Master's thesis

Transposon mutagenesis in the Lyme disease pathogens *B. afzelii* and *B. burgdorferi*

Laboratory of Molecular Ecology of Vectors and Pathogens

INSTITUTE OF PARASITOLOGY

Biology Center, ASCR

Bc. Nora Andrea Hagleitner, BSc.

Supervisor: Ryan O. M. Rego, PhD

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Annotation:. The aims of this thesis were to create the first transposon mutant in the European Lyme disease spirochete *B. afzelii* and to investigate transposon mutants created by Dr. Rego (unpublished data), in the infectious background *B. burgdorferi* s.s derivative B31-A3. Furthermore, transformation of the fully sequenced *B. burgdorferi* derivative B31-A3 with a GFP containing shuttle vector and passaging of the already fluorescent strains 297 *(B. burgdorferi)* and CB43-dsRED *(B. afzelii)* was done.

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Abstract

Since its first use in *B. burgdorferi* s.s., transposon mutagenesis has enabled the investigation of many previously uncharacterized genes in spirochete morphology, physiology, growth rate and pathogenesis. In this master's thesis, I examined three transposon mutants, created in an infectious *B. burgdorferi* s.s. background (B31-A3, Rego et al., unpublished) and studied the function of the disrupted gene loci in pathology and biology of *B. burgdorferi*. During the course of the project I focused on the most promising candidate of the three transposon mutants, which showed defects in growth rate, motility, morphology and infectivity. The disrupted gene locus of this transposon mutant is part of a big operon, which encodes multiple flagellar proteins, being essential for proper flagellar assembly, further emphasizing the role of the periplasmic flagellum in bacterial shape, motility and infectivity of this complex pathogen.

Transposon mutagenesis has been widely applied in *B. burgdorferi* s.s., however, other *Borrelia* species, including *B. afzelii*, the primary agent of Lyme disease in Europe, remain mostly unexplored. Therefore, I tried to create the first transposon mutant in an infectious *B. afzelii* background via adaption of the protocol used by Stewart et al. in 2004.

Visualization of *B. burgdorferi* s.s. is another genetic tool which has greatly improved our understanding of this organism. The use of fluorescent proteins expressed in *B. burgdorferi* s.s. enlightened our understanding of spirochetal gene expression during different stages of the infectious cycle as well as the route this organism undertakes during dissemination from the tick vector to the vertebrate host. Therefore, I attempted to create the first green fluorescent *B. burgdorferi* strain B31-A3 via electroporation using a GFP containing shuttle vector. Furthermore, I was the first to perform continuous in vitro-passaging of the already green fluorescent strain 297 (*B burgdorferi* s.s) and the red fluorescent CB43-dsRed strain (*B. afzelii*) to acquire plasmid loss. The final passages of both fluorescent strains were plated on solid media and several clones were picked. In future studies our lab wants to assess the plasmid profile of these clones obtained and observe if loss of certain plasmids has an effect on the interaction of those clones with their tick vector or their vertebrate host.

Acknowledgement

To begin with, I would like to thank my supervisor Dr. Ryan Rego for the opportunity to work in his lab, for his confidence, patience, support and advice. Furthermore, I would like to thank all people of our lab, who offered their help whenever it was needed, including Lisa Hain BSc., Mgr. Martin Strnad, Mgr. Zuzana Vavrušková, Mgr. Helena Rohaková, and Peter Weber. I would also like to acknowledge RNDr. Marie Vancová, Ph.D. who conducted the electron microscopy work. Moreover, I would like to thank my friends and fellow students Paul Strasser, Helmut Stanzl, Katharina Böttinger and Lisa Hain. We always studied together and everybody of us is good in slightly different things, but together we have been a pretty good team for more than two years now. I also want to thank my family for supporting me all the years financially, mentally, and who always believed in my skills. Another person I want to thank is my Karate trainer Hubert Havlik, who taught me in his own but affectionate way self-discipline, endurance, and the steady will to surpass oneself - features which helped me a lot during the course of my study.

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1. Literature research

1.1 Lyme disease

Lyme disease, or Lyme borreliosis, is a widespread, multistage, systemic infection. Over the last two decades, Lyme disease is recognized as the most common arthropod borne illness in temperate regions all over the Northern hemisphere, including North America, Europe and Asia (1, 2). In these regions, Lyme disease is a major public health concern due to the steadily increasing number of new infections (1).

Lyme disease was first recognized as a separate entity in 1976, due to the geographical clustering of children, who were misdiagnosed with juvenile rheumatoid arthritis, around a small town called Lyme, in Connecticut, United States. This first clinical diagnosis was rejected as it became apparent that Lyme disease is a tick transmitted, multisystemic bacterial infection (1). The unknown infectious agent of Lyme disease was first isolated from nymphal *Ixodes scalpularis* ticks in 1981 by Willy Burgdorfer and his co-workers (3). It was a spirochetal bacterium which was denoted as *B. burgdorferi*, after its co-discoverer. Later on, the same spirochete was cultured from the skin, blood and cerebrospinal fluid of early Lyme disease patients, proving that *B. burgdorferi* is the causative agent of Lyme disease (1, 3).

1.2 *Borrelia*

B. burgdorferi, the causative agent of Lyme disease is a tick borne obligate parasite of the genus *Borrelia*, belonging to the eubacterial phylum of *Spirochaetes* (1, 2). *Borrelia* spirochetes are divided into two major phylogenetic groups, the causative agents of Lyme disease and those responsible for relapsing fever (3). Based on phylogenetic analysis, Lyme disease spirochetes have been further subdivided into 18 distinct species, which are all collectively referred to as the *B. burgdorferi* sensu lato complex (4).Members of this complex are transmitted to their vertebrate host, including humans, by specific hard ticks of the *Ixodes spp.* complex. However, only three genospecies of the *B. burgdorferi* senus lato (s.l.) complex are responsible for human cases of Lyme disease, namely, *B. burgdorferi* sensu stricto (s.s.) in the United States and Western Europe, and *B. afzelii* and *B. garinii* in Eurasia. Different clinical manifestations of Lyme disease are associated with each genospecies, explaining geographical variations of human Lyme disease symptoms between regions where those pathogens are endemic (5). For instance, *B. garinii* is the most neurotropic, while *B. burgdorferi* s.s. is the primary agent of carditis and arthritis, and *B. afzelii* is mostly associated with dermatological manifestations (6).

1.3 Phylogeny and Morphology

B. burgdorferi belongs to the ancient eubacterial phylum of *Spirochaetes.* Members of this phylum are either free living or host associated. Several spirochetes are pathogenic to humans and other animals, including *Treponema pallidum* (syphilis), *Borrelia spp.* (Lyme disease and relapsing fever), *Brachyspira hyodysenteriae* (swine dysentery) and pathogenic *Leptospira spp.* (leptospirosis) (2).

Spirochetes represent a group of vigorously motile bacteria that are either helically shaped or have a flat sinusoidal or meandering waveform. Spirochetes have a unique morphology, since in addition to the typical inner bacterial plasma membrane, which is surrounded by the peptidoglycan composed cell wall, they also possess an outer lipid bilayer, which is designated as membrane sheath. The space within the protoplasmic cell cylinder, consisting of the cytoplasm, cell membrane and cell wall, and the outer membrane sheath, is called protoplasmic space or periplasm. The flagella are embedded within the periplasm, between the protoplasmic cell cylinder and the outer membrane sheath. The periplasmic flagella are attached to either one terminus of the protoplasmic cell cylinder and traverse through the periplasm towards the opposite terminus of the protoplasmic cell cylinder (Figure 1) (7–10). Different members of the spirochetal phylum can vary in size, their number of flagella and whether their flagella overlap at the cell cylinder (7, 9).

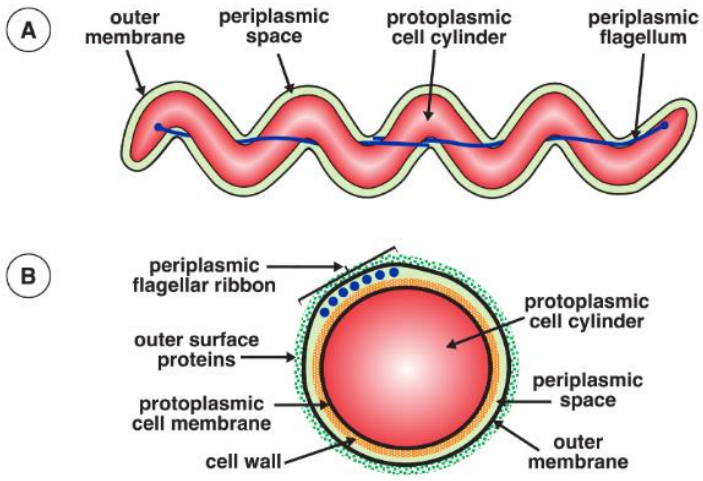

Figure 1*:* (A) Longitudinal diagram of a typical spirochete. The periplasmic flagella are shown in blue, overlapping at the cell centre. (B). Cross sectional diagram of *B. burgdorferi*. (Charon et al. 2012)

The intracellular location of the spirochetal flagellum is unique in the bacterial world as bacterial flagella are normally located externally at the surface of the bacterial cell (11). These exceptional morphological characteristics, as well as their unusual mode of motility, provides all members of the spirochetal phylum with distinct advantages (8, 9). For instance, spirochetes can traverse

through highly viscous, gel like media that would normally slow down or even stop externally flagellated bacteria (12). In contrast to other bacteria, spirochetes are not that heavily affected by their environment, such as pH, salt conditions or flagellar specific antibodies of their hosts immune system (13–15). In addition, spirochetes are able to colonize ecological niches, uninhabitable for other bacteria, disseminate within their host and penetrate specific host connective tissues (16–18).

The importance of motility as a virulence factor has already been implicated in several spirochetal species, including *B. burgdorferi* (19–21). Being 0.33 µm in diameter and 10–20µm in length, *B. burgdorferi* is a relatively large spirochete (22). They possess a flat-wave morphology with a wavelength of 2.83 µm, and a peak-to-peak amplitude of 0.78 µm (22, 23). In 2015, Sultan et al. (24) created *B. burgdorferi* mutants, missing flagellin by knocking out the gene encoding the major periplasmic filament forming protein *FlaB.* Compared to the wild type, which is motile and has a flat-wave morphology, mutant cells were non-motile and rod-shaped. Furthermore, these non-motile mutants were unable to establish an infection in mice via needle inoculation and had a decreased viability in infected ticks. Consequently, periplasmic flagella have cytoskeletal functions and contribute to cell motility, which is essential for the infectious lifecycle of *B. burgdorferi (24).*

1.4 Infectious cycle

B. burgdorferi persists in nature via an enzootic cycle between ticks of the *Ixodes spp.* complex and a wide range of mammalian reservoir hosts, including small mammals, lizards and birds (Figure 2) (25). Four different hard tick species of the *Ixodes spp.* complex are capable of transmitting *B. burgdorferi*: *I. ricinus* in Europe, *I. scalpuaris* and *I. pacificus* in Eastern and Western North America, and *I. persulcatus* in Asia (26, 27).

Ixodid ticks undergo a three-stage lifecycle: larvae, nymph and adult, with only one blood meal per stage. No transovarial transmission of *B. burgdroferi* occurs. Therefore, each generation of *Ixodid* ticks must acquire the infection anew. Consequently, larval ticks are not primary vectors of Lyme disease. Spirochetes are usually acquired by larvae during their first bloodmeal when they feed on an infected vertebrate host. The infection then persists in the ticks during their subsequent moults. Uninfected mammals acquire spirochetes when bitten by infected nymphal ticks, which feed on a similar host range as larval ticks, infecting the naive animals and continuing the enzootic cycle. Adult ticks generally feed on larger animals, including deer. Humans are accidental dead end hosts, which are not part of the previously described enzootic cycle (28).

In unfed infected ticks, the spirochetes are located in the tick midgut. During feeding of the infected nymph on a vertebrate host, the spirochetes respond to the incoming bloodmeal and the rise in temperature in several ways (29). Firstly, the spirochete population expands and undergoes phenotypic changes by altering the expression of outer surface proteins (30, 31). The spirochetes then move out of the tick midgut and enter the homocoel. From there, the spirochetes navigate towards the tick salivary glands and pass with the saliva under the skin of the mammalian host at the tick bite site (3, 32, 33). In the early stage of infection, the spirochetes multiply at the tick bite site and frequently cause a localized skin rash called erythema migraines (29, 34). After two days, the spirochetes then penetrate blood vessels, entering the bloodstream and spreading to more distinct tissues, including heart, joints, nervous system and skin. Several factors including motility and adherence to host molecules via outer surface lipoproteins are major strategies of *B. burgdoferi* to evade the hosts immune response and establish a persistent infection (3, 35).

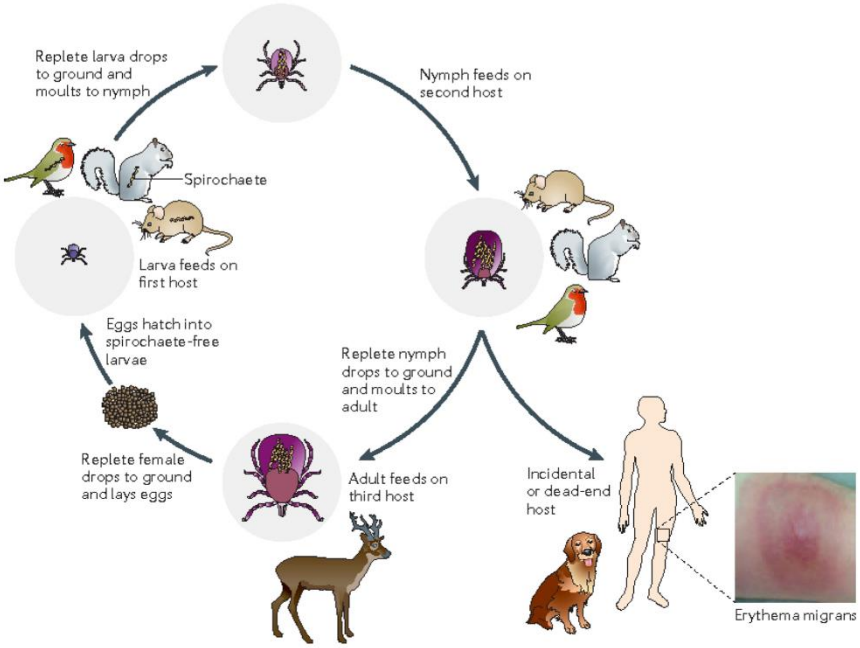

Figure 2*:* The enzootic life cycle of the Lyme disease spirochete *B. burgdorferi* (Radolf et al. 2012).

1.5 Genome

Borrelia possess one of the most complex genome structures in the bacterial world. The genome of the *B. burgdorferi* s.s. strain B31 has been already elucidated. The relatively small and highly segmented genome, consists of a small linear chromosome, with an approximate size of 910 bp, and a large complement of linear (lp) and circular (cp) plasmids, with sizes ranging from 9 bp to 62 bp (36, 37). More than 90% of the *B. burgdorferi* chromosome is comprised of coding regions and the majority of chromosomal genes are also found in other bacterial genomes (36). By contrast,

plasmids usually encode genes with no known homologues in other organisms, indicating that they are unique to the genus *Borrelia (37).*

Genes needed for the infectious cycle of *Borrelia* are located on the chromosome as well as on the plasmids (38–41). During in vitro passaging and continuous in vitro growth, some plasmids are frequently lost, while others are quite stable. The loss of certain plasmids, such as the linear plasmid lp25 and lp28-1, is accompanied with a loss of infectivity, indicating that these plasmids encode genes, which are essential for the viability and virulence of the bacterium in vivo (41). For instance, lp 25 encodes a nicotinamidase, which is involved in NAD biosynthesis, and BptA, a protein essential for colonization of the tick and for mammalian infection (42, 43). Lp 28-1 encodes the antigenic variation locus *vlse*, which includes the gene encoding the variable surface lipoprotein VlsE. It is required by spirochetes to evade the hosts immune response (44). Furthermore, loss of plasmids lp25 and lp56 correlates with an increased transformation efficiency of *B. burgdorferi* strains, as both plasmids encode putative restriction modification genes, which hinder the introduction of foreign DNA by preventing its methylation and subsequently cleaving it at its respective restriction sites. These two gene loci were denoted as *bbe02* on lp25, and *bbq67* on lp56 (45).

The plasmid profile of different genospecies within the *B. burgdorferi* sensu lato complex, including *B. afzelii*, *B. garinii*, and *B. burgderfori* s.s., is highly heterogenic (39, 40). The genetic diversity between members of the *B. burgdorferi* sensu lato complex was first recognized in 2013, after elucidating the full genome sequences of the other European *Borrelia* species, including *B. garinii, B. bavariensis and B. afzelii*, which is the Lyme disease spirochete that is responsible for most cases of human Lyme disease in Europe (46). Interestingly, a high degree of conservation exists between the linear chromosomes of *B. burgdorferi* s.s and *B. afzelii*. However, some plasmids of *B. burgdorferi* s.s. are missing in *B. afzelii* genomes, and the corresponding virulence genes encoded on those plasmids are encoded in *B. afzelii* on other more unrelated plasmids. For example, the previously mentioned PncA, a nicotinamidase, encoded on lp25 in *B. burgdorferi* s.s, is located on lp28-1 in *B. afzelii*. However, identical to the genome of *B. burdorferi* s.s and *B. garinii*, the chromosome of *B. afzelii* as well as its plasmids cp26, cp32 and lp54 seem to be the evolutionary most stable part of its genome, while the linear plasmids seem to be the evolutionary most unstable, undergoing the highest content of reorganization and therefore containing the highest number of degraded genes (47).

