Construction and use of GFP and DsRed expressing vectors and transformation in *Borrelia afzelii*

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Annotation: The aim of this thesis was to create a GFP containing *Borrelia* shuttle vector and DsRed expressing strains of a European *Borrelia* species, *Borrelia afzelii* to further our understanding of the complex interactions of this European Lyme Disease pathogen with its hosts: ticks and mammals.

Affirmation: I hereby declare that I have worked on the submitted bachelor thesis independently. All additional sources are listed in the bibliography section. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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1. Introduction

1.1. *Borrelia* and Lyme Disease

Lyme disease is a global public health problem in the northern hemisphere, which is caused by a spirochete bacterium. The spirochetes are transmitted to mammalian hosts by ticks. The tick attaches to their host for a blood meal and by doing that, it allows the spirochetes to enter the hosts' body (Radolf *et al*., 2012). In humans, transmission of the spirochetes usually does not occur until 36 to 48 hours of the tick being attached to a human host (U.S. Department of Health&Human Services, 2015).

Borrelia is a bacterium that belongs to the phylum of spirochetes, and shares with other spirochetes some common structural features: they are helically shaped and have three modes of movement. The outer cell membrane surrounds a protoplasmic cylinder complex consisting of the inner cell membrane and the peptidoglycan. They also have flagella similar to other bacteria's flagella. However, *Borrelia's* flagella are located between the outer cell membrane and the protoplasmic cylinder complex (Barbour A.G. & Hayes S. F., 1986). Even though *Borrelia* are Gram-negative bacteria, they lack lipopolysaccharide in their outer membrane (Hyde *et al*., 2011).

The occurrence of Lyme disease in different regions is caused by different genospecies of *Borrelia* and it is, despite improvements in prevention, diagnosis and treatment, still a large problem in temperate regions of the Northern Hemisphere. Lyme borreliosis is the most-common vector-borne disease in this region with very diverse clinical manifestations that range from *erythema migrans* to severe arthritis, cardiovascular or neurological manifestations (Rudenko *et al*., 2011; Rizzoli *et al.,* 2011; Allen C. Steere, 2001). Some studies show a link between differences in clinical manifestations of Lyme borreliosis with different genospecies of *Borrelia* (Coipan *et al*., 2016).

1.2. *Borrelia burgdorferi* s. l.

Analysis of *Borrelia's* genetic material allows for the division into different subgroups. Two major phyletic groups have been established, one containing species capable of causing relapsing fever, the other containing species that are causing Lyme disease. The latter is commonly referred to as the *Borrelia burgdorferi* sensu lato (s. l.) complex (Radolf *et al*., 2012).

The *B. burgdorferi* s. l. complex contains 20 named spirochete species as well as an unnamed group referred to as Genomospecies 2 (Becker *et al*., 2016; Rudenko *et al*., 2011). Out of these 20 genospecies 3 are known to predominately infect humans: *B. burgdorferi* sensu stricto (s. s.), *B. afzelii*, and *B. garinii*. With the renaming of *B. bavariensis* – formerly thought to be part of *B. garinii* – and the discovery of *B. spielmanii* – two more species were identified as pathogenic to humans (Margos *et al*., 2009). While *B. burgdorferi* s. s. is located both in the USA as well as in Western Europe, *B. afzelii*, *B. garinii*, *B. bavariensis* and *B. spielmanii* are restricted to Eurasia (Sykes *et al*., 2014). Even though the different species are geographically distributed into certain regions, the overall distribution of spirochetes and therefore Lyme disease is thought to be expanding both towards higher altitudes and latitudes (Rizzoli *et al*., 2011).

1.3. Tick Mouse Infectious Cycle

All *Borrelia* species are transmitted to vertebrate hosts by blood-feeding arthropods (Barbour A. G. & Hayes S. F., 1986). *B. burgdorferi* s. l. is transmitted by ticks of the genus *Ixodes*. Four species of *Ixodes* ticks are prevailingly transmitters: *Ixodes ricinus* in Europe, *Ixodes scapularis* and *Ixodes pacificus* in the USA as well as *Ixodes persulcatus* in Asia (Radolf *et al*., 2012; Allen C. Steere, 2001).
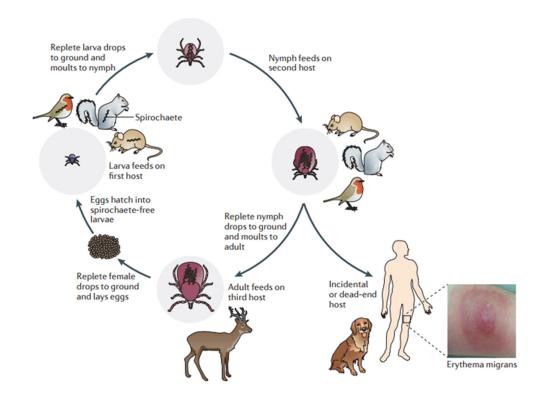

Figure 1: Graphic showing the dynamic tick-host infectious cycle of *Borrelia* at different stages of tick life cycle (adapted from Radolf *et al.,* 2012)

The ticks undergo three life stages: larvae, nymph and adults; each life stage consists of one blood meal on a vertebrate host followed by molting into the next stage. The host for each stage of tick ranges from small animals like mice, squirrels or birds to bigger species like deer and also humans. Larvae are generally uninfected as they hatch from eggs, thus the vector takes up the spirochetes by feeding on an infected vertebrate host. During the next blood meal the spirochetes are transmitted to the next vertebrate host, where other vectors can feed and obtain the spirochetes. This forms the dynamic bacteria-tick-mouse infectious cycle of *B. burgdorferi* s. l. (Radolf *et al*., 2012).

1.4. Genome of *B. burgdorferi* s. l.

The genome of *B. burgdorferi* s. l. is very unique compared to genomes of other bacteria. The first genome of a *Borrelia* species displayed a linear chromosome of approximately 900 kilo base pairs (kbp), around 12 linear plasmids (lp) as well as 9 circular plasmids (cp) ranging in sizes between 5 and 56 kbp in the strain B31 of *B. burgdorferi* s. s. (Fraser *et al.*, 1997). Even though species of *B. burgdorferi* s. l. all share this unique genome structure, the plasmid profiles show diversity among species of *Borrelia* from different geographical regions and biological sources which make the plasmid profiles of other species of the *B. burgdorferi* s. l. complex different from the plasmid profile of *B. burgdorferi* s. s. (Purser *et al*., 2000; XU *et al.,* 1996; Brisson *et al.,* 2013)

The plasmids play an important role in the genome of *B. burgdorferi,* as many important genes are located there. It is suggested that some plasmids contain genes important for survival *in-vivo*, however not important *in-vitro*, which frequently leads to loss of these plasmids in *in-vitro* studies. The loss of plasmids and thus the loss of certain genes can thereby also lead to loss of infectivity so that no infection occurs in mice and/or ticks (Grimm *et al.*, 2003).

Furthermore, it has been shown that the loss of plasmids leads to heterogeneous populations of the *Borrelia*, bearing some spirochetes that retain infectivity and others that have lost it. *B. burgdorferi* tends to lose plasmids upon passaging of isolates and plasmid stability is also affected by freezing and thawing of stocks (Grimm *et al.*, 2003).
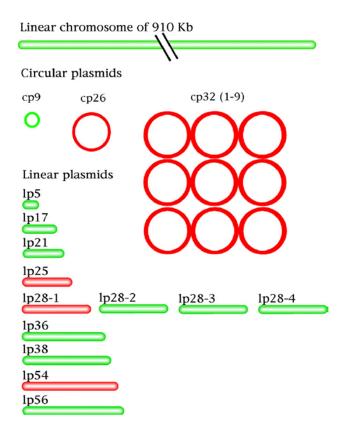
Figure 2: An illustration of the genome of *B. burgdorferi* s. s. (adapted from Stewart *et al.,* 2005)

The genetic diversity within the *B. burgdorferi* s. l. complex widened after complete genomes of different strains of *B. afzelii* and *B. garinii* were sequenced in 2011 (Casjens *et al.,* 2011). It was found that overall the linear chromosome showed a high degree of conservation within several *B. afzelii* strains and a *B. burgdorferi* s. s. strain. It was found that the sequence identity between *B. afzelii* strains, *B. garinii* strains and *B. bavariensis* is higher than the sequence identity of *B. afzelii* with *B. burgdorferi* s. s. (Schüler *et al.*, 2015).

