Přírodovědecká fakulta
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Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

Adapting Genetic Methods For Transposon Mutagenesis And Fluorescent Markers In Borrelia

Bachelor Thesis

Laboratory of Molecular Ecology of Vectors and Pathogens
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České Budějovice, 2020

Bibliography: Weber, P., 2020: "Adapting Genetic Methods For Transposon Mutagenesis And Fluorescent Markers In Borrelia", Bachelor of Science Thesis – 39p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

Annotation: The aim of this Bachelor thesis was the examination and generation of different mutants in various *Borrelia* species. While several assays were used to examine aspects of *B. burgdorferi* transposon mutants, genetic tools were applied in order to generate fluorescent markers in *B. afzelii* and a vector construct in *B. burgdorferi*. This work should provide an overview of various genetic methods and their methodology that have been developed in *Borrelia* bacteria.

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České Budějovice, 22.05.2020 Peter Weber

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1 Literature research

1.1 Lyme disease

Having emerged about 40 years back, Lyme disease (LD) has developed from a local phenomenon occurring in southeastern Connecticut into a serious health threat for people living across the Northern Hemisphere (1) (2). LD is a vector-borne disease transmitted by ticks from within the *Ixodes ricinus* complex. The causative is a bacterium that is of the genus *Borrelia*, hence it is also known as Lyme borreliosis (3).

Since the disease transmission depends on the vectors exposure and abundance, diagnosis of acute LD is at a maximum in June and July, the months with the strongest tick activity due to environmental factors, in many central and northern European countries (4). Once injected into the skin of a mammalian host, LD is understood as being mediated by the inflammatory response evoked by the host (2).

Clinical manifestations in the host can be differentiated into three phases, varying depending on the *Borrelia* species causing the infection. Stage I includes the characteristic inflammatory skin rash for LD known as erythema migrans at the injection site in 60-80% of the cases, and influenza-like symptoms (4). It is important to consider, that stage II can develop without manifesting the symptoms of stage I. During stage II, the inflammation is following the disseminating *Borrelia* through the host's body towards their organs; which may include joints, heart and nervous system. When LD remains undetected and untreated it might advance to stage III; which can include the development of chronic arthritis, neuroborreliosis and skin disorders (1).

Estimates report 65.000 cases per year in Europe and 300.000 cases annually in the U.S. (4) (5). These reports might be underestimated considering the inaccuracy of diagnosing symptoms leading to an uncertainty in reporting. Treatment usually involves the administration of antibiotics, however there is no European consensus on a specific treatment (4).

1.2 *Spirochaetes – Borrelia*

The phylum of *Spirochaetes* represents a distinctive and easily identifiable bacterial group inclusive of free living and parasitic bacteria. The phylum can be differentiated into three families, *Brachyspiraceae*, *Leptospiraceae* and *Spirochaetaceae*. Depending on life strategy and family affiliation, the distinctive spiral cellular expansion of the bacteria can vary from 0.09 µm – 0.75 µm in diameter and 3 µm – 500 µm in length. Their motility is based on a

flagellum running alongside the wave-like body and being encapsulated by the inner and outer membrane and thus called periplasmic flagellum (1). On the basis of being the causative agent of human pathogens, three spirochetal species deriving out of the *Spirochaetaceae* family became the major research target, *Treponema* being syphilis associated, *Leptospira* transmitting leptospirosis, and *Borrelia* causing relapsing fever and LD (1) (6).

1.2.1 Phylogenetics of *Borrelia*

For *Borrelia,* the state-of-the-art research can be divided into two major groups according to the phylogenetic branches of *Borrelia* spirochaetes, relapsing fever and LD. *Borrelia* causing LD are usually assigned to the *Borrelia burgdorferi sensu lato* (s.l.) species complex. Among more than 20 known species, several are considered as human pathogens; in particular *B. mayonii* in the US, *B. burgdorferi* sensu stricto (s.s.) in the US and Western Europe; and *B. bavariensis***,** *B. garinii* and *B. afzelii* in Europe and Eurasia (7) (8).

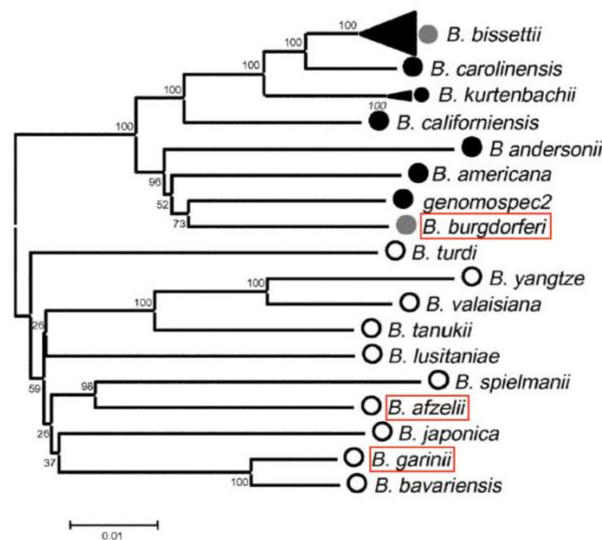


Figure 1: Neighbor joining tree extrapolated from MLSA housekeeping genes. Black dots indicate occurence in North America, circels indicate occurence in Eurasia, grey dots indicate occurrence on both continents. Major pathogenic species are indicated by red fringe. (modified) (10).

However, individual infections have been reported to be caused by either *B. spielmanii*, *B. bissettii*, *B. lusitaniae* and *B. valaisiana* (8). Depending on the required environmental reservoir and the species-specific global distribution, four different hard ticks from within the *Ixodes ricinus* complex serve as hosts for LD transmitting *Borrelia. I. scapularis* serving as host for *B. burgdorferi s.s* at the Eastern Coast and *I. pacificus* at the Western Coast of the US. *I. persulcatus* in Asia and Russia transmitting *B. afzelli* and *B. garinii* and *I. ricinus* transmitting *B. afzelli, B. bavariensis, B. burgdorferi s.s. and B. garinii* in Europe (9).

1.2.2 Infectious Cycle

The life cycle of ticks from within the *I. ricinus* complex is comprised of three stages; larva, nymph and adult, with one blood meal per stage. As transovarial transmission rarely ever occurs for LD *Borrelia*, these blood meals are pivotal for acquiring *Borrelia* from an infected host (2) (3).

Infection is predominantly obtained by their first larval blood meal on either small rodent or bird species and the infection is retained during their molting (3). Prior to the nymphal feeding, the *Borrelia* do not spread the infection to other tissues of the tick and retain in the midgut. Through the nymphal feeding of the tick, the *Borrelia* replicate extensively and change from nonmotile organisms to motile ones and relocate to the salivary glands from where they are transmitted by saliva (1) (10). Feeding in the adult stage is dictated by the sex of the tick. While male ticks do not feed in their adult stage, female ticks favorably feed on larger incompetent hosts leading to the nymphal blood meal being the main cause of accidental infection of humans with increasing likelihood after a tick attachment of above 48 hours (2) (9) (10) (11). This leads to LD being considered as a zoonosis due to the fact that the *Borrelia* are acquired and maintained in a natural infectious cycle (1).

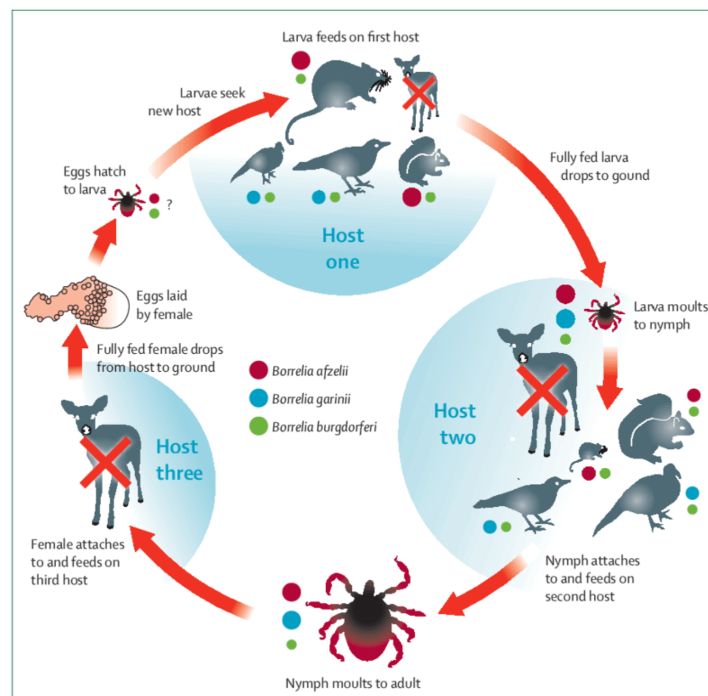


Figure 2: The natural infectious cycle of European genospecies. Size of the colored circles expresses the relative involvement of the different vertebrate reservoirs for the different genospecies. A red cross indicates non-reservoir host (12).

The fact, that *Spirochaetes* have to face various distinctly different environments through their life cycle, requires them to have a complex system of up and down regulation of their gene expression.

An example is a modification of their outer membrane protein composition directly before and during transmission from tick to their new host. As the pH is lowered and temperature elevates, the predominantly expressed outer surface protein (*Osp*) gene's transcription is altered, leading to a change from *OspA* to *OspC* (13). *OspC* is upregulated during mammalian infection, as the mentioned condition changes are observed during that period of transmission (13).

