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Genetic and biochemical characterization of the roles of two putative purine transporters in the infectious cycle of Borrelia burgdorferi

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GENETIC AND BIOCHEMICAL CHARACTERIZATION OF THE ROLE(S) OF TWO PUTATIVE PURINE TRANSPORTERS IN THE INFECTIOUS CYCLE OF *BORRELIA BURGDORFERI*

by

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ABSTRACT

Lyme disease, the most common tick borne disease in United States, is caused by the bacterial pathogen *Borrelia burgdorferi*. In nature, *B. burgdorferi* exists in an enzootic infectious cycle between an arthropod vector and mammalian hosts. Identification and characterization of the genes essential for *B. burgdorferi* survival throughout its infectious cycle is an important step toward understanding the molecular mechanisms involved in *B. burgdorferi* pathogenesis. *B. burgdorferi* contains a small genome, which lacks the genes encoding for the enzymes required for *de novo* synthesis of amino acids, fatty acids and nucleic acid precursors. Therefore, the spirochete is dependent upon the host environment for the uptake of these essential nutrients. Purines are required for the synthesis of nucleotides for the biosynthesis of DNA and RNA. Due to the lack of *de novo* purine synthesis, the ability of *B. burgdorferi* to salvage purines from its host environments is essential to its survival. While the enzymes critical for the *B. burgdorferi* purine salvage pathway are known, the transporters involved in the uptake of purines from the host environments are not. The work in this thesis is focused on identification of the genes encoding purine permeases in *B. burgdorferi* and genetic and biochemical characterization of their functions in the infectious cycle of *B. burgdorferi*. Here, we demonstrate that homologous genes *bbb22* and *bbb23* present on circular plasmid 26 encode for purine permeases, which are important for transport of hypoxanthine, adenine and guanine. Furthermore, genes *bbb22-23* together were essential for *B. burgdorferi* infection in mice. BBB22 and BBB23 share 78% amino acid identify. And although, individually both BBB22 and BBB23 were found to be capable of purine transport, BBB22 has higher affinity for

hypoxanthine and adenine compared to BBB23. Moreover, the *bbb22* gene alone was sufficient to restore mouse infectivity to spirochetes lacking both *bbb22* and *bbb23*, whereas, *bbb23* was not. Nonetheless, the spirochete loads in the tissues of mice infected with *B. burgdorferi* carrying *bbb22* alone were significantly reduced compared to *B. burgdorferi* carrying both *bbb22* and *bbb23*, demonstrating the importance of the two genes together for the spirochetes to achieve wild type levels of infection. In ticks, genes *bbb22* and *bbb23* were dispensable for spirochete survival but contributed to spirochete replication in fed larvae. The replication of spirochetes lacking *bbb22-23* in larval ticks was restored to wild type levels by the reintroduction of the low affinity purine transporter encoded by *bbb23* alone. Overall, we have identified a purine transport system in *B. burgdorferi,* which is essential for spirochete survival in the mammalian host and contributes to spirochete replication in the tick vector. As *B. burgdorferi* lacks typical virulence factors and toxins, these studies highlight the critical role of physiological functions in the virulence of this pathogen. Moreover, the BBB22-23 *in vivo* essential transport system may represent a novel therapeutic target to deliver antimicrobial drugs to treat Lyme disease.

I want to dedicate this dissertation to my parents, relatives and friends for their faith and support, which always encouraged me to pursue my goals.

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CHAPTER 1: INTRODUCTION

1.1 Lyme disease, Pathophysiology, Prevalence, Diagnosis and Treatment

Lyme disease, first recognized in 1975, is a zoonotic disease and the most prevalent tick borne infection in United States [1, 2]. Lyme disease is a multi-stage inflammatory disease in the humans that affects the joints (arthritis), heart (carditis) and nervous system (encephalitis) etc. According to recent reports from the Centers for Disease Control and Prevention, approximately 300,000 cases of Lyme disease are diagnosed every year which is 10 times higher than the actual number of cases reported [3].

Lyme disease can be caused by a number of *Borrelia* species like *B. burgdorferi*, *B. afzelli*, and *B. garinii* [4]. All these *Borrelia* species are together called as *B. burgdorferi senso lato*. Among these, *B. garinii* and *B. afzelli* are endemic to Europe, whereas *B. burgdorferi* is mainly distributed in North America [5]. In the United States Lyme disease is most prevalent in the northeast and the upper midwest states.

Lyme disease pathogenesis has been classified into 3 stages: 1) early localized infection, 2) early disseminated infection and 3) late disseminated infection [6]. Early localized infection involves the inflammatory reaction at the site of tick bite on the skin and the development of an erythema migrans (EM), a bulls eye rash most typically characterized with Lyme disease [7]. This circular rash at the site of an infectious tick bite is a result of inflammatory responses as spirochetes begin disseminating, creating the appearance of a bull's eye. Usually, the EM appears between 3 to 30 days after an

infected tick feeding [8]. However, not every case of Lyme disease shows this bull's eye rash, and with only approximately 70-80% of the infected population developing EM [9].

Early localized infection also displays flu like symptoms such as fever, headache, muscle pain and discomfort. History of a tick bite, an appearance of bull's eye rash and flu like symptoms together represent a symptomatic diagnosis of Lyme disease. During early dissemination, spirochetes start moving from the original site of localized infection to other tissues via blood circulation. At this stage, patients can develop EMs on other parts of the body. Other more severe symptoms include signs of the nervous system impairment like facial palsy, meningitis, memory loss and heart impairment (carditis) and arthritic problems [10]. At late stage dissemination, the spirochetes are disseminated into deep tissues. In these later stages spirochetes evade host immune responses by decreasing the expression of immunogenic outer surface proteins [11, 12], resulting in continued persistence of *B. burgdorferi*. At this point many organs such as heart, joints, brain, and eyes start showing the late stage disease symptoms and can lead to permanent functional impairments.

Lyme disease diagnosis is based upon the symptoms, tick bite history, and serology analysis of *B. burgdorferi* specific antibodies. Since quantitative diagnosis is dependent on antibody production, identification of an infection during the early stages of an infection is difficult. Western blot and ELISA, are the two most commonly used lab tests for the Lyme disease diagnosis. The CDC recommends two-tiered test that includes an initial ELISA based assay and a followed by a western blot. The ELISA is a sensitive but less specific assay. Positive ELISA results are confirmed by western blot, which is more specific [13]. Levels of IgM and IgG-specific antibodies are measured to

diagnose Lyme disease. Antibiotic treatment of Lyme disease is successful if diagnosed and treated in early stages of infection. In later stages of infection, while spirochetes are killed by antibiotics, complete recovery may be hindered because of the inflammatory damage that has already occurred as a result of the *B. burgdorferi*. Treatment of Lyme disease depends upon many factors such as disease stage, age and immune system of the patient. According to the CDC, patients diagnosed with Lyme disease and treated in the early stages show complete recovery [14]. So, early diagnosis is a critical step in the treatment of Lyme disease.

1.2 *Borrelia burgdorferi* and its infectious cycle

Borrelia burgdorferi is a bacterial pathogen, which causes Lyme disease in the humans and animals [15]. The infectious cycle of *B. burgdorferi* includes a vertebrate host, typically the white-footed mouse (*Peromyscus leucopus*), and a tick vector, mainly *Ixodes scapularis* [16-19]. Ticks may also feed on the lizards, birds and small and large mammals, all of which may contribute to the environmental reservoir of *B. burgdorferi* (Figure 1). Hard ticks of the genus *Ixodes* are main ticks that transmit Lyme disease. *I. scapularis* is the major tick vector that transmits Lyme disease in United States and is most prevalent in the northeastern and midwestern United States. *Ixodes pacificus* serves as the Lyme disease vector in California and other western states [20]. *Ixodes scapularis,* also known as blacklegged tick or deer tick, has three life stages; larvae, nymphs and adults. These ticks prefer warm and humid environments and feed only once at each tick stage before molting to the next stage. Female adult stage ticks lay eggs in the late spring, which hatch a few months later in the summer. Transovarial transmission of spirochetes has not been demonstrated, so hatched larvae are not

infected with spirochetes and must feed on an infected host to acquire spirochetes [21]. Larvae mostly feed on the small mammals and birds and can acquire *B. burgdorferi* from these infected hosts. Larval ticks subsequently transmit spirochetes to an uninfected host during second and third blood meals as nymphal and adult ticks, respectively. The fed larvae are approximately close to the size of mustard seed. The larval stage ticks can survive for a long time even without a blood meal. Once they have a blood meal, they molt into nymphs during the next spring. The nymphs have 8 legs and they feed on the small and large mammals. Humans are more prone to Lyme disease transmission by the nymphs [22] because of their small size and the tick secretions released during the bite that prevents itching sensation [23]. Due to these reasons, nymphs mostly remain undetected while feeding on humans and feed longer. Nymphs, like all life stages of *Ixodes scapularis*, are slow feeders. The nymphs feed continuously for 2-5 days depending upon the host blood circulation where the nymphs are embedded. It takes 36-48 hours of tick feeding to start spirochete transmission to the host [24]. After the blood meal, nymphs increase in size but these are still hard to notice. Humans are not reservoir hosts for *B. burgdorferi* but acquire spirochetes inadvertently when bitten by an infected tick [25]. Nymphal stage ticks show peak activity in the summer months and at this time human exposure to the Lyme disease is greatest. Fed nymphs molt into adult ticks during the fall. The adult ticks feed mostly on the large mammals, typically deer [26]. Adult ticks mate and female ticks lay eggs in the spring, all in all resulting in a two year life cycle.

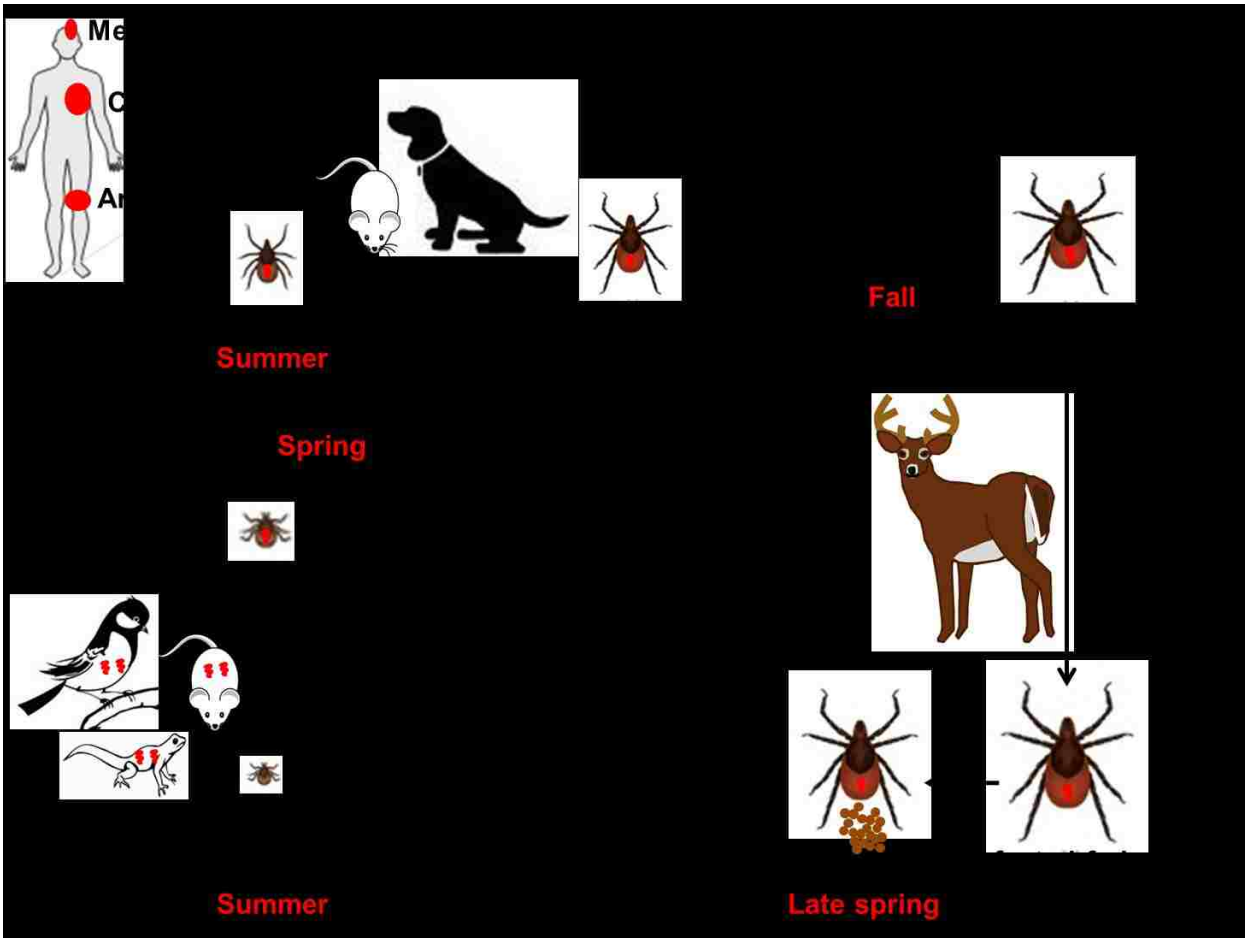

Figure 1. Infectious cycle of *Borrelia burgdorferi*.

Infectious cycle of *B. burgdorferi* includes a tick vector and multiple vertebrate hosts. Adult female ticks lay eggs in the late spring. In summer, the larvae hatch from the eggs and feed mostly on small vertebrate host. In next spring larvae molt into nymphs. Nymphs feed on uninfected host and transmit Lyme disease to them. Human, not a natural host for *B. burgdorferi*, but can acquire Lyme disease from nymphs. Nymphs molt in to adult in Fall. Adult ticks further feed on next host and mate with adult male ticks and hatch eggs in late spring [27].

Due to their motility and spiral morphology, *B. burgdorferi* is classified as a spirochete and are placed in family Spirochaetaceae. Like other members of the family Spirochaetaceae, *B. burgdorferi* possesses 7-11 flagella, which run along the entire length of spirochete between the inner and outer membrane within the periplasmic space [28]. As a result of the periplasmic localization of the flagella, *B. burgdorferi* move in a corkscrew type motion [29]. This type of motility allows the spirochete to move

through viscous materials like connective tissue and the tick midgut. Although categorized as gram negative bacteria, *B. burgdorferi* and other spirochetes are unique in that these species lack lipopolysaccharide and do not stain gram negative in a gram stain [30] . *B. burgdorferi* are 5-25 μm in length and 0.2-0.5 μm in width. They are microaerophilic and have a slow *in vitro* replication time of approximately 6-8 hours compared to the division times of other bacterial species such as *E. coli* (20 minutes) [31], *Bacillus subtilis* (2 hours) [32] and *Staphylococcus aureus* (24 minutes) [33].

1.3 *Borrelia burgdorferi* genome

Compared to some other free-living bacteria, *B*. *burgdorferi* has a relatively small and highly complex genome. *B. burgdorferi* contains one linear chromosome, 12 linear plasmids and 9-11 circular plasmids [34-36] (Figure 2). The name of each plasmid represents the approximate size of that particular plasmid in kilobase pairs. The size of the linear chromosome is 910,725 base pairs [37]. Plasmid sizes vary from 5kb (linear plasmid 25) to 56 kb (linear plasmid 56). Linear plasmids are 12 in number as following lp5, lp17, lp21, lp25, lp28-1, lp28-2, lp28-3, lp28-4, lp36, lp38, lp54 and lp56. Circular plasmids are 9-11 in number as following cp9, cp26, cp32-1, cp32-2, cp32-3, cp32-4, cp32-5, cp32-6, cp32-7, cp32-8 and cp32-9 [38]. *B. burgdorferi* chromosome contains 853 open reading frames, among which the function for most of them is unknown. About 60% of these open reading frames are assigned some biological functions. 12% of ORFs show identity to hypothetical proteins from other organisms and 29% of these ORFs represent new genes with unknown functions.

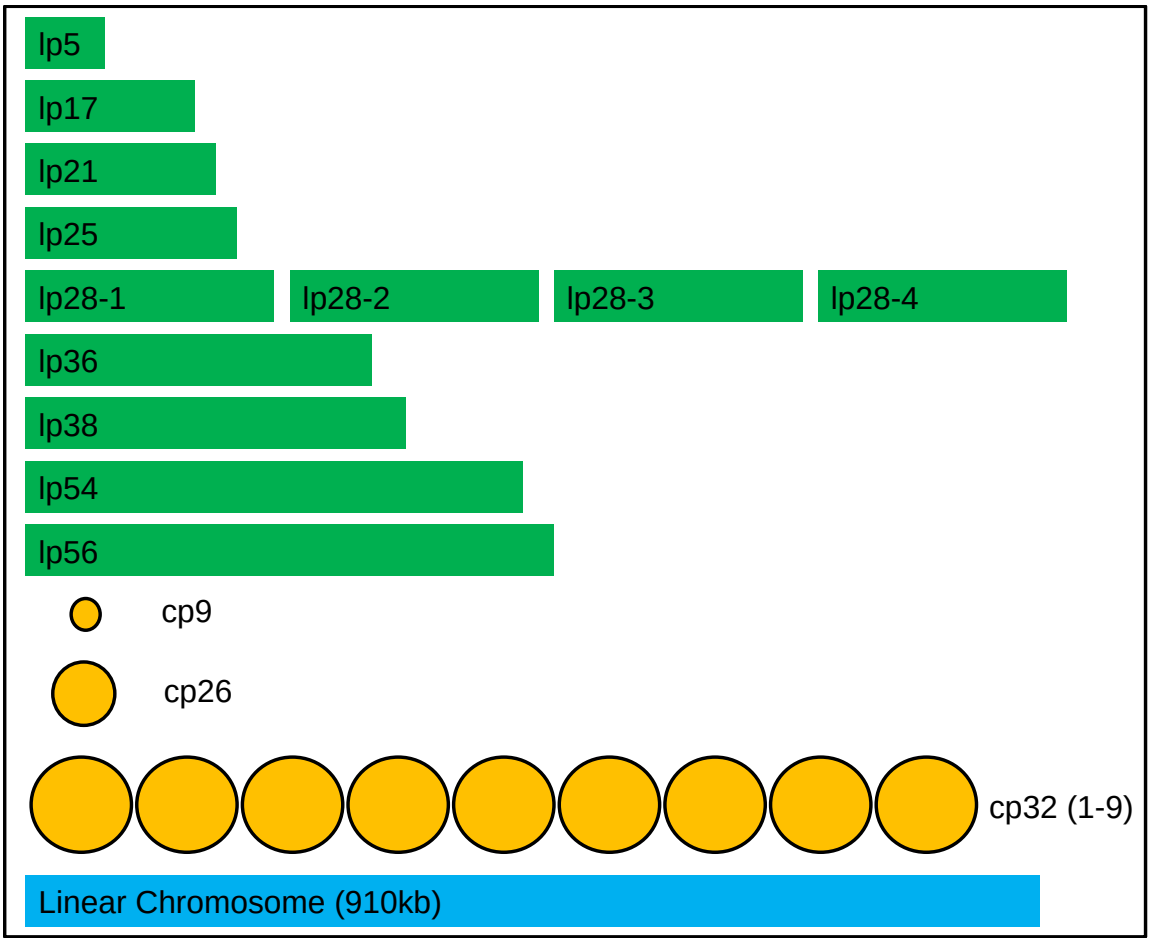

Figure 2. *B. burgdorferi* genome.

B. burgdorferi genome contains a linear chromosome, 12 linear plasmids and 9-11 circular plasmids. Linear chromosome is shown in blue, linear plasmids are shown in green and circular plasmids are shown in yellow. The name of individual plasmid represents the size of the plasmid [38].

Sequence analysis of plasmids reveals that 175 gene families, mostly unique to the *Borrelia* species and very few genes have predicted function. About 60% of genes found on *B. burgdorferi* plasmids are not present in the other organisms and are unique to *Borrelia*. Actual coding sequences on plasmid comprise 71% of the total plasmid DNA, which is less compared to the chromosome. This decreased coding sequence compared to the linear chromosome suggests these plasmids contain longer non-

coding inter-genic sequences. Each plasmid in *B. burgdorferi* B31 strain contains genes predicted to be essential for plasmid replication and compatibility. The number of these genes vary from 1-4 with most of the plasmids having a set of 4 genes [39].

B. burgdorferi has the capacity to lose plasmids during *in vitro* propagation, and the infection studies with high passage spirochetes show that the loss particular plasmids correlates with the loss of pathogenicity [40]. This was the first evidence that *B. burgdorferi* contains the plasmid encoded genes essential for the mouse infection. Some of the plasmids are ubiquitous and can never be lost by *B. burgdorferi*, like the circular plasmid 26 [41]. Some of the genes present on *B. burgdorferi* plasmids represent physiological functions. The presence of some plasmids like lp25 and lp56 make *Borrelia burgdorferi* transformation very difficult [42]. These plasmids contain genes that encode restriction and/or modification system that may result in the low *B. burgdorferi* transformation frequencies [42]. It was later found that a gene *bbe02* present on lp25 makes *B. burgdorferi* transformation difficult and knocking out just *bbe02* from lp25 and *bbq67* from lp56, makes the *B. burgdorferi* transformation easier [42].

Some of the genes on the *B. burgdorferi* plasmids are essential for mouse infection such as *guaA*, *guaB* [43], *bbb26* and *bbb27* [41] on cp26, *pncA* on lp25 and *bbk17* on lp36. *bbe22* alone can restore mouse infectivity when added back to spirochetes missing the entire lp25 plasmid suggests other genes on lp25 are not essential for mouse infection [44, 45]. *bbe16*, another gene on lp25, is required for tick colonization [46]. lp28-1 is one of the linear plasmids, absence of which leads to the reduced *B. burgdorferi* infectivity in mice [47]. lp28-1 contains VMP like sequence (vls)

which is involved in the antigenic variation [48]. So, if this plasmid is missing, spirochetes will not able to modifiy antigens to escape the host immune response [48, 49].

B. burgdorferi genes show differential expression with changes in the environment [50]. Throughout the complex infectious cycle of *B. burgdorferi*, which involves various hosts like small rodents, lizards, birds and large mammals and a tick vector, the spirochetes are exposed to the different environmental stresses (*i.e.*,differences in temperature, pH and nutrients). In ticks, spirochetes reside in the midgut, which has basic pH and a temperature of ~25°C [51]. In the mammalian host, blood pH is almost neutral and the temperature is 37°C [52]. To overcome these basic environmental differences, *B. burgdorferi* regulates its gene expression in response to each host. In particular *B. burgdorferi* alters expression of its outer surface proteins in response to distinct environmental signals [53, 54]. Spirochetes express outer surface proteins for dissemination, virulence and immune evasion [55]. OspA is an outer surface protein of *B. burgdorferi*, which helps *B. burgdorferi* to colonize in the tick midgut [25]. Upon a blood meal, spirochetes begin downregulating the production of OspA and increasing production of the mammalian specific OspC [54]. The blood meal serves as the trigger of a genetic switch in the tick colonized *B. burgdorferi,* altering the pH and temperature of the midgut environment.

Many of the molecular mechanisms of *B. burgdorferi* pathogenesis remain unknown. *B. burgdorferi* is not known to produce toxins but is still able to penetrate host tissues and cause infection [37, 39]. To understand *B. burgdorferi* pathogenesis in

depth, it is important to study the molecular mechanisms involved in the survival of these spirochetes in different host environments.

B. burgdorferi has a reduced genome that lacks the genes required for the synthesis of necessary biological molecules such as amino acids, fatty acids, enzyme co-factors and nucleotides [56]. Due to the lack of biosynthetic pathways, *B. burgdorferi* depends upon the host environment for the uptake of these essential nutrients [36, 43, 57, 58]. Barbour Stoenner Kelli II (BSKII) culture media is used for *in vitro* growth of *B. burgdorferi*. This media is highly rich in nutrients and is supplemented with the rabbit serum. A variety of genetic studies have shown that various plasmid encoded genes play essential roles in the nutrient acquisition. The *guaA* and *guaB* genes present on circular plasmid 26 encode for GMP synthase and IMP dehydrogenase respectively, which play an essential role in the *B. burgdorferi* purine salvage pathway [43]. PncA on *bbe22* (lp25) encodes for a nicotinamidase and is required for the biosynthesis of NAD [59, 60].

1.4 Purine metabolism

Purines and pyrimidines are the nucleotide base components of deoxyribonucleotides and nucleotides, which are the building blocks of DNA and RNA, respectively [56]. Each nucleotide is composed of a nitrogenous base that can be a purine (adenine, guanine) or pyrimidine (cytosine, thymidine), a five-carbon sugar (oxyribose for RNA, deoxyribose for DNA) and a phosphate group. The number of phosphate groups can vary between 1-3, depending upon the nucleotide e.g. nucleotides used in cell signaling like cAMP, cGMP have one phosphate group, whereas nucleotides playing role in the metabolism such as ATP, GTP CTP and UTP

are having three phosphate groups [61]. Nucleic acid contains five nucleobases such as adenine, guanine, thymidine, cytosine and uracil, among which adenine and guanine are classified as purines. Hypoxanthine is a purine derivative that is converted into IMP in the nucleotide biosynthesis pathway [43]. Purines consist of a pyrimidine ring and an imidazole ring. Apart from their crucial role in the DNA and RNA synthesis, purines are also important components of other biomolecules such as ATP, GTP as energy sources, cAMP as secondary messenger in biological processes and NADH as coenzyme in living cells [61-63].

Nucleotides are critical for the survival of all living cells and can be synthesized either *de novo* or through the purine salvage pathways. Ammonia, CO_2 and amino acids, serve as sources for the components used in the synthesis of these nucleotides. During the process of DNA and RNA synthesis, some nucleic acids undergo repair and degrade into purines and pyrimidine. It has been shown in some bacteria, like *Helicobacter pylori,* that the mechanisms for *de novo* nucleotide synthesis are absent, requiring purines for nucleic acid synthesis [64-67]. Purine metabolism is critical for the virulence of most of the bacterial pathogens in the mammalian host [68-70]. Degradation of the host DNA can serve as purine sources for bacterial pathogens [65].

1.5 Purine salvage in *Borrelia* species

Borrelia species have a reduced genome and lack *de novo* synthesis of purines to biosynthesize nucleotides. As a result, *Borrelia* species are dependent upon the host environments for purine and pyrimidine salvage. *Borrelia* species differ in these salvage pathways. *B. burgdorferi*, *B. afzelli* and *B. garinii* genomes lack genes involved in classic purine salvage pathway such as *purA* (adenylosuccinate synthase), *purB*

(adenylosuccinate lyase), *hpt* (hypoxanthine-guanine phosphoribosyltransferase), *nrdI* (auxiliary protein), *nrdE* (ribonucleotide-diphosphase reductase alpha subunit), *nrdF* (ribonucleotide-diphosphase reductase beta subunit), and a locus encoding ribonucleotide reductase (RNR) [37, 56, 71-73]. While, the relapsing fever spirochetes, *Borrelia hermsii* and *B. turicatae* possess all these genes. Ribonucleotide reductase, present in *Borrelia hermsii*, synthesizes deoxynucleotides to be utilized in DNA synthesis [73]. *B. burgdorferi* lacks RNR, and therefore requires either external sources of deoxyribonucleosides or alternative pathways to synthesize them. *B. burgdorferi* has a unique enzyme called deoxyribosyltransferase encoded by *bb0426* that is involved in the transfer of a deoxyribosyl moiety from deoxynucleosides to hypoxanthine to convert it into deoxyinosine (dI), and another enzyme deoxynucleotide kinase to convert dI into dIMP to synthesize DNA (Figure 3). Likewise, *B. burgdorferi* lacks *purA* and *purB* genes, so hypoxanthine utilization is limited to the synthesis of dGMP and GMP, unlike in *B. hermsii*, where hypoxanthine can also be utilized in the synthesis of dAMP and AMP. This suggests a requirement for host driven adenine nucleosides in *B. burgdorferi*, but not in *B. hermsii*. Phosphoribosyl transferase (PRT) transfers a phosphate group to a purine base for the synthesis of nucleoside monophosphates such as AMP, GMP, IMP or XMP [56]. Inosine monophosphate (IMP) is an important intermediate for the biosynthesis of nucleotides like AMP and GMP [43, 56]. Previous studies have shown that PRT activity in *B. hermsii* is 7 fold higher than in *B. burgdorferi* [73]. *B. hermsii* PRT has a similar activity for hypoxanthine and guanine, whereas *B. burgdorferi* does not have PRT activity with the guanine substrates little activity with hypoxanthine [73]. Rather, *B. burgdorferi* has an adenine deaminase enzyme, lacking from *B. hermsii*, that

converts adenine into hypoxanthine [57], which is then believed to be converted to IMP by PRT [56]. Due to high hypoxanthine-PRT activity in *B. hermsii*, direct incorporated hypoxanthine is sufficient to get converted into IMP. It has been observed that *B. hermsii* incorporate 9 fold higher hypoxanthine compared to *B. burgdorferi* [73]. Hypoxanthine is the most abundant purine in human plasma compared to the other purine bases, with hypoxanthine at 8.2 ± 1.3uM, xanthine at 2.5 ± 0.6uM, adenine at 0.3 ± 0.15uM and adenosine at0.6 ± 0.2uM) [74]. This most likely attributes to higher hypoxanthine PRT activity in *B. hermsii* compared to *B. burgdorferi*, as relapsing fever spirochetes have ability to achieve very high concentrations in blood by scavanging the available hypoxanthine [73].