When the plasmid number and the plasmid encoded genes of *B. burgdorferi* s. s. were compared to the plasmids of *B. afzelii* as well as *B. garinii* strains, it was found that there are significant differences. Certain plasmids and the overall gene contents on them are rather similar within all of the strains, e.g. plasmids cp26 and lp54. On the other hand it was found that some genes are found on completely different plasmids and *B. afzelii* generally has less plasmids than *B. burgdorferi* s. s.. *B. afzelii* strains have plasmids lp17, lp38, lp54, cp26 and varying numbers of cp32 and lp28, whereas plasmids like cp9, lp5, lp21, lp25 and lp36 that are all found in *B. burgdorferi* B31 are generally not present in *B. afzelii* strains.

Several important genes for infectivity, like the adenine deaminase, *adeC*, is found on lp36 in *B. burgdorferi* B31. This plasmid is not found in *B. afzelii* strains, but the *adeC* gene homolog is found to be on lp38. Another gene needed for infectivity in mice, *pncA*, is found on lp28-2 for *B. afzelii* strains, whereas it is found on lp25 in *B. burgdorferi* s. s.. The membrane lipoprotein *VlsE*, which is part of the immune escape mechanism of *Borrelia*, is another important gene for infectivity. In *B. afzelii* this gene is located on lp28-8, whereas in *B. burgdorferi* s. s. it is found on lp28-1. The virulence factor *bbk32*, which is on lp36 in *B. burgdorferi* s. s. and which also plays a role in pathogenicity, is located on lp17 in *B. afzelii* strains (Schüler *et al.,* 2015).

1.5. Genetic Tools and Manipulation

Genetic manipulation is the initiated change in the genome of organisms, brought about by genetic tools. It is used to provide insight into role and function of different genes which gives further knowledge of the pathogenicity and physiology of the organism.

Since *Borrelia* spirochetes are genetically very different from other bacteria, genetic manipulation in *Borrelia* had turned out to be, and still is, challenging, considering the evolutionary distance from other bacteria and the complex growth conditions. However, there are now several genetic tools available that have been developed by different groups of researchers over the years (Hyde *et al.,* 2011; Rosa *et al.,* 2010).

Several of these techniques are standard procedures in the lab nowadays; the preparation of growth media for cultivation, the use of shuttle and suicide vectors, transformation by electroporation, gene inactivation, antibiotic resistance markers and growth conditions of different strains of *Borrelia* (Tilly *et al*., 2000; Hyde *et al*., 2011).

Even though many genetic tools have been developed already, there are still many things unknown about the morphology and physiology of the spirochetes so there is still a strong need for more genetic tools in the future research of *Borrelia*.

Genetic manipulation concerning European species of the *B. burgdorferi* s. l. complex, *B. afzelii, B. garinii, B. bavariensis* and *B. spielmanii*, is not as thoroughly developed as in *B. burgdorferi* s. s.. Most of the work that has been done using genetic manipulation methods has been done on *B. burgdorferi* s. s. due to *B. burgdorferi* being discovered earlier and the early publication of the full genome.

1.6. Genetic Tools for Visualization

In addition to classical genetic tools, like gene inactivation and complementation, genetic tools for visualization by use of fluorescent proteins have been applied by some research teams in the *Borrelia* field. Fluorescent proteins were introduced into bacteria, in order to observe gene expression in *in-vitro* or *in-vivo* studies via observable fluorescent proteins, or in order to monitor the movement of the *Borrelia* by microscopy in model systems representing the environments and hosts of the *Borrelia* (Carroll *et al.,* 2003; Dunham-Ems *et al*., 2009).

For research on *Borrelia* it is especially important to identify essential proteins in the infectious cycle. This is made possible by monitoring gene expression along the way at different stages of the infectious cycle. One technique is to use a quantitative reporter system for gene expression, and it has been proven to work for different bacteria to fuse promoter regions with gene encoding green fluorescent protein (GFP) as such a reporter system. This system has been used both during *in-vitro* and *in-vivo* studies. In *B. burgdorferi* for such a gene expression reporter system the constitutive and strong *Borrelia* promoter *flaB* has been used. Gene expression in response to different environmental factors like pH or temperature, that resemble the conditions the spirochetes are exposed to *in-vivo*, could be assessed by this technique (Carroll *et al*., 2003).

Live imaging of the dissemination of *Borrelia* into the midguts and salivary glands of ticks was made possible by integration of a GFP reporter into the genome of the infectious *B. burgdorferi* s. s. (Dunham-Ems *et al*., 2009). By this novel technique important information on the interaction of *Borrelia* with their arthropod-vector was gained. Instead of incorporating the GFP gene into a shuttle vector that is transformed into the *Borrelia*, the gene was integrated into its genome, which promised constitutively expressed GFP without risk of getting lost.

Another novel technique for visualization is the correlative cryo-fluorescence and cryo-scanning electron microscopy (Strnad *et al*., 2015). This tool allows for the visualization of the interaction between *Borrelia* and its host. Fluorescently tagged structures are identified and targeted and are further subsequently imaged by high resolution microscopy. This technique has been tested for a *B. burgdorferi* s. s. isolate expressing GFP.

Most of the genetic tools for *Borrelia* have first been developed for strains of *B. burgdorferi* s. s., just like now new techniques for visualization are mainly developed for and tested on the strains of *B. burgdorferi* s. s.. The whole genome of the first *B. burgdorferi* s. s. isolate B31 had been published in 1997 (Fraser *et al*., 1997). In 2011 the genomes of 13 additional isolates of *B. burgdorferi* s. s. were published, leading to a focus on *B.*

burgdorferi s. s. for research (Schutzer *et al*., 2011). The first whole genomes of important European genospecies of *Borrelia* were published in December 2011: the genomes of two *B. afzelii* and two *B. garinii* isolates (Casjens *et al.*, 2011).

Since there are differences between the different genospecies within the *B. burgdorferi* s. l. complex, it is important to create more tools for European genospecies for further understanding of the complex life of these European pathogens, as well as for the understanding of the differences between the different genospecies.

2. Aims

- ➢ DsRed project:

  - o Obtaining fluorescent *Borrelia* by transformation using an already existing DsRed containing shuttle vector into infectious and non-infectious clones of CB43 (*B. afzelii*), and an infectious strain of PKo (*B. afzelii*)

  - o Injection of infectious, red fluorescing strains of CB43 and PKo into naïve mice for the creation of a tick animal model to verify the infectivity of the DsRed containing strain

- ➢ GFP project:

  - o Constructing a GFP containing shuttle vector

  - o Use of this shuttle vector containing GFP in a similar manner to the DsRed construct

3. Materials and Methods

3.1.1. Bacterial Species and Strains

The focus of this project was to work with European species of *B. burgdorferi* s. l., as no genetic tools for visualization are available for species other than *B. burgdorferi* s. s.. *B. afzelii* served as the European genospecies. The strain CB43 was used mainly, together with PKo for some experiments. From the CB43 strain, clonal population 6 (CB43-6) was used and for preliminary transformations certain passages of CB43-6 were used: CB43-6/5 and CB43-6/24.

Special thanks go to Dr. Melissa Caimano from the Department of Medicine, Connecticut, USA, for providing the *B. burgdorferi* s. s. 297, to Professor Joppe Hovius from the Academisch Medisch Centrum in the Netherlands for providing *B. afzelii* PKo, to Professor Jan Kopecky from the University of South Bohemia for providing *B. afzelii* CB43 and to Dr. Patricia Rosa from the National Institute of Health, USA, for providing pBSV2G-DsRed shuttle vector.

