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Differentiating the Adhesion Properties of Surface Proteins between Various *Borrelia* Species

Master's Thesis

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Annotation: The aim of the thesis was to gain a better understanding of decorin binding proteins A and B (DbpAB) of various *Borrelia* species my different means. Overexpression of DbpA or DbpB in *B. afzelii* was addressed, examining phenotypic changes in mutants. Also, it was aimed to create shuttle vectors to complement DbpAB of different European *Borrelia* species as well as suicide vectors.

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, 13.12.2021 Anna-Celine Danklmaier

Acknowledgement

First of all, I want to express my gratitude for my supervisor Ryan Rego, PhD who always supported me during my time in the lab. During these two years we faced a lot of challenges, but he never had doubts and encouraged me to not give up. Also, when I think about the great chats we had not only about the project, but about everything and anything, I can say it was a great time and I am happy about it. I also want to thank my colleague Martin Strnad, PhD who was also involved in this project. He was always helping me out when questions arose and moreover the time in the office was very fun and enjoyable. Thanks also to Helena Rohackova, our lab technician who made is possible for me to conduct a lot of my work. Concerning the electron microscopy work I am grateful for RNDr. Marie Vancová, PhD and Mgr. Martina Tesařová for assisting me.

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1. Introduction

1.1. Lyme Disease

Lyme disease is considered as the most common arthropod-borne disease in the Northern Hemisphere, including North America, Europe and Asia [1,2]. In North America there are more than 476,000 cases reported annually [3] and in Europe the number of documented cases exceeds 200,000 [4]. Also referred to as Lyme borreliosis, it is defined as a multisystem inflammatory disorder caused by spirochetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex [5]. The transmitting vector is the hard tick of the genus *Ixodes* [6].

1.2. *Borrelia*

The species of *Borrelia* belong to the phylum *Spirochaetes*. Together with other members of this phylum like *Leptospira* and *Treponema* they cause pathogenic disease in humans. The latter two genera are causative agents of, respectively, leptospirosis (*Leptospira interrogans*) and either syphilis (*Treponema pallidum*) or periodontal disease (*Treponema denticola*) [7–9]. *Borrelia* species are subdivided into two major phylogenetic groups, one related to relapsing fever and the other to Lyme disease, which is also called Lyme borreliosis. Its causative agents constitute the *Borrelia burgdorferi* sensu lato (s.l.) complex, consisting of more than 20 species [5,10] that are transmitted by ticks of the genus *Ixodes* [2,11]. However, primarily *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii* and *B. bavariensis* are known to be pathogenic in humans. Though there are other genospecies that occasionally cause human Lyme disease: *B. bissettii*, *B. mayonii*, *B. lusitaniae*, *B. spielmanii* and *B. valaisiana* [4,12–14]. Also, there are differences concerning the geographical distribution of the species. While *B. afzelii*, *B. garinii* and *B. bavariensis* cause human disease in Europe and Asia, *B. burgdorferi* is the predominant agent in North America [12].

1.3. Genome Complexity of *Borrelia*

The borrelial genome is one of the most complex genomes among bacteria due to its unique segmentation. It consists of a linear chromosome and a set of circular and linear plasmids (denoted cp and lp respectively). *B. burgdorferi* B31 was the first sequenced *Borrelia* strain, which genome comprises one large linear chromosome of about 910 kilo base pairs (kbp) and 21 extrachromosomal plasmids (12 lps and 9 cps) which range in size from 5 to 56 kpb

approximately, with a total of around 610 bp [15,16]. Two additional plasmids were found to be lost from the sequenced strain due to *in-vitro* cultivation [17].

