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Generation of GFP producing *Borrelia afzelii*, the Lyme Disease pathogen, and its evaluation using a tick-mouse model

Bachelor Thesis

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Annotation: The aim of this thesis was to create a GFP expressing *Borrelia afzelii* strain and to use it together with an already obtained DsRed expressing strain of this species for confirming their infectivity using a tick-mouse model for infection. This work is the basis for providing a method for visualizing an European Borrelia species for further studies, focusing on the investigation of *Borrelia*-host interactions.

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.

České Budějovice, 13.08.2018 Anna-Celine Danklmaier

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1. Introduction

1.1. Lyme Disease

Lyme disease is the most common arthropod-borne disease in temperate regions of the Northern Hemisphere. (Dennis and Hayes, 2002; Margos *et al.*, 2011). Also called Lyme borreliosis in Europe and Asia, it is caused by the pathogenic bacterial species of *Borrelia burgdorferi* sensu lato (s. l.). The transmission occurs during the blood meal of an infected tick which becomes increasingly likely after approximately 48 hours of tick attachment to the host (Piesman *et al.*, 1987; Rosa *et al*., 2005; Radolf *et al.*, 2012).

This multisystem inflammatory disorder, which is caused by the host's immune response, is characterized by three stages. After transmission of the spirochetes into the skin by a tick bite, early infection (stage I) occurs, causing a local skin lesion called erythema migrans (EM) as well as influenza-like symptoms. Hereby it has to be noted, that neither EM or influenza-like symptoms do not occur in all patients. Further on, the bacteria disseminate through the body, causing infections of the nervous system, heart and joints (stage II). The localized and early-disseminated infection can be usually treated with antibiotics in a 2-4-week course. However, if the disease is untreated or antibiotics are inefficient, a late-persistent infection (stage III) develops with more severe clinical manifestations as chronic arthritis, neuroborreliosis or skin disorders (Steere, 2001; Rosa *et al.*, 2005).

1.2. *Borrelia*

The bacteria of the genus *Borrelia* belong to the phylum of spirochetes, sharing similar morphological characteristics. These comprise a helical cell shape and three types of movement. The protoplasmic cylinder complex, which consists of cytoplasm, an inner cell membrane and peptidoglycan, is surrounded by an outer cell membrane. Spirochetes also have flagella located in the periplasmic space, between the outer cell membrane and the protoplasmic cylinder (Barbour and Hayes, 1986; Steere, 2001). Although *Borrelia* are characterized as Gram-negative bacteria, they lack lipopolysaccharides (LPS) in their outer membrane (Schröder *et al.*, 2008).

1.3. *Borrelia burgdorferi* sensu lato

The species of *Borrelia* can be divided into two major groups, related to their ability to cause a specific disease. Due to phylogenetic analysis it was found that one group consists of spirochetes causing relapsing fever and the other causing Lyme disease, also known as the *Borrelia burgdorferi* s. l. complex (Piesman and Schwan, 2010; Radolf *et al*., 2012).

To date, the *B. burgdorferi* s. l. complex consists of more than 20 species (Rudenko *et al.*, 2011; Margos *et al.*, 2015; Becker *et al*., 2016), wherein at least 5 species are known to be pathogenic in humans: with *B. burgdorferi* sensu stricto (s. s.), *B. afzelii* and *B. garinii* as the most common ones as well as *B. bavariensis* and *B. spielmanii*. Another 3 species which have occasionally been isolated from humans are *B. lusitaniae*, *B. valaisiana* and *B. bissetii* (Margos *et al.*, 2009; Rizzoli *et al.*, 2011; Stanek *et al*., 2012; Coipan *et al*., 2016). The only *Borrelia* species known to cause human Lyme disease in North America is *B. burgdorferi*. However, all of the 5 pathogenic species are prevalent in Europe (Stanek *et al*., 2012).

Even though Lyme disease is characterized by specific clinical manifestations, the different genospecies are associated with distinct ones: *B. burgdorferi* s. s. most commonly causes arthritis and neuroborreliosis, whereby the latter is also related to *B. garinii*. Skin disorders like acrodermatitis chronica atrophicans (ACA) are known to be primarily caused by *B. afzelii* (Rizzoli *et al*., 2011).

These varying clinical manifestations can be traced back to epidemiological changes of *Borrelia* due to host specialization they have undergone. The vector and host adaptions happening are related to the geographical location and hence depend on the present ecological niche. This in turn influences the species and population distribution of *Borrelia*. Regarding these facts, there is a dynamic interplay during the infectious life cycle between *Borrelia* and its vectors and hosts (Kurtenbach *et al*., 2006; Margot *et al*., 2011).

1.4. Infectious cycle of *Borrelia*

The transmission of *Borrelia burgdorferi* s. l. occurs via hard ticks of the genus *Ixodes* (Burgdorfer *et al*., 1982; Barbour and Hayes, 1986). Generally, the four species *Ixodes pacificus*, *I. persulcatus*, *I. ricinus* and *I. scupularis* are vectors for this *Borrelia* complex. In

Europe, *I. ricinus* is the main transmitter, in North America it is *I. pacificus* together with *I. scupularis* and in Asia *I. persulcatus* (Piesman and Schwan, 2010; Radolf *et al*., 2012).
Since there is no transovarial transmission occurring, larval ticks are uninfected when hatching. Thus, *B. burgdorferi* is acquired by obtaining the first blood meal as larvae from an infected reservoir host. Such hosts comprise vertebrates, ranging from small mammals like mice, squirrels or birds to bigger ones like deer and humans. Ixodes ticks feed once per live stage which comprise larval, nymphal and adult. Every blood meal is followed by a molt into the next stage. Thus, transmission to a naïve host usually occurs at the nymphal stage during the blood meal. After the mating of adult ticks, the cycle begins again from uninfected larvae which hatch from eggs (Radolf *et al*., 2012).

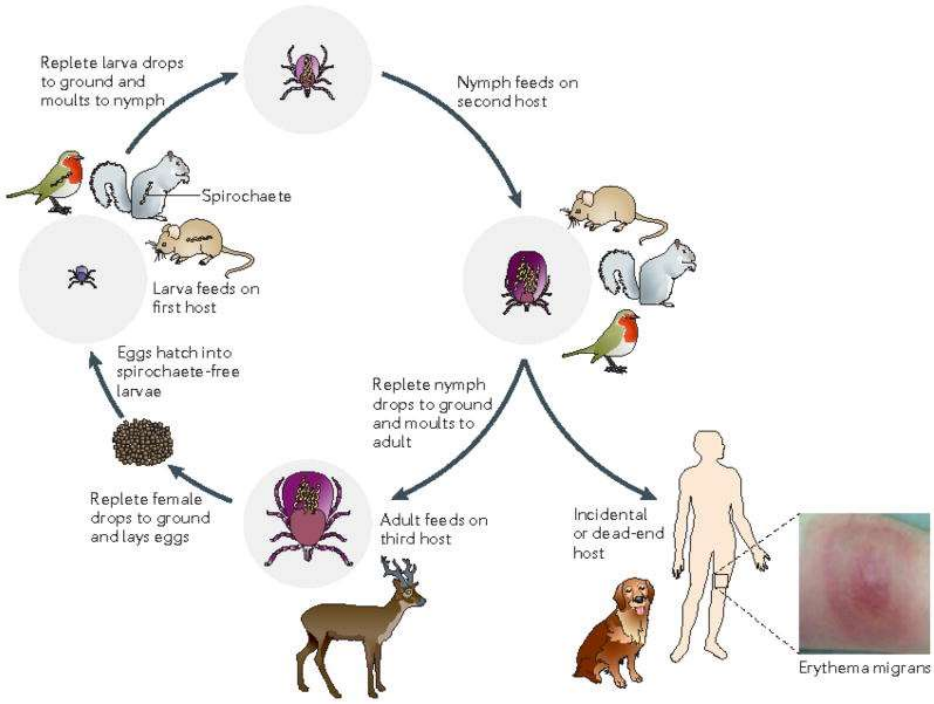

Figure 1: Graphical illustration of the infectious life cycle of *Borrelia* (from Radolf *et al.*, 2010)

1.5. Genome of *Borrelia*

The first genome sequenced of a *Borrelia* species consisted of a linear chromosome of about 900 kilo base pairs (kbp) together with approximately 12 linear plasmids (lp) and 9 circular plasmids (cp) with sizes between around 5 and 56 kbp (Fraser *et al*., 1997; Casjens *et al*., 2000).

This unique segmented genome structure is shared by all species in the *B. burgdorferi* s. l. complex. However, even the use of adapted primers for strains different than the first sequenced isolate *B. burgdorferi* B31 did not show the same results for plasmid profiling. Hence, complete sequences are needed for the exact determination of plasmids in strains other than B31, due to the heterogeneity in plasmid content of different *Borrelia* species (Rosa *et al*., 2010).

