University of South Bohemia in České Budějovice
Faculty of Science

The Interaction of *Borrelia* Outer Surface Proteins with Tick Salivary Proteins

Bachelor's Thesis

Laboratory of Molecular Ecology of Vectors and Pathogens
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Annotation: The aim of this thesis was to investigate the interaction of the salivary gland protein, Salp15, in relation to the outer surface protein C, OspC, using in silico analysis, as well as trying to amplify Salp15 from different tick species found in same geographical areas and observing the growth of *Borrelia* in the midgut and salivary glands of these different tick species *in vitro*.

Affirmation: I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 14.12.2021 Aylin Paktan

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1. INTRODUCTION

1.1 LYME DISEASE

Lyme disease (LD), or Lyme borreliosis (LB), is the most frequent vector-borne infection caused by genospecies of the *Borrelia burgdorferi* sensu lato complex. LB is a multisystem disease occurring in North America, Europe, and Asia and includes a number of different manifestations, which can lead to a wide array of complications, if not recognized or treated appropriately. The symptoms of Lyme disease vary but share some common features such as flu-like illness and erythema chronicum migrans (skin rash). Eventually, symptoms like arthritis, skin disorders and various neurological complications may occur [1,2,3,4]. Lyme borreliosis was first recognized in 1975 in Old Lyme, Connecticut (USA) and was therefore named after the town [1,5]. It is assumed that the geographical border shifts constantly due to the climate changes towards higher altitudes and latitudes [6,7], making it an even bigger health concern. Lyme borreliosis is hardly fatal. In general, it is treated with prescription of antibiotics for two to four weeks [2].

1.2 ANATOMICAL AND GENOMIC ORGANISATION OF *BORRELIA*

Originally it was assumed that pathogens of Lyme disease were a homogeneous group of bacteria. They were referred to as *Borrelia burgdorferi (B. burgdorferi)*, named after its discoverer Willy Burgdorfer [8,9]. However, it was later shown that it was a heterogeneous group of bacteria [1,10,11]. In the meantime, a distinction is made between *B. burgdorferi* sensu lato (*B. burgdorferi* s. l., "in the broader sense") and *B. burgdorferi* sensu stricto (*B. burgdorferi* s. s, "in the narrower sense"). *B. burgdorferi* s. l. describes the heterogeneous species complex, which includes all previously known *Borrelia* genospecies of LD causing spirochetes, while *B. burgdorferi* s. s. refers to the originally discovered species of this complex. Molecular genetic analyzes have been able to classify 22 different genospecies so far, whereby six of these species were classified to have human pathogenic potential [12]. Therefore, within the *Borrelia genus* a distinction can be made between different human pathogenic species. The most important are the species causing LD and the pathogens causing relapsing fever [1,13].

Borrelia are bacteria with a length of 20- 30 μm and a diameter of 0.2-0.5 μm. They are spirally wound bacteria that move in a helical manner and belong to the phylum Spirochetes, class Spirochaete, order Spirochaetales and family Spirochaetaceae. Unlike other bacteria, spirochetes have so-called endoflagella to move around (Figure 1). This structure leads to the helical appearance and the helical movement of *Borrelia*, which enables locomotion in highly viscous media such as the host tissue [14,15,16,17]. Because of its double-membrane envelope, *B. burgdorferi* are often mistakenly classified among gram negative bacteria. But their outer cell membrane is lacking lipopolysaccharides and the presence of major surface lipoproteins is what distinguishes them evidently from gram negative species. Notably, they matured to be independent of iron, which is a limiting factor for bacterial pathogens in their hosts [18,19].

The genome of *Borrelia* is segmented and consists of an approximately 1Mbp long linear chromosome and multiple smaller, linear and circular plasmids [20]. The linear chromosome is comparatively small within the enormous majority of bacteria, including other spirochetes and carries largely the housekeeping genes [21]. These genes are involved in fundamental processes such as replication, transcription, translation or metabolic processes. They therefore serve to preserve cells and are subject to little evolutionary change. Plasmid-encoded genes have been reported to be essential for transmission and inhibition of the immune response of the host [1,4]. The genes present on plasmids encode for most of the outer-surface proteins, or enzymes and proteins.

The reason why the essential factors for survival within a mammalian host of *B. burgdorferi* have been under the scope of science is that these outer membrane proteins (OMPs) suggest themselves as potential vaccine candidates. OMPs comprise a broad range of proteins and their derivatives (eg. lipoproteins), including outer surface proteins (Osp) [4], which will be the focus in the next section.

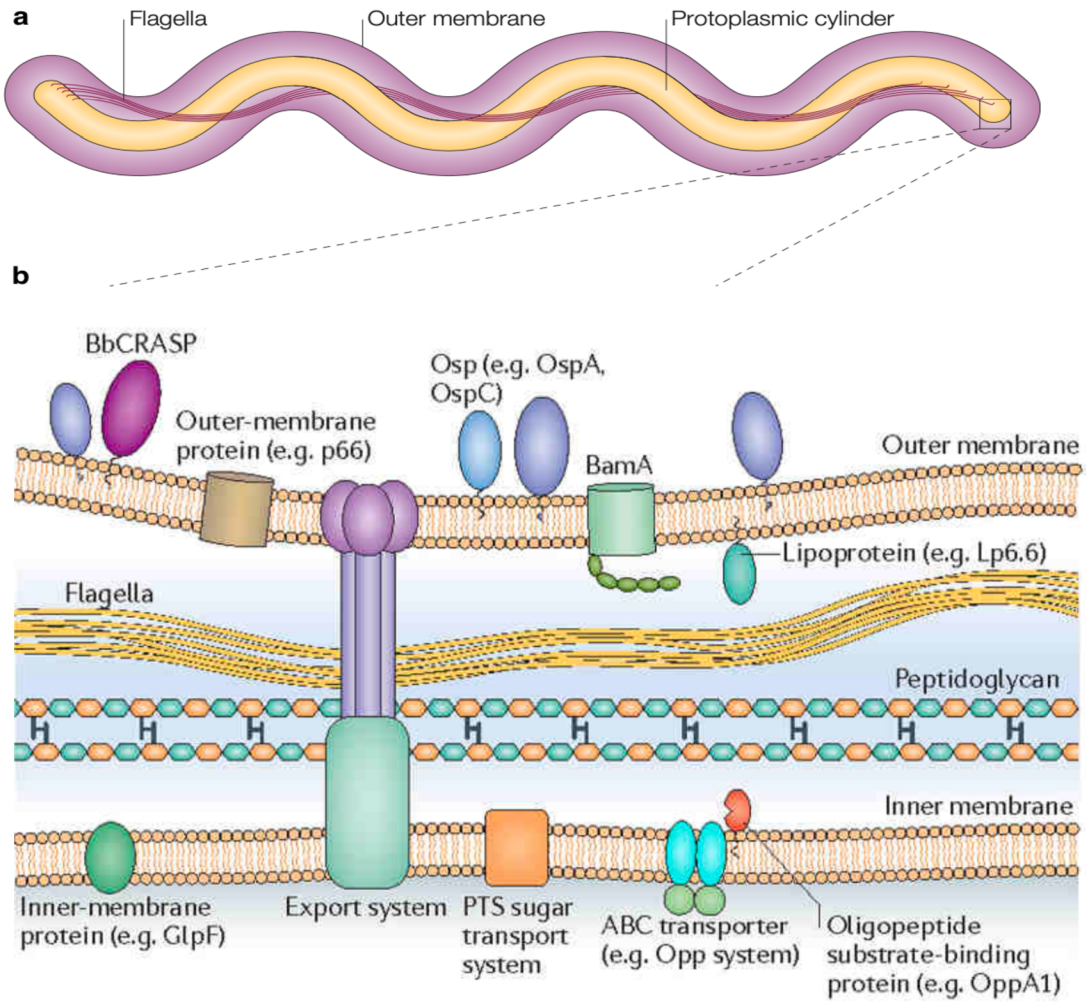

Figure 1: Structure and morphology of *Borrelia burgdorferi*. a) diagram of the spirochaete. b) overview of the borrelial cell envelope. (adapted from P.A. Rosa *et al*. 2005 and from Radolf *et al*. 2012)

1.3 THE IMPACT OF OUTER SURFACE PROTEINS OF *BORRELIA*

The genome of *B. burgdorferi* encodes high number of lipoproteins. Different lipoproteins are expressed at different stages of the spirochete life cycle and play an important role in the process of tick and mammalian infection [4,22].