Even though *B. burgdorferi* lacks some of the enzymes involved in the classic purine salvage pathways, these spirochetes are still able to salvage purines from the host environment and are able to survive in the host. *B. burgdorferi* possess IMP dehydrogenase (GuaB) and GMP synthase (GuaA), which are two critical enzymes in purine salvage pathways (Figure 3) [43]. GuaA and GuaB are essential for the survival of *B. burgdorferi* in a mouse and play an important role in the spirochete growth in the tick vector [43]. Also, in conditions of stress, the enzymatic activities of GuaA and GuaB are sufficient to synthesize GMP and dGMP, even when deoxyguanine nucleosides are low [73]. *In vitro* transport assays have shown that *B. burgdorferi* transport deoxynucleotides (dCMP, dGMP and dAMP) and oxynucleotides (CMP, GMP and AMP) that are incorporated into the DNA and RNA, respectively [56].

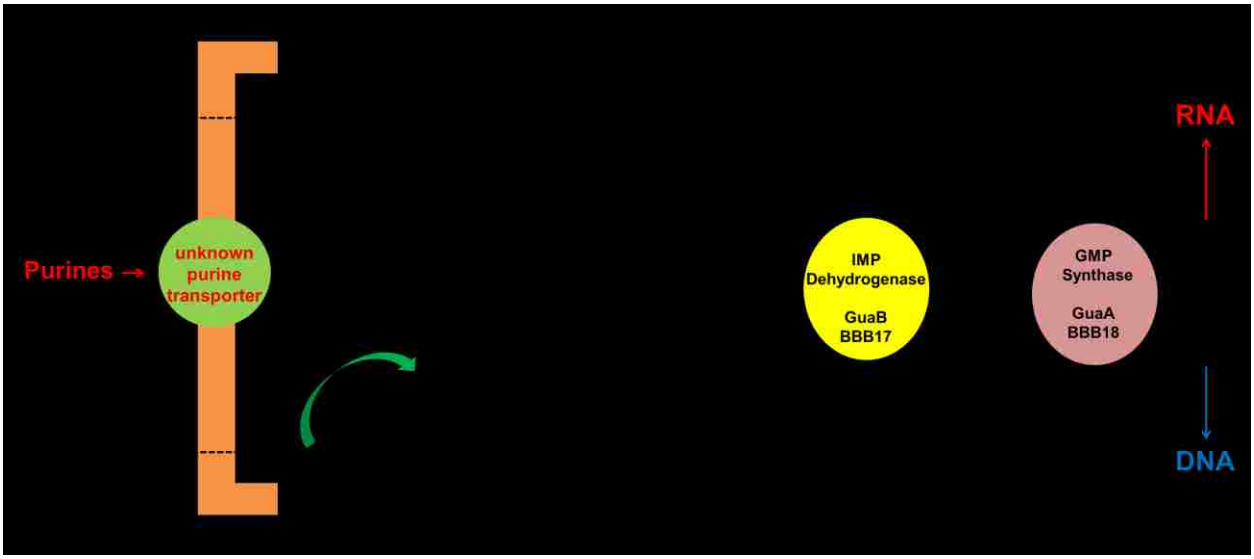

Figure 3. Novel purine salvage pathway in *B. burgdorferi*.

Adenine, hypoxanthine and guanine are transported from the host environment through unknown purine transporter/s. Once these purines are incorporated into the cytoplasm, adenine is converted into hypoxanthine by adenine deaminase [43, 56, 73]. Hypoxanthine is converted into IMP by phosphoribosyltransferase [43, 56]. Hypoxanthine to dIMP conversion involves deoxyribosyltransferase followed by deoxynucleotide kinase. IMP is further converted into GMP through IMP dehydrogenase (GuaB) and GMP synthase (GuaA). GMP and dGMP are utilized in the synthesis of RNA and DNA, respectively. *B. burgdorferi* also uptakes nucleotides and deoxynucleotides which are dephosphorylated prior to transport. Later these nucleosides and deoxynucleosides are phosphorylated again and are directly utilized in the synthesis of RNA and DNA. Deoxynucleosides also serve as a source of deoxy group to hypoxanthine to synthesize deoxyinosine [56].

1.6 Purine Transporters

Uptake of purines from the external environment can be important for cell survival, as these purines can be utilized as building blocks for DNA and RNA [56], or as carbon and nitrogen metabolic sources [75]. Specific membrane transport proteins are required to bring purines into the cell. The nucleobase/ascorbate transporter (NAT) family includes prokaryotic membrane transport proteins for purines and pyrimidines and eukaryotic ascorbate transport proteins [76, 77]. In mammals, NAT transporters are sodium symporters, whereas in bacteria these are proton symporter [77-79]. Bacterial

and fungal putative purine transporters fall under one of the two nucleobase cation symporter families nucleobase cation symporter 1 (NCS1) and nucleobase cation symporter 2 (NCS2). To date only a few purine transporters have been functionally characterized, with the majority belonging to the NCS2 family [80]. The NCS2 superfamily contains two clusters, COG2233 and COG2252. COG2233 cluster contains well studied permeases for xanthine and/or uric acid permeases, like xanthine permease (XanQ) of *E. coli* [80], uric acid permease (UacT) of *E. coli* [80], and xanthine/uric acid permease (UapA) of *Aspergillus nidulans* [80, 81]. COG2252 cluster contains purine transporters for hypoxanthine, adenine and guanine [80]. AzgA is a purine transporter of *Aspergillus nidulans*, which transports hypoxanthine, adenine and guanine [82]. It was the first characterized member of the membrane proteins [82]. *E. coli* contains four purine transport proteins that are similar in sequence to AzgA and characterized under the COG2252 cluster, PurP, YjcD, YgfO and YicO [80]. YjcD and YgfQ are high affinity hypoxanthine-guanine permeases and PurP and YicO are high affinity adenine permeases [80]. *Bacillus subtilis* contains two purine transporters PbuO and PbuG under COG2252 cluster. Both PbuO and PbuG are hypoxanthine-guanine permeases [80]. *Borrelia* burgdorferi lacks the genes required for the *de novo* synthesis of purines and to date, no purine transporters have been functionally characterized in any of the *Borrelia* species.

1.7 Unique features of circular plasmid 26 in *B. burgdorferi*

The circular plasmid 26, unlike some of the other circular plasmids, has never been observed to be lost through *in vitro* passaging of *B. burgdorferi* cultures [41, 83-85]. This suggests cp26 contains the genes essential for the survival of these spirochetes. There

are 29 ORFs present on cp26 and 14 of these show sequence homology with the genes from other organisms with known functions. The *resT* gene present on cp26 encodes for a telomere resolvase, which is required for resolution of the replicated telomeres of linear chromosome and linear plasmids [36]. Similarly, *bbb18*, *bbb17,* and *bbb19* encoding GuaA, GuaB, and OspC, respectively, are essential for spirochete survival in the mouse [43, 86]. As *B. burgdorferi* lacks *de novo* synthesis of purines, so it is dependent on purine salvage from the host environment. Purine permeases transporting these purines into *B. burgdorferi* are yet unknown. Genes *bbb22* and *bbb23* present adjacent to each other on cp26 are annotated to encode putative xanthine/uracil permeases under nucleobase cation symporter 2 (NCS2) family.

1.8 Hypothesis

The transport activities of the proteins encoded by *bbb22* and *bbb23* play a critical role in *B*orrelia *burgdorferi* survival and/or growth in an infectious cycle between the mammalian host and tick vector due to their roles in purine salvage.

CHAPTER 2: *BORRELIA BURGDORFERI* HARBORS A TRANSPORT SYSTEM ESSENTIAL FOR PURINE SALVAGE AND MAMMALIAN INFECTION

2.1 Preface

The first complete draft of the chapter was written by SJ. Comments from MWJ and reviewers were incorporated into the final version presented here. This chapter was published previously and is reprinted here with permission. Jain S., Sutchu S., Rosa PA., Byram R. and Jewett MW. 2012. *Borrelia burgdorferi* harbors a transport system essential for purine salvage and mammalian infection. Infection and Immunity. 80(9):3086-93. DOI: 10.1128/IAI.00514-12.

2.2 Abstract

Borrelia burgdorferi is the tick borne bacterium that causes the multistage inflammatory disease, Lyme disease. *B. burgdorferi* has a reduced genome and lacks the enzymes required for *de novo* synthesis of purines for synthesis of RNA and DNA. Therefore, this obligate pathogen is dependent upon the tick vector and mammalian host environments for salvage of purine bases for nucleic acid biosynthesis. This pathway is vital for *B. burgdorferi* survival throughout its infectious cycle as key enzymes in the purine salvage pathway are essential for the ability of the spirochete to infect mice and critical for spirochete replication in the tick. The transport of pre-formed purines into the spirochete is the first step in the purine salvage pathway and may represent a novel therapeutic target and/or means to deliver anti-spirochete molecules to the pathogen. However, the transport systems critical for purine salvage by *B. burgdorferi* have yet to be identified. Herein, we demonstrate that genes *bbb22* and

bbb23 present on *B. burgdorferi*'s essential plasmid circular plasmid 26 (cp26) encode key purine transport proteins. BBB22 and/or BBB23 are essential for hypoxanthine transport and contribute to the transport of adenine and guanine. Furthermore, *B. burgdorferi* lacking *bbb22-23* were non-infectious in mice up to a dose of $1x10^7$ spirochetes. Together our data establish that *bbb22-23* encode purine permeases critical for *B. burgdorferi* mammalian infectivity, suggesting that this transport system may serve as a novel anti-microbial target for the treatment of Lyme disease.

2.3 Introduction

Borrelia burgdorferi, the pathogenic spirochete that causes Lyme disease, is an obligate parasite that cycles in nature between the tick vector, *Ixodes sp.*, and mammalian host, typically a small rodent. Transmission of *B. burgdorferi* to humans by tick bite, although not part of the zoonotic infectious cycle of the spirochete, results in Lyme disease. Unlike many other bacterial pathogens, *B. burgdorferi* is not known to harbor classic virulence factors, such as toxins or secretion systems to deliver bacterial effector molecules [35, 37]. Clinical manifestations associated with Lyme disease are believed to result from the immune response to the spirochetal infection [87]. Therefore, genes encoding physiological functions that allow for growth within the infected host are important virulence determinants [43, 57, 60], as survival is a critical component of *B. burgdorferi* pathogenesis.

B. burgdorferi lacks the enzymes required for *de novo* synthesis of purines and therefore relies absolutely upon salvage of purines from its environment for nucleic acid biosynthesis [43, 56, 73, 88]. *B. burgdorferi* has evolved a novel purine salvage pathway that in addition to scavenge of purine bases, involves the salvage of

deoxynucleotides from the host environment. Purine metabolism in *B. burgdorferi* also utilizes interconversion of purine bases to deoxynucleosides by the deoxyribosyl transferase BB0426, and the catalysis of inosine monophosphate (IMP) to guanosine monophosphate (GMP), and deoxyinosine monophosphate (dIMP) to deoxyguanosine monophosphate (dGMP), by GuaA and GuaB, respectively [56]. This pathway is vital for *B. burgdorferi* survival throughout its infectious cycle as key enzymes in this purine salvage pathway are essential for the ability of the spirochete to infect mice and critical for spirochete replication in the tick [43, 57]. The transport of pre-formed purines into the spirochete represents the first step in the purine salvage pathway; however, the transport systems critical for purine salvage by *B. burgdorferi* have yet to be identified.

Circular plasmid 26 (cp26) is the only plasmid that is present in all *B. burgdorferi* isolates examined to date and is not lost during *in vitro* propagation. This plasmid carries genes essential for survival of the spirochete during both *in vitro* and *in vivo* growth [36, 41]. Cp26 harbors the essential gene *resT*, which encodes the telomere resolvase required for replication of linear plasmids and the linear chromosome [36], and the *ospC* gene, which is critical for mammalian infection [86, 89-91]. Furthermore, cp26 carries the genes encoding IMP dehydrogenase (GuaB) and GMP synthase (GuaA), two key enzymes in the *B. burgdorferi* purine salvage pathway, as described above [43, 56, 92]. In addition to genes involved in peptide transport (*oppAIV*) [58], chitobiose transport (*chbA, chbB* and *chbC*) [93] and glucose transport (*bbb29*) [36], cp26 harbors two adjacent genes, *bbb22* and *bbb23*, both of which are hypothesized to encode putative purine permeases [36].

Herein we establish that the BBB22 and BBB23 proteins function to transport hypoxanthine, adenine and guanine. Moreover, our results demonstrate that this transport system is absolutely required for survival of *B. burgdorferi* during mammalian infection.

2.4 Materials and methods

2.4.1: Bacteria clones and growth conditions.

All low-passage *B. burgdorferi* clones were derived from infectious clone A3-68ΔBBE02, which lacks cp9, lp56 and gene *bbe02* on lp25 [94]. *B. burgdorferi* was grown in liquid Barbour-Stoenner-Kelly (BSK) II medium supplemented with gelatin and 6% rabbit serum [95] and plated in solid BSK medium as previously described [96, 97]. All spirochete cultures were grown at 35°C supplemented with 2.5% CO2. Kanamycin was used at 200g/ml, streptomycin at 50g/ml and gentamicin at 40g/ml, when appropriate. All cloning steps were carried out using DH5α E. coli. E. coli cultures were grown in LB broth or on LB agar plates containing 50g/ml kanamycin, 300g/ml spectinomycin or 10g/ml gentamycin.

2.4.2: Deletion of *bbb22-23.*

A 3.8 kb DNA fragment encompassing the BBB22 and BBB23 ORFs along with 500 bp flanking region from both sides was amplified from the *B. burgdorferi* B31 clone A3 genomic DNA using the Expand Long PCR system (Roche) and primers 1 and 2 (Table 1) and was cloned into the TOPO XL vector (Invitrogen), creating plasmid TOPO XL *bbb22-23*+500. The 2.8 kb DNA sequence encoding the BBB22 and BBB23 ORFs was removed by inverse PCR using the Expand Long PCR system (Roche) and primers

7 and 8, generating linear p∆*bbb22-23* with SalI ends. Spectinomycin/streptomycin resistance cassette, *flaB$_p$-aadA* [41] with XhoI ends, was amplified using Taq polymerase and primers 3 and 4. The *flaB$_p$-aadA* product was treated with XhoI and ligated with SalI and DpnI-digested linear p∆*bbb22-23*, yielding the allelic exchange plasmid p∆*bbb22-23-flaB$_p$-aadA*. *B. burgdorferi* A3-68∆BBE02 was transformed with 20ug of p∆*bbb22-23-flaB$_p$-aadA* purified from *E. coli* as previously described [97-99]. Streptomycin-resistant colonies were confirmed to be true transformants by PCR using primer pairs 1 and 2 and 5 and 6. Positive ∆*bbb22-23-flaB$_p$-aadA* clones were screened with a panel of primers for the presence of all of the *B. burgdorferi* plasmids of the parent A3-68∆BBE02 clone [94], and a single clone was selected for further experiments.

2.4.3: Complementation of the ∆*bbb22-23* mutant.

A DNA fragment encompassing the *bbb22-23* genes and upstream putative promoter sequence was amplified from B31 A3 genomic DNA using Phusion enzyme (Thermo Scientific) and primers 5 and 6 (Table 1), which introduced BamHI and HindIII sites at the 5' and 3' ends, respectively. The BamHI+HindIII-digested PCR product was ligated into BamHI+HindIII-cut *B. burgdorferi* shuttle vector pBSV2G [98]. The plasmid structure and sequence were confirmed by restriction digest and DNA sequence analysis. The ∆*bbb22-23* mutant was transformed with 20 µg of pBSV2G *bbb22-23* or pBSV2G isolated from *E. coli* and positive transformants selected as previously described [57]. The clones that retained the *B. burgdorferi* plasmid content of the parent clone were selected for use in further experiments.

2.4.4: *In vitro* growth analysis.

Wild type (A3-68ΔBBE02), Δ*bbb22-23*/vector and Δ*bbb22-23/bbb22-23*$^{+}$ spirochetes were inoculated in triplicate at a density of 10^5 spirochetes/ml in 5 ml of BSK II medium. Spirochete densities were determined every 24 hours under dark field microscopy using a Petroff-Hausser chamber over the course of 96 hours.

2.4.5: RNA isolation from *in vitro* grown spirochetes.

Wild type (B31 A3) spirochetes were grown in triplicate in 5 ml of BSKII pH7.5 at 35°C to a density of $2x10^8$ spirochetes/ml. A total of $1x10^7$ spirochetes was harvested from each culture and resuspended in 25 µl of RNA*later* (Life Technologies) and stored at -80°C until processing. Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA was resuspended in 100 µl DEPC-treated dH_2O. RNA was treated with TURBO DNAse (Life Technologies) to remove any contaminating genomic DNA. 1 µl of Riboguard (40U/µl) RNAse inhibitor (Epicentre) was added to all samples and RNA stored at -80°C.

2.4.6: RNA isolation from infected mouse tissue.

B. burgdorferi infected mouse hearts (see Mouse infection experiments below) were manually macerated on ice using sterile scalpels and transferred to a 2 ml tube containing lysing Matrix D (MP Biomedicals). 1 ml of RNA pro solution (FastRNA Pro Green kit, MP Biomedicals) was added to each sample on ice. Tissues were homogenized using a PowerGen High-Throughput Homogenizer (Fisher Scientific) following six cycles of beating for 45 sec and 2 minutes on ice. Samples were centrifuged at 13,000 rpm for 5 minutes at 4°C. The upper aqueous phase was

transferred to new tubes and incubated for 5 minutes at room temperature. 500 µl of 1-bromo-3-chloropropane (Sigma) and 45 µl of 5M sodium acetate were added to each sample and samples were incubated for an additional 5 minutes at room temperature. Samples were centrifuged at 13,000 rpm for 5 minutes at 4°C. The upper aqueous phase was transferred to new tubes and RNA precipitated with the addition of 500 µl of absolute ethanol and 1 µl of GlycoBlue (Life technologies). RNA was pelleted by centrifugation at 13,000 rpm for 10 minutes at 4°C. RNA was washed with 70% ethanol in DEPC treated-H_20 and resuspended in 100 µl of DEPC treated-H_20. RNA was treated with TURBO DNAse (Life Technologies) to remove any contaminating genomic DNA. 1 µl of Riboguard (40U/ µl) RNAse inhibitor (Epicentre) was added to all samples and RNA stored at -80°C.

2.4.7: Gene expression analysis.

cDNA was synthesized from all RNA samples using the qScript Flex cDNA synthesis kit (Quanta BioSciences) with random hexamer primers according to the manufacturer's instructions. Parallel cDNA reactions were carried out in the absence of reverse transcriptase. Real-time quantitative PCR (qPCR) reactions were prepared using 5 □l cDNA and Perfecta qPCR Fast Mix (Quanta BioSciences). Using an Applied Biosystems 7500 instrument, samples were assayed for the *flaB* [43], *bbb22* and *bbb23* transcripts using primers pairs 13 and 14 and 15 and 16, respectively (Table 1) and probes 17 and 18, respectively (Table 1). The *flaB* transcript was used as the endogenous reference to which the transcripts of the target genes were normalized. The *bbb22* and *bbb23* primers were confirmed to be specific for their respective gene targets. The amounts of the *flaB*, *bbb22* and *bbb23* transcripts were determined using a

standard curve for each gene target that was generated using genomic DNA isolated from 10^6,10^5, 10^4, 10^3, 10^2 and 10^1 spirochetes. Samples were analyzed in triplicate with at least two biological replicates and gene expression reported as the number of gene transcripts per *flaB* mRNA copy. The amplification of samples lacking reverse transcriptase was similar to that of the no-template control. Data sets were compared using the nonparametric Kruskal-Wallis test with Dunn's multiple-comparison test using GraphPad Prism 5.0 for Windows (GraphPad Software).

2.4.8: Radioactive transport assays.

B. burgdorferi clones were grown in 40 ml of BSKII to a density of 8 x 10^7 spirochetes/ml, washed twice with HN buffer (50 mM Hepes, 50 mM NaCl, pH 7.6) and resuspended in a final volume of 2.5 ml of HN buffer. 500 µl (~ 5x10^8 spirochetes) aliquots of spirochetes were combined with 20 µl of 300 mM glucose (6 mM final concentration) and an additional 480 µl of HN buffer. Spirochetes were preincubated at 37°C for 30 minutes. 250 nM [^{3}H]hypoxanthine monohydrochloride (specific activity 20 Ci/mmol) (Perkin Elmer) or 33 nM [2,8-^{3}H]adenine (specific activity 30 Ci/mmol) (Perkin Elmer) was added to the reaction and 100 µl aliquots of the reaction (5x10^7 spirochetes) were removed at various time points following the addition of radioactivity and filtered onto cellulose acetate filter membranes using a 10 place filtration manifold (Hoefer, Inc.) and immediately washed with 10 ml of HN buffer. Filter-bound spirochetes were collected in scintillation vials containing 3ml of ScintiVerse BD cocktail scintillation fluid (Fisher Scientific) and radioactivity counted in a Microbeta^2 with an efficiency of 52% (Perkin Elmer). The rates of uptake of hypoxanthine and adenine (picomoles/10^7 spirochetes/minute) were calculated by first converting CPMs to picomoles assuming 1

CPM = disintegrations per minute (dpm) x an efficiency of .52 and 1 Ci = $2.2x10^{12}$ dpm. The rates corresponding to the time points for the linear portion of each uptake curve were averaged to determine the average rate of uptake.

Spirochetes for [^{3}H]hypoxanthine monohydrochloride uptake assays in the presence of cold competitors were grown and prepared as described above. 1 mM stock solutions of each cold competitor: hypoxanthine, adenine, guanine, xanthine, cytosine, thymine, uracil, ascorbic acid, and 5'-deoxy-5'-(methylthio)adenosine (MTA) were prepared in 8 mM NaOH. Reactions were initiated with the addition of 50 □l of competitor (50 µM final concentration, 200-fold excess) or 50 µl of 8 mM NaOH alone and 250 nM [^{3}H]hypoxanthine monohydrochloride. A 100 µl aliquot ($5x10^7$ spirochetes) of each reaction was removed 10 minutes following the addition of radioactivity and filtered onto cellulose acetate filter membranes using a 10 place filtration manifold (Hoefer, Inc.) and immediately washed with 10 ml of HN buffer. Filter-bound spirochetes were collected in scintillation vials containing 3ml of ScintiVerse BD cocktail scintillation fluid (Fisher Scientific) and radioactivity counted in a Microbeta^2 (Perkin Elmer). A3-68ΔBBE02 and A3-68ΔBBE02 heated to 95°C for 10 minutes served as the wild type control and the heat killed negative control, respectively, for all transport assays. Each transport assay was repeated in triplicate.

2.4.9: Mouse infection experiments.

The University of Central Florida (UCF) is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Protocols for all animal experiments were prepared according to the guidelines of the National Institutes of Health and approved by UCF's Institutional Animal Care and Use

Committee. For gene expression studies, four mice (C3H/HeN, 6-8 week old females, Harlan) were needle inoculated, 80% intraperitoneal and 20% subcutaneous, with *B. burgdorferi* clone B31 A3 at a dose of $1x10^5$ spirochetes. For infectivity studies, groups of 6 mice each (C3H/HeN, 6-8 week old females, Harlan) were needle inoculated, 80% intraperitoneal and 20% subcutaneous, with clone Δ*bbb22-23*/vector or Δ*bbb22-23*/*bbb22-23*$^+$ at a dose of $1x10^4$ or $1x10^7$ spirochetes. The number of spirochetes inoculated into mice was determined using a Petroff-Hausser counting chamber and verified by colony-forming unit (cfu) counts in solid BSK medium. Ten colonies per inoculum were verified by PCR for the presence of the virulence plasmids lp25, lp28-1 and lp36 in at least 90% of the individuals in the population. Further, total plasmid content of each inoculum was confirmed to be as expected [57]. Mouse infection was assessed three weeks post inoculation by serology and reisolation of spirochetes from ear, bladder and joint tissues, as previously described [57]. Heart tissues were harvested from B31 A3 inoculated mice for RNA isolation and gene expression studies.

2.5 Results

2.5.1: The BBB22 and BBB23 open reading frames on cp26.

The BBB22 and BBB23 open reading frames (ORFs) are in the same orientation and separated by 109 bp [35, 37]. The *bbb22* and *bbb23* genes (1356 bps and 1392 bps, respectively) and their annotated proteins (451 amino acids and 463 amino acids, respectively) are 79.8% and 78.3 % identical at the nucleotide and amino acid levels, respectively, suggesting that these genes may be paralogs and their encoded proteins may have overlapping functions in *B. burgdorferi*. Both BBB22 and BBB23 are annotated as guanine/xanthine permeases and have a conserved domain COG2252

(xanthine/uracil/vitamin C permease) that is a distant subfamily of the NCBI CD superfamily cI00967 NAT (Nucleobase/Ascorbate Transporter) family [100]. BBB22 and BBB23, similar to other COG2252 domain proteins, are predicted to both localize to the bacterial inner membrane and contain 12 transmembrane domains [82, 101-104] (Fig. 4). Current annotation of the BBB23 ORF suggests that translation of the BBB23 protein initiates at the alternative start codon TTG and produces a 463 amino acid protein that is 12 amino acids longer than the predicted 451 amino acid size for the BBB22 protein [35, 37] (Fig. 4). Upon further analysis of the BBB23 ORF sequence, we identified an ATG start codon 36 nucleotides within the current annotation of the BBB23 ORF, which is preceded by a reasonable Shine-Dalgarno sequence five base pairs upstream. Our prediction of this ATG residue as the putative true start codon for the BBB23 protein resulted in the additional prediction that the BBB23 ORF produces a 451 amino acids protein, as is predicted for BBB22.

Figure 4. The BBB22 and BBB23 proteins demonstrate a high level of identity.

Shown is an amino acid alignment of the BBB22 (NP_047008.1) and BBB23 (NP_047009.2) proteins, according to current annotation. Identical amino acids are shaded. Amino acid sequences were aligned using the Clustal W algorithm in the MegAlign program from the DNASTAR Lasergene suite. The putative sequences of the 12 transmembrane domains were predicted using the TMHMM Server v. 2.0 [105] and are indicated with gray underlines. Divergent sequences in putative periplasmic loops 4 and 6 are indicated with black underlines. An alternative prediction for the start codon of BBB23 is indicated with a black arrow.

2.5.2: The *bbb22-23* genes are essential for *B. burgdorferi* mouse infectivity.

Given the high degree of amino acid identity between the BBB22 and BBB23 proteins, it is reasonable to predict that these two proteins could have overlapping functions and in the absence of one, the other may be able to functionally compensate. Moreover, we have found that both genes are expressed during mammalian infection (Fig. 5), suggesting that both proteins may be important for survival in the mouse.