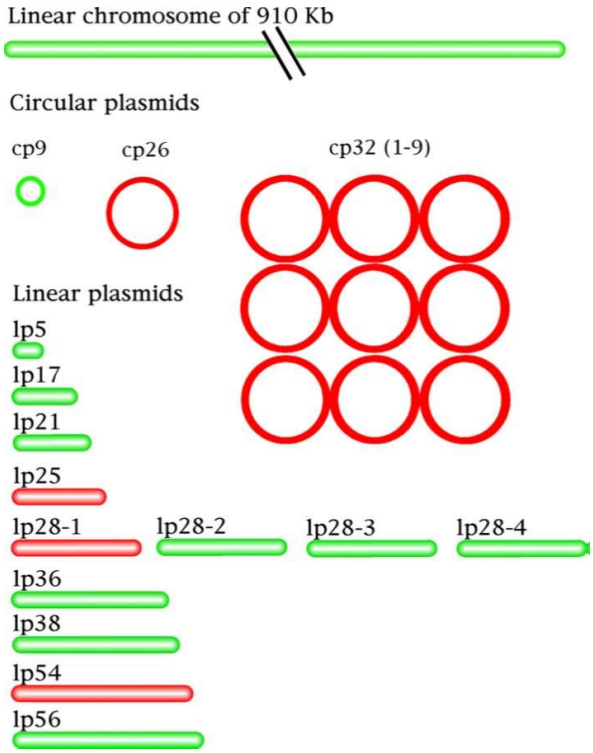

Figure 2: A graphic showing the genome of *Borrelia burgdorferi* s. s. (from Stewart *et al.*, 2004)

An essential point in the Eurasian *Borrelia* field was the sequencing of the whole genomes of various strains of *B. afzelii* and *B. garinii* (Casjens *et al*., 2011). A comparison between several *B. afzelii* strains has shown a high degree of identity in genome sequences. There is also a high conservation to *B. burgdorferi* B31. Nevertheless, the sequence similarity between strains of *B. afzelii*, *B. garinii* and *B. bavariensis* is even higher (Schüler *et al*., 2015).

Examining the presence of certain plasmids, it can be observed that fewer plasmids exist in *B. afzelii* than in *B. burgdorferi* s. s.. The plasmid composition in different *B. afzelii* strains is relatively homogeneous, however, compared to *B. burgdorferi* s. s. there are substantial differences. In *B. afzelii* the plasmids lp17, lp38, lp54, cp26 and a varying number of lp28 and cp32 can be found, unlike plasmids like lp5, lp21, lp25, lp36 and cp9, which are all present in

B. burgdorferi B31 (Schüler *et al*., 2015). Truncation or elongation of plasmids carried by *B. afzelii* and the resulting differences in size compared to *B. burgdorferi* s. s. also leads to different designations of plasmids. Hence certain plasmids are not completely lost, but are present in a different form.

The genome of *Borrelia* is important to study since each plasmid carries different genes which are encoding for particular functions and more than 70% of the genome is only found in *Borrelia*. One example is *pncA*, which is an essential gene, needed for the infectivity in mice. In *B. burgdorferi* B31 it is located on lp25, whereas it can be found on lp28-2 in *B. afzelii* strains. Another gene which is important for infectivity in mammals is adenine deaminase, *adeC*, which is found on lp36 in *B. burgdorferi*. However, the gene homolog to *adeC* is located on lp38 in *B. afzelii* strains (Schüler *et al.*, 2015).

Even though the plasmids contain important genes necessary for *in-vivo* survival, they vary in stability when propagated *in vitro*. This leads to the fact that even if *in-vitro* growth is guaranteed, the loss of certain plasmids leads to the loss of infectivity *in vivo* (Casjens *et al.*, 2000; Stewart *et al.*, 2004).

In accordance to that, there are various factors which lead to a loss of certain plasmids in *in-vitro* studies. One of these is the loss of plasmids in *B. burgdorferi* by passaging of clonal isolates as well as the thawing and freezing of glycerol stocks which leads to instability. Hence, such loss in plasmid content is responsible for heterogeneity in the outgrowth population of *Borrelia* (Grimm *et al*., 2003). It is also suggested that the original *B. burgdorferi* B31 isolate contained more plasmids and may have lost some due to *in-vitro* propagation before the first actual sequencing had been taking place. (Casjens *et al.*, 2000).

Nevertheless, there are a few plasmids, one of them being circular plasmid 26 (cp26), which is present in every sequenced genome of *Borrelia* and is never lost during *in-vitro* propagation and genetic manipulations. This ubiquitous plasmid comprises genes which are encoding for essential functions, like cell viability (Byram *et al*., 2004). This unique characteristic of cp26 can thus be applied to improve the already existing tools for genetic manipulation of *Borrelia*.

1.6. Genetic Manipulation of *Borrelia*

Special considerations are necessary for genetic modification of *Borrelia*, which is due to special features like the unique structure of the genome, complex growth conditions which have to be fulfilled as well as their big genetic difference to other bacteria (Rosa *et al.*, 2010).

However, there have been various tools for genetic manipulation developed by now. Some of these are the transformation by electroporation together with the preparation of electrocompetent cells, the use of antibiotic resistance markers and the construction and use of shuttle and suicide vectors, where the latter is used for gene inactivation and complementation. Also the development of methods for transposon mutagenesis, media preparation and growth conditions for different *Borrelia* strains have been accomplished (Tilly *et al.*, 2000; Hyde *et al.*, 2011).

1.7. Visualization of *Borrelia* by Genetic Manipulation

In addition to general genetic tools for manipulation, techniques for visualizing *Borrelia* have gained higher importance. Therefore, fluorescent proteins can be used for monitoring gene expression at different environmental conditions as well as for studies which focus on the dissemination of the bacteria. The latter comprises either the observation as live-imaging in ticks or the propagation of *Borrelia* in the vasculature of a living host (Carrol *et al*., 2003; Dunham-Ems *et al*., 2009; Moriarty *et al.*, 2008). Hence, there is a wide range of possibilities how visualizing *Borrelia* can be used for studying their virulence.

In order to get a better understanding of the infectious cycle of *Borrelia*, it is necessary to identify proteins which are essential in this process. A useful genetic technique is the utilization of a quantitative gene expression system. Therefore, the gene encoding green fluorescent protein (GFP) is fused with a promoting region, which was confirmed to work in different bacteria and can be used *in vivo* and *in vitro*. To obtain such a reporter system in *B. burgdorferi*, the strong *Borrelia* promoters *ospC, ospA* and *flaB* have been used. Subsequently, the constructed shuttle vector was transformed into *B. burgdorferi* stains. For simulating the gene expression of *Borrelia* as occurring *in vivo*, the bacteria were exposed to various environmental conditions, like pH and temperature (Carrol *et al*., 2003).

A constructed GFP reporter system as a shuttle vector transformed into *B. burgdorferi* s. s. can be also used for imaging the spirochetes in the living mammalian host. Therefore, high resolution 3D imaging has been used in order to visualize the adherence and escape of *Borrelia* from the microvasculature of the animal (Moriarty *et al.*, 2008).

Another technique of developing a GFP reporter system is to stably integrate the gene, which is encoding for GFP, into the genome. This was accomplished by homologous recombination with the stable plasmid cp26, guaranteeing the constitutive expression of GFP. This system was used for live imaging of the dissemination of infectious *Borrelia burgdorferi* s. s. within the midgut and salivary glands of *I. scupularis* ticks (Dunham-Ems *et al.*, 2009). Using this method gave substantial information about the interactions between *Borrelia* and the transmitting vector.

For observing *Borrelia* interacting with its host cells, an advanced technique was developed where fluorescence and electron microscopy are combined in order to gain finer details about pathogen-host cell interactions. This so-called correlative cryo-fluorescence and cryo-scanning electron microscopy works in a way where fluorescently tagged structures are identified and subsequently visualized at high resolution, made possible due to cryo-conditions (Strnad *et al.*, 2015).

Nevertheless, most of these techniques are developed for *B. burgdorferi* s. s. since its discovery and sequencing of the genome occurred much earlier than the European species of *Borrelia*. Thus more emphasis on developing methods for genetic engineering to better understand the physiology and virulence of major European species is needed. Also, tools for visualizing *Borrelia* have to be further developed to improve the knowledge about these bacteria and differences between the species.

2. Aims

2.1. GFP Project

- Creation of green fluorescing and infectious *B. afzelii* CB34-6 by transformation of an already constructed shuttle vector, containing GFP
- Confirming the infectivity of the obtained strain by injection into naïve mice to develop a tick-mouse model

2.2. DsRed Project

- Development of a tick-mouse model for already created infectious *B. afzelii* CB43-6 SH6
- Reisolation of this strain by plating together with subsequent plasmid profiling
- Generation of a new, red fluorescing and infectious *B. afzelii* CB43-6 strain by transformation of a DsRed containing shuttle vector

3. Materials and Methods

3.1. *Borrelia* Species and Strains

The main species used in the project was *B. afzelii* since the aim was to provide genetic visualization tools for European species of *B. burgdorferi* s. l.. Concerning transformations of *Borrelia* the strain CB43 was used, specifically its clonal population CB43-6. The strain used to further investigate the tick-host cycle was CB43-6 SH6 which was created by Sophie Honeder (2017), who did the transformation and plasmid profile.