1.3 Genome of *Borrelia*

The genome of *Spirochaetes* from within the *B. burgdorferi s.l.* complex is unique among prokaryotes in terms of its complexity, as it is composed of a linear chromosome and a varying number of linear plasmids (lp) and circular plasmid (cp).

The linear chromosome is between 910 and 920 kilo base pairs (kbp) long mostly containing housekeeping genes not unique to *Borrelia* and inserts at the lower end of prokaryotic chromosome sizes (580-9300 kbp (14)) (15).

The opposite refers to the plasmid part of the genome. LD *Borrelia* contain the largest number of plasmids among known prokaryotes, varying in their size and a significant part of the encoded genes being unique for *Borrelia* (16) (3). However, the presence of particular plasmids is depending on the *Borrelia* strains and the degree of cultivation the culture has undergone (16). For *B. burgdorferi* strain B31, the plasmids range from lp5 - lp56 and cp9 - cp32, while it is carrying several copies of cp32 (1-9) and lp28 (1-4) (17). The number is referring to the relative molecular weight of the plasmids in kbp (17). Only 8% of the expected genes located on the 21 plasmids of *B. burgdorferi* B31 coincide with genes from other bacteria (15). The presence of particular plasmids, is varying among and within species; compared to strain B31, *B. afzelli* strain PKo and ACA-1 contain 7 and 4 copies of cp32 respectively (18).

The high redundancy and variation of genetic material specifically on the plasmids is part of the genome's unique properties; aspects like linear replicons , large tracts of directly repeated short DNA sequences and the evidence for numerous and recent exchanges of DNA among plasmids are unusual and remain a target for research as they have not been completely understood (19) (15). Another aspect corresponding to the periplasmic flagella mentioned in section 1.2, is the importance *Borrelia* emphasize on motility and chemotaxis as virulence factors. More than 6% of the linear chromosome are connected to motility and chemotaxis. *Borrelia* are equipped with the most redundant set of the chemotaxis gene *che (cheA, cheB, cheR, cheW and cheY*) in the whole eubacteria genus (19). This high redundancy might be of

need for the *Borrelia* in order to function at the different environments they face, mentioned in section 1.2.2.

1.4 Genetic Manipulation of *Borrelia*

Although it has been over 30 years since the original isolation of *B. burgdorferi*, the development of successful genetic tools for manipulation of the borrelial genome is conscientious, but significant progress has been made. The first successful transformation of a *Borrelia* species was conducted in 1994 in *B. burgdorferi* B31 by *Samuels et al.* This transformation included insertion of coumermycin resistance via electroporation (20).
In spite of the fact that coumermycin was identified to be an ineffective marker and has consequently been replaced by other antibiotic resistance genes, the basic protocol for transformation remains the same (1). Since 2000, different research groups introduced erythromycin, streptomycin, kanamycin and gentamycin as antibiotic selective marker by creating different cloning vectors (21) (22) (23) (24). Alongside the use of these selectable markers, these attempts revealed central new aspects important for the use of shuttle vectors as potential tools for genetic manipulations in *Borrelia* species.
A different approach for genetic modification is transposon mutagenesis, which is described in further detail below.

1.4.1 Green Fluorescent Protein

The Green Fluorescent Protein (GFP) was discovered in 1961 as a by-product of aequorin from the jellyfish *Aequorea victoria* and since then has developed into an important protein marker in molecular biology (25) (26).
GFP was first introduced into *B. burgdorferi* B31 via an exogeneous plasmid in 2000 alongside erythromycin resistance as part of the aim to develop the wide-range plasmid pGK12 as basis for cloning and the development of additional molecular genetic systems in *Borrelia* (21). Successful introduction of GFP into B31 without the use of exogenous DNA was accomplished by using pCE310, a shuttle vector based on the remarkable stable cp32. Creation of the shuttle vector was based on autonomous replication and the GFP was cloned into the vector once with and once without the *B. burgdorferi flab* promoter (27).
The previous described methods did involve a specific purpose of GFP in terms of monitoring gene expression and were additionally conducted to present the ability of introducing and using GFP as a marker for gene expression as part of shuttle vector. Applications of GFP for monitoring gene expression in strain B31, were conducted by transcriptionally fusing GFP to

promoters controlling the expression of *OspA, OspC* and *flaB*. These constructs were consequently cloned into the shuttle vector pBSV2 and monitored for their expression in response to pH and temperature changes (28). Besides monitoring gene expression, GFP was used for tracking of *B. burgdorferi 297* within their tick-vector during the nymphal feeding. A construct of GFP and a gentamycin-resistance cassette, promoted by *flaB* and *flgB* respectively, was introduced via the suicide plasmid pMC1916 into the endogenous cp26 by a double cross-over event (10).

The methods for the use of fluorescence proteins in *Borrelia* recently developed, provide a new opportunity alongside more classical molecular tools for monitoring different processes inside *Borrelia* and at the tick-host interface.

1.4.2 Transposon Mutagenesis

Transposon mutagenesis utilizes foreign heterologous DNA for the random disruption of genetic elements by inserting the DNA into the chromosome, yielding a large number of mutants by a fast and efficient method which can be easily mapped through the transposon insertion at the gene loci (29) (30).

Transposon mutagenesis in *Borrelia* is based on highly active mutants of the *Himar1* transposable element of the *mariner* family, originally isolated from *Drosophila mauritiana* (31). Elements of the *mariner* family are useful in respect of their simplicity as they perform transposition without the need of host cofactors and their transposition is practically random, in only requiring a TA dinucleotide for target specificity (30). Specifically *Himar1* has already been in wide use as a genetic tool in a diverse set of prokaryotes (32) (33).

Various vector constructs were developed as a delivery suicide vector of the transposon (30) (34). The vector pMarGent consisting of *Himar1* fused to a *flgB* promoter, ColE1 origin of replication and a gentamycin-resistance gene, yielded transposon insertion near saturating levels and was subsequently used in non-infectious and infectious strains of *B. burgdorferi* for the identification of unknown factors affecting infectivity or growth rate (30) (35). pMarGent was further modified by the addition of kanamycin resistance as second selectable marker yielding the vector pGKT (31). This modification significantly increased the vector stability and allows simple extension of characteristics for future applications (for example addition of signature tags) (31).

2 Aims

2.1 Gene BB_0267

- Construction of a shuttle vector for complementation of a transposon mutant in the *B. burgdorferi* B31background (gene BB_0267) using Gibson Assembly

2.2 GFP

- Generation of a *B. afzelii* infectious clone constitutively expressing GFP by creating a suicide construct using Gibson Assembly and transformation of the strain CB43

2.3 Plasmid Screening

- Plasmid screening of fluorescently labeled *B. burgdorferi 297* after passaging

2.4 Swarm motility assay

- Swarm motility assay of *B. burgdorferi* B31 transposon mutants

3 Materials and Methods

3.1 *Borrelia* strains and cultivation

Depending on the specific project, either a low-passage, infectious strain B31-A3 (*B. burgdorferi s.s.*) and its transposon mutants, or strain *297* of *B. burgdorferi s.s* were used. For cultivation, a pipette tip was used to scratch pieces of solid glycerol stock, stored at - 80°C, which were added to 7 mL of either liquid in-house produced Barbour-Stoenner-Kelly (BSK) II or in liquid BSK-H (Sigma-Aldrich), both enriched with 6% rabbit serum (RS). *Borrelia* were allowed to multiply at 34°C for 1 ½ to 2 weeks to reach an optimal density of 10^7 *Borrelia*/mL.

3.2 Genomic DNA isolation

Genomic DNA (gDNA) was isolated from 7 mL of *Borrelia* at optimal density. Optimal density was determined via the Marienfeld-Superior counting chamber (depth = 0.1 mm, 0.0025 mm^2). When high density was expected, the cultures were diluted 1:10 (50 µL culture:450 µL media) and 3µL of the solution were applied onto the counting chamber. The number of *Borrelia* inside 5 squares (x) were counted and transformed to *Borrelia*/mL according to Equation 1.

Equation 1: Calculation of concentration of *Borrelia* per 1 mL, 10^7 is taking a 1:10 dilution into account.

$$number\ of\ \left[\frac{Borrelia}{mL}\right] = \frac{x}{5} * 1.25x10^7$$

For gDNA isolation, a commercially available isolation kit was used, and isolation was performed according to the protocol provided by the manufacturer (Wizard®Genomic DNA Purification Kit, Promega).

For isolation, *Borrelia* cultures were transferred into a 15 mL plastic tube, vortexed, and centrifuged for 10 min at 8,000 rpm and 20°C. The supernatant was decanted and 600 µL of Nucleic Lysis Solution were added for resuspending the pellet. The resulting suspension was transferred into 1.5 mL Eppendorf tubes and vortexed for 1 min. Lysis of cells was induced by incubation of the tubes at 80°C for 5 min. Once the tubes cooled to room temperature, 3 µL of RNase were added and the solution was carefully mixed by inverting the tubes several times. After incubation at 37°C for 15 min and subsequent cooling to room temperature, 200 µL of Protein Precipitation Solution were added and carefully mixed. The sample was

cooled on ice for 5 min, followed by centrifugation for 10 min at 13,000 rpm and 10°C. The obtained supernatant was transferred to a tube containing 600 µL of isopropanol and mixed by gentle inversion of the tube. After centrifugation with the same settings, the supernatant was decanted, where it was ensured that as much supernatant as possible was removed. To the residue, 600 µL of room-temperature 70% ethanol was added and centrifuged with the same settings as above. The supernatant was carefully removed using a pipette, absorbent paper and evaporation. The final product was dissolved in 100 µL of DNA Rehydration Solution and stored in the fridge at 4°C.