The relatively small genome of *B. burgdorferi* fits its lifestyle as an obligate parasite. No biosynthetic machinery is encoded by the genome of *B. burgdorferi* meaning that spirochetes

totally depend on the hosts biosynthetic machinery for the synthesis of compounds like fatty acids, nucleic acids, amino acids and enzyme cofactors. Furthermore, its genome does not encode any known toxin, permitting that *B. burgdorferi* is a non-toxic, pervasive and persistent pathogen that causes infection via triggering its host's immune response (36).

1.6 Genetic manipulation of *B. burgdorferi*

Many efforts have been made to identify genes important in biology and pathogenesis of Lyme disease spirochetes, however, these efforts have been greatly hindered due to the lack of proper genetic tools applicable in *B. burgdorferi*. In recent years, strong advances have been made in the development of appropriate methods allowing genetic manipulations of this unique bacterium (45).

The first successful transformation and genetic exchange in *B. burgdorferi* was achieved in 1994, by introducing a point mutation in the endogenous *gyrB* gene of *B. burgdorferi*, rendering mutant cells resistant against coumermycin A_1 (48). Since 2004, four additional antibiotic selective markers have been developed: kanamycin, erythromycin, gentamycin and streptomycin (49–52). The development of these antibiotic selectable markers has been pivotal in the development of suicide and shuttle vectors, introducing techniques such as transformation, targeted gene activation and the restoration of those genes via complementation (52–54). However, the identification of potential virulence genes candidates has been greatly hampered. Firstly, due to the low transformation efficiency of *B. burgdorferi*, especially in low passage strains that still contain both linear plasmids lp25 and lp56 and secondly, the loss of plasmids that frequently occurs during long time cultivation and due to genetic manipulations, such as transformation. Till 2012, only 50 genes have been disrupted by site directed mutagenesis in infectious *B. burgdorferi* strains (55). Another approach, allowing the creation of large deletions within the *B. burgdorferi* genome, thereby allowing to study the contribution of neighbouring genes on overall spirochetal infectivity, was utilized in 2010, by Bestor et al. and is a slightly modified form of the Cre-lox recombination system (56).

Another approach, described in more detail below, is transposon mutagenesis. Transposon mutagenesis allows the random mutation of the bacterial genome. Furthermore, a small library of transposon mutants can be created via one transformation experiment, allowing the investigation of multiple genes at once (55).

1.6.1 Transposon mutagenesis

Transposons are mobile DNA elements that have the capability to randomly transposase or insert into foreign DNA via a cut and paste mechanism. Transposons thus provide a powerful tool for the random mutagenesis of bacterial genomes to study genes of known and unknown function. A wide range of naturally occurring mobile DNA elements exist, however, most transposable elements are host specific, restricting their applications to a certain host range, and limiting their implementation as a universal genetic tool (57). Transposons of the *mariner* family are not restricted to a certain host range, making them an ideal tool for mutagenesis in a wide range of organisms including eukaryotes, archaea and prokaryotes. They are widespread in nature and are found in range of eukaryotes. Besides, from their indefinite host range, these transposons provide further advantages as they do not relay on host cofactors for transposition. All the major transposition steps are carried out by the transposon itself (58). Moreover, *mariner* transposons randomly insert into foreign DNA at any TA dinucleotide sequence. No other sequence restrictions for this family of transposable elements exists (59).

Two widely known and frequently used *mariner* transposons are *Mos1*, which was originally isolated from the blowfly, *Drosophila mauritiana*, and *Himar1*, which was isolated from the horn fly *Haematobia irritans* (59). While *Mos1* is frequently used for transposon mutagenesis of eukaryotes, *Himar1* is extensively used in prokaryotes. In 1999, Lampe et al., (60) created hyperactive forms of *Himar1* via substitution of two amino acids. The higher transposition frequency of those hyperactive forms of *Himar1* compared to the wild type, greatly improved the applicability of transposon mutagenesis in prokaryotes, allowing its application in the Lyme disease spirochete *B. burgdorferi* (60, 61).

In 2004, Stewart et al. (62) reported the development of a *Himar1* based transposon suicide vector, referred to as pMarGent (Figure 3). The transposable element of pMarGent is flanked by two *Himar1* inverted tandem repeats, and is composed of a *$flgB_p$:aacC1* cassette, which provides gentamicin resistance, and ColE1, the *E. coli* origin of replication. The incorporation of ColE1 into the transposon permits plasmid replication in *E. coli* and rescue of the transposon insertion site. The *Himar1* transposase is transiently expressed from a strong borrelial *$flgB_p$*, promoter, ensuring high transposition frequency in *B. burgdorferi*. Once the transposition has occurred, the transposable element is randomly integrated into the bacterial genome, but not the transposase gene. The transposase gene is left on a foreign fragment of DNA, which will be recognized and degraded by nucleases, enhancing the likelihood of only one transposition event per cell. Stewart

et al. showed that pMarGent randomly inserts into the *B. burgdorferi* genome at near saturating levels (62).

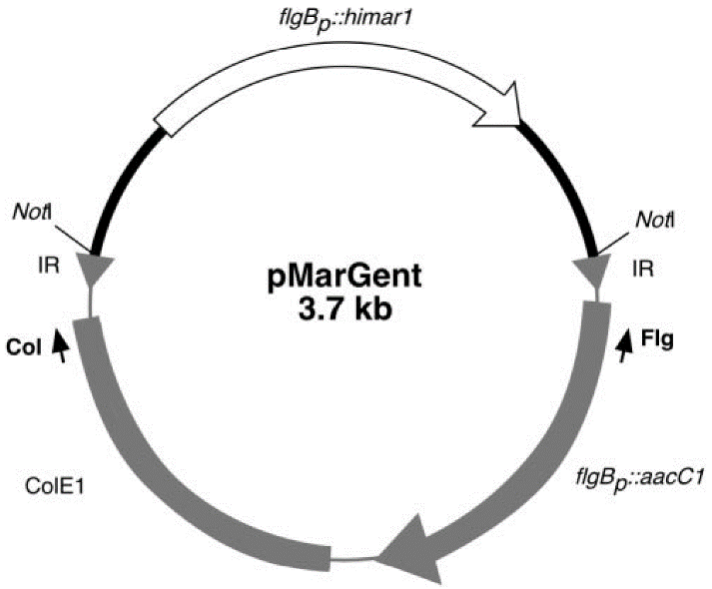

Figure 3*:* Plasmid map of pMarGent. The transposable element consists of a flgBp: aacC1 cassette, providing gentamycin resistance, ColE1, *E. coli* origin of replication, and short oligonucleotides for sequencing after rescue in *E. coli*. The transposable element is flanked by inverted repeats. The *Himar1* is expressed from a borrelial $flgB_p$ promoter.

Transposons mutants were obtained in the low-passage-number but non-infectious strain A3-89 as well as the non-infectious high-passage-number strain B31-AchbC72. Both strains lack linear plasmids lp25 and lp56, which encode the putative restriction modification genes *bbe02* and *bbq67*. The creation of transposon mutants in A3-89 and B31-AchbC72 allowed to investigate the function of many previously uncharacterized open reading frames. Several genes involved in morphology, physiology and cell division of *B. burgdorferi* were identified and further characterized (Figure 4). However, transformation attempts in low-passage, infectious *B. burgdorferi* strains A3 and N40, which both contain lp25, with pMarGent, were unsuccessful (62).

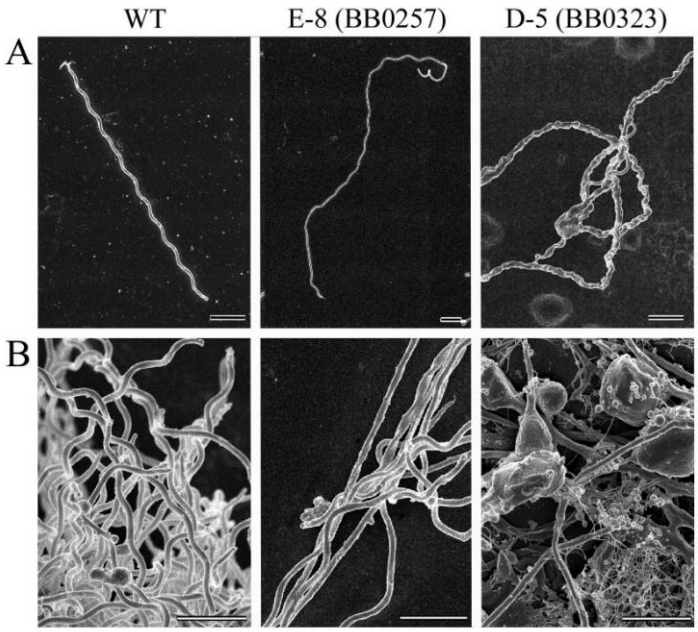

Figure 4*:* Scanning electron micrographs of *B. burgdorferi* cells. Images of wild-type (WT) *B. burgdorferi*, cell division mutant E-8 (BB0257), and hypothetical ORF mutant D-5 (BB0323). E-8 (BB0257) mutant forms long chains of incompletely separated spirochetes, suggesting a defect in cell division, supporting the role of BB0257 in cell division. The function of the D-5 (BB0323) ORF remains unknown, but the transposon insertion has a sgnificant impact on the ability of *B.burgdorferi* cells to grow individually and form an intact outer membrane

In the very same year, Kawabata generated the two infectious, transformable *B. burgdorferi* strains, 54ANP1 and 5A18NP1, by inactivating the putative restriction modification gene *bbe02*, on lp25. Strain 5A18NP1 also lacks lp56, which encodes the *bbq67* gene locus. In 2006, Botkin and colleagues used pMarGent to transform 5A18NP1, leading to the first successful transposon mutagenesis in an infectious *B. burgdorferi* strain. 33 gentamycin resistant clones were obtained. Some of these transposon mutants were fully infective in mice, while some showed reduced or no infectivity at all, firstly establishing a direct correlation between the function of some genes and spirochetal infectivity. However, no complementation studies were carried out. Nevertheless, this study showed the feasibility of transposon mutagenesis for large scale studies in *B. burgdorferi* (63).

In 2007, Lin et al. started the accumulation of a comprehensive siganture tagged mutagenesis (STM) mutant libary using a modified version of pMarGent, referred as pGKT. The pGKT vector was devloped later and contains a second selectable marker, a kanamycin resistance cassette, in the non-transposable backbone in addition to the gentamycin resistance cassette, which greatly increases its stability and facilitates additional alterations. They further introduced 12 different 7 bp "signature tags" into the transposable element of pGKT. The introduction of this unique DNA elements into the transposable backbone of pGKT, permitted the coinfection of animal models with

multiple transposon mutants, each equipped with a different STM 'tag'. Till 2014, a sequence-defined library of nearly 4500 mutants was created. Through analysis of these mutants several genes playing a role in morphology, physiology and virulence of *B. burgdorferi* were identified. For example, mutations in genes involved in motility, chemotaxis, the phosphoenolpyruvate phosphotransferase system, and other transporters, as well as putative plasmid maintenance genes resulted in severe loss of infectivity, indicating the importance of these gene classes in infectivity of Lyme disease spirochetes (55, 64).

1.6.2 Visualization *B. burgdorferi*

In addition to the classical genetic tools such as site directed mutagenesis, transposon mutagenesis and complementation, other genetic tools, utilizing fluorescent proteins, have been developed in recent years and have been used for different purposes in *B. burgdorferi* (32, 65).

For example fluorescent proteins have been applied to study gene expression of *B .burgdoferi* in response to changes in pH and temperature, which spirochetes also face during their infectious lifecycle in vivo, when alternating between their arthropod vector and vertebrate host, therefore allowing the identification of genes being essential during this process (65). In addition, fluorescent proteins enabled the investigation of *B. burgdorferi* during dissemination within the tick midgut and salivary glands via live imaging using a fluorescence microscope. For the first time, the routes and mechanisms by which *B. burgdorferi* disseminates within the tick has been elucidated, suggesting that this process is biphasic. In this experiment, no shuttle vector was used, instead the GFP gene was incorporated into the genome, allowing strong constitutive expression of GFP, without the risk of losing it during long time cultivation, in vitro passaging or during genetic manipulations. Consequently, fluorescent proteins facilitate investigations of *Borrelia* morphology, physiology, interaction with its tick vector or vertebrate host and changes in gene expression during its infectious life cycle (32).

Most of the genetic tools, including site directed mutagenesis, transposon mutagenesis and fluorescence techniques are developed for *B. burgdorferi* s.s. For the European Lyme disease species, most of these genetic tools need to be modified to allow the investigation of genes participating in spirochete physiology, morphology and virulence.

1.7 The bacterial cytoskeleton-Bactofilins

The prokaryotic cytoskeleton is composed of several cytoskeletal elements that ensure the proper temporal and spatial organization of the prokaryotic cell and its cellular machinery. Several components of the prokaryotic cytoskeleton have cytoskeletal protein homologues in eukaryotes including the tubulin homolog FtsZ, the actin homologue MreB, and the intermediate filament (IF) like proteins (66–68). These elements of the prokaryotic cytoskeleton play a role in several cellular processes including morphogenesis, polarity determination and cell division (69–72). Several other cytoskeletal polymer forming proteins exist which have no protein homologues in eukaryotes, meaning that they are unique to bacteria (70).

Several years ago, an additional group of cytoskeletal polymer forming proteins in bacteria, had been identified (73) and denoted as bactofilins. Bactofilins are a class of proteins that are widespread in gram-positive as well as gram-negative bacteria, with many species containing paralogous copies. Bactofilins are small proteins, having a unique beta helical structure and polymerizing into protein sheets and bundles without the help of any nucleotide cofactors (73–79). Bactofilins are characterized by a conserved protein domain of unknown function also denoted as DUF-domain flanked by variable terminal regions. They are predicted to serve as cytoskeletal scaffold in various cellular pathways. For example, *C. crescentus* possess two bactofilin paralogous that assemble into a polar scaffold that recruits a peptidoglycan synthase participating in pole morphogenesis (73). In *Heliobacter pylor*i a single bactofilin is responsible for maintaining cell shape, whereas in *Bacillus subtilis* two proteins of this family ensure the proper flagellar assembly (80, 81).

In 2018 Jackson et al. characterized the role of bactofilins in the morphology and physiology of the spirochetal bacterium *Leptospira biflexia*. Based on phylogenetic analyses, they identified five different bactofilin homologues in *L. biflexia*. One protein of this family, denoted as *LbbD*, was further investigated by knocking out this gene. Mutants lacking *LbbD*, possess a unique compressed helical morphology, a reduced motility and their cell wall integrity was weakened which made them more sensitive to osmotic stress and cell wall targeting antibiotics compared to the wild type. The change in helical spacing together with the defects in motility and cell wall integrity indicates the relationship and coevolution of motility and cell shape in those spirochetes. The role of bactofilins in other spirochetes, including *B. burgdorferi*, remain still unknown (82).

2. Aims

2.1 Transposon mutagenesis of *B. afzelii*

- Obtaining the first transposon mutant in the infectious strain CB43 (*B. afzelii).*

2.2 Analysis of three S4 (B31-A3) transposon mutants

- Getting information about the 3 disrupted gene loci and the proteins they encode on NCBI, BioCyc, Database Collection and STRING: functional protein association
- Plasmid profiling of transposon mutants
- Analysis of the protein expression of transposon mutants
- Studying growth rate of transposon mutants in liquid BSK-H media at 34°C
- Comparing growth of transposon mutants on solid media
- Investigating physiology and morphology of transposon mutants using dark-field microscopy
- Using a tick-mouse model for all transposon mutants to investigate if the disrupted gene loci plays a role in infectivity of *B. burdorferi* s.s.

2.3 Passaging of green fluorescent strain 297 and red fluorescent strain CB43-dsRed

- Plasmid loss of both fluorescent strains during continuous in vitro passaging
- Plating the final passages and picking several colonies for inoculation in liquid media
- Investigating physiology and morphology of clones using dark-field microscopy
- Analysing the plasmid profile of those clones revealing altered morphology and motility

2.4 GFP project

- Obtaining green fluorescent spirochetes in the *B. burgdorferi* s.s strain B31-A3 by transformation using an already designed GFP containing shuttle vector provided by Dr. Melissa J. Caimano (Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut, USA).

3. Materials and Methods

3.1 General procedures

3.1.1 Protein Lysate preparation

The bacterial culture was centrifuged for 10 minutes at 8000 rpm, 20°C. The supernatant was discarded, and the bacterial pellet was resuspended in 1 ml of cold HN-Buffer. The mixture was transferred to a 1.5 ml eppendorf tube followed by centrifugation for 10 minutes at 13000 rpm, 10°C. The supernatant was removed by pipetting and the washing step was repeated. The bacterial pellet was then lysed in 200 µl B-PER™ (Bacterial Protein Extraction Reagent, ThermoFisher Scientific™). The suspension was left standing at room temperature (RT) for 10 minutes and the protein concentration was determined by using Nanodrop. Prior to use, the protein lysate was diluted 1:1 by adding a mixture composed of 190 µl 2x Laemmli Sample Buffer (Bio-Rad) and 10 µl β-mercaptoethanol.

3.2.1 SDS-page gel electrophoresis and Coomassie Brilliant Blue staining

SDS-page gels were prepared by using the TGX™ and TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad). The gels were made using 0.75 mm glass plates according to the manufacturer's instructions as depicted in Table 1.

Table 1: Reagents and the respective amounts of each reagent needed for SDS-Page gel preparation

Reagents	Separation Gel	Stacking Gel
Resolver A	2 ml	-
Resolver B	2 ml	-
Stacker A	-	1 ml
Stacker B	-	1 ml
TEMED (N,N, N',N'-tetramethylethylenediamine)	2 µl	2 µl
10% APS (Ammonium persulfate)	20 µl	10 µl

After polymerization, the SDS-Page gel was put into running buffer. The protein lysates were incubated for 10 minutes at 100°C and 20 µl of hot protein lysate were loaded into the respective wells alongside with 8 µl of Protein Marker VI (AppliChem). SDS gel electrophoresis was conducted at 120 V for approximately 80 minutes till the ladder reached the bottom of the gel. Afterwards, the gel was shaken for 30 minutes in a protein fixation solution (70% methanol, 10% acetic acid and 20% distilled water). After protein fixation, the gel was washed three times

for 15 minutes in distilled water and stained for 2 hours in 50 ml of Coomassie Brilliant Blue (BioRad). Subsequently, the gel was shaken for 1 hour in distilled water. For removal of excess dye, the distilled water was exchanged once more, and the gel was shaken overnight.