The most thoroughly studied borrelial lipoproteins are the outer surface protein A (OspA) and the outer surface protein C (OspC). OspC is much more polymorphic than OspA, and consist as a dimer, predominantly composed of alpha-helixes. The ospC gene is located on a circular plasmid, denoted cp26 [4,23]. OspA remains a major surface lipoprotein during the gut colonization [24]. TROSPA (Tick receptor for OspA) has been identified in the tick gut, which mediates spirochete attachment to gut epithelial cells, enabling the spirochete to survive the molting process and to persist throughout the tick's next life-stage. [25].

Schwan *et al.* (1995) characterised an inverse relationship of the genes encoding OspA and OspC during the transmission cycle of spirochetes. It was reported that while OspA is downregulated during tick feeding, *ospC* gene is activated [26]. In the midgut of an unfed tick, *Borrelia* produce high levels of OspA protein, whereas OspC production is almost undetectable [27]. Within 36–48h of a blood meal, spirochetes in the tick downregulate their production of OspA, and OspC production is generated [26]. OspC is allowing spirochetes to leave the gut in order to reach the hemocoel and therefore implying to be a very important component enabling spirochetes to invade the tick salivary glands. It indicates, that OspC is an essential virulence factor required in transmission and survival during early stages of host colonization [4,28,29].

OspB, another surface-exposed lipoprotein, is structurally similar to OspA. Additionally, both are encoded on the same linear plasmid, namely lp54. It is reported that OspB-deficient spirochetes, similarly to OspA- deficient bacteria, have limited ability to survive in the tick gut [30]. Moreover, OpsD has also been noted to have a certain impact in the adherence of spirochetes to the tick gut cells. It is reported that OspD expression is dominant on the *B. burgdorferi* surface shortly after tick feeding and detachment and is encoded on linear plasmid lp38,of *B. burgdorferi* [31].

The differential expression of surface proteins allows spirochetes to adapt to the tick or mammalian host environment as needed and is therefore important in the parasitic strategy of *B. burgdorferi*. For instance, fibronectin-binding protein (BBK32), a family of anticomplement proteins (Erp's), a decorin-binding protein (DbpA), and several VlsE proteins in mammals are synthesized by *B. burgdorferi*, leading to promote spirochete dissemination and survival within the host [28].

Borrelia burgdorferi also produces OspE and OspF on its outer surface. The surface lipoprotein OspE is identified to be the first binding protein to the soluble host serum proteins factor H (FH) [47,48]. FH binding proteins expressed by the spirochetes are referred to as complement regulator-acquiring surface proteins (CRASPs) [49]. It was noted that OspE expression is upregulated by temperature raise *in vitro* and during tick feeding and mammalian infection and it also has been suggested that it may promote spirochetes to spread [50,51]. OspF is also shown to be upregulated during mammalian infection and suggested to be important in tissue tropism during mammalian infection [50,52].

Numerous outer surface lipoproteins have been identified in recent years. In table 1 some more important adhesion proteins are listed.

Table 1: Important *Borrelia burgdorferi* adhesins

***B. burgdorferi* adhesins**	**Function**
P66	Adhesion and activation to host cell
BB0690	Persistence in tick during starvation
BBE31	Adhesion to tick salivary glands
BBA52	Tick gut/ salivary gland migration
BBA64	
BBA65	
BBA66	
BBA03	Tick host transmission
BBA07	
BBA74	Tick environment adaption

1.4 TICKS

Around 900 different species of ticks can be found worldwide and are of public health significance because they transmit numerous bacterial, viral, and protozoan disease agents [32]. Ticks cause great economic losses to livestock globally and have damaging effects on their livestock hosts in several ways [33]. Most of the ticks can be referred to one of the two main large families, the *Argasidae*, also known as soft ticks and the *Ixodidae*, also known as hard ticks. There also exists the family *Nuttalliellidae*, but this one is monotypic and only contains one rare African species. Individuals from the families *Argasidae* and Ixodidae differ in their life cycles, morphology and physiology [34,35]. Depending on the type of tick, different *Borrelia* species are transmitted. Transmitters of human pathogens are called “vectors" [5].

Ticks can feed on a variety of hosts, which accounts for the geographical span and the way of spreading of *B. burgdorferi s.l.* A detailed table of the vectors of different *Borrelia* species and the host preferences of these vectors is given in table 2.

Table 2: Main hosts of *Ixodes* ticks and primarily transmitted pathogens and distribution

Tick	Main host	Pathogen	Geographical distribution
Ixodes ricinus [53]	Small rodents and mammals; red deer, roe deer, birds	*B. burgdorferi s. s.*, *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae* and *B. spielmanii*	Eurasia
Ixodes persulcatus [54]	Small rodents, livestock, goats and birds	*B. garinii*, *B. afzelii*, *B. valaisiana*, *B. bissettiae*	Russia and Asia
Ixodes scapularis [55]	especially white-footed mouse, small mammals, birds and white-tailed deer	*B. burgdorferi s. s.*	East Coast of the USA
Ixodes pacificus [56]	especially reptiles, roe deer, dogs, coyotes, wild boars, horses, small rodents, birds and black-tailed deer	*B. burgdorferi s. s.*	West Coast of the USA

1.5 TICK SPECIES *IXODES RICINUS*

I. ricinus, the castor bean tick, is a three-host tick and can feed on humans and animals. It is the primary tick vector of Lyme borreliosis spirochetes in Europe and transmits a vast range of bacterial pathogens to both, animals, and humans, with variable degrees of pathogenicity. It is found mainly in cool humid environments (e.g., forests). Moreover, tick-borne encephalitis (TBE), a viral infection of the central nervous system, is spread by this tick species [36,37]. The major restricting factor for the geographical distribution of *I. ricinus* is the climate. Unfed nymphs, like diapausing engorged larvae of *I. ricinus* are not able to survive longer than 30 days at -10°C. The adult ticks start to be active in March and end their activity in October. However, the peak of activity is in the months of May-June [38].

1.6 TICKS SPECIES *DERMACENTOR MARGINATUS* AND *DERMACENTOR RETICULATUS*

Dermacentor species belong to the subfamily of the *Rhipicephalinae*, family *Ixodidae*, order *Ixodida*, subclass Acari, class *Arachnida* [35]. *Dermacentor* species usually follow the three-host life-cycle, as is *I. ricinus,* and the entire life cycle can be completed in one year. *Dermacentor marginatus*, also known as ornate sheep tick, is next to *D. reticulatus,* one of the two European *Dermacentor* species. The species *D. marginatus* and *D. reticulatus* are of particular importance due to transmission of pathogens and causing economical losses on livestock in Eurasia. Pathogens transmitted through *Dermacentor* spp. are either viral, causing e.g., Colorado tick fever (CTFV) and tick-borne encephalitis, or bacterial causing e.g., Rocky Mountain Spotted Fever (RMSF) (*Rickettsia rickettsii*), tularemia, also known as rabbit fever (*Francisella tularensis*) and Siberian tick typhus disease (*R. siberica*). Furthermore, this tick species as well transmits pathogens of protozoan origin, like the tick-borne disease canine babesiosis (*Babesia canis*) to animals [39]. In contrast to the *Ixodes* spp., the activity of adult *Dermacentor* spp. is during the end of autumn through into winter [36]. *Dermacentor* spp. are found throughout various geographical regions that overlap areas where there is an incidence of human Lyme disease [40]. Even though they are the second most reported tick species after *Ixodes ricinus* in central Europe, they are not able to successfully maintain and transmit the causative agent of Lyme disease.

1.7 TICK LIFE CYCLE

Besides the egg, ticks (male or female) undergo three different life stages: namely larva, nymph and adult. Both, soft and hard ticks undergo those life stages, but when they evolve is different. Soft ticks differ in the fact, that they have more than three developmental stages [41]. The life cycle for *Ixodes* ticks lasts usually two to three years. Ticks are fully dependent on their single nutrition source – the blood and are born uninfected. After dropping to the ground, they start laying thousands of eggs. After hatching from eggs in leaf litter, larvae acquire the spirochete by feeding on an infected reservoir host and spread into the vegetation and parasitise the first host. Once engorged, they drop off the host and moult to nymphs. At this stage, they have eight legs. Nymphs seek a second host, feed and moult again, which can then transmit the pathogen onto naïve reservoir hosts. For the transmission to occur the ticks need to be feeding on an animal for at least 48 hours. When the nymph falls off its host and transitions into an adult, it will look for its third and final host, which prey generally on bigger animals like deer, cattle, or human pets like dogs. After detachment females find an optimal environment, lay eggs and die [1].