3.3 Gel electrophoresis

Based on the number of samples, either 1 g or 2 g of Agarose (Sigma-Aldrich) was weighed in on a balance and dissolved in 100 mL or 200 mL, respectively, of 1x Tris-acetate-EDTA (TAE) buffer. The suspension was heated until all particles were dissolved and allowed to cool for 10 min. During cooling, 2,5 µL/100 mL of GelRed (Biotium) was added for visualization of DNA within the gel. After 10 min, the solution was poured in the gel caster and allowed to solidify for 30 min. The solid gel was placed into the gel electrophoresis chamber which was filled up with 1x TAE buffer. The respective wells of the gel were filled with 8 µL of 1 Kb Plus Ladder (InvitrogenTM) and 10 µL of sample. Separation was performed at 120 V for 30 or 60 min depending on the gel size. For image analysis following the gel electrophoresis, the ChemiDoc MP Imaging System (Bio-Rad) was used.

3.4 Design of Primers

Designing primers was based on the *B. burgdorferi* B31 sequence submitted to NCBI by *Fraser et al.* in relation to their paper “Genomic sequence of a LD spirochaete, *Borrelia burgdorferi*” (NCBI Accession Number: AE000783.1) (19). For primer design, the free primer3 software, NCBI’s primer-BLAST and NEBuilder® Assembly Tool (v2.2.7) were used. Primers were ordered from Generi Biotech. After receiving, the primer pellet was dissolved in Milli-Q H_2O were ½ of the volume stated on the description of each tube was used. To obtain the working solution, the primer solution was diluted 1/10 in Milli-Q H_2O to acquire a final volume of 200 µL with a concentration of $2x10^{-5}$ µm/µl of the primer. The working solution was stored at 4°C and the stock solution at -20°C.

3.5 Construction of a shuttle vector for complementation of a transposon mutant in the *B. burgdorferi* B31 background (gene BB_0267)

3.5.1 Restriction Digestion and Ligation of BB_0267 and flg

For determination of whether the phenotypic difference of the transposon mutant S4-7 obtained by Dr. Rego (unpublished work) compared to its parental strain is due to disruption of the BB_0267 gene locus or not, the gene was to be added back to S4-7 by complementation. A restriction digestion was used in order to create sticky ends at gene BB_0267 and the *flg* promoter for ligation according to Table 1. The gDNA used, was provided by Nora Hagleitner which she isolated in course of her master thesis (36). Once the reaction mixture was prepared, it was incubated for 1 hour at 37°C.

Table 1: Reaction mixture for restriction digestion of BB_0267 and *flg* promoter. Abbreviation: NEB = New England Biolabs.

Reagents	**Volume / µL**	
	BB_0267	**flg**
Enzymes (NEB)	**SalI, ClaI**	**XmaI, ClaI**
1 µg of DNA	4.50	2.70
Enzymes	1 of each	1 of each
10x CutSmart Buffer (NEB)	3.00	3.00
Milli-Q H2O	21.5	23.3

After incubation, two ligations were performed with different gene to promoter ratios according to Table 2. The two reaction mixtures were incubated overnight at 16°C.

Table 2: Reaction mixture for ligation of BB_027 and *flg* promoter.

Reagents	**Volume / µL**	
	BB_0267 : flg (1:3)	**BB_0267 : flg (3:1)**
BB_0267	2.5	7.5
Flg	7.5	2.5
T4 DNA Ligase Buffer (NEB)	2	
T4 DNA Ligase (Invitrogen)	1	
Milli-Q H_2O	7	

3.5.1.1 Complementation into pCRTM 2.1-TOPOTM vector

The ligation products were directly complemented into pCRTM 2.1-TOPOTM vector (Invitrogen) without checking their successful ligation by agarose gel electrophoresis. The reaction mixture was prepared according to Table 3 and incubated for 30 min at room temperature.

Table 3: Reaction mixture for complementation of flg-BB_0267 fragment into pCRTM 2.1-TOPOTM vector.

Reagents	Volume / µL
Ligation product	4
Salt solution	1
Milli-Q H_2O	1
pCRTM TOPOTM 2.1 vector	1

3.5.1.2 Transformation into NEB® 5-alpha Competent *E. coli*

A tube containing NEB® 5-alpha Competent *E. coli* (High Efficiency) cells was removed from the -80°C freezer and thawed on ice. 50 µL of the thawed *E. coli* cells were then transferred to a 1.5 mL Eppendorf tube. 1.5 µL of the complementation reaction was added to the Eppendorf tube and allowed to rest on ice for 30 min. Afterwards, the reaction mixture was heat shocked for 30 s at 42°C in the heating block and immediately placed on ice. After an incubation of 2 min, 250 µL of SOC Outgrowth Medium (NEB®) was added. The mixture was shaken at 37°C and 250 rpm for 60 min.

3.5.1.3 Plating of *E. coli*

Plating was performed on two carbenicillin plates per sample, which were warmed in the incubator at 30°C for 90 min prior to the plating. After cooling at RT for 5 min, 40 µL of XGal solution (Thermo ScientificTM) was spread on the plates using a sterile glass spreader and was allowed to air dry for 10 min. The sterile glass spreader was then used to spread 75 µL and 150 µL of each *E. coli* solution on the respective plates. Once solidified, the plates were transferred to the incubator and remained there at 37°C overnight.

3.5.1.4 Plasmid DNA isolation of *E. coli*

In preparation for plasmid DNA isolation, 5 white *E. coli* colonies of each plate were picked, dissolved in 7 mL liquid Lysogeny Broth (LB) media and allowed to multiply at 37°C while shaken at 250 rpm overnight.

For the plasmid DNA isolation, a commercially available isolation kit was used, and isolation was performed according to the protocol provided by the manufacturer (NucleoSpin® kit, Macherey-Nagel).

The tubes containing the *E. coli* cells in LB media were centrifuged for 30 s at 11,000 rpm, and the supernatant was discarded. For cell lysis, 250 µL of Buffer A1 and Buffer A2 were added successively. After addition of the latter, the solution was mixed by inversion of the tube several times, and incubated at room temperature for 5 min. Subsequently, 300 µL of Buffer A3 were added and mixed thoroughly by inversion of the tube until blue residues turn completely colorless. This suspension was centrifuged for 5 min at 11,000 rpm. The clear supernatant was transferred into a NucleoSpin® Plasmid/Plasmid (NoLid) Column, which was placed in a 2 mL Collection Tube. After centrifugation for 1 min at 11,000 rpm, the flow through was discarded and the silica membrane was washed with 600 µL of Buffer A4 by centrifugation for 1 min at 11,000 rpm. In order to dry the silica membrane, the empty column was centrifuged for 2 min at 11,000 rpm. For elution of the DNA, the Collection Tube was exchanged by an Eppendorf Tube and 50 µL of Buffer AE were added onto the membrane, incubated for 1 min at room temperature and centrifuged for 1 min at 11,000 rpm. The obtained plasmidDNA was stored in the fridge at 4°C.

3.5.1.5 Restriction digestion of the flg-BB_0267 fragment

A restriction digestion was performed to remove the flg-BB_0267 fragment from the plasmid in order to check for the correct size via gel electrophoresis according to chapter 3.3. The reaction mixture was prepared as stated by Table 4 for each tube and incubated for 60 min at 37°C.

Table 4: Reaction mixture for restriction digestion of flg-BB_0267 fragment.

Reagents	Volume / µL
plasmidDNA	4.0
Enzyme: EcoRI (NEB)	1.0
Buffer: 10 samples with 10x CutSmart Buffer (NEB) 10 samples with 10x FastDigest Buffer (Thermo Scientific™)	2.0
Milli-Q H_2O	13

3.5.1.6 Sequencing

In order to prepare a sample for sequencing, one tube with 5 µL of the DNA sample and 5 µL of forward primer and a second tube with the same amount of DNA sample and 5 µL of the reverse primer were prepared. Samples were sequenced by eurofins Genomics.

3.5.2 Gibson Assembly of BB_0267 and fla promoter

To increase the chance of successful joining of the two fragments, Gibson Assembly was performed. To facilitate the incorporation into the pCRTM TOPOTM2.1 vector (Invitrogen), an overhang (AATTCGGCTT) complementary to the pCRTM TOPOTM2.1 vector (Invitrogen) was added to the forward primer of the *fla* fragment and the reverse primer of the BB_0267 fragment. On the second primer of each fragment, a 10-15 base pair long overhang of the other fragment was added.

Table 5: Primers and their corresponding sequence for the Gibson Assembly of *fla* promoter and the gene BB_0267.

Primer	**Sequence (5‘ to 3‘)**
Forward fla	AATTCGGCTTATGAGGGAAGCGGTGATC
Reverse fla	TGTCATCCATTTATTTGCCGACTACCTTGG
Forward BB_0267	CGGCAAATAAATGGATGACAGGGCTATAAATTTTTC
Reverse BB_0267	AATTCGGCTTTCACCTAGACTTAAAAGGAATATAG

Primers were prepared according to chapter 3.4 and the correct design of the primers was assessed using PCR and gel electrophoresis according to chapter 3.3.