In contrast to other bacteria, the genome appears to be rather small what corresponds to the parasitic lifestyle of *B. burgdorferi*, relying mainly on the biosynthetic machinery of its host. Genes necessary for the synthesis of amino acids, fatty acids, enzyme cofactors and nucleotide are almost completely absent [15,18,19]. Also, even though the structure of the borrelial genome is unusual, most genes encoded on the linear chromosome of *Borrelia* are also found in other bacterial genomes [15]. Reversely, only a fraction of plasmid-encoded genes shows similarities to genes in other genera [16]. The majority of housekeeping genes of *Borrelia* are located on the chromosome, while the plasmids carry most genes that encode surface lipoproteins that are necessary for the transition through the enzootic cycle. However, both, chromosome and plasmids carry genes that are important for infectivity or the maintenance of the enzootic cycle. The exact functions of these genes differ; some encode surface proteins necessary for host cell interactions, immune evasion, or basic cellular and regulatory functions [16,19–22]. For example, the plasmid-encoded nicotinamidase PncA, which is involved in NAD biosynthesis, was found to be essential for mammalian infection. In *B. burgdorferi* B31 *pncA* is found on lp25, which absence is associated with a loss of infectivity in mice [23–27] and ticks [28]. Another virulence gene is present on lp25, namely *bptA*, which is essential for the survival of *B. burgdorferi* in its tick vector [29]. Also, *adeC*, encoding for an adenine deaminase, was shown to be required for infectivity in mice, and is located on lp36 in B31 [30]. An essential mechanism for immune evasion of is the recombination at the antigenic variation locus *vls*, located on lp28-1in B31. It consists of 15 silent cassettes and the expression site *vlsE*, encoding for the variable surface lipoprotein VlsE [31]. It was confirmed that mutants lacking lp28-1 do not cause a persistent infection in mice [23,25,32].

Since a complete genome sequence was only available for *B. burgdorferi* B31 for a long time, most of the research done concerning genetic manipulations of *Borrelia* has been conducted with *B. burgdorferi* s.s. strains. With the release of the genome sequences of *B. afzelii* and *B. garinii* [33], the study of European species had become feasible. Also, the sequencing methods have been improved, so that more genome sequences had been published during the last years [21,22,34]. However, there is still only a moderate number of genetic studies carried out for European *Borrelia* species [35]. Additionally, the research involving genetic manipulations can be difficult, since *B. burgdorferi* s.l. species exhibit heterogenicity in their gene content, but also in number and types of plasmids they possess. The linear chromosomes were found to be relatively constant in gene content and organization throughout the species

[16,19–22,36]. Some plasmids also show a high degree of conversation among the sequenced isolates. Particularly cp26 and lp54 are present in all genomes with high sequence identities [16,21,22,37–39]. Nevertheless, these plasmids carry genes important for infection and host adaption, which are highly variable. The outer surface lipoprotein OspC, encoded exclusively on cp26, is expressed during early stage of a mammalian infection and is essential for establishment of an infection [26,40]. It is one of the most variable single-copy genes in the *B. burgdorferi* s.l. genome [41] and seems to have experienced several horizontal exchange events [42–45]. Similarly, decorin binding proteins A and B (DbpAB), encoded on lp54, show low sequence identity across *Borrelia* species [46]. The surface-expressed proteins facilitate adhesion to host tissues and are found to be important for virulence in mice [47–51]. The variations of the sequences contribute to differences in tissue tropism [52].

Since the number and types of plasmids is not identical across the species, certain genes are located on different plasmids. For instance, the previously mentioned virulence genes *pncA* and *bptA* are found on lp28-2 in *B. afzelii* strains, instead of partially related lp25 in *B. burgdorferi* [36] and *B. garinii* [22]. Similarly, *B. afzelii* does not possess lp36, which encodes for *adeC*, as in *B. burgdorferi*. However, the *adeC* homolog is located on lp38 [22,36] and on lp25 in *B. garinii* [22]. In Table 1.3.1 a summary of important virulence genes in different *B. burgdorferi* s.l. species is shown.

Table 1.3.1: Important genes for infectivity of chosen *B. burgdorferi* s.l. species and their respective location in the genome (adapted from Casjens *et al.* 2018 [22] for all strains except PBi which information was retrieved from NCBI). *In *B. bavariensis, vlsE* was found to be present on lp28, but it is unclear which one exactly [38].