For the cloning and transformation into *E. coli,* NEB® 5-alpha Competent *E. coli* (High Efficiency) chemically competent cells and DH5α™ (Invitrogen®) competent cells as well as competent cells prepared by CaCl$_2$ method were used.

3.1.2. Media and Growth Conditions

For the growth of *Borrelia*, glycerol stocks of *Borrelia* were put into Barbour-Stoenner-Kelly (BSK) II media containing 6% rabbit serum by scraping off pieces of the frozen glycerol stock with a pipette tip and inserting it into the media. The media was kept at 34°C, which is the optimal temperature for *Borrelia* growth. After appropriate density of the *Borrelia* was reached (mid-log phase: 10^7 *Borrelia*/ml) the tubes were removed from the incubator and were used for genomic DNA isolation, lysate preparation or for the inoculation of larger volumes for preparation of competent *Borrelia* for transformation.

For the growth of *E. coli*, competent cells were put into LB (lysogeny broth) media and kept at 37°C in a shaker for approximately 12 hours.

For the preparation of the LB media, 10 g of sodium chloride, 10 g of Trypton and 5 g of Yeast Extract are dissolved in 1 L of deionized water. The solution was then autoclaved.

For the preparation of BSKII media, 50 g of BSA, 5 g of Neopeptone and 9.7 g of 10x CMRL powder were weighed in and dissolved in 1 L of water by stirring for approximately

2-3 hours. Subsequently, 2 g of Yeastolate, 6 g of Hepes acid, 5 g of Glucose, 0.7 g of Sodium citrate, 0.8 g of Sodium pyruvate, 0.4 g of N-acetyl glucosamine and 2.2 g of Sodium bicarbonate were added and stirred until all has dissolved. Finally, the pH is adjusted to 7.6 by addition of NaOH. The obtained media is filtered through 0.2mm filter and aliquoted into 500ml bottles for storage. 30 ml of rabbit serum (6%) was added per 500ml and frozen at -20°C.

For the preparation of 1.5x BSK$^+$ media, 69.4 g of BSA are fully solubilized in 1 L of water. Subsequently 6.9 g of Neopeptone, 8.3 g of Hepes acid, 6.9 g of Glucose, 1 g of Sodium citrate, 1.1 g of Sodium pyruvate, 0.6 g of N-acetyl glucosamine, 6.4 g of Sodium bicarbonate, 3.5 g of Yeastolate and 12.7 g of 10x CMRL powder are added and stirred for approximately 2-3 hours until fully dissolved. The pH is adjusted to 7.5 with NaOH. The media is filtered through 0.2mm filter and 300 ml are aliquoted into 500ml bottles. 12 ml of rabbit serum (6%) was added per 300ml and frozen at -20°C.

3.1.3. Genomic DNA Isolation

The genomic DNA of *Borrelia* needed was either available in the lab or was prepared from respective *Borrelia* cultures from existing glycerol stocks. For the gDNA isolation from cultures the Wizard Genomic DNA Purification Kit (Promega®) was used for DNA isolation adapting the manufacturer's protocol for Gram Positive and Gram Negative Bacteria as follows:

Up to 10 ml of *Borrelia* culture in a 15 ml centrifuge tube were centrifuged at 7800rpm for 10 minutes at 20°C. The supernatant was carefully removed and the pellet suspended in 600 µl of Nucleic Lysis Solution and transferred into a 1.5 ml Eppendorf tube. After incubation at 80°C for 5 minutes and on ice for 2 minutes 3 µl of RNase were added and inverted to mix. After 15 minutes incubation at 37°C and cooling on ice for a few seconds 200 µl of Protein Precipitation Solution were added and all was mixed by inversion. After 5 minutes incubation at room temperature the sample was centrifuged at 13000rpm for 10 minutes at 4-8°C. The supernatant was removed carefully to keep the pellet. 600 µl of room temperature 70% Ethanol were added and the sample was centrifuged again at 13000rpm for 10 minutes at 4-8°C. The ethanol was removed carefully by pipette and small Ethanol residue drops were removed using a fine paper towel. The tubes were kept open to allow the pellet to dry for 10 minutes. At the end, 100 µl of DNA Rehydration Solution was added and the sample was stored in the fridge for minimum of 1 day before further use.

3.2. GFP Project

The main task in the GFP project was the creation of a shuttle vector containing GFP together with a strong *Borrelia* promoter, which can then be used for introduction into various strains of *Borrelia*.

3.2.1. Polymerase Chain Reaction (PCR)

Genes of interest were amplified by PCR using the appropriate primers, polymerase, buffers as well as PCR settings.

For the GFP project a P_{flaB}-gfp cassette was amplified using P_{flaB}-gfp cassette primers. HotStarTaq *Plus* DNA polymerase from Qiagen® was used with the corresponding 10x PCR buffer from Qiagen®. The master mix composition as well as the PCR program settings is shown in Table 1.

The P_{flaB}-gfp casette was amplified from a *B. burgdorferi* s. s. strain 297 that was obtained from Dr. Melissa Caimano, where the P_{flaB}-gfp casette had been introduced previously (Dunham-Ems *et al.,* 2009).

Master Mix 2 – Standard MM	Amounts	PCR Settings 2 – Onetaq			
10x Qiagen® PCR buffer	2 µl	#	PCR step	T [°C]	t [sec]
dNTP's	0.4 µl	1c	Denaturation in cycle	95	45
Primer F	1 µl	2c	Annealing in cycle	48	45
Primer R	1 µl	3c	Elongation in cycle	68	60
HotStar Taq DNA Polymerase	0.1 µl	5	Final Elongation	68	600
MilliQ H_2O	12.5 µl	6	Hold	16	∞
DNA	3 µl				
Total	20 µl				

Table 1: PCR Master Mix and PCR settings for PCR of P_{flaB}-gfp casette

3.2.2. Molecular Cloning into *E. coli*

For the creation of the shuttle vector containing the P_{flaB}-gfp casette, first the P_{flaB}-gfp sequence was amplified by PCR using the appropriate primers. These P_{flaB}-gfp casette primers also inserted a HindIII restriction site for restriction digestion. After checking if the PCR product was obtained by gel electrophoresis. The P_{flaB}-gfp PCR product was directly used for ligation into pCR®2.1-TOPO® vector (Invitrogen®) using the TOPO® TA Cloning® kit. The reaction mixture for cloning into the TOPO vector is depicted in table 2.

pCR®2.1-TOPO® vector	
Reagent	Amount
PCR product	2 µl
Salt Solution	0.5 µl
TOPO vector	0.5 µl

Table 2: TOPO reaction mixture

The reaction mixture was prepared and incubated at room temperature for 5 minutes. After incubation, the ligation mixture was transformed into *E. coli* competent cells. 1.5 µl of the TOPO reaction mixture was added to 25 µl of thawed competent cells in a 1.5 ml Eppendorf tube that were kept on ice. The mixture was mixed by flicking the tube and then incubated on ice for 30 minutes. In the meantime a water bath of 42°C was prepared. After the incubation time the competent cells/TOPO mixture was heat shocked by dipping the Eppendorf tube into the 42°C water bath for 30 seconds. The cells were placed back on ice immediately. After 2 minutes 250 µl of S.O.C outgrowth media was added and the mixture was placed in a horizontal shaker with 200rpm at 37°C for approximately 1 hour. While the cells were in the shaker LB plates with carbenicillin were prepared and warmed in the incubator at 37°C.

Approximately 15 minutes before the competent cells were ready for use and taken out of the shaker the LB plates were taken out and treated with X-Gal by adding 40 µl of 20mg/mL X-Gal (Thermo Scientific ® #R0941) to each plate. 70 µl and 140 µl of cells were added. The plates were kept in the 37°C incubator overnight.