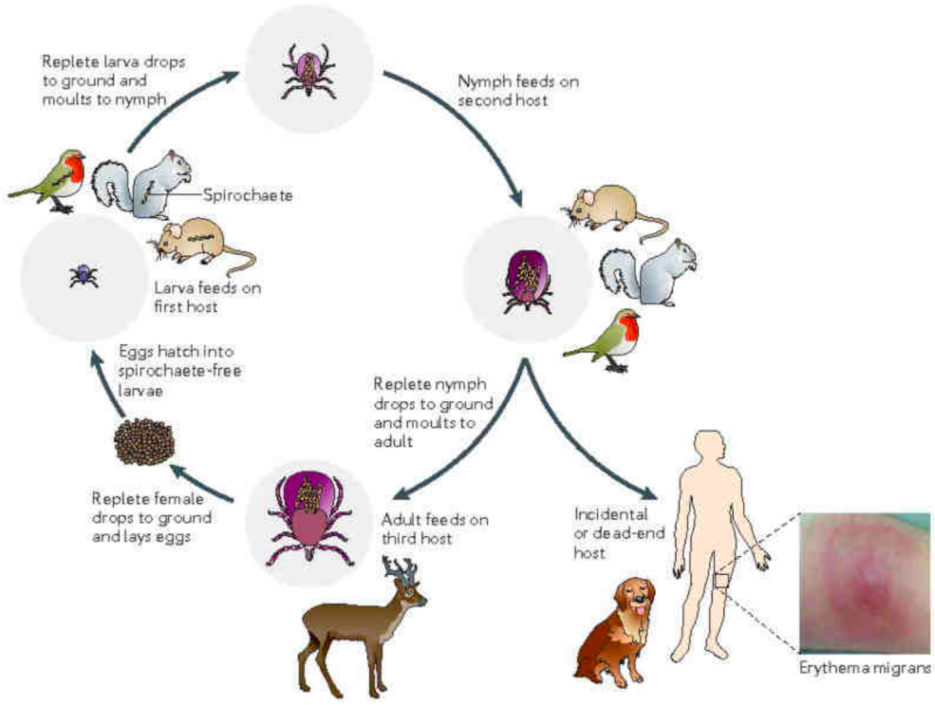

Figure 2: The three-stage life cycle of *Ixodes* ticks (adapted from Radolph *et al.* 2012).

1.8 TICK SALIVARY PROTEINS

Tick saliva is abundantly secreted into the host during feeding. The salivary glands are a multifunctional and morphological complex mixture of peptidic and non-peptidic molecules and known to be critical for the biological success during the feeding periods on the host obtaining their blood meal, as well as during the lengthy periods off their hosts. Thanks to the hygroscopic fluid production of the salivary glands, ticks remain hydrated during the long interval between bloodmeals [42,43]. Therefore, tick salivary proteins significantly affect the enzootic cycle of *B. burgdorferi*. Such an extension of pathogen transmission via the action of tick saliva is called as saliva-activated transmission (SAT) [44]. The classes of components in tick saliva include enzymes involved in preventing clotting of blood, enzyme inhibitors of coagulation enzymes, host protein homologues which inhibit different arms of the immune response, immunoglobulin-binding proteins, and cytokine expression modulators such as Salp15 [57]. Especially interesting is the multifunctional protein Salp15. It is a 15-kDa tick salivary gland protein and was originally discovered as one of the many of antigenic salivary proteins isolated from feeding Ixodid ticks [42]. Interestingly, in no other genus of ticks except *Ixodes*, the Salp15 has been found till now. Further studies showed that Salp15 protected *B. burgdorferi s.s.* from antibody-mediated killing by binding to OspC of the spirochete. It was observed that Salp15 has been able to suppress host immunity through complicated mechanisms, such as binding to CD4 to inhibit $CD4^+$ T cell activation, preventing dendritic cell activities, and altering the expression level of cytokines in the vertebrate host [28,45]. Due to the mentioned properties, scientists have suggested that Salp15 could be a potential target for the development of vaccines against Lyme spirochetes [46]. Important to mention is also the fact, that there are various other tick salivary proteins which could be important for pathogen transmission. As mentioned in the review of Rego *et al.* (2019) the tick mannose-binding lectin inhibitor (TSLPI), as well as the tick histamine release factor (tHRF) present in the tick saliva, influence the biological success of the spirochete. The expression of TSLPI is noted to be increased in the salivary glands of infected ticks and therefore be important for the survival of the bacteria while passing to the vertebrate host. tHRF plays an important role in the late phases of tick feeding since the expressions shown to be increased and thus enabling the tick to uptake more blood while rapid engorgement by stimulating the release of histamine from basophiles. Studies showed that a knockdown of tHRF impaired tick feeding on mice, leading to decreased *B. burgdorferi* transmission [70].

2 HYPOTHESIS

Why are *Dermacentor marginatus* and *Dermacentor reticulatus* non-competent vectors?

3 OBJECTIVES

I. To amplify and sequence Salp15 from the ticks *Dermacentor marginatus*, *Dermacentor reticulatus*, *Amblyomma americanum* and Ix*odes ricinus*.
II. To make a molecular and phylogenetic characterization of Salp15 and OspC
III. To look at the bacterial growth in tick salivary glands and guts of the species *Dermacentor marginatus*, *Dermacentor reticulatus* and *Ixodes ricinus*.
IV. To find the closest 3D model of given OspC protein and analyse the binding potential to specific Salp15 models

4 MATERIAL AND METHODS

Diverse techniques of molecular biology were used in the experimental work of this thesis while trying to amplify and sequence Salp15 from *Dermacentor marginatus*, *Dermacentor reticulatus*, *Amblyomma americanum*, as well as *Ixodes ricinus*. Isolation of their genomic DNA, establishing an alignment of all available Salp15 sequences gathered from the NCBI database as a template for designing adequate Primers, followed by cloning and transformation in *Escherichia coli* and sequencing was performed. Furthermore, the growth of the *Borrelia* strain, Bb914, in tick tissue of the mentioned species employing Fluorescence Microscopy was performed. Bioinformatic tools were used to obtain a 3D model of a given OspC- sequence for advanced protein structure analysis and phylogenetic trees of OspC and Salp15 were constructed.

4.1 TICK SPECIES

Ticks of the species *D. marginatus, D. reticulatus*, *A. americanum* and *I. ricinus* were maintained in the housing facility of the Institute of Parasitology, Biology Centre of Sciences of the Czech Republic in České Budějovice.

4.2 BACTERIAL STRAINS

In case of covering different *Borrelia* genospecies, the strains *B. afzelii* (PKoA, PKoB, RU1, RU2, CB43), *B. burgdorferi s.s.* (Bb914) were used. Transformation to *E. coli* using Subcloning Efficiency™ DH5α™ competent cells (Invitrogen™) was performed for cloning and transformation.

4.3 BACTERIAL GROWTH CONDITION

For the growth of *Borrelia,* glycerol stocks of *Borrelia* were put into Barbour- Stoenner- Kelly (BSK) H complete media containing 6% of rabbit serum at 34°C, which is the optimal temperature for *Borrelia* growth, in an incubator till the mid-log phase (10^7 *Borrelia* /ml) had been reached. 1x *Borrelia* Antibiotics were added as needed. After appropriate density was reached the tubes were removed from the incubator and were used for tissue dissection and genomic DNA isolation.
For the growth of *E. coli,* lysogeny broth (LB) media at 37°C in an orbital shaker was used.

4.4 DISSECTION OF TICK SALIVARY GLANDS AND MIDGUT

For the dissections adult female ticks of each species, *D. marginatus*, *D. reticulatus*, A. *americanum* and *I. ricinus*, were used. The collection of the guts and the salivary glands were kept in a GFP *Borrelia* culture of the strain Bb914. Glycerol stocks of *Borrelia* were started from glycerol stocks as described in the chapter before.