Strain	*adeC*	*dbpAB*	*ospC*	*pncA*	*vlsE*
***B. burgdorferi* B31**	lp36	lp54	cp26	lp25	lp28-1
***B. afzelii* PKo**	lp38	lp54	cp26	lp28-2	lp28-8
***B. garinii* PBr**	lp25	lp54	cp26	lp25	lp28-3
***B. bavariensis* PBi**	lp25	lp54	cp26	lp25	lp28*

The described uniqueness of the borrelial genome (as well as the variations in genome content between the species) makes genetic manipulation a challenging task. In general, the lack of biosynthetic pathways requires a complex growth medium for *in-vitro* cultivation of *Borrelia*, like for example the addition of mammalian serum to the culture medium [15,20]. Also, transformations of *Borrelia* can be difficult due to low transformation efficiencies. In contrast to other bacteria which only need nanogram amounts of DNA to be transformed, *Borrelia*

require larger quantities in the microgram region [53]. This arises from the plasmid-encoded restriction modification enzymes which degrade foreign unmethylated DNA. It was observed that the loss of plasmids lp25 and lp56, which carry the putative restriction modification genes, leads to an increased transformation efficiency of *B. burgdorferi* [54]. Furthermore, it is essential to monitor the segmented borrelial genome during genetic manipulations, importantly after the selection of mutants, to ensure that wild type and mutants remain isogenic except for directed genetic changes [55]. Certain plasmids of the potentially unstable genome are frequently lost during *in-vitro* studies. For example, the plasmids lp25 and lp28-1 of *B. burgdorferi*, which are essential for the enzootic cycle, are commonly lost [56–58]. In general, passaging of cultures as well as multiple thawing and freezing of glycerol stocks can lead to loss of multiple plasmids, hence heterogeneity in the outgrowth population [59]. One exception is the circular plasmid cp26, which is never lost during genetic studies like *in-vitro* propagation [60,61].

1.4. Transmission Dynamics

B. burgdorferi s.l. species are maintained in their natural enzootic cycles through the transmission by tick vectors to their vertebrate reservoir hosts and vice versa. Hard ticks of the genus *Ixodes* can acquire and transmit the bacteria. There are four principal species that are vector competent: *Ixodes scupularis* and *I. pacificus* in eastern and western North America, *I. ricinus* in Europe and *I. persulcatus* in Asia [6,62]. The ticks tend to feed on variable vertebrate hosts, which include rodents, birds, and lizards, but also larger vertebrates like deer. These host species differ in their capability to acquire *B. burgdorferi* s.l. from infected ticks and to further transmit it to uninfected ticks. It depends on the *Borrelia* species whether a host is reservoir competent [63]. Consequently, this leads to the interconnection of the ecological niches of *Borrelia* [6]. Rodents like mice and voles serve as reservoir for *B. afzelii*, whereas *B. garinii* depends on birds. In contrast *B. burgdorferi* relies on both, rodents, and birds. *B. bavariensis* is associated with mice as reservoir hosts [6,64].

During the infectious cycle of *B. burgdorferi* s.l. the hard tick vectors undergo three developmental stages (larva, nymph, adult) over the course of a two-year life cycle. As depicted in Figure 1.4.1, one blood meal is obtained per life stage of the ticks. Larvae must acquire *B. burgdorferi* via the first blood meal from an infected reservoir host, like small rodents or birds. *B. burgdorferi* is retained during the subsequent life stages. Infected nymphal ticks, which have a similar host range as larvae, transmit the spirochetes during the second

blood meal to a naïve host [65]. That larvae and nymphs feed on the same animal species is essential for the persistence of the spirochetes throughout different tick generations [5]. Since adult females feed primarily on large animals like deer, which are non-competent host, they are not important for the maintenance of *B. burgdorferi* [6]. Still, adults generally mate on deer what makes them essential for sustaining the tick populations. Mostly infected by nymphal ticks, humans are considered as incidental, suspectedly dead-end hosts, and are not part of the enzootic cycle [65].

