroplastic [Solanum tuberosum] | 17% | 34% | 3e-04 | XP_006341150.1 |
| *Trichomonas vaginalis* | Protozoa; Causative agent of STD trichomoniasis | hypothetical protein [Trichomonas vaginalis G3] | 21% | 39% | 4e-04 | XP_001583435.1 |
| *Wolbachia* endosymbiont of *Culex molestus* | Bacteria; Endosymbiont of insect Culex molestus, London underground mosquito | ankyrin repeat domain protein [Wolbachia endosymbiont wPip_Mol of Culex molestus] | 16% | 43% | 4e-04 | CQD12293.1 |
| Dokdonia sp. | Bacteria; Marine | hypothetical protein [Dokdonia sp. PRO95] | 22% | 38% | 5e-04 | WP_035333977.1 |
| *Zea mays* | Grass; Corn, maize | Ankyrin repeat domain-containing protein EMB506 chloroplastic [Zea mays] | 17% | 34% | 5e-04 | AQK60863.1 |
| None | Synthetic ANK motif | Chain A, Crystal Structure Of Engineered Protein. Northeast Structural Genomics Consortium Target Or264 | 28% | 28% | 5e-04 | 4GPM_A |
| *Zea mays* | Grass; Corn, maize | unknown [Zea mays] | 17% | 34% | 5e-04 | ACF80469.2 |
| *Zea mays* | Grass; Corn, maize | uncharacterized protein LOC100191181 [Zea mays] | 17% | 34% | 5e-04 | NP_001130088.1 |
| *Citrus clementina* | Tree; Clementine tree | hypothetical protein CICLE_v10013529mg [Citrus clementina] | 17% | 34% | 6e-04 | XP_006431025.1 |

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| *Sorghum bicolor* | Grass; Sorghum, great millet, durra, jowari, milo processed as food, animal feed, and ethanol | ankyrin repeat domain-containing protein EMB506, chloroplastic [Sorghum bicolor] | 17% | 34% | 6e-04 | XP_002451459.1 |
| *Macrostomum lignano* | Animal; flatworm found in various aquatic/semi-aquatic environments | hypothetical protein BOX15_Mlig004077g1 [Macrostomum lignano] | 21% | 39% | 6e-04 | PAA47570.1 |
| *Wolbachia* endosymbiont of *Culex molestus* | Bacteria; Endosymbiont of insect Culex molestus, London underground mosquito | ankyrin repeat domain-containing protein [Wolbachia endosymbiont of Culex molestus] | 15% | 45% | 7e-04 | WP_087740701.1 |
| Agrilus planipennis | Insect; Emerald ash borer | PREDICTED: ankyrin repeat domain-containing protein 39-like isoform X2 [Agrilus planipennis] | 33% | 26% | 7e-04 | XP_018329392.1 |
| Candidatus *Amoebophilus* sp. | Bacteria; Bioreactor metagenome | hypothetical protein BGO68_00130 [Candidatus Amoebophilus sp. 36-38] | 25% | 32% | 7e-04 | OJW67418.1 |
| *Dichanthelium oligosanthes* | Grass; Heller's rosette grass, fewanther obscure grass, few-flowered panic grass | Ankyrin repeat domain-containing protein EMB506, chloroplastic [Dichanthelium oligosanthes] | 17% | 34% | 7e-04 | OEL16152.1 |

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| *Wolbachia* endosymbiont of *Culex pipiens* | Bacteria; Endosymbiont of insect Culex pipiens, Northern house mosquito | ankyrin domain protein ank12 [Wolbachia endosymbiont of Culex pipiens] | 15% | 45% | 8e-04 | CAM59627.1 |
| *Wolbachia* endosymbiont of *Folsomia candida* | Bacteria; Endosymbiont of insect Folsomia candida, white rat springtail (springtails may have been used in biological warfare during the Korean War) | hypothetical protein ASM33_07545 [Wolbachia endosymbiont of Folsomia candida] | 18% | 37% | 8e-04 | APR99226.1 |
| *Mizuhopecten yessoensis* | Animal; Japanese scallop, Yesso scallop, ezo giant scallop | E3 ubiquitin-protein ligase MIB2-like [Mizuhopecten yessoensis] | 20% | 31% | 9e-04 | XP_021361486.1 |
| *Wolbachia* endosymbiont of *Culex pipiens molestus* | Bacteria; Endosymbiont of insect Culex molestus, London underground mosquito | ankyrin repeat domain-containing protein [Wolbachia endosymbiont of Culex pipiens molestus] | 15% | 45% | 0.001 | WP_019078795.1 |
| *Wolbachia* endosymbiont of *Culex quinquefasciatus* | Bacteria; Endosymbiont of insect Culex quinquefasciatus, Southern house mosquito | ankyrin repeat domain-containing protein [Wolbachia endosymbiont of Culex quinquefasciatus] | 15% | 45% | 0.001 | WP_038227557.1 |
| *Wolbachia* endosymbiont of *Diaphorina citr*i | Bacteria; Endosymbiont of insect Diaphorina citri, Asian citrus psyllid | ankyrin repeat domain-containing protein [Wolbachia endosymbiont of Diaphorina citri] | 15% | 45% | 0.001 | WP_017531746.1 |