3.1.3 Western Blotting

SDS–Page gel was prepared according to Table 1, and gel electrophoresis was conducted as described in subsection 3.2.1. After gel electrophoresis was completed, the proteins were transferred from the gel on a nitrocellulose paper for 30 minutes at 25 V, using a semidry blotting machine (BioRad). After protein transfer was completed, the nitrocellulose membrane was shaken for 2 hours in 5% blocking solution, which was prepared by dissolving 5 g of dehydrated milk in 100 ml of 1x TBS-Tween 20. The primary antibody solution was prepared by mixing 990 µl of blocking solution with 10 µl of the respective mouse sera (dilution 1:100). After the blocking step was finished, the membrane was cut into strips, each one corresponds to a certain well on the SDS gel and thus a certain protein lysate. The strips were sealed in plastic bags together with 1 ml of the respective primary antibody solution and stored in the fridge overnight. Next, plastic bags were shaken for 1 hour, opened, and the membrane pieces were washed three times for 15 minutes with 1x TBS-Tween 20. The secondary antibody solution was prepared by mixing 50 ml blocking solution with 5 µl of anti-mouse IgG (dilution 1:10000). After washing was finished, the membrane strips were shaken for 1 hour in the secondary antibody solution, and again washed three times for 15 minutes with 1x TBS-Tween 20. During the last wash, the Pierce™ ECL Western Blotting Substrate (ThermoFisher Scientific™) was prepared by mixing 1 ml of each detection reagent (1 and 2). The membrane strips were incubated for 5 minutes in the substrate solution, arranged in the appropriate order on a transparent foil, residual liquid was removed, and the membrane was visualized using Chemi-Doc system (Bio-Rad).

In case of a high number of samples, the Mini-PROTEAN® II Multiscreen Apparatus (Bio-Rad) was used for incubation of the primary antibody solution. In that case, the SDS gel was prepared as described above, but with a 1 well comb, in which 150 µl of the respective protein lysate was loaded. SDS gel electrophoresis, electroblotting and blocking were performed as described above. The uncut membrane was clamped into the Mini-PROTEAN® II Multiscreen Apparatus (Bio-Rad), the apparatus was slightly tilted and 600 µl of primary antibody solution was loaded into the respective capillary from the bottom to the top, avoiding air bubbles. The set-up apparatus was put in the fridge overnight. The apparatus was shaken for 1 hour and the capillaries were washed with

distilled water and the membrane was removed from the apparatus. The next steps were performed in the same manner as mentioned above.

3.1.4 Genomic DNA isolation

Genomic DNA (gDNA) from spirochetal cultures was isolated with the Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's protocol for Gram-Positive and Gram-Negative Bacteria. Briefly, the bacterial culture was centrifuged for 10 minutes at 8000 rpm, at 20°C and the supernatant was discarded. The bacterial pellet was resuspended in 600 µl of Nucleic Lysis Solution, transferred to a 1.5 ml eppendorf tube and incubated for 5 minutes at 80°C. Afterwards, the mixture was put on ice for 3 minutes to cool to RT. 3 µl RNase was added, the tube was inverted 2-3 times and then incubated at 37°C for 15 minutes. The mixture was left at RT for 5 minutes, 200 µl of Protein Precipitation Solution was added and the tube was inverted a few times. The mixture was centrifuged for 10 minutes at 10°C and at 13000 rpm, the supernatant was transferred to a 1.5 ml eppendorf tube containing 210 µl of isopropanol and inverted a few times followed again by centrifugation for 10 minutes at 13000 rpm and at 10°C. The supernatant was discarded, the tube was inverted on a paper towel and 600 µl of 70% ethanol were added. The centrifugation step was repeated a third time and the supernatant was removed using a pipette. The DNA pellet was air dried for 5-7 minutes to get rid of the residual ethanol and afterwards, 100 µl of DNA Rehydration solution were added without mixing. The DNA sample was stored at 4°C overnight and pipetted up and down before using it.

3.2 Transposon mutagenesis

All experiments for transposon mutagenesis are listed at the end of this section in Table 3.

3.2.1 Bacterial strains and growth conditions

B. afzelii (CB43, CB43 Clone 24 missing plasmids), *B. bavariensis* (PBi), *B. burgdorferi* s.s. (B31-A3, B31 A3-68-1, green fluorescent 297) and *B. finlandensis* (SV1), were inoculated from frozen glycerol stocks and cultivated either in liquid Barbour-Stoenner-Kelly (BSK) II media (Barbour, 1984), or liquid BSK-H Medium (complete, sterile-filtered, with 6% rabbit serum, Sigma Aldrich) at 34°C.

3.2.2 Methylation of pGKT

CpG Methyltransferase (M.SssI) from New England Biolabs was used for methylation of pGKT prior to ethanol precipitation. DNA concentration of pGKT was determined by measuring absorbance at 240 nm, and the reaction set up-was prepared according to Table 2. The required quantity of each reagent was adapted to the number of micrograms of pGKT needed for the respective transformation of *Borrelia.*

Table 2: Optimum reaction mixture for methylation of the pGKT vector according to New England Biolabs

Reagent	Amount
Nuclease-free Water	up to 50 µl
Methyltransferase Reaction Buffer (10×)	5 µl
Diluted SAM	5 µl
DNA	1 µg
Methyltransferase	4 – 25 units (1 µl)

The reaction mixture was mixed by pipetting up and down, followed by incubation for 1 hour at 37°C. The CpG Methyltransferase was heat inactivated by incubation at 65°C for 20 minutes. Afterwards, ethanol precipitation of the methylated pGKT vector was performed.

3.2.3. Ethanol precipitation

First, DNA concentration of pGKT vector was measured using absorbance at 240 nm. According to the microgram of pGKT needed for the respective transformation, the appropriate volume of DNA was transferred to a new 1.5 ml eppendorf tube. Afterwards, 1/10 of the volume (of pGKT

in µl) of 3M Sodium acetate as well as 2 times the volume of 100% ethanol was added. The tube was inverted 6-8 times and centrifuged for 10 minutes at 13000 rpm, at 4°C. The supernatant was removed by pipetting and 1 time the volume of 70% ethanol was added. The tube was inverted 6-8 times and centrifuged for 10 minutes at 10000 rpm and at 4°C. The supernatant was removed by pipetting and the DNA pellet was air-dried for 5-7 minutes. The DNA pellet was resuspended in 15 µl Milli-Q water to reach the desired concentration of pGKT in µg/µl needed for the respective transformation.

3.2.4. Transformation of *Borrelia*

The *Borrelia* strains were inoculated from frozen glycerol stocks in 7 ml fresh BSK-H medium. The spirochetes were grown to exponential phase (5×10^7 to 1×10^8 cells/ml) and the exact cell density was determined using a Petroff-Hauser counting chamber. Afterwards, the original culture was diluted in 50 ml of fresh BSK-H medium to reach a cell density of 5×10^7cells/ml after one week. The fresh 50 ml cultures were incubated at 34°C and after 7 days, centrifuged at 4000 g for 20 minutes at 10°C. The supernatant was discarded and 25 ml of cold electroporation solution (EPS) was added to the bacterial pellet. The bacterial pellet was resuspended, and the tube was again centrifuged at 4000 g for 10 minutes at 10°C. The supernatant was again discarded, and the bacterial pellet was dissolved in 100 µl of cold EPS. The bacterial cell density in the suspension was investigated under the dark-field microscope. In the case of too crowded or clumped cells, additional EPS was added, and the cell density checked again under the dark-field microscope. Subsequently, 15 µl of previously precipitated pGKT vector was added and the whole reaction mixture was transferred into 0.2 mm electroporation cuvette (Bio-Rad) using a 200 µl pipette and electroporated (2,5 kV; 25 µF, 200 Ω, 2 mm cuvette diameter). After electroporation, a small volume of warm BSK-H medium was added into the electroporation cuvette (Bio-Rad) and the whole mixture was then transferred to a tube containing 7 ml of fresh BSK-H medium. The cells were kept in the incubator for 1 or 2 days, depending on the type of strain used. In the case of B31-A3, B31 A3-68-1, 297, only one-day incubation was enough, whereas in the case of SV1, PBi and CB43, 2 days of incubation were necessary. After the appropriate incubation period, the transformants were either selected by plating or by limiting dilution.

3.2.5. Plating of transformants

The cell density of transformants was determined by counting using a Petroff-Hauser counting chamber. After calculating the exact cell density, the cultures were diluted to 1×10^4 cells/ml by

serial dilution. Subsequently, 18 ml of Rabbit Serum was added to 300 ml of 1.5×BSK medium and heated to 53°C. 200 ml of autoclaved liquid 1.7% plating agarose (SeaKem®) was added to the mixture and kept at 53°C to avoid solidification. Afterwards, 10 ml of the agarose media mixture was poured onto each plate, establishing the bottom layer. The plates were allowed to solidify in a flow box. Then, two control plates without any antibiotics were prepared by transferring 200 µl of the final dilution (10^4cells/ml) into a 50 ml falcon tube, adding 40 ml of the agarose media mixture and pouring 20 ml onto each plate, leading to an expected concentration of 100 *Borrelia* per plate. Afterwards, kanamycin and gentamycin were added to the remaining agarose-media mixture to reach a concentration of 40 µg/ml of gentamycin and 200 µg/ml of kanamycin. Six plates with 2×500 µl, 2×1 ml, and 2×1.5 ml of the original culture were prepared by transferring 1 ml, 2 ml, 3 ml of the start culture to a 50 ml falcon tube. 40 ml of agarose media mixture were added to each tube and 20 ml were poured onto each plate. The plates were put into an air tight box with CO_2 satchets (GasPak™) and incubated at 34°C for 3 weeks.

The remaining liquid cultures were filled up with fresh BSK-H and incubated for 2 days at 34°C. After two days, gentamycin and kanamycin were added at the appropriate concentration and liquid cultures were again incubated at 34°C for 1 week. After 1 week, the presence of viable spirochetes was checked using a dark-field microscope.

3.2.5.1 Picking *Borrelia* colonies

Borrelia colonies on antibiotic plates were picked using a 1 ml micropipette and transferred to 7 ml of fresh BSK-H medium. The bacterial cultures were then incubated at 34°C for 3 days, after which gentamycin and kanamycin was added to the liquid cultures at an appropriate concentration (40 µg/ml gentamycin and 200 µg/ml of kanamycin). Afterwards, the liquid cultures were incubated again for one more week, and the viability of spirochetes was examined using a dark-field microscope. Depending on the viability of cells and the cell density, the liquid cultures were either thrown away, further incubated, or genomic DNA isolation was performed using Wizard® Genomic DNA Purification Kit (Promega) by following the manufacturer's protocol for Gram-Positive and Gram-Negative Bacteria as described in subsection 3.1.4. Successful transformation was further assessed by checking for the presence of kanamycin resistance cassettes via PCR using the appropriate primer pairs.

3.2.6 Limiting dilution of transformants

The recovered transformants were diluted in 90 ml of BSK-H and antibiotics were added at a concentration of 40 µg/ml in the case of gentamycin, and 200 µg/ml of kanamycin. 250 µl of the

culture medium were distributed into each well of a 96 well plate. The plates were then incubated at 34 °C in humidified atmosphere containing 1.5% to 5% CO_2.

Table 3: List of all transposon experiments, -✓ methylated pGKT vector, × non-methylated pGKT, - technique was not carried out, + technique was performed

***Borrelia* Strain**	**Migrogramm of pGKT [µg]**	**Methylation of pGKT**	**Plating**	**Limiting dilution**
CB43	20	×	+	-
	40	×	+	-
	20	✓	+	-
	20	✓	-	+
297	20	✓	+	-
	20	✓	-	+
PBi	20	✓	-	+
SV1	20	✓	-	+
B31 A3-68-1	80	×	+	-
CB43, Clone 24	50	×	+	-

3.3 Identification of S4 (B31-A3) transposon mutants

3.3.1 Bacterial strains and growth conditions

The *bbe02* mutants (Rego et al., 2011), obtained in the B31-A3 background obtained were denoted as S4 and were further transformed with the transposon suicide vector pGKT (unpublished), which was methylated, prior to transformation, using the CpG methyltransferase M.SssI (New England BioLabs or Zymo Research, Irvine, CA). Characterization of transposon mutants was carried out as per the protocol published by Stewart and colleagues (62).

Three of those S4 (B31-A3) transposon mutants created by Dr. Rego were then selected for further analysis. The function of the genes with a transposon insertion in morphology, physiology and virulence of *B. burgdorferi*, have not been investigated up to now. Information about the disrupted gene loci as well as on the proteins they encode was received from NCBI. Furthermore, more detailed information of protein function was assessed using the BioCyc Database Collection and STRING: functional protein association network. The three S4 transposon mutants and the disrupted gene loci are listed in Table 4. The protein association network of two gene loci are shown in Figure 5.

Table 4: List of S4 transposon mutants and their disrupted gene loci

Transposon mutant	Gene locus (NCBI)
S4-7	BB_0267
S4-8	BB_F02
S4-19	BB_0602

Glycerol stocks of the respective S4 transposon mutants were provided by Dr. Rego. S4 transposon mutants as well as B31-A3, which served as positive control in all experiments were inoculated from frozen glycerol stocks and cultivated either in liquid BSK-II media, or liquid BSK-H medium at 34°C.

3.3.2 Plasmid analysis

The previously described transposon mutants were inoculated from frozen glycerol stocks in 6 ml of fresh BSK-H. The spirochetes were grown to exponential phase (5×10^7 to 1×10^8 cells/ml) and genomic DNA isolation was performed using the Wizard® Genomic DNA Purification Kit (Promega) by following the manufacturer's protocol for Gram-Positive and Gram-Negative Bacteria according to subsection 3.1.4. Since plasmid loss frequently occurs during transformation the full plasmid profile of transposon mutants S4-7, S4-8 and S4-19 was checked by PCR, using primers designed for the 12 linear and 11 circular plasmids of the *B. burdoferi* s.s. strain B31.

For all PCRs the OneTaq® Hot Start Quick-Load® 2x Master Mix with Standard Buffer (New England Biolabs®) was used for preparation of a master mix. Its composition as well as the temperature program can be found in Table 5. The denaturation, annealing and elongation step was repeated 30 times in a cycle for all programs.

Table 5: PCR settings used for screening plasmid content of S4 transposon mutants

Master Mix	**Amount [µl]**		**PCR Settings 2 – OneTaq MM**			
OneTaq HS Quick-Load 2x	10		**#**	**PCR Step**	**T [°C]**	**t [sec]**
Millli-Q H2O	6		**1**	Initial Denaturation	94	30
Forward Primer	1		**2c**	Denaturation in cycle	94	30
Reverse primer	1		**3c**	Annealing	59	30
DNA	2		**4c**	Elongation in cycle	68	60
Total	20		**5**	Final Elongation	68	600
			6	Hold	16	∞

3.3.3 Protein profile comparison

All three transposon mutants as well as B31-A3 were cultured from frozen glycerol stocks, inoculated into 6 ml of fresh BSK-H medium and grown to mid log-phase (5×10^7 to 1×10^8 cells/ml). Protein lysates were prepared as mentioned in subsection 3.1.1, and protein concentration was determined. To compare the protein profiles of S4-7, S4-8, and S4-19, SDS gel electrophoresis was performed and the gel was subsequently stained in Coomassie Brilliant Blue as described in subsection 3.1.1.

3.3.4 Growth curve

All three transposon mutants as well as B31-A3 were started from frozen glycerol stocks and inoculated in 6 ml of fresh BSK-H medium. The bacterial cultures were grown to exponential phase (5×10^7 to 1×10^8 cells/ml) and the exact cell density of each culture was determined by using a Petroff-Hauser counting chamber. Afterwards, the appropriate volume of each starting culture was inoculated into 6 ml of fresh BSK-H to obtain a cell density of 1×10^5 cells/ml. The cultures were incubated at 34°C and counted every day with a Petroff-Hauser counting chamber until a cell density of 5×10^7 cells/ml was reached. Each experiment was performed in triplicate.

3.3.5 Plating transposon mutants

The bacterial cultures of B31-A3, S4-7, S4-8 and S4-19 were grown to exponential phase (5×10^7 to 1×10^8 cells/ml), the exact cell density of each culture was determined by using a Petroff-Hauser counting chamber and each culture was diluted to 10^4 cells/ml. Preparation of the medium-agarose mixture was performed as in subsection 3.2.5. 10 ml of the medium-agarose mixture served as bottom layer of each plate. 200 µl of the final dilution (10^4 cells/ml) into a 50 ml falcon tube. Then 40 ml of the agarose media mixture was added, and 20 ml was poured onto each plate, leading to a concentration of 100 *Borrelia* per plate. The plates were put into an air tight box with CO_2 satchets (GasPak™) and incubated at 34°C for 3 weeks.