The growth of the cultures was observed by microscopy using a Petroff-Hauser counting chamber (Hauser Scientific). A 1:10 dilution of the *Borrelia* culture was prepared and 6 μl of the dilution was placed on the Petroff-Hausser counting chamber slide and covered with a cover slip. The number of all *Borrelia* occurring on five squares were denoted and the *Borrelia* cells per ml were calculated using equation 1, whereby x represents the number of *Borrelia* counted within 5 squares of the counting chamber.

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

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

A soon as the appropriate density of the *Borrelia* was reached the dissection of the midgut and the salivary glands was performed. The number of *Borrelia* used in the first dissection was 1.568×10^7 *Borrelia* per ml.

For the first dissection, six 1.5 ml eppendorf tubes were filled with 50 μl of the BSK-H complete (Sigma-Aldrich) media including 1x *Borrelia* antibiotics. The adult female ticks were taken out of their moist environment carefully. For surface sterilization a 3% hydrogen peroxide solution and 70% Ethanol was used. Five of each species were stuck with caution on the microscope slides and fixed on with tape. Each tick was covered with a droplet of 1x phosphate buffered saline (PBS) onto the microscope slide and viewed under a dissecting microscope (1X Objective, 10X eyepiece, 3.5X magnification). Fine tipped forceps supported the stabilization of the tick by holding the basis capitulum (mouth parts). By gently inserting the fine tipped forceps into the rear of the tick and slicing up the tick's dorsum using dissection scissors the organs were exposed. At this time the removal of midgut could be performed and transferred to a fresh pool of PBS on a microscope slide. This washing

step was repeated several times to remove any external microorganisms and tick debris. Moreover, the pair of salivary glands (grape- like clusters) located bilaterally alongside the legs of the tick were collected and equally transferred to a fresh pool of PBS. After the washing step was done, 5 tick tissues of each species were carried into the prepared Eppendorf tubes filled with BSK-H complete media in addition of 1x *Borrelia* antibiotics. By pipetting up and down with a sterile 10 µl pipette, the collected organs were crushed in the media. Subsequently, 10 µl of the *Borrelia* culture were added in each tube and the samples were kept for 48 hours at 34°C in an incubator. The following step was to fill up the incubated 1.5 ml Eppendorf tubes with BSK-H complete media by adding the remaining value of 1.44 ml of the media till the meniscus reached the 1.5 ml mark. Further work was done after one week of incubation at 34°C. Counting of the growth density of the spirochetes by Bright- Field microscopy, as well as Fluorescence microscopy, was denoted and the samples were put again into the incubator at 34°C. For the growth density determination approximately all *Borrelia* occurring on 5 different spots were counted with use of the Marienfeld- Superior counting chamber (Depth: 0.02mm, 0.025mm^2) by placing 3µl of culture onto the slide and the average stated.
The second dissection was prepared as previously described by preparation of a new GFP *Borrelia* culture of the strain Bb914. The steps were followed as stated above. The number of *Borrelia* used was 1.625*10^7 *Borrelia* per ml and the density enumerated employing the Bright -Field Microscopy.

A third dissection was started, incubating the organs in GFP *Borrelia* culture of the strain CB43, and performed as previously described, but the samples showed too much contamination for further work up.

4.5 FLUORESCENCE MICROSCOPY

The incubated tick tissues were stained by a fluorescent intercalating agent, Propidium iodide (PI) (Invitrogen™) to differentiate between the healthy and dead cells. Since the Propidium iodide cannot cross the membrane of live cells, the dead spirochetes were able to be differentiated by colour. Dead cells were exposed in red and the alive ones in green. The samples were prepared by adding 3 µl of a 60 µg/ml concentrated PI in 1 ml (0.18 µg/ml) sample. The glass slides including 5 µl of the labelled samples were inspected in the fluorescence microscope Olympus BX- 60. The number of all *Borrelia* at several spots was determined and the average was stated.

4.6 DESIGNING PRIMERS

For the amplification of the salivary gland protein Salp15 an alignment of all available amino acid sequences was downloaded from the NCBI databank as fasta files The sequences were aligned with the help of the Multiple Alignment Program for amino acid or nucleotide sequences (Mafft version 4). The analysis of the already aligned sequences was done using the Molecular Evolutionary Genetics Analysis software version 6.0 (MEGA6). Out of this alignment 2 different types of Primer sets were designed and additional available primers for *D. marginatu*s (HQ645110.1) and *D. reticulatus* (FJ196390.1) were chosen from the NCBI nucleotide database and synthesized at Generi Biotech. Final primers are shown in table 3.

Table 3: Primers used for amplifications

Primer	Sequence 5` —> 3`	Product size of the primer pair
FullSalp15 F	ATGAAGGTGGTGTGCATA	393 bp
FullSalp15 R	GCATGTCTGTCCATTCGGTCCACAA	
Salp15 F	TAACACTGCGATAAACGACAAACA	142 bp
Salp15 R	GCATGTCTGTCCATTCGGTCCACAA	
DM act F	TCCTGTGGTGGACAATGGGT	369 bp
DM act R	GGGGTGTTGAAGGTCTCGAA	
DR comp F	CAGACTGGGCTTCAACCGAT	126 bp
DR comp R	TGTCAACGTGGCAGTGATGA	

4.7 RNA EXTRACTION

RNA was extracted from uninfected female adult ticks of *D. marginatus*, *D. reticulatus*, *A. ammericanum* and *I. ricinnus* using the RNAeasy kit (Qiagen GmbH, Hilden, Germany). The isolation was done at room temperature. Before crushing the ticks, 700 μl of a guanidine isothiocyanate buffer supplied with the RNAeasy kit (RLT Buffer), to support the binding of RNA to the silica membrane, and 7 μl of 2- mercaptoethanol were prepared in a 1.5 ml micro-centrifuge tube. The ticks were washed in a collection tube by addition of 1ml of 70% ethanol and dried on a filter paper. After transferring them into the already prepared solution, they were crushed until the lysate turned into a homogeneous solution with a minimum of solid tick residues. After crushing the ticks, the lysate was centrifuged for 3 minutes around 12 000- 13 000 rpm and the supernatants pipetted out into new micro-centrifuge tubes. Subsequently 700 μl of 70% ethanol was added to the lysates and mixed by pipetting up and down. Next, 700 μl of the homogenates were transferred to RNeasy spin columns, which were placed in a 2 ml collection tube. After centrifuging the samples for 15 seconds at 9000 rpm, the flow- through was discarded and the RNeasy spin columns were washed with 700 μl of RW1 buffer for efficiently removal of biomolecules such as carbohydrates, proteins, fatty acids etc., that are non-specifically bound to the silica membrane. Followed by another centrifugation at 9000 rpm for 15 seconds, the collection tubes were carefully removed from the RNeasy spin columns without getting in contact with the flow- through. Moreover, 500 μl of RPE buffer, which is a mild washing buffer with the main function to remove traces of salts, which are still on the column due to buffers used earlier, was added and centrifuged at 9000 rpm for 2 minutes before the RNeasy spin column was placed into a new collection tube and centrifuged again for 1 minute at 13 000 rpm. The final step was to place the RNeasy spin column into a new 1.5 ml collection tube and centrifuging for one minute at 9000 rpm, after eluting the membrane with 30 μl of RNAse free water. Total RNA concentration was measured using NanoDrop 3300 (Thermo Fisher Scientific, Carlsbad, USA). The RNA was stored at -20°C until further use.

Table 4: Constituents of the buffers used for the RNA extraction

Company	Buffers	Constituents
Qiagen	RLT	High concentration of Guanidine isothiocyanate
	RW1	Guanidine salt and Ethanol
	RPE	Confidential proprietary of RNeasy

4.8 GENOMIC DNA ISOLATION

Genomic DNA from *Borrelia* cultures was either available in the lab or was prepared from respective *Borrelia* cultures from present glycerol stocks. For the isolation of the gDNA was the Wizard Genomic DNA Purification Kit (Promega®) was used, following an adapted version of the manufacturer's protocol "Isolation of Genomic DNA from Gram Positive and Gram Negative Bacteria". The procedure was as followed:

8 mL of *Borrelia* culture was centrifuged at 8000 rpm for 10 minutes at 20°C in a 1.5 ml centrifuge tube. The supernatant was carefully removed and the pellet resuspended in 600 µl of Nucleic Lysis Solution and the solution was pipetted into a 1.5 ml Eppendorf tube. The tube was incubated at 80°C for 5 minutes and subsequently placed on ice for 2 minutes. 3 µl of RNase Solution was added and the tube was inverted to mix. Afterwards, the mixture was incubated at 37 °C for 15 minutes and then placed on ice for a few seconds to cool. 200 µl of Protein Precipitation Solution was added and all was mixed by inversion. The sample was cooled on ice for 5 minutes and then centrifuged at 13000 rpm for 10 minutes at 4-8°C. The supernatant was pipetted carefully into a 1.5 ml tube, containing 600 µl of room temperature 70% Ethanol. The tube was mixed via inversion for 4-5 minutes and centrifuged again at 13000 rpm for 10 minutes at 4-8°C. The supernatant was removed by pipetting and small Ethanol residue drops were drained on absorbent paper and the pellet was allowed to air-dry for 10 minutes. At the end, 100 µl of DNA Rehydration Solution was added and mixed again via inversion. The sample of the prepared gDNA was stored at 2-8°C for a minimum of 1 day before further use.