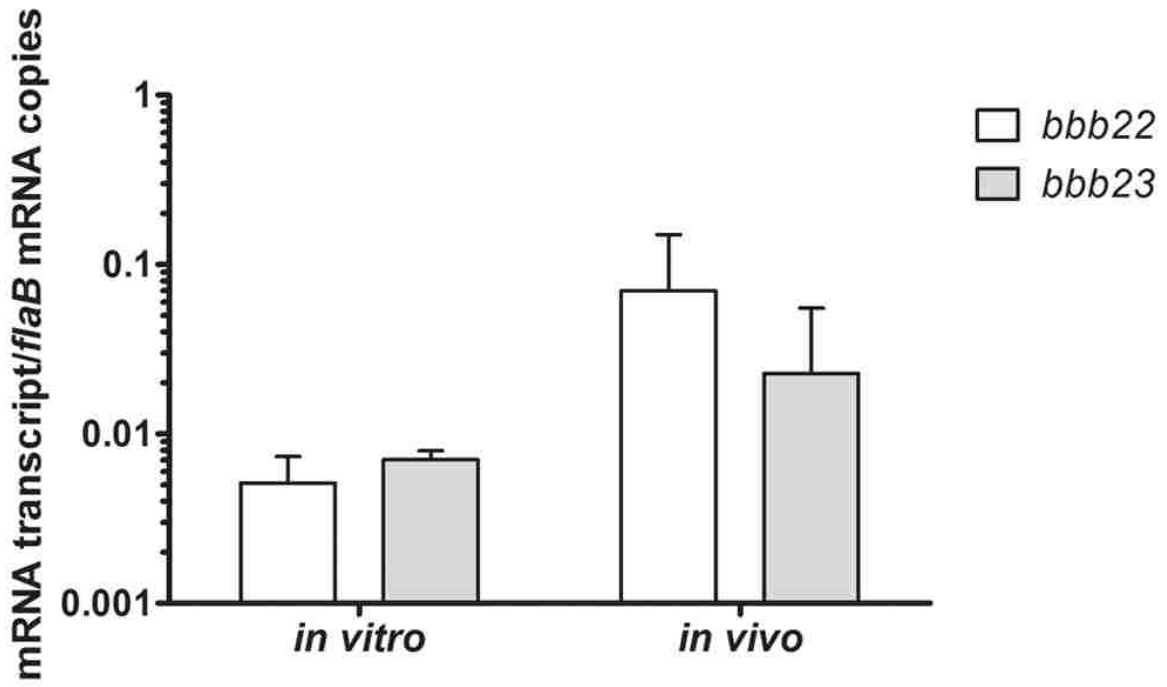

Figure 5. The *bbb22* and *bbb23* genes are expressed during murine infection.

Spirochete RNA was isolated from log phase ($3x10^7$ spirochetes/ml) *B. burgdorferi* clone B31 A3 grown in BSKII medium, pH 7.5 at 35°C (*in vitro*). Total RNA was isolated from spirochete infected heart tissue harvested 2 weeks post intradermal inoculation of C3H/HeN mice with $1x10^5$ *B. burgdorferi* clone B31 A3 (*in vivo*). Expression of genes *flaB*, *bbb22* and *bbb23* was quantified by reverse transcriptase qPCR. The data are expressed as the number of *bbb22* (open bars) or *bbb23* (gray bars) gene transcripts per *flaB* mRNA copies. The *in vitro* data represent the average of two biological replicates. The *in vivo* data represent the average of four biological replicates. Error bars represent the standard deviation from the mean. All data sets were compared pairwise using the Kruskal-Wallis nonparametric rank test followed by Dunn's multiple-comparison test (GraphPad Prism 5.0) and were found to have no statistical difference.

Therefore, in order to examine the combined functional role of the BBB22 and BBB23 proteins during mammalian infection, the sequence encompassing both open reading frames was deleted from low passage, infectious *B. burgdorferi* and replaced with the *flaB*$_p$-*aadA* spectinomycin/streptomycin resistance cassette [41] by allelic

exchange (Fig. 6). Successful recovery of Δ*bbb22-23* spirochetes demonstrates that the function of the encoded proteins is not essential for *B. burgdorferi* survival in *in vitro*. The Δ*bbb22-23* spirochetes were genetically complemented *in trans* on the shuttle vector pBSV2G with wild type copies of the *bbb22* and *bbb23* genes and102 bps of 5' flanking sequence encompassing the presumed promoter [98]. *In vitro* growth analysis demonstrated no difference in growth between Δ*bbb22-23*, Δ*bbb22-23*/*bbb22-23*$^{+}$ and wild type spirochetes over the course of 96 hours (data not shown), suggesting that the function of the gene products are not required for spirochete replication in nutrient rich BSKII *in vitro* growth medium.

Table 1: List of primers and probes used in this study.

Primer/Probe	Designation	Sequence (5'-3')
1	BBB22 -500	CAGTTAAAGAGCTTACAAGCCC
2	BBB23 + 500	AGGATACCTTGCAATATGATCC
3	aadA-3' Xho1	CCGCTCGAGTTATTTGCCGACCTACCTTGG
4	flaB- 5' Xho1	CCGCTCGAGCTGTCGCCTCTTGTGGCTTC
5	BBB22-3' HindIII	CCCAAGCTTGACTCTTTTTTATGATTTATAATTTAGG
6	BBB23-5' BamHI	CGGGATCCTATTTTCAAACTTTACCTGACAGCG

Primer/Probe	Designation	Sequence (5'-3')
7	BBB22F (17918) Sall 3'	ACGCGTCGACCCTAAATTATAAATCATAAAAAAGAGTC
8	BBB23R (20824) Sall 5'	ACGCGTCGACAATAATTATTAGGCTTTTAATTCTTTTTAAAGGCC
9	BBB22-Fwd Taqman	AAGAAATTATCGGTGGTATTACCACTTT
10	BBB22-Rev Taqman	CAATTGGCATACCTGTGCTAGATAATAT
11	BBB23-Fwd Taqman	GCACTAGTTACCGCAACCTGTCT
12	BBB23-Rev Taqman	CTCATTCCAGAAGCCAATGCT
13	BBB22 Probe	AGCATGGCATACATAATAGCTGTTAATCCGGC
14	BBB23 Probe	CTAATGGGACTTTATACCAATACGCCTT

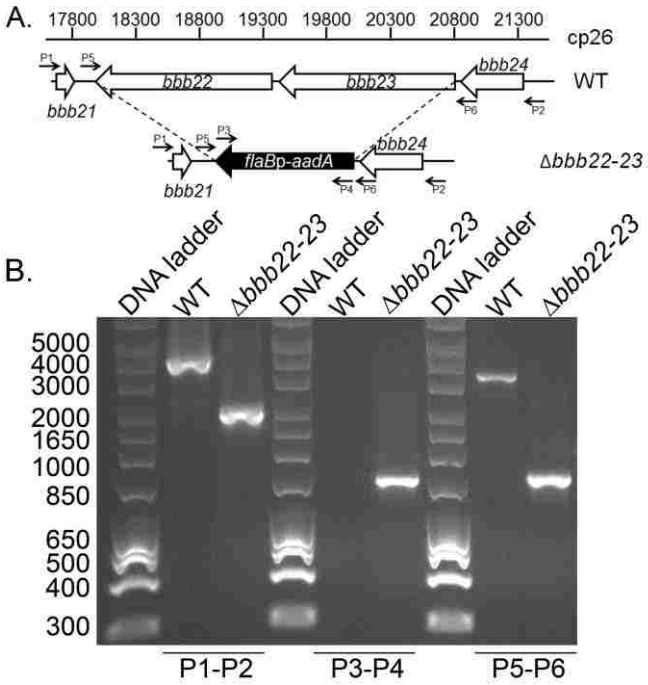

Figure 6. Generation of the Δ*bbb22-23* mutant in *B. burgdorferi*.

(A) Schematic representation of the wild type (WT), Δ*bbb22-23* loci on cp26. The sequence of the BBB22-23 open reading frame was replaced with a *flaB*$_p$-*aadA* antibiotic resistance cassette. Locations of primers for analysis of the mutant clones are indicated with small arrows and labels P1-P6. Primer sequences are listed in Table 1. The boundaries of the deletion construct are indicated by broken lines. (B) PCR analysis of mutant clones. Genomic DNA isolated from WT and Δ*bbb22-23* spirochetes served as the template DNA for PCR analyses. DNA templates are indicated across the top of the gel image. The oligonucleotide pairs used to amplify specific DNA sequences are indicated below the gel image and correspond to target sequences as shown in A.

The contribution of the *bbb22-23* genes to *B. burgdorferi* mammalian infectivity was assessed by inoculation of mice with mutant spirochetes lacking *bbb22-23* or the isogenic *bbb22-23*$^+$ complemented clone at a dose of 1x10^4 spirochetes. None of the mice inoculated with the Δ*bbb22-23* mutant spirochetes became infected, as assessed by serology and reisolation of spirochetes from tissues 3 weeks post inoculation (Table 2). Conversely, six out of six mice inoculated with the *bbb22-23*$^+$ complemented clone were seropositive and spirochetes were reisolated from tissues (Table 2). To further

explore the role of these genes in *B. burgdorferi* murine infection, mice were inoculated with $1x10^7$ mutant or complemented spirochetes. Strikingly, no mice inoculated with spirochetes lacking *bbb22-23* were infected, even at this high dose, whereas all mice inoculated with the complemented spirochetes became infected (Table 2). Together these data demonstrate that *bbb22* and/or *bbb23* are absolutely required for *B. burgdorferi* mouse infectivity by needle inoculation.

Table 2: Genes *bbb22* and/or *bbb23* are required for *B. burgdorferi* mouse infectivity.

Clone	Spirochete dose per mouse	Infected mice[a]
Δ*bbb22-23*/pBSV2G	$1x10^4$	0/6
	$1x10^7$	0/6
Δ*bbb22-23*/pBSV2G *bbb22-23*	$1x10^4$	6/6
	$1x10^7$	6/6

[a] Number of infected mice/ number of mice analyzed determined 3 weeks post inoculation by positive serology against *B. burgdorferi* proteins and reisolation of spirochetes from ear, bladder and joint tissues.

2.5.3: BBB22 and/or BBB23 are hypoxanthine, adenine and guanine transporters.

Transport and incorporation of hypoxanthine and adenine by wild type *B. burgdorferi* have been demonstrated previously [56, 73, 88]. Contrary to the annotated function of COG2252 domain proteins, none of the biochemically characterized members of this family has been shown to transport xanthine, uracil or vitamin C. Instead, they have all been found to function as purine permeases capable of transporting adenine, hypoxanthine and/or guanine [82, 106-108], supporting the hypothesis that BBB22 and BBB23 are responsible for transport of purines in *B.*

burgdorferi. BBB22-23^{+} and BBB22-23^{-} spirochetes were examined for transport of hypoxanthine and adenine using a radioactive filter-binding assay [109]. Transport of [^{3}H]hypoxanthine was found to be dependent on the BBB22 and/or BBB23 proteins as the Δ*bbb22-23* mutant clone, similar to heat killed wild type spirochetes, was incapable of hypoxanthine uptake (Fig. 7A). Conversely, wild type and complemented spirochetes demonstrated increased accumulation of [^{3}H]hypoxanthine over a time period of approximately 15 minutes (Fig. 7A). Unlike transport of [^{3}H]hypoxanthine, transport of [^{3}H]adenine was not completely BBB22 and/or BBB23-dependent (Fig. 7B). The Δ*bbb22-23* mutant clone demonstrated transport of [^{3}H]adenine. The [^{3}H]average rate of adenine uptake for the Δ*bbb22-23* mutant spirochetes (0.015 ± 0.006 pmol/10^{7} spirochetes/min) was reduced 68-fold relative to that of wild type and complemented spirochetes (0.99 ± 0.02 pmol/10^{7} spirochetes/min). A time period of approximately 15 minutes was required for the mutant spirochetes to accumulate a maximum level of [^{3}H]adenine equivalent to spirochetes harboring functional BBB22-23 proteins. Wild type and complemented spirochetes achieved maximum [^{3}H]adenine uptake within the first one minute of the assay (Fig. 7B). The average rate of [^{3}H]adenine uptake by wild type and complemented spirochetes was 29-fold faster than the average rate of [^{3}H]hypoxanthine uptake by the same two clones (0.035 ± 0.005 pmol/10^{7} spirochetes/min). [^{3}H]Adenine transport by wild type spirochetes was greater than 80% inhibited in the presence of 200-fold excess of unlabeled adenine (data not shown). Together these data demonstrate that BBB22 and/or BBB23 are responsible for salvage of hypoxanthine; whereas, these proteins contribute to uptake of adenine by *B.*

burgdorferi but the spirochetes appear to harbor additional transport systems important for this function.

To further elucidate the function of the BBB22-23 transport system, we took advantage of the finding that BBB22 and/or BBB23 is the only transporter system for hypoxanthine uptake. The ability of these proteins to transport other substrates was determined by measuring [^{3}H]hypoxanthine uptake in the presence of 200-fold excess unlabeled competitors, including purine derivatives, pyrimidines and ascorbic acid. As expected unlabeled adenine and hypoxanthine competed for [^{3}H]hypoxanthine uptake. The percent [^{3}H]hypoxanthine uptake in the presence of unlabeled adenine or hypoxanthine was not statistically difference from that of heat killed or Δ*bbb22-23* mutant spirochetes (adenine: *P*=0.1, *P* =0.95, respectively; hypoxanthine: *P* =0.69, *P* =0.27, respectively; unpaired t-test) (Fig. 7C). Furthermore, these analyses established that guanine is an additional substrate for BBB22 and/or BBB23, as the percent [^{3}H]hypoxanthine uptake in the presence of excess unlabeled guanine was similar to that of heat killed and Δ*bbb22-23* mutant spirochetes (*P* =0.38, *P* =0.11, respectively; unpaired t-test) (Fig. 7C). The presence of xanthine, cytosine, thymine, uracil or ascorbic acid had no inhibitory effect on [^{3}H]hypoxanthine uptake, indicating that these metabolites are not transported by BBB22 or BBB23 (Fig. 7C). All in all, these data clearly establish BBB22 and/or BBB23 function as hypoxanthine-adenine-guanine permeases.

5'-Methylthioadenosine (MTA) is a sulfur-containing nucleoside produced from S-adenosylmethionine (SAM) during polyamine synthesis in mammalian tissues [110] and polyamine, autoinducer-1 and biotin synthesis in bacteria [111]. Bacterial 5'-

methylthioadenosine/S-adenosyl homosysteine (MTA/SAH) nucleosidases (Pfs) catalyze the cleavage of MTA into 5'-methylthioribose (MTR) and adenine [112]. *Borrelia burgdorferi* harbors three *pfs* homologues, BB0588 (*bgp*/*pfs-2*), BB0375 (*pfs-1*) and BBI06 (*mtnN*). Bgp and Mtn are believed to be present on the surface of the spirochete, whereas Pfs-1 is predicted to localize to the cytoplasm [111, 112]. Both Bgp and Pfs-1 have demonstrated MTA nucleosidase activity [112] and together these proteins are predicted to contribute to adenine salvage from spirochete and host metabolism [111]. To test the hypothesis that MTA may provide a source of adenine for salvage by *B. burgdorferi*, MTA was examined for its ability to compete for [^{3}H]hypoxanthine transport by BBB22-23. In the presence of MTA [^{3}H]hypoxanthine transport was significantly reduced relative to transport in the absence of competitor (*P*=0.0006; unpaired t-test); however, MTA was not sufficient to reduce [^{3}H]hypoxanthine transport levels equivalent to that of heat killed or Δ*bbb22-23* mutant spirochetes (*P*=0.0012, *P*=0.0030, respectively; unpaired t-test) (Fig. 7C). These data support the hypothesis that exogenous MTA can serve as a source of adenine for salvage by *B. burgdorferi*.

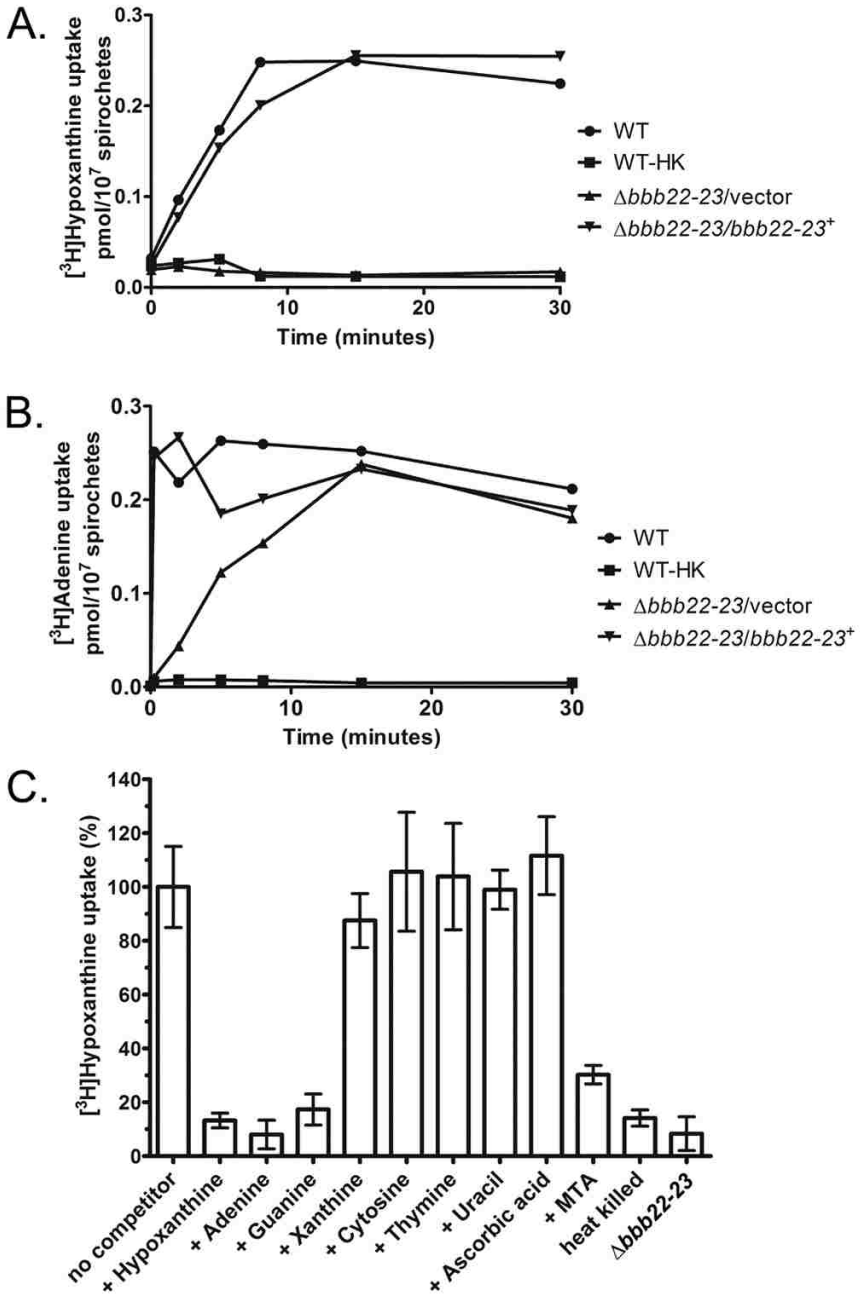

Figure 7. BBB22 and/or BBB23 are hypoxanthine, adenine and guanine permeases.

[^{3}H]Hypoxanthine uptake (A) and [^{3}H]adenine uptake (B) by wild type (WT), heat-killed WT (WT-HK), Δ*bbb22-23* mutant (Δ*bbb22-23*/vector) and Δ*bbb22-23* complemented (Δ*bbb22-23*/*bbb22-23*$^+$) spirochetes was measured over a 30 minute time course. Uptake of radiolabeled purine is expressed in picomoles/10^7 spirochetes/minute. The final concentrations of labeled hypoxanthine and adenine in the reactions were 250nM and 33nM, respectively. The specific activities of labeled hypoxanthine and adenine were 20 Ci/mmole and 30 Ci/mmole, respectively. Data shown are representative

results of one of three replicate experiments. (C) [^{3}H]Hypoxanthine (250nM, 20 Ci/mmole) uptake by wild type spirochetes was measured in the absence (no competitor) or presence of 200-fold excess (50 µM) unlabeled competitor as indicated. Heat killed wild type (heat killed) and Δ*bbb22-23* mutant (Δ*bbb22-23*) spirochetes in the absence of competitor served as negative controls. Radioactive uptake was measured 10 minutes after the addition of [^{3}H]Hypoxanthine with or without unlabeled competitor. Radioactive uptake by wild type spirochetes in the absence of competitor was taken as 100%. All other data are represented as the percent uptake relative to wild type spirochetes in the absence of competitor. Error bars represent the standard deviation of triplicate experiments. Statistical analyses were performed using the unpaired t-test (GraphPad Prism 5.0).

2.6 Discussion

During its infectious cycle *B. burgdorferi* experiences environments with potentially distinct purine availability. Hypoxanthine is the most abundant purine in mammalian blood [113], and it is presumed to be available for salvage by *B. burgdorferi* during the blood meal of an infected tick and during the spirochete's transient presence in the mammalian bloodstream. During mammalian infection *B. burgdorferi* resides in various tissues, which have been shown to be rich in adenine [114]. Guanine is present at low levels in mammalian blood and tissues [113, 114]. It has been demonstrated previously that *B. burgdorferi* is able to transport and incorporate adenine, hypoxanthine, nucleosides and deoxynucleosides into DNA and RNA [56, 73]. However, little to no transport of guanine or xanthine has been previously observed for *B. burgdorferi* [73]. We now establish that the homologous proteins BBB22 and/or BBB23, encoded on cp26, transport hypoxanthine, adenine and guanine and are necessary for *B. burgdorferi* mammalian infection. The *bbb22* and/or *bbb23* gene products were found to be essential for hypoxanthine uptake. At least one of these transporters is also important for adenine uptake; however, our data revealed that an additional, as of yet unknown, protein(s) also contributes to this function. Nonetheless, the function of the additional adenine transporter(s) was not sufficient to allow full or even attenuated

survival of Δ*bbb22-23* mutant *B. burgdorferi* in the mouse, as spirochetes lacking only *bbb22-23* were unable to infect mice up to a dose of $1x10^7$ bacteria. These data suggest that transport of hypoxanthine, rather than adenine, may be critical during the initial stages of *B. burgdorferi* mammalian infectivity, consistent with the transient dissemination of spirochetes through the blood early in infection, while adenine salvage may be critical for persistent survival of *B. burgdorferi* in tissues. In addition to direct scavenge of adenine from host tissues, our data suggest that catabolism of MTA produced by the host may provide a source of exogenous adenine for purine salvage. *B. burgdorferi* harbors two MTA nucleosidase homologs, Bgp and BBI06, which have been shown or predicted, respectively, to localize to the spirochete's outer surface, suggesting that the biological role of these enzymes is to catabolize host derived MTA [112]. Spirochetes lacking *bgp* maintain wild type virulence in mice [112], indicating that BBI06 is able to compensate for the loss of Bgp activity or MTA catabolism contributes little to *B. burgdorferi* survival in the mammal. Hypoxanthine transport by BBB22 and/or BBB23 was competitively inhibited by guanine, demonstrating for the first time that *B. burgdorferi* is able to transport guanine and this likely occurs via BBB22 and/or BBB23. Contrary to annotation of BBB22 and BBB23 as xanthine/uracil/vitamin C permeases, none of these metabolites were able to compete for hypoxanthine transport. Furthermore, BBB22 and BBB23 function did not include pyrimidine transport, as neither cytosine nor thymine competed for hypoxanthine transport. These data are consistent with the function of the protein members of a new class of nucleobase transporters containing COG2252 defined by the *Aspergillus nidulans* hypoxanthine-adenine-guanine permease, AzgA [82]. Additional characterized members of this

subfamily include the *E. coli* adenine permease, PurP [106, 107] and the two *Bacillus subtilis* guanine-hypoxanthine permeases, PbuG and PbuO [108, 115, 116].

Despite the critical roles of BBB22 and/or BBB23 in purine uptake *in vivo*, spirochetes lacking *bbb22-23* do not exhibit a growth defect *in vitro*. The rich, complex *B. burgdorferi in vitro* growth medium provides excess purines and is likely a source of nucleosides and deoxynucleosides. In the presence of excess nutrients the unidentified, additional adenine transporter system may provide a mechanism for the spirochetes to salvage sufficient amounts of adenine for survival in the absence of BBB22 and BBB23. We have demonstrated previously that adenine is converted to hypoxanthine by the adenine deaminase activity of BBK17 (AdeC), which may relieve the requirement for hypoxanthine salvage in environments high in adenine [57]. *B. burgdorferi* has been shown to import and incorporate into DNA and RNA exogenous nucleosides and deoxynucleosides [56]. Direct salvage of nucleosides and deoxynucleosides by a transport system other than BBB22 and BBB23 may allow the spirochetes to bypass the requirement for hypoxanthine, adenine and/or guanine transport to synthesize DNA and RNA. This mechanism has also been suggested to explain the absence of an *in vitro* growth defect for spirochetes lacking the *in vivo* essential, purine salvage genes *guaA* and *guaB* [43].

The high degree of sequence identity shared between *bbb22* and *bbb23* suggests that these two genes have evolved as the result of a duplication event. Moreover, this prediction raises the question of why *B. burgdorferi* has two purine permeases encoded by adjacent, paralogous genes on cp26. One possibility is that the two proteins have distinct substrate specificities. The BBB22 and BBB23 proteins are

predicted to traverse the spirochete's inner membrane and therefore the amino acid sequences of the six periplasmic loops of each protein are likely important determinants of the substrates that are transported by these purine permeases. Two of the six putative periplasmic loops within BBB22 (amino acids 207-254 and 418-431, respectively) and BBB23 (amino acids 219-266 and 430-443) (Fig. 4) are predicted to be highly divergent between the two proteins, suggesting that these domains may contribute to differential substrate specificity between the two proteins, if any. Alternatively, BBB22 and BBB23 may have similar substrate specificities but each protein functions in response to distinct environmental cues. The sequences and synteny of the BBB22 and BBB23 homologs appear to be conserved among the Lyme disease *Borrelia sp.*. Conversely, the relapsing fever spirochetes appear to only harbor distantly related BBB23-like sequences (data not shown). Furthermore, the Lyme disease spirochetes lack the key purine salvage enzymes ribonucleotide reductase, hypoxanthine-guanine phosphoribosyltransferase (Hpt), adenylosuccinate synthase (PurA) and adenylosuccinate lyase (PurB) [56, 73, 88] that are present in the relapsing fever spirochetes [56, 73, 88] and the presence or absence of BBB22 and BBB23 homologs may reflect the distinct purine salvage requirements between the two groups of pathogens.

The *bbb22* and *bbb23* transcripts were detected in spirochetes grown in the *in vivo* mammalian host environment. Although not statistically significant, the data suggest that the levels of *bbb22* and *bbb23* expression *in vivo*, as determined by reverse transcription quantitative real time PCR, may be increased relative to the levels of expression of these genes in nutrient rich *in vitro* growth conditions. Furthermore,

these data imply that the expression of the encoded permeases may be upregulated in response to potentially nutrient limited growth conditions in which purine salvage is likely critical for *B. burgdorferi* survival. Key purine salvage enzymes, IMP dehydrogenase (GuaB) and GMP synthase (GuaA) for the synthesis of GTP and dGTP, have been shown to be indispensable for *B. burgdorferi* survival throughout its infectious cycle [43], suggesting that the tick vector and mammalian host environments are limited for direct scavenge of GTP and dGTP for RNA and DNA biosynthesis. Moreover, despite evidence presented herein that guanine can be transported by BBB22 and/or BBB23, guanine and guanine nucleotide precursors are thought to be limiting for *B. burgdorferi* survival *in vivo* [43, 56]. *B. burgdorferi* has evolved a unique purine salvage pathway in the absence of the "classic" purine salvage pathway enzymes [56, 73, 88], in order to generate dGTP from salvaged hypoxanthine or hypoxanthine derived from deaminated salvaged adenine [43, 56, 57]. Based on these data, it is intriguing to speculate that expression of *bbb22* and/or *bbb23* may be controlled by the availability of guanine nucleotide precursors leading to increased hypoxanthine and/or adenine transport when guanine nucleotide pools are low. In support of this notion, purine nucleotide metabolism in *E. coli* and *B. subtilis* has been shown to be controlled by the availability of purine nucleotides from the environment [107, 108, 117]. The relative contributions of BBB22 and BBB23 individually to purine salvage and spirochete survival *in vivo*, as well as the regulatory mechanism(s) controlling *bbb22* and *bbb23* expression, are currently under investigation.

We have demonstrated that BBB22 and BBB23 function in the critical first step of the purine salvage pathway in *B. burgdorferi* and in turn, are a gateway into the

spirochete for the acquisition of essential nutrients. These transporters may also provide a novel means to deliver potential anti-spirochete molecules. Understanding the molecular determinants of substrate specificity and regulation of BBB22 and BBB23 may provide the framework for rational design of inhibitors or anti-metabolites targeted against the *B. burgdorferi* purine salvage pathway resulting in new treatments for Lyme disease.