3.3.6. Experiment mouse model

All three S4 transposon mutants together with B31-A3 were inoculated from frozen glycerol stocks in 6 ml fresh BSK-H medium. The spirochetes were cultured to mid log-phase, counted using a Petroff-Hauser counting chamber and then diluted to 4×10^3 cells/ml, 4×10^4 cells/ml, 4×10^7 cells/ml and 4×10^8 cells/ml. 250 µl were injected intraperitonedly (150 µl) and subcutaneously (100 µl) into each mouse. A group of 3 three mice was used for each transposon mutant and B31-A3. After 10 days a small biopsy of the ear of each mouse was taken, genomic DNA was isolated using NucleoSpin® Tissue from Machery-Nagel following the manufacturer's instructions, and infection was verified by PCR, using a primer pair specific for strain B31. Successful infection has been further verified by performing a serological test in form of a Western Blot after 3 weeks post infection. 4 weeks after inoculation the mice were euthanized. Re-isolation of spirochetes from the mouse ear, bladder, heart and joint were performed. Mouse tissues were incubated for up to 21 days in BSK-H growth medium and the presence of spirochetes was tested by dark-field microscopy.

3.3.7. Tick infection studies

To test whether those S4 transposon mutants can be acquired by larval ticks during their first bloodmeal, naive *I. ricinus* or *I. scalpularis* larvae where fed on previously mentioned needle inoculated mice. 100 larvae were put on each mouse. The larvae were fed to repletion for 3 days and collected once they dropped off the mice. Ten days after feeding to repletion the successful acquisition of transposon mutants was tested by re-isolation of *Borrelia* either by inoculation of crushed ticks in liquid BSK-H or by plating them on solid media.

Approximately two months after moulting, 15 nymphs infected with either one of the transposon mutants or B31-A3 were allowed to feed to repletion on naive mice. 3 naive mice per transposon mutant and B31-A3 were used. The nymphs fed to repletion for 4 days and were collected once they dropped of the mice. Infectivity of the respective nymphs was tested by re-isolation of *Borrelia* either by inoculation of crushed ticks in liquid BSK-H or by plating 10 days after feeding. Furthermore, the successful transmission of spirochetes from infected nymphs to naive mice was tested as well. Successful transmission of spirochetes from nymphs to mice was then performed as mentioned in subsection 3.3.6.

3.3.7.1 Re-isolation of *Borrelia* from ticks

Re-isolation of *Borrelia* from ticks was performed by separating the respective ticks and transferring them in 1.5 ml eppendorf tubes. The ticks were washed by adding 200 µl of 3% H_2O_2 for 15 minutes, making sure that the ticks were completely immersed. The liquid was removed, and the ticks were washed again by immersing them in 200 µl of 70% ethanol. The liquid was discarded using a pipette and the tubes were kept open for 10 minutes in a flow-box to evaporate the residual ethanol. Afterwards, 500 µl of warm BSK-H medium was added and the ticks were crushed using a pestle. The pestle was washed with 500 µl of warm BSK-H, adding up to a total volume of 1 ml, and 10 µl of *Borrelia* antibiotics were added, reaching a dilution of 1:100.

Subsequently, two plates per tick were prepared for plating. The agarose-medium mixture was well as the bottom layer of the plates was prepared as in subsection 3.2.4. Afterwards, 250 µl and 500 µl of the previously prepared liquid tick cultures were transferred to separate 15 ml falcon tubes. The falcon tubes were filled up with 10 ml of the agarose media mixture and poured onto the bottom layer of the plate. The plates were stored in air tight boxes with anaerobic satchets (GasPak™) at 34°C for 3 weeks.

The remaining liquid tick cultures were filled up again to 1 ml by adding 750 µl of BSK-H. 75 µl of *Borrelia* antibiotics was added. The tick cultures were incubated for 2 weeks at 34°C and the presence of spirochetes was examined using a dark-field microscope.

3.3.8 Further investigation and complementation of transposon mutant S4-7

3.3.8.1 Phylogenetic analysis

The nucleotide and amino acid sequence of BB_0267 was obtained from NCBI, in addition to the BB_0267 amino acid sequence of other *Borrelia* species, including some causing relapsing fever. The sequences were aligned via Geneious and a phylogenetic tree was constructed, showing the

relationship between different species. The accession numbers and protein sequences of each species are summarized in Table 6.

Table 6:List of *Borrelia* species and their amino acid accession numbers

Species	Amino acid accession number
Borreliella burgdorferi [B31]	WP_002657709.1
Borrelia mayonii [MN14-1539]	WP_075551972.1
Borreliella bissettii [DN127]	WP_014023515.1
Borreliella garinii [PBr]	WP_031539180.1
Borreliella spielmanii [A14S]	WP_006433408.1
Borreliella bavariensis [PBi]	WP_011193606.1
Borreliella afzelii [PKo]	WP_011600927.1
Borreliella japonica [ATCC:51557]	WP_091972651.1
Borreliella valaisiana [Tom4006]	WP_044006444.1
Borreliella chilensis [VA1]	AJA90096.1
Candidatus Borrelia tachyglossi	WP_108729058.1
Borrelia turcica [IST7]	AYE36154.1
Borrelia miyamotoi [LB-2001]	WP_020954657.1
Borrelia duttonii [CR2A]	WP_038366132.1
Borrelia recurrentis [A1]	WP_012538787.1
Borrelia parkeri [SLO]	WP_025407461.1
Borrelia turicatae [91E135]	WP_011772224.1
Borrelia anserine [BA2]	WP_025419471.1
Borrelia persica [No12]	WP_024653997.1
Borrelia hermsii [DAH]	WP_012422041.1
Borrelia hispanica [CRI]	WP_024655530.1
Borrelia crocidurae [DOU]	WP_040236116.1
Borrelia coriaceae [Co53]	WP_025407926.1
Treponema azotonutricium [ZAS-9]	WP_015711742.1

The amino acid sequence of BB_0267 was aligned with three hypothetical bactofilins encoded by the *B. burgdorferi* strain B31: BB_0321, BB_0245, and BB_0538. All four proteins contain the conserved DUF342 domain, being a characteristic feature of most bactofilins. Furthermore, bactofilin homologues encoded by the genome of *L. biflexia* were also used for comparison with gene locus BB_0267. An identical protein of gene locus BB_0267 in *L. biflexia* was identified using the STRING database protein association network and also utilized for alignment. The accession numbers and protein sequences of those proteins are given in Table 7.

Table 7: List of different bacterial species and their amino acid accession numbers

Species	Amino acid accession number
B. burgdorferi [B31]	AE000783.1
B. burgdorferi [B31]	AAC66618.1
B. burgdorferi [B31]	AAC66633
B. burgdorferi [B31]	AE000783.1
Leptospira biflexa serovar Patoc str. ATCC 23582	LEPBI_I1431
Leptospira biflexa serovar Patoc str. ATCC 23582	LEPBI_I0947

Furthermore, 3D models of those proteins were computed using the Phyre2 server, followed by analysis using the Chimera structure modelling program.

3.3.8.2 Electron Microscopy

Due to the observation of morphological phenotype for transposon mutant S4-7 under the dark-field microscope, its spirochetal morphology was investigated. Therefore, transposon mutant S4-7and wild type B31-A3 were inoculated from frozen glycerol stocks in 7 ml fresh BSK-II medium. The spirochetes were grown to exponential phase and the bacterial cultures were sent to the Institute of Electron Microscopy to Dr. Marie Vancová, who prepared the samples and conducted the electron microscopy work

3.3.8.2 Swarm motility assay

Difference in motility between transposon mutant S4-7 and the wild type B31-A3 was assessed via a swarm motility assay. 34.2 ml of 1.5xBSK was mixed with 1.7 ml of Rabbit Serum. 24 ml of this solution was transferred to a new flask. 9.19 ml of 5% fresh sodium bicarbonate, 15 ml of 10×CMRL-1066 and 144 ml of Dulbecco's PBS were added. The solution was then heated to 50°C and 48 ml of softened 1.7% liquid autoclaved agarose (SeaKem®) was added. After mixing, 35 ml of the swarm plate mixture were transferred to one plate. The plates were allowed to solidify in the flow-box. Then, the bacterial cultures of B31-A3 and S4-7 were counted using a Petroff-Hauser counting chamber. The bacterial cells were collected by centrifugation for 20 minutes at 4000 g and 25°C. The supernatant was decanted carefully, and the bacterial pellet was gently resuspended in BSK×1,5 medium so that 5 µl contained 1×10^6 cells. This was then inoculated under the agarose surface by using a micropipette so that the tip did not touch the bottom surface of the plate. 2×5 µl of B31-A3 as well as 2×5 µl of S4-7 were inoculated on each plate, 6 plates in total, ensuring equal conditions and thus comparability of the obtained results. The plate lids were kept open inside the laminar fume hood for 10 minutes and then incubated for 6 days in air tight boxes with anaerobic

satchets (GasPak™) at 34°C. After six days the swarm diameter of each colony was measured. Swarm motility assay was performed according to the protocol described in Motaleb et al., 2012 (83).

3.3.8.3 Microdilution assay

Microdilution testing was performed using 96-well, round-bottom microtiter plates. Each plate included positive controls (bacterial culture without antimicrobials), negative controls (BSK-II medium only), and serial twofold dilutions of each of the two-cell wall targeting antibiotics namely Amdinocillin, a drug that specifically targets Penicillin Binding Protein-2 (PBP-2), and A22, which inhibits ATP binding to MreB, all in BSK-II medium. Cell wall targeting antibiotic containing wells included final concentrations of each drug, ranging from 250 μg/ml to 0.01 μg/ml in the case of A22 hydrochloride and 25 μg/ml to 0.01 μg/ml for Pivmecillinam. B31-A3 and S4-7 inoculum was produced from cultures grown to mid-log phase at 34°C. The exact cell density of each spirochetal culture was determined using of a Petroff-Hauser counting chamber. Following the addition of a 100 μl inoculum containing 2×10^6 spirochetes/ml to the antimicrobial-containing and positive control wells, the plates were incubated with CO_2 satchets (GasPak™) at 34°C. The plates were examined four days after inoculation under the dark-field microscope and cell density of wells containing highest and lowest antimicrobial concentration were counted using Petroff-Hauser counting chamber. MIC was performed in triplicate. Protocol for microdilution assay using cell wall targeting antibiotics was adapted from Murray et al., 2004 (84).

3.3.8.4 Creation of complementation construct for BB_0267 in S4-7

To determine whether the observed phenotype of transposon mutant S4-7 was caused by disruption of the BB_0267 gene locus, complementation was performed in order to re-establish the wild type. First of all, the borrelial $flgB_p$ promoter and the BB_0267 gene locus was amplified by PCR using self-designed primer pairs. These primers pairs were equipped with restriction sites on the 5' and the 3' end. Both fragments were amplified by PCR using the respective primer pairs listed in Table ***8***. The PCR products were cloned into the pCR®2.1-TOPO® vector (Invitrogen®) using the TOPO® TA Cloning® kit and the pCR®2.1-TOPO® vector was transformed into competent *E. coli* cells. Plasmid DNA of *E. coli* cells was isolated using a NucleoSpin® Plasmid Kit (Machery-Nagel), double digested using the appropriate pair of restriction enzymes for each fragment (*XmaI/ClaI* for borrelial $flgB_p$-promoter and *ClaI/SalI* for the BB_0267 gene locus) and visualized via agarose gel electrophoresis. The size of the restriction products was controlled, and

the respective gel pieces were cut out of the agarose gel using QIAquick Gel Extraction Kit from QIAGEN by following the manufacturer's instructions.

Table 8: List of primer designations, primer sequences and attached restriction sites for complementation

Task	Primer	Restriction enzyme (NEB)	Sequence 5'-3'
Amplification of BB_0267 gene locus	BB_0267-FP	*ClaI*	CTATCGATATGGATGACAGGGCTATAAATTTTTC
	BB_0267-RP	*SalI*	CAGTCGACTCACCTAGACTTAAAAGGAATATAG
Amplification of borrelial flB_p promoter	$flgB_p$-FP	*XmaI*	ATCCCGGGTACCCGAGCTTCAAG
	$flgB_p$-RP	*ClaI*	GCATCGATATGGAAACCTCCCTCATTTAAAATTGCTTTTAAC
Amplification of $flgB_p$-BB_0267 fragment	$flgB_p$-BB_0267-FP	*XmaI*	ATCCCGGGTACCCGAGCTTCAAG
	$flgB_p$-BB_0267-RP	*SalI*	CAGTCGACTCACCTAGACTTAAAAGGAATATAG

3.3.8.4.1 Molecular Cloning and Transformation into *E. coli*

The BB_0267 gene locus and the borrelial $flgB_p$ promoter were amplified by PCR using the respective primer pairs. After confirming the purity and the correct size of the PCR product via agarose gel electrophoresis, the PCR product was directly used for ligation into the pCR™2.1-TOPO® vector (Invitrogen™) using the TOPO® TA Cloning® Kit. The composition of the reaction mixture is shown in Table ***9***.

Table 9: Reaction mixture set up for cloning of the DNA fragments into the pCR®2.1-TOPO® vector

pCR®2.1-TOPO® vector	
Reagent	**Amount**
Salt solution	0.5µl
PCR product	2µl
TOPO vector	0.5µl
MilliQ water	3µl

The reaction mixture was prepared and incubated for 10 minutes at room temperature (RT). NEB® 5-alpha Competent *E. coli* (High Efficiency) cells were put on ice for 5 minutes. After incubation for 10 minutes, 2 µl of the of the ligation reaction were added to 25 µl *E. coli* cells in a 1.5 ml eppendorf tube, mixed by flicking the tube and incubated for 30 minutes on ice. Afterwards, *E. coli* cells were heat-shocked for 20 seconds at 42°C and put on ice for 2 minutes. Subsequently,

250 µl of SOC Outgrowth Medium (New England Biolabs®) was added and the tube was fixed to a shaking incubator and shaken for 90 minutes at 37°C.

Liquid broth (LB) plates with carbenicillin were warmed in an incubator at 30°C. Approximately 15 minutes before the cells were ready for use, the LB plates were put out of the incubator, rested for 5 minutes at RT and each treated with 40 µl (20mg/mL) X-Gal (Thermo Scientific ® #R0941) for blue-white selection. The plates were allowed to rest for 10 minutes, 75 µl and 150 µl of cells were added to two separate plates, and then incubated overnight at 30°C.

3.3.8.4.2 Picking of Colonies and Plasmid DNA Isolation

White colonies were picked from the plates using, a pipette tip and transferred to 15 ml falcon tubes, filled with 7 ml of liquid broth (LB) medium at RT. The tubes were incubated for 16 hours in the horizontal shaker at 37°C and at 200 rpm.

After the incubation, the plasmid DNA was isolated using the NucleoSpin® Plasmid Kit from Machery-Nagel. Briefly, the *E. coli* LB culture was centrifuged for 8 minutes at 7843 rpm, at 20°C, the supernatant was discarded and 250 µl of Buffer A1 were added to the bacterial pellet. The bacterial pellet was resuspended, and the mixture was transferred to a 1.5 ml eppendorf tube. 250 µl of Buffer A2 was added, the tube was inverted 6-8 times and let stand on RT for 5 minutes. Afterwards, 300 µl of Buffer A3 was added and the tube was again inverted 6-8 times. The mixture was centrifuged for 10 minutes at 11000 rpm and at 25°C. 750 µl of the supernatant was loaded onto NucleoSpin® Plasmid Column in a 2 ml Collection Tube and centrifuged for 11000 rpm for 1 minute. The flow through was discarded and the column was put back into the collection tube, 500 µl of washing Buffer AW was added and again centrifuged for 1 minutes at 11000 rpm. The flow through was discarded, 600 µl of Buffer A4 was added again and the column was centrifuged for 1 minute at 11000 rpm. Finally, the flow through was discarded and the silica column was dried by centrifuging for 2 minutes at 11000 rpm. The column was put into a 1.5 ml eppendorf tube and 50 µl of elution buffer EB was added to the centre of the column. After incubation for 1 minute, centrifugation for 1 minute at 11000 rpm was performed and DNA was stored in the fridge.

3.3.8.4.3 Restriction digestion

For the verification of successful ligation of the BB_0267 gene locus and the borrelial $flgB_p$-promoter into the pCR®2.1-TOPO® vector, restriction digestion with the appropriate enzymes was performed. The composition of the reaction mixture is shown in Table 10.

Table 10: Reaction mixture set up for restriction digestion of the pCR®2.1 TOPO® vector

Reagent	Amount
Restriction Enzyme 1	1 µl
Restriction Enzyme 2	1 µl
MilliQ water	Filled up to50 µl
DNA	1µg
Cut Smart Buffer	5 µl

The reaction mixture was incubated for 1 hour at 37°C. Subsequently, enzymes were deactivated by incubation at 65°C for 20 minutes. The restriction digest was then visualized via agarose gel electrophoresis. The size of the restriction products was controlled, and the respective gel pieces were cut out of the agarose gel using QIAquick Gel Extraction Kit from QIAGEN by following the manufacturer's instructions.

3.3.8.4.4. Ligation of the borrelial $flgB_p$ promoter with the BB_0267 gene locus

The following reaction mixture given in Table 11 was prepared for ligation of the of the borrelial $flgB_p$ promoter with the BB_0267 gene locus after the two DNA fragments were extracted from the agarose gel.

Table 11: Reaction mixture for ligation of the BB_0267 gene locus and the flgBp promoter

Reagent	Amount
BB_0267	5 µl
$flgB_p$	5 µl
T4 DNA ligase	1 µl
MilliQ water	7 µl
10×T4 Ligase Buffer	2 µl

The reaction mixture was incubated overnight at 16°C. Successful ligation was proven by PCR using the $flgB_p$ -FP and the BB_0267-RP.

3.3.8.5 Creation of construct for protein expression

To generate antibodies against the BB_0267 gene locus, the gene would be transferred into the pQEt expression vector. After the successful expression of the protein, the protein will be injected into rabbit for immunization.

First of all, the BB_0267 gene locus was amplified by PCR using a self-designed primer pair, listed in Table 12 equipped with restriction sites at its 5' and 3' end and the PCR product was then ligated

into the pGEM®-T-vector. The BB_0267 gene and the pQE30 expression vector were double digested using the two restriction enzymes *KpnI*-HF and *BamHI*-HF (New England BioLabs) and the restriction digest was visualized via agarose gel electrophoresis. The size of the restriction products was controlled, and the respective gel pieces were cut out of the agarose gel using QIAquick Gel Extraction Kit from QIAGEN, following the manufacturer's instructions. The two DNA fragments were joined by ligation using T4-ligase (Promega Cooperation).