The DNA fragments with the desired lengths were isolated using the QIAquick PCR Purification Kit (Qiagen). Extraction was performed according to the manufacturer’s manual. NEB is stating, that a PCR clean-up step is not required, however, in order to increase the chance of success, one Gibson Assembly was performed on the base of the fragments from the gel extraction, and a second one was performed directly with the PCR product. For the reaction, the Gibson Assembly® Cloning Kit (NEB) was used.

Table 6: Reaction mixtures for Gibson Assembly of TOPO vector and fla/BB_0267 fragments.

Reagents	**Volume / µL**		
	After Gel Extraction	**After PCR**	**Positive Control**
pCRTM TOPOTM 2.1 vector (Invitrogen)	2.0	3.0	10

Fla	4.0	3.5	
BB_0267	4.0	3.5	
Gibson Assembly Master Mix (2x)	10	10	10
Total Volume	20	20	20

Once the samples were prepared, they were incubated in the heating block at 50°C for 15 min. 2 µL of each reaction was transformed into NEB® 5-alpha Competent *E. coli* (High Efficiency) cells according to chapter 3.5.1.2; with the deviation, that 950 µL of room temperature SOC medium was added to each tube. For plating, 50 µL of XGal (Thermo Scientific™) was applied on the selection carbenicillin plates followed by 100 µL of the cells. For the positive control, ampicillin plates were used.

3.5.3 QuantitativePCR to examine the different gene expression of *B. burgdorferi* transposon mutant S4-7 and *B. burgdorferi* wild-type B31

3.5.3.1 RNA isolation of S4-7 transposon mutants and wild-type B31

RNA was isolated from two cultures in BSK H and BSK II for the mutant as well as for the wild type. For the isolation, the NucleoSpin RNA/Protein Mini kit for RNA and protein purification (Macherey-Nagel) was used. RNA isolation was performed according to the manufacturer's manual.

For cell lysis, 350 µL of Buffer RP1 and 3.5 µL β-mercaptoethanol were added to the bacteria pellet. The lysate was filtered by transferring the lysate into a NucleoSpin® filter, which had been placed into a Collection Tube, and centrifugation for 1 min at 11,000 rpm. Subsequently 350 µL of 70% ethanol were added to the Collection Tube and gently mixed by pipetting. This solution was loaded into a NucleoSpin® RNA/Protein Column and centrifuged for 30 s at 11,000 rpm. For desalination of the silica membrane containing the RNA, 350 µL MDB were added and centrifuged for 1 min at 11,000 rpm. To remove residual DNA, a rDNase reaction mixture containing 10 µL of rDNase and 90 µL Reaction Buffer for rDNase was prepared in a sterile tube. 95 µL of this mixture were transferred onto the silica membrane and incubated for 15 min. The RNA on the silica membrane was washed 3x successively with 200 µL Buffer RA2, 600 µL Buffer RA3, and 250 µL Buffer RA3, with centrifugation for 2 min at 11,000 rpm in between each wash. To elute the RNA, 60 µL of RNase-free water were applied onto the silica membrane and centrifuged for 1 min at 11,000 rpm, and the resulting solution

was stored at 4°C. After the isolation, the RNA concentration was determined using the NanoDrop 1000 (Thermo Scientific).

3.5.3.2 Reverse transcription for the generation of cDNA

Reverse transcription was performed for S4-7 grown in BSK II and for both wild type samples based on the measured RNA concentrations. For reverse transcription, the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) was used according to the protocol provided by the manufacturer.

The volume of RNA added to the mix was calculated in order to contain 1 µg of RNA for the transposon mutant, and 0.5 µg of RNA for the wild type. The RNA-primer solution was prepared according to Table 7, and the structure was denatured by heating the sample at 65°C in a block cycler with a heated lid. After 10 min, the tube was removed and immediately cooled on ice. The components listed in Table 8 were added to the mixture in the order of Table 8, gently mixed and centrifuged to collect the sample at the bottom. The reaction mixture was then incubated at 55°C. After 30 min, the tube was removed and stored in the freezer.

Table 7: Composition of the 1st master mix for the reverse transcription.

<table>
<tr><td rowspan="4">S4-7</td><td>Reagent</td><td>Volume / µL</td><td rowspan="4">Wild type B31</td><td>Reagent</td><td>Volume / µL</td></tr>
<tr><td>RNA</td><td>6.4</td><td>RNA</td><td>7.3</td></tr>
<tr><td>Primer</td><td>1.0</td><td>Primer</td><td>1.0</td></tr>
<tr><td>RNase-free H_2O</td><td>4.0</td><td>RNase-free H_2O</td><td>3.1</td></tr>
</table>

Table 8: Composition of the 2nd master mix for the reverse transcription, same volumes for both samples.

Reagents	Volume / µL
Reaction buffer	4
Protector RNase inhibitor	0.5
DNTPs mix	2
DTT (Dithiothreitol)	1
Transcriptor High Fidelity Reverse Transcriptase	1.1

3.5.3.3 QuantitativePCR with TaqMan probe

For examination of the different gene expression of the transposon mutant S4-7 and the B31 wild type, the TaqMan probe approach for quantitativePCR (qPCR) was used. Specifically, the expression of BB_0267 in the mutant and *fla* in B31 was examined. Primer and the probe were designed using the Real-time PCR (TaqMan) Primer and Probes Design Tool from GenScript® (37), ordered from generi biotech and are summarized in Table 9. Correct design of the primers was examined by PCR and gel electrophoresis. Reaction mixtures were prepared on ice according to Table 10, the probe was shielded from light to ensure ideal fluorescent properties and transferred on a 96-well plate. For the mutant and wild type, identical triplets were analyzed alongside a negative control. qPCR was performed on the LightCycler® 480 (Roche) with two different cycler setting summarized by Table 11, in both attempts, amplification underwent 50 cycles.

Table 9: Primers and their corresponding sequence for the qPCR of fla promoter and the gene BB_0267.

Primer	Sequence (5' to 3')
Fla (Amplicon Size = 169 bp)	
Forward primer	TTTCAGGGTCTCAAGCGTCT
Reverse primer	GCTCCTTCCTGTTGAACACC
Probe	ACTGCTCAGGCTGCACCGGT
BB_0267 (Amplicon Size = 124 bp)	
Forward primer	TGTTGGACCTGCTACTGGAG
Reverse primer	GCACCGACCCACTAATCCTT
Probe	ACATTGCCCTGCACAAGAACCATTCCA

Table 10: Reaction mixture for qPCR for measuring different gene expression in S4-7 and B31.

Reagents	Volume / µL
FastStart Universal Probe Master (ROX) (Roche)	12.50
Probe	0.125
Forward primer	0.225
Reverse primer	0.225
Sample	3.000
Milli-Q H_2O	8.925

Table 11: Cycler Settings for the LightCycler® 480 for Probe qPCR.

	1st attempt		**2nd attempt** (38)	
	Temperature / °C	Time / s	Temperature / °C	Time / s
Initial Denaturation	95	600	95	300
Amplification	95	15	95	30
	50	30	60	60
	72	20		
Cooling	40	30	40	30

3.5.3.4 qPCR with SYBR Green protocol

In order to increase the chance for a successful measurement, a protocol for the SYBR Green method was tested. Whether or not the cycler settings worked was assessed by preparing reactions for two different primer sets for the *fla* promoter. The reaction mixture was prepared according to Table 12 and transferred into individual wells on a 96-well plate. Triplets for both sets were analyzed, and a negative control was used for each. The same cycler was used as in 3.5.3.3, with the settings from Table 13. 50 cycles of the amplification step were performed.

Table 12: Reaction mixture for qPCR using the SYBR Green method.

Reagents	**Volume / µL**
LightCycler® FastStart DNA Master SYBR Green I	12.50
Forward primer	0.225
Reverse primer	0.225
Sample	3.000
Milli-Q H_2O	10.00

Table 13: Cycler Settings for the LightCycler® 480 for SYBR Green qPCR.

	Temperature / °C	**Time / s**
Initial Denaturation	95	600
Amplification	95	5
	62	5
	72	8

Cooling	40	30

3.6 Swarm Motility Assay of B31 transposon mutants

A swarm motility assay (SMA) was used to examine the difference in motility of the transposon mutants described in 3.1. The cultures were prepared in 7 mL of BSK-H + 6% RS.

3.6.1 Preparation of Reagents

3.6.1.1 10x CMRL-1066

For the preparation of 10x CMRL-1066, 9.741 g of its solid form (Sigma-Aldrich) were weighted in and completely dissolved under stirring in 80 mL Milli-Q H_2O. 2.2 g of $NaHCO_3$ (Sigma-Aldrich, solid) were added to the solution and stirred. 20 mL of Milli-Q H_2O were added, the resulting solution was stirred, filtered and placed at 4°C in the fridge until application.

3.6.1.2 5% Na_2CO_3

5 g of Na_2CO_3 (Sigma-Aldrich, solid) were dissolved in 100 mL of Milli-Q H_2O under stirring and placed at 4°C in the fridge until application.

3.6.1.3 1.7% plating agarose and BSK 1.5 + RS

3.5 g of plating agarose were dissolved in 200 mL of Milli-Q H_2O under stirring and sterilized by autoclaving. In-house prepared BSK 1.5 and RS were removed from the -20°C freezer and allowed to thaw. Once thawed, 68.4 mL of BSK 1.5 were mixed with 3.6 mL RS.

3.6.1.4 Culturing of mutants

All bacterial cultures of the transposon mutants from Table 14 were counted using the Marienfeld-Superior counting chamber and enumerated using Equation 1 from chapter 3.2. All *Borrelia* were accumulated by centrifuging the culture for 20 min at 4,000 rpm and 25°C. The supernatant was drained in order to obtain a concentration of 2×10^5 cells/µL by gentle resuspending of the bacterial pellet in the appropriate amount of BSK 1.5 using a pipette.