4.9 PCR - POLYMERASE CHAIN REACTION

The amplification of the Salp15 protein with designed Primer sets was performed by PCR from total RNA purified from various *B. burgdorferi* uninfected tick species, as well as from already prepared and isolated genomic DNAs and cDNA samples. Moreover, the amplification of the OspC protein with two different sets of Primers was performed by PCR from the *B. afzelii* strains of the cultures PKoA, PKoB, RU1 and RU2. This procedure was tested with diverse PCR programs and polymerases. Nevertheless, best results could be obtained only with OneTaq® Hot Start Quick-Load® 2x Master Mix with the corresponding buffer from New England BioLabs®. The combination of this polymerase with the buffers E or F of the Fail Safe PCR Kit from Epicenter showed the best amplification results for Salp15. The composition of the Master Mix together with the PCR program settings are listed below.

Table 5: PCR master mix composition and PCR program used for the amplifications

Master Mix 1	**Amount [µl]**
OneTaq HS Quick-Load 2x MM	10
Primer Forward	1
Primer Reverse	1
MiliQ water	6
DNA	2
Total	20

	PCR machine program		
#	PCR step	T [°C]	t [sec]
1	Initial Denaturation	94	120
2c	Denaturation in cycle	94	45
3c	Annealing in cycle	55	45
4c	Elongation in cycle	72	60
5	Final Elongation	72	300
6	Hold	16	∞

Table 6: PCR master mix composition and PCR program used for the amplifications

Master Mix 2 FailSafe	**Amount [µl]**
Fail Safe Buffer	10
One Taq Hot Start Polymerase	0.2
Primer Forward	1
Primer Reverse	1
MiliQ water	5.8
DNA	2
Total	20

	PCR machine program		
#	PCR step	T [°C]	t [sec]
1	Initial Denaturation	95	120
2c	Denaturation in cycle	95	45
3c	Annealing in cycle	55	45
4c	Elongation in cycle	72	90
5	Final Elongation	72	600
6	Hold	4	∞

4.10 GEL ELECTROPHORESIS

Visualization of the successful amplification of the desired PCR product or restriction digests was done by gel electrophoresis using a 1% of agarose gel concentration and 1x TAE as running buffer. For 1% agarose gels, 1g of agarose powder was weighed in a heat resistant bottle and dissolved in a 100 ml of Tris-acetate-EDTA (TAE) buffer by microwaving for around 5 minutes. The hot gel solution was poured into a corresponding form and allowed to cool and solidify. Before solidification a comb of appropriate size was immersed into the gel mixture to create loading wells. The comb was carefully removed after complete solidification of the gel. Afterwards the gel was placed in the electrophoresis apparatus and submerged in TAE buffer.

The wells were loaded with 10 µL of the PCR sample mixed with 2 µL of Loading Dye containing 10xSYBR and run together with 1kb+ DNA ladder from Invitrogen. The visualisation of the gel was performed under UV light. The expected size for the amplifications is shown in table 3.

4.11 MOLECULAR CLONING AND TRANSFORMATION INTO *E. COLI*:

After confirming that the PCR products had the expected sizes by agarose gel electrophoresis, they were cloned into a vector and transformed into *E.coli*. The Invitrogen's pCR®2.1-TOPO® vector was used in this procedure. The setup of the cloning was performed by the following reaction mixture listed in table 7.

Table 7: TOPO reaction mixture

pCR®2.1-TOPO® vector	
Reagent	Amount [µl]
DNA insert	3
Salt Solution	0.5
TOPO vector	0.5
MiliQ H_2O	2

Before proceeding with the transformation in *E.coli.*, the TOPO reaction mixture was incubated for 5-10 minutes at room temperature. NEB 5-alpha competent *E. coli* cells were used for the transformations. Before use, they were thawn on ice for 5 minutes and a mixture of 25 μl of cells, together with 2 μl of Ligation reaction, was prepared and mixed by flicking. Afterwards the mixture was incubated on ice for 30 minutes. The membrane was heat shocked for 30 seconds at 42°C and subsequently kept on ice for 2 minutes. 250 μl of S.O.C.- media was added to the reactions and kept in the shaking incubator at 37°C for 60 - 90 minutes. The pre - warmed agar plates containing the antibiotic Carbenicillin were layed with 40 μl of X-Gal before plated with 75 μl and 150 μl cells. The plates were kept at room temperature for 5 minutes and incubated overnight at 37°C.

Using blue-white screening, colonies containing the vector where chosen, grown and the plasmid DNA was isolated. These steps were followed by restriction digestion to verify the presence of the vector carrying the insert in the isolated DNA. The detailed procedures for these steps are described as in the following chapters

4.12 PLASMID DNA ISOLATION:

Plasmid DNA was isolated using QIAprep Spin Miniprep kit and NucleoSpin® Plasmid DNA Purification kit (Machery-Nagel) following the protocols provided by the manufacturers. LB media was removed from fridge and left standing for at least 45 minutes. 6 ml of media were aliquoted into 15 ml centrifugation tubes. For the isolation of the plasmid DNA, the white *E. coli* colonies from incubated plates were picked with small pipetting tips and inserted into the LB media tubes. The tubes were incubated in a horizontal shaker (200rpm) overnight at 37°C. The following day plasmid DNA isolation was performed as follows:

The LB culture tubes were centrifuged for 30 seconds at 8000 rpm. The supernatant was removed and 250 µl of A1 buffer (Resuspension buffer) was added and the pellet was resuspended by pipetting. 250 µl of A2 buffer (Lysis buffer) was added, mixed in by inversion of the tube 6-8 times and left standing at room temperature for 5 min. 300 µl of A3 buffer (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 and the supernatant was pipetted into a NucleoSpin® Plasmid Column and placed in a 2 ml collection tube. Another 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. 500 µl of AW buffer (Wash buffer) was added and the tubes were centrifuged again (8 000 rpm, 60 sec, 20°C). The flow-through was disposed and 600 µl of A4 buffer (Wash buffer) was pipetted into the column and the tubes were centrifuged again for another minute. 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 columns were placed into new 1.5 ml microcentrifuge tubes and 50 µl of AE buffer (Elution buffer) was pipetted directly onto the membrane. After 1 minute of incubation, centrifugation was performed for 1 minute at same conditions as previously described. Spin Columns were discarded and the plasmid DNA was stored at 2-8°C in the refrigerator.

4.13 RESTRICTION DIGESTION:

For the verification of successful ligation of the inserts in the isolated plasmid DNA samples into the pCR®2.1-TOPO® vector, restriction digestion with NEB's EcoR I restriction enzyme was performed. The reactants (Table 9) were carefully mixed in a microcentrifuge tube and incubated for at least 30 minutes at 37°C. The enzyme was heat inactivated at 80°C for 20 minutes. The restriction digestion products were visualized using gel electrophoresis.

Table 9: Reaction Mixture Restriction Digestion EcoR I

Restriction Digestion	Amount [µl]
EcoR I Buffer	2 µl
EcoR I Enzyme	0.5 µl
MiliQ H_2O	14.5 µl
DNA	3 µl

After verification of the insert, the cloned products were sequenced. 5 μl of the plasmid DNA sample containing the insert was mixed with 5 µl of either T7 or M13R (concentration 5 pmol/µL) sequencing primers, which are listed in table 8. The samples were labeled and sent to GATC Biotech Lightrun sequencing service to sequence from both directions.