3.2. Media and Bacterial Growth Conditions

3.2.1. *Borrelia*

To prepare Barbour-Stoenner-Kelly (BSK) II media, the appropriate amounts (see Table 1) of 10x CMRL, Neopeptone and BSA were weighed first and Milli-Q water was added. The mixture was stirred for 2-3 hours to fully dissolve the ingredients. All other compounds which are found in Table 1 were added and stirred until everything was dissolved. Afterwards the pH was measured and adjusted to 7.6 using 10 M NaOH solution. The media was filtered through a 0.2 mm pore filter and aliquoted into 500 ml bottles. To each bottle 30 ml of rabbit serum (6%) was added. For storage the bottles were kept at -20°C.

The 1.5x BSK^+ media was prepared by first weighing the needed amount of BSA (see Table 1) and dissolving it in the required amount of Milli-Q water. Further on, all other compounds were weighed according to Table 1 and stirred until completely dissolved. The pH was then adjusted to 7.5 with 10 M NaOH solution and the media was filtered through a 0.2 mm pore filter. Then 300 ml were aliquoted into 500 ml bottles and 12 ml of rabbit serum was added to each one. The aliquotes were stored at -20°C.

Substance	BSKII – mass [g/l]	1.5x BSK^+ - mass [g/l]
10x CMRL	9.7	12.7
Neopeptone	5.0	6.9
BSA	50.0	69.4
Yeastolate	2.0	3.5
Hepes acid	6.0	8.3
Glucose	5.0	6.9
Sodium citrate	0.7	1.0
Sodium pyruvate	0.8	1.1
N-acetyl glucosamine	0.4	0.6
Sodium bicarbonate	2.2	6.4

Table 1: The ingredients and their concentrations needed for media preparation

The *Borrelia* cultures were prepared with glycerol stocks which are added to BSKII or BSK-H complete (Sigma-Aldrich) media, depending on the requirements. This was done by scraping off a tiny bit of the frozen glycerol stock with a pipette tip and transferring it to the media by resuspending it. Then the cultures were incubated at 34°C. Depending on the *Borrelia* strain antibiotics were added. For *B. afzelii* CB43-6 SH6, 20 µg/ml gentamycin was added after 3-4 days of incubation to maintain its shuttle vector carrying DsRed. Usually 40 µg/ml of gentamycin are added, however the spirochetes seemed to have a too high sensitivity against it, wherefore the used concentration had been changed. In contrast, 200 µg/ml kanamycin was added to *B. afzelii* CB43-6 AD 1-4 right away after starting the culture. The cultures were removed from incubation after an appropriate density of *Borrelia* had acquired, which lies at 10^7 *Borrelia*/ml.

3.2.2. *E. coli*

The preparation of lysogeny broth (LB) media was done by weighing 10 g/l of sodium chloride, 10 g/l of Trypton and 5 g/l of Yeast extract. The compounds were dissolved in distilled water and afterwards autoclaved.

For growing *E. coli*, competent cells were put into LB media and kept in the shaking incubator at 37°C for approximately 12 hours.

3.3. Ethanol Precipitation

Before transformation of plasmid DNA into *Borrelia*, ethanol precipitation of pBSV2K-GFP and pBSV2G-DsRed had to be performed to obtain a higher concentration of DNA. In order to accomplish a successful transformation about 40µg of vector DNA would be needed at an approximate concentration of 2µg/µl. The amount of 3 M Sodium acetate (pH 6) added was 1/10 of the volume of the particular plasmid DNA. Also 2 times the volume of 100% ethanol was added and the mixture was mixed by inversion of the tube, followed by centrifugation at 12000 rpm for 10 minutes at 12°C. The supernatant was removed with a pipette and 1 volume of 70% ethanol was added without resuspending. The mixture was centrifuged again at the same conditions. Then the supernatant was removed by pipetting and the pellet was allowed to dry for approximately 5 minutes. In the end, 20 µl of Milli-Q water were added to obtain a final concentration of 2 µg/µl.

3.4. Transformation of *Borrelia*

For the transformation of both, the plasmids pBSV2K-GFP and pBSV2G-DsRed, the infectious clonal population CB43-6 was used. In the beginning, *Borrelia* cultures were started according to chapter 3.2. Media and Bacterial Growth Conditions and were allowed to grow to approximately 10^7 *Borrelia*/ml.

The number of *Borrelia* was obtained by counting, using the Marienfeld-Superior counting chamber (depth = 0.02 mm; 0.0025 mm^2). Due to the expected high *Borrelia* density a 1:10 dilution was prepared by filling up 100 µl of the respective culture with 900 µl of BSKII. Then 3 µl of the vortexed culture (1:10 dilution) was added onto the counting chamber. The *Borrelia* present within 5 squares were counted. The number was noted and by using of the equation depicted below, the number of *Borrelia* per milliliter was determined. The variable x represents the number of *Borrelia* counted occurring on 5 squares.

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

The required concentration of *Borrelia* for an effective transformation is $5x10^7$ *Borrelia*/ml. From the count of the *Borrelia* cultures and the known doubling time of *B. afzelii* CB43-6, which is approximately 12 hours an appropriate volume of culture was added to 100ml of BSKII and then incubated at 34°C for 5 days.

After the appropriate *Borrelia* density has been reached, the culture was centrifuged for 10 minutes at 7800 rpm and 10°C and the supernatant was discarded. The pellet was resuspended in 25 ml of cold electroporation solution (EPS). Then the cells were centrifuged again for 10 minutes at 7800 rpm, 10°C. The supernatant was removed and the washing step was repeated. After the second washing, the pellet was resuspended in 250 µl EPS and transferred into a 1.5 ml Eppendorf tube and put on ice. The contents of EPS are 93.1 g/l of Sucrose and 150 g/l of Glucose in Milli-Q water. The mixture is filtered through a 0.2 mm pore filter and kept at 4°C. The cells were resuspended gently using a 200 µl pipette to disperse them. This was checked under the microscope and more EPS was added if there was too much clumping of bacteria.

The competent *Borrelia* cells were vortexed and added to the previously prepared plasmid DNA. Then the mixture was transferred to electroporation cuvettes (VWR) and electroporated. The cells after electroporation were transferred to fresh BSKII and stored at 34°C for approximately 48 hours before plating.

3.5. Plating of *Borrelia* Cultures after Transformation

Concerning the plating of *Borrelia*, the electroporated cell cultures were counted and the density was determined. Two plates without antibiotics were prepared in order to check for correct enumeration of the culture and if the plating was performed well, with 100 *Borrelia* per plate. For that reason, a dilution series was made in order to obtain 10^3 *Borrelia*/ml, which is the desired concentration of 1 *Borrelia*/µl.

The agarose media was prepared by mixing a 1.7% liquid autoclaved agarose (SeaKem®) with 300 ml of 1.5x BSK^+ media and adding 5 ml of *Borrelia* antibiotics (containing 2 mg/ml phosphomycin, 5 mg/ml rifampicin and 250 mg/ml amphotericin B, dissolved in 20% DMSO) to obtain a 1:100 ratio. The mixture was then placed in a water bath at 55°C to avoid solidifying.

Then 10 ml of the agarose media was poured onto each plate and allowed to solidify. 40 ml of the agarose media was mixed with 200 µl of the respective 10^3 *Borrelia*/ml culture in a 50 ml

falcon tube and 20 ml of that mixture was put onto the prepared 10 ml plates. The remaining plates were prepared in order to be selective to either the transformed pBSV2K-GFP or pBSV2G-DsRed vector by adding 200 µg/ml of kanamycin or 20µg/ml of gentamycin to the agarose media. Again 40 ml of the agarose media was added to three falcon tubes and 1, 2 or 3 ml of the original *Borrelia* culture was added to each one. Then 20 ml were added to the already solidified plates as before.

Afterwards, the solidified plates were placed into air tight boxes with anaerobic sachets (GasPak™) and stored at 34°C. Colonies appeared after 4 weeks and were plugged into BSKII media. This was done by poking a colony with a 1 ml pipette tip and sucking it up. Then the piece of colony within the pipette was pushed into BSKII media in an 8 ml tube. The cultures were grown according to chapter 3.2. Media and Bacterial Growth conditions, except that kanamycin and gentamycin respectively were added six days after picking the colonies. After an approximate *Borrelia* density of 10^7 *Borrelia*/ml had been reached, the cultures were checked for fluorescence, gDNA was isolated and glycerol stocks were made. Also, lysates were prepared and mice were injected with the cultures for further serological studies.

3.6. Lysate Preparation

From the cultures which were used for injecting into mice, lysates were prepared to determine the infectivity of the *Borrelia* by performing Western Blotting.