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CHAPTER 3: MOLECULAR DISSECTION OF A *BORRELIA BURGDORFERI IN VIVO* ESSENTIAL PURINE TRANSPORT SYSTEM

3.1 Abstract

Borrelia burgdorferi, the causative agent of Lyme disease, lacks the enzymes required for *de novo* synthesis of purines for the biosynthesis of RNA and DNA. As a result, purine salvage from the host environment is critical for the survival of this pathogen. Previously, our group has shown that genes *bbb22* and *bbb23*, present on circular plasmid 26 (cp26), encode for purine permeases, which together are important for the transport of hypoxanthine, adenine and guanine. Additionally, genes *bbb22* and *bbb23* together were found to be essential for *B. burgdorferi* mouse infection. Herein, our results demonstrate that individually *bbb23* and *bbb22* each is capable of transporting hypoxanthine, adenine and guanine. However, BBB23 demonstrates a lower affinity for hypoxanthine and adenine compared to that of BBB22. Spirochetes harboring *bbb23* alone were non-infectious in mice; whereas spirochetes harboring only *bbb22* were proficient for infection in mice. Although, the spirochete loads in the tissues from mice infected with spirochetes expressing *bbb22* alone were low relative to the number of spirochetes in the tissues isolated from mice infected with spirochetes expressing both *bbb22* and *bbb23*. These data suggest that both genes *bbb22* and *bbb23* are critical for *B. burgdorferi* to achieve maximal infection of mice and the differences in the capabilities of the two transporters may reflect distinct purine salvage needs that the spirochete encounters throughout its nature infectious cycle.

3.2 Introduction

Purine nucleobases are required for the synthesis of DNA and RNA. Consequently, purine biosynthesis and/or transport are critical processes across all kingdoms of life. Moreover, nucleobase transporters represent possible therapeutic targets for cancer and infectious diseases [118].

Borrelia burgdorferi , the causative agent of Lyme disease, has a limited genome and lacks the enzymes required for *de novo* purine biosynthesis [35, 37, 56, 73, 88]. Therefore, purine salvage from host environments is important for *B. burgdorferi* survival and pathogenesis [41, 43, 119]. Recently, our laboratory established that genes *bbb22* and *bbb23*, on *B. burgdorferi* essential circular plasmid (cp) 26, together encode a purine transport system that is required for hypoxanthine transport and contributes to adenine and guanine transport [119]. Spirochetes lacking both *bbb22* and *bbb23* are non-infectious in mice [119]. Conversely, these genes are dispensable for *B. burgdorferi* growth in nutrient-rich medium *in vitro* [119].

The *bbb22* and *bbb23* genes encode proteins within cluster COG2252 of the nucleobase cation symporter-2 superfamily (NCS2) [119], which includes permeases found in bacteria, fungi and plants that are specific for adenine, hypoxanthine and/or guanine [80, 82, 115, 120-123]. The *Aspergillus nidulans* AzgA transporter, the founding member of this family of transporters, has specificity for adenine, hypoxanthine and guanine [82, 120, 122]. In other species, however, specific purine transport functions have been attributed to distinct proteins. For example, *E. coli* harbors two high affinity adenine permeases, PurP and YicO and two high affinity hypoxanthine-guanine permeases, YjcD and YgfQ [80, 121]. Similarly, *B. subtilis* has distinct uptake systems

for hypoxanthine-guanine and adenine [115, 124]. The Arabidopsis proteins AtAzg1 and AtAzg2 have been shown to be plant adenine-guanine transporters [123] although hypoxanthine uptake has yet to be examined for these proteins. The *B. burgdorferi* BBB22 and BBB23 open reading frames share 79.8% and 78.3% identity at the nucleic acid and amino acid levels, respectively, and are adjacent genes on cp26, suggesting that the two genes may be paralogs [119]. While the transport activities conferred by *bbb22* and *bbb23* together closely resemble the adenine-hypoxanthine-guanine permease function of AzgA [82, 119, 122], the individual contributions of *bbb22* and *bbb23* to the uptake of specific purines remains unknown. Although the overall amino acid sequences of the BBB22 and BBB23 proteins are highly conserved, sufficient sequence divergence may occur within two of the six putative periplasmic loops, which may allow for differential substrate specificity between the two proteins. In addition, it is possible that both genes may confer the same transport functions but are differentially expressed.

Here, we analyzed the individual roles of *bbb22* and *bbb23* in purine transport and mammalian infection. Our findings demonstrated that BBB22 and BBB23 were each capable of hypoxanthine, adenine and guanine uptake, albeit with differing affinities. Moreover, *bbb22*, but not *bbb23*, alone restored murine infectivity to Δ*bbb22-23* spirochetes. Although, the spirochetes carrying *bbb22* alone were unable to achieve the spirochete loads in the infected mouse tissues equivalent to that of *bbb22-23*$^{+}$ spirochetes. Together these data suggest that both genes *bbb22* and *bbb23* are critical for *B. burgdorferi* to achieve wild type levels of purine transport and infection of mice.

3.3 Materials and methods

3.3.1: Bacterial clones and growth conditions.

Escherichia coli strain DH5α, were grown in LB broth or LB agar plates at 37ºC. Gentamicin and spectinomycin were used at 10 µg/ml and 300 µg/ml, respectively. All *B. burgdorferi* clones were generated in the low passage B31 A3-68-ΔBBE02-Δ*bbb22-23* clone, which lacks genes *bbb22-23* and *bbe02* and plasmids cp9 and lp56 [119]. *B. burgdorferi* clones were grown in Barbour-Stoenner-Kelly (BSK) II medium at 35ºC. Gentamicin, streptomycin and kanamycin were used at 40 µg/ml, 50 µg/ml and 200 µg/ml, respectively.

3.3.2: 5' RACE.

Total RNA was isolated with TRIzol reagent (Life Technologies) from 50 ml culture of *B. burgdorferi* clone B31 A3 grown in BSKII to a density of $4x10^7$ spirochetes/ml (log phase) or 5 ml culture at a density 2×10^8 spirochetes/ml (stationary phase). 5' Rapid Amplification of cDNA Ends (5'RACE) using the 5' RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Life Technologies) was performed according to the manufacturer's instructions. 4 µg of RNA was used per reaction and along with gene specific primers 1297, 1298, and 1299 for *bbb22*, primers 1316, 1317 and 1318 for the transcript within *bbb23* and primers 1300, 1301 and 1302 for *bbb23* (Table 3). Nested PCR products were purified using a PCR purification kit (Qiagen) and the 5' end of each transcript determined by sequence analysis (Genewiz).

Table 3. Primers and probes used in this study.

Primer number	Primer name	Sequence (5'-3')
1006	BBB23 ORF 1392-BamHI-Fwd	**CCCGGGGGATCC**TTGAAAATATTTTTAAACAATAAAAAGGAAA GTTT
1007	BBB23 ORF 1356-Fwd-BamHI	**CCCGGGGGATCC**ATGGGTAAGTATGTAAAAGGTTTATTTTTT
1011	BBB23 ORF 1392-Rev-SalI	**CCCGGGGTCGAC**TTACAGATCTTCTTCAGAAATAAGTTTTTGT TCATAGCC
1123	recA-F	AATAAGGATGAGGATTGGTG
1124	recA-R	GAACCTCAAGTCTAAGAGATG
1137	*flaB*-Taqman-FWD	TCTTTTCTCTGGTGAGGGAGCT

Primer number	**Primer name**	**Sequence (5'-3')**
1037	pUC18F	CCCAGTCACGACGTTGTAAAAC
1138	*flaB*-Taqman-REV	TCCTTCCTGTTGAACACCCTCT
1140	*Nid*-Taqman-FWD	CACCCAGCTTCGGCTCAGTA
1141	*Nid*-Taqman-REV	TCCCCAGGCCATCGGT
1166	BBB23 Taqman Fwd	GCACTAGTTACCGCAACCTGTCT
1167	BBB23 Taqman Rev	CTCATTCCAGAAGCCAATGCT
1168	BBB22 Taqman Fwd	GAATCAATCCAAAGAAACATTGTTAT

Primer number	Primer name	Sequence (5’-3’)
1169	BBB22 Taqman Rev	AGTTGCAGTAACTAATGCGCCAAT
1297	BBB22 GSP-1A (663)	TACTGCGCTTTCTGGAC
1298	BBB22 GSP-1B (388)	AATTTGCAATACTTTCCCTAGC
1299	BBB22 GSP-1D (188)	GCTGATGTTAAGCAAGTTGCAG
1300	BBB23 GSP-1A (700)	CCTGGGCACCTTCTAAAT
1301	BBB23 GSP-1B (424)	AGTTTATAATTTGCTCCCTTACTC
1302	BBB23 GSP-1D (222)	TGCTGTTAGACAGGTTGCG

Primer number	Primer name	Sequence (5'-3')
1316	BBB22 IVET RACE GSP1 (1089)	GGCAAAAAAAACTGCAAATAGAAATAATG
1317	BBB22 IVET RACE GSP2 (945)	GCCGTCTACCAGTAATATTTTTTTTGC
1318	BBB22 IVET RACE GSP3 (837)	ATTGAACAAGAGAATAAAAACTATTGATATAAAAGTCC
1525	BBB23-cmyc-HindIII Rev	**CCCGGGAAGCTT**TTACAGATCTTCTTCAGAAATAAGTTTTTGTT CATAGCCATAAATAAATTTAATAATAAAAATTAAGC
1526	BBB23 promoter KpnI FW	**CCCGGGGGTACCTA**TTTTCAAACTTTACCTGACAGCG
1527	BBB23 promoter BamHI R	**CCCGGGGGATCC**TAATAATTATTAGGCTTTTAATTCTTTTTAAA
1528	BBB22 BamHI-Fwd	**CGGGATCC**TCTCCTGTACTGCTAATATTATGC

Primer number	Primer name	Sequence (5'-3')
1529	BBB22 HindIII- Rev	**CCCAAGCTT**GACTCTTTTTTATGATTTATAATTTAGG
1577	*flaB*p-fwd-KpnI	**CCCGGGGGTACC**TGTCTGTCGCCTCTTGTGGCT
1578	*flaB*p-Rev-BamHI	**GGGGGATCC**GATTGATAATCATATATCATTCCT
1668	pUC18R	AGCGGATAACAATTTCACACAG
1139	*flaB* Taqman probe	6-FAM-AAACTGCTCAGGCTGCACCGGTTC-TAMRA
1142	*Nid* Taqman probe	6-FAM-CGCCTTTCCTGGCTGACTTGGACA-TAMRA
1575	BBB22 Taqman probe	6-FAM-AGCATGGCATACATAATAGCTGTTAATCCGGC-TAMRA

Primer number	Primer name	Sequence (5’-3’)
1576	BBB23 Taqman probe	6-FAM-CTAATGGGACTTTATACCAATACGCCTT-TAMRA

3.3.3: Generation of plasmids containing *bbb22* and *bbb23*.

A region of 107 base pairs sequence upstream of the BBB23 ORF (23_p) [119], and *flaB* promoter sequence were amplified from *B. burgdorferi* B31 clone A3 genomic DNA using Phusion enzyme (Thermo Scientific) and primer pairs 1526 and 1527, and 1577 and 1578 (Table 3), respectively. The *B. burgdorferi* shuttle vector pBSV2G [125] and promoter fragments were digested with restriction enzymes KpnI and BamHI, ligated and cloned in *E. coli*. Plasmids were confirmed by restriction digestion and DNA sequence analysis. The annotated BBB23 ORF sequence was amplified from B31 A3 genomic DNA using Phusion enzyme (Thermo Scientific) and primer pair 1006 and 1525. Subsequently PCR using primer pair 1006 and 1011 (Table 3) and the PCR product derived from primer pair 1006 and 1525 as a template was used to replace the 3' HindIII restriction site with SalI. The *bbb23* gene was ligated into pBSV2G 23_p and pBSV2G $flaB_p$ plasmids using restriction enzymes BamHI and SalI and cloned in *E. coli*. Plasmids pBSV2G 23_p-*bbb23* and pBSV2G $flaB_p$-*bbb23* were confirmed by restriction digestion and DNA sequence analysis. DNA fragments containing the BBB22 ORF and 1466 bp of upstream sequence ($22dist_p$) or the BBB22 ORF and 160 bp of upstream sequence ($22prox_p$) were amplified from B31 A3 genomic DNA using Phusion enzyme (Thermo Scientific) and primer pairs 1007 and 1529 and 1528 and 1529, respectively. The DNA fragments were ligated into the pBSV2G vector using BamHI and HindIII restriction sites and the plasmids cloned in *E. coli*. Plasmids pBSV2G $22dist_p$-*bbb22* and pBSV2G $22prox_p$-*bbb22* were confirmed by restriction digestion and DNA sequence analysis (Genewiz).

3.3.4: Reintroduction of *bbb22* and *bbb23* into *bbb22-23* mutant spirochetes.

B. burgdorferi clone B31 A3-68-ΔBBE02-Δ*bbb22-23* was transformed with 20µg of plasmid pBSV2G 23_p-*bbb23*, pBSV2G $flaB_p$-*bbb23*, pBSV2G $22prox_p$-*bbb22* or pBSV2G $22dist_p$-*bbb22* and the transformants were selected in semi-solid BSKII 1.5X medium containing 1.7% agarose and 40 µg/ml gentamicin at 37ºC, 2.5% CO_2 for 6-8 days. *B. burgdorferi* transformants were confirmed by colony PCR using primers 1037 and 1668. The endogenous plasmid content of each new *B. burgdorferi* clone was confirmed by PCR analysis [57] to contain the plasmid content of the B31 A3-68-ΔBBE02-Δ*bbb22-23* parent clone [119].

3.3.5: RNA extraction from *in vitro* grown cultures.

B. burgdorferi clones *bbb22-23*$^+$, Δ*bbb22-23*/vector, 23_p-*bbb23*$^+$, $flaB_p$-*bbb23*$^+$, $22prox_p$-*bbb22*$^+$ and $22dist_p$-*bbb22*$^+$ were grown to a density of 2 x 10^8 spirochetes/ml in BSKII medium containing antibiotics. 2 x 10^8 spirochetes were harvested at 5800 xg for 10 minutes. RNA was isolated using TRIzol reagent (Life Technologies) or Direct-zol RNA MiniPrep Kit (Epigenetics) and resuspended in 50µl of DEPC-treated dH_2O. RNA samples were DNAse treated using Turbo DNA-free (Life Technologies) and confirmed to be free of *B. burgdorferi* genomic DNA contamination by PCR using primers 1123 and 1124. 1µl of SUPERaseIn RNase inhibitor (Life Technologies) was added to prevent RNA degradation and samples were stored at -80ºC.

3.3.6: Gene expression analysis.

400 ng of *B. burgdorferi* RNA, extracted from late stationary phase spirochetes, as described above, was used to synthesize cDNA with the iScript Select cDNA

synthesis kit (Bio Rad) using random hexamer primers. Reactions lacking reverse transcriptase were run in tandem as negative controls. Taqman real time quantitative PCR was performed using 400 ng cDNA, IQ Super Mix (Bio Rad) and primer/probe sets 1168, 1169 and 1575 (*bbb22*), 1166, 1167 and 1576 (*bbb23*) and 1137, 1138 and 1139 (*flaB*), respectively (Table 3). mRNA transcript copies for *bbb22* and *bbb23* were normalized with *flaB* mRNA copies. All the data sets were compared using unpaired t-test using GraphPad Prism 5.0.

3.3.7: Radioactive transport assays.

B. burgdorferi clones were grown to late stationary phase at density of $2x10^8$ spirochetes/ml. Radioactive transport assays were performed as previously described [119]. Briefly, spirochetes were harvested and washed twice in HN buffer (50 mM Hepes, 50 mM NaCl, pH 7.6) and resuspended in HN buffer to a density of $1x10^9$ spirochetes/ml. $5x10^8$ spirochetes were pre-incubated at 37ºC for 30 minutes in 1 ml reaction volumes containing HN buffer, 3% rabbit serum (Pelfreez) and 6mM glucose. 1.25 µM [^{3}H]hypoxanthine monohydrochloride (specific activity 20 Ci/mmol) (Perkin Elmer) or 0.25 µM [2,8-^{3}H]adenine (specific activity 30 Ci/mmol) (Perkin Elmer) were added to the reactions. $5x10^7$ spirochetes were removed at 10 seconds and 15 minutes following the addition of radioactivity and filtered onto cellulose acetate filter membranes (Fisher Scientific) using a 10 place filtration manifold (Hoefer, Inc.) pre-loaded with 10 ml of HN buffer and washed again with 10 ml of HN buffer. Filter-bound spirochetes were collected in scintillation vials containing 3ml of ScintiVerse BD cocktail scintillation fluid (Fisher Scientific) and radioactivity counted in a Microbeta^2 with an efficiency of

52% (Perkin Elmer). The amounts of hypoxanthine and adenine uptake were converted to femtomoles/ $5x10^7$ spirochetes as previously described [119].

For *B. burgdorferi* clones for [^{3}H]hypoxanthine uptake assays in the presence of cold competitors were grown and prepared as described above. Stock solutions (1 mM) of each cold competitors hypoxanthine, guanine and adenine and cytosine were prepared in 25 mM NaOH. All the reactions were carried out in a 1 ml volume containing $1x10^9$ spirochetes, 250 nM [^{3}H]hypoxanthine and either 5 µl of cold competitor (5 µM final concentration, 20-fold excess) or 5µl of 25 mM NaOH alone . 100 µl aliquots containing 10^8 spirochetes were removed 15 minutes following the addition of radioactivity and collected as described above. The percent [^{3}H]hypoxanthine uptake for each reaction condition was calculated relative to the amount of [^{3}H]hypoxanthine transport in the absence of competitor. *bbb22-23*$^+$ spirochetes heated to 95ºC for 10 minutes (heat killed) served as the negative control for all transport experiments.

3.3.8: Mouse infection experiments.

University of Central Florida is accredited by International Association for Assessment and Accreditation of Laboratory Animal Care. All the mice experiments were done according to the guidelines of National Institutes of Health (NIH) and approved by Institutional Animal Care and Use Committee of UCF. Groups of 6-8 week old C3H/HeN female mice (Harlan) were inoculated with *B. burgdorferi* clone *bbb22-23*$^+$, Δ*bbb22-23*/vector, *23*$_p$*-bbb23*$^+$, *flaB*$_p$*-bbb23*$^+$, *22prox*$_p$*-bbb22*$^+$ or *22dist*$_p$*-bbb22*$^+$. Mice were inoculated intraperitoneally (80%) and subcutaneously (20%) with a total dose of $1x10^4$ spirochetes. Inoculum cultures were analyzed for the endogenous plasmid

content by PCR, plated on solid BSK medium and individual colonies from each population analyzed for the presence of virulence plasmids lp25, lp28-1 and lp36 [41]. All inoculum cultures carried the expected endogenous plasmid content (Table 4) and 70-100% of individual colonies from each clone were confirmed to contain all three virulence plasmids. Blood for serological analysis was collected prior to inoculation and 3 weeks post-inoculation. Mouse infection was determined by serology and spirochete reisolation from tissues as previously described [119].

3.3.9: Quantitation of spirochete loads in mouse tissues.

Ear, heart and joint tissues were collected for DNA extraction from infected mice. DNA extraction was performed as previously described [57]. Real time quantitative PCR was performed using 100 ng DNA from infected mouse tissues. *B. burgdorferi flaB* copies and mouse *nid* copies were quantified using *flaB* primers/probe and mouse *nid* primers/probe, respectively (Table 3) and IQ supermix (Bio Rad), as previously described [57]. *B. burgdorferi flaB* copy number was normalized with 10^{10} *nid* copies. Unpaired t-test statistical analysis was performed using GraphPad Prism 5.0.

3.4 Results

3.4.1: The *bbb22* and *bbb23* genes are separate transcripts.

We previously demonstrated that genes *bbb22-23,* present on circular plasmid 26 (cp26), encode for purine permeases, which together are essential for *B. burgdorferi* mouse infection [119]. However, the individual contributions of *bbb22* and *bbb23* to purine transport and mouse infection remains unknown. The annotated BBB22 and BBB23 open reading frames are separated by a 109 bp intergenic region [37],

suggesting that these two genes may be transcribed separately and therefore may play distinct roles in ability of *B. burgdorferi* to salvage purines. Rapid amplification of cDNA ends (RACE) was used to identify the 5' ends of the *bbb22* and *bbb23* transcripts. The transcription start site for *bbb23* was found to be 260 bps upstream of the annotated BBB23 open reading frame (ORF), which corresponds to nucleotide 21082 on cp26 and is 225 bps within the 3' end of BBB24 (Figure 8). Whereas, the transcription start site for *bbb22* (*bbb22prox*) was found to be 16 base pairs upstream of the annotated BBB22 ORF at nucleotide 19338 on cp26 (Figure 8). In addition, an *in vivo* expression technology (IVET)-based genetic screen performed by our laboratory to identify *B. burgdorferi* sequences that are expressed during murine expression [126] identified a candidate promoter sequence (*Bbive103*; nucleotides 20322-20238 on cp26) within and in the same orientation to the BBB23 ORF. Consistent with this finding, 5' RACE analysis validated the presence of a transcription start site at nucleotide 20219, 17 bps downstream of the *Bbive103* sequence (*bbb22dist*) (Figure 8). The double sequence observed in the sequence analysis of the *bbb22dist* 5' RACE PCR product (Figure 8B) reflects detection of both the transcript derived from the putative distal promoter for *bbb22* as well as the *bbb23* transcript itself. Together these data indicate that *bbb23* and *bbb22* have distinct transcription start sites and that an additional transcription start site is present 897 bps upstream of *bbb22* within *bbb23*.

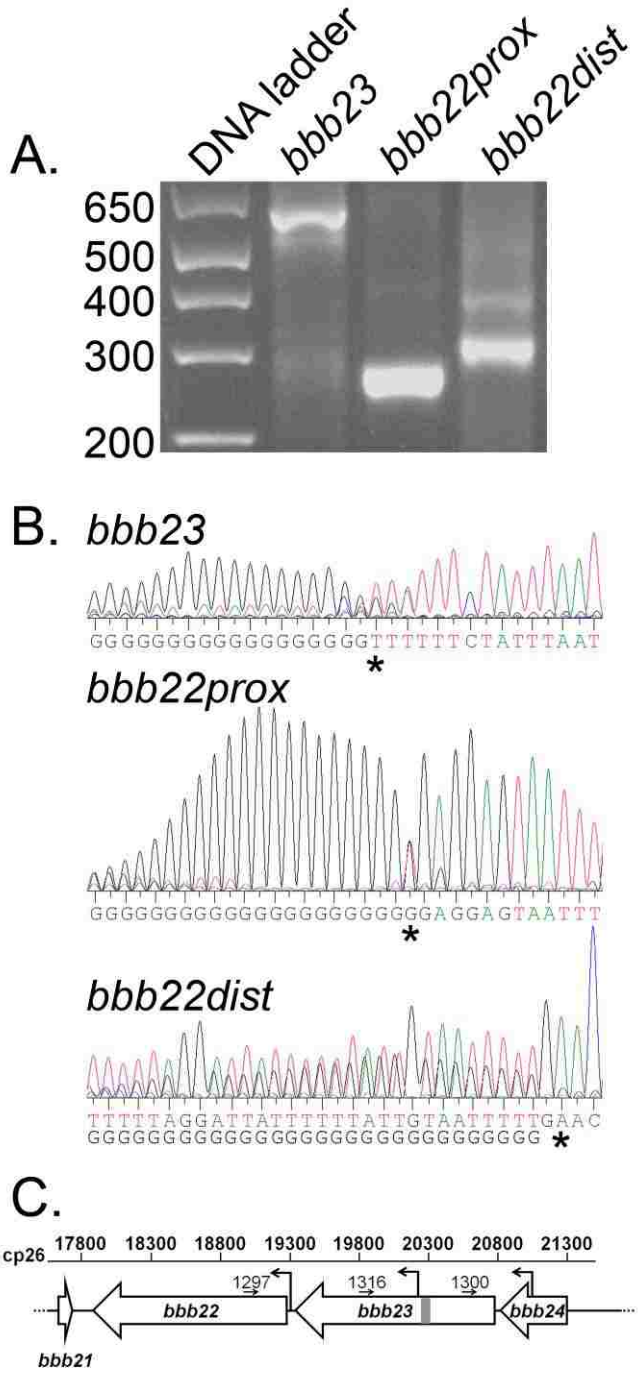

Figure 8. Genes *bbb22* and *bbb23* each harbor their own promoter.

Rapid Amplification of cDNA Ends (5' RACE) analysis of the transcription start sites for the *bbb23*, *bbb22prox* and *bbb22dist* transcripts using RNA extracted from B31 clone A3 *B. burgdorferi*. (A) Transcript specific 5' anchored nested PCR products generated from the 5' RACE analysis. Data shown are representative of two replicate experiments. The DNA ladder is shown in base pairs. (B) DNA sequence chromatographs of the

DNA sequence of the 5' RACE products shown in A. Both sequences detected for *bbb22dist* are shown. The 5' nucleotide of each transcript is indicated with a star. (C) Schematic diagram of the genetic organization of genes *bbb22* and *bbb23*. Transcription start sites identified in B are shown as bent arrows. The BbIVET *in vivo* active promoter sequence within the BBB23 ORF is shown as a light gray box. The location and numbers of the transcript specific primers used to generate the cDNA for the 5' RACE analysis are indicated. A ruler for the nucleotide coordinates on cp26 is shown.

3.4.2: A long 5'UTR within *bbb23* is required for wild type expression of *bbb22*.

Toward the goal of understanding the individual roles of *bbb22* and *bbb23* a panel of plasmids was engineered using the *B. burgdorferi* shuttle vector pBSV2G [125] and the BBB23 ORF along with its endogenous promoter sequence (*23*$_p$-*bbb23*$^+$), the BBB23 ORF along with the constitutive *flaB* promoter, the BBB22 ORF along with the proximal promoter sequence (*22*prox$_p$-*bbb22*$^+$) or the BBB22 ORF along with the proximal and distal promoter sequences (*22*dist$_p$-*bbb22*$^+$) (Table 4). In order to generate spirochetes that only harbored *bbb22* or *bbb23*, the shuttle vector plasmids carrying *bbb23* or *bbb22* were each transformed into a low passage *B. burgdorferi* clone that lacks both *bbb22* and *bbb23* [119]. All *B. burgdorferi* transformants were verified to contain all of the endogenous plasmid replicons of the Δ*bbb22-bbb23* mutant parent (Table 4). The *in vitro* gene expression of genes *bbb22* and/or *bbb23* was analyzed in the *B. burgdorferi* clones harboring *bbb22* alone or *bbb23* alone, along with the *B. burgdorferi* Δ*bbb22-bbb23* mutant carrying both *bbb22* and *bbb23* (*bbb22-23*$^+$) [119] or pBSV2G alone (Δ*bbb22-bbb23*/vector) [119] (Table 4) each grown to late stationary phase, a growth condition we have previously demonstrated to induce purine permease activity [119] (Figure 9). The expression level of *bbb22* under the control of the proximal promoter alone (*22prox*$_p$-*bbb22*$^+$) was significantly reduced compared to that of the level

of *bbb22* expression in the *bbb22-23*$^{+}$ positive control spirochetes (P = 0.025) (Figure 9A). Interestingly, however, the level of *bbb22* expression in *22dist*$_{p}$-*bbb22*$^{+}$ spirochetes was similar to that in *bbb22-23*$^{+}$ spirochetes (P = 0.338) (Figure 9A). Together, these data suggest a possible role for the long 5' UTR in the *22dist*$_{p}$-*bbb22*$^{+}$ construct in the regulation of wild type levels of *bbb22*. There was no significant difference detected between the levels of *bbb23* transcription in *23*$_{p}$-*bbb23*$^{+}$ spirochetes compared to *bbb22-23*$^{+}$ spirochetes (P = 0.113) (Figure 9B), suggesting the upstream sequence included in this construct was sufficient to drive *bbb23* expression to wild type levels. It was observed that in late stationary phase growth the level of *bbb23* expression in *bbb22-23*$^{+}$ spirochetes (Figure 9B) was approximately 100-fold lower than the level of *bbb22* expression in the same clone (Figure 9A). A similar significant difference between the levels of late stationary phase expression of *bbb22* and *bbb23* was detected in wild type spirochetes (data not shown). We found that by driving expression of *bbb23* under the control of the constitutive promoter *flaB*, the level of *bbb23* expression in late stationary phase could be increased to a level similar to that of *bbb22* under its endogenous promoter (Figure 9).

Table 4. *B. burgdorferi* clones used in this study.