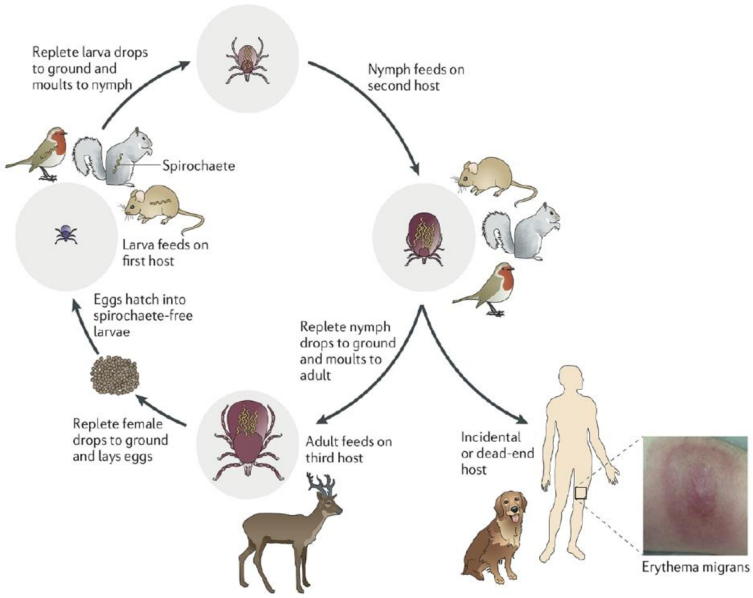

Figure 1.4.1: Graphical depiction of the enzootic life cycle of *B. burgdorfer*i (from Radolf *et al.* 2020 [66]).

1.5. Morphology

As *Borrelia* belong to the phylum of spirochetes, they show similar morphology, like the distinctive helical cell shape and flat-wave morphology (see Figure 1.5.1). Instead of external flagellar filaments that are found in most motile bacteria, spirochetes possess periplasmic flagella. These are located within the periplasmic space between the outer membrane and the peptidoglycan layer which surrounds the protoplasmic cylinder that is enclosed by a cytoplasmic membrane [67,68]. Also, the outer membrane possesses a number of different surface-exposed lipoproteins [68]. Often, *B. burgdorferi* is referred to as gram-negative-like bacterium, however it lacks lipopolysaccharides on the outer membrane [15]. The periplasmic flagella are attached via the flagella motors to both terminal ends of the protoplasmic cylinder, the innermost compartment of spirochetes [68,69]. Depending on the species, the morphology can differ in terms of size, number of flagella and if these overlap in the middle of the cell

[68]. In the case of *B. burgdorferi* there are 7-11 periplasmic filaments inserted at the cell poles which wind around the protoplasmic cylinder, in a ribbon-like structure, and overlap in the middle [67,70]. Also, *B. burgdorferi* is considered as a comparatively large spirochete with a length of 10-20 µm and a diameter of 0.33 µm [71]. Its shape is characterized to be a planar flat wave with an amplitude of 0.78 µm and a wavelength of 2.83 µm [71,72]. Not only are periplasmic flagella essential for the motility, but also partly for the distinct morphology of the spirochetes. It was found that *B. burgdorferi* mutants which are deficient in *flaB*, encoding for the major filament forming protein FlaB, are non-motile and rod-shaped [73]. However, it was also shown that FlaB alone is not leading to the characteristic flat-wave morphology, but also motor rotation. Upon inactivation of *motB*, which codes for the flagellar motor stator MotB, *B. burgdorferi* loses its motility as well as the characteristic morphology, while retaining periplasmic flagella. Complementation restored the phenotype of the wild type [74]. Additionally, both mutants are unable to establish a murine infection by needle inoculation, but also via transmission from infected ticks. A reduced viability in fed ticks was observed as well [74,75].