The cultures were centrifuged for 10 minutes at 7800 rpm and at 20°C. The supernatant was discarded and 1 ml of cold HN buffer was added. The pellet was resuspended and transferred to a 1.5 ml Eppendorf tube. The centrifugation step was repeated with the same conditions and the supernatant was removed by pipetting. Then, again washing with 1 ml of cold HN buffer and the same centrifugation settings was performed. After discarding the supernatant, 200 µl B-PER™ (Bacterial Protein Extraction Reagent from ThermoFisher Scientific™) were added and the pellet was resuspended. The suspension was then kept at room temperature for 10 minutes. Further on, 200 µl Laemmli buffer was added, which is a mixture of 950 µl of 2x Laemmli Sample Buffer (Bio-Rad) and 50 µl of β-mercaptoethanol.

3.7. Genomic DNA Isolation

The genomic DNA (gDNA) from cultures was isolated with the Wizard® Genomic DNA Purification Kit (Promega) by following the manufacturer's protocol for Gram Positive and Gram Negative Bacteria with some exceptions.

The *Borrelia* cultures were centrifuged in 15 ml falcon tubes for 10 minutes at 7800 rpm. The supernatant was discarded and 600 µl of Nuclei Lysis Solution was added. Then the pellet was resuspended and transferred to a 1.5 ml Eppendorf tube. To lyse the cells, they were incubated at 80°C for 5 minutes and afterwards put on ice for 2 minutes. 3 µl RNAse were added to the cell lysate and the tube was inverted 2-5 times for mixing. After incubation for 30 minutes at 37°C followed by cooling down to room temperature, 200 µl of Protein Precipitation Solution were added. The mixture was incubated on ice for 5 minutes and centrifuged at 13000 rpm for 5 minutes. Next the supernatant was transferred to a clean 1.5 ml Eppendorf tube containing 600 µl isopropanol. The tube was gently inverted until thread-like strands of DNA formed a visible mass and was centrifuged at for at 13000 rpm for 10 minutes. The supernatant was carefully removed by pipetting, the tube drained on a clean paper towel and air-dried to get rid of small residues of isopropanol. Afterwards 600 µl of 70% ethanol was added and the tube was again centrifuged at 13000 rpm for 10 minutes. The supernatant was removed in the same way as isopropanol. In the end, 100 µl of DNA Rehydration Solution was added without mixing and the sample was stored at least 1 day prior to processing it.

3.8. Polymerase Chain Reaction (PCR)

After obtaining *B. afzelii* CB43-6 clones AD 1-4 and CB43-6 DsRed clones 2, 4 and 5 by transformation and plating, the presence or loss of plasmids was confirmed by PCR for guaranteeing their infectivity. The same was done for the plated CB43-6 SH6.

The plasmid profile was done for 16 linear plasmids and 5 circular plasmids, where primers designed for *B. afzelii* PKo were used. These primers are not published yet (please contact Ryan Rego, PhD for further information). As a positive control served CB43-6 SH6, a *Borrelia* strain for which the presence of all plasmids was already verified.

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

Master Mix	**Amount [µl]**
OneTaq HS Quick-Load 2x MM	10
Millli-Q H2O	7
Primer F+R	1
DNA	2
Total	20

PCR Settings 1 – Plasmid Profile			
#	**PCR Step**	**T [°C]**	**t [sec]**
1	Initial Denaturation	95	30
2c	Denaturation in cycle	95	45
3c	Annealing in cycle	52	45
4c	Elongation in cycle	68	60
5	Final Elongation	68	300
6	Hold	16	∞

Table 2: Master Mix with given amounts per reaction and PCR reaction settings for plasmid profiling

3.9. Experimental Mouse Model

3.9.1. Western Blotting

The green-fluorescing cultures of *B. afzelii* CB43-6 clones AD 1-4 were injected into 3 BALB/c mice each and later also into 3 C3H/HeN mice each. Also, a red-fluorescing culture of CB43-6 SH6 was injected into 6 BALB/c mice. In order to inject 10^6 *Borrelia* into one mouse, 250 µl of culture with a concentration of 4000 *Borrelia*/µl was used. After 3 weeks, Western Blotting was performed to check whether the strains are infectious, wherefore the sera of the injected mice were used as primary antibodies. The larval ticks which fed on SH6 infected mice

were kept 2 months allowing them to molt to nymphs. From these, 2-4 ticks were put on naïve C3H/HeN mice each. Again Western Blotting was conducted after 3 weeks.

First SDS-page gels were prepared by using the TGX™ and TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad). The gels were made using 0.75 mm glass plates with amounts of reagents depicted in Table 3.

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

Table 3: Reagents which were used for the preparation of each SDS-PAGE gel

Before loading 20 µl of the respective lysate into the well, the lysate was incubated for 10 minutes at 100°C. Then SDS-page gel electrophoresis was conducted at 120 V for approximately 1 hour. Next the blotting set-up was built like the following: blotting paper, nitrocellulose membrane, gel, blotting paper. The nitrocellulose membrane was soaked in 1x blotting buffer (1:10 dilution of 25 mM Tris-base and 192 mM glycine distilled water) for 5 minutes prior to its use. The blotting paper was also shortly soaked with the same buffer. The transfer was run at 25 V for 30 minutes.

For blocking of the membrane, a 5% blocking solution was prepared by dissolving 10 g of dehydrated milk in 200 ml of 1x TBS Tween 20. After the completed transfer, the membrane was removed from the assembly and put into blocking solution for 2 hours on a shaking platform. In the meantime, primary antibody solutions were prepared by mixing 995 µl of blocking solution with 5 µl of the respective mouse sera (1:200 ratio). When blocking was finished the membrane was cut into single lines for each lysate and put into plastic bags. The corresponding primary antibody was added and the bag was tightly sealed without air bubbles

and stored overnight in the fridge. After incubation the bags were opened and the membrane pieces were washed three times with 1x TBS Tween 20 for 15 minutes each. The secondary antibody was prepared by adding 5 µl of Anti-Mouse antibody IgG (Sigma Aldrich, St. Louis, MO) to 50 ml of blocking solution (1:10000 ratio). The membranes were shaken in secondary antibody solution for 1 hour. Then they were washed again three times with 1x TBS Tween 20 for 15 minutes. During the last wash, the Pierce™ ECL Western Blotting Substrate (ThermoFisher Scientific™) was prepared by mixing 1 ml of each detection reagent (1 and 2). Then the membranes were incubated for 5 minutes in the substrate solution. Finally, the pieces of membrane were assembled on a transparency and the results visualized with the Chemi-Doc system (Bio-Rad).

In the case of a high number of samples, the Mini-PROTEAN® II Multiscreen Apparatus (Bio-Rad) was used for incubation of the primary antibody. For that the SDS-page gel was prepared as described above, using a 2-well comb1-well, where 150 µl of lysate was loaded into the big well provided. The SDS-page and the electroblotting was run with the same conditions as already described. Also the blocking step was performed in the same way. Then, the uncut membrane was clamped into the apparatus in a way that the wells overlap with the lysate to an appropriate extent. The incubation apparatus was slightly tilted and 600 µl of primary antibody was loaded into each well from the bottom. It was ensured that no air bubbles were incorporated. The set-up apparatus was put on a shaking platform for 1-2 hours and afterwards incubated in the fridge overnight. The subsequently performed steps were the same as without using the blotter.

3.9.2. Genomic DNA Isolation from Tissue

To further verify whether mice were infected three weeks after injection, gDNA isolation was performed. Also, to confirm if *I. ricinus* ticks, which were put on infected mice, have acquired *Borrelia* after feeding the same method was used. In the beginning this was accomplished by adapting the Spin-Column Protocol for Purification of Total DNA from Animal Tissues from the DNeasy Blood & Tissue Kit (Quiagen). Later the isolation of gDNA was performed using the similar kit, NucleoSpin® Tissue from Machery-Nagel for Genomic DNA from tissue. This was done by adapting the Standard protocol for human or animal tissue and cultured cells as follows:

In the first step the animal tissue had to be chopped into small pieces. For the mouse tissue, which was taken from the ear, this was done by placing it onto Parafilm and cutting it with a sterile scalpel. For sufficient isolation of DNA, approximately 25 mg of tissue was used. The tissue was then transferred to a 1.5 ml Eppendorf tube and 180 µl of Buffer T1 and 25 µl of Proteinase K were added.

For purifying DNA from ticks, either at least 2 larval ticks or 1 nymphal tick were put into a 1.5 ml Eppendorf tube. Then 180 µl of Buffer T1 was added and the ticks were crushed into at least 2 visible pieces using a pestle and 25 µl of Proteinase K was added.

The tubes for both, the isolation of gDNA from mouse and tick, were vortexed and incubated at 56°C overnight, including additional vortexing if needed. Care was taken that all of the sample was covered with the lysis solution while incubating.

Before further following the procedure for the mouse sample, the hair has been removed by centrifugation of the sample at 2000 rpm for 10 minutes and carefully pipetting of the supernatant.