Table 14: All bacterial cultures used for the SMA.

Mutant	Numbering of glycerol stock
S4	1-10, 12 13, 15, 16
S4-LC	1-4

3.6.2 Plating of B31 transposon mutants

The reagents mentioned above were prepared for 20 plates and mixed according to Table 15. The reagents were mixed in order of Table 15 in a 1000 mL bottle that has been sterilized by autoclaving, and subsequently heated to 50°C in a water bath. 35 mL of the mixture was transferred into each plate and allowed to solidify with open lid in the fume hood under laminar air flow for 10 min 5 µL of the BSK 1.5-*Borrelia* mixture was pipetted just below the surface without the tip touching the bottom of the plate. Each mutant was pipetted four times on each plate. After additional 10 min of air drying in the fume hood, the plates were closed and incubated at 34°C for two weeks in airtight boxes with anaerobic sachets (GasPak™). After the incubation period, the diameters of each colony were determined using the ChemiDoc MP Imaging System (Bio-Rad).

Table 15: Mixture of plating media for the SMA of B31 transposon mutants.

Reagents	Volume / mL
BSK 1.5 + RS	72.0
5% Na_2CO_3	28.0
10x CMRL-1066	46.0
1x Dulbecco's PBS (Sigma-Aldrich)	432
1.7 % plating Agarose (heated to 50°C before addition)	144

3.7 Plasmid screening of green fluorescent *B. burgdorferi 297* after passaging

The clones in Table 16 are the result of passaging 23x GFP expressing *B. burgdorferi* strain 297. 10 out of 25 clones were chosen for an investigation of their plasmids after expressing morphological changes under the dark field microscope, all conducted by Nora Hagleitner as part of her master thesis (36). The profile was based on using the Purser-Norris primers for *B. burgdorferi* strain B31-A3 (39). As pointed out in chapter 1.3, different strains can contain a different number of plasmids, particularly of the plasmid cp32, which can be present in up to nine copies (1). While these plasmids are genetically nearly identical, some gene loci vary giving each copy a unique and important structure (1). In this strain, it was screened for the copies 1-4 and 6-9 of cp32 and not for cp32-5, because no primers were available.

Table 16: The used clones and the corresponding plasmids which were screened for.

Clones	7, 8, 11, 15, 18, 22, 23, 25
Circular plasmids (cp)	9, 26, 32 (all copies but cp32-5)
Linear plasmids (lp)	17, 21, 25, 28 (1-4), 36, 38, 54, 56, 25

3.7.1 PCR assay for plasmid screening

The available primers were used for screening of their corresponding plasmids. For verification, gDNA of *B. burgdorferi* strain B31 was used as a positive control and Milli-Q H_2O as a negative control for each plasmid. For lp28-1 one additional primer set was used for verification, for lp25, a total of three primer sets were used. The reaction mixtures were prepared on ice according to Table 17 with deviations for the positive and negative control. For the positive control, only 0.5 µL of DNA and 7,5 µL of Milli-Q H_2O were used because of the high concentration of the DNA sample and for the negative control 8 µL of Milli-Q H_2O were used.

Table 17: Composition of the PCR reaction mixtures for the plasmid screening.

Reagents	**Volume / µL**
OneTaq® Quick-Load® 2x Master Mix with Standard Buffer (NEB)	10
Forward primer	1
Reverse primer	1
DNA	2
Milli-Q H_2O	6

For the PCR, 30 cycles of segment 2 - 4 were performed with the settings summarized by Table 18. After the PCR, results were examined either on an agarose gel according to chapter 3.3, or on a fast gel running at 220 V for 15 min.

Table 18: PCR settings used for plasmid screening of 297 clones.

Segment		**Temperature / °C**	**Time / min**
1	Initial Denaturation	95.0	05:00
2c	Denaturation in cycle	94.0	01:00
3c	Annealing in cycle	57.0	01:00

4c	Elongation in cycle	72.0	01:00
5	Final Elongation	72.0	10:00
6	Hold	16.0	--:--

3.7.2 Swarm motility assay (SMA)

SMA was used to examine the difference in motility of the clones mentioned in Table 16. The preparation of cultures for the SMA as well as the procedure was conducted in the same way as described in Chapter 3.6.1 and 3.6.2, with the deviation that the plates were incubated for 8 days. For this assay, only 10 plates were required, therefore the reagent volumes of Table 15 were halved.

3.8 Generation of a *B. afzelii* infectious clone constitutively expressing GFP by creating a suicide construct using Gibson Assembly and transformation of the strain CB43

3.8.1 Designing primers and checking them with Taqman and Hotstar MM

In order to join the flaGFP and flgGENT fragments using Gibson Assembly and prepare them for homologous recombination with cp26, the primer required the respective overhangs. To the forward primer of the flgGENT fragment and the reverse primer of the flaGFP fragment, the sequence of the recognition site of the AseI restriction enzyme (~ATTAAT~) was added. AseI is a two-cutting enzyme in the sequence of cp26.

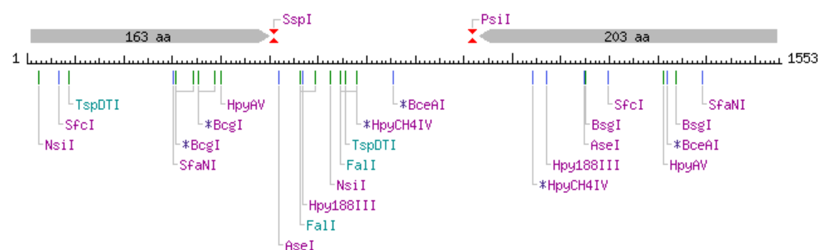


Figure 3: DNA sequence of cp26 with the respective two-cutters inclusive AseI. NEBcutter V2.0. Retrieved March 20, 2020, from http://nc2.neb.com/NEBcutter2/cutshow.php?name=98e4d549-.

On the second primer of each fragment, a 10-15 base pair long overhang of the other fragment was added.

Primers were designed and prepared according to chapter 3.4. After preparation, it was checked if the primers lead to the correct DNA fragment size by PCR with three different 297

gDNA samples. The first and second gDNA differed only in quality, while the third one had GFP already introduced. The PCR reaction mixtures were prepared with OneTaq® Quick-Load® 2x Master Mix with Standard Buffer (NEB) according to Table 17. After being unsuccessful in the first run, the primers were additionally checked with the HotStarTaq® Plus Master Mix (Qiagen) according to Table 19.

Table 19: Composition of the PCR reaction mixture to check correct primer design.

Reagents	**Volume / µL**
HotStarTag® Plus Master Mix	10
Forward primer	1
Reverse primer	1
DNA	2
Milli-Q H_2O	6

The cycler was set according to Table 18 for both reaction mixtures. For validity of the results, a negative control was used. After completion of the PCR, the DNA fragments were visualized according to chapter 3.3.

3.8.2 Gel extraction

In order to purify the DNA fragments from the agarose gel, the fragments were extracted using the QIAquick Gel Extraction Kit (Qiagen). Extraction was performed according to the manufacturer's manual. The DNA fragment was cut from the gel using a clean and sharp scalpel. The fragment was weight (1 gel volume), and 3 volumes Buffer QG were added and incubated at 50°C until the gel fragment dissolved, leading to a yellow mixture. To this mixture, 1 volume of isopropanol was added and mixed. The sample was then applied onto the membrane of a QIAquick column and centrifuged for 1 min. The DNA was washed by application of 750 µL Buffer PE onto the membrane of the column and subsequent centrifugation for 1 min. For elution of the DNA, the QIAquick column was placed in an Eppendorf tube and 30 µL of Buffer EB were applied on the center of the membrane and centrifuged for 1 min.

3.8.3 Gibson Assembly

For fragment joining, Gibson Assembly was used. NEB states that for a Gibson Assembly, no PCR clean-up is required, however, in order to increase the chance of success, one Gibson

Assembly was performed on the base of fragments from the gel extraction of chapter 3.8.2, and a second one was performed directly with the PCR product after checking the correct design with the primer. For the reaction, the Gibson Assembly® Cloning Kit (NEB) was used. The amount of each fragment was calculated in order to achieve the recommended 0.02-0.05 pmols of DNA and the reaction mixtures were prepared as stated by Table 20.

Table 20: Reaction mixtures for Gibson Assembly of flgGENT and flaGFP fragments.

Reagents	**Volume / µL**		
	After Gel Extraction	**After PCR**	**Positive Control**
flgGENT	2	4	10
flaGFP	5	4	
Milli-Q H2O	3	2	
Gibson Assembly Master Mix (2x)	10	10	10

Once prepared, the samples were incubated in the heating block at 50°C for 15 min. 2 µL of each reaction were transformed into NEB® 5-alpha Competent *E. coli* (High Efficiency) cells according to chapter 3.5.1.2; with the deviation, that 950 µL of SOC medium room-temperature SOC medium was added to each tube and 100 µL of the cells were spread onto the selection plates. For the positive control, ampicillin plates were used.