Table 8: Sequencing primers

Primer	**Sequence**
T7	5'-TAA TAC GAC TCA CTA TAG GG-3'
M13R	5'-CAG GAA ACA GCT ATG AC-3'

4.14 SEQUENCE ANALYSIS

After retrieving the sequences from GATC Biotech Lightrun sequencing service, first the sequences were trimmed to only contain the gene of interest and not the plasmid sequence. To confirm that they were sequences of the Salp15 protein, the trimmed sequence was compared with other sequences by BLAST in the NCBI database to check for sequence identity.

4.15 PHYLOGENETIC, MOLECULAR AND STRUCTURAL CHARACTERISATION

A phylogenetic tree was constructed to investigate how conserved the sequence of the OspC protein is in different *Borrelia* spp., which can be found in various geographic regions. This analysis was also done for the Salp15 protein, by investigating how conserved the sequences in different geographical distributed *Ixodes* spp. are. Additionally, to get a molecular and structural characterisation, secondary structure prediction and protein modelling was done.

4.15.1 PHYLOGENETIC ANALYSIS

To ease the construction of a phylogenetic tree, first homologues nucleotide sequences of the OspC protein from *Borrelia* spp., as well as sequences of the Salp15 protein from *Ixodes* ticks from different regions needed to be collected. This was done by the use of Blastn tool from BLAST [58]. To align all selected sequences for each tree, MAFFT 7 alignment server [59] with default settings was used to confirm nucleotide identity. The phylogenetic trees were then computed by Maximum Likelihood method based on Le Gascuel 2008 model using MEGA V6.0 for Salp15 and later MEGA X for OspC. Initial trees for experimental search were acquired automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model. To model evolutionary rate differences among sites a distinct gamma distribution was applied. All positions containing gaps and missing data were removed [60].

4.15.2 PROTEIN MODELLING

In order to elect the tertiary structure of OspC, the amino acid sequence was submitted to multiple servers which were I-tasser (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) [61], Phyre 2 (www.sbg.bio.ic.ac.uk/~phyre/) [62], Robetta (robetta.bakerlab.org) [63] and Swiss Model (https://swissmodel.expasy.org/) [64,65]. In parallel, the target protein sequence was submitted to BLASTp [58] to perform to PSI- to find more adjoining homologues, using a non-redundant database and five iterations. Moreover, as template for modelling, homologues with known crystal structures as were identified by obtaining the position specific scoring matrix (PSSM) and submitted to the Protein databank (https://www.rcsb.org/). The "maximum matches in a query range" was set to 10. The closest homologue observed was used to operate homologues protein modelling using UCSF Chimera (https://www.cgl.ucsf.edu/chimera/) software program [66]. Subsequently, the obtained models were submitted to structure evaluation tools, namely: ModFOLD 4 (http://www.reading.ac.uk/bioinf/ModFOLD/) [67], RESPROX (https://omictools.com/resprox-tool) [68] and Qmean (https://swissmodel.expasy.org/qmean/) [69]. The observed scores were examined between all the models and finally the most likely protein structure was selected for further analysis.

5 RESULTS

5.1 SALIVARY GLAND AND GUT DISSECTION

The growth of *Borrelia* in each species was observed *in vitro* with the guts and salivary glands of *D. marginatus, D. reticulatus* and *I. ricinus,* after incubating them with *Borrelia* at 34°C for one week. The observed amounts of *Borrelia* in tick tissues are listed in the tables below. The average of approximately all *Borrelia* at 5 different spots were noted and the average stated. The abbreviation S stands for salivary glands and the G for the gut of the ticks.

Table 10: Growth of *Borrelia* in tick tissue with *Borrelia* antibiotics

Tick tissues	**Results**	**Results**
Gut and Salivary glands (5 ticks per species)	Start	After one week
D. marginatus G	7	68
D. marginatus S	6	64
D.reticulatus G	6	53
D. reticulatus S	5	62
I.ricinus G	30	76
I.ricinus S	24	73

Table 11: Growth of *Borrelia* in tick tissue without antibiotics

Tick tissues	**Results**	**Results**
Gut and Salivary glands (5 ticks per species)	Start	After one week
D. marginatus G1	14	40
D. marginatus S1	12	36
D. marginatus G2	16	44
D. marginatus S2	11	34
D.reticulatus G1	10	26
D. reticulatus S1	7	23
D.reticulatus G2	12	31
D. reticulatus S2	8	26
I.ricinus G1	40	70
I.ricinus S1	46	80
I.ricinus G2	40	74
I.ricinus S2	57	80

In all species borrelial growth was visible. Without antibiotics, the number was twice as high as with antibiotics, except for *Dermacentor* spp; the growth of the *Borrelia* was observed to be lesser than with antibiotics. Moreover, pathogen invasion in the salivary glands was seen to be less than in the guts.

5.2 IDENTIFICATION AND SEQUENCING OF SALP15

To amplify Salp15 from *D. marginatus*, *D. reticulatus*, *I. ricinus* and *A. amblyomma* the first approach we used was to design degenerate primers. Even if the PCR products showed clear bands at approximately the required sizes (table 3), no homogeneity to Salp15 could be observed from the obtained sequences. The primer sets were not able to amplify Salp15 in any species.

5.3 PHYLOGENETIC TREES

To learn more about the relationship between OspC and Salp15, two phylogenetic trees were constructed (Figure 3 and 4). All sequences obtained in this study have been collected from the GenBank database collaborations from NCBI with the accession numbers listed next to the abbreviations of the species. A key for the abbreviations is given in table 12. The Salp15 phylogeny analysis involved 33 nucleotide sequence. There were a total of 317 positions in the final database. The phylogenic tree shows a significant difference in the branches from the *Ixodes holocyclus*, which are all from Australia, to the others (*Ixodes ricinus, I. persulcatus, I. scapularis, I. sinensis*). The OspC tree were conducted with MEGAX and the phylogeny analysis involved 34 nucleotide sequences with a total of 624 positions in the final dataset.

Table 12: Key for the Phylogenetic trees a) Salp15 b) OspC

a)

Species	
IPSC	*Ixodes persulcatus*
IPAC	*Ixodes pacificus*
ISPC	*Ixodes scapularis*
IAF	*Ixodes affinis*
IRI	*Ixodes ricinus*
ISI	*Ixodes sinensis*
IHO	*Ixodes holocyclus*

Countries	
CH	China
USA	USA
CZ	Czech Republic
NE	Netherlands
JP	Japan
GE	Germany
AUSTR	Australia

b)

Species	
Ba	*Borrelia afzelli*
Bb	*Borrelia burgdorferi*
Bg	*Borrelia garinii*
Bv	*Borrelia valaisiana*
By	*Borrelia yangtzensis*

Countries	
CZ	Czech Republic
JA	Japan
USA	Usa
NE	Netherlands
KOREA	Korea
CH	China
FI	Finnland