3.2.3. Plugging of Colonies and Plasmid DNA Isolation

7 ml of room temperature LB media was aliquoted into 15ml tubes. White colonies from the plates were chosen and picked with the 10µl pipette tip. Afterwards the tip was put into the media and left there to allow the colonies to grow in the liquid LB media. The tubes were kept in the horizontal shaker at 37°C overnight.

The next day, plasmid DNA isolation was performed using the Macherey Nagel® NucleoSpin® Plasmid kit following the manufacturer's instructions for Plasmid protocols as follows:

The *E. coli* LB culture was centrifuged for 30 seconds at 11,000 x g. The supernatant was removed and 250 µl of Buffer A1 (Resuspension buffer) was added and the pellet was resuspended by pipetting up and down. 250 µl of Buffer A2 (Lysis buffer) were added and mixed by inverting the tube 6-8 times. After 5 minutes of incubation at room temperature, 300 µl of Buffer A3 (Neutralization buffer) was added and mixed by inverting the tube 6-8 times. Centrifugation for 5 minutes at same centrifugation conditions as previously was performed at room temperature. The supernatant was loaded into a NucleoSpin® Plasmid Column in a 2ml Collection Tube and centrifugation for 1 minute was performed at same centrifugation conditions as previously. The flow-through was discarded and the column was placed back into the collection tube. For washing, 500 µl of Buffer AW (Wash buffer) was added and centrifugation for 1 minute was performed. The flow-through was discarded and 600 µl of Buffer A4 (Wash buffer) was added and centrifugation for 1 minute was performed. The flow-though was discarded and the empty column was put back into the collection tube. Centrifugation was performed for 2 minutes to dry the silica membrane. The column was put into a new 1.5ml microcentrifuge tube and DNA was eluted by adding 50 µl of Buffer AE (Elution buffer). After 1 minute of incubation, centrifugation was performed for 1 minute at same conditions as previously described.

3.2.4. Restriction Digestion and Sequencing

For the verification of successful ligation of the P_{flaB}-gfp cassette into the pCR®2.1-TOPO® vector, restriction digestion with EcoRI restriction enzyme was performed. The reaction mixture is describes in Table 3. Restriction digestion was also performed with HindIII restriction enzyme.

The reaction mixture was prepared according to table 3, incubated for 1 hour at 37°C followed by a heat inactivation of the enzyme at 80°C for 20 minutes.

The restriction digestion products were visualized by gel electrophoresis together with an undigested plasmid as a control. The cloned products were sequenced and the sequences were analyzed with GENIOUS and compared with other sequences by BLAST in the NCBI database.

Restriction digestion with EcoRI or HindIII	
Reagent	Amount
EcoRI or HindIII	0.5 µl
EcoRI or HindIII buffer	1 µl
MilliQ H_2O	6.5 µl
plasmid DNA	2 µl
Total	10 µl

Table 3: EcoRI/HindIII digestion reaction mixture

3.2.5. Gel electrophoresis

For the visualization of PCR amplified products or results from restriction digests, gel electrophoresis was performed. For 1% agarose gels, 1g of agarose powder was weighed in and dissolved in 100 ml of water by microwaving for around 5 minutes. The hot and liquid gel was poured into a corresponding form and allowed to cool and solidify. A comb of appropriate size was inserted before solidification for creation of wells. After complete solidification of the gel, the comb was removed and 1x TAE buffer was added to fill the electrophoresis apparatus. 10 µl of the PCR sample was then mixed with 2 µl of Loading Dye containing 10xSYBR and together loaded into the wells. Several ladders were used for visualization of different experiments: 1kb from Fermentas®, 1kb+ and 100bp from Invitrogen®. The gels were visualized using UV light.

3.2.6. Gel Extraction

For the creation of a shuttle vector containing the P_{flaB}-gfp casette, the shuttle vector pBSV2K (R. Rego) with kanamycin resistance and a size of approximately 5.4 kb was chosen. The plasmid DNA containing the P_{flaB}-gfp cassette in TOPO was digested with HindIII restriction enzyme according to the procedure in chapter *3.2.4. Restriction Digestion and Sequencing*. 24 µl of the digestion product were added to each of 4 wells

and run on a gel, using GelRed™ for visualization to avoid the use of SYBR, which has caused troubles with gel extraction whenever performed previously. Gel extraction of the HindIII digested P_{flaB}-gfp product was performed using the QIAquick® Gel Extraction kit from Qiagen® according to the user's manual.

3.2.7. Ligation into shuttle vector

For the ligation into the pBSV2K shuttle vector, pBSV2K was digested with HindIII. Alkaline Phosphatase was added to the restriction digested pBSV2K to remove the 5' phosphate group for higher chance of ligation. For removal of the 5' phosphate group FastAP Thermosensitive Alkaline Phosphatase as well as 10X Fast AP buffer from ThermoFisher Scientific® were used. A reaction mixture according to table 4 was prepared.

Alkaline Phosphatase	
Reagent	Amount
pBSV2K (rest. dig. Mix)	35 µl
FastAP Thermosens. Alkaline Phosphatase	1.75 µl
10X FastAP buffer	3.5 µl
Total	**40.25 µl**

Table 4: Alkaline Phosphatase reaction mixture

The mixture was mixed thoroughly and spun down briefly. Incubation at 37°C for 10 minutes was followed by alkaline phosphatase inactivation at 65°C for 15 minutes.

For the ligation into pBSV2K, T4 ligase and T4 ligation buffer from New England BioLabs® were used. Ligation mixtures of 4:1 and 8:1 of insert (P_{flaB}-gfp casette) to vector (pBSV2K) ratios were used with the ligation mixtures depicted in Table 5.

The ligation mixture was prepared according to table 5, incubated at 16°C overnight and inactivated by incubation at 65°C for 20 minutes. Transformation into *E. coli* according to the procedure chapter 3.2.2. *Molecular cloning into E. coli* was performed. Instead of 1.5 µl, 5 µl of the ligation reaction was added to 25 µl of DH5α™ *E. coli* competent cells (Invitrogen®). The cells were kept in the 42°C warm water bath for 45 seconds and kept

on ice for 3 minutes afterwards. The rest of the procedure stayed the same; the cells were plated on LB plates containing kanamycin.

Ligation of pBSV2K and P$_{flaB}$-gfp casette		
Ratio	4:1	8:1
Reagent	Amount	Amount
Insert DNA (P$_{flaB}$-gfp cassette)	8 µl	16 µl
Vector DNA (pBSV2K)	2 µl	2 µl
Buffer T4	2 µl	2 µl
Ligase T4	1 µl	1 µl
MilliQ H$_2$O	7 µl	-
Total	**20 µl**	**21 µl**

Table 5: Ligation reaction mixture of 2 different ratios

3.2.8. Primers

The Primers used for the amplification of P$_{flaB}$-gfp casette as well as the T7 primer that was used for sequencing are shown in Table 6.

Primer	Sequence
T7	5' – TAA TAC GAC TCA CTA TAG GG – 3'
P$_{flaB}$-gfp casette F	5' – GCA GCT AAT GTT GCA AAT CTT TTC – 3'
P$_{flaB}$-gfp casette R	5' – GGG TAA GCT TTC CGT ATG TAG C – 3'

Table 6: Primers used for sequencing and amplification of the P$_{flaB}$-gfp casette

3.3. DsRed Project

For the DsRed project, a shuttle vector, pBSV2G, containing DsRed, had been provided by Dr. Patricia Rosa (NIH). The experimental work could be started with transformation of this vector into *Borrelia* right away after creation of a plasmid profile for the *Borrelia* populations used.

3.3.1. Polymerase Chain Reaction (PCR)

For the DsRed Project PCR was performed for the verification of plasmids present or plasmids lost after transformation. For the plasmid profile of *B. afzelii* strain CB43-6 and passages of it, primers designed for *B. afzelii* strain PKo were used. The PCRs screened for 14 linear plasmids as well as for 7 circular plasmids. For the plasmid profile New England BioLabs® Taq DNA polymerase was used together with 10x ThermoPol® buffer. The addition of $MgCl_2$ yielded better looking results. The composition of the Master Mix as well as the PCR program settings are shown in Table 7.