Next, 200 µl of Buffer B3 was added to the sample, followed by vortexing and subsequent incubation at 70°C for 10 minutes. Then 210 µl of 96% ethanol was added and the tube was vortexed thoroughly. To bind the DNA, a NucleoSpin® Tissue Column was placed into a Collection Tube and the sample was applied. It was then centrifuged at 13000 rpm for 1 minute. The flow-through was discarded and the column was placed into a new Collection Tube. For washing, 500 µl of Buffer BW was added and the sample was centrifuged at 13000 rpm for 1 minute. Again, the flow-through was discarded and a second washing step was performed, adding 600 µl of Buffer B5 and centrifuging at 13000 rpm for 1 minute. The flow-through was removed and the membrane was dried by centrifuging at the same conditions as before. The column was then placed into a 1.5 ml Eppendorf tube and 100 µl of Buffer BE was added. The sample was kept at room temperature for 1 minute to elute the DNA with further centrifugation at 13000 rpm for 1 minute.

3.9.3. Polymerase Chain Reaction (PCR)

After isolating gDNA from mice or ticks, PCR was used to confirm the presence of *Borrelia*. The primers used for looking at *Borrelia* in mice were *adeC*, mouse actin (to identify that the

isolation was correct) and kanamycin primers (Rego *et al*., 2011). For the tick DNA the same primers were used, but instead of mouse actin a tick actin primer was used. The positive control utilized was DNA from mouse or tick respectively, which was already confirmed to be infected.

For all PCRs the OneTaq® Hot Start Quick-Load® 2x Master Mix with Standard Buffer (New England Biolabs®) was used for preparation of a master mix. Its composition as well as the temperature program can be found in Table 4. The denaturation, annealing and elongation step was repeated 30 times in a cycle for all programs.

Master Mix	**Amount [µl]**		**PCR Settings 2 – OneTaq MM**			
OneTaq HS Quick-Load 2x MM	10		**#**	**PCR Step**	**T [°C]**	**t [sec]**
Millli-Q H2O	7		**1**	Initial Denaturation	94	30
Primer F+R	1		**2c**	Denaturation in cycle	94	30
DNA	2		**3c**	Annealing in cycle	55	45
Total	20		**4c**	Elongation in cycle	68	60
			5	Final Elongation	68	600
			6	Hold	16	∞

Table 4: Master Mix with given amounts per reaction and PCR reaction settings all PCRs different than plasmid profiling

3.9.4. Re-isolation of *Borrelia* from Ticks in Media

After the blood meal of ticks on CB43-6 SH6 infected mice, re-isolation of *Borrelia* was performed by crushing ticks in BSKII media to confirm that the respective ticks have acquired the *Borrelia*. When isolating from larval ticks, at least three per tube had to be used to guarantee a sufficient amount of *Borrelia*.

First the ticks were put into 1.5 ml Eppendorf tubes and covered with 500 µl of a 3% Hydrogen peroxide solution for 15 minutes, making sure that all the ticks are immersed. The liquid was removed by pipetting and 500 µl of 70% ethanol was added. After 15 minutes the liquid was

removed again by pipetting and the tubes were kept open to allow the remaining EtOH to evaporate for about 5 minutes. Then 150 µl of warm BSKII media was added, wherein the ticks were crushed using a pestle. 850 µl of BSKII media was used to wash the pestle and to fill up the tube to 1000 µl. Afterwards, 10 µl of *Borrelia* antibiotics was added to obtain a 1:100 dilution.

After 6-7 days, 20 µg/ml gentamycin had to be added in order to maintain the shuttle vector. The cultures were kept at 34°C for at least 10 days and then checked from time to time for *Borrelia* growth.

3.9.5. Re-isolation of *Borrelia* from Mouse Tissue in Media

After euthanizing mice, which were used in tick-host model experiments, their tissues and organs (ear, bladder, joint and heart) were put into 7 ml of BSKII media, containing 70 µl of *Borrelia* antibiotics (1:100 dilution). For re-isolation of CB43-6 SH6, gentamycin with a concentration of 20 µg/ml was added after around 6-7 days of incubation at 34°C. In contrast, after 4 days, 200 µg/ml of kanamycin were added for re-isolating CB43-6 AD 1-4.

3.9.6. Artificial Infection of *I. ricinus* larvae

Since larval ticks of the species *I. ricinus* were already proven to acquire CB43-6 SH6 by feeding on infected mice, artificial infection was performed with about 200 larvae (adapted from Policastro and Schwan, 2003). This was done in order to skip acquisition of the borrelia from infected mice. Still, these ticks had to feed on naïve mice after infecting them artificially to let the *Borrelia* propagate inside the larvae. Also, these larvae could have been used after molting for subsequent feeding on naïve mice.

The ticks were split up and put into 3 separate 1.5 ml screw cap microcentrifuge tubes. These tubes were desiccated at lowered humidity with a sat. $(NH_4)_2SO_4$ solution to increase the percentage of lavae that take up the *Borrelia* during immersion. After 2 days, the larvae were immersed in 1 ml of culture with a density of 1.75×10^7 *Borrelia*/ml, instead of the recommended 1×10^7 *Borrelia*/ml. The tubes were gently vortexed and incubated for 1 hour at 32°C, making

sure that all ticks are immersed into the culture. Every 10 minutes during incubation the vortexing was repeated. Centrifugation was performed at 1500 rpm for 30 seconds to pellet the ticks for an easier removal of the *Borrelia* culture. After pipetting off the culture, the larvae were washed 2 times with 1 ml of cold 1x PBS (4°C). The buffer was removed and the tubes were dried with filter paper to avoid fungal infections. The tubes were perforated and put into a desiccator together with a sat. KCl solution to provide 90% humidity for 1 day. The usual time of incubation is 1-3 days until the ticks are allowed to feed on mice. After incubation, the ticks were put on 3 naïve C3H/HeN mice to feed. Also DNA isolation and re-isolation of *Borrelia* by crushing the ticks in BSKII was done after the completed blood meal.

3.9.7. Plating of Ticks

Plating was performed in order to re-isolate *Borrelia* from larvae, which either fed on infected mice (CB43-6 clones AD 1 and 4) or were artificially infected with CB43-6 SH6. The procedure was also performed with nymphs, which fed as larvae on infected mice (CB43-6 SH6).

First the ticks were prepared as for the re-isolation in culture (see chapter 3.9.4. Re-isolation of *Borrelia* from Ticks in Media), except that no antibiotics at all were added before the plating was done. For each tick-culture, 4 plates were prepared, with 2 of them being without antibiotics. The other 2 plates were prepared to be selective for the respective shuttle vectors.
The agarose media was prepared in the same way as for the plating of usual *Borrelia* cultures. The detailed description can be found in chapter 3.5. Plating of *Borrelia* Cultures after Transformation.

Afterwards, 10 ml of the mixture was poured on each plate and allowed to solidify. For the non-antibiotic plates 40 ml of agarose media was mixed with 250 ml of tick-culture in a 50 ml falcon tube. Then 20 ml of the obtained mixture was poured onto a plate containing the solidified 10 ml and were also allowed to solidify. The other two plates were prepared to be selective for either the pBSV2K-GFP or pBSV2G-DsRed vector, depending on the crushed ticks, by adding 200 µg/ml of kanamycin or 20µg/ml of gentamycin to the agarose media. Again 40 ml of the agarose media were added to a falcon tube together with 250 ml of tick-culture. Subsequently, 20 ml of the mixture were added to the solidified plates as before.

In the end, the plates were stored in air tight boxes with anaerobic sachets (GasPak™) at 34°C for 4 weeks. Unfortunately, no colonies appeared so there was no propagation done.

The remaining tick-cultures were filled up to 1500 µl with BSKII media and 15 µl *Borrelia* antibiotics was added to obtain a ratio 1:100. Then the tubes were incubated at 34°C and after around 6-7 days 20 µg/ml gentamycin was added to the cultures of the ticks, infected with CB43-6 SH6. After around 4 days 200 µg/ml kanamycin was added to the tick cultures for re-isolating CB43-6 AD 2 and 4. The cultures were checked after 10 days and afterwards from time to time for *Borrelia* growth.

3.10. Molecular Cloning and Transformation of *E. coli*

To further confirm whether mice, injected with CB43-6 AD 1-4, were infected gDNA isolation was performed together with PCR. The amplified kanamycin sequence had to be additionally verified by sequencing, wherefore cloning and transformation into *E. coli* was accomplished.

First it was checked whether a pure product was obtained by gel electrophoresis. Afterwards the kanamycin PCR product was used for ligation into the pCR™2.1-TOPO® vector (Invitrogen™) using the TOPO® TA Cloning® Kit. The reaction mixture was prepared by mixing 0.5 µl of Salt Solution, 0.5 µl of vector and 2 µl of PCR product. Then incubation was conducted for 10 minutes at room temperature.