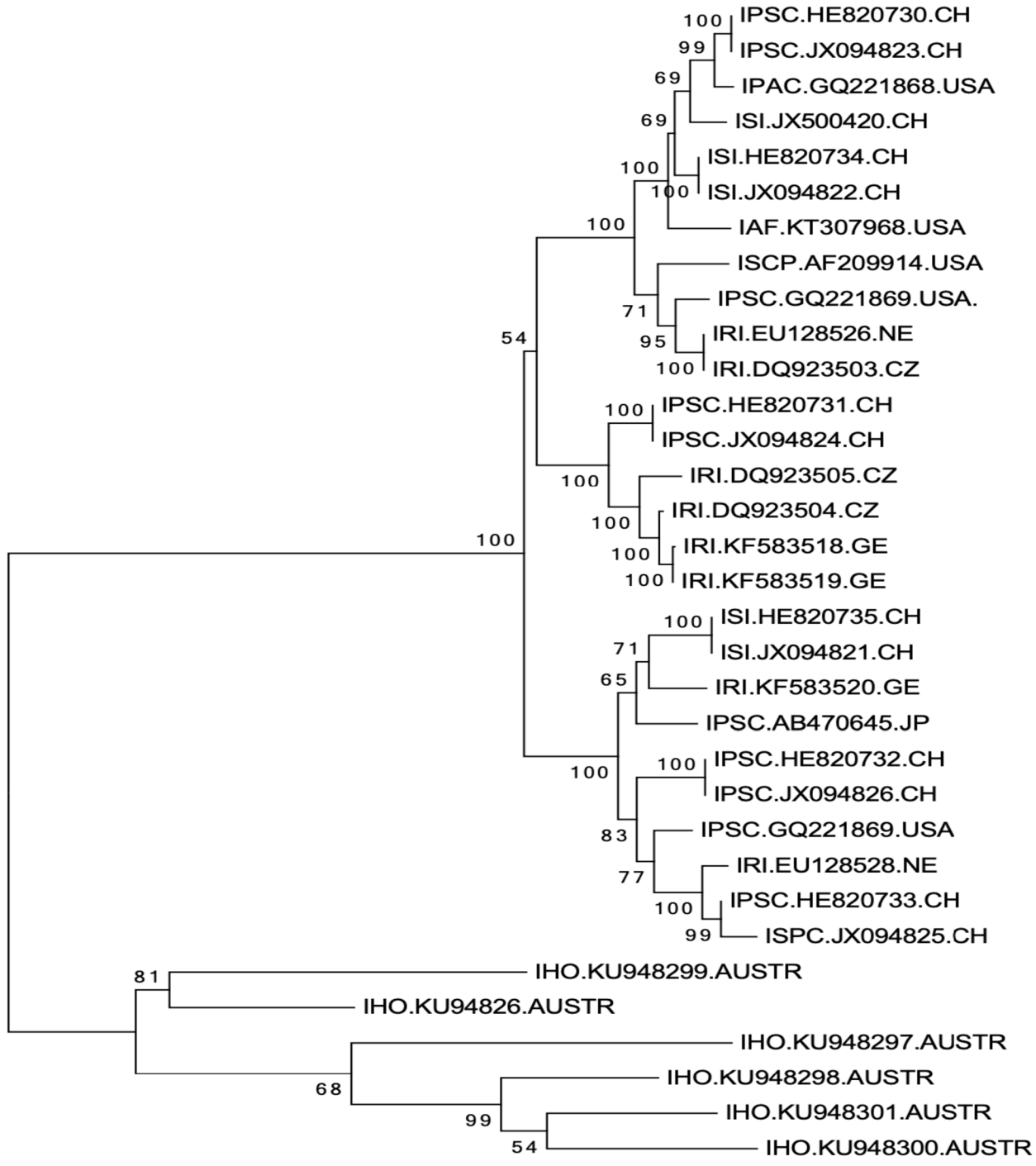

Figure 3: Maximum likelihood phylogenetic tree of Salp15. Name of the species, accession numbers and the locations are presented for each sequence used. The numbers at the tree nodes indicate the percent of bootstrap value from 1000 replicates. Bootstrap values more than 50% are indicated.

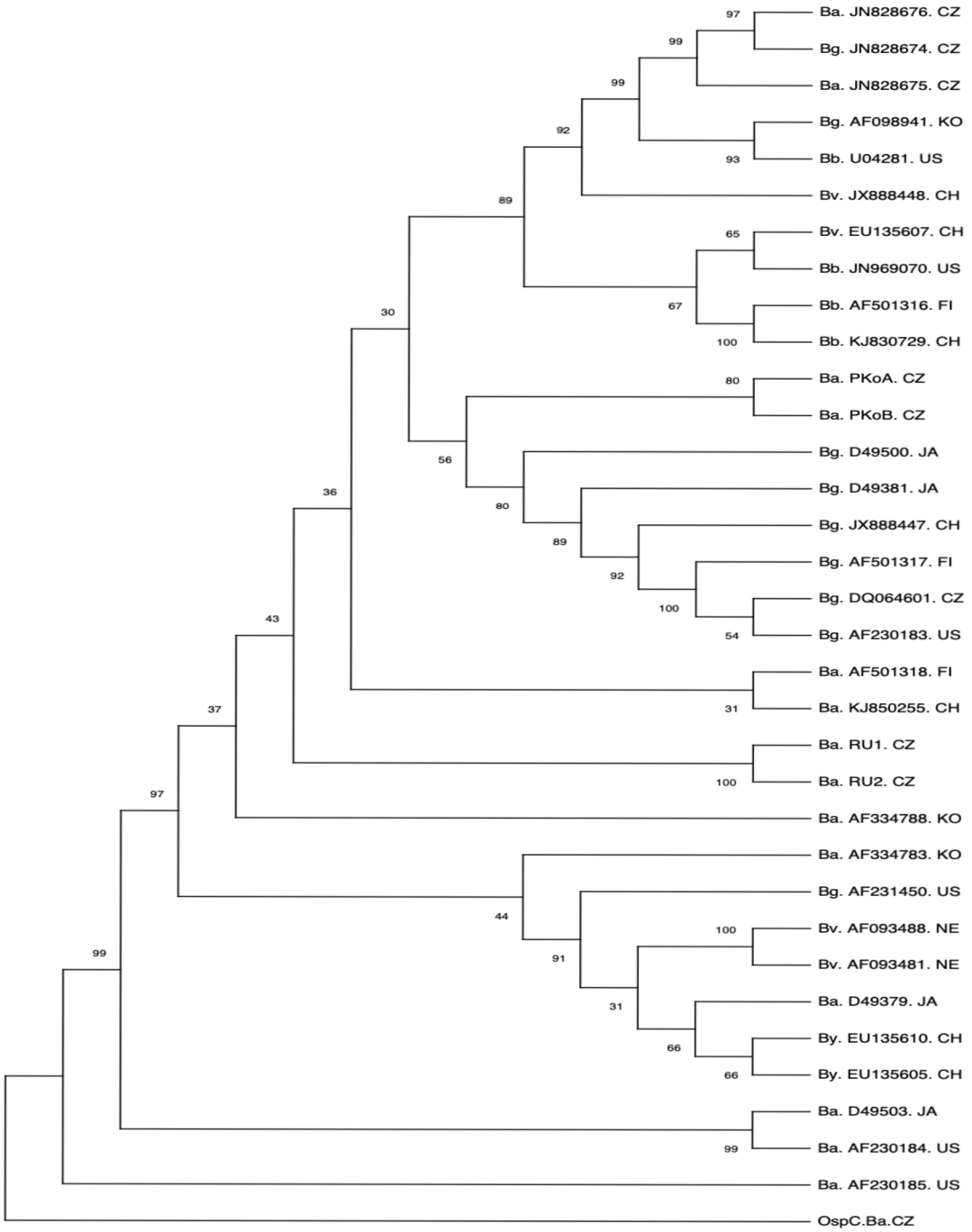

Figure 4: Maximum likelihood phylogenetic tree of OspC. Name of the species, accession numbers and the locations are presented for each sequence used. The numbers at the tree nodes indicate the percent of bootstrap value from 1000 replicates. Bootstrap values more than 50% are indicated.

5.4 PROTEIN HOMOLOGY MODELING OF OspC

Several tools were used to gain a homology model of OspC of *B. afzelii* sequence as template (PDB code 1GGQ) as described before in the methods' section "Protein modelling". The aim was to look at the interactions of Salp15 with OspC, but unfortunately no Salp15 model was available. The three-dimensional structure of OspC is predominantly helical, with four long helices plus a short fifth helix. It is known that the interaction in the dimeric interface is almost completely hydrophobic. The variable regions in these sequences are mainly in the loop regions of the molecule.