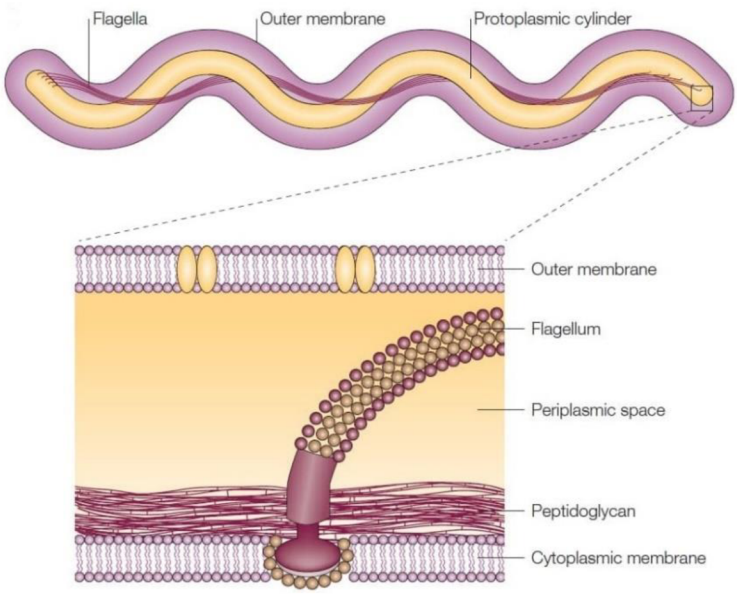

Figure 1.5.1: Structure and morphology of *B. burgdorferi*. (Adapted from Rosa *et al.* 2005 [76])

1.6. Clinical Manifestations

Lyme disease (or Lyme borreliosis) is a multisystem inflammatory disorder with diverse clinical presentations depending on the *B. burgdorferi* s.l. species [5,77]. In general, the course of infection can be divided into three stages: early localized, disseminated, and late infection [5,78]. However, this division is rather theoretical and is in many patient cases not strictly observed [79]. The transmission of the spirochetes by a tick bite leads to a localized infection, that may result in the characteristic skin lesion erythema migrans (EM) after days to weeks [5,80–82]. It is conventionally described as the so-called bull's eye rash: an expanding, annular erythematous skin lesion with or without central clearing [78,82]. Besides EM, *B. burgdorferi* s.s. might cause flu-like symptoms as for example malaise, fatigue, headache, and fever during the early stage of infection in the United States [78,83]. In Europe, EM caused by species of *B. burgdorferi* s.l. is usually not accompanied by other symptoms [5]. Infection of *B. garinii*, in contrast to *B. afzelii* and *B. burgdorferi* s.s., leads to a faster expanding skin lesion as well as more symptomatic lesions, commonly causing burning, itching and pain [84,85]. In rare cases, the skin manifestation borrelial lymphocytoma is observed in Europe, which is mostly caused by *B. afzelii* [5,80,86].

If the infection has not been treated with antibiotics, the spirochetes will migrate from the site of the tick bite within days to weeks. Depending on the species, different but sometimes overlapping clinical manifestations, develop during the early and late disseminated phase. Also, the phases must not be strictly separated from each other [2,5,80]. *B. burgdorfer*i s.s. is commonly associated with arthritis and neuroborreliosis, but also carditis [78]. In contrast, *B. afzelii* is known to mainly cause skin conditions like acrodermatitis chronica atrophicans [79,87,88]. However, an infection with *B. garinii* or *B. bavariensis* leads to neuroborreliosis in most cases [79]. Additionally, even the different strains within a single species display variations in symptom severity and dissemination activity [89,90].

The mechanisms behind the observed species-specific differences in tissue tropism are not well understood yet. However, the adhesion of *Borrelia* via surface-expressed proteins to host tissues is known to be an important step for dissemination and colonization [91]. Even though the borrelial genome is overall rather similar, there are certain virulence genes encoding for such surface proteins which are highly variable between the species. Hence, the sequence heterogenicity is potentially contributing to the specific tissue tropisms.