The sequences of the primers used for PCRs of the plasmid profile (PKo primers) are not published yet (for further information please contact Dr. Rego, Thesis Supervisor).

Master Mix 1 – Plasmid Profile	Amounts		PCR Settings 1 – PP NEB			
		#	PCR step		T [°C]	t [sec]
10x ThermoPol® buffer	2 µl	#	PCR step		T [°C]	t [sec]
dNTP's	0.4 µl	1	Initial Denaturation		95	30
Primer F + R	1 µl	2c	Denaturation in cycle		95	45
$MgCl_2$	0.2 µl	3c	Annealing in cycle		52	45
NEB Taq DNA polymerase	0.1 µl	4c	Elongation in cycle		68	60
MilliQ H_2O	15.3 µl	5	Final Elongation		68	300
DNA	1 µl	6	Hold		16	∞
Total	20 µl					

Table 7: PCR Master Mix and PCR settings for plasmid profile PCR

3.3.2. Ethanol precipitation of DNA

For transformation of the appropriate vector into *Borrelia*, the plasmid DNA needed to be at a concentration of 2µg/µl. The concentration of plasmid DNA was measured and the appropriate amount of DNA was taken to have approximately 40 µg of plasmid DNA. To the DNA, 1/10 of the volume (of plasmid DNA used in µl) of 3M Sodium acetate as well as 2 times the volume of 100% Ethanol was added. The mixture contents were mixed by inverting the tube a few times and centrifuged at 13000rpm for 10 minutes at 4°C. The supernatant was removed by pipette and 1 volume of 70% Ethanol was added and

centrifuged at13000 rpm for 10 minutes at 4°C again. The supernatant was removed and the tube was allowed to dry for approximately 5 minutes. To the pellet, 20 µl of MilliQ water were added which should give 40 µg of DNA in 20 µl of water, to give a total concentration of 2µg/µl.

3.3.3. Transformation and Plating of *Borrelia*

For the first round of transformations of the DsRed vector, the non-infectious clones of CB43-6 : CB43-6/5 and CB43-6/24 - were used. For the second round of transformations the infectious clonal population CB43-6 was used as well as infectious PKo which is a *B. afzelii* strain.

The CB43-6/5 and CB43-6/24 clones were assumed to be non-infectious, due to the loss of plasmids that contain genes important for infectivity. The plasmid profiles are provided in Table 9.

In the first step *Borrelia* cultures were started from glycerol stocks as described in chapter 3.1.2. *Media and Growth Conditions*. The *Borrelia* were allowed to grow to approximately 10^7 *Borrelia*/ml. For successful transformation the density of *Borrelia* should be 5×10^7 *Borrelia*/ml.

The *Borrelia* were counted using the Petroff-Hausser counting chamber (Hausser Scientific). A 1:10 dilution of the *Borrelia* culture was prepared by adding 50 µl of the culture to 450 µl of media and 6 µl of the diluted culture was placed onto the slide (dilution factor 10). All *Borrelia* occurring on 5 squares were counted and the number was denoted. The number of *Borrelia* cells per ml was calculated using Equation 1, where x represents the number of *Borrelia* counted within 5 squares of the counting chamber.

$$number \ of \ borrelia = \frac{x}{5} * 1.25 * 10^6 * dilution \ factor \ (10) \quad [\frac{Borrelia}{ml}]$$

Equation 1: calculation of the number of *Borrelia* in a culture

From the number of *Borrelia* per ml the number of *Borrelia* per µl was determined and it was assessed what volume of the culture has to be added to 100 ml of fresh BSKII media until the appropriate *Borrelia* density of 5×10^7 *Borrelia*/ml is reached. To 100 ml of BSKII media the calculated volume of the *Borrelia* culture is added and incubated at 34°C.

5-7 days later, the prepared media was centrifuged for 10 minutes at 7800rpm, 20°C. The supernatant was discarded and the pellet was resuspended in 25ml of cold electroporation solution (EPS). The tubes were centrifuged again for 10 minutes at 7800rpm, 10°C. The

supernatant was discarded and the pellet of *Borrelia* cells was re-suspended in approximately 250 µl EPS and transferred into a 1.5 ml Eppendorf tube. The contents of EPS are 93.1 g of Sucrose and 150 g of Glycerol in 1 L of water that is filtered through a 0.2mm filter and then kept at 4°C. The pellet was re-suspended carefully by slowly pipetting up and down with a 200 µl pipette. The density of *Borrelia* cells was checked under the microscope. The perfect density for transformation is when the cells are connected but not too packed and no clumps are visible. More EPS was added if the cells looked too clumpy or dense.

To 100 µl of the suspended *Borrelia* cells 10 µl of the previously precipitated vector DNA was added. The mixture of *Borrelia* and plasmid DNA was transferred into 0.2mm electroporation cuvettes (Bio-Rad) and electroporated. After electroporation the cells were transferred into 8ml tubes containing BSKII and kept in the 34°C incubator until the next day.

For the plating of *Borrelia*, first the electroporated *Borrelia* was checked and counted with use of the counting chamber. Non-antibiotic plates were prepared in a way that 100 *Borrelia* per plate should be plated for checking if the counting of *Borrelia* was approximately right and that the plating conditions were good for normal *Borrelia* growth. If e.g. one *Borrelia* was counted on the counting chamber:

$$\frac{1}{5} * 1.25 * 10^6 * 10 = 2.5 * 10^6 \ \left[\frac{Borrelia}{ml}\right] = 2.5 * 10^3 \left[\frac{Borrelia}{\mu l}\right]$$

40µl of the $2.5*10^6$ *Borrelia*/ml was added to 960µl of BSKII media resulting in a 10^5 *Borrelia*/ml culture. 10µl of this culture was added to 990µl of BSKII media resulting in the desired 1000 *Borrelia*/ml which is 1 *Borrelia*/µl.

The next step was the preparation of the agarose media by mixing 200 ml of liquid autoclaved agarose (Seachem) with 300 ml of 1.5x BSK⁺ media and kept in a water bath (55°C) to avoid solidifying.

10 ml of the agarose/1.5xBSK⁺ mixture was poured onto each plate and allowed to solidify. In a 50 ml falcon tube 40 ml of agarose/1.5xBSK⁺ mixture was mixed with 200 µl of the 1000 *Borrelia*/ml culture (= 1 *Borrelia*/µl culture). 20 ml of this was then added on top of the already solidified 10ml. For the preparation of the plates that should be selective for *Borrelia* transformed with the pBSV2G vector bearing a gentamicin antibiotic resistance cassette, gentamycin was added to the agarose/1.5xBSK⁺ mixture at a 40 µg/ml

concentration. Three 50 ml falcon tubes with 40 ml agarose/1.5xBSK⁺mixture were prepared and to each 1, 2 or 3 ml of the original *Borrelia* transformation culture were added. Again, 20 ml of each of the mixtures were added on top of already solidified media in plates.

The readily solidified plates were put into air tight boxes with anaerobic satches (GasPak, BD) and kept at 34°C for 4 weeks before colonies appeared that could be plugged.

After colonies appeared, they were cultivated again in BSKII media, by poking the colony with a 1ml pipette tip and sucking it up into the pipette and thoroughly rinsing the pipette with the BSKII media in an 8ml tube to allow the growth of *Borrelia* under conditions mentioned in chapter *3.1.2. Media and Growth Conditions.* After 7-10 days the cultures were checked for fluorescence and gDNA isolation was performed.

3.3.4. Lysate Preparation

In order to perform Western Blots to check the infectivity of the obtained *Borrelia* in mice, lysates of the cultures that were injected into mice were prepared.