4 Results

4.1 Construction of a shuttle vector for complementation of a transposon mutant in the *B. burgdorferi* B31 background (gene BB_0267)

After restriction digestion and ligation of the *flg* promoter and the BB_0267 gene, the ligated fragment was directly complemented into pCR™2.1-TOPO™ vector and subsequently transformed into NEB® 5-alpha Competent *E. coli* (High Efficiency) cells. Plating yielded white colonies conveying that transformation was successful. The obtained plasmidDNA was reprocessed by restriction digestion with EcoRI, in order to cut the ligated fragment of promoter and gene out of the pCR™2.1-TOPO™ vector. Gel electrophoresis resulted in 5 of 20 samples expressing two bands with approximately the desired sizes, however pointed out by Figure 4, the gel showed impurities with a smaller molecular mass in all 5 samples.

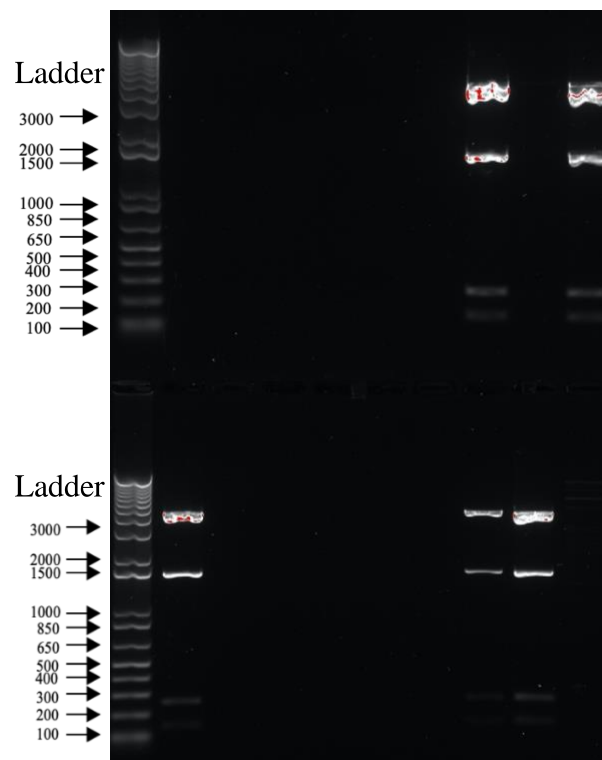


Figure 4: Agarose gel after restriction digestion of the obtained plasmidDNA. Two sample in the top row resulted from the plate with cultures containing 3:1 promoter to gene ratio, bottom row resulted from the plate with cultures containing 1:3 promoter to gene ratio. Ladder labeling in bp.

After sending these 5 samples for sequencing, only two showed similarity with *B. burgdorferi* B31 when using the Basic Local Alignment Search Tool (BLAST) of NCBI.

As this was not a clear result to judge whether or not the procedure was successful, the new strategy of joining the fragments was Gibson Assembly. For this purpose, the primers were proven to be correct by PCR as the bands showed the desired lengths, however the well were the BB_0267 fragment was administrated showed two instead of one desired band, pictured in Figure 5. The lower band must be an impurity. Therefore, the QIAquick Gel Extraction Kit was used to extract the upper band.

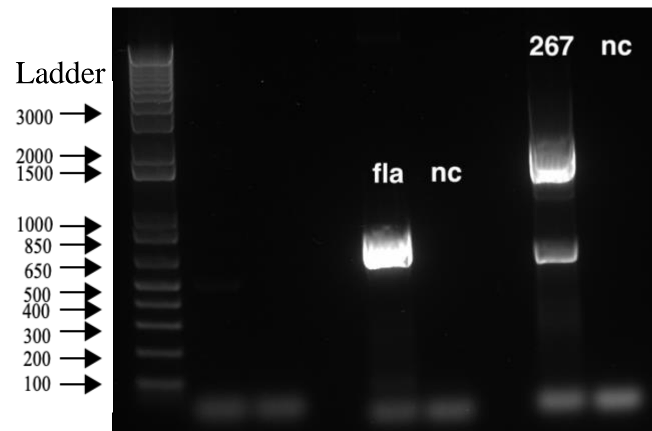


Figure 5: Agarose Gel after PCR to check for correct design of the primers. Ladder labeling in bp.

Based on these primers, Gibson Assembly was conducted as described in chapter 3.5.2 alongside a positive control. After allowing the plates to rest in the incubator, this procedure turned out to be unsuccessful for both attempts. No *E. coli* cultures had grown on the plates of the samples, but the plate of the positive control showed *E. coli* cultures, proving that the procedure was performed correctly.

4.1.1 QuantitativePCR to examine the different gene expression of *B. burgdorferi* transposon mutant S4-7 and *B. burgdorferi* wild-type B31

Primers were proven to be designed correctly by PCR and gel electrophoresis from Figure 6, but the negative control remained contaminated until the end of my project. Even after ordering the primers a second time and making sure, that the used Milli-Q H_2O was not contaminated, the origin of this contamination remained unclear.

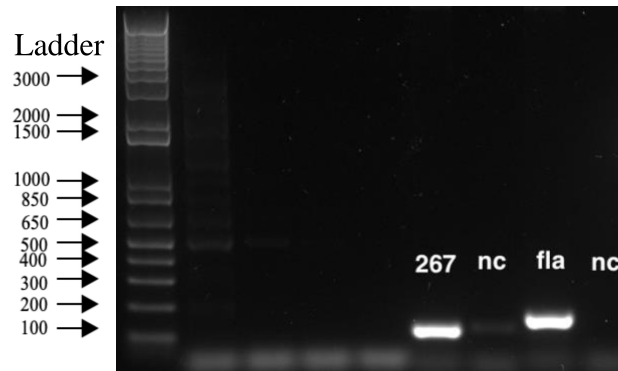


Figure 6: Agarose gel after PCR to prove correct design of the primers. First four rows from the left can be neglected. Ladder labeling in bp.

Independent of the protocol and settings used, no successful qPCR could be performed with this set of primers, therefore I was unable to determine a difference in the gene expression of the BB_0267 gene in the transposon mutant S4-7 and the *fla* promoter gene in wild type B31.

4.2 Swarm Motility Assay of B31 transposon mutants

The transposon mutants examined in this assay were created by Dr. Ryan Rego during his post doc. The SMA was used to determine whether the mutation disrupted a gene associated with motility of the *Spirochaetes* or not. Examination was based on measuring and comparing the diameters of the colonies on the plates after allowing them to replicate for two weeks at 34°C. Plating of the positive control, wild type B31-A3 was unsuccessful, but based on the results of the Swarm Motility Assay and the growth curve by Nora Hagleitner as part of her Master Thesis, an average diameter of 2,16 cm can be estimated for the wild type B31 culture after incubation of 14 days (36). The diameters of the mutant colonies are summarized by Graph 1.