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| *Ictalurus punctatus* | Fish; Channel catfish | PREDICTED: acyl-CoA-binding domain-containing protein 6 [Ictalurus punctatus] | 22% | 29% | 0.001 | XP_017322820.1 |
| *Hydra vulgaris* | Animal; Fresh-water polyp | PREDICTED: serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit B-like [Hydra vulgaris] | 24% | 33% | 0.001 | XP_012554050.1 |
| *Wolbachia* endosymbiont of *Culex quinquefasciatus* | Bacteria; Endosymbiont of insect Culex quinquefasciatus, Southern house mosquito | ankyrin repeat domain protein [Wolbachia endosymbiont of Culex quinquefasciatus JHB] | 15% | 45% | 0.002 | EEB55260.1 |
| *Brachyspira hyodysenteriae* | Bacteria; Causative agent of swine dysentery and a spirochete | ankyrin [Brachyspira hyodysenteriae] | 22% | 33% | 0.002 | KLI56385.1 |
| *Trichomonas vaginalis* | Protozoa; Causative agent of STD trichomoniasis | ankyrin repeat protein [Trichomonas vaginalis G3] | 19% | 37% | 0.002 | XP_001327937.1 |
| *Wolbachia* endosymbiont of *Folsomia candida* | Bacteria; Endosymbiont of insect Folsomia candida, white rat springtail | hypothetical protein ASM33_05675 [Wolbachia endosymbiont of Folsomia candida] | 16% | 38% | 0.002 | APR98703.1 |
| *Capsicum annuum* | Plant; Peppers, peppers of all types | PREDICTED: ankyrin repeat domain-containing protein EMB506, chloroplastic [Capsicum annuum] | 17% | 32% | 0.002 | XP_016553864.1 |

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| *Wolbachia* endosymbiont of *Drosophila santomea* | Bacteria; Endosymbiont of insect Drosophila santomea, fruit fly | ankyrin domain protein [Wolbachia endosymbiont of Drosophila santomea] | 14% | 49% | 0.002 | AFV33507.1 |
| *Mariprofundus ferrinatatus* | Bacteria; Marine | ankyrin repeat domain-containing protein [Mariprofundus ferrinatatus] | 22% | 31% | 0.002 | WP_100265207.1 |
| *Wolbachia* | Bacteria; Arthropod endosymbiont | MULTISPECIES: hypothetical protein [Wolbachia] | 21% | 35% | 0.003 | WP_082941437.1 |
| *Trichomonas vaginalis* | Protozoa; Causative agent of STD trichomoniasis | hypothetical protein [Trichomonas vaginalis G3] | 14% | 37% | 0.003 | XP_001323115.1 |
| *Wolbachia* endosymbiont of *Nomada flava* | Bacteria; Endosymbiont of insect Nomada flava, bee | hypothetical protein [Wolbachia endosymbiont of Nomada flava] | 21% | 35% | 0.003 | WP_082941371.1 |
| *Bacillus sp*. | Bacteria | ankyrin repeat domain-containing protein [Bacillus sp. EB01] | 21% | 32% | 0.003 | WP_043930594.1 |
| *Wolbachia* | Bacteria; Arthropod endosymbiont | MULTISPECIES: ankyrin repeat domain-containing protein [Wolbachia] | 25% | 35% | 0.003 | WP_007301895.1 |
| *Spirochaetae* bacterium | Bacteria; Groundwater metagenome | hypothetical protein CVV51_02635 [Spirochaetae bacterium HGW-Spirochaetae-7] | 22% | 31% | 0.003 | PKL09672.1 |
| *Wolbachia* endosymbiont of *Culex pipiens* | Bacteria; Endosymbiont of insect Culex pipiens, Northern house mosquito | ankyrin domain protein ank12 [Wolbachia endosymbiont of Culex pipiens] | 26% | 35% | 0.003 | CAM59626.1 |

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| *Beta vulgaris* subsp. vulgaris | Plant; Chard, Swiss chard | PREDICTED: ankyrin repeat domain-containing protein EMB506, chloroplastic [Beta vulgaris subsp. vulgaris] | 37% | 26% | 0.003 | XP_010688297.1 |
| *Wolbachia* endosymbiont of *Culex quinquefasciatus* | Bacteria; Endosymbiont of insect Culex quinquefasciatus, Southern house mosquito | ankyrin repeat domain protein [Wolbachia endosymbiont of Culex quinquefasciatus Pel] | 25% | 35% | 0.003 | CAQ54560.1 |
| *Wolbachia* | Bacteria; Arthropod endosymbiont | MULTISPECIES: ankyrin repeat domain-containing protein [Wolbachia] | 16% | 36% | 0.004 | WP_065094613.1 |
| Candidatus *Amoebophilus asiaticus* | Bacteria; Amoeba symbiont | hypothetical protein [Candidatus Amoebophilus asiaticus] | 41% | 28% | 0.004 | WP_012473465.1 |
| *Paenibacillus polymyxa* | Bacteria; Non-pathogenic, found in various terrestrial and aquatic sediments | hypothetical protein [Paenibacillus polymyxa] | 24% | 31% | 0.004 | WP_068940871.1 |
| *Bacillus psychrosaccharolyticus* | Bacteria; Adapted to cold environment (misleading because it wouldn't survive in North Dakota) | hypothetical protein [Bacillus psychrosaccharolyticus] | 27% | 27% | 0.005 | WP_051387238.1 |