The tubes with culture were centrifuged at 7800rpm for 10 minutes at 20°C and the supernatant was removed. 1 ml of cold HN-buffer was used for re-suspension of the pellet and transfer into a 1.5 ml Eppendorf tube. Centrifugation at same conditions was performed again. The supernatant was removed by pipette and the same procedure with the cold HN-buffer and centrifugation was performed. After discarding the supernatant again 200 µl of B-Per buffer (Bacterial Protein Extraction from ThermoFisher Scientific®) were used for careful re-suspension of the pellet. The solution was incubated at room temperature for 10 minutes. Then 200 µl of Laemmli buffer were added. Laemmli buffer was prepared after adding 25 µl of ß-Mercapto Ethanol to 475 µl of 2X Laemmli buffer.

3.3.5. Western Blot

From the red-fluorescing cultures obtained of CB43-6, 2 mice for each DsRed expressing clone were injected with 10^5 *Borrelia* and checked for infectivity by Western Blot using the sera of the infected mice as primary antibodies. For the gel preparation amounts depicted in table 8 were used together with 0.75mm plates.

Western Blot Gel Preparation		
Reagent	**Stacking Gel – Amounts**	**Separation Gel - Amounts**
30% Acrylamide	0.165 ml	2 ml
Stacking/Separation Buffer	0.25 ml	1.25 ml
MilliQ H_2O	0.575 ml	1.7 ml
10% APS (Ammonium persulfate)	10 µl	50 µl
TEMED (N,N,N',N',-tetramethylethylenediamine)	1 µl	2 µl

Table 8: Amounts used for gel preparation for Western Blots

The lysate samples were added to the wells and an SDS-page electrophoresis was run at 120V for 1 hour. In the Blotting apparatus, the Blotting set-up was assembled as follows: Blotting Paper, nitrocellulose membrane, Gel, Blotting Paper. The nitrocellulose membrane was incubated in 1X Transfer/Blotting Buffer (25mM Tris-HCl, 192mM glycine, 20% (v:v) methanol) for 5 minutes before usage and the blotting paper was soaked in the same buffer. The transfer was run for approximately 60 minutes at 25V.

For a 5% blocking solution 10 g of dried milk was dissolved in 200 ml of 1x TBS Tween 20 buffer. The membrane was removed from the set-up, cut into corresponding pieces of single lines for each lysate and put into blocking solution for 2 hours. Primary antibody solutions were prepared by adding 10 µl of corresponding mouse sera of the infected mice into 2 ml of blocking solution (1:200 ratio). The strips of membrane were put into ziplock bags and the corresponding primary antibody was added and the bag was tightly sealed and kept in the fridge overnight. The next day, the bags were opened and the membranes were washed three times with 1X TBS Tween 20 for 15 minutes. Secondary antibody solution was prepared by adding 5 µl of Anti-Mouse antibody IgG (Sigma-Aldrich, St. Louis, MO) conjugated with peroxidase into 50 ml of blocking solution (1:10000 ratio). The membranes were incubated with the secondary antibody solution for 2 hours and washed with TBS Tween 20 again three times for 15 minutes. Substrate solution – Pierce® ECL Western Blotting Substrate (ThermoFisher Scientific®) – was prepared taking 1ml of each of the detection reagents (1 and 2) and mixing them. The membranes were put into substrate solution right away and were shaken for 5 minutes. Then the membranes were assembled on a transparency and the result was visualized using the Chemi-Doc system (Bio-Rad).
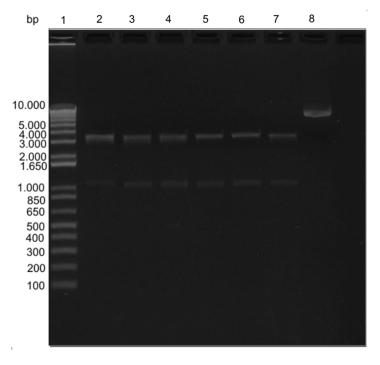
4. Results

4.1. GFP Project

For the GFP Project, first a P_{flaB}-gfp cassette was amplified by PCR using P_{flaB}-gfp cassette primers. The PCR product was directly inserted into pCR®2.1-TOPO® vector (Invitrogen®).

After transformation and plating of the ligated DNA, 6 *E. coli* colonies were plugged, grown in LB media and subsequently the DNA of the vector containing the inserted gene was isolated by mini preps. The mini prep DNA (P_{flaB}-gfp cassette in TOPO 1-6) was then restriction digested with help of the HindIII restriction enzyme, which is present on the pCR®2.1-TOPO® vector. The desired products of the restriction digestion are the P_{flaB}-gfp cassette gene with HindIII restriction sites on both ends and the open-cut TOPO vector. Gel electrophoresis was performed to visualize the size of the obtained products from the restriction digestion (Figure 3).
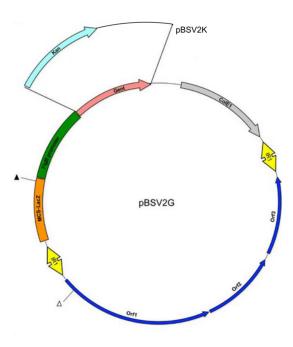
From figure 3 it is visible that all of the P_{flaB}-gfp cassette in TOPO mini prep DNAs (1-6) that were digested, both the P_{flaB}-gfp cassette product (~1000 bp) as well as the open cut TOPO vector (~3.9 kbp) were obtained.

Figure 3: HindIII restriction digestion of P_{flaB}-gfp casette in TOPO: **1** – 1 kb plus DNA ladder (Invitrogen); **2-7** - P_{flaB}-gfp cassette in TOPO 1-6, **8** - undigested P_{flaB}-gfp cassette in TOPO 1
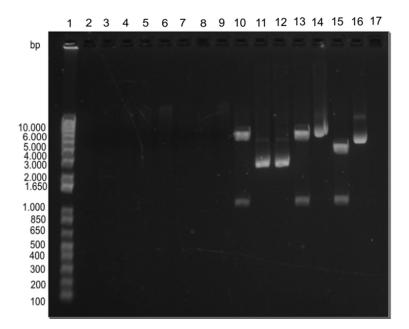
In addition to the gel electrophoresis result the obtained DNA was sent for sequencing to verify that the inserted gene is the P_{flaB}-gfp cassette. A T7 site is present on the TOPO vector, hence a T7 primer was used for sending the DNA to sequencing. The results from sequencing were compared by BLAST in the NCBI database and prove was obtained that the inserted piece of DNA was the P_{flaB}-gfp casette.

A larger amount of P_{flaB}-gfp cassette in TOPO DNA was digested again and the band at length 1000 bp was extracted. This P_{flaB}-gfp piece that then contained HindIII restriction sites at its end was used for ligation into the pBSV2K vector (as well as into the pBSV2G vector).

Figure 4: pBSV2G/pBSV2K shuttle vector (G and K represent the corresponding antibiotic resistance cassette, gentamicin or kanamycin); approximate size: ~5.8kbp (adapted from Rego *et al.,* 2011)

The pBSV2K-P_{flaB}-gfp cassette ligation as well as the pBSV2G-P_{flaB}-gfp cassette ligation were again transformed into *E. coli* and grown on kanamycin or gentamycin LB plates respectively. From each of the ligations, four colonies were plugged and the DNA was isolated by Mini Prep DNA isolation. The obtained DNA from the Mini Preps were restriction digested with HindIII and the obtained gel electrophoresis results is shown in figure 5.
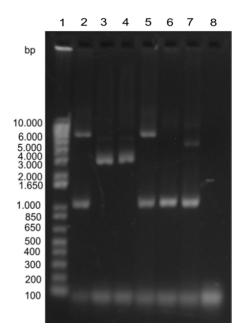
Figure 5: HindIII restriction digestion products: **1** - 1 kb plus DNA ladder (Invitrogen); **2-3** - P_{flaB}-gfp pBSV2G 1-2 from earlier plates; **4** - non-digested P_{flaB}-gfp pBSV2G 1 from earlier plates; **5-8** - P_{flaB}-gfp pBSV2G 1-4 from plates (04.07.2016); **9** - uncut P_{flaB}-gfp pSV2G 1 from plates (04.07.2016); **10-13** - P_{flaB}-gfp pBSV2K 1-4 from plates (04.07.2016); **14** - non-digested P_{flaB}-gfp pBSV2K1 from plates (04.07.2016); **15** - digested P_{flaB}-gfp cassette in TOPO; **16** - non-digested pBSV2G

From figure 5 it is visible that only the pBSV2K vector worked for successful ligation with the P_{flaB}-gfp cassette. Additionally, a PCR with the P_{flaB}-gfp cassette primers was used on the same Mini Preps (P_{flaB}-gfp pBSV2K 1-4) to check if the P_{flaB}-gfp cassette was inserted properly and can still be amplified by PCR. As a positive control, *B. burgdorferi* 297 DNA was used, which was used initially to obtain the P_{flaB}-gfp cassette for ligation into the TOPO vector. The results are shown in figure 6.