Further on, transformation into *E. coli* was performed using Subcloning Efficiency™ DH5α™ competent cells (Invitrogen™). 1.5 µl of the TOPO reaction was added to 50 µl of thawed cells in a 1.5 ml Eppendorf tube. After keeping it on ice for 30 minutes, heat shock of the competent cells/TOPO mixture was performed by incubating it 30 seconds at 42°C and then immediately putting it on ice. 2 minutes later, 250µl of SOC Outgrowth Medium (New England Biolabs®) was added and the tube was placed on a horizontal shaker at 200 rpm at 37°C for 1 hour.

3.11. Plating of *E. coli* and Picking of Colonies

During shaking of the cells in the incubator, LB plates with carbenicillin were warmed up at 37°C. The plates were treated with 40 µl of X-Gal solution (ThermoFisher Scientific™) each, 15 minutes before the competent cells were ready to plate. Then 100 µl and 200 µl of cells were added. The plates were kept at 37° overnight.

For plugging the colonies, 7 ml of room temperature LB media was aliquoted into 15 ml falcon tubes. 10 µl pipette tips were used to pick chosen colonies and insert them into the media together with the tips. The tubes were kept in the horizontal shaker at 37°C for at least 12-14 hours.

3.12. Plasmid DNA isolation

After the incubation on the horizontal shaker had been finished, plasmid DNA isolation was accomplished using the NucleoSpin® Plasmid Kit (Machery-Nagel). The NucleoSpin® Plasmid protocol for isolation of high-copy plasmid DNA from E. coli was applied as follows:

The *E. coli* culture was centrifuged at 13000 rpm for 30 seconds. As much as possible of supernatant was removed and 250 µl of Buffer A1 was added. The pellet was resuspended and transferred into a 1.5 ml Eppendorf tube. Then 250 µl of Buffer A2 was added and the tube was gently inverted 6-8 times for mixing reasons. The mixture was incubated for 5 minutes at room temperature and afterwards 300 µl of Buffer A3 was added and mixed by thoroughly inverting the tube 6-8 times. The sample was centrifuged for 5 minutes at 13000 rpm and room temperature. If the supernatant was not clear this step was repeated. Then, 750 µl of the supernatant was loaded onto a NucleoSpin® Plasmid Column which was placed in a 2 ml Collection Tube. The tube was spun down at 13000 rpm for 1 minute, the flow-through was discarded and the column was placed back into the collection tube. For washing, 600 µl of Buffer A4 was added and centrifugation at the same condition as previously was performed. The flow-through was discarded and the column was placed into a new collection tube. The membrane was dried by centrifugation for 2 minutes at 13000 rpm. Next the column was placed into a 1.5 ml Eppendorf tube and 50 µl of Buffer AE was added. After 1 minute of incubation at room temperature the tube was centrifuged at 13000 rpm for 1 minute.

3.13. Restriction Digest and Sequencing

Restriction digestion was performed to confirm the successful ligation of the kanamycin sequence into the pCR™2.1-TOPO® vector, using EcoRI restriction enzyme and NEBuffer™ 2.1, both from New England Biolabs®. The reaction mixture was prepared as described in Table 5 and incubated for 1 hour at 37°C.

Gel electrophoresis was used to visualize the obtained products of the restriction digestion together with an undigested vector as control. Chosen products were sequenced and the sequences were examined by comparison to other sequences in the NCBI database.

Restriction Digest with EcoRI	
Reagent	Amount [µl]
EcoRI	0.5
NEBuffer 2.1	2.0
DNA	2.0
Milli-Q water	15.5
Total	20.0

Table 5: Used amounts of reagents per reaction for a restriction digestion

3.14. Plating of *B. afzelii* CB43-6 SH6

Due to unfortunate contamination of glycerol stocks, containing CB43-6 SH6, plating of a selected stock, which was the least contaminated was done. For that reason, cultures of that stock were started in BSKII as well as BSK-H complete media. Further on, plugging of the obtained colonies was done together with subsequent gDNA isolation of the culture to obtain a plasmid profile.

It was assumed that the cultures to be plated contained *Borrelia* with a density of 10^7 *Borrelia*/ml. In order to plate 200 *Borrelia* cells per plate, a concentration of 10^3 *Borrelia*/ml or 1 *Borrelia*/µl was obtained. This was done by stepwise dilution, whereby

10 µl of culture were filled up to 1000 µl with warm BSKII or BSK-H compete respectively to reach a concentration of 10^5 *Borrelia*/ml. After vortexing this was repeated in order to get the needed *Borrelia* density for plating.

The further plating procedure was performed as for the usual plating of *Borrelia* cultures, described in chapter 3.5. Plating of *Borrelia* Cultures after Transformation.

After four weeks of incubation at 34°C colonies appeared, which were propagated in BSK-H complete medium in order to avoid contamination. This was accomplished by poking a colony with a 1 ml pipette tip and inserting it into the media in an 8 ml tube. Then the pipette tip was rinsed with the media by pipetting up and down. Five days later, gentamycin was added with a concentration of 20 µg/ml. As the BSK-H media was not supporting the growing conditions of the *Borrelia afzelii* strain, 1 ml of the culture was transferred into self-made BSKII media, containing *Borrelia* antibiotics with a 1:1000 ratio. The new cultures and the ones with BSK-H complete, were incubated at 34°C until a concentration of 10^7 *Borrelia*/ml had been reached. Then the cultures were checked for fluorescence, glycerol stocks were made and gDNA isolation was performed.

4. Results

4.1. GFP Project

The first part in this project was to transform a pBSV2K-GFP shuttle vector, provided by Sophie Honeder (2017), into *B. afzelii* CB43-6.

To verify if the obtained clones are expressing the GFP gene, fluorescence microscopy was used. Also dark-field microscopy was performed using the same glass slide of the respective *Borrelia* culture as for the fluorescence microscopy. This was done to see approximately how many *Borrelia* of the culture maintained the shuttle vector. All *Borrelia* cultures obtained from the four clones, named CB43-6 AD 1-4, were confirmed to fluoresce green in a quite high amount.

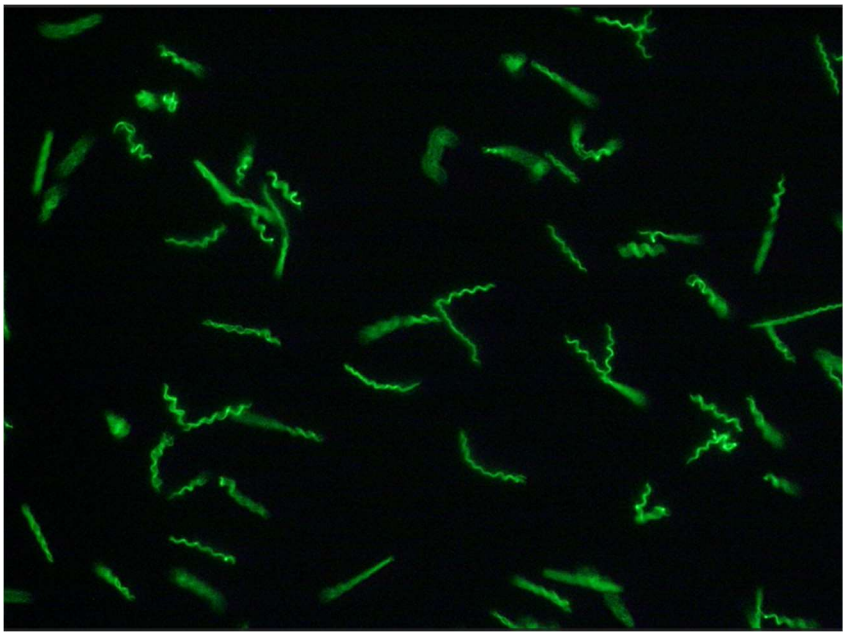

Figure 3: *Borrelia afzelii* CB43-6 expressing GFP (40x magnification)

During the gDNA isolation of the *Borrelia* cultures it had been observed that the pelleted cells had a green color, giving an additional promise of a successful transformation (see Figure 4).

Figure 4: Pelleted *Borrelia* cells during gDNA isolation

Then a plasmid profile of four clonal populations was done using PKo primers. This was conducted to confirm whether no plasmids were lost during transformation in order to verify the infectivity of the clones.

Plasmid	CB43-6 AD1	CB43-6 AD2	CB43-6 AD3	CB43-6 AD4
lp17	+	+	+	+
lp25	+	+	+	+
cp26	+	+	+	+
cp26+27	+	+	+	+
lp28	+	+	+	+
lp28-2	+	+	+	+
lp28-3	+	+	+	+
lp28-4	+	+	+	+
lp28-7	+	+	+	+
lp28-8	+	+	+	+
cp30	+	+	+	+
lp32	+	+	+	+
cp32-5	-	-	-	-
cp32-7	+	+	+	+
lp32-9	+	+	+	+
lp32-10	+	+	+	+
lp34	+	+	+	+
lp38	+	+	+	+
lp54	+	+	+	+
lp60	-	-	-	-
lp60-2	+	+	+	+

Table 6: Plasmid profiles of GFP expressing clones CB43-6 AD 1-4; the grey areas indicate missing plasmids

As seen in Table 6, all the four clones do not carry cp32-5. However, since other types of this plasmid are detectable it can be assumed that cp32 is still present in the genome.