B. burgdorferi **clone**	**Genotype**	**Shuttle Vector**	**Reference**
Δ*bbb22-23*/vector	B31 A3-68 *bbe02*::*flgB*$_p$-*kan*, *bbb22-23*::*flaB*$_p$-*aadA*, cp9$^-$, lp56$^-$	pBSV2G	[119]
bbb22-23$^+$	B31 A3-68 *bbe02*::*flgB*$_p$-*kan*, *bbb22-23*::*flaB*$_p$-*aadA*, cp9$^-$, lp56$^-$	pBSV2G *bbb22-23*$^+$	[119]
23$_p$-*bbb23*$^+$	B31 A3-68 *bbe02*::*flgB*$_p$-*kan*, *bbb22-23*::*flaB*$_p$-*aadA*, cp9$^-$, lp56$^-$	pBSV2G *23*$_p$-*bbb23*$^+$	This work
flaB$_p$-*bbb23*$^+$	B31 A3-68 *bbe02*::*flgB*$_p$-*kan*, *bbb22-23*::*flaB*$_p$-*aadA*, cp9$^-$, lp56$^-$	pBSV2G *flaB*$_p$-*bbb23*$^+$	This work
22prox$_p$-*bbb22*$^+$	B31 A3-68 *bbe02*::*flgB*$_p$-*kan*, *bbb22-23*::*flaB*$_p$-*aadA*, cp9$^-$, lp56$^-$	pBSV2G *22prox*$_p$-*bbb22*$^+$	This work
22dist$_p$-*bbb22*$^+$	B31 A3-68 *bbe02*::*flgB*$_p$-*kan*, *bbb22-23*::*flaB*$_p$-*aadA*, cp9$^-$, lp56$^-$	pBSV2G *22dist*$_p$-*bbb22*$^+$	This work

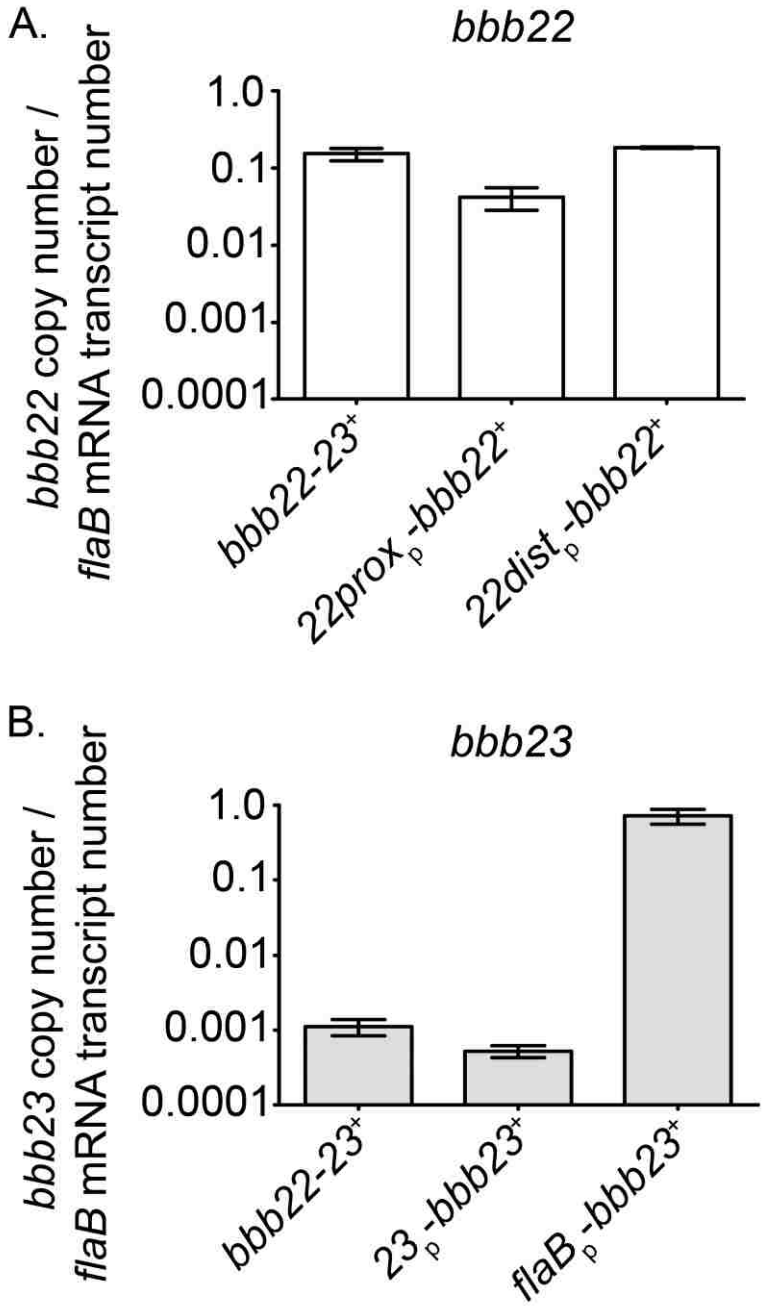

Figure 9. *In vitro* gene expression of *bbb22* and *bbb23* in *B. burgdorferi* clones.

RNA was extracted from *bbb22-23*$^{+}$, *23*$_{p}$-*bbb23*$^{+}$, *flaB*$_{p}$-*bbb23*$^{+}$, *22prox*$_{p}$-*bbb22*$^{+}$ and *22dist*$_{p}$-*bbb22*$^{+}$ *B. burgdorferi* clones at late stationary phase (2x10^{8} spirochetes/ml) grown at 37°C. Gene expression for *bbb22*, *bbb23,* and *flaB* was quantified by reverse transcriptase qPCR. (A) *bbb22* expression was analyzed for clones *bbb22-23*$^{+}$, *22prox*$_{p}$-*bbb22*$^{+}$ and *22dist*$_{p}$-*bbb22*$^{+}$. *bbb22* mRNA transcripts were normalized to the *flaB* mRNA copies. (B) *bbb23* expression was analyzed for clones *bbb22-23*$^{+}$, *23*$_{p}$-

bbb23^{+} and *flaB*$_{p}$-*bbb23*$^{+}$. *bbb23* mRNA transcripts were normalized to the *flaB* mRNA copies. Data represent the average value of three biological replicates. Error bars represent the standard deviation from the mean. Statistical analyses were performed using the unpaired t-test (GraphPad Prism 5.0).

3.4.3: The *bbb22* gene allows spirochetes to uptake wild type steady state levels of hypoxanthine.

We previously demonstrated the combined roles of *bbb22* and *bbb23* in hypoxanthine transport [119]. To determine the ability of spirochetes harboring either *bbb22* or *bbb23* alone to transport hypoxanthine, uptake assays were performed using the panel of *B. burgdorferi* clones carrying either *bbb22* or *bbb23*. The samples were measured 10 seconds and 15 minutes following the addition of [^{3}H]hypoxanthine as a means to assess the initial rate of transport as well as the ability of the spirochetes to take up hypoxanthine overtime. The amounts of [^{3}H]hypoxanthine detected in the spirochetes carrying only *bbb22* or *bbb23* at the 10 second time point were significantly reduced compared to that of the *bbb22-23*$^{+}$ clone (Figure 10A, $P \leq 0.01$), suggesting that at this initial time point neither gene alone provided *B. burgdorferi* with the ability to transport hypoxanthine to the level conferred by both genes together. Interestingly, at the 15 minute time point spirochetes carrying *bbb22* alone driven by the long 5' UTR sequence (*22dist*$_{p}$-*bbb22*$^{+}$) achieved a level of hypoxanthine uptake equivalent to that of spirochetes carrying both genes (Figure 10A, $P = 0.96$). Whereas, the hypoxanthine uptake at 15 minutes by spirochetes carrying *bbb22* alone under the control of the proximal promoter (*22prox*$_{p}$-*bbb22*$^{+}$) was found to be no different than that of the Δ*bbb22-23* mutant (Δ*bbb22-23*/vector) (Figure 10A, $P = 0.19$), further suggesting a role of the long 5' UTR sequence in *bbb22* function. Similar to what was observed for *22prox*$_{p}$-*bbb22*$^{+}$ spirochetes, the *bbb23* gene under the control of the *bbb23* promoter

(*23*$_p$-*bbb23*$^+$) was not sufficient to provide the Δ*bbb22-23* mutant with the ability to transport hypoxanthine (Figure 10A, *P* = 0.66). Furthermore, although expression of *bbb23* under the control of the constitutive *flaB* promoter resulted in a significant increase in hypoxanthine relative to that of the Δ*bbb22-23* mutant (*P* = 0.04), the level of hypoxanthine uptake by *flaB*$_p$-*bbb23*$^+$ spirochetes did not achieve that of the *bbb22-bbb23*$^+$ or *22dist*$_p$-*bbb22*$^+$ clones (*P* = 0.01 and *P* = 0.03, respectively). Together these data indicated that initial uptake of hypoxanthine was only accomplished by spirochetes harboring both *bbb22* and *bbb23*, suggesting a synergistic effect of the two genes together. The *bbb22* gene, when expressed under the control of the long 5' UTR, was sufficient to attain the level of hypoxanthine uptake of *bbb22-bbb23*$^+$ spirochetes at the 15 minute time point. Although the *bbb23* gene alone conferred modest hypoxanthine transport function at the 15 minute time point, this fuction was only detectable when *bbb23* was constitutively expressed.

3.4.4: Gene *bbb22*, but not *bbb23*, contributes to the ability of *B. burgdorferi* to transport adenine.

It has been previously demonstrated that the ability of *B. burgdorferi* to undergo rapid transport of adenine is due to genes *bbb22-23* [119]. In addition, *B. burgdorferi* is able to transport adenine in the absence of *bbb22-23,* albeit at a much slower rate, likely due to the function of an additional, as of yet unknown, adenine transporter(s) [119]. To understand the individual contributions of *bbb22* and *bbb23* to adenine uptake *B. burgdorferi* clones were analyzed 10 seconds and 15 minutes following the addition of [^{3}H]adenine. Similar to what was observed for hypoxanthine transport at 10 seconds, clones carrying either *bbb23* or *bbb22* alone were not able to achieve the level of

adenine transport comparable to that of the *bbb22-23*$^{+}$ clone (Figure 10B, $P < 0.02$). However, unlike clones *23*$_{p}$-*bbb23*$^{+}$, *flaB*$_{p}$-*bbb23*$^{+}$ and *22prox*$_{p}$-*bbb22*$^{+}$, clone *22dist*$_{p}$-*bbb22*$^{+}$ demonstrated a significant increase in adenine uptake compared to the *bbb22-23* mutant clone (Figure 10B, $P = 0.003$). Furthermore, at the 15 minute time point there was no statistical difference between the adenine uptake by *22dist*$_{p}$-*bbb22*$^{+}$ spirochetes and spirochetes harboring both genes (Figure 10B, $P = 0.07$). Whereas, the adenine uptake by all other clones was found to be no different than that of the double mutant at the same time point (Figure 10B, $P < 0.5$). Together these data suggest that alone *bbb23* does not contribute to adenine transport under the conditions of this assay. The *bbb22* gene when expressed under the control of the long 5' UTR was sufficient to restore adenine uptake to the level of *bbb22-23*$^{+}$ spirochetes. However, the initial rate of adenine transport by *22dist*$_{p}$-*bbb22*$^{+}$ spirochetes was significantly reduced, suggesting that both *bbb22* and *bbb23* are required for *B. burgdorferi* to achieve a rapid initial rate for adenine uptake.

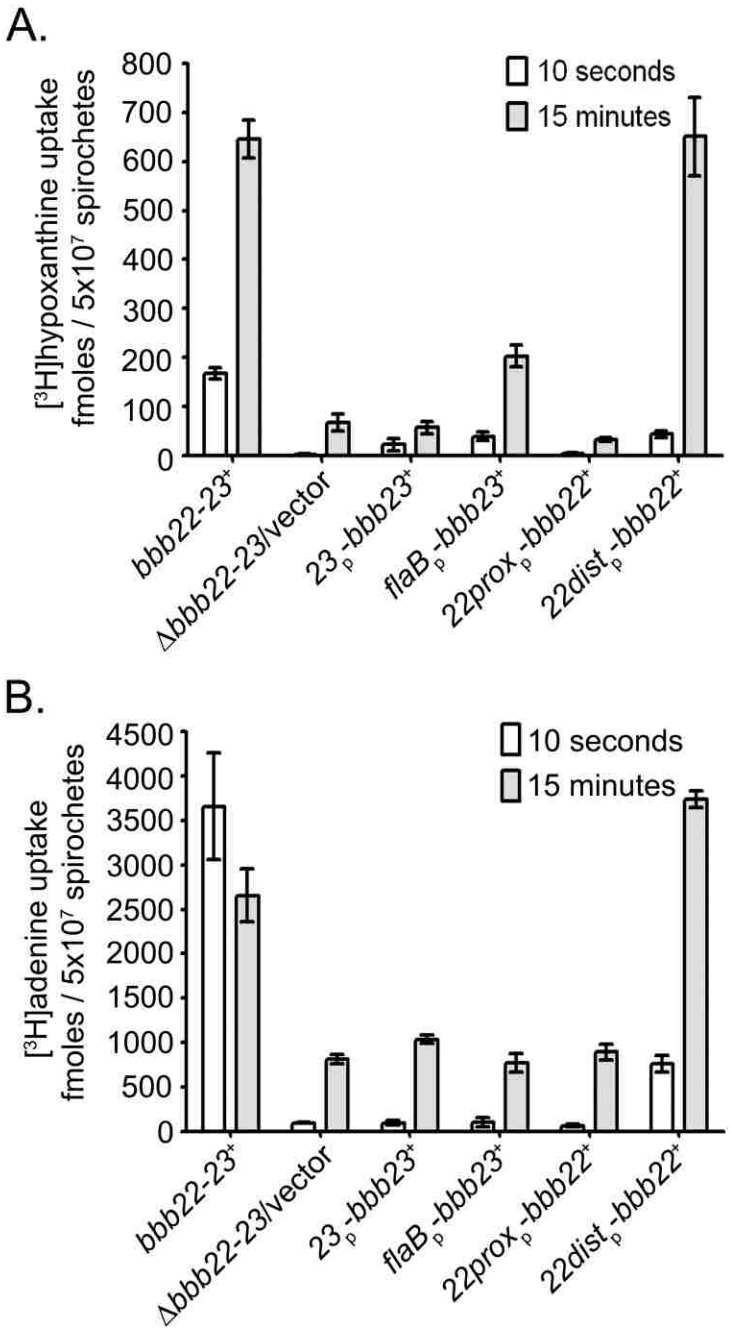

Figure 10. The *bbb22* and *bbb23* alone provide spirochetes with distinct abilities to uptake purines.

Radioactive purine uptake by *B. burgdorferi* clones *bbb22-23*$^+$, Δ*bbb22-23*/vector, *23*$_p$-*bbb23*$^+$, *flaB*$_p$-*bbb23*$^+$, *22prox*$_p$-*bbb22*$^+$ and *22dist*$_p$-*bbb22*$^+$ was measured at 10 seconds (white bars) and 15 minutes (gray bars) following the addition of (A) 1.25 µM [^{3}H]hypoxanthine or (B) 0.25 µM [2,8-^{3}H]adenine. Uptake of radiolabeled purine is expressed in fentomoles/5x10^7 spirochetes. The specific activity of the labeled

hypoxanthine used was 20 Ci/mmole. The specific activity of the labeled adenine used was 30 Ci/mmole. Error bars represent the standard deviation from the mean for at least two biological replicates. Statistical analyses were performed using the unpaired t-test (GraphPad Prism 5.0).

3.4.5: Adenine and guanine compete with hypoxanthine for uptake by spirochetes harboring either *bbb22* or *bbb23* alone.

Taking advantage of the finding that spirochetes harboring *bbb22* alone (*22dist*$_p$-*bbb22*$^+$) or *bbb23* alone (*flaB*$_p$-*bbb23*$^+$) were capable of transporting [^{3}H]hypoxanthine (Figure 10B), we sought to determine the ability of unlabeled hypoxanthine, adenine and guanine at 20 fold excess to compete with this function. Consistent with our data demonstrating that *bbb22* is functional to transport adenine as well as hypoxanthine, [^{3}H]hypoxanthine transport was reduced to nearly 3% and 20% in the presence of excess cold adenine and cold hypoxanthine, respectively (Figure 11). Furthermore, *bbb22* provided the spirochetes with the ability to transport guanine, as [^{3}H]hypoxanthine transport by the *22dist*$_p$-*bbb22*$^+$ clone was reduced to 37% in the presence of excess cold guanine. Similarly, *flaB*$_p$-*bbb23*$^+$ spirochetes demonstrated a reduction in [^{3}H]hypoxanthine transport in the presence of excess cold hypoxanthine, adenine and guanine (13%, 16% and 5%, respectively) (Figure 11). The finding that that excess cold adenine was able to compete for [^{3}H]hypoxanthine transport by *flaB*$_p$-*bbb23*$^+$ spirochetes was surprising given that direct transport of [^{3}H]adenine by this clone was not detected (Figure 10B) and suggested that *bbb23* is capable of transporting adenine only when the substrate is present in high concentrations. As expected hypoxanthine transport by either of these clones was unaffected by the presence of the pyrimidine cytosine (Figure 11). Overall these data suggest that individually both *bbb22* and *bbb23* are able to provide *B. burgdorferi* with the ability

transport to hypoxanthine, adenine and guanine. Moreover, these data suggest a trend in which adenine is a stronger competitor than guanine for hypoxanthine transport by *bbb22*$^{+}$ spirochetes (P = 0.06) and guanine is a stronger competitor than adenine for hypoxanthine transport by *bbb23*$^{+}$ spirochetes (P = 0.02).

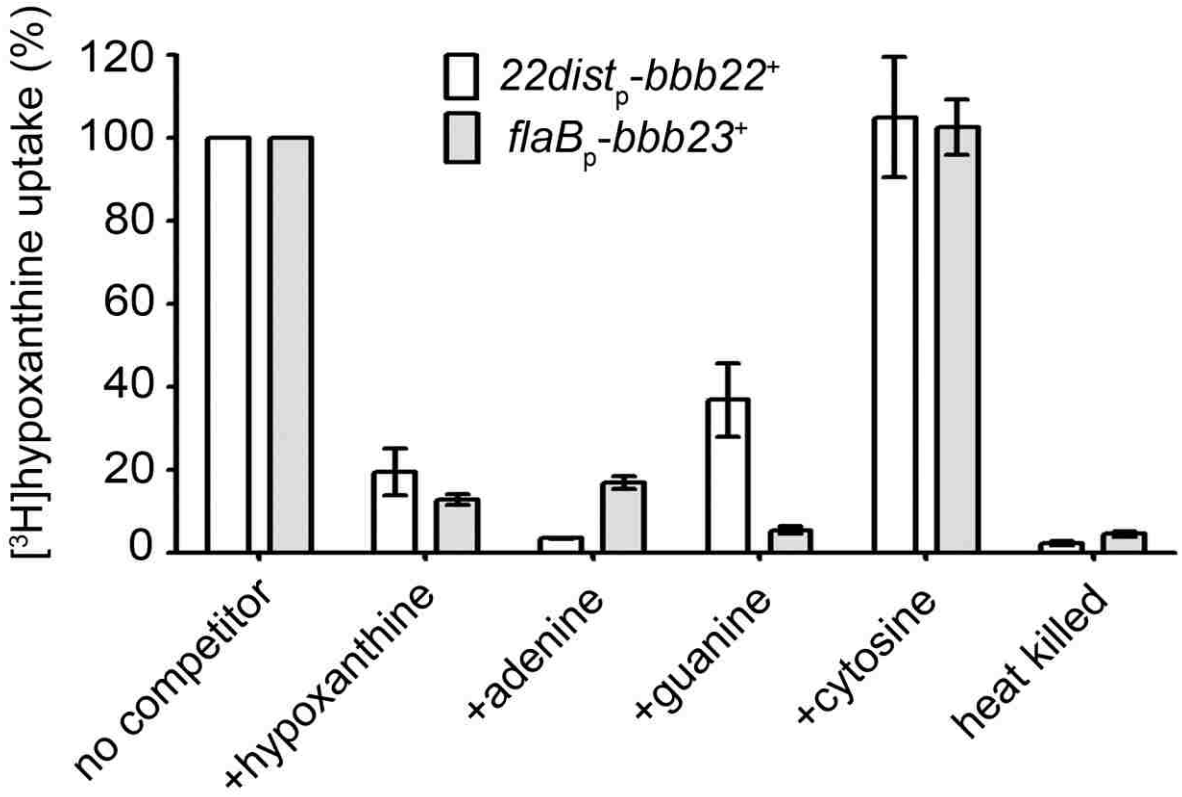

Figure 11. Adenine and guanine compete with hypoxanthine for transport by both BBB23 and BBB22 individually.

[^{3}H]hypoxanthine (0.25 µM, 20µCi) uptake by *22dist*$_s$-*bbb22*$^{+}$ (white bars) and *flaB*$_p$-*bbb23*$^{+}$ (gray bars) spirochetes was determined at 15 minutes following addition of radiolabeled purine in the absence (no competitor) or presence of 20 fold excess (5 µM) unlabeled competitor as indicated. [^{3}H]hypoxanthine uptake by *22dist*$_p$-*bbb22*$^{+}$ or *flaB*$_p$-*bbb23*$^{+}$ spirochetes in the absence of competitor was taken as 100%. All other data are represented as the percent uptake relative to *22dist*$_p$-*bbb22*$^{+}$ or *flaB*$_p$-*bbb23*$^{+}$ spirochetes, respectively, in the absence of competitor. Heat killed spirochetes (heat killed) served as the negative controls.

3.4.6: *bbb22,* but not *bbb23*, is critical for mouse infectivity.

Together genes *bbb22* and *bbb23* are essential for *B. burgdorferi* infection in mice [119]. We have now demonstrated that individually *bbb22* and *bbb23* vary in their contributions to the ability of *B. burgdorferi* transport purines (Figures 3 and 4). To investigate the individual contributions of *bbb22* and *bbb23* to mouse infection, mice were needle inoculated with *B. burgdorferi* clones *bbb22-23*$^{+}$, Δ*bbb22-23*/vector, *23*$_{p}$-*bbb23*$^{+}$, *flaB*p-*bbb23*$^{+}$, *22prox*$_{p}$-*bbb22*$^{+}$, and *22dist*$_{p}$-*bbb22*$^{+}$ at a dose of 10^4 or 10^7 spirochetes. Mouse infection was assessed three weeks post inoculation by serology and spirochete reisolation from tissues. Of the mice inoculated with *B. burgdorferi* clones carrying *bbb23* or *bbb22* alone, only the mice inoculated with *22dist*$_{p}$-*bbb22*$^{+}$ spirochetes acquired infections (Table 5). These data suggest that *bbb22* is sufficient for *B. burgdorferi* infection of mice; whereas, *bbb23* is not. In support of this notion, mice infected with spirochetes harboring *bbb23* alone (*flaB*$_{p}$-*bbb23*$^{+}$ and *23*$_{p}$-*bbb23*$^{+}$) were found to be seronegative and negative for reisolation of spirochetes from tissues (Table 5). Serology and reisolation of spirochetes from mouse tissues are qualitative measures of *B. burgdorferi* infectivity.

Table 5. Spirochetes carrying *bbb22* alone demonstrate qualitative measures of mouse infectivity.

Clone	Spirochete dose per mouse	Infected mice[a]
bbb22-23$^+$	1x10^4	6/6
	1x10^7	6/6
bbb22-23$^+$	1x10^4	0/6
	1x10^7	0/6
flaB$_p$-*bbb23*$^+$	1x10^4	0/6
	1x10^7	0/6
22dist$_p$-*bbb22*$^+$	1x10^4	6/6
	1x10^7	6/6

[a] Number of infected mice/ number of mice analyzed determined 3 weeks post inoculation by positive serology against *B. burgdorferi* proteins and reisolation of spirochetes from ear, bladder and joint tissues.

To identify quantitative differences between the *B. burgdorferi* infections by *22dist*$_p$-*bbb22*$^+$ and *bbb22-23*$^+$ spirochetes the spirochete loads in mouse tissues inoculated with the two different clones were evaluated by quantitative PCR. The analysis was also carried out for mouse tissues inoculated with clones *flaB*p-*bbb23*$^+$ and Δ*bbb22-23*/vector, both of which were found to be non-infectious by the qualitative measures of infection (Table 5). Our results demonstrated that spirochetes loads in ear and heart tissues infected with the *22dist*$_p$-*bbb22*$^+$ clone were significantly reduced compared to the spirochete burdens in the ear and heart tissues of mice infected with *bbb22-23*$^+$ spirochetes (Figure 12A and 12B, $P = 0.04$ and $P = 0.01$). Although no

statistical difference was detected between the spirochete loads in the joint tissues infected with the two clones, the data suggested a trend toward reduced spirochete numbers in the joint tissues of mice infected with spirochetes harboring *bbb22* alone (Figure 12C). As expected there was no quantitative detection of spirochetes in the tissues of mice infected with clones *flaB*p-*bbb23*$^{+}$ and Δ*bbb22-23*/vector. Together these data suggest that spirochetes harboring *bbb22* alone are able to infect mice. However, the *bbb22* gene alone is not sufficient to allow the spirochetes to maintain the level of *B. burgdorferi* burden in tissues achieved by spirochetes that carry both the *bbb22* and *bbb23* genes together.

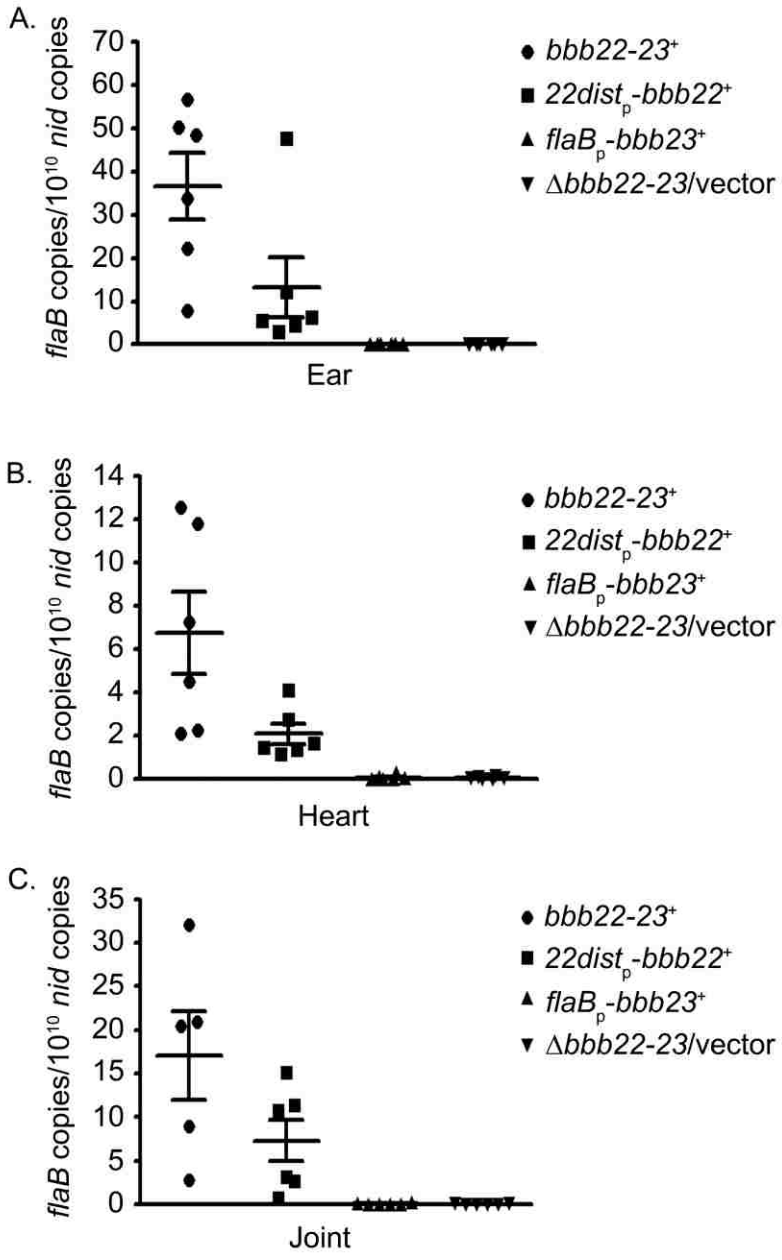

Figure 12. The *bbb22* gene alone is not sufficient to maintain wild type levels of spirochete loads in infected mouse tissues.

DNA was isolated from ear, heart and joint tissues of C3H/HeN mice inoculated with 1x10^4 *bbb22-23*$^+$ (●), Δ*bbb22-23*/vector (○), *flaB*$_p$-*bbb23*$^+$ (□) or *22dist*$_p$-*bbb22*$^+$ (Δ) spirochetes. Samples were analyzed for spirochete *flaB* and murine nidogen DNA copies by qPCR. The data are presented as *B. burgdorferi flaB* copies per 10^{10} nidogen (*nid*) copies. Each data point represents the average of triplicate measures from the DNA of an individual mouse. The mean value for each group is indicated by a

horizontal line. Error bars represent the standard deviation from the mean. Statistical analysis was performed using the unpaired t-test (GraphPad Prism 5.0).