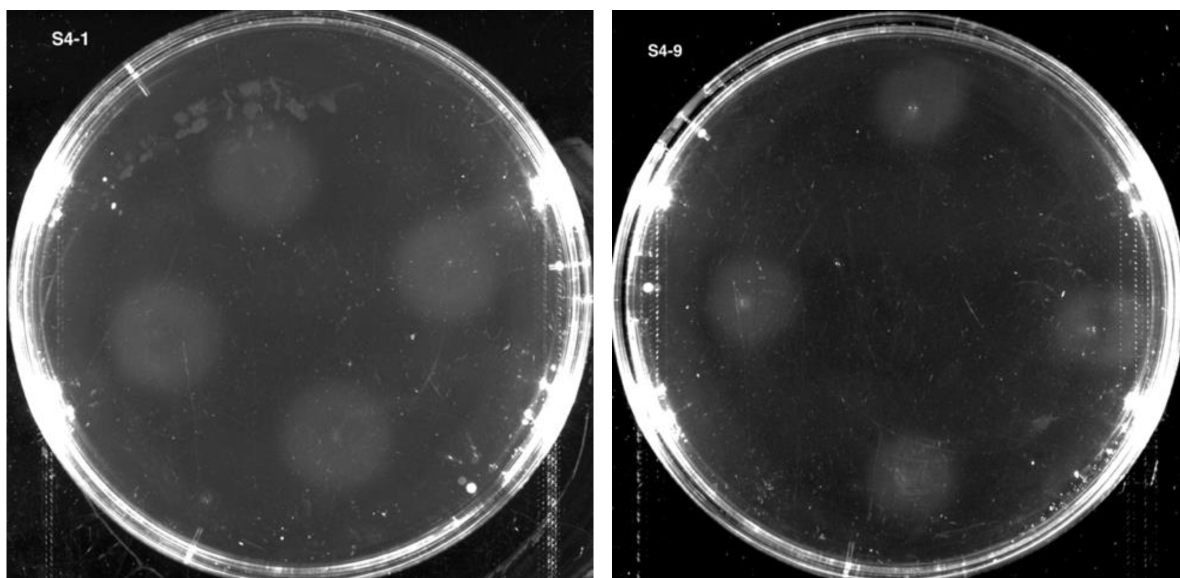
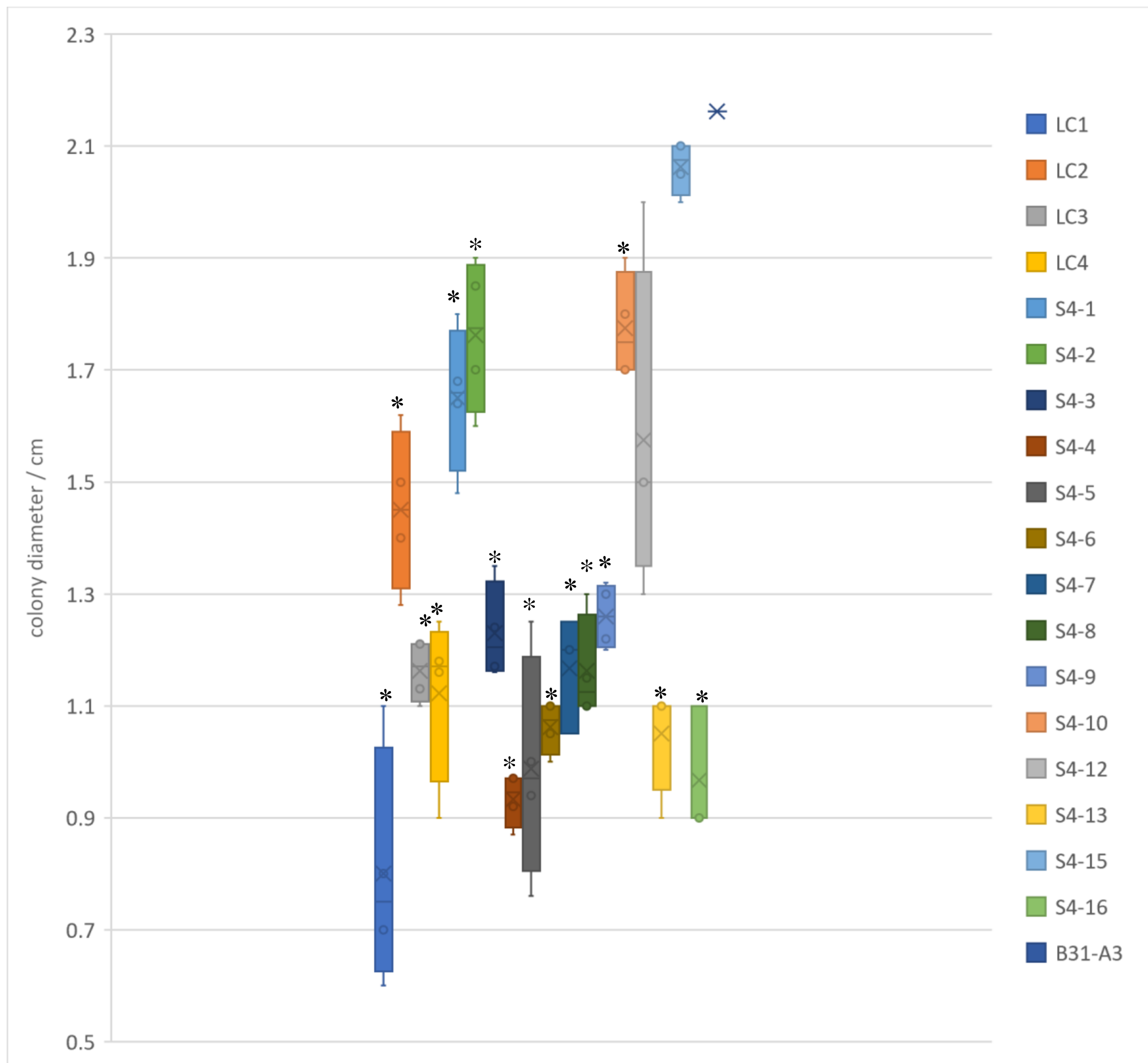


Figure 7: Plate of the SMA after an incubation period of 2 weeks. Left: Plate of S4-1 with a mean diameter of 1.65 cm. Right: Plate of S4-9 with a mean diameter of 1.26 cm.



Graph 1: Summarized mean diameter of transposon mutants alongside standard deviation bars, statistical significance was established using the Student Paired t-test and is symbolized by an asterix ($p<0.001$).

By comparing these values to the assumed value of the B31-A3 culture, it was determined that the mutation induced by transposon mutagenesis lead to the disruption of gene loci associated with the motility of the *Spirochaetes* leading in turn to a significant decrease in diameter of the spirochaete colony in 17 out of 20 clones. This is in accordance to literature, where it has been reported that disruption of genes associated with the spirochetal motility, leads to a decreased swarm motility. However, the literature mutants were created by insertion of kanamycin into the respective gene and not transposon mutagenesis (40).

4.3 Plasmid screening of green fluorescent *B. burgdorferi 297* clones after passaging

Screening for a variety of linear and circular plasmids was performed to assess the number of present plasmids after passaging. The experiment was conducted with the appropriate primers of Purser-Norris (39).

Table 21: Evaluation of plasmid screening. Plasmids with green coloring are present in the genome of the respective clone. The presence of plasmids with red coloring remains undetermined as the positive control was negative.

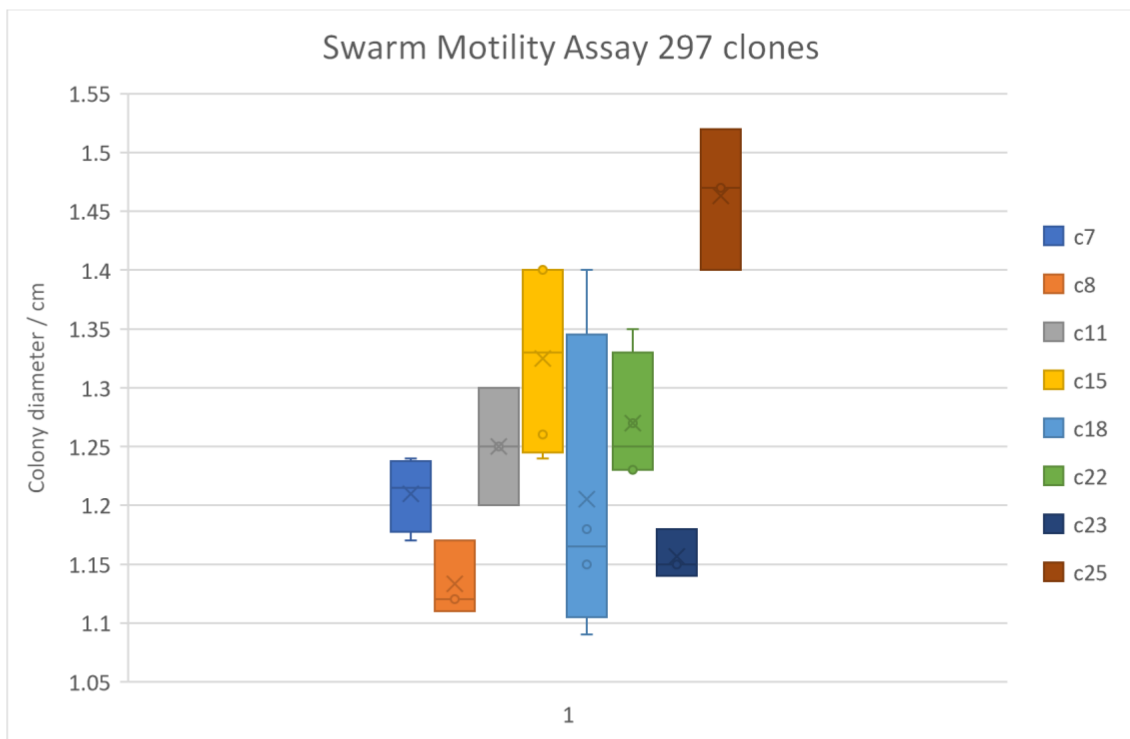
Plasmids	**pc**	**c7**	**c8**	**c11**	**c15**	**c18**	**c22**	**c23**	**c25**
Cp26	+	+	+	+	+	+	+	+	+
Cp32-1	+	+	+	+	+	+	+	+	+
Cp32-2	+	+	+	+	+	+	+	+	+
Cp32-3	-	-	-	-	-	-	-	-	-
Cp32-4	-	-	-	-	-	-	-	-	-
Cp32-6	-	-	-	-	-	-	-	-	-
Cp32-7	-	-	-	-	-	-	-	-	-
Cp32-8	-	-	-	-	-	-	-	-	-
Cp32-9	+	+	+	+	+	+	+	+	+
Cp9	-	-	-	-	-	-	-	-	-
Lp17	+	+	+	+	+	+	+	+	+
Lp21	+	+	+	+	+	+	+	+	+
Lp25	+	-	-	-	-	-	-	-	-
Lp25 (2)	+	-	-	-	-	-	-	-	-
Lp25 RR/FF	+	+	+	+	+	+	+	+	+
Lp28-1	-	-	-	-	-	-	-	-	-
Lp28-1 (2)	-	-	-	-	-	-	-	-	-
Lp28-2	-	-	-	-	-	-	-	-	-
Lp28-3	+	+	+	+	+	+	+	+	+
Lp28-4	+	+	+	+	+	+	+	+	+
Lp36	+	+	+	+	+	+	+	+	+
Lp38	-	-	-	-	-	-	-	-	-

Lp54	+	+	+	+	+	+	+	+	+
Lp56	-	-	-	-	-	-	-	-	-

Results of the screening are summarized by Table 21. All plasmids which did not express bands on the agarose gel were associated with a failure of the positive control and I am unable to ascertain whether the plasmid is not present, or the used primers do not work. For lp25, three primer pairs were used to ensure presence of the plasmid, as the first two primer sets resulted in absence of the plasmid.

4.3.1 Swarm motility assay

The motility of the clones was investigated by a SMA. Examination was based on measuring and comparing the diameters of the cultures on the plates after allowing them to replicate for eight days at 34°C.



Graph 2: Summarized mean diameter of the colonies of different 297 clones alongside standard deviation bars.