Figure 5: 3D- Model of OspC protein from *B. burgdorferi* (adapted from Kumaran *et al.*, 2001)

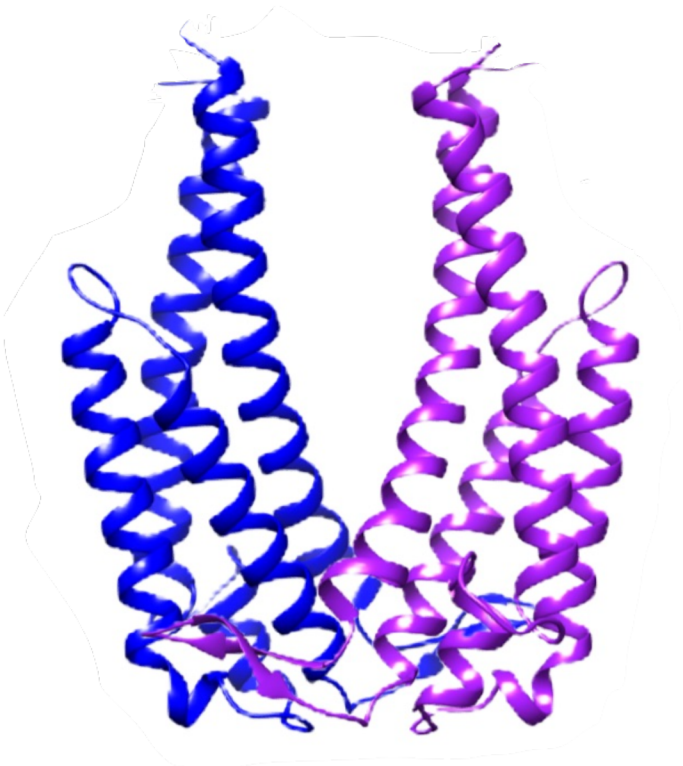

Figure 6: 3D- Model of OspC from *B. afzelii*

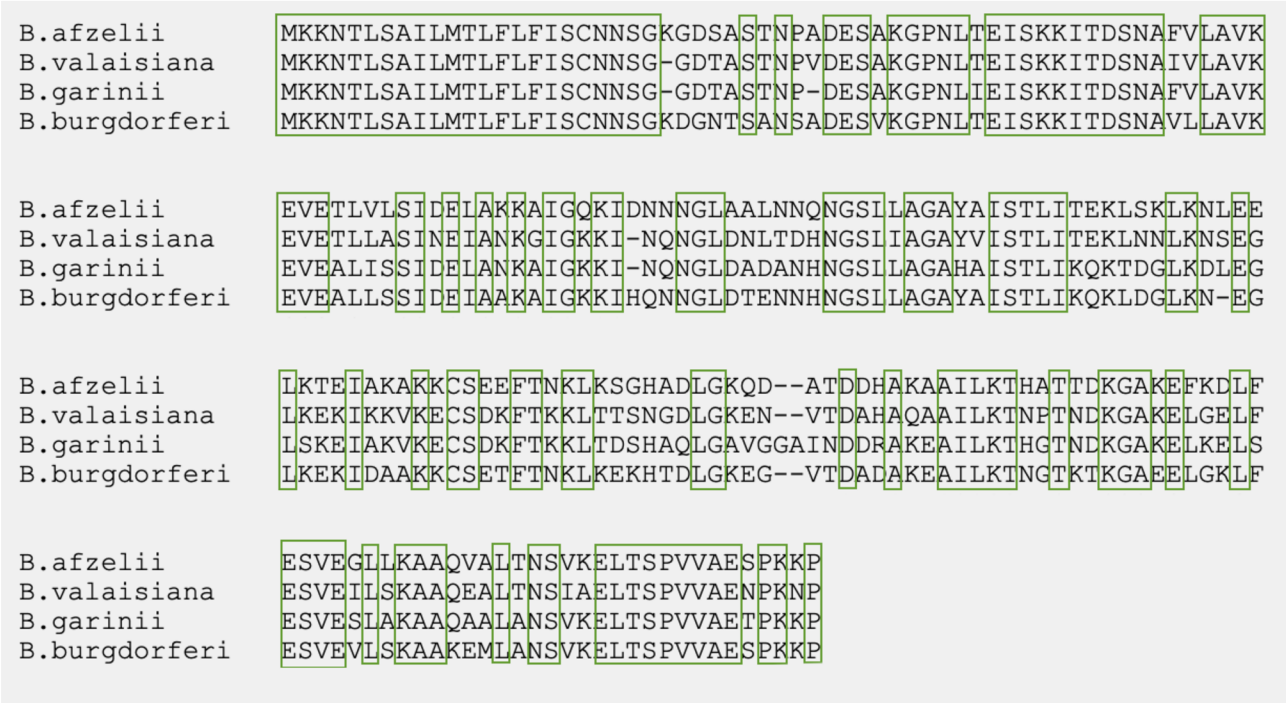

Figure 7: Alignment of four types of OspC amino acid sequences. Conserved residues demonstrated and highlighted in green.

6 DISCUSSION

So far, 22 different *B. burgdorferi s. l.* species are identified and six of these species have been classified as pathogenic to humans [12]. Depending on the *Borrelia* species, different infection rates of patient isolates and ticks were found [1,13]. Various hypotheses could be made as reasons for the different infection rates of patient isolates and ticks of *B. burgdorferi s. l.* in Europe. Hanincova *et al.* analyzed over 1000 ticks from Central Europe for infection with *B. burgdorferi s. l.* As a result they detected *B. afzelii* (50.6%), followed by *B. valaisiana* (26.5%) and *B. garinii* (19.1%, without differentiation from *B. bavariensis*) as the most frequently. In European ticks, *B. afzelii* was considered to be the predominant species [71]. The second most reported tick species after *Ixodes ricinus* in central Europe are the *Dermacentor variabilis* tick [40]. In this research the question arose why *Dermacentor variabilis* ticks are not able to successfully maintain and transmit the causative agent of Lyme disease. Under the stimulus of a new blood meal and following tick attachment, spirochetes begin to express OspC and move from the midgut through the haemolymph to the salivary glands, where they encounter Salp15, which protects them from antibody- and complement-mediated killing and promotes their transmission and replication in the host skin [24-29, 42-45]. The first attempt was to observe if the *Dermacentor marginatus* and *D. reticulatus* spp. lack the salivary gland protein Salp15. Despite various PCR modifications with designed primer sets, no desired product was obtained. Changing the reaction conditions, re- designing the PCR primers, as well as making sure the used template DNA is not degraded, especially when working with cDNA, and of course avoiding any type of contamination and finding out the right temperature conditions, would be attempts for further investigations. In order to elucidate the borrelial growth in different tick tissues (salivary glands and midgut) of the species *I. ricinus*, *D. marginuts* and *D. reticulatus,* dissection was performed. After one week of incubation with *Borrelia*, the number of *Borrelia* present in the salivary glands and midgut was noted to be quite similar for all 3 species. The borrelial growth in the guts was seen to be higher than in the salivary glands, suggesting that less individuals are able to reach the salivary glands. In the *I. ricinus* tissues a significant higher number was observed, than in the *Dermacentor* spp., as well in the beginning of the incubation, as after incubating for one week., showing that the salivary gland and midgut of *I. ricinus* is offering a better environment for Borrelia to growth *in vitro*. This observation may be due to the lack of Salp15 protein in *Dermacentor* ticks, which could make it possible that *Borrelia* do not survive in the *Dermacentor* ticks neither *in vivo*.

The challenges of identifying protective saliva gland antigens are increased due to salivary gland gene differences between tick species [72]. In this research phylogenetic analyses were used to gain more information about the relationship between the Salp15 protein isolates of *Ixodes* spp. found in the NCBI database, since in no other genus of ticks Salp15 has been found till now. A clear protruding clustering of the *Ixodes holocyclus* species from Australia to all other species could be observed.

Many putative lipoproteins encoded by *B. burgdorferi* have been implicated in supporting tick vector colonization and disease transmission, however, precise mechanisms and function of most of these remains largely unknown [4, 22]. In this thesis our focus was on the outer surface protein C (OspC). Seinost *et al.* noted that only some types of OspCs are responsible for invasive human disease. Distinctions regarding the surface potential were observed from the farthest point from the surface of the membrane to be highly negative for OspCs from invasive strains [73]. Therefore, it is speculated that this region's interaction with a positively charged ligand, such as fibronectin or a similar molecule may play an important role in the pathogenesis of Lyme disease. In this work protein homology modelling of *B. afzelii* OspC sequence as template (PDB code 1GGQ) was used for further molecular and structural characterization analysis. In order to develop an effective OspC-type vaccine, more knowledge about the representative 3D- structures of few OspCs, especially those from the invasive strains, is of importance, particularly regarding the binding ability to Salp15 proteins. There are differences that could be important for how the European species bind to *Ixodes ricinus* OspC, when looking at the 3D models shown in the results. Additionally phylogenetic analysis of OspC protein sequences of different *Borrelia* spp found in various geographical areas was performed. Neither special geographical, nor species dependent clustering was observed, which may result due to not trimmed sequences from the start codon.

Understanding why *Ixodes* ticks are Lyme disease vectors and the *Dermacentor* ticks are not, even if they share the same geography, could provide important information about the survival and moreover insights between the interaction of the lipoprotein layer functioning of *Borrelia* with ticks' proteins, especially the salivary gland proteins, which can potentially provide important targets for interrupting *B. burgdorferi* infectious cycle and development of vaccine against Lyme disease.

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