3.5 Discussion

Borrelia burgdorferi is dependent upon purine salvage from the environment for the synthesis of DNA and RNA [43, 56, 57, 73, 88]. We have previously shown that *bbb22* and *bbb23* present on circular plasmid 26 encode for purine permeases, which together are essential for *B. burgdorferi* infection in mice [119]. In this study we now demonstrate the individual contributions of *bbb22* and *bbb23* to purine transport and *B. burgdorferi* mouse infectivity. To understand the individual roles of *bbb22* and *bbb23* we first undertook transcriptional analysis to identify the transcription start sites for both genes. 5' RACE analysis identified a distinct transcription start site for *bbb22*. Surprisingly however, reverse transcriptase PCR analysis across the intergenic region between the annotated BBB23 and BBB22 ORFs indicated the presence of a continuous transcript across the two genes (SJ and MWJ unpublished). Together these data suggested the possibility of an additional long *bbb22* transcript that originates either from co-transcription with *bbb23* or a second *bbb22* transcription start site within the BBB23 ORF. Indeed, the 5' end of a transcript was identified upstream of *bbb22* within *bbb23* just downstream of a promoter sequence identified in our BbIVET screen. The long 5' UTR sequence produced from the *bbb22* distal promoter ($22dist_p$) was found to be important for *bbb22* expression as well as *bbb22*-dependent purine transport and murine infectivity. A long 5' UTR was also identified upstream of the BBB23 ORF. However, the *bbb22-23 trans* complementation construct carrying only 110 bp of *bbb23* upstream sequence was sufficient to restore wild type *in vitro* purine

transport and mouse infectivity to Δ*bbb22-23* spirochetes under the experimental conditions tested, raising the question as to the importance of the *bbb23* 5' UTR (this work and [119]). Regulation of genes that transport and metabolize purines has been shown to occur by riboswitch-mediated control [127, 128]. For purine-sensing riboswitches this type of regulation typically occurs through the binding of guanine or adenine to the riboswitch sequence in the long 5' UTR sequences of the regulated genes [129]. Currently, there are four known classes of purine-sensing riboswitches; however, additional classes may remain yet undiscovered [129]. While, the extended leader sequences detected upstream of *bbb22* and *bbb23* suggests the possibility of a riboswitch mechanism, bioinformatics analysis of the *bbb22* and *bbb23* 5' UTR sequences did not identify one of the four classes of purine-sensing riboswitches. The functional roles of the 5' UTR sequences of both the *bbb22* and *bbb23* genes are currently under investigation.

Interestingly, the expression level of the *bbb23* gene in spirochetes grown to late stationary phase was found to be approximately 100-fold less than that of the *bbb22* gene. This disparity in the level of expression of the two genes in late stationary phase was eliminated when the *bbb23* ORF was expressed under the control of the constitutive *flaB* promoter. Standardization of the *bbb23* and *bbb22* expression levels allowed direct comparison of *in vitro* transport function(s) conferred by each gene alone. The *bbb22* gene (*22dist*$_p$-*bbb22*$^+$) enabled Δ*bbb22-23* spirochetes to uptake [^{3}H]hypoxanthine and [^{3}H]adenine. Furthermore, [^{3}H]hypoxanthine uptake by *22dist*$_p$-*bbb22*$^+$ spirochetes was inhibited by excess cold hypoxanthine and adenine and to a lesser extend guanine. Whereas, the *bbb23* gene (*flaB*$_p$-*bbb23*$^+$) enabled Δ*bbb22-23*

spirochetes to uptake a detectable amount of [^{3}H]hypoxanthine but not [^{3}H]adenine. Surprisingly, however, [^{3}H]hypoxanthine uptake by *flaB*$_p$-*bbb23*$^+$ spirochetes was inhibited by excess cold hypoxanthine, adenine and guanine. These data suggest that individually both *bbb22* and *bbb23* can provide Δ*bbb22-23* spirochetes with the ability to transport hypoxanthine, adenine and guanine. However, the transport function conferred by each gene is quantitatively distinct. Unlike the *bbb22* gene, *bbb23* was not sufficient to achieve the level of hypoxanthine uptake afforded by the *bbb22-23*$^+$ spirochetes at the 15 minute time point. Constitutive expression of *bbb23* was required to determine the transport function conferred by this gene, as the uptake of hypoxanthine and adenine by *23*$_p$-*bbb23*$^+$ spirochetes was undetectable. Furthermore, detectable adenine transport by *flaB*$_p$-*bbb23*$^+$ spirochetes required a 20 fold higher concentration of adenine compared to the amount of adenine required for transport by *22dist*$_p$-*bbb22*$^+$ spirochetes. Together these findings demonstrate that the purine transport function encoded by the *bbb22* gene alone is greater than the purine transport function encoded by the *bbb23* gene alone. Nonetheless, neither gene alone restored the initial rate of hypoxanthine or adenine uptake to that achieved by *bbb22-23*$^+$ spirochetes, demonstrating that both genes are required to provide wild type purine transport function.

The amino acid sequences of BBB22 and BBB23 are 78.3% identical; however, the two proteins demonstrate distinct abilities to transport hypoxanthine, adenine and guanine. This suggests that the differential transport functions of the two proteins may be dictated by a few keys differences in amino acid residues. Recently, structure-function analyses of the *E. coli* adenine-specific permeases PurP and YicO and

hypoxanthine-guanine-specific permeases YjcD and YgfQ as well as the *A. nidulans* adenine-hypoxanthine-guanine permease AzgA identified key amino acid residues critical for purine uptake activity [80, 122]. Both of these studies identified an aspartic acid and a glutamic acid residue (Asp339 and Glu394 in AgzA) to be essential for the transport function for all five COG2252 family members [80, 122]. These residues are conserved in BBB22 and BBB23. Amino acid Thr275 in YjcD was found to be critical for hypoxanthine-guanine transport [80, 122]. Interestingly, this residue is conserved in the other COG2252 proteins [80, 122] including BBB22; however, an isoleucine is present at this position in BBB23. Furthermore, Gly129 has been shown to be important for AzgA substrate binding [80, 122]. This residue is conserved in BBB22 and the *E. coli* adenine-specific permeases; whereas, BBB23 and the *E. coli* hypoxathine-guanine-specific permeases have a serine at this position [80, 122]. Future mutational analysis of the BBB22 and BBB23 proteins, guided by the current understanding of the molecular interactions important for the transport activities of COG2252 family members, will provide insight into the molecular basis for the functional differences between these two highly related transporters.

Of the *B. burgdorferi* clones carrying only *bbb22* or *bbb23*, only *22dist*$_{p}$-*bbb22*$^{+}$ spirochetes demonstrated mouse infectivity as measured by serology and reisolation of spirochetes from infected tissues. This finding, while perhaps expected based on the near wild type purine transport function of this clone *in vitro*, highlights the requirement for the *bbb22* 5' UTR sequence for *bbb22* function and the distinct individual capabilities of the *bbb22* and *bbb23* genes. Hypoxanthine is abundant in mouse blood [113] and adenine in mouse tissues [114]. *B. burgdorferi* is transiently present in the blood during

the initial stages of mammalian infection and then rapidly disseminate to distal tissues [130-132] Although *flaB*$_p$-*bbb23*$^+$ spirochetes demonstrated some ability to transport purines *in vitro*, the finding that *flaB*$_p$-*bbb23*$^+$ spirochetes are non-infectious in mice suggests that the purine transport affinity of this clone is not sufficient to support spirochete survival. While, *22dist*$_p$-*bbb22*$^+$ spirochetes demonstrated a higher affinity for hypoxanthine and adenine uptake compared to *flaB*$_p$-*bbb23*$^+$ spirochetes, the initial rate of purine uptake by *22dist*$_p$-*bbb22*$^+$ spirochetes was significantly reduced relative to spirochetes carrying both genes. Our data suggest that this deficiency resulted in a quantitative difference in the infectious phenotype of *22dist*$_p$-*bbb22*$^+$ versus *bbb22-23*$^+$ spirochetes. Ear and heart tissues isolated from mice infected with *22dist*$_p$-*bbb22*$^+$ spirochetes demonstrated a significantly reduced spirochete load as compared to those tissues isolated from mice infected with *bbb22-23*$^+$ spirochetes. A similar trend was also detected in joint tissues. These data suggest that even though *bbb23* is dispensable for *B. burgdorferi* survival in mice, this gene is required, in addition to *bbb22,* for *B. burgdoreri* to achieve maximal spirochete loads in infected mouse tissues.

We have established that individually both BBB22 and BBB23 are capable of transporting hypoxanthine, adenine and guanine. However, the contribution of BBB22 to the purine transport function of *B. burgdorferi* is greater than that of BBB23. In addition to the direct involvement of BBB23 in purine transport, the BBB23 ORF contains regulatory sequence critical for *bbb22* expression. The combined critical nature of the *bbb22* and *bbb23* genes to *B. burgdorferi* biology is highlighted by the fact that these genes are encoded on the essential cp26 plasmid. Cp26, unlike the approximately 20 linear and circular plasmid of *B. burgdorferi* is present in all natural

isolates examined [41], ensuring that the essential *bbb22* and *bbb23* genes will be maintained in the *B. burgdorferi* population. All in all, we conclude that *B. burgdorferi* requires both *bbb22* and *bbb23* for purine transport and mouse infectivity.

3.6 Acknowledgements

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CHAPTER 4: PURINE TRANSPORT CONTRIBUTES TO *BORRELIA BURGDORFERI* GROWTH IN *IXODES SCAPULARIS* TICKS

4.1 Abstract

Borrelia burgdorferi in the tick vector is dependent mainly upon the blood meal to uptake nutrients. These spirochetes persist through the molt and persist in unfed tick between feeding. *B. burgdorferi* has a limited genome and lacks *de novo* synthesis of purines. We have previously shown that *bbb22* and *bbb23* present on circular plasmid 26 encode for hypoxanthine-adenine-guanine permeases. Spirochetes missing both *bbb22-23*, do not survive in murine infection by needle inoculation [119]. BBB22 has high affinity for hypoxanthine and adenine compared to BBB23 (Chapter 3). BBB23 is not critical for spirochete murine infectivity but it is required along with BBB22 for maximum infection in the mouse (Figure 12). Here we demonstrate that collectively *bbb22* and *bbb23* are not essential for but contribute to spirochete growth in fed *Ixodes scapularis* larvae. Spirochetes missing both *bbb22* and *bbb23* have significantly low spirochete loads compared to wild type spirochetes in fed larvae. In fed nymphs spirochetes missing both *bbb22* and *bbb23* are able to replicate to wild type levels, thus spirochete replication in fed nymphs is not affected in the absence of both *bbb22* and *bbb23*. This suggests *bbb22-23* mutant spirochete replication to wild type spirochete levels in fed nymphs might be due to an increased availability of nucleotides and deoxynucleotides in blood meal compared to fed larvae, which can bypass the requirement of *bbb22* and *bbb23* for the salvage of nucleotide precursors. We have also found that spirochetes harboring BBB23 only are sufficient for spirochete growth in fed larvae to wild type levels, suggesting that BBB23, a low affinity hypoxanthine-adenine-

guanine permease, alone is sufficient to transport adequate amounts of purines required to maintain spirochete loads to wild type levels in fed larvae. Overall our data suggest that *bbb22* and *bbb23* contribute to spirochete replication in fed larvae but are not essential for survival in the tick and BBB23 alone, which is low affinity purine permease, is sufficient to maintain wild type levels of spirochete loads in ticks. Studying the requirements for purine transport in *B. burgdorferi* survival in the tick vector helps in the understanding of how spirochetes survival varies as a result of the availability of purine bases, nucleotides and/or deoxynucleotide molecules.

4.2 Introduction

Borrelia burgdorferi, the causative agent of Lyme disease, is maintained in a complex infectious cycle, involving a tick vector and vertebrate hosts. Lyme disease is the most commonly reported arthropod borne disease in United States. *Ixodes scapularis* (deer tick or black legged tick) and *Ixodes pacificus* (western-blacklegged tick) are the major tick vectors responsible for Lyme disease transmission in the United States [133]. The *Ixodes scapularis* tick has three distinct developmental stages (larval, nymphal and adult) in its life cycle. Ticks acquire spirochetes during the larval stage while feeding on infected hosts [134]. The white-footed mouse (*P. leucopus*) is the most common feeding host for larval ticks and an important reservoir for *B. burgdorferi* [135]. Immature *Ixodes scapularis* larvae also feed on other small rodents, birds and lizards [136-138]. After completion of a blood meal, larvae molt into nymphal ticks (environmental reservoir), which is the life stage mostly responsible for transmission of spirochetes to humans [22]. Transovarial transmission of spirochetes to newly hatched

larvae has not been reported [22]. *Ixodes scapularis* ticks are slow feeders and can feed up to 5-7 days. Laboratory studies have shown that nymphs rarely transmit spirochetes on initial attachment to host, but with the majority of transmission occurring between 48-72 hours of feeding [139].

Due to limited biosynthetic machinery, *B. burgdorferi* is dependent upon the host environment for nutrient uptake [2, 139-142]. In an infected, unfed tick, spirochetes stay in the lumen midgut and are present in low number. When these ticks feed on a mammalian host, blood enters the midgut, resulting in change in pH and temperature, and ultimately leading to exponential spirochete growth [50]. From the midgut, these spirochetes migrate to salivary glands for transmission to the host [2].

In the tick vector, spirochetes acquire nutrients from the blood meal. *B. burgdorferi* lacks *de novo* synthesis of purines and the spirochete is dependent upon salvage of purines and other nucleotide precursors from blood meal. *B. burgdorferi* genes *bbb22* and *bbb23* present on circular plasmid 26 encode for purine permeases [119]. Individually both BBB22 and BBB23 are capable of transporting hypoxanthine, adenine and guanine (Figure 10, Figure 11). BBB22 has a greater affinity for hypoxanthine and adenine uptake compared to BBB23. We have also demonstrated that BBB22 is critical for *B. burgdorferi* survival in murine infection, but not BBB23 (Table 5). Here we have studied the collective role of *bbb22* and *bbb23* in spirochete survival in larval and nymphal ticks. We demonstrate that *bbb22* and *bbb23* are not essential for spirochete survival in the tick vector, but affect the spirochete load in the midgut, suggesting the role of the genes purine salvage from the blood meal.

Furthermore, addition of *bbb23* alone to *bbb22-23* mutant spirochetes was sufficient to restore wild type level of spirochete loads in fed larvae.

4.3 Materials and methods

4.3.1: *B. burgdorferi* clones and growth conditions.

B. burgdorferi clones, Δ*bbb22-23*/vector, *bbb22-23*$^{+}$ and *flaB*$_{p}$-*bbb23*$^{+}$ made previously by our lab were used in these tick studies ([119], Chapter 3). Δ*bbb22-23*/vector spirochetes are missing *bbb22-23* from cp26, *bbb22-23*$^{+}$ spirochetes are *trans*-complements for *bbb22-23* and *flaB*$_{p}$-*bbb23*$^{+}$ spirochetes are *trans*-complements for *bbb23* in *bbb22-23* mutant background spirochetes. *B. burgdorferi* cultures were grown in BSKII medium at 35ºC. BSK1.5X medium and 1.7% agarose were used as plating media. Gentamicin was used at a concentration of 40 µg/ml.

4.3.2: Mouse infection experiments.

University of Central Florida is accredited by International Association for Assessment and Accreditation of Laboratory Animal Care. All the mice experiments were conducted according to the guidelines of National Institutes of Health (NIH) and approved by Institutional Animal Care and Use Committee of UCF. 6-8 week old C3H/HeN female mice were anesthetized with 2.5% isoflurane. 100-200 µl of pre-inoculation blood was collected by retro-orbital bleeding for serology studies. Mice were inoculated intraperitoneally (80%) and subcutaneously (20%) with a total dose of $1x10^{4}$ B31 clone A3 *B. burgdorferi* in a total 250µl BSKII medium. Blood was re-collected 21 days post inoculation. Mouse infection was determined by serology and the results were

further confirmed by spirochete reisolation from infected mouse tissues as described previously [119].

4.3.3: Feeding uninfected larvae on mice.

Newly hatched naive *Ixodes scapularis* larvae were received from Dr. Michael Levin at the Centers for Disease Control through the BEI Resources/National Institute for Allergy and Infectious Diseases repository and were maintained under ~98% humidity a bell jar containing using a saturated solution of potassium sulfate. 4-5 month old larvae were used for mouse feeding. Larvae were dehydrated in a bell jar containing saturated solution of ammonium sulfate (relative humidity ~81%) for 48 hours prior to feeding on mice. Two *B. burgdorferi* infected mice (see above) were anesthetized by intraperitoneal injection of an 80 µl mixture of ketamine and xylazine (ketamine - 25mg/ml, xylazine - 7.6mg/ml). 100-150 larvae were placed onto a shaved area on the back of each mouse. Once mice recovered from anesthesia they were put individually into perforated plastic tubes covered with a piece of cloth and both the ends were closed with rubber stoppers. Mice were able to turn around in the tube but were not able to scratch their backs. These tubes were kept in cages for 4 hours to give sufficient time for larvae to attach to mouse skin. After 4 hours mice were removed from tubes and were transferred to the wire bottom feeding cages sitting over a pan of water. Fed larvae were collected from water every day for 7 days. Once all the larvae were collected, mice were transferred to regular cages.

4.3.4: RNA isolation from infected ticks.

Collected fed larvae (see above) were transferred to saturated potassium sulfate chambers. After 4 weeks, these larvae were molted into nymphs. Two groups having 20 unfed nymphs in each group were homogenized with a small pestle in BSKII media. Homogenized ticks were spun at 5800G for 10 minutes. Pellets were dissolved in 1 ml of TRIzol reagent (Life Invitrogen). TRIzol mix was transferred to Phase Lock Gel Heavy tubes (5 Prime) and was incubated for 10 minutes at room temperature. 300 µl chloroform was added to TRIzol mix and tubes were shaked vigorously for 15 seconds. Tubes were spun at 13000 G for 5 minutes at room temperature. Top layers of RNA were transferred to another tubes and RNA samples were precipitated using 500 µl isopropanol. Samples were spun at 15300 G at 4ºC for 10 minutes. RNA pellet was washed twice with 200 µl of 70% ethanol. RNA pellet was dissolved in 100 µl DEPC water. RNA samples were treated with TURBO DNase (Life Technologies) as described previously ([119], Chapter 3). RNA samples were treated with a total of 1 ul Riboguard RNase inhibitor 40 U/ µl (Epicenter).

4.3.5: RNA isolation from *in vitro* grown *B. burgdorferi* cultures.

B31 A3-68-ΔBBE02 Wild type spirochetes was grown to a density of 4 x 10^7 spirochetes/ml in BSKII media at 37ºC. 107 spirochetes were spun at 5800G for 10 minutes. BSKII media was removed and RNA was isolated from spirochete pellet using 1 ml of TRIzol reagent (Life Technologies) as described above. RNA pellet was dissolved in 100 µl of DEPC treated water. 1ul of RNase inhibitor was added to prevent RNA degradation by RNase. RNA sample was treated with TURBO DNase (Life

Technologies) as described previously ([119] Chapter 3). 1ul of RiboGuard RNase inhibitor (Epicenter) was added to prevent RNA degradation.

4.3.6: Gene expression by real time quantitative PCR.

400-600 ng of RNA was used to make cDNA using qScript cDNA Supermix (Quanta Biosciences) as per manufacturer's guidelines. Reactions missing reverse transcriptase were run as control. 2000 ng of cDNA was taken for each Real Time Quantitative PCR reaction. Expression of *B. burgdorferi* genes *bbb22* and *bbb23* was determined using primer-probe sets 1-2-5 and 3-4-6 respectively (Table 6). *bbb22* or *bbb23* transcript copy number was normalized with *B. burgdorferi flaB* mRNA copy number. RNA from *in vitro* grown *B. burgdorferi* clone A3 was taken for comparison.

Table 6. Primers and probes used in this study.

Primer number	Primer name	Primer sequence
1137	*flaB*-Taqman-FWD	TCTTTTCTCTGGTGAGGGAGCT
1138	*flaB*-Taqman-REV	TCCTTCCTGTTGAACACCCTCT
1166	BBB23 Fwd - Taqman	GCACTAGTTACCGCAACCTGTCT
1167	BBB23 Rev - Taqman	CTCATTCCAGAAGCCAATGCT
1168	BBB22 Fwd - Taqman	AAGAAATTATCGGTGGTATTACCACTTT
1169	BBB22 Rev - Taqman	CAATTGGCATACCTGTGCTAGATAATAT
1575	BBB22 Taqman probe	6-FAM-AGCATGGCATACATAATAGCTGTTAATCCGGC-TAMRA
1576	BBB23 Taqman probe	6-FAM-CTAATGGGACTTTATACCAATACGCCTT-TAMRA
1139	*flaB* Taqman probe	6-FAM-AAACTGCTCAGGCTGCACCGGTTC-TAMRA

4.3.7: Feeding artificially infected larvae on mice.

4-5 month old larvae were kept in saturated ammonium sulfate container for dehydration for 24-48 hours. After dehydration larvae were immersed in 20 ml of *B. burgdorferi* clone *bbb22-23*$^{+}$, Δ*bbb22-23* or *flaB*p-*bbb23*$^{+}$, at a density of 2x10^{7}

spirochetes/ml. *B. burgdorferi* clones were tested for the presence of virulence plasmids lp25, lp28-1 and lp36. More than 70% of the populations for all *B. burgdorferi* clones were positive for these virulence plasmids. Larvae were co-incubated in the *B. burgdorferi* cultures for 50 minutes at 37ºC and samples were inverted every 10 minutes. After 50 minutes tubes were centrifuged at low speed and the *B. burgdorferi* cultures were removed. Larvae were washed twice with cold 1X phosphate buffered saline (PBS) buffer. Tubes containing larvae were dried using filter paper and tubes were returned to potassium sulfate container. Artificially infected larvae were allowed to recover for two days prior to feeding on mice. At least 3 uninfected 6-8 week old female C3H/HeN mice per group were fed upon by artificially infected larvae as described above and fed larvae were collected for 7 days. Fed larvae were transferred to the saturated potassium sulfate container and a portion of each population was reserved to molt into nymphs. Following the molt *B. burgdorferi* nymphs were allowed to feed on at least 3 uninfected mice per group at a dose of 25 nymphs/mouse. Blood was collected from all mice before tick feeding and 21 days after start of tick feeding.

4.3.8: Determination of spirochete loads in ticks.

Individual unfed larvae, fed larvae, unfed nymphs and fed nymphs were surface sterilized in 1 ml of 3% H_2O_2 for 5-10 minutes followed by 1 ml of 70% ethanol for 5-10 minutes. Ticks were air dried and individual ticks were homogenized using a pestle in BSKII medium containing 40ug/ml gentamicin. BSK plating medium was prepared to contain the 100 X *B. burgdorferi* antibiotic cocktail including 2mg/ml phosphomycin, 5mg/ml rifampicin and 250 µg/ml amphotericin. Spirochete loads per tick were

determined by plating each tick homogenate and quantifying the number of colony forming units.

4.4 Results

4.4.1: *B. burgdorferi* express *bbb22* and *bbb23* in the tick vector.

The *B. burgdorferi* genes *bbb22* and *bbb23* present on circular plasmid 26 are expressed both *in vitro* and during a murine infection [119]. To determine whether *bbb22* and *bbb23* are expressed in unfed nymphal ticks, uninfected larvae were fed upon on mice infected with *B. burgdorferi* clone A3. Fed larvae were collected and were kept in a container having saturated solution of K_2SO_4 until they molted into nymphs. cDNA was made using RNA extracted from unfed infected nymphal ticks. Real time quantitative PCR was performed to determine *bbb22* and *bbb23* expression in the tick vector. Here we show that spirochetes express both *bbb22* and *bbb23* in unfed nymphs and that *bbb22* expression is 3.7 fold higher than *bbb23* ($P = 0.0086$) (Figure 14) *In vitro* grown *B. burgdorferi* did not show a significant gene expression difference in between *bbb22* and *bbb23*.

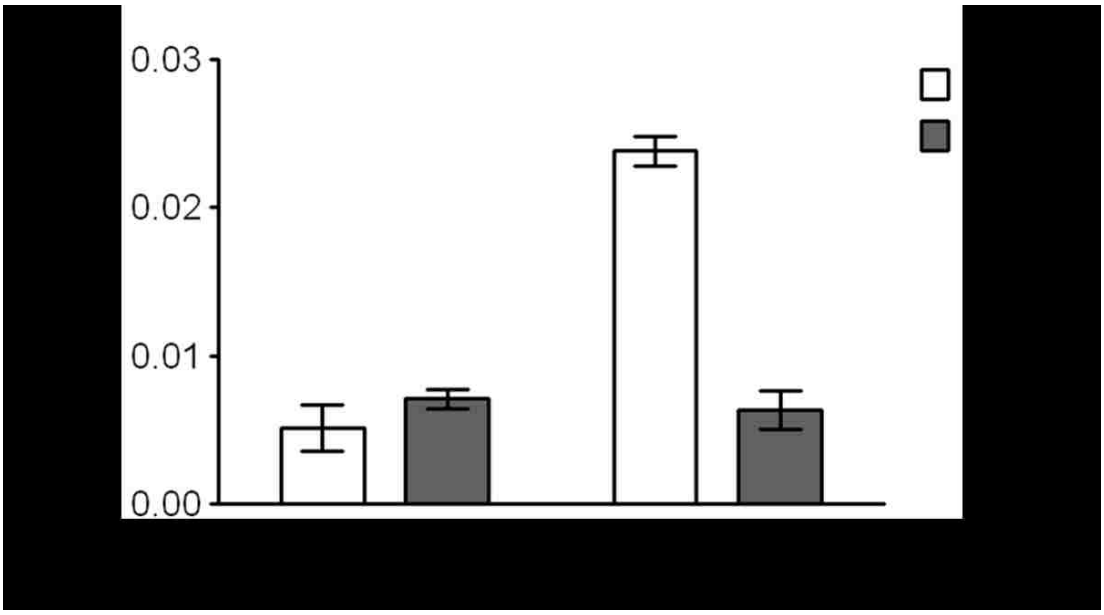

Figure 13. *B. burgdorferi* express *bbb22* and *bbb23* in unfed *Ixodes scapularis* nymphs.

Total RNA was isolated from *in vitro* grown B31 A3 spirochetes at a density of 4 x 10^7 spirochetes/ml. *B. burgdorferi* grown in BSKII media with pH 7.5 at 35°C. Total RNA from unfed nymphs infected with B31 A3 spirochetes, was isolated from a group of 20 unfed nymphs. Expression of *bbb22* and *bbb23* was determined by Real Time Quantitative PCR. *bbb22* and *bbb23* gene transcript copies were normalized to *flaB* transcript copied. *In vitro* and unfed nymph data represents an average of two biological replicates. Error bars represent mean standard deviation. Statistical analysis was done using unpaired t-test (Graph Pad Prism 5.0).

4.4.2: *bbb22* and *bbb23* are not essential for *B. burgdorferi* survival in the tick but play an important role in *B. burgdorferi* growth in fed larvae.

B. burgdorferi genes *bbb22* and *bbb23*, encode for purine permeases [119]. BBB22 and BBB23 are both hypoxanthine-adenine-guanine permeases (Chapter 3) and together are required for *B. burgdorferi* mouse infection [119]. To determine if *bbb22* and *bbb23* together have a role in spirochete survival or growth in *Ixodes scapularis* naïve larvae were artificially infected with *B. burgdorferi* clones Δ*bbb22-23*/vector or *bbb22-23*$^+$ and the spirochete loads in the tick analyzed throughout the tick-mouse infectious cycle (Figure 14). Individual unfed and fed ticks (larvae and nymphs) were

homogenized and were plated for assessment of the number of spirochete colony forming units per tick. These data demonstrated that spirochetes lacking both *bbb22* and *bbb23* are able to survive throughout larval and nymphal tick stages, suggesting *bbb22* and *bbb23* are not essential for spirochete survival in *Ixodes scapularis* (Figure 15A). The number of spirochetes per unfed larva were the same for both the *bbb22-23* mutant and *bbb22-23*$^{+}$ spirochetes, indicating artificial infection of the unfed larvae resulted in equal infection with both clones. Interestingly, the number of *bbb22-23* mutant spirochetes in the fed larvae was significant reduced compared to that of the *bbb22-23*$^{+}$ spirochetes (Figure 15A). Spirochete loads in fed larvae infected with *bbb22-23*$^{+}$ spirochetes were same as fed larvae infected with wild type spirochetes indicating that the *bbb22-23*$^{+}$ clone retains the wild type phenotype (data not shown Fed larvae were molted into nymphs and these nymphs were fed upon uninfected mice. Surprisingly, there was no difference in spirochete loads in fed nymphs between *bbb22-23* mutant spirochetes and *bbb22-23*$^{+}$ spirochetes (Figure 15B).