In order to assess whether or not the motility of the clones is restricted, I would rely on a working 297 positive control. However, I was unable to grow an uncontaminated culture for 297 as a positive control and hence had no positive control. I did not find a value in the literature but given that the plasmid content is equal compared to the 297 parental strain, one can assume that there is no difference and the variability between all is not significant.

4.4 Generation of a *B. afzelii* infectious clone constitutively expressing GFP by creating a suicide construct using Gibson Assembly and transformation of the strain CB43

For the Gibson Assembly, two sets of primer were tested as illustrated by Figure 8 and Figure 9.

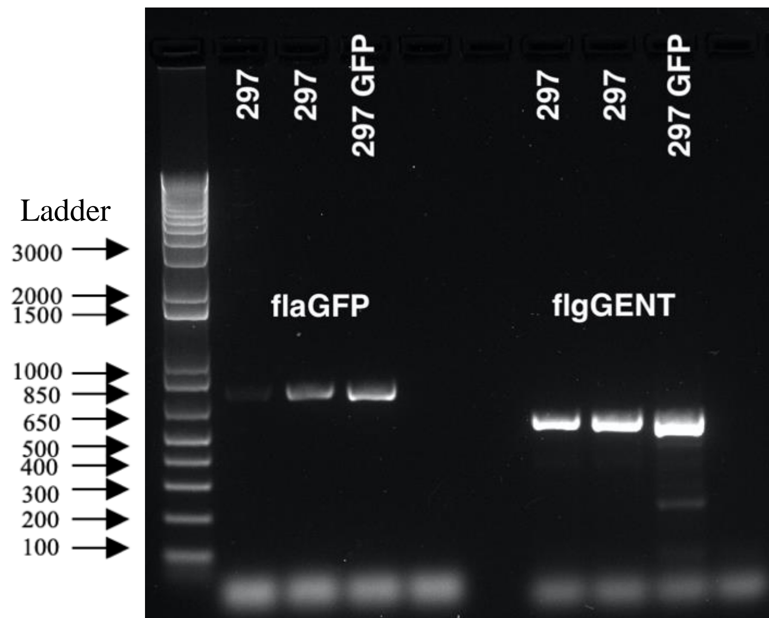


Figure 8: Test of correct design of primer set 1.

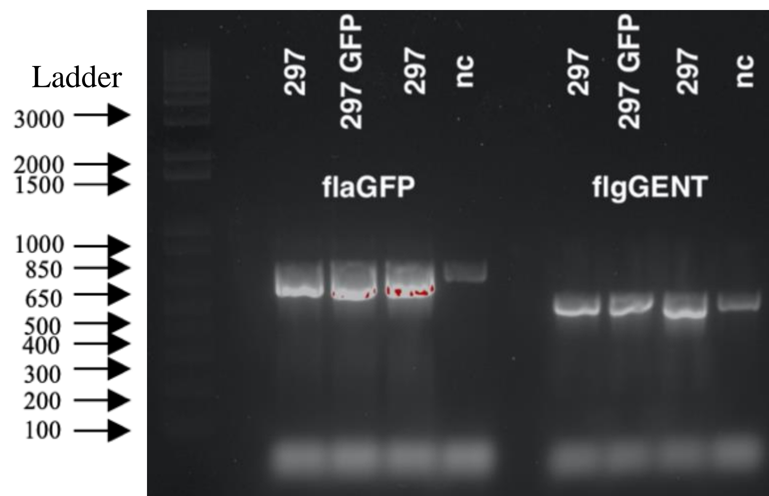


Figure 9: Test of correct design of primer set 2.

The desired fragment size would have been 717 bp for flaGFP and 534 bp for flgGENT. However, fragments from primer set 1 were of considerable bigger size. For primer set 2, it

remains unclear were the contamination in the negative control originates from and it was not further investigated due to time reasons.

The Gibson Assembly based on primer set 1 was unsuccessful, only the positive control showed *E. coli* cultures after transformation. Fragments created with primer set 2 have not yet been used for Gibson assembly due to time reasons. Their size suggests a more promising result for the Gibson assembly as they are closer to the desired size.

5 Discussion

Since the identification of *Borrelia* bacteria to be the causative agent of LD almost 40 years ago, tremendous progress has been made in the generation and application of genetic tools in order to reveal the unique structural properties of the borrelial genome. Specifically the plasmid component of the genome has been the target for many research groups (15) (16) (18) (19) (41) (42). It has been determined, that plasmid loss is connected to in vitro propagation and/or genetic manipulation of the bacteria, it is consequently necessary to screen cultures ,which have been passaged extensively, for their plasmid content. Passaging was conducted to obtain clones with a decreased number of plasmids compared to the original strain. The absence of certain plasmids expresses itself by a phenotype of the respective spirochaete. The fact that the investigated *Borrelia* express GFP, allowed using a microscope for investigation of *Borrelia* at the tick-animal interface and determination of the effect of the particular plasmid on the *Borrelia'*s ability to cause an infection. Within the context of this thesis, the plasmid content of *B. burgdorferi* strain 297 has been determined after 23x passaging. All examined clones exhibited the same number of plasmids. Specifically a screen with a different primer for lp28-1 should be repeated, as that plasmid is associated with reduced infectivity and would be a significant loss for the particular strain (43). The primers were designed for the genome of strain B31 and there may be inherent sequence differences in primer design for some of the plasmids given that the genomes are different (44). The importance *Borrelia* emphasizes on its motility is represented by the high percentage of genes associated with it compared to the overall genome (6%) (19). This importance makes motility a topic targeted by application of different genetic tools. In order to gain a better understanding of underlaying mechanisms it is important to determine whether or not particular genes are associated with motility of *Borrelia.* The gene BB_0267 is denoted as a hypothetical protein in NCBI, however has shown similarity to the flagellar assembly protein A (*FapA*) in the BioCyc Database Collection (36). *FapA* is rarely expressed in bacteria outside of the family *Vibrionaceae* and is crucial for the biosynthesis of the flagellar in these bacteria. This is emphasized by literature reports, that show a complete loss of motility for mutants of *V. vulnificus,* whose *fapA* gene has been knocked out (44). Gene locus BB_0267 is a transposon mutant which was studied by Nora Hagleitner as part of her Master Thesis, describing different morphology and motility (36). I was unable to obtain a shuttle vector that could complement the gene and check whether these morphological changes were associated with the disruption of gene BB_0267. Additionally, it was not possible to determine a difference in gene expression of BB_0267 in the mutant and in *B. burgdorferi* wild type strain B31 even after application of several different qPCR

protocols. Both experiments should be repeated in the future if time allows, specifically joining of the gene locus should be pursued in the future to be able to assign a particular function to gene BB_0267, which would in turn lead to a better understanding of mechanisms associated with motility in *Borrelia*.

A more recently developed technique for genetic manipulation ,already in extensive use in *Borrelia* species, is transposon mutagenesis (30) (31). For this experiment, mutants were created by Dr. Ryan Rego by using the pGKT vector based on a transposable element of the mariner family. The difference between these mutants and those from earlier studies is that these clones retain the lp56 plasmid while transposon mutants that have been obtained from an infectious parental strain lack plasmid lp56. All clones showed a significantly decreased diameter for their colonies obtained by the SMA compared to the parental strain obtained by Nora Hagleitner's master thesis (36) for the positive control, meaning that insertion of the transposable element occurred and lead to disruption of positions in the genome which encode information required for smooth implementation of the motility. Interpretation of how strong the motility of the *Borrelia* is restricted due to induced mutations and therefore the colony diameter, is limited, as the positive control was used from a previously conducted experiment (36). Even though the procedure was performed in exactly the same way as in the previous experiment, colonies might still show a different growing behavior. It is consequently recommended to repeat this experiment with a working positive control in order to ensure the propriety of the results obtained by this experiment.

The introduction of fluorescent properties by inserting GFP into the genome opened up new possibilities for research to exploit. Fluorescent techniques, compared to traditional staining techniques using a dye, have an advantage in not having competition for binding sites, because no dye is required as the fluorescent properties are expressed by the bacteria itself (45). This was utilized by Vancova *et al*. for evaluation of changes induced by exposing GFP-expressing *Borrelia* to physiological stress (45) (46). Changes in morphology and viability were assessed using the cryo-fluorescence and cryo-SEM approach (45). An experimental setup examining the interactions in terms of routes and mechanisms of *B. burgdorferi* and tick during their nymphal blood meal was developed by Dunham-Ems *et al.* (10). Fluorescent properties induced by incorporation of GFP into cp26 revealed that *Borrelia* maneuver through the tick during its nymphal blood meal via a biphasic model. The first phase involves nonmotile *Spirochaetes* while the second one is based on motile *Borrelia* (10). Describing these experiments supports the importance of introduction of fluorescent properties into *Borrelia* species. Building on these experiments, I was unsuccessful in producing a GFP-expressing

infectious *Borrelia* clone. Gibson Assembly of the flgGENT and flaGFP fragments was not successful. This might be explained by to short overhangs which were attached to the primers or simply wrong DNA fragments. As the fragments of primer set 2 have not yet been used for Gibson assembly, it is suggested for repetition of this experiment with these primers, as PCR results suggest a more promising Gibson assembly result.

With the advance of LD as a health threat for humans in the Northern Hemisphere and by being its causative agent, the application of known techniques and development of new methods concerning *Borrelia* should proceed. This contributes to improve the knowledge regarding borrelial properties in vector and host, which in turn is helpful for reaching the ultimate goal of developing a vaccination.

6 Literature

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