1.7. Borrelial Adhesion and Dissemination

For *Borrelia* it is essential to cause a disseminated, persistent infection in a vertebrate host to maintain its enzootic cycle [92]. The crucial step in the infection process is the adhesion to host tissues via surface expressed proteins. It is necessary that binding occurs with sufficient strength: too extensive binding hinders the dissemination through the host which results in the clearance of the infection. Reversely, the inability to bind prevents the infection to happen in the first place [91]. Therefore, *Borrelia* possesses a wide range of adhesive proteins that are expressed on the surface to interact with specific host molecules [91–96].

The extracellular matrix (ECM) on the host cell surface is a major adhesive target of the spirochete. The ECM consists of different types of molecules which *Borrelia* can utilize to interact with the host tissues, like proteoglycans, glycosaminoglycans (GAGs), fibronectin, laminin and collagen. Additionally, *Borrelia* can bind to integrins of host cells and to components of host serum and extracellular fluids, like plasminogen and complement regulatory proteins [91–96]. Essentially important in the adhesion process appear to be the GAGs, which are large, linear, and negatively charged polysaccharide chains. The negative charge arises from sulfurylation of the repeating disaccharide units, the most common being chondroitin-4-sulfate (type A), dermatan sulfate (type B) and chondroitin-6-sulfate (type C). The GAGs decorate certain ECM proteins to form together the so-called proteoglycans (e.g. decorin, biglycan, aggrecan), which are either inserted into the cell membrane or localized to the cell surface ECM [97]. The proteoglycan decorin associates with collagen via its collagen binding core and its amino acid sequence is rich in leucine. Also, a single GAG side chain is bound to it, being either dermatan sulfate or chondroitin-6-sulfate [98–100]. Decorin has not only an important function as structural molecule of the ECM, but also acts as a cell signaling molecule [101]. Another relevant ECM component is fibronectin which is a large plasma glycoprotein that is involved in various processes like cell adhesion and migration, cell-cell signaling, ECM remodeling and more [102]. Laminin is also a glycoprotein component of the ECM, consisting of three subunits. It serves a cellular scaffolding function and interacts with integrins and other ECM constituents [103]. Finally, there are also integrins involved which are glycosylated transmembrane signaling molecules which possess a heterodimeric structure. They mediate cell-cell as well as cell-matrix interactions [104].

Lyme disease *Borrelia* encode a range of various surface-expressed adhesion proteins that participate during the multi-stage infectious process of mammalian hosts. The specific functions of all yet to known proteins are not perfectly understood still and some even seem multifunctional [105]. An outline of borrelial surface proteins is given in Table 1.7.1 with

respective substrate molecules in the mammalian host. Some of the adhesion proteins were found to play a role in borrelial motility and dissemination in the host. For example, BBK32 which binds fibronectin was determined to mediate three different types of interaction during vascular dissemination [106,107]. DbpA promotes only transient interactions with endothelial cells [108]. In contrast, P66 and OspC were found to be necessary for the transmigration through the vessel wall [109,110].

Table 1.7.1: Summary of important adhesion proteins during mammalian infection and their respective ligands in the host.

Borrelial adhesin	Host substrate molecule	Reference
BBK32	Fibronectin, GAGs	[111,112]
Bgp	Dermatan sulfate, GAGs, aggrecan	[113,114]
CspA/Z	Fibronectin, laminin, plasminogen, complement regulatory proteins	[115]
DbpA/B	Decorin, biglycan dermatan sulfate, GAGs, laminin	[108,116–119]
ErpA/C/P	Complement regulatory proteins, plasminogen	[120,121]
ErpX	laminin	[122]
OspC	Plasminogen, fibrinogen, dermatan sulfate	[110,123]
P66	integrins	[124]
RevA/B	Fibronectin, laminin (RevA)	[107,125]