Table 12: List of primer pair designations, primer sequences and attached restriction sites for antibody generation

Task	Primer	Restriction Enzyme (NEB)	Sequence 5'-3'
Amplification of BB_0267 gene locus	BB_0267-FP	*BamHI*-HF	CTGGATCCATGGATGACAGGGCTATAAATTTTTC
	BB_0267-RP	*KpnI*-HF	ATGGTACCTCACCTAGACTTAAAAGGAATATAGG

3.3.8.5.1 Molecular Cloning and Transformation into *E. Coli*

The BB_0267 gene locus was amplified by PCR. After confirming the purity of the PCR product via agarose gel electrophoresis, the PCR product was directly used for ligation into the pGEM®-T vector (Promega Cooperation). The reaction mixture set up is shown in Table 13.

Table 13: Reaction mixture set up for ligation of BB_0267 into the *pGEM®-T vector*

Reagent	Amount
2x Rapid Ligation Buffer	2.5µl
pGEM®-T easy vector	0.5 µl
DNA insert	1.5 µl
T4 DNA Ligase	0.5 µl

The reaction mixture was incubated overnight and the next day it was proceeded as described in subsection 3.3.8.4.1. *E. coli* colonies were picked, and plasmid DNA was isolated as mentioned in subsection 3.3.8.4.2.

3.4 CB43-dsRED and green fluorescent 297 project

3.4.1 Bacterial strains and growth conditions

B. burgdorferi s.s (green fluorescent strain 297) provided by Dr. Melissa J. Caimano (Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut, USA). and *B. afzelii* (CB43-dsRed) which was created by Sophie Honeder (University of South Bohemia) in

2016, were inoculated from frozen glycerol stocks and cultivated either in liquid BSK-II media or liquid BSK-H Medium at 34°C

In the case of *B. burgdorferi* s.s (green fluorescent 297), the $flgB_p$-GFP cassette was incorporated into the genome at the circular plasmid cp26, which greatly reduces the risk of losing green fluorescence during long time cultivation, in vitro-passaging and other genetic manipulations. On the other hand, the $flgB_p$-dsRED cassette was introduced into *B. afzelii* strain CB43 via a shuttle vector, which means the risk of losing the fluorescence reporter during in vitro-cultivation, in vitro-passaging and any other genetic manipulation is higher. Therefore, gentamycin was added to the culture at a concentration of 40 µg/ml to avoid loss of $flgB_p$-dsRED cassette carrying plasmid.

3.4.2 Passaging

Both strains were inoculated from frozen glycerol stocks in 7 ml of fresh BSK-H or BSK-II.

As soon as the bacterial cultures reached a cell density of 10^7 spirochetes/ml, they were examined under the fluorescence microscope to check for fluorescence. After the fluorescence of spirochetes was confirmed, the bacterial cultures were diluted in a new tube to a final concentration of 10^5 spirochetes/mL, yielding 7 mL of passaged culture. In the case of CB43-dsRed, gentamycin was used in every passage at a concentration of 40 µg/ml. From every passage a protein lysate was prepared, and a glycerol stock was prepared from every third passage.

3.4.3. Comparing plasmid profiles

To see whether protein expression of the two strains has changed during the course of in vitro-passaging, a SDS-Page gel was prepared for each strain. Protein lysates of the start culture, the final passage and a passage between start and end of in vitro passaging was loaded on each gel. SDS gel electrophoresis was performed and subsequently stained in Coomassie Brilliant Blue as described in subsection 3.2.1.

3.4.4. Plating of final Passages

Final passages of green fluorescent 297 and of CB43-dsRed were inoculated from frozen glycerol stocks in 7 ml of fresh BSK-H or BSK-II medium. The spirochetes were allowed to grow to exponential phase (5×10^7 to 1×10^8 cells/ml) and counted using a Petroff-Hauser counting chamber. Depending on the respective cell density of each culture, a serial dilution was performed so that the cell density in the final dilution accounted for 1×10^4 spirochetes/ml.

The preparation of the medium-agarose mixture and the bottom layer of each plate was prepared as in subsection 3.2.5. Gentamycin was supplemented to the agarose-media mixture to acquire a gentamycin concentration of 40 µg/ml. 200 µl of the final dilution were transferred to a 50 ml falcon tube. The 50 ml falcon tube was filled with 40 ml of the agarose-media mixture and 20 ml were poured onto the bottom layer of the plate, resulting in 100 bacterial cells per plate. 6 plates per strain were prepared. The plates were stored in air tight stored with anaerobic satchets (GasPak™) at 34°C for 3 weeks. After 3 weeks of incubation, 20 colonies of each strain were picked from the plates and inoculated in 7 ml of fresh BSK-II medium. 3 days following this gentamycin was added to the liquid cultures at a concentration of 40 µg/ml. 10 days after inoculation, viability, cell density, morphology and fluorescence of spirochetes was checked using dark-field and fluorescence microscopy. Genomic DNA was isolated from those spirochetes which showed altered morphology and motility using the Wizard® Genomic DNA Purification Kit (Promega) according to subsection 3.1.4.

3.5 GFP-project

3.5.1 Bacterial strains and growth conditions

Two *B. burgdorferi* s.s. strains B31-A3 and B31 A3-68-1 were used. B31 A3-68-1 misses lp25 and lp54, which greatly increases its transformation efficiency, but renders it non-infectious. Both strains were inoculated from frozen glycerol stocks and were either cultivated in liquid BSK-II media, or BSK-H Medium at 34°C.

3.5.2 Transformation of B31-A3 and B31 A3-68-1

For the GFP project, a suicide vector, pMC2498, containing GFP, had been provided by Dr. Melissa J. Caimano (Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut, USA). Ethanol precipitation of the pMC2498 vector was performed as described in subsection 3.2.3. Transformation of both *B. burgdorferi* s.s. strains B31-A3 and B31 A3-68-1 was carried out as in subsection 3.2.4. Plating of *Borrelia* was also performed as mentioned in subsection 3.2.5. However, only gentamycin was added to the antibiotic plates at a concentration of 40 µg/ml. The remaining liquid cultures were filled up with fresh BSK-H and incubated at 34°C. After 2 days, gentamycin was added at the appropriate concentration. Liquid cultures as well as plates were examined after 3 weeks of incubation and bacterial colonies on antibiotic plates were picked and inoculated in 7 ml of fresh BSK-H medium as described in subsection 3.2.5.1. Again, only gentamycin was added to the liquid cultures three days after

inoculation. In the case of the GFP-project, the bacterial cultures were examined after 10 days of inoculation under the dark-field microscope and the fluorescence microscope to prove viability and fluorescence of transformants. After reaching the appropriate cell density, the bacterial cells were spinned off and a DNA thermolysate was prepared.

3.5.3 Preparing DNA thermolysates

Bacterial cells were pelleted by centrifugation for 10 minutes at 13000 rpm. The supernatant was removed using a pipette and 100 µl of Elution Buffer from QIAGEN was added to the bacterial pellet. The mixture was incubated at 100°C for 10 minutes and then put on ice for 10 minutes. Centrifugation for 10 minutes at 10°C and at 13000 rpm was performed and the supernatant was transferred to a new 1.5 ml eppendorf tube. The presence of the $flgB_p$::aacC1 and the $flgB_p$-GFP cassette was assessed via PCR using the appropriate primer pairs. The transformations carried out are listed in Table 14.

Table 14: List of transformations performed using the GFP vector

Strain	Microgram of GFP vector
B31-A3	25
	50
B31 A3-68-1	50

4. Results

4.1 Transposon mutagenesis

Different species within the *B. burgdorferi* spp. complex were transformed under various conditions to find the optimal procedure for obtaining the first transposon mutant in the European Lyme disease *B. afzelii* strain CB43. All transposon experiments and their results are listed in Table 15.

Table 15: Detailed list of all transposon mutagenesis experiments plus the corresponding results. Index of characters used in the table: ✓ methylated pGKT vector; × non-methylated pGKT vector; – no colonies on solid media or liquid cultures.

Plating of transformants					
Strain	**pGKT [µg]**	**Methylation of pGKT**	**Colonies on control plates (average out of 2)**	**Colonies on plates with antibiotics (Gent+Kan)**	***Borrelia* in liquid cultures with antibiotics (Gent+Kan)**
CB43	20	×	123	-	-
	40	×	120	-	-
	20	✓	79	-	-
297	20	✓	80	-	-
B31 A3-68-1	80	×	106	4	-
CB43, Cl. 24	50	×	140	-	-

Limiting dilution of transformants			
Strain	**pGKT [µg]**	**Methylation of pGKT**	**Liquid cultures (96 well plates)**
CB43	20	+	-
297	20	+	-
PBi	20	+	-
SV1	20	+	-

Transformation of *B. burgdorferi* s.s strain B31 A3-68-1, which misses both plasmids lp25 and lp56, rendering those spirochetes non-infectious, but significantly increasing their transformation efficiency, with pGKT, resulted in four antibiotic resistant colonies. In addition, the presence of the kanamycin resistance cassette in the genome of all colonies was successfully proven via PCR. Mutagenesis of strain B31 A3-68-1 resulted in a transformation frequency of $1{,}5\times10^{-5}$. Successful transformation of B31 A3-68-1 confirmed the full functionality of the used pGKT vector.

No transposon mutants were obtained in the fully infectious strains of green fluorescent 297, PBi, SV1 and CB43. After transformation, the transformants were either recovered by subsurface

plating on solid BSK agar plates or by limiting dilution. In the case of plating, no colonies grew on the antibiotic plates, however, the successful growth of spirochetes on control plates showed that the plating procedure itself was carried out properly. Consequently, either the transformation was unsuccessful, or the transposon mutants were unable to grow on solid medium. Therefore, limited dilution was carried out as an alternative, as this technique also allows to recover transposon mutants which exhibit a growth defect on solid media. Nevertheless, after 5 weeks of incubation no colour change of the liquid media was observed in any well of the 96 well plates prepared and investigation of those wells under the dark-field microscope revealed only dead *Borrelia*. Those results indicate that in all experiments the transformation step was inefficient, leading to the suggestion that either the pGKT vector is not suited for application in European Lyme disease *Borrelia*, or that transformation is hindered by the presence of the two putative restriction modification gene loci *bbe02* and *bbq67* in fully infectious strains.

As transformation in B31 A3-68-1 was successful, we concluded that transformation of CB43 Clone 24 might be promising, as it misses several plasmids, including lp17, carrying the putative restriction modification locus *bbe02.* Transformation efficiency generally increases by several folds in the absence of this gene. Albeit absence of that gene locus, transformation of CB43 Clone 24 with pGKT remained was unsuccessful.

4.2 Identification of S4 (B31-A3) transposon mutants

4.2.1 Identification of the protein function of the disrupted gene loci

Information about the disrupted gene loci of the three S4 (B31-A3) transposon mutants as well as the proteins they encode was received from NCBI and BioCyc Database Collection. The collected data is listed in Table 16.

Table 16: Disrupted gene loci of S4 transposon mutants and the function of the proteins they encode

Transposon mutant	Gene locus (NCBI)	Protein function (NCBI)	Identical protein groups (NCBI)	BioCyc
S4-7	BB_0267	Hypothetical protein	DUF342 domain-containing protein	FapA : Flagellar Assembly Protein A
S4-8	BB_F02	Hypothetical protein	Hypothetical protein	-
S4-19	BB_0602	Chaperonin	Chaperonin, J-domain containing protein	DnaJ : DnaJ domain

According to the NCBI database, the protein encoded by the gene locus BB_0267 is recorded as a hypothetical protein, possessing an evolutionary conserved DUF342 domain. These domains are frequently observed in proteins denoted as bactofilins, which are components of the bacterial cytoskeleton. On the other hand, on the BioCyc Database Collection, the protein encoded by the gene locus BB_0267, is denoted as the flagellar assembly protein *FapA*, a filamentous protein that is part of a big operon, encoding proteins essential for proper flagellar assembly. The protein function of gene locus BB_0267 was further assessed via a protein association network (Figure 5) using the STRING database, which emphasized the results predicted on the BioCyc Database Collection as many proteins associated with gene locus BB_0267, are either involved in flagellar biosynthesis, namely *flhA* and *flhB*, or flagellar motor switch like proteins such as *fliM*. The BB_0602 gene locus is listed as a chaperonin encoding open reading frame on both NCBI and the BioCyc Database Collection. This was also confirmed via the STRING protein association network in Figure 5, where BB_0602 is connected with several chaperonins, as well as a cysteine t-RNA ligase. No information on the protein encoded by gene locus BB_F02 was found on any of those three databases.

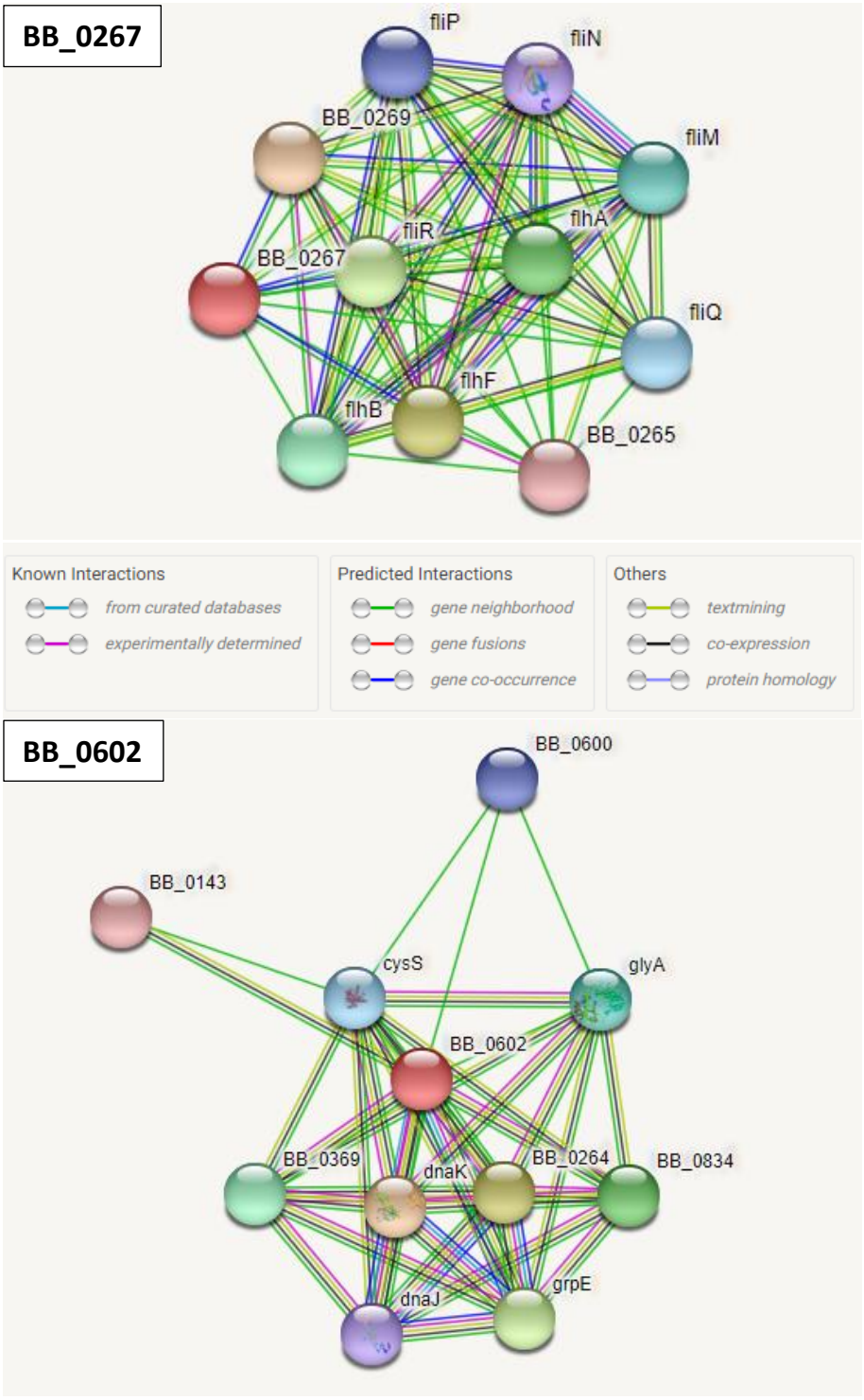

Figure 5: Protein association network of gene locus BB_0267 and BB_0602. Legend defining different forms of protein associations is given.

4.2.2 Plasmid analysis

Plasmid profile of transposon mutants S4-7, S4-8 and S4-19 was established by a PCR, using primers designed for the 12 linear and 11 circular plasmids of the *B. burdoferi* s.s. strain B31. All three transposon mutants contain the full plasmid profile except for the circular plasmid cp9, which

was expected since *B. burgdorferi* strain B31-A3 is an infectious, clonal derivative of the B31 strain, containing all plasmids except for circular plasmid cp9.

4.2.3 Protein profile comparison

SDS-PAGE revealed no differences in protein expression between wild type B31-A3 and transposon mutants S4-7, S4-8 and S4-19 as shown in Figure 6.