The results from figure 6 are in accordance with figure 5, showing that P_{flaB}-gfp pBSV2K 1 and P_{flaB}-gfp pBSV2K 4 have a band at ~1000bp representing the P_{flaB}-gfp cassette, as well as a band at ~6kbp representing the vector, whereas P_{flaB}-gfp pBSV2K 2 and P_{flaB}-gfp pBSV2K 3 did not properly insert the P_{flaB}-gfp cassette and a band at ~ 3kbp is visible.

The mini prep DNA of P_{flaB}-gfp pBSV2K 1 and P_{flaB}-gfp pBSV2K 4 was sent for sequencing using P_{flaB}-gfp cassette F primers. The results showed that the P_{flaB}-gfp cassette was properly inserted in the correct orientation into the pBSV2K vector.
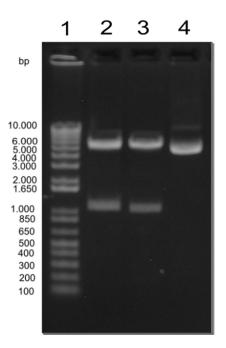
Figure 6: PCR with P$_{flaB}$-gfp cassette primers: **1** - 1 kb plus DNA ladder (Invitrogen); **2-5** - P$_{flaB}$-gfp pBSV2K 1-4; **6** - *B.b.* 297 DNA (positive control); **7** - P$_{flaB}$-gfp cassette in TOPO; **8** - negative control

Figure 7: HindIII restriction digestion: **1** – 1 kb plus DNA ladder (Invitrogen); **2** - P$_{flaB}$-gfp pBSV2K 1 mini prep DNA; **3** - P$_{flaB}$-gfp pBSV2K 1 maxi prep DNA; **4** - P$_{flaB}$-gfp pBSV2K 1 maxi prep DNA non-digested

P$_{flaB}$-gfp pBSV2K 1 was then grown in a larger volume of LB media which was inoculated using a glycerl stock of P$_{flaB}$-gfp pBSV2K 1. A maxi prep was performed to obtain a larger amount of DNA. Both the P$_{flaB}$-gfp pBSV2K 1 mini prep as well as the P$_{flaB}$-gfp pBSV2K 1 maxi prep were restriction digested and the results of restriction digestion of the P$_{flaB}$-gfp pBSV2K 1 mini prep as well as the P$_{flaB}$-gfp pBSV2K 1 maxi prep and the undigested P$_{flaB}$-gfp pBSV2K 1 maxi prep are shown in figure 7.

4.2. DsRed Project

For the transformation of the shuttle vector pBSV2G-DsRed into *B. afzelii,* different *Borrelia* strains were cultivated. For the selected *Borrelia* strains, a plasmid profile was created using a PKo plasmid primer set.

In table 9 the plasmids that were checked for the plasmid profiles by PCR are depicted together with the results of the PCRs for both the *Borrelia* strains that were used in the first round of transformations (species: *B. afzelii,* strain: CB43, clonal population: 6, passages: 5 and 24) as well as for the ones that were used in the second round (low passage *B. afzelii* clonal population CB43-6).

Plasmid	CB43-6/5	CB43-6/5 +DsRed	CB43-6/24	CB43-6/24 +DsRed	CB43-6	CB43-6 SH 2	CB43-6 SH 4	CB43-6 SH 6
lp17	-	-	-	-	+	+	+	+
lp25	-	-	-	-	+	+	+	+
cp26	+	+	-	-	+	+	+	+
c26+27	+	+	-	-	+	+	+	+
lp28	+	+	-	-	+	+	+	+
lp28-2	-	-	+	+	+	+	+	+
lp28-3	-	-	-	-	+	+	+	+
lp28-4	+	+	-	-	+	+	+	+
lp28-7	-	-	-	-	+	+	+	+
lp28-8	+	+	+	+	+	-	-	+
cp30	+	+	+	+	+	+	+	+
lp32	+	+	+	+	+	+	+	+
cp32-5	-	-	-	-	-	-	-	-
cp32-7	-	-	-	-	+	+	+	+
cp32-9	+	+	-	-	-	-	-	-
cp32-10	+	+	+	+	+	+	+	+
lp34	-	-	+	+	+	-	+	+
lp38	+	+	+	+	+	-	+	+
lp54	+	+	+	+	+	+	+	+
lp60	-	-	-	-	-	-	-	-
lp60-2	+	+	-	-	+	+	+	+

Table 9: plasmid profiles of parental strains and various clones transformed with DsRed; PCRs performed using PKo primers

Plasmid profiles were created to verify that no plasmids were lost in the process of transformation, and to check if specific plasmids that are known to be important are maintained.

For the first round of transformations clones obtained from passages of CB43-6 were used that were already lacking important plasmids like lp28-2 or lp17, which contain genes important for virulence. The *Borrelia* that were obtained from these experiments were not further used for a tick-mouse infectious cycle as it was assumed that these clonal populations were not infectious to begin with, even if no plasmids had been lost during transformation. These transformations were performed to get better feeling for the difficulty of the transformation of the DsRed gene on the pBSV2G vector into *B. afzelii* CB43-6 as well as PKo. Since certain plasmids were lost the chances for successful insertion of another circular plasmid were assumed to be higher than for a passage that had not lost any plasmids/genes including those responsible for restriction-modification (Rego *et al.*, 2011).

After successful insertion of the vector into two of the passages of CB43-6, in the second round of transformations, it was tried to transform the DsRed containing vector into CB43-6 and PKo directly.

Verification of expression of the DsRed gene was performed by fluorescent microscopy. The *Borrelia* cultures obtained from the colonies on the *Borrelia* plates that were visible under the fluorescent microscope were expressing the DsRed gene. These cultures were named CB43-6 SH 2, 4, 6 and 9 as well as PKo + DsRed 2 for the corresponding colony on the plates. Also colonies of *Borrelia* that did not express DsRed were obtained: CB43-6 SH 1, 3, 5, 7, 8, 10 as well as PKo + DsRed 1 did not show up red under the microscope.
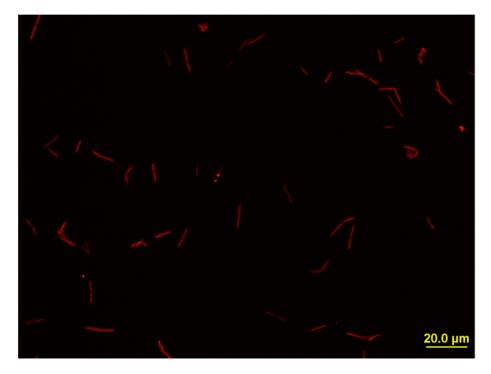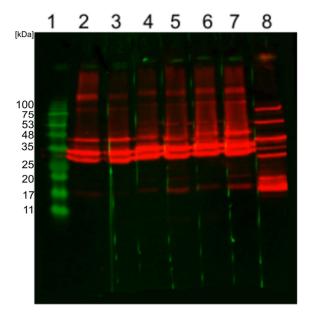

Figure 8: DsRed expressing *B. afzelii* CB43-6

Unfortunately, CB43-6 SH 2 lost lp28-8, lp34 as well as lp38 in the course of transformation and plating and also CB43-6 SH 4 lost lp28-8. However, cultures of all of these *Borrelia* populations were injected into naïve Balb/c mice. Two Balb/c mice each were used for the injection of CB43-6 SH 2, 4 and 6 cultures. 19 days post injection, tissue DNA isolation was performed with ear tissue of all of the mice. Western Blots were performed with sera of the mice approximately three weeks post inoculation.
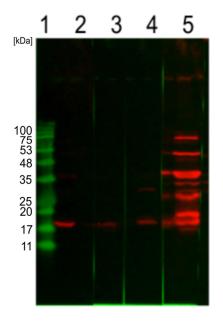
Larval ticks were put onto mice approximately one month after injection of the cultures.