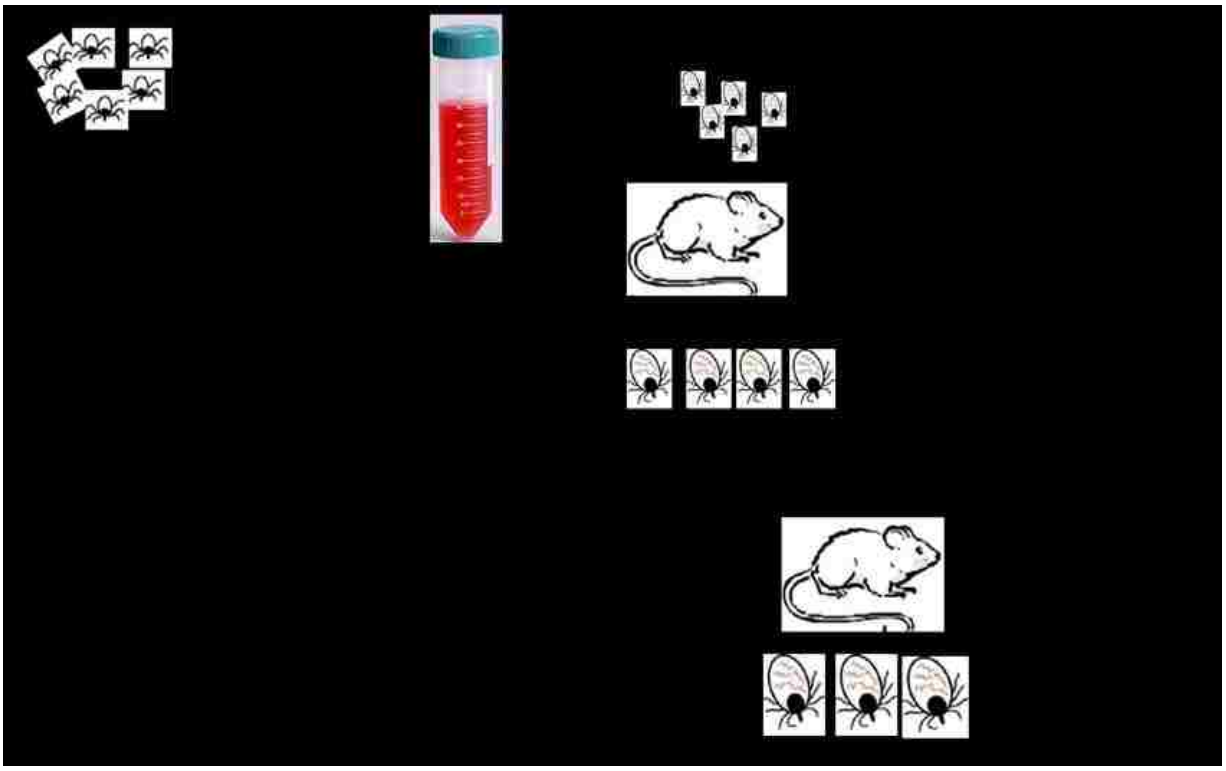

Figure 14. Experimental design of tick-mouse transmission of *B. burgdorferi*.

Ixodes scapularis unfed larvae were immersed in *B. burgdorferi* cultures of Δ*bbb22-23*/vector or *bbb22-23*$^{+}$ spirochetes grown at a density of 2 x10^{7} spirochetes/ml. Tubes were incubated at 35°C for 45 minutes. 100-150 artificially infected larvae were fed on each mouse. Fed larvae were collected for 5-6 days. To determine mouse infection, serology studies and spirochete reisolation experiments were performed on mice fed on by uninfected larvae after 3 weeks. Some of the individual fed larvae were plated in BSK plating media to count the spirochetes loads. Larvae were molted into nymphs and nymphs were fed on uninfected mouse. Infection in mice fed upon by nymphs and spirochete load in individual nymphs were determined as described for larvae [43].

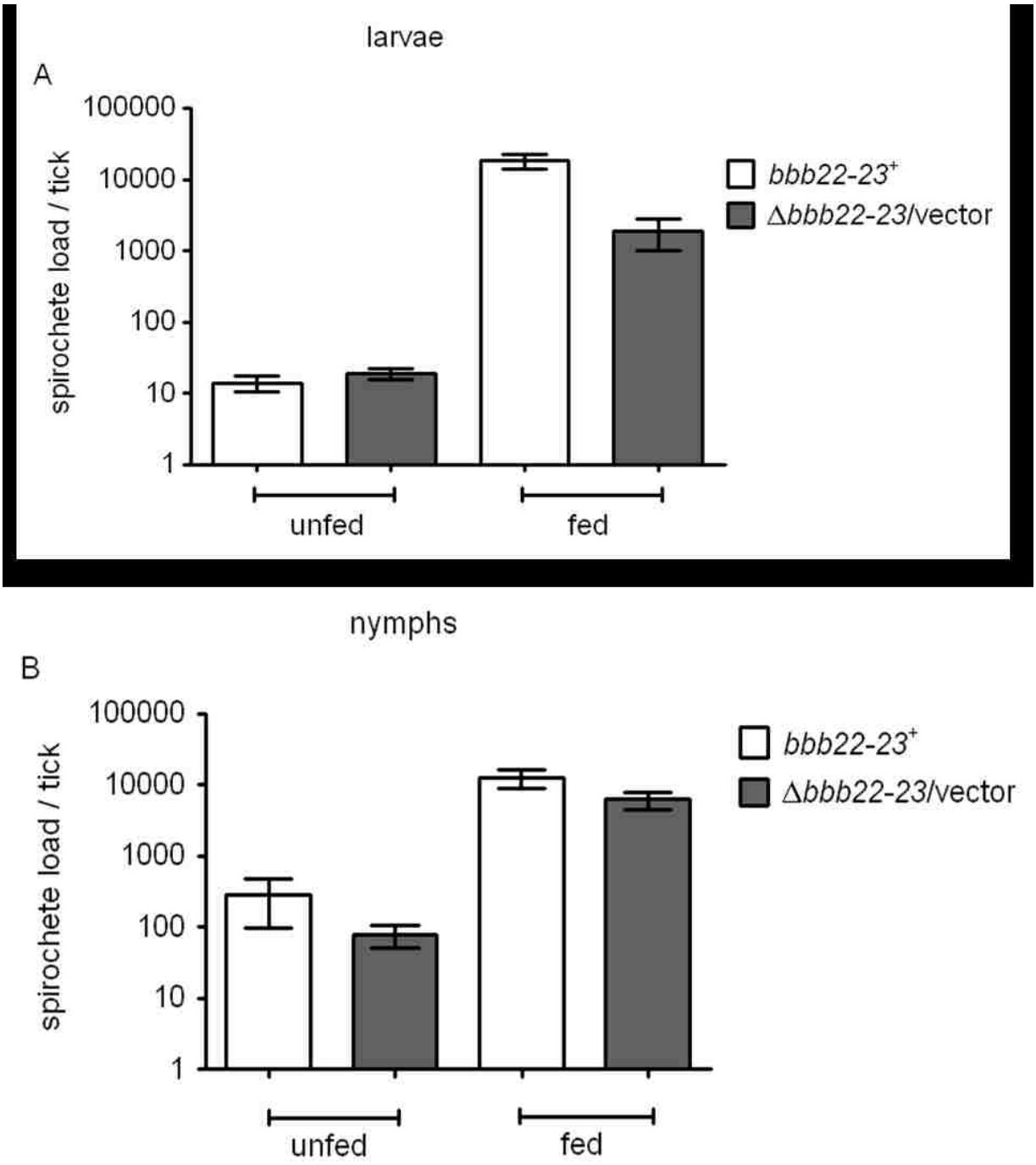

Figure 15. *bbb22-23* play critical role in *B. burgdorferi* growth in fed larvae.

Unfed larvae were artificially infected with *bbb22-23*$^{+}$ or Δ*bbb22-23*/vector spirochetes by immersing these larvae in *B. burgdorferi* cultures at a density of 2x10^{7} spirochetes/ml. 100-150 artificially infected larvae for each clone were fed on uninfected mice with 4 mice each *B. burgdorferi* clone. (A) Seven days following larval feeding, the number of

spirochetes per unfed and fed larva were determined by plating on solid of homogenized ticks on solid medium. (B) Following the molt, nymphal ticks were fed on mice and the number of spirochetes per unfed and fed nymph were determined as described. The data represents the average of at least 5 individual unfed larvae, 25 fed larvae, 25 unfed nymphs and 20 fed nymphs. Error bars represent standard deviation from mean.

4.4.3: *bbb22-23* are required by *B. burgdorferi* in tick to mouse transition.

Previous data from our group has shown that *bbb22-23* are essential for mouse infection by needle inoculation. Here we found that *bbb22-23* are not essential for spirochetes survival in tick vector. To study if *bbb22-23* mutant spirochetes persist in mice on tick transmission, serology studies and spirochete reisolation experiments were done on mice fed upon by infected unfed larvae. Unfed larvae and nymphs infected with *bbb22-23* mutant or *bbb22-23*$^{+}$ spirochetes were fed on mice. The mice fed upon by larvae infected with *bbb22-23* mutant spirochetes did not show immune response. Results were confirmed by spirochete reisolation experiment from mouse tissues. Mice fed upon by *bbb22-23*$^{+}$ spirochetes showed immune response and showed spirochete growth from mouse tissues (Table 7). Serology studies showed no immune response in mice fed upon with unfed larvae or unfed nymphs infected with *bbb22-23* mutant spirochetes (Table 7). So, *bbb22-23* are also essential for mouse infection by tick transmission.

Table 7: Spirochetes lacking *bbb22-23* are non-infectious in mice by tick bite transmission.

Ticks	Clone	number of infected mice/total[a]
Larvae	Δ*bbb22-23*/vector	0/4
	bbb22-23^{+}	2/2
Nymphs	Δ*bbb22-23*/vector	0/4
	bbb22-23^{+}	3/3

[a] Data is from two separate tick experiments. Infection was assessed by immunoblot analysis with *B. burgdorferi* lysate at 3 weeks post tick feeding and by spirochete reisolation from mouse ear, bladder and joint tissues collected from mice inoculated with *B. burgdorferi* clones.

4.4.4: The *bbb23* gene is sufficient to restore *bbb22-23*$^{+}$ spirochete loads in fed larvae.

We have previously shown that BBB23 is a low affinity purine permease (Chapter 3). Constitutive expression of the *bbb23* gene (*flaB*p-*bbb23*$^{+}$) allowed hypoxanthine uptake by Δ*bbb22-23* mutant spirochetes (Chapter 3). Because hypoxanthine may be the predominate purine available to *B. burgdorferi* for salvage from the tick blood meal this suggested that constitutive expression of *bbb23* may be sufficient to restore wild type spirochete loads to Δ*bbb22-23* mutant spirochetes in fed larvae. Naïve larvae were artificially infected with *B. burgdorferi* clone Δ*bbb22-23*, *flaB*$_{p}$-*bbb23*$^{+}$ or *bbb22-23*$^{+}$ and the infected ticks allowed to feed on mice, as previously describe. Spirochetes loads ticks were quantified. The data indicates that spirochetes harboring *bbb23* alone

are able to replicate to numbers equivalent to that of *bbb22-23*+ spirochetes in fed larvae (Figure 16).

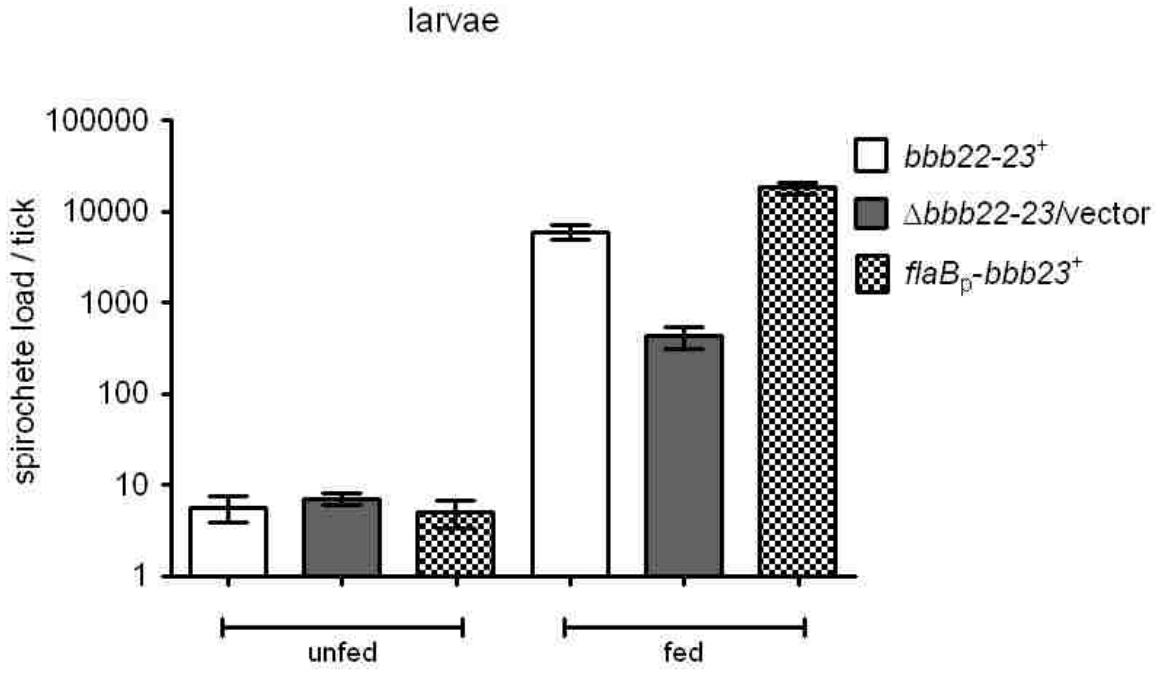

Figure 16. *bbb23* alone is sufficient to maintain spirochete loads to wild type levels in fed larvae.

Unfed larvae were artificially infected with *bbb22-23*$^{+}$, Δ*bbb22-23*/vector and *flaB*p-*bbb23*$^{+}$ as discussed above (Figure 15A). Infected larvae were fed on mice and fed larvae were collected and spirochete loads were calculated in individual larval tick as discussed above (Figure 15A).

4.5 Discussion

Borrelia burgdorferi is dependent upon purine salvage to synthesize its DNA and RNA. Previously we showed *bbb22* and *bbb23* are purine transporter genes present on circular plasmid 26 and are essential for survival of spirochetes in the mouse [119]. *B. burgdorferi* gene expression changes with environmental conditions such as temperature, pH, mammalian host, tick vector and availability of nutrients to allow *B.*

burgdorferi to survive in unique environmental niches [143]. Some of the well-studied genes in *B. burgdorferi* such as *ospA* and *ospC* show differential gene expression in the tick vector and the murine host. OspA and OspC are outer surface proteins in *B. burgdorferi* which these spirochetes express in tick vector and mammalian host, respectively [54]. Gene expression of *ospC* goes down in ticks whereas *ospA* expression goes down in mammalian host. So, as *B. burgdorferi* demonstrates changes in gene expression with changes in growth conditions, we were interested to determine the level of *bbb22* and *bbb23* gene expression in the tick vector. We have shown previously that *bbb22* and *bbb23* are expressed both *in vitro* and during mouse infection. To further study the role that spirochete purine salvage plays in tick vector, we first demonstrated that both these genes are expressed in unfed nymphal *Ixodes scapularis*, suggesting that these genes may contribute to *B. burgdoreri* persistence in the tick.

B. burgdorferi has also been shown to transport nucleotides and deoxynucleotides such as CMP, dCMP, AMP, dAMP, GMP and dGMP [56]. Nucleotides and deoxynucleotides are taken up by *B. burgdorferi* and are utilized directly to synthesize DNA and RNA [56]. Availability of sufficient amounts of nucleotides and deoxynucleotides in an external environment may bypass the requirement of both *bbb22 and bbb23*, as purine salvage may not be critical under these growth conditions. Indeed, Δ*bbb22-23* mutant spirochetes grow normal in BSKII medium, which is a complex, nutrient rich growth environment [119]. The inability of Δ*bbb22-23* mutant spirochetes to survive in the mouse suggests that the *in* vivo environments in the mammalian host contain insufficient levels of nucleotides and deoxynucleotides

spirochete survival in the absence of purine salvage. To study the role that *bbb22* and *bbb23* play in spirochete survival in the tick vector, spirochete survival and replication was examined in the tick developmental stages throughout the *B. burgdorferi* infectious cycle. The data demonstrated that genes *bbb22* and *bbb23* are not required for *B. burgdorferi* survival in larval and nymphal ticks, but contribute to the spirochete growth in larval ticks. The survival of *bbb22-23* mutant spirochetes in tick vectors might be due to the availability of nucleotides and/or deoxynucleotides during a blood meal, which can bypass the requirement for salvage of purine nucleobases. As the tick blood meal may only provide finite amounts of nucleotides and/or deoxynucleotides that can be utilized by spirochetes, having the ability to salvage purines through purine transporters may promote spirochete replication. Because of the difference in body size between larvae and nymphs, the size of the larval blood meal may be smaller than that of nymphs perhaps resulting in reduced amounts of available nucleotides and deoxynucleotides. This suggests that the advantage of purine transporters for spirochete replication may be more critical in larvae compared to nymphs. This reasoning may provide an explanation for why *bbb22-23* mutant spirochetes have low spirochetes load in larval ticks but not in nymphal ticks compared to the wild type spirochetes. Furthermore, the addition of *bbb23* to *bbb22-23* mutant spirochetes allows purine scavenging in fed larvae, restoring the spirochetes ability to replicate to levels similar to that of wild type spirochetes. To date no defined, minimal medium is available for *B. burgdorferi* growth *in vitro*. The use of minimal medium would allow experimental determination of concentrations of purines and other nucleotide precursors needed for spirochete survival and how *bbb22* and *bbb23* expression and function changes based

upon the availability of different amounts of purines. Future studies are focused on studying the role of BBB22 purine transporter in the growth of spirochetes harboring *bbb22* alone in larval ticks.

CHAPTER 5: CONCLUSIONS AND FUTURE STUDIES

5.1 Conclusions

B. burgdorferi has a unique infectious cycle that includes a variety of vertebrate hosts and a tick vector. Even though these spirochetes are not known to produce toxins [37, 39], they are still capable of invading the host tissues, potentially causing a disease state for months to years. These spirochetes cause disease by triggering the body's immune response, and resulting in the activation of various inflammatory reactions [144]. *B. burgdorferi* experiences various environmental changes during its infection cycle [145], therefore it is important to understand the molecular mechanisms involved in the survival of *B. burgdorferi* throughout its infectious cycle. *B. burgdorferi* shows changes in its gene expression in response to the different the environments it encounters such as the tick vector, mammalian host as well as distinct *in vitro* growth conditions [145]. Identifying and functionally characterizing the genes essential for *B. burgdorferi* survival *in vivo* can be helpful in identifying new therapeutic targets to treat Lyme disease.

Due to the reduced genome and limited metabolic capabilities, these spirochetes cannot survive in nature without a host, and require a complex, high nutrient medium for *in vitro* growth. *B. burgdorferi* lacks genes required for the synthesis of amino acids, fatty acids and nucleotides [146]. As nucleotides and deoxynucleotides are required for the synthesis of RNA and DNA, respectively, *B. burgdorferi* possess a purine salvage pathway for obtaining the nucleotide and deoxynucleotide precursors in place of *de novo* synthesis. Identifying the genes involved in the purine salvage pathway and understanding their importance in the infectious cycle of *B. burgdorferi*, will help in

better understanding the molecular mechanisms involved in Lyme disease pathogenesis.

Genes *bbb22* and *bbb23,* present on cp26, are annotated to encode for xanthine-uracil permeases and belong to the nucleobase cation symporter 2 (NCS2) superfamily. *bbb22* and *bbb23* are 79.8% and 78.3% identical at the nucleotide and amino acid levels, respectively. Both BBB22 and BBB23 appear to possess 12 transmembrane domains. The successful deletion of both *bbb22* and *bbb23* together showed that both of these genes are not essential for the survival of *B. burgdorferi in vitro* (Chapter 2) [119]. The *in vitro* purine transport assay results show that spirochetes require *bbb22-23* for hypoxanthine uptake (Chapter 2, Figure 7A) [119]. No hypoxanthine uptake by the *bbb22-23* mutant spirochetes suggests that there is no additional high affinity hypoxanthine transporter in *B. burgdorferi*. The data from a similar experiment with the use of [^{3}H]adenine in place of [^{3}H]hypoxanthine showed that BBB22-23 contribute to, but are not essential for, adenine uptake; as even in the absence of *bbb22-23*, spirochetes showed slow uptake of adenine (Chapter 2, Figure 7B) [119]. The competition assays were also performed to see if these transporters also transport xanthine, uracil (as annotated function) and pyrimidines. The competition assays were performed using 200 fold higher concentration of the various unlabeled competitors compared to [^{3}H]hypoxanthine to identify the substrates being transported by BBB22-23. Data suggests that only the unlabeled hypoxanthine, adenine and guanine among various purines and pyrimidines, compete with [^{3}H]hypoxanthine for their uptake by BBB22-23. This suggests that the BBB22-23 do not actually operate their annotated

function as a xanthine/uracil permease, but rather are purine permeases for hypoxanthine, adenine and guanine transport (Chapter 2) [119].

The BBB22-23 transport system belongs to COG2252 cluster and shows a similar function to the AzgA purine transporter in *Aspergillus nidulans*. AzgA specifically transports hypoxanthine, adenine and guanine [82]. To understand the importance of BBB22-23 in *B. burgdorferi*-mouse infection, the mice were inoculated with spirochetes missing *bbb22-23*. Data from a mouse needle inoculation experiments demonstrated that spirochetes missing *bbb22-23* did not survive in the mouse, suggesting that the purine permease activities of BBB22-23 are essential for *B. burgdorferi* survival in the mammalian host (Chapter 2, Table 2) [119]. The purine permease activities of BBB22-23 are not essential for the *in vitro* survival of *B. burgdorferi. B. burgdorferi* has previously shown to uptake of nucleosides and deoxynucleosides that are directly used in the synthesis of RNA and DNA [56]. The spirochetes missing *bbb22-23* can grow normal *in vitro* because the BSK II medium is highly rich in nutrients, and the availability of the nucleotides and deoxynucleotides might be sufficient to bypass the requirement for *bbb22* and *bbb23* (Chapter 2, Figure X) [119]. This is in contrast to the environment in mice, where the availability of the nucleotides, deoxynucleotides and adenine to *B. burgdorferi* is not sufficient for its survival. These spirochetes therefore have to rely upon purine salvage to survive in the mouse but not in BSKII medium *in vitro*. *bbb22-23* mutant spirochetes do not transport hypoxanthine, but still do transport adenine at a very slow transport rate compared to the wild type. After uptake, the adenine is converted into hypoxanthine by adenine deaminase [43]. However, this slow adenine uptake by *bbb22-23* mutant spirochetes is not sufficient for spirochete survival in a

mouse, suggesting that *bbb22-23* mutant spirochetes might require high amounts of adenine at the initial stages of mouse infection to compensate for the lack of hypoxanthine uptake for their survival.

B. burgdorferi quickly start disseminating from the inoculation site into the mouse tissues via the blood stream [147]. Adenine is known to be abundant in the mouse tissues and hypoxanthine in the blood [114]. During the initial stages of infection, the spirochetes are found in blood stream for a very short period where hypoxanthine is abundant. Because the *bbb22-23* mutant spirochetes are not able to uptake hypoxanthine, these spirochetes likely die in the early stages of mouse infection. Indeed, all the mice inoculated with the different doses of *bbb22-23* mutant spirochetes were seronegative (Chapter 2, Table 2), further suggesting that *bbb22-23* mutant spirochetes were cleared from the mice in the early stages of infection and before the immune response started. In conclusion, the *in vivo* phenotype of the *bbb22-23* mutant spirochetes highlights the importance of purine salvage for the ability of *B. burgdorferi* to survive in the mammalian host.

These results led us to further investigate the individual roles of the *bbb22* and *bbb23* genes. A few questions we wanted to answer were: 1) Are the BBB22 and BBB23 both purine transporters? 2) Do BBB22 and BBB23 each have distinct substrate specificities for the dfferent purines? 3) Does one of the two purine transporters have a great affinity for purine uptake compared to the other? 4) Does *B. burgdorferi* require both the *bbb22* and *bbb23* for mouse infection? To answer all of these questions, we made *B. burgdorferi* clones, in which only one of the purine permease encoding genes (*bbb22* or *bbb23*) was expressed in the spirochetes. To dissect the BBB22-23 purine

transport system, it was important to identify the transcription start sites for *bbb22* and *bbb23*. Our results demonstrate that *bbb22* and *bbb23* are separate transcripts and that the transcription start sites for *bbb22* and *bbb23* are located at 16 and 260 base pairs upstream of their respective open reading frames, respectively. (Chapter 3, Figure 8). Previously, our group found an *in vivo* active promoter sequence within the BBB23 ORF sequence. Initially, we speculated that this sequence may function as a promoter sequence for the *bbb22* transcript. Our 5' RACE data demonstrated that there is an transcript starting downstream of this promoter sequence (Chapter 3, Figure 8). This long upstream promoter (*22dist*$_p$) regulates the expression of *bbb22* and is required for the expression of *bbb22* (Chapter 3, Figure 9A). Another short sequence promoter, referred to herein as the proximal promoter (*22prox*$_p$), was not sufficient to maintain *bbb22* expression to wild type levels (Chapter 3, Figure 9A). It remains unclear how the long sequence including *22dist*$_p$ upstream of BBB22 ORF regulates *bbb22* expression. Purine transport assays were performed to study the individual roles of BBB22 and BBB23. Results showed that both the BBB22 and BBB23 are capable of hypoxanthine uptake but their affinities for the hypoxanthine uptake are different. Uptake of hypoxanthine by BBB23 alone was only detectable when *bbb23* was expressed under a constitutive promoter, and this transport was still at a slow rate compared to spirochetes carrying *bbb22* alone (Chapter 3, Figure 10). Our data indicate that BBB22 is the more efficient hypoxanthine transporter compared to BBB23. BBB22 can individually transport hypoxanthine when expressed under the long upstream sequence including the distal promoter (*22dist*$_p$) but not under the short upstream sequence including the proximal promoter (*22prox*$_p$). *In vitro* hypoxanthine assay results showed that in the initial stages,

the hypoxanthine uptake by both transporters individually was siginificantly reduced compared to the BBB22-23 together. At later time points, BBB22 mediated-hypoxanthine uptake levels were comparable to the hypoxanthine uptake levels of BBB22-23 together (Chapter 3, Figure 10A). Data from the adenine uptake assays suggest that BBB22 alone can transport, adenine, but adenine uptake is slow compared to the BBB22-23 together (Figure 10B). This slow adenine uptake by BBB22 alone compared to the BBB22-23 together, suggests BBB23 also contributes to adenine uptake. But, results from adenine uptake assays with spirochetes carrying *bbb23* alone under control of a constitutive promoter did not show any adenine uptake by this clone at the concentration of adenine examined. These data suggest that BBB23 alone is not sufficient for the adenine transport or BBB23-dependent adenine uptake is below detection limits and therefore, BBB22 is required along BBB23 for the adenine uptake. BBB22 has high affinity for the adenine uptake but BBB22 alone is still not sufficient to transport adenine to levels similar to BBB22-23 together. So, BBB23 is required along with BBB22 to achieve wild type levels of adenine uptake.

To confirm if BBB23 does indeed function as an adenine transporter or if only the *bbb23* transcript is needed for the full activity of BBB22 in adenine uptake, competition assays were performed using the spirochetes harboring only *bbb23* under a constitutive promoter (*flaB*$_p$-*bbb23*$^+$). Results clearly show that the uptake of [^{3}H]hypoxanthine in these spirochetes was significantly reduced in the presence of 20 fold excess unlabeled adenine (Chapter 3, Figure 11B). So, BBB23 is a purine permease with a low affinity for adenine uptake. Competition assay results from *B. burgdorferi* clones harboring only *bbb22* or *bbb23* also demonstrated that both BBB22 and BBB23 are guanine

permeases. Overall these data demonstrate that the BBB22 and BBB23 are both purine permeases in which BBB22 has higher affinity for the uptake of both adenine and hypoxanthine compared to BBB23. It has been suggested that there may be insufficient guanine available in the murine host to allow *B. burgdorferi* survival [43], so, hypoxanthine and adenine salvage from the mouse is critical for *B. burgdorferi* to synthesize guanine nucleotides and deoxynucleotides during infection.