In the second part of this project, cultures of all 4 clonal *Borrelia* populations were injected into 3 BALB/c mice each. After 20 days, Western Blotting was performed with the sera of all mice together with gDNA isolation from the ear tissue of every mouse 22 days post inoculation, to confirm if the mice were infected.

Unfortunately, the mice were not verified to be infected by Western Blotting. In comparison to the positive control, the bands of the mouse samples were only a few, suggesting that the mice have cleared the infection (see Figure 5). Simultaneously, gDNA was isolated from each mouse and all samples were positive for kanamycin primers, indicating the presence of the shuttle vector carrying the GFP.

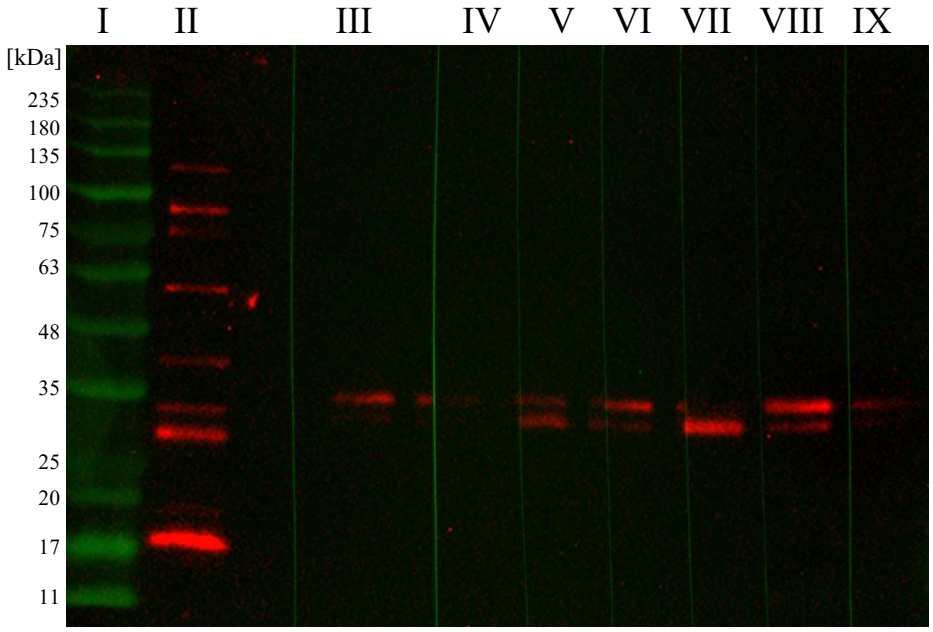

Figure 5: Western blot - lysate loaded in all wells from infectious CB43-6; Lane: **I** – protein marker IV (AppliChem); **II** – positive control: serum from mouse infected with CB43-6; **III-IX** – sera of mice

Additionally, to the isolation of gDNA, transformation and cloning together with sequencing of a purified PCR product of kanamycin was done to make sure the amplification by PCR was correct. Sequences were not obtained for the cloned PCR product.

One month after injection of the mice, larval *I. ricinus* ticks were put on 3 mice, which had shown the highest degree of infection, in terms of intensity of the bands of the Western Blot. One of the chosen mice was injected with CB43-6 AD 2, and two mice were injected with CB43-6 AD 4. The larvae were collected after feeding, stored in tubes and allowed to molt. Three days after collecting them, 6 ticks per mouse, split up into 3 ticks per 1.5 ml Eppendorf tube, were crushed in media and plated in order to re-isolate *Borrelia*. The remainders of the crushed tick media were filled up with BSKII media and kept in the incubator for isolation of *Borrelia* in media. Approximately 20 days after feeding of the ticks, gDNA isolation was performed on 4 ticks per mouse, each divided into groups of 2, followed by PCR to verify whether the fed ticks acquired the *Borrelia*.

From the plates of the crushed ticks, no colonies at all were growing and the cultures of those ticks did not give a positive result either possibly due to contamination. Unfortunately, the isolated gDNA from the fed larvae came up to be negative for *adeC* and kanamycin.

Two months post injection of the *Borrelia* cultures, the mice were euthanized and one ear of each mouse was put into BSKII media for re-isolating *Borrelia*. Those cultures did not give a positive result, possibly because the media did not support the growing conditions of *Borrelia*.

Again, cultures of CB43-6 AD 1-4 were injected into 3 mice each, however, into mice of the strain C3H/HeN. Then 3 weeks post inoculation, the mice were bled and the sera were used for Western Blotting. Unfortunately, the same result was obtained as for the BALB/c mice, since only 1-2 have shown up for the mouse samples, suggesting that the infection got cleared.

After 8 weeks, the mice were euthanized without keeping any tissues and organs due to the fact that the clones were not infectious, after the second attempt at infecting mice.

4.2. DsRed Project

4.2.1. Experimental Tick-Mouse Model

The aim in this project was to investigate the infectivity of DsRed expressing *B. afzelii* CB43-6 SH6 using a tick-mouse model. On one hand this was done by injecting mice together with letting *I. ricinus* larvae feed on the mice, after they were proven to be infected. Then the ticks were allowed to molt to nymphs and put on naïve mice afterwards. Since it has been shown that larval ticks can acquire the *Borrelia* with their first blood meal, the first steps of the infectious cycle were left out due to lack of time with the project and to try out a different method for infecting larvae. Therefore, artificial infection on larval ticks was conducted. These ticks were then allowed to feed on naïve mice.

At first, 6 BALB/c mice were infected by inoculation with a culture of CB43-6 SH6. After 20 days the mice were bled and Western Blotting was conducted. For all samples three bands have shown up, however the positive control did not work, leading to inconclusive results.

Then larvae were put on the mice for feeding, one month after injecting the mice with the culture. Seven days after the blood meal had been finished, gDNA isolation was performed on 4 ticks per mouse, split up into two ticks per 1.5 ml Eppendorf tube. At the same time, 6 ticks per mouse, split up into 3 ticks per tube were used for re-isolating *Borrelia* in media.

The isolated DNA was positive for gentamycin primers, except for one of the two samples of mouse number 1. Unfortunately, the ticks in media were negative due to contamination of the tubes.

The mice were euthanized after 2 months and 11 days, when their organs and tissues (ear, bladder, joint and heart) were put into media for re-isolation of *Borrelia*. Also, gDNA isolation was performed on pieces of the ears which were not used for the cultures. The remaining pieces of ear were kept in the freezer.

The media, containing the organs and tissues, did not give a positive result, because of contamination reasons and the media as such, which was probably not supporting the growing conditions. The isolated DNA gave no positive results for *adeC* and gentamycin.

The fed larvae were allowed to molt for approximately 2 months. Unfortunately, the majority of those ticks was lost due to desiccation. Hence, experiments were conducted in a different way than originally planned for providing a tick-mouse model, since a lower number of ticks

was given. The ticks were put on naïve C3H/HeN mice with different numbers (2-4 ticks) as depicted in Table 7. After the finished blood meal, the nymphs were collected and stored at appropriate conditions. Approximately 18 days after feeding, gDNA isolation was performed with one tick per mouse. All samples except one were shown to be positive for gentamycin primers, indicating that the DsRed shuttle vector was present.

Then Western Blotting was done with the sera of the mice, around 20 days post feeding. Only 2-3 bands showed up for each mouse suggesting that the *Borrelia* did not survive the tick molt and hence the mice did not take up the infection via the blood meal of the ticks.

The remaining individual ticks (Table 7) were crushed in media and used for plating, approximately 5 weeks post feeding. The cultures which were left after plating were filled up with BSKII and used for re-isolating *Borrelia* in media. These tubes were checked from time to time for the growth of *Borrelia*.

No colonies at all showed up on the plates and also the tick cultures were negative for the presence of *Borrelia*.

I	II	III	IV	
Naive Mice	**# of Nymphs per Mouse**	**# of Nymphs used for gDNA Isolation**	**# of Nymphs used for Plating**	
1	3	1	-	
2	2	1	1	
3	2	1	-	
4	3	1	2	
5	3	1	2	
6	4	1	3	
7	2	1	1	
8	3	1	2	
9	3	1	1	
10	4	-	4	
11	3	1	2	
11	**32**	**10**	**18**	**total**

Table 7: I – Mice injected with CB43-6 SH6; **II** – Remaining nymphs from injected mice; **III –** Groups of remaining nymphs; **IV –** Naïve mice used for feeding of nymphs; **V -** Number of used nymphs per mouse; **VI -** Nymphs used for gDNA isolation; **VII –** Nymphs used for plating; the two ticks which were not used for platin (mouse number 1 and 3) died within the course of the experiments

As mentioned above, an attempt was made to artificially infect *I. ricinus* larvae with CB43-6 SH6. After this was conducted the larvae were put for feeding on 3 naïve C3H/HeN mice. Around 12 days post feeding, gDNA isolation was done with 3 of the fed larvae. The tick DNA was found to be positive when gentamycin primers were used.