Two months after inoculation of the mice with the *Borrelia*, the mice were sacrificed and mouse tissues and organs (ear, joint, heart and bladder) were saved. Pieces of ear, joint and bladder were placed into 12ml of BSKII media and pieces of ear, joint and heart were kept in 1.5ml Eppendorf tubes in the freezer. The tissues in the BSKII media were checked from time to time for the presence of *Borrelia*.

The larval ticks from mice were collected and kept in 1.5ml Eppendorf tubes. Tick DNA isolation was performed to check if *Borrelia* were acquired from the infected mice into the ticks. Unfortunately, the media with tissues and organs of the sacrificed mice were not proven to contain *Borrelia,* nor did the DNA isolated from tissue show positive results. However, the isolated tick DNA was positive when gentamicin primers were used.

Figure 9: Western blot – (lysate for all wells from infectious CB43-6) – Lane: **1** - protein marker VI (AppliChem); **2-7** - sera from mice; **8** - positive control: sera from mouse infected with infectious CB43-6

Figure10: Western blot – (lysate for all wells 2-4 from infectious PKo; lysate for well 5: CB43-6) – Lane: **1** – protein marker VI (AppliChem); **2-4** - sera from mice; **5** - positive control: sera from mouse infected with infectious CB43-6

If the mice produced specific antibodies against the antigens of the *Borrelia* that were injected, these antibodies bind to the lysate proteins that were separated by gel electrophoresis. The secondary antibody (anti-mouse antibody) is conjugated with a signal-giving molecule, needed for detection. The membrane in figure 9 shows a number of bands, suggesting that the injected cultures CB43-6 SH 2, 4 and 6 have maintained their infectivity.

The obtained red fluorescing *B. afzelii* PKo +DsRed 2 were injected into 3 C3H/HeN mice. The serum was taken from the mice approximately four weeks later and a western blot was performed.

From the picture of the western blot membrane in figure 10 it is seen that only a very small amount of antibodies were produced from the mice infected with the PKo + DsRed 2 strains of *Borrelia*, whereas the positive control shows several bands. This leads to the assumption that this pathogenic PKo strain has lost its infectivity.

5. Discussion

The aim of this thesis was to create infectious *B. afzelii* strains that are fluorescent, for their use in visualizing their properties and functions within a mammalian or arthropod host, as well as for visualization in *in-vitro* studies.

The technique of using fluorescent proteins to visualize bacterial behavior has been widely used in several studies. Fluorescence imaging allows for non-invasive detection of viable microorganisms within living tissue and thus allows for *in-vivo* studies for better insight on bacterial behavior (Van Zyl *et al*., 2015).

In 2001, Kadioglu and colleagues used GFP for the incorporation into *Streptococcus pneumoniae* to trace pneumococcal adherence and invasion in broncho-epithelial cells. This was the first time pneumococcal invasion and cellular translocation in bronchial epithelial cells was reported. They used confocal fluorescent microscopy to study the properties of the bacteria and could characterize the penetration and the internalization of these pneumococci over time (Kadioglu *et al.,* 2001).

Also in 2001, Bettina Hammer and colleagues performed FISH (fluorescence in situ hybridization) on *I. recinus* ticks body sections to visualize *B. burgdorferi* s. l. within the ticks as well as on gerbil skin biopsies to visualize the *Borrelia* in the mammalian host. (Hammer *et al.,* 2001) They were able to label the *Borrelia* with 16S-rRNA-directed oligonucleotides that were specific for *B. burgdorferi* s. l. With this technique it was able for them to observe the typical morphology of *Borrelia* within the tick body section. However, this method only allowed the visualization of *Borrelia* after the tissue or tick sample was treated with the fluorescent label, which requires for the tick or mammal to be dissected before.

In 2009, a new method was used to visualize *B. burgdorferi* s. s. within isolated tick midguts and salivary glands (Dunham-Ems *et al*., 2009). GFP was stably introduced into the genome of the infectious *B. burgdorferi* s. s. strain 297, onto cp26. This was advantageous since the GFP was introduced on the very stable cp26 and not on a vector that could get lost upon replication. The *Borrelia* were live-imaged by confocal fluorescent microscopy to visualize their progress throughout the feeding tick. The question on the dissemination of the *Borrelia* within the ticks was unraveled and it was found to be different than thought. Contrary to previous belief the spreading of the bacteria was not solely motility driven, but also included a non-motile period in which the *Borrelia* were adhering onto different kinds of epithelial cells on their way throughout the mid-gut toward the basolateral epithelium. In the second phase, where the *Borrelia* transition into motile

bacteria is happening, they penetrate the mid-gut and relocate into the salivary glands, from where it is easy to be transmitted into the vertebrate host.

In 2014 Bockenstedt and colleagues were investigating the migration of green fluorescent *B. burgdorferi* s. s. with the use of a two-photon real-time imaging of ticks that are feeding on a host (Bockenstedt *et al.*, 2014).

To get an even better insight on what is happening at the microscopic level when *Borrelia* interact with its hosts, a new microscopy technique was developed by Strnad and colleagues (Strnad *et al.*, 2015). The microscopy technique uses both light and electron microscopy as well as fluorescently tagged *Borrelia* to obtain near-to-nanometer resolution of the *Borrelia* interacting with its host cells.

For all of the techniques that should provide further knowledge on bacteria host interactions, it is crucial to have fluorescently labeled bacteria that can be easily visualized by fluorescent microscopy, either within the tick or the mammalian host tissue. These new techniques have already been used on *B. burgdorferi* s. s.. However, other European strains of *B. burgdorferi* s. l. have not been investigated. Therefore, the creation of fluorescent European species of *Borrelia* is crucial for discovering the differences between the *Borrelia* species within the mammalian host and the tick during their infection. Furthermore it is important to understand interaction of European *Borrelia* with the mammalian host and their movement and behavior within the tick.

The auto fluorescence of certain tick tissue can be of advantage and disadvantage. Since ticks are generally auto fluorescent this fluorescence provides for a good histological orientation when tick sections are examined (Hammer et al., 2001). However, if green fluorescent *Borrelia* are examined in ticks, the *Borrelia* cannot be distinguished from the tick tissue properly. Thus, the use of *Borrelia* that are expressing a protein that is fluorescing in a different color, like DsRed, is of advantage.

During the work of my thesis project, successful incorporation of DsRed into strains of *B. afzelii* was achieved and infectious red fluorescent strains were obtained. This is the first successful transformation of fluorescence expressing shuttle vector into an infectious European *Borrelia* species. Additionally, a shuttle vector containing a P_{flaB}-gfp cassette was created for *Borrelia* transformations.

The use of new life-imaging methods (Dunham-Ems *et al.*, 2009; Bockenstedt *et al.*, 2014) is a good approach to gain more detailed understanding of the pathogen-vector interaction during the very critical transmission process. It is crucial to understand how these microorganisms gain access to their vertebrate hosts during the blood meal of the tick.

Additionally, the use of the cryo-fluorescence and cryo-scanning electron microscopy (Strnad *et al.*, 2015; Vancova *et al.,* 2017) does provide another straightforward tool for the determination and study of *Borrelia* and host interactions at a very high resolution.

These techniques could be used on the newly acquired red fluorescing *Borrelia* obtained in the course of this project.

6. Literature

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