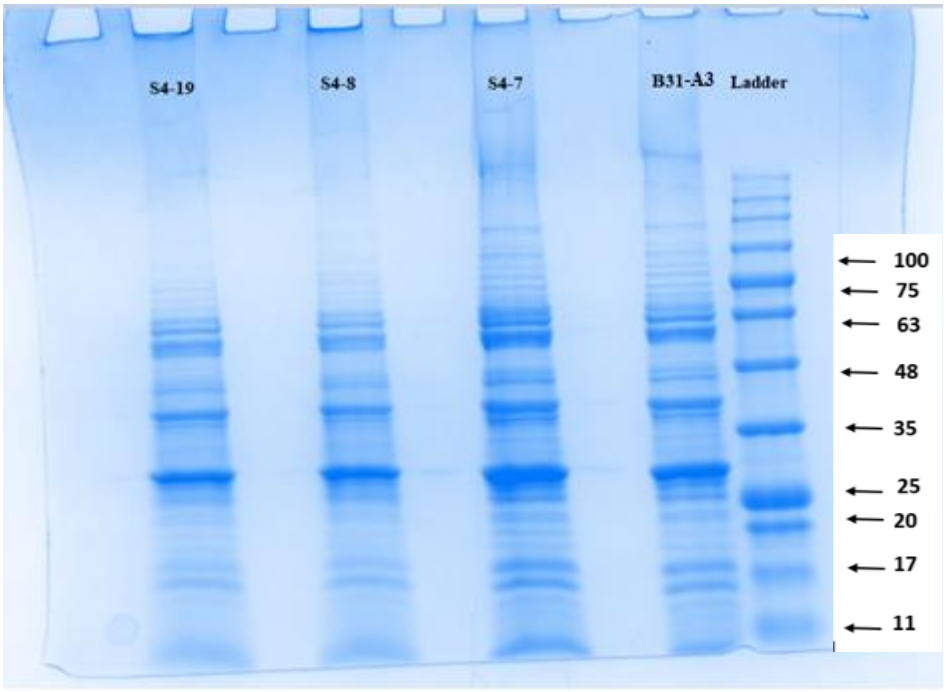

Figure 6: Protein expression profile of transposon of transposon mutant S4-7, S48, S4-19 and wild type B31-A3

4.2.4 Growth curve

While transposon mutants S4-8 and S4-19 grew at the approximately same rate than wild type B31-A3, transposon mutant S4-7 grows considerably slower. The statistical significance regarding the difference in the growth rate of B31-A3 and each transposon mutant was calculated using a Paired Student t-Test. The results show that the difference in growth rate of transposon mutant S4-7 and the wild type B31-A3 is statistically significant (p-value<0.001). Growth curves of all three transposon mutants as well as B31-A3 are displayed in Figure 7.

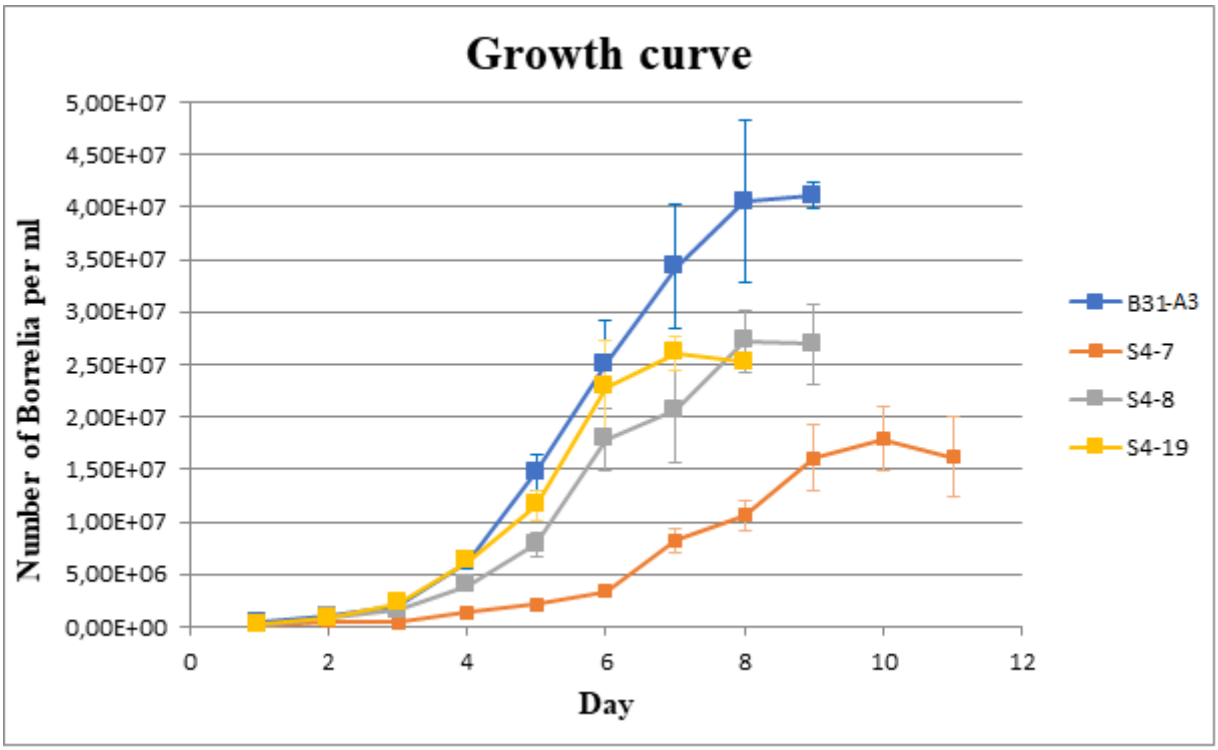

Figure 7: Growth rates of wild type B31-A3, transposon mutants S4-7, S4-8 and S4-19. Mean values and standard deviation bars are indicated. The difference in growth rate of S4-7 and the wild type B31-A3 is statistically significant, which was determined by conducting a Paired Student t-Test (p-value<0.001).

4.2.5 Plating transposon mutants

Morphology of wild type B31-A3 and transposon mutants S4-7, S4-8, S4-19, was further assessed by growing them on sold media as it is described in section 3.3.5. Three weeks after incubation, pictures (Figure 8) of all plates (transposon mutants and wild type) were taken using ChemiDocTM MP Imaging System. Subsequently, size and shape of colonies formed by transposon mutants S4-7, S4-8, and S4-19 were compared to colonies formed by wild type B31-A3. While no morphological differences in the colonies of transposon mutants S4-8 and S-19 can be observed (bigger colonies of transposon mutant S4-8 are caused by the lower number of *Borrelia* plated), the colonies of transposon mutant S4-7 show clear morphological changes compared to wild type B31-A3. Furthermore, colonies formed by transposon mutant S4-7 are non-uniform in shape and size. These results further emphasize that disruption of gene locus BB_0267 causes morphological changes, which was also observed under the dark-field and electron microscope.

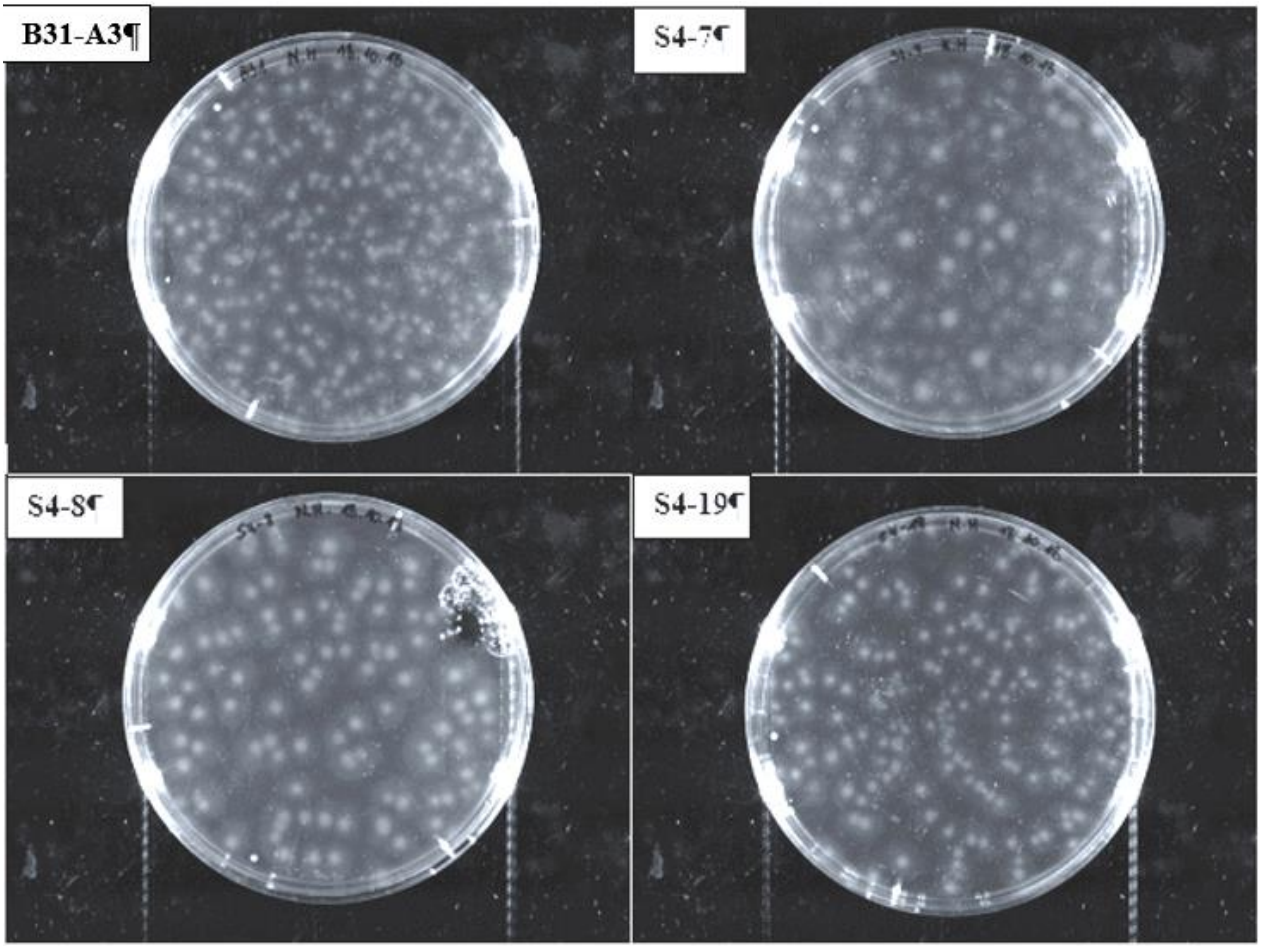

Figure 8: Colonies of wild type B31-A3 and transposon mutants S4-7, S4-8, and S4-19. Clear morphological changes can be observed for transposon mutant S4-7.

4.2.6 Mouse infection studies

The pathogenesis of transposon mutants was tested by needle infection of naive mice. 10 days post-infection, successful infection of mice was verified by PCR, using a primer pair specific for strain B31. Furthermore, serological tests were conducted in the form of a Western Blot. After four weeks post-infection, ear, joints and heart were taken, inoculated in 7 ml of fresh BSK-H medium and tissue outgrowth was checked using a dark-field microscope. PCR and tissue outgrowth results for each infective dose are listed in Table 17. The results obtained from Western blotting were not conclusive in any experiment due to the low number of bands observed, even in those mice, which were shown to be fully infectious due to outgrowth of spirochetes from all tissues. However, positive control always revealed full number of bands, indicating that procedure of Western blotting itself was successful.

All three transposon mutants were first injected at an initial infective dose of $1x10^4$ borrelia/ml. Transposon mutant S4-8 revealed full infectivity, being isolated from 24/24 sites, whereas

transposon mutants S4-7 and S4-19 showed reduced infectivity phenotypes. Transposon mutant S4-7 infected only 2/5 mice, being re-isolated only from ear tissues but not affecting any other organs, while transposon mutant S4-19 infected 2/6 mice, growing out of all tissues. Therefore, infectivity of transposon mutants S4-7 and S4-19 was further assessed at a dose of 1×10^7 borrelia/ml. Both transposon mutants infected 6/6 mice at a dose of 1×10^7 borrelia/ml, however, infectivity of transposon mutant S4-7 was still reduced as spirochetes were re-isolated from 5/12 sites, only from ears and joints. Transposon mutant S4-7 was also injected at a dose of $1x10^8$ borrelia/ml to assess if accomplishment of the stationary growth phase has any effects on infectivity of this transposon mutant. However, results were not conclusive. 2/3 mice were infected according to PCR, 2/3 mice showed up to be positive by Western Blot, but no spirochetes were growing out of any tissues after dissection of those mice. The lack of tissue outgrowth, after PCR and Western blotting data showed that those mice are infected, might be due to hindered in vitro growth of spirochetes. All these experiments were carried out in female Balb/c mice and are listed in Table 17.

For transposon mutants S4-7 and S4-19, the infection studies were continued in a different mouse strain, namely female C3H mice. Two infectious doses were tested for both transposon mutants, 1×10^3 borrelia/ml and 1×10^7 borrelia/ml. At a low infectious dose of 10^3 borrelia/ml, both transposon mutants were non-infectious in mice. However, at the higher infectious dose of 10^7 borrelia/ml, transposon mutant S4-7 infected 4/4 mice, being re-isolated only from the ear tissues, while transposon mutant S4-19 infected 3/3 mice and spirochetes grew out of all tissues. These results more or less assort with the results obtained in Balb/c mice. Experiments were conducted as described in section 3.3.6 and are listed in Table 17.

Table 17: Results of mouse infection studies. Index of characters used in the table: X stands for non-investigated; f for female.

						Tissue outgrowth			
***Borrelia* strain**	**Mouse strain**	**Mouse gender**	**Number of mice**	**Dose**	**PCR**	**Ear**	**Bladder**	**Heart**	**Joint**
B31-A3	Balb/c	f	3	10^5	X	3/3	3/3	3/3	3/3
	C3H	f	3	10^5	3/3	3/3	3/3	3/3	3/3
S4-7	Balb/c	f	5	10^4	5/5	2/5	0/5	0/5	0/5
			3	10^7	3/3	3/3	0/3	2/3	0/3
			3	10^8	2/3	0/3	0/3	0/3	0/3
	C3H	f	4	10^3	0/4	0/4	0/4	0/4	0/4
			4	10^7	4/4	4/4	0/4	0/4	0/4
S4-8	Balb/c	f	6	10^4	3/6	6/6	6/6	6/6	6/6
S4-19	Balb/c	f	6	10^4	3/6	2/6	2/6	2/6	2/6
			3	10^7	3/3	3/3	3/3	3/3	3/3
	C3H	f	3	10^3	3/3	0/3	0/3	0/3	0/3
			3	10^7	3/3	3/3	3/3	3/3	3/3

4.2.7 Tick infection studies

To assess if the transposon mutants can be acquired from ticks during feeding, naive *I. ricinus* or *I. scaluplaris* larvae were put on mice, infected either with B31-A3 or one of the three transposon mutants S4-7, S4-8, S4-19. 100 larvae per mouse were allowed to feed for 2-3 days, 3 mice per positive control and per transposon mutant. Larvae were allowed to feed to repletion. 10 days after dropping from their host, 3 full feed larvae per mouse were washed and subsequently crushed in liquid media. For each larva, a liquid culture as well as 2 plates were prepared as described in section 3.3.7. Different hard tick species were used due to the low number of *I.scalpularis* larvae available over the course of the experiment. Furthermore, only one mouse infected with B31-A3 was used in the case of *I.sclapularis* larvae, as the other two mice of this group died during the course of the experiment. The number of larvae that got infected during feeding is listed in Table ***18***.

Table 18: Assessment of larvae infected by feeding on mice infected either with B31-A3, S4-7, S4-8 or S4-19

Borrelia **Strain**	**Number of infected mice**	**Mouse strain**	**Mouse gender**	**Tick species**	**Naive larvae per mouse**	**Total number of larvae crushed**	**Total number of infected larvae**
B31-A3	1	Balb/c	f	*I.scalpularis*	100	2	1
S4-8	3	Balb/c	f	*I.scalpularis*	100	9	5
B31-A3	3	Balb/c	f	*I.ricinus*	100	9	4
S4-7	3	Balb/c	f	*I.ricinus*	100	9	0
S4–19	3	Balb/c	f	*I.ricinus*	100	9	0

The results show that wild type B31-A3 was successfully acquired by both *I. scaluplaris* and *I. ricinus* larvae during feeding on infected mice. In the case of the three transposon mutants, only transposon mutant S4-8 was re-isolated from full fed *I.scaluplaris* larvae, while no successful acquisition was shown for transposon mutants S4-7 and S4-19. However, as we only crushed 15 of the 100 ticks we put on each mouse, we decided to continue the experiment by assessing if the transposon mutants can be transmitted from their arthropod tick vector to a vertebrate host. Therefore, we allowed the remaining *I. scalpularis* and *I. ricinus* larvae to moult to nymphs. Then, we put 15 nymphs infected with either one of the transposon mutants or B31-A3 on one naive mouse, 3 mice per transposon mutant and B31-A3. The infected nymphs were allowed feed to repletion and 10 days after dropping from their host, 5 full feed nymphs per mouse were washed and subsequently crushed in liquid media. For each nymph, a liquid culture as well as one plate were prepared as mentioned in section 3.3.7, to check if the respective nymphs were infected. The results are listed in Table 19.

Table 19: Checking nymphs infected with B31-A3, S4-7, S4-8 and S4-19, for infectivity after feeding on naive mice

Borrelia	**Number of naive mice**	**Mouse train**	**Mouse gender**	**Tick species**	**Infected nymphs per mouse**	**Total number of nymphs crushed**	**Total number of infected nymphs**
B31-A3	3	Balb/c	f	*I.scalpularis*	10	12	0
S4-8	3	Balb/c	f	*I.scalpularis*	15	15	3
B31-A3	3	C3H	m	*I.ricinus*	10	15	2
S4-7	3	GGtal	m	*I.ricinus*	15	15	0
S4–19	3	GGtal	m	*I.ricinus*	15	15	0

No wild type B31-A3 was re-isolated from any of the *I.scalpularis* nymphs crushed, however, it was successfully isolated from *I. ricinus* nymphs. In the case of transposon mutant S4-8 at least some of the crushed nymphs were infected, while no spirochetes were re-isolated from nymphs

that should be infected with transposon mutant S4-7 or S4-19, suggesting that acquisition of those transposon mutants was not successful during larval feeding.