Our data indicate that BBB22, a high affinity purine transporter compared to the BBB23, is able to salvage sufficient amounts of adenine and hypoxanthine to allow for *B. burgdorferi* survival in the mouse. Because of the low hypoxanthine and adenine uptake affinities of BBB23, spirochetes harboring only BBB23 were unable to survive in the mouse. So, *bbb22* is critical for mouse infection but *bbb23* is not (Chapter 3, Table 5). Spirochete loads in mouse tissues were determined for the spirochetes harboring *bbb22* and were compared with mice inoculated with spirochetes having both *bbb22* and *bbb23*. We found that the mice inoculated with the spirochetes harboring only *bbb22*, showed a significant reduction in spirochete loads in mouse tissues compared to the spirochete loads for *bbb22-23*$^{+}$ spirochetes. The low spirochete loads for *bbb22*$^{+}$ spirochetes are believed to be due to the reduced affinity of purine uptake by BBB22 alone compared to BBB22-23. A diminished capacity to salvage purines will result in decreased nucleotides synthesis for DNA, RNA and the other metabolic processes, and thus low spirochetes numbers compared to when both the *bbb22* and *bbb23* are present. As *bbb22* alone is sufficient for mouse infection, it is not clear yet why *B. burgdorferi* possesses two purine transporters with seemingly the same function but different purine uptake affinities. One possible explanation is that due to the complex

infectious cycle of *B. burgdorferi*, the availability of the purines may vary with the change in host environments. So, in some host environments due to low purine availability, both BBB22 and BBB23 are required for the maximum uptake, whereas in others with a high abundance of purines, only one or the other purine transporter may be necessary for the spirochete survival. To be able to study the effects of varying amounts of purines on the activities of these purine transporters, a defined, minimal medium would be critical for *in vitro* growth analysis. At present such an experimental system does not exist for *B. burgdorferi.* Future studies are focused on identification of a defined, minimal medium for growth of *B. burgdorferi*. An alternate strategy to study the roles of varying amounts of purines on BBB22 and BBB23 activity is using *E. coli* clones expressing *bbb22* or *bbb23* or both *bbb22* and *bbb23* together, and an *E. coli* minimal media. The amounts of different purines can be controlled manually and it would be studied that how BBB22 and BBB23 work in the low and high concentrations of adenine or hypoxanthine or guanine. *bbb22* and *bbb23* expression will be studied at different concentration of these purines.

Ticks acquire spirochetes by feeding upon an infected host. Spirochetes persist in the tick midgut and obtain nutrients from the tick blood meal [50]. As some bacteria can use red blood cell (RBC) as a source of purine salvage like *Plasmodium falciparum* [148], so *B. burgdorferi* may also obtain nutrients from degraded RBCs found within the tick midgut. After a blood meal from an infected host, tick larvae molt into nymphs, which can then transmit spirochetes from the salivary glands to an uninfected host during a subsequent feeding [2]. To study the combined role of *bbb22-23* in spirochete survival in the tick vector, expression analysis was performed for *bbb22* and *bbb23* to

see if *B. burgdorferi* express both the genes while residing in the tick vector. These data showed that both *bbb22* and *bbb23* are expressed in the tick vector with *bbb22* expression 4 fold higher than *bbb23*. This difference may or may not be significant (Chapter 4, Figure 13). The roles of *bbb22-23* were studied in the spirochete survival and growth in larval and fed ticks. As *bbb22-23* mutant spirochetes do not transport hypoxanthine, the survival and growth of *bbb22-23* mutant spirochetes in the tick vector will likely depend upon the availability of adenine, nucleotides and deoxynucleotides during blood meal. Our results show that *bbb22-23* mutant spirochetes survive in both larval and nymphal ticks. However, statistically fewer *bbb22-23* mutant spirochetes were detected in fed larvae as compared to the number of *bbb22-23*$^{+}$ spirochetes. As the size of a fed nymph is much bigger compared to a fed larva, this suggests that the volume of the blood meal and therefore the availability of the purines, nucleotides and DNA from the degraded blood may be more in fed nymphs. Wild type spirochetes in fed larvae have access to all these nutrients along with hypoxanthine and guanine but *bbb22-23* mutant spirochetes cannot uptake hypoxanthine. The diminshed availability of nutrients for the salvage of purines and nucleotides likely accounts for the finding that *bbb22-23* mutant spirochetes replicate to a less number compared to the wild type spirochetes in fed larvae (Chapter 4, Figure 15A). In fed nymphs due to the possible larger volume of the blood meal, the nutrients available to the *bbb22-23* mutant spirochetes may be sufficient to allow the spirochetes to replicate to levels equivalent to *bbb22-23*$^{+}$ spirochetes (Chapter 4, Figure 15B). We also found that reintroduction of *bbb23* alone to *bbb22-23* mutant spirochetes was sufficient to restore spirochete replication to wild type levels (Chapter 4, Figure 16). Overall, our data suggest that

spirochetes have access to more nutrients in the tick blood meal compared to the initial stages of mouse infection and thus *bbb22-23* contribute to but are not essential for spirochete survival and growth in fed larvae. We also studied if there are any other unknown genetic mechanisms, which are activated in the *bbb22-23* mutant spirochetes inside the tick vector and to allow the spirochetes to infect mice by tick bite transmission. Serology studies and spirochete reisolation experiments were done on mice that were fed upon by *bbb22-23* mutants spirochete infected unfed larvae and nymphs. The *bbb22-23* mutant spirochetes were unable to infect mice by tick bite transmission, indicating that no compensatory mechanisms were activated during the tick to mouse transmission, which could allow the *bbb22-23* spirochetes to survive in the mouse.

Overall, this study has shown that purine transport system in *B. burgdorferi* is essential for the mammalian infection. No essential role of BBB22 and BBB23 in B. burgdorferi survival in tick vector suggests the availability of nutrients in blood meal in ticks might be more compared to mammalian host and due to that *bbb22-23* mutant spirochetes bypass the requirement of bbb22-23 and grow normal in tick vector; whereas it is not true in mammalian host.

5.2 Future studies

1) Understanding the mechanisms of gene regulation of *bbb22* and *bbb23***:**

Our lab has previously used an *In Vivo* Expression Technology (IVET) based method to identify for the first time *B. burgdorferi* genes that are actively expressed during a mouse infection [126]. One of the identified *in vivo* active promoter sequences of *B. burgdorferi*, was found to be within the BBB23 ORF sequence. In this thesis, the data

shows a transcription start site downstream of this *in vivo* active promoter sequence contained within the BBB23 ORF sequence. *bbb22* expression and function is dependent on the presence of a long sequence including this *in vivo* active promoter sequence upstream of the BBB22 ORF. In constructs lacking this long upstream sequence *bbb22* expression goes down (Chapter 3, Figure 9) and spirochetes carrying only *bbb22* in the absence this upstream sequence lose the ability to transport purines (Chapter 3, Figure 10). To further investigate this transcript, we performed PCR analysis using cDNA made from extracted RNA of *in vitro* grown wild type spirochetes. PCR was done using various primer sets to determine the length of this new unknown transcript associated with an *in vivo* active promoter sequence (Table 8). PCR results showed that transcription downstream of the *in vivo* active promoter sequence from BBB23 ORF sequence at the 3' end runs into the *bbb22* ORF sequence (Figure 17).

Table 8: Primers used in this study.

Primer number	Primer name	Primer sequence
1006	BBB23 ORF (1392)-F	CCCGGGGGATCCTTGAAAATATTTTTAAACAATAAAAAGGAAAGTTT
1009	BBB23 ORF-Reverse	CCCGGGAAGCTTTTAATAGCCATAAATAAATTTAATAATAAAAATTAAGC
1172	BBB22 Start-Rev	GAAATAACAATGTTTCTTTGGATTGAT
1383	BBB23 IVET out 409-Fwd	GAGCAAATTATAAACTCTATTCCGA
1384	BBB23 IVET in 667- Fwd	ATATATGCAATCTTTAATTTAGAAGGTG

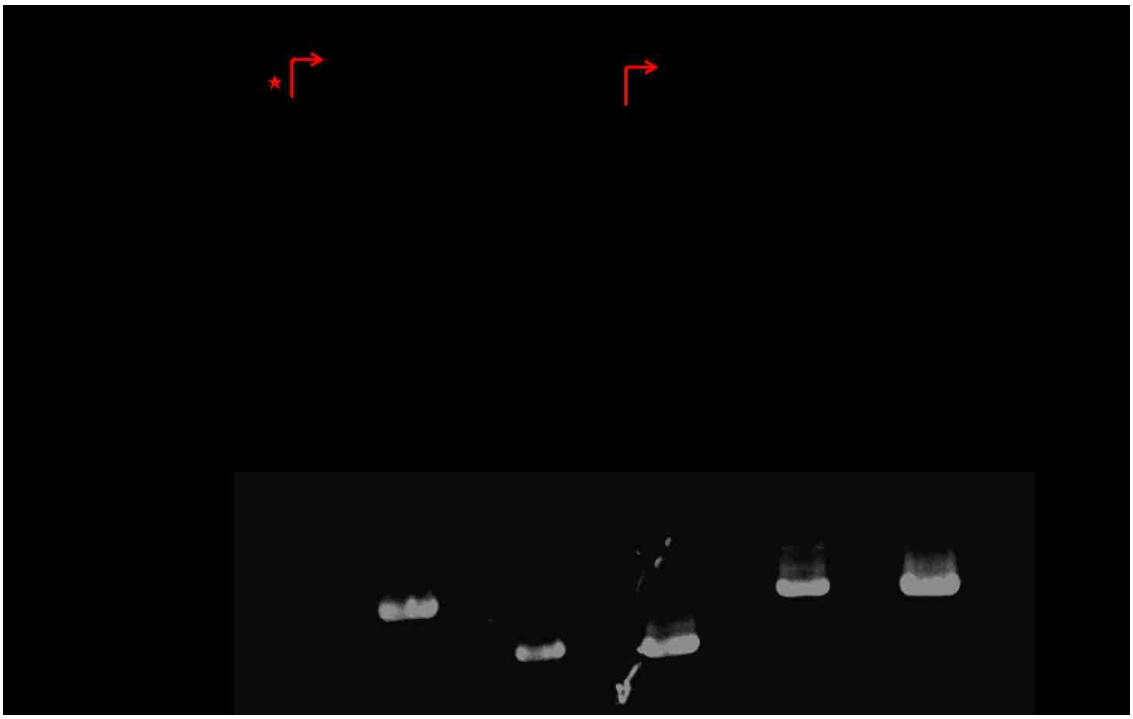

Figure 17. PCR analysis of a transcript across *bbb23* and *bbb22*.

(A) Schematic representation of various primers used to determine intragenic transcript within *bbb23* ORF. Primers used are shown as arrows in black. IVET promoter sequence site is shown as a start in red. Transcription start site for *bbb22* and IVET promoter within *bbb23* ORF are show as arrows in red. (B) PCR analysis of transcript from IVET promoter. PCR results with primer sets 1383 and 1172, 1384 and 1172 and 1006 and 1009 are shown in figure. cDNA template is used to study transcript from IVET promoter. RT- represents cDNA control template without reverse transcriptase. gDNA represents the genomic DNA template, taken as PCR control for primers. gDNA- represents no genomic template as a negative control to determine reagent contamination.

Future experiments will be done to determine the 3' end of this transcript by 3' RACE or walking reverse primer towards 3' end into *bbb22* ORF sequence or determining transcript size by Northern blot. Once the size of this transcript is known, then it can be determined if only a small part of this long transcript or the entire transcript is required for *bbb22* expression and function. Future studies investigating the regulation of *bbb22* and *bbb23* are focused on understanding the mechanisms involved

in the regulation of *bbb22*, specifically by means of this distal promoter sequence (*22dist*$_p$).

To study the gene regulation of purine permease encoding genes, it is important to have defined *in vitro* growth medium. This will allow experimental examination of expression of the transport genes in the presence of various concentrations of purines. In the case of *B. burgdorferi*, BSKII medium is not defined and is highly rich in nutrients. Due to the complex nature of BSKII medium, it would be difficult to reduce the purine levels. Another way to study the affect of purine concentration of the regulation of the purine transport genes could be to add additional adenine, hypoxanthine or guanine to *B. burgdorferi* cultures growing in the BSKII medium. Supplementing the *B. burgdorferi* cultures with an excess of adenine, hypoxanthine and guanine and giving sufficient time for spirochetes to grow, could result in a change in the gene expression of *bbb22* and *bbb23*. *B. burgdorferi* is expected to show lower expression levels for *bbb22* and *bbb23* in the presence of excess of purines.

It is also important to study *bbb22* and *bbb23* gene regulation in log and stationary growth phases of spirochetes because our quantitative PCR analysis for the gene expression of *bbb22* and *bbb23* in the wild type spirochetes show that *bbb23* expression in log phase spirochetes increases by 36-fold compared to the stationary phase spirochetes; whereas *bbb22* expression does not show a significant difference. So, identifying the mechanisms involved in the change in gene expression of *bbb23* but not *bbb22* under different growth stages, are under investigation. Purine transport

genes have shown to be controlled by riboswitches by binding of adenine and guanine to the riboswitch present in 5' UTR of transport genes. So finding possible riboswitches in 5' UTR of *bbb22* and *bbb23* studying their role in gene regulation is also under investigation.

2) Using BBB22 and BBB23 as potential therapeutic targets to treat Lyme disease:

BBB22 and BBB23 are purine transporters, which can be used as potential targets for drug development or drug delivery to prevent the *B. burgdorferi* growth and thus to treat Lyme disease. These purine permeases could be used as therapeutic targets in two ways, 1) delivering antimetabolites through these permeases or 2) using drugs that inhibit purine permease function [149] (Figure 18). Antimetabolites mimic the structure the true metabolites and prevent the use of essential metabolites during metabolism. Antimetabolites such as purine analogues, mimic purines in structure and will prevent purine incorporation into DNA or RNA. As a result, these antimetabolites will eventually shut down nucleic acid synthesis. Many of the purine and pyrimidine antimetabolites approved by FDA are being used as therapeutics [150]. Most of the purine transporters in microbial organisms are different from the purine transporters in humans and mammals. So, using these microbial purine transporters as a therapeutic target may successfully treat the various infectious diseases [77, 79, 151]. Using BBB22 and BBB23 as therapeutic targets may hold a number of possible advantages such as having the ability to use lower minimum inhibitory concentrations of drugs due to the drugs being delivered through specific permeases as opposed to the drugs being delivered through diffusion process, which may require higher concentrations. In

addition, drug delivery through these permeases will be more specific to kill or inhibit bacteria but not host cells. Targeting *B. burgdorferi* through BBB22 and BBB23 will be helpful to treat Lyme disease more effectively by increasing safety range for drug use thus expanding therapeutic window.

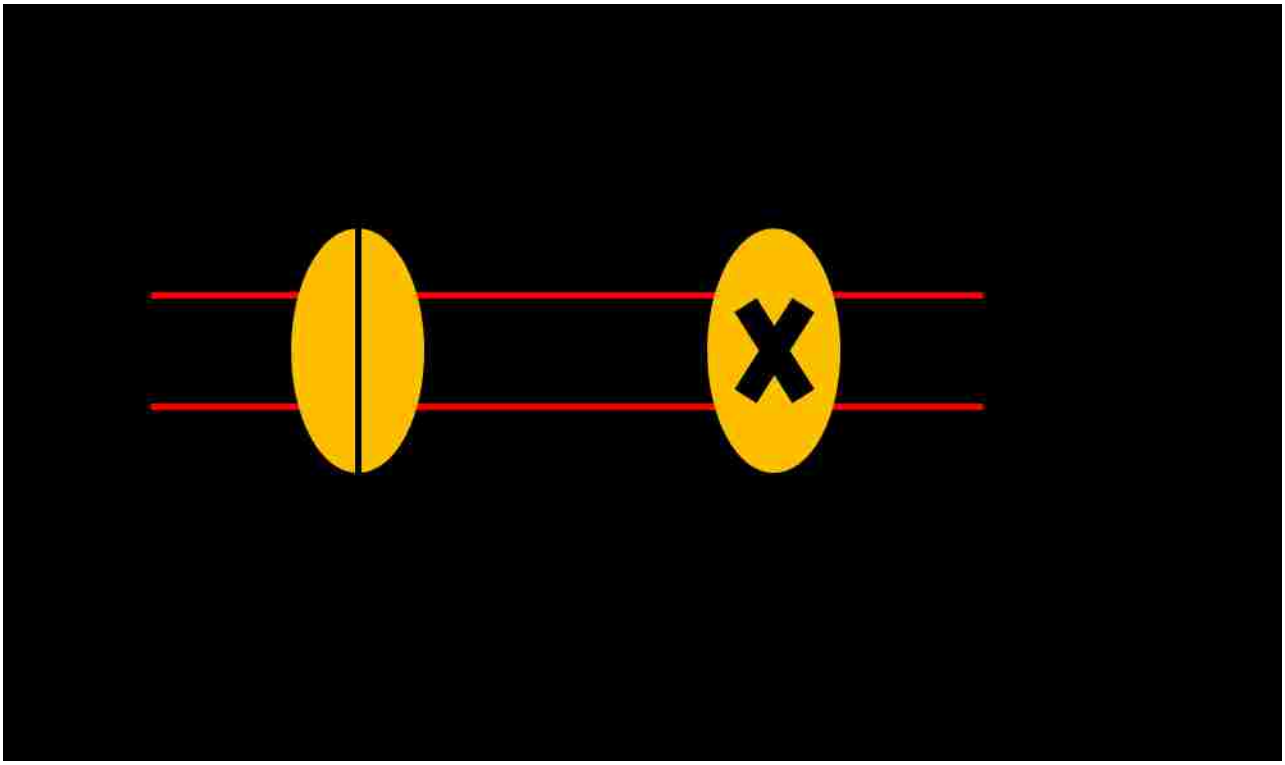

Figure 18: Use of purine permeases as therapeutic targets.

BBB22-23 purine permeases are shown as yellow oval. Two possible ways to use BBB22-23 purine transporters as therapeutic targets has been shown. Using BBB22-23 transporters to deliver various antimetabolites such as hypoxanthine, adenine or guanine analogues are shown as part A. Another way to target BBB22-23 is by preventing purine uptake in *B. burgdorferi* by inhibiting BBB22-23 with different purine permease inhibitors as shown in part B.

Various antimetabolites can be that can be targeted for transport through the *B. burgdorferi* purine permeases include purine analogues to hypoxanthine such as allopurinol, 6-mercaptopurine and 8-aza-2, 6-diaminopurine, purine analogues to

adenine such as 2-fluroadenine, 6-methyl purine, 2, 6-diaminopurine, 2-aminopurine and 8-aza-adenine, and purine analogues to guanine such as 6-thioguanine, 8 azoguanine and 2-amino-6-chloropurine. Purine analogues are FDA approved drugs used to treat cancer and other disease [150, 152]. The *E. coli* hypoxanthine-guanine specific purine permease, YjcD, has been shown to be responsible for bacterial sensitivity to purine analogue toxicity [121]. Using purines permeases to deliver toxic analogues would therefore aid in the prevention of bacterial infections in the host. Plasmodium falciparum and Trypanosoma has previously shown to be sensitive to the toxicity of purine analogues [153, 154]. Similar studies can be performed in *B. burgdorferi* to see if the BBB22 and BBB23 purine permeases transport purine analogues. *B. burgdorferi in vitro* growth assays can be performed using various concentrations of these analogues. We preliminarily examined the purine analogues 2-fluroadenine, 8-aza-guanine, 6-mercaptopurine and 6-thioguanine to determine if these molecules are transported through BBB22 and/or BBB23 and are able to inhibit *B. burgdorferi in vitro* growth (Table 9).

Table 9. *B. burgdorferi* growth inhibition by purine and nucleoside analogues.

Analogues	*B. burgdorferi* clone	700uM[a]	70uM[a]	7uM[a]
6-mercaptopurine	Wild type	NI	NI	NI
	Δ*bbb22-23*/vector	NI	NI	NI
	bbb22-23^{+}	NI	NI	NI
2-fluoroadenine	Wild type	CI	PI	NI
	Δ*bbb22-23*/vector	CI	PI	NI
	bbb22-23^{+}	CI	PI	NI
8-azaguanine	Wild type	NI	NI	NI
	Δ*bbb22-23*/vector	NI	NI	NI
	bbb22-23^{+}	NI	NI	NI
6-thioguanine	Wild type	CI	PI	NI
	Δ*bbb22-23*/vector	CI	PI	NI
	bbb22-23^{+}	CI	PI	NI
6-Azaxanthine monohydrate	Wild type	NI	NI	NI
	Δ*bbb22-23*/vector	NI	NI	NI
	bbb22-23^{+}	NI	NI	NI
2-fluoroadenosine	Wild type	PI	NI	NI
	Δ*bbb22-23*/vector	PI	NI	NI
	bbb22-23^{+}	PI	NI	NI

[a]CI = Complete Inhibition, PI = Partial Inhibition, NI = No Inhibition

The growth assay results showed that *B. burgdorferi* has minimal sensitivity to 2-fluoroadenine, 6-thioguanine and 2-fluoroadenosine. We did not see any clear differences between the wild type and *bbb22-23* mutant spirochetes in the toxicity of the purine analogues tested. It is unclear from these data if BBB22 and BBB23 contribute in the transport of purine analogues such as 2-fluoroadenine and 6-thioguanine. Our studies showed that *B. burgdorferi* do not transport hypoxanthine in the absence of BBB22 and BBB23, suggesting these are the only high affinity purine transporters in *B. burgdorferi* to transport hypoxanthine. Data from adenine uptake assays also suggests the presence of another unknown adenine transporter/s in *B. burgdorferi*, but these data cannot discount another guanine transporter(s) being present in *B. burgdorferi* apart from BBB22 and BBB23. Therefore it is possible that BBB22 and BBB23 may transport these antimetabolites but, if there are other unknown transporters for adenine and guanine, these could also be responsible for the transport of the purines analogues in *B. burgdorferi*. This might be the reason that *bbb22-23* mutant spirochetes are also sensitive to 2-fluoroadenine and 6-thioguanine. 6-mercaptopurine, a hypoxanthine analogue, was expected to show toxic effects in wild type spirochetes but not in the *bbb22-23* mutant spirochetes because in *B. burgdorferi*, hypoxanthine is being transported by BBB22 and BBB23 only. Results show that both types of spirochetes grow normal in the presence of 6-mercaptopurine and do not show any toxic effects on *in vitro* growth. This may be due to either low phosphoribosyltransferase activity for hypoxanthine in *B. burgdorferi* [73] or rapid oxidation of mercaptopurine as seen in the other bacteria such as *Pseudomonas aeruginosa* [155]. Allopurinol and 8-aza-2,6-diaminopurine are the other hypoxanthine analogues, which could additionally be

studied to see if these are being transported by BBB22 and BBB23 and are toxic only to the wild type spirochetes but not the *bbb22-23* mutant spirochetes. As *B. burgdorferi* has a minimal purine salvage pathways lacking many of the enzymes that are involved in purine salvage pathway. So it is not only important to identify the analogues being transported by these purine transporters but it is also important to make sure if *B. burgdorferi* has enzymes to metabolize these analogues. Identifying such drug candidates that are transported through BBB22 and BBB23 will further provide information about the presence of enzymes in *B. burgdorferi* that act on these metabolites. These studies will also provide information on if the enzymatic activities of these enzymes in *B. burgdorferi* are sufficient to metabolize these antimetabolites and show toxic effects.

3) Amino acid sequence alignment of BBB22 and BBB23 with other purine transporters of COG2252 cluster, and identifying putative amino acids essential for the transport activities of BBB22 and BBB23:

BBB22 and BBB23 are closely identical to each other in amino acid sequence as well as the number of putative transmembrane domains. It remains unclear as to why *bbb22* expression and its encoded protein activity are high compared to *bbb23*. We were unable to detect hypoxanthine and adenine uptake by BBB23 alone in spirochetes carrying *bbb23* expressed under the control of its own promoter. Even when *bbb23* expression was increased under the control of a constitutive promoter to the native *bbb22* levels, only minimal hypoxanthine uptake and no adenine uptake by BBB23 were

detected. This suggested to us to look closely at the amino acid sequence of BBB22 and BBB23 to work to understand the purine permease affinity differences between BBB22 and BBB23. Amino acid sequence alignment of various purine permeases in COG2252 such as PurP, YicO, YjcD and YgfQ of *E. coli*, PbuG and PbuO of *Bacillus subtilis* and AzgA of *Aspergillus nidulans* have some amino acids within its transmembrane domains that are either fully conserved (dark green) or partial conserved (light green) [80]. These amino acid positions were determined based upon the positions of fully conserved or partial conserved amino acids in well studied purine permeases of COG2233 cluster [80]. COG2233 cluster belongs to nuclebase cation symporter 2 (NCS2) superfamily and includes well studied xanthine and/or uric acid permeases. Conservation of these amino acids occurs between the members of COG2252, but the members of COG2233 are well conserved for amino acid residues at same locations among their members but are distinct from the COG2252 members. Point mutations in these conserved amino acids of COG2252 members has identified key residues critical for hypoxanthine, adenine, and guanine uptake activity [80]. We have found that amino acid sequence alignment of BBB22 and BBB23 with other members of COG2252 cluster using the ClustalW algorithm show that both BBB22 and BBB23 also possess these fully conserved (shown as dark green) and the partially conserved (light green) amino acid residues (Figure 19). These residues are expected to play important roles in the substrate specificity and binding affinity as seen in the purine permeases in *E. coli* [80]. Interestingly, we have found that residues of BBB22 and BBB23 corresponding to residue 271 of PurP show a change in the amino acid residue from a threonine in BBB22 similar to the other members of COG2252, to an

isoleucine in BBB23 (Figure 19). It has been studied that threonine in YjcD corresponds to residue 271 of PurP is required for hypoxanthine/guanine transport. We speculate that differences in the activities of BBB22 and BBB23 may be due, in part, to the difference in this residue. Future experiments include point mutations to replace the isoleucine in BBB23 with a threonine and perform purine transport assays with these mutation clones. We hypothesize that BBB23 transport activity will increase after replacement of the key isoleucine with threonine.

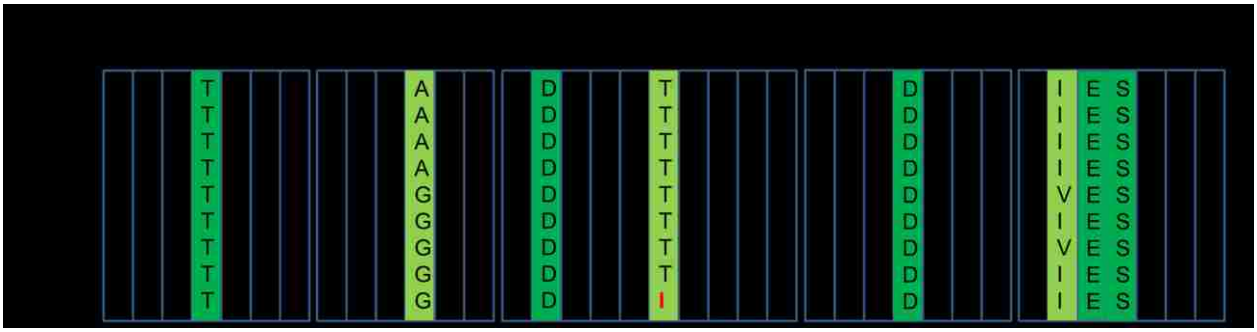

Figure 19. BBB22 and BBB23 show amino acid variation at one of the conserved residues.

Amino acid sequences of BBB22 and BBB23 were aligned with other members of COG2252 cluster using Clustal W method. Sequence alignment of transmembrane loops is shown as TM. Conserved amino acids are highlighted as dark green and semi-conserved amino acids as light green. Residue numbers refer to PurP purine transporter in *E. coli* [80]. BBB23 amino acid Isoleucine in Transmembrane 8, varies from BBB23 and other members of COG2252 cluster, is shown in red.

5.3 Final conclusion

Collectively, we have identified a purine transport system in *B. burgdorferi*, which is essential for *B. burgdorferi*-mammalian infection. Identification such a purine transport system in *B. burgdorferi*, and genetic and biochemical characterization of the roles of these purine transporters in the infectious cycle of *B. burgdorferi* improves our

understanding of the essential molecular mechanisms involved in the virulence of this Lyme disease pathogen. Based upon our experimental data, we hypothesize a putative amino acid residue in BBB23 that is responsible for its low transport activity compared to BBB22.

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