Three weeks after feeding, the mice sera were used for performing Western Blotting. Unfortunately, the were no bands showing up for the mice in comparison to the positive control, indicating that the mice have cleared the infection.

Also plating was carried out with the ticks, 24 days post feeding. Therefore, 6 ticks per mouse split up into groups of 3 were used for crushing the ticks in media. The obtained cultures were used for the plating and the remainders were filled up with BSKII. For both, the plating and the tick cultures, it did not work to re-isolate *Borrelia*.

4.2.2. Plating of *B. afzelii* CB43-6 SH6

Since the glycerol stocks of CB43-6 SH6 were contaminated, it was decided to plate a culture of the least contaminated stock again. Afterwards, colonies were plugged and grown in media. At a concentration of approximately 10^7 *Borrelia*/ml, the cultures were checked for fluorescence, gDNA was isolated and glycerol stocks were made. Then a plasmid profile was created of all clonal isolates using PKo primers in order to check, whether all plasmids are still present in the genome.

Plasmid	SH6-2	SH6-3	SH6-4	SH6-5	SH6-6	SH6-7	SH6-8	SH6-9	SH6 IV
lp17	+	+	+	+	+	+	+	+	+
lp25	+	+	+	+	+	+	+	+	+
cp26	+	+	+	+	+	+	+	+	+
cp26+27	+	+	+	+	+	+	+	+	+
lp28	+	+	+	+	+	+	+	+	-
lp28-2	+	+	+	+	-	+	+	+	+
lp28-3	+	+	+	+	+	+	+	+	+
lp28-4	+	+	+	+	+	+	+	+	+
lp28-7	+	+	+	+	+	+	+	+	+
lp28-8	+	+	+	+	-	+	+	+	+
cp30	+	+	+	+	+	+	+	+	+
lp32	+	+	+	+	+	+	+	+	-
cp32-5	-	-	-	-	-	-	-	-	-
cp32-7	+	+	+	+	+	+	+	+	-
lp32-9	+	+	+	+	+	+	+	+	+
lp32-10	+	+	+	+	+	+	+	+	+
lp34	+	+	+	+	+	+	+	+	-
lp38	+	+	+	+	+	+	+	+	+
lp54	+	+	+	+	+	+	+	+	-
lp60	-	-	-	-	-	-	-	-	-
lp60-2	+	+	+	+	+	+	+	+	+

Table 8: Plasmid profile of the plated and re-isolated CB43-6 SH6; the grey areas indicate missing plasmids

According to Table 8, it can be found that all clones have lost cp32-5. As for the GFP clones it can be assumed that this plasmid is still present, even though a specific type of them is missing. Additionally, clone SH6-6 is lacking in plasmids lp28-2 and lp28-8 and clone SH6-IV is missing lp28, lp32, lp32-7, lp34 and lp54.

4.2.3. Generation of DsRed Expressing *B. afzelii* CB43-6

For the reason that glycerol stocks of previously transformed *B. afzelii* with pBSV2G-DsRed were contaminated, it was repeated to generate a red fluorescing strain of *B. afzelii.* Also, these stocks have undergone several thawing and freezing steps, which probably led to a loss in plasmids.

First, the clones were verified to carry the shuttle vector containing the DsRed by the use of fluorescence microscopy. Also glycerol stocks were made and plasmid profiling performed to confirm whether the clones have lost certain plasmids (Table 9).

Plasmid	CB43-6 Ds2	CB43-6 Ds4	CB43-6 Ds5
lp17	+	+	+
lp25	+	+	+
cp26	+	+	+
cp26+27	+	+	+
lp28	+	+	+
lp28-2	+	+	+
lp28-3	+	+	+
lp28-4	+	+	+
lp28-7	+	+	+
lp28-8	+	+	+
cp30	+	+	+
lp32	+	+	+
cp32-5	-	-	-
cp32-7	-	-	-
lp32-9	+	+	+
lp32-10	+	+	+
lp34	+	+	+
lp38	+	+	+
lp54	+	+	+
lp60	-	-	-
lp60-2	+	+	+

Table 9: Plasmid profile of DsRed expressing clones of CB43-6; the grey areas indicate missing plasmids

As visible in Table 9, all the three clones have lost cp32-5 and cp32-7. Even though this types of cp32 are not carried, it is assumed that it is still present, as for the GFP expressing clones.

5. Discussion

To better understand the various *Borrelia* species in Europe together with their differences and their interactions with their tick vector, one important step is to develop new visualization techniques. These can be used for instance to investigate the dissemination of *Borrelia* under different environmental conditions *in vitro* as well as *in vivo.*

In 2008, Moriarty and colleagues managed to use GFP for investigating the dissemination of *Borrelia* in the microvasculature of a living mammalian host by constructing a GFP expressing shuttle vector which was transformed into different infectious and non-infectious *B. burgdorferi* B31 strains. These were inoculated into various mice strains for further examination. The use of different microscopy techniques, like conventional and spinning-disk confocal intravital microscopy, allowed them to observe the bacteria in real-time as well as in 3D. It was found that the hematogenous spreading is a multi-stage process which comprises temporary tethering to the endothelium, followed by dragging along the vessel wall and stationary adhesion, mostly observed at endothelial junctions. Also the further extravasation (escape from the vasculature) of the spirochetes was most commonly seen at those positions (Moriarty *et al.*, 2008). This was the first report of using high resolution 3D imaging for dissemination studies of pathogenic bacteria overall.

The investigation of *Borrelia's* essential proteins needed for their viability, which are in turn important for their virulence, is also necessary to get a better insight into its interactions with both, the arthropod and the mammalian host.

A novel technique, first used in 2009, was to stably integrate the gene encoding for GFP into the ubiquitous plasmid cp26 of the *B. burgdorferi* s. s. strain 297. Further on, the obtained fluorescing strain was used to study the dissemination of the *Borrelia* within the isolated midgut and salivary glands of the engorged arthropod vector (Dunham-Ems *et al*., 2009). By using live imaging of the bacteria by confocal fluorescent microscopy, a biphasic mode of dissemination within the ticks was revealed which is contrary to previous believes. It was found that the spirochetes at first progress through the midgut towards the basolateral epithelium in a non-motile manner, whereby they adhere to different kinds of epithelial cells. The second phase comprises the transition of the *Borrelia* into motile organisms that penetrate the membrane of the midgut to migrate into the salivary glands.

A technique which provides a more optimized imaging in terms of resolution coupled with fluorescently tagged structures was developed by Strnad and colleagues in 2015. This microscopy method improves the ability to observe pathogen-host interactions on a cellular level by combining fluorescence and electron microscopy at cryo-conditions to observe fluorescent *Borrelia* strains at high resolution (Strnad *et al.*, 2015). This novel technique was also applied on GFP expressing *B. burgdorferi* 297 strain to better understand its response at different stress conditions. Therefore, the spirochetes were exposed to medium, sera and water in order to observe morphological changes at nano-scale (Vancova *et al.*, 2017).

The fact that ticks are auto-fluorescing green might be of a disadvantage when working with *Borrelia* which are expressing GFP. For that reason, it is of advantage to express a different fluorescing color like red fluorescing *Borrelia*, which have DsRed or monomeric red fluorescing protein (mRPF) incorporated. Therefore, it would be easy to distinguish the bacteria from the tick vector. Nevertheless, *Borrelia* which is fluorescing green can be used for studying interactions within a mammalian host, since those tissues can be viewed with a different color.

The creation of GFP expressing *Borrelia* by transformation was successfully accomplished during this project. The fact that the obtained clones appear to be not infectious in mice, even though all plasmids are present, means that more transformations have to be performed in the future to obtain an infectious GFP expressing clone.

The investigation of DsRed expressing *Borrelia* in mice and ticks gave rather inconclusive results, suggesting that also these experiments need to be repeated. Also the re-isolated CB43-6 SH6 clones as well as the generated CB43-6 Ds2, Ds4 and Ds5 need further investigation in terms of infectivity, even though plasmid profiles were obtained and their fluorescence was confirmed.

Overall, most of the techniques for studying the interactions of *Borrelia* and its hosts are developed for *Borrelia burgdorferi* s. s. strains. Therefore, it is of high importance to generate fluorescing European strains of the *Borrelia burgdorferi* s. l. complex and to use them to further understand the virulence factors of the pathogen for infecting the mammalian as well as its arthropod host. It is also necessary to improve the knowledge about the different European strains existing in order to get a better insight into their varying ecological niches and hence into their differing mechanisms of survival within their hosts. This work is the first to obtain a GFP expressing European species of Borrelia and provides hope for further success while working with this and other European Borrelia species.

6. Literature

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