The successful transmission of B31-A3 and the three transposon mutants from their tick vector to naive mice was assessed as mentioned in section 3.3.6. Two mice, where we put on nymphs infected with transposon mutant S4-19, died during the course of the experiment. The results are listed in Table 20.

Table 20: Assessing successful transmission from nymphs infected with B31-A3, S4-7, S4-8 and S4-19 to naive mice.

						Tissue outgrowth			
Borrelia	**Mouse strain**	**Number of naive mice**	**Tick species**	**PCR**	**Western Blot**	**Ear**	**Bladder**	**Heart**	**Joint**
B31-A3	Balb/c	3	*I.scalpularis*	3/3	1/3	1/3	1/3	1/3	1/3
S4-8	Balb/c	3	*I.scalpularis*	3/3	0/3	0/3	0/3	0/3	0/3
B31-A3	C3H	3	*I.ricinus*	1/3	0/3	0/3	0/3	0/3	0/3
S4-7	Ggtal	3	*I.ricinus*	1/3	0/3	0/3	0/3	0/3	0/3
S4-19	Ggtal	3	*I.ricinus*	1/3	0/3	0/3	0/3	0/3	0/3

In the case of B31-A3 the full transmission cycle was proven using *I. sclapularis* ticks. However, only 1/3 mice where we allowed infected nymphs to feed on got infected, which might be explained by the fact that our *I. scalpularis* population was already quite old. For transposon mutant S4-8, the acquisition of spirochetes from naive larval ticks to infected mice was shown, in larvae as well as nymphs, however, no transmission of spirochetes occurred from infected nymphs to naive mice, indicating that a phenotype in transmission might exist for transposon mutant S4-8. The experiment should be repeated using a new population of *I. scalpularis* larvae and more mice. In the case of transposon mutants S4-7 and S4-19 we used *I. ricinus* larvae. We were not able to re-isolate spirochetes either from larvae nor from nymphs, indicating that acquisition of both transposon mutants did not work. For B31-A3, which we used as positive control, we showed that spirochetes were successfully acquired during feeding of larvae on infected mice, however, also in the case of B31-A3 no successful transmission of spirochetes occurred, which might be due to the fact that *I. ricinus* is not the natural arthropod tick vector for *B. burgdorferi* s.s.

4.2.8 Further analysis of transposon mutant S4-7 (B31-A3)

4.2.8.1 Phylogenetic analyses

Using the sequences listed in Table 6, a phylogenetic analysis was performed using the Geneious software. First of all, a multiple alignment of all sequences was conducted using as a cost matrix a BLOSUM 62 matrix. Afterwards, the phylogenetic tree was created with the previously created sequence alignment using Jukes Cantor as the genetic distance model and UPGMA (Unweighted Pair Group Method with Arithmetic mean) as a tree build method. The phylogenetic tree of the protein encoded by gene locus BB_0267 was conducted to learn more about its evolutionary relationship within the different species of the *Borrelia* genus.

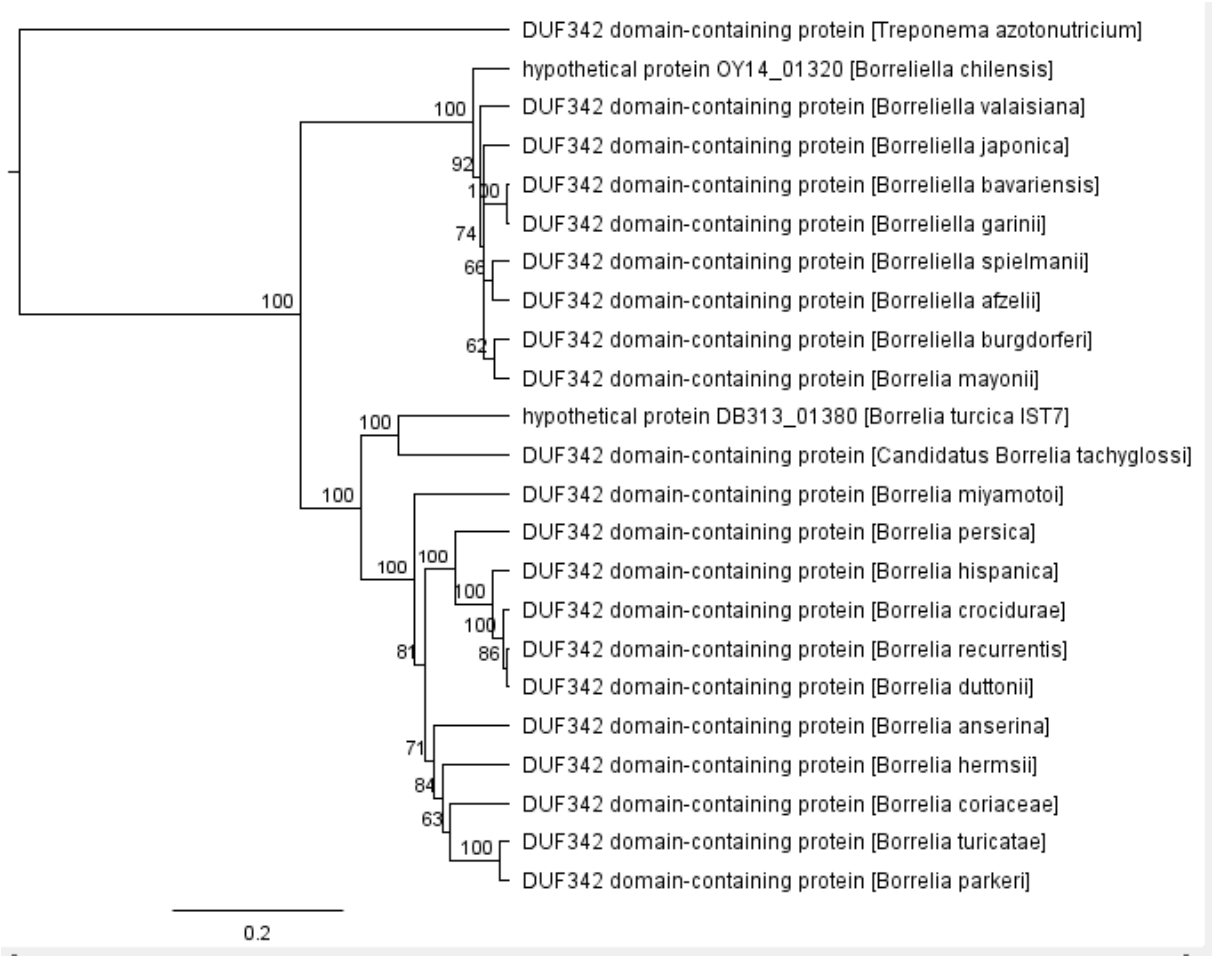

Figure 9: Phylogenetic tree including Lyme disease and relapsing fever spirochetes encoding a DUF342 domain containing protein

Figure 9 shows the expected the existence of two distinct phylogenetic groups, the upper branch, being composed of the European, American and Asian Lyme disease spirochetes and the lower branch, being composed of the African spirochetes, which are responsible for relapsing fever.

To identify if gene locus BB_0267 encodes a protein that could be a bactofilin, which at the same time participates in proper flagellar assembly, a phylogenetic tree (Figure 10) of the amino acid sequences listed in Table 7 was generated using the parameters described above. Furthermore, the 3D-structures of those proteins were visualized using the Chimera programme (Figure 10).

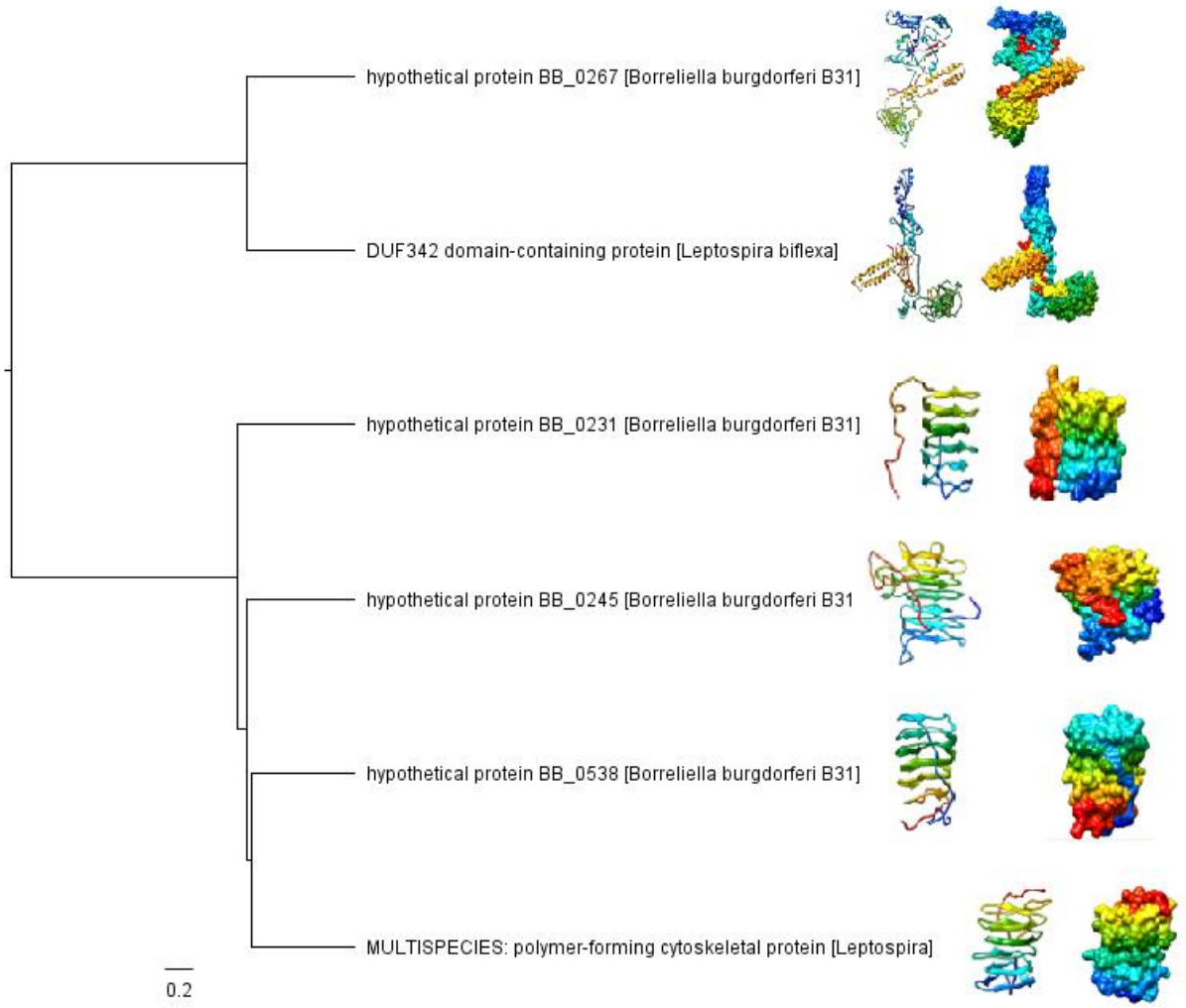

Figure 10: Phylogenetic tree of gene locus BB_0267 as well as three bactofilin homologues in *B. burgdorferi* and one bactofilin homologue in *L. biflexia.* Identical DUF342 domain containing in *L. biflexia* was also incorporated in the phylogenetic tree. 3D structures of the bactofilin homologues in *B. burgdorferi* and *L. biflexia* as well as of the DUF342 domain containing protein (BB_0267) in *B. burgdorferi* and its homologue in *L. biflexia* were created.

The phylogenetic tree shows that the bactofilin homologues and the DUF342 domain containing proteins of *B burgdorferi* and *L. bilfexia* cluster in two distinct branches, indicating different function of those two protein families. In addition, 3D analysis using the Chimera programme reveals that the 3D structures of the protein encoded by gene locus BB_0267 and the different bactofilins in *B. burgdorferi* and *L. biflexia* are significantly different. Bactofilins are relatively small proteins composed of 200-300 amino acids, which are mainly composed of β-strands. On the

other hand, the DUF342 domain containing protein encoded by gene locus BB_0267 is significantly larger, being composed of more than 500 amino acid residues, which is built up by several structural units, including α-helices, β-strands and β-loops.

4.2.8.2 Electron Microscopy

Due to the observation of changes in morphology as well motility of transposon mutant S4-7 under the dark-field microscope, the morphology of transposon mutant S4-7 was further investigated in more detail using electron microscopy. Selected electron microscopic pictures of transposon mutant S4-7 and wild type B31-A3 are illustrated in Figure 11.

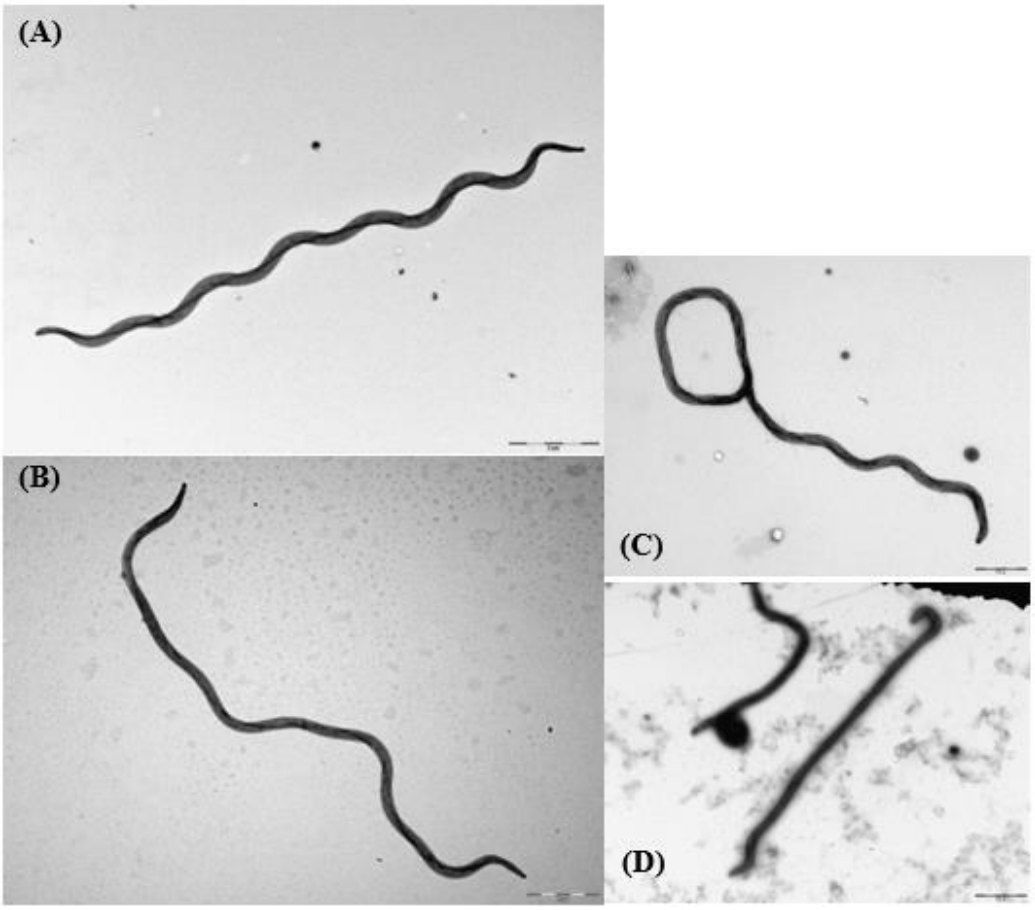

Figure 11: Electron microscopy pictures of wild type B31-A3 and transposon mutant S4-7. (A) Wild type B31-A3; (B) Transposon mutant S4-7; (C) Loop formation at one end of the protoplasmic cell cylinder of transposon mutant S4-7; (D) Formation of hook like structure at one end of protoplasmic cell cylinder of transposon mutant S4-7.

Electron microscopic pictures show that wild type B31-A3 and transposon mutant S4-7 differ in morphology in regard to their helical pitch distance. In comparison to the wild type, the overall number of helical pitches in transposon mutant S4-7 seems to be decreased, due to the enlarged distance between two consecutive helical pitches. Furthermore, the formation of a hook or loop

like structure at one terminus of the protoplasmic cell cylinder during the spirochetal course of movement was frequently observed under the dark-field microscope and these morphological changes were also captured during the specimen preparation and final imaging using the electron microscope.

4.2.8.3 Swarm motility assay

As we observed distinct changes in morphology and motility of transposon mutant S4-7 under the dark-field microscope, motility of S4-7 was further assessed by conducting a soft agar motility assay, using B31-A3 for comparison. In six biological replicate plates S4-7 cells formed significantly smaller colonies with an average diameter of 650 mm, while the wild type B31-A3 colonies were larger with an average diameter of 1350 mm. The result suggests that the inactivation of the BB_0267 gene locus results in impaired motility. The statistical significance was calculated using a Student Paired t-Test. (p-value<0.0001). Results are illustrated in Figure 12.

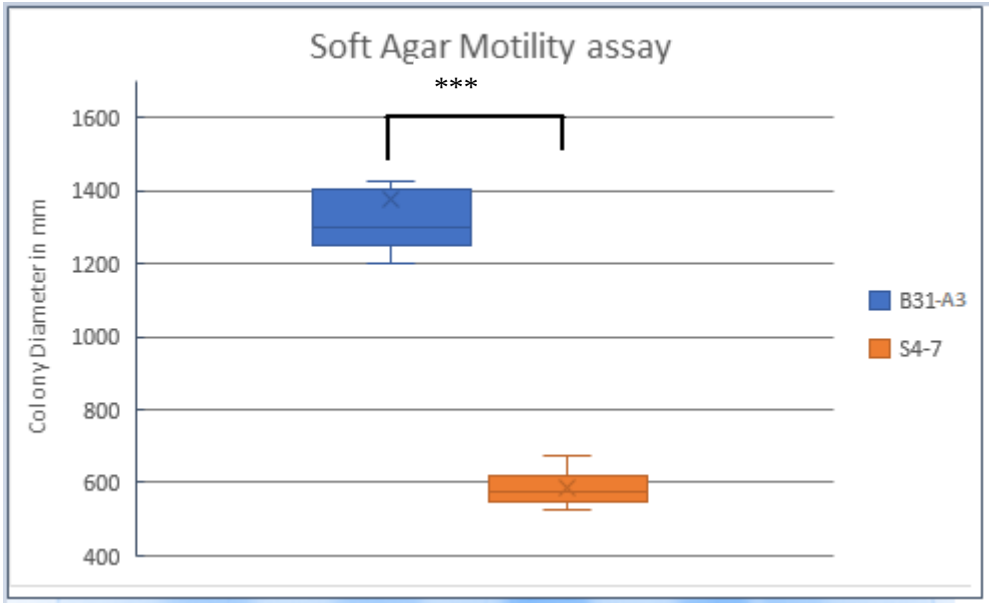

Figure 12: Loss of BB_0267 reduces spirochete motility. Colony size was measured for the wild type B31-A3, and transposon mutant S4-7 following a 6-day incubation period. Mean values and standard deviation bars are shown. Statistical significance was determined using Student Paired t-Test. (p-value<0.0001).

4.2.8.4 Microdilution assay

Previous studies showed that deletion of the bactofilin homologues *BacM* in *M. xhantus*, and *LbbD* in *L. biflexia* lead to an increased sensitivity of those prokaryotic cells to cell wall targeting antibiotics (82). To check if the BB_0267 gene locus encodes a bactofilin, we assessed the sensitivity of transposon mutant S4-7 to the cell wall targeting antibiotics Amdinocillin, and

Pivmecillinam, to observe if gene locus BB_0267 and its encoded protein play a role in spirochetal cell wall integrity. Strain B31-A3 served as a positive control. For both antibiotics, the concentration range was initially set to 25 µg/µl to .0.01 µg/µl. As no effect on viability of spirochetal cells was observed in the case of A22 at this concentration range, the upper limit was raised to 250 µg/µl. Nevertheless, viability of both B31-A3 and S4-7 was not influenced by cell wall targeting antibiotic A22 at any concentration. Differences in cell density between B31-A3 and S4-7 in antibiotic wells was caused by the slower growth rate of S4-7. Both strains were sensitive to the highest concentration of Pivmecillinam, which might be also caused by DMSO in which the antibiotic was dissolved. In all cases, S4-7 was not sensitive to cell wall targeting antibiotics, indicating that the protein encoded by gene locus BB_0267 does not contribute to cell wall integrity and is no classical bactofilin.

4.2.8.5 Creation of complementation construct for BB_0267 in S4-7

The BB_0267 gene locus and the borrelial $flgB_p$-promoter were successfully amplified by PCR using the primer pairs listed in Table 8. The two amplified PCR products were then cloned into TOPO® TA Cloning® kit and the pCR®2.1-TOPO® vector was transformed into competent *E. coli* cells. Transformation was successful and white colonies were picked. Plasmid DNA was isolated and restriction digestion with the respective enzymes listed in Table 8 was performed. The restriction digest was visualized via agarose gel electrophoresis and proven to be correct due to the presence of only two DNA fragments (the TOPO vector and the BB_0267 gene locus) and the appropriate size of the respective bands. DNA was isolated from the agarose gel using QIAquick Gel Extraction Kit from QIAGEN by following the manufacturer's instructions. The isolated DNA was then used for performing ligation, however, after ligation we were not able to amplify the whole sequence of $flgB_p$-BB_0267 via PCR.

4.2.8.6 Creation of construct for protein expression

The BB-0267 gene locus was successfully amplified via PCR using the primer pairs listed in Table ***12*** . DNA fragments were visualized via agarose gel electrophoresis and the size of the respective bands was controlled. The PCR product was cloned into the pGEM®-T-vector and transformed into *E. coli*. Colonies were screened via white blue selection. White colonies were picked, plasmid DNA was isolated, and restriction digestion was performed. Restriction digest was controlled via agarose gel electrophoresis, the bands had the right size and were extracted from the gel using the Qiagen kit. The pQET30 vector and the BB_0267 gene locus was then ligated using T4 ligase (Promega cooperation) and transformed into NEB-alpha cells. Resulting white colonies were

picked and DNA was isolated, restriction digestion with the appropriate enzymes was performed to see if the BB_0267 gene was successfully ligated into the pQET30 vector; however, no DNA fragment was cut out of the vector, indicating that ligation was ineffective.

4.3 CB43-dsRED and green fluorescent 297 project

The green fluorescent *B. burgdorferi* s.s strain 297 was passaged 23 times, whereas red fluorescent CB43-dsRed was passaged only 10 times, due to the slower growth rate of CB43-dsRed. The protein expression profile of both strains was investigated during the course of passaging by performing SDS-PAGE gel electrophoresis, however, no changes in protein profile was observed between the different passages, for both 297 and CB43-dsRed. The final passages of both strains were plated and in the case of green fluorescent 297, 25 clones were picked and inoculated in 6 ml of fresh BSK-H medium. After 10 days glycerol stocks of all 25 clones were prepared. In addition, all 25 clones were investigated under the dark-field microscope and the fluorescence microscope, checking if clones are fluorescent and exhibit changes in morphology or motility. Based on phenotypic changes, 10 out of 25 clones were selected for further investigation. Finally, their genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's protocol for Gram-Positive and Gram-Negative Bacteria and their plasmid profile will be established using the primers designed for the 12 linear and 11 circular plasmids of the *B. burdoferi* s.s. strain B31.

4.4 GFP project

The *B. burgdorferi* s.s. strains B31-A3 and B31 A3-68-1 were transformed with a GFP containing vector construct in order to create fluorescent B31-A3. All transformation experiments using the GFP vector and their outcome are listed in Table 21.

Table 21: Transformation experiments using the GFP vector. – stands for no living *Borrelia*

Strain	GFP vector [µg]	Colonies on control plates	Colonies on plates with antibiotics (Gent)	Liquid cultures with antibiotics (Gent)	Transformation frequency
B31-A3	25	25	7	-	9.3×10^{-5}
	50	150	17	-	3.7×10^{-5}
B31 A3-68-1	50	50	24	-	1.6×10^{-4}

The gentamycin resistant colonies of both *B. burfdorferi* strains B31-A3 and B31 A3-68-1 were picked from the antibiotic plates, inoculated in 6 ml of fresh BSK-II medium and incubated at 34°C. Gentamycin was added at an appropriate concentration of 40 µg/ml and after 10 days of incubation the viability of bacterial cells was investigated under the dark-field microscope. Those bacterial cultures which survived addition of gentamycin to liquid media were further investigated under the fluorescence microscope. However, no gentamycin resistant clone was fluorescing green. DNA of all bacterial cultures was isolated using a quick DNA thermolysis protocol. Nevertheless, no bacterial pellet showed any greenish colouring, further indicating the absence of GFP expression in those clones. Clones were further checked for gentamycin resistance cassette $flgB_p$:: aacC1 via PCR using the appropriate primers. All 24 clones of transformed B31-A3 were gentamycin positive. 3 clones yielding a very strong signal in the PCR investigating the presence of gentamycin, were further screened for the presence of the green fluorescent gene GFP via PCR, yielding a very faint band. Afterwards, the GFP vector was further assessed for the presence of the whole region, containing the gentamycin resistance cassette as well as the GFP expressing gene using the appropriate primer pairs. Green fluorescent strain 297 was used as a positive control. The vector was positive for gentamycin and GFP, however, amplification of the whole region was not possible in contrast to the positive control of green fluorescent 297, where the whole region could be amplified.

6. Discussion

Since its first discovery and cultivation in 1981, *B. burgdorferi* has been the subject of intense study to identify previously unidentified genes important in spirochete biology and pathology. These efforts lead to tremendous progress, resulting in the development of various genetic tools, including shuttle and suicide vectors, enabling genetic manipulations such as transformation, site-directed mutagenesis and complementation. Another exciting development was the random tagged mutagenesis of the *B. burgdorferi* genome by development of the *Himar1*-based transposon suicide vectors, pMarGent and pGKT. Insertion of the *Himar1* transposase, belonging to the mariner family of transposable elements, is random and occurs at near saturating levels within the *B. urgdorferi* genome. Since its first application, transposon mutagenesis lead to the creation of large mutant libraries, enabling the identification many open reading frames, participating in spirochete morphology, motility and infectivity (64). However, most studies were conducted in *B. burgdorferi s.s.* B31 derivates lacking either lp 25 and lp56, or lacking lp56 plus selectively inactivated the *bbe02* gene locus (62, 64, 85). In 2011, Rego and colleagues transformed *bbe02* mutants in an infectious background B31-A3 with pGKT (unpublished), obtaining the first transposon mutants in an infectious B31 derivative, containing both linear plasmids lp25 and lp56 (86). Besides transposon mutagenesis, another major success in investigating the molecular biology and pathology of *B. burgdorferi* was the application of fluorescent proteins, allowing to study the interaction of this complex organism with its arthropod tick vector and vertebrate host (32). However, most of the genetic tools which are currently available, including site directed mutagenesis, transposon mutagenesis and fluorescence techniques have been developed for *B. burgdorferi* s.s., while the European Lyme disease strain *B. afzelii* remained pretty uninvestigated.

In this project, we analysed three transposon mutants created by Dr. Rego, where the transposon insertion site was known, however, the function of the disrupted genes has not been investigated.
Gene locus BB_0267 is listed as a protein participating in flagellar assembly, containing a widely conserved DUF342 domain. Proteins that frequently contain a DUF342 domain include the recently discovered class of prokaryotic cytoskeletal forming proteins denoted as bactofilins. Bactofilins are proteins acting in various cellular pathways. For example, in *B. subtillis* two bactofilins are required for proper flagella assembly (80, 81). To identify, if gene locus BB_0267 encodes a protein being a bactofilin and participating in proper flagella assembly, a phylogenetic analysis was conducted by comparing the sequence similarity of the protein encoded by gene locus BB_0267 with various bactofilin homologues within the *B. burgdorferi* as well as the *L. biflexia* genome.

The phylogenetic tree revealed no clustering of the protein encoded by BB_0267 with any of the bactofilin homologues used. Moreover, classical bactofilins are relatively small proteins, ranging in size from 100 to 200 amino acids, usually possessing a unique secondary structure that is mainly composed of β-helices, forming a unique rigid β-helical core (82). The protein encoded by gene locus BB_0267 is significantly larger, encompassing 634 amino acids, being composed of various structural elements, including α-helices, β-strands etc.

Jackson et al. (2018), investigated a bactofilin homologue in the spirochete *L. biflexia* by creating deletion mutants. Compared to wild type cells, the cell wall integrity of those deletion mutants was weakened when coping with cell wall targeting antibiotics. The sensitivity of BB_0267 mutant cells to cell wall targeting antibiotic was investigated, however, no difference in cell wall integrity of BB_0267 mutant cells relative to wild type B31-A3 was observed. This assay together with phylogenetic analyses and comparison of 3D-structures suggests that the protein encoded by BB_0267 contains a DUF342 domain but is not a bactofilin.

The insertion into the BB_0267 gene locus, resulted in bacterial cells revealing defects in growth rate, morphology, motility and infectivity. BB_0267 lacking spirochetes grow significantly slower and show a decreased ability to move through the soft agar medium in comparison to wild type B31-A3. Furthermore, electron microscopic pictures of BB_0267 mutants revealed spirochetes with altered helical morphology, often forming hook or loop like structures at one end of the protoplasmic cell cylinder. Infectivity studies revealed a reduced infectivity phenotype of BB_0267 mutant cells. At low infectious doses (10^3-10^4 *Borrelia*), only 2 out of 9 mice got infected and re-isolation of spirochetes occurred only from the ear. At higher concentrations 7 out of 7 mice got infected, however, tissue outgrowth occurred only from the ears and the joints, underlining the importance of gene locus BB_0267 in infectivity during needle inoculation. During tick infection studies, BB_0267 mutant cells were not acquired during feeding of naive *I ricinus* larvae on infected mice, suggesting also an acquisition phenotype for this transposon mutant. These results coincide with published data from Lin et al. 2014, where mutation in genes participating in flagellar assembly, namely *fliH, fliI*, and *flbA*, resulted in reduced motility, division defects, structural changes in the flagellar motor and a low infectivity phenotype.

The creation of a shuttle vector for complementation to restore the wild type and prove that the observed phenotype is really caused by disruption of gene locus BB_0267, was not successful; however, it is the goal of future projects in our laboratory. The same accounts for the creation of an expression vector, enabling protein expression and antibody generation, which might allow the localization of the antigen in the spirochete by immune transmission electron microscopy. Furthermore, as the naive *I. ricnus* larvae were not able to acquire transposon mutant S4-7 during

feeding on infected mice, we were not able to investigate if transmission of spirochetes from their arthropod tick vector to naive mice works. Therefore, naive *I. scalpularis* larvae should be artificially infected with BB_0267 mutant and then allowed to feed on naive mice. Another goal for the future, is to determine how BB_0267 lacking spirochetes cope with osmotic stress.

The second disrupted gene locus denoted as BB_F02 is listed as a hypothetical protein on any of the previously mentioned databases. BB_F02 mutant cells revealed no morphological changes and no difference in the growth rate in comparison to wild type B31-A3. Transposon mutant S4-8 was fully infective at low (10^4 *Borrelia*) and high (10^7 *Borrelia*) infectious doses. Furthermore, spirochetes were successfully acquired by *I. scaluplaris* larvae during feeding on infected mice. However, there was no successful transmission of spirochetes during feeding of infected moulted nymphs on naive mice. For the wild type B31-A3 transmission worked, however, only one of three mice got infected. Consequently, the experiment should be repeated with a higher number of naive mice and a fresher population of *I,scalpularis* ticks to either complete the full infectious cycle for this transposon mutant or to prove that a phenotype in transmission exists.

The protein encoded by BB_0602 was listed in all databases as chaperonin. BB_0602 mutant cells revealed no morphological changes and no difference in growth rate relative to wild type B31-A3. Mice infectivity studies showed a reduced infectivity phenotype of transposon mutant S4-19 at low infectious doses (10^3 and 10^4 *Borrelia*), where only 2 out of 9 mice got infected. However, when infection occurred, spirochetes were growing out of all tissues. Higher doses (10^7 *Borrelia*) displayed full infectivity. In tick infection studies, BB_0602 lacking cells were not successfully acquired by naive *I. ricinus* larvae during their first bloodmeal on infected mice, suggesting an infectivity and acquisition phenotype. Our results suggest that gene locus BB_0602 plays a role in infectivity especially at low infectious doses. However, the exact role of this gene locus during infectivity has to be investigated in the future. Furthermore, transmission should be also assessed via artificial infection of naive *I. scapularis* ticks followed by feeding on naive mice.

Transformation of the Lyme disease spirochete *B. afzelii* strain CB43 using the pGKT vector was not successful, independent of the micrograms of pGKT that were used or its methylation status. However, in all previous studies, transposon mutagenesis was performed in *B. burgdorferi* B31 derivatives, missing either lp25 and lp56 or lacking lp56 plus selectively inactivated the *bbe02* gene locus (62, 64, 85). Therefore, it might be beneficial to use a clone of CB43 missing plasmids whose loss positively affects transformation efficiency, or by mutagenesis of *bbe02* restriction modification locus as it was performed by Rego et al. in 2011 in the infectious clonal derivative B31-A3.

Transformation of the *B. burgdorferi* strain B31-A3 and B31-A3-68-1 using the GFP vector, resulted in several colonies growing on the selective solid BSK agar plates. However, none of the colonies picked, revealed green fluorescence under the fluorescence microscope after 10 days of incubation at 34°C in liquid BSK-H medium. In addition, no greenish colour was observed after decantation of the bacterial pellet prior to genomic DNA isolation. PCR using primers pairs either amplifying the gentamycin resistance or the GFP encoding gene revealed bands for both products in most of the mutants picked, however, very faint ones. Amplification of the whole region, including both genes from the provided vector construct by PCR remained ineffective, however, the positive control (amplifying the same construct from the already green fluorescent 297) was successful, suggesting that the functionality of the used vector should be investigated in depth.

Successful in vitro passaging of the already fluorescent strains 297 (*B. burgdorferi*) and CB43-dsRED (*B. afzelii*) was performed. Final passages were plated out on solid BSK agar plates. Plasmid analysis of the picked clones is still outstanding. Promising clones can be either used for transposon mutagenesis or to study interaction of respective clones with their arthropod tick vector and their vertebrate host.

7. Conclusion

The analysis of the three transposon mutants created in the infectious *B. burgdorferi* derivative B31-A3 (Rego et al., unpublished) revealed two promising gene candidates. Firstly, gene locus BB_0267, encoding a protein ensuring proper flagella assembly, influencing spirochetal growth rate, morphology, motility and infectivity. Secondly, gene locus BB_0602 which encodes a chaperonin. BB_0602 mutant cells revealed no changes in growth rate, morphology and motility, however it showed a reduced infectivity phenotype. For both gene loci, BB_0267 and BB_0602, complementation is part of future projects.

Transposon mutagenesis in the fully infectious *B. afzelii* strain CB43 was not successful. However, it might be promising to the experiment using CB43 clones that either miss some of the plasmids, influencing transformation efficiency or by selective disruption of the putative restriction modification genes *bbe02* and *bbq67*.

8. Refernces

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