1.7.1. Decorin binding proteins A and B

The decorin binding proteins A and B (DbpAB) were first identified by Guo and colleagues, who demonstrated that *B. burgdorferi* can bind decorin, a proteoglycan found in the ECM in mammals. The approximately 20 kDa large proteins were shown to be encoded in a bicistronic operon on lp54 [116,117]. It was shown that DbpA is a helical bundle protein consisting of five helices held together by a strong hydrophobic core. Portions of two helices form a basic patch together with two flexible linkers. Upon solving the DbpA structure of *B. burgdorferi* B31, insight into the biophysical interactions with GAGs has been gained [126]. Although dermatan sulfate is the GAG mostly associated with decorin, DbpA is able to bind

also other GAGs like heparin and heparan sulfate [127]. Additionally, DbpA shows even a higher binding affinity for heparin than dermatan sulfate [126]. Further, it can be stated that DbpA is a bifunctional protein, able to interact with both, the protein core of decorin as well as with the GAG side chain, dermatan sulfate, however with different affinities [117,128,129].

Later on, the DbpAB structures of *B. burgdorferi* N40 and *B. garinii* PBr had been solved (see **Fehler! Verweisquelle konnte nicht gefunden werden.**). Interestingly, the DbpAs of different *B. burgdorferi* s.l. species are highly variable up to approximately 40-60%, while DbpB is conserved between the species [46,128,130]. Accordingly, the structures of DbpA display conformational differences as well as varying substrate binding affinities. Specifically, the C-terminus of the PBr DbpA has a different orientation as found in the DbpAs of B31 and N40. Also, the flexible linker between helices one and two of the latter is mostly unstructured, while the linker in PBr DbpA adopts a helical conformation [131].

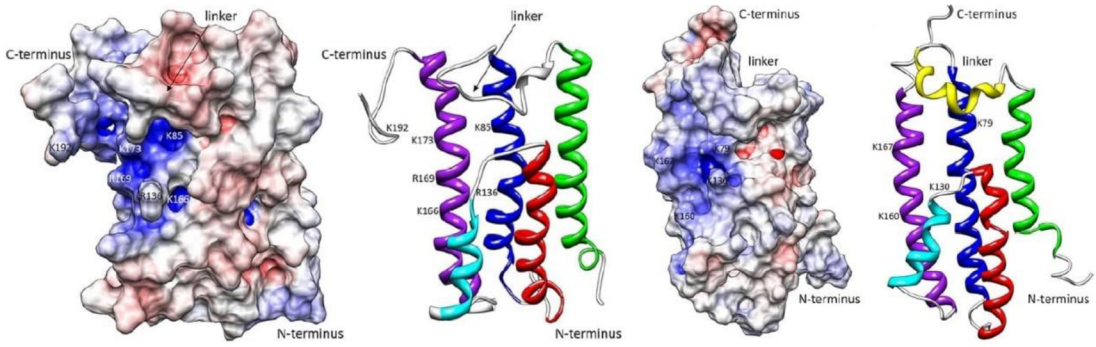

Figure 1.7.1: Electrostatic potential surface and ribbon diagram for N40 DbpA (left) and PBr (right).

As a functional consequence the PBr DbpA contains at least two independent GAG-binding epitopes, instead of only one found in the DbpAs of B31 and N40 and the retracted helical linker in PBr DbpA provides more space for the GAG ligands to bind at the epitopes [131]. Accordingly, the binding affinities of the PBr DbpA for decorin and dermatan sulfate are the highest, followed by B31 and N40 DbpA. The DbpAs of other strains were also assessed, including *B. afzelii* VS461. In contrast to the others, DbpA of VS461 promoted differential binding to the two substrates, the dermatan sulfate affinity laying between B31 and N40 DbpA, while the decorin affinity being greater than both [128]. Also, the group of Salo examined decorin binding of *B. burgdorferi*, *B. garinii* and *B. afzelii*. DbpA of *B. garinii* and DbpB of *B. garinii* and *B. burgdorferi* facilitate strong binding to decorin. Lower binding affinities were observed for DbpA of *B. burgdorferi* and both individual Dbps of *B. afzelii*. Whole spirochetes expressing *B. afzelii* Dbps were not able to bind decorin at all, in contrast to *B. garinii* and

B. burgdorferi. It was suggested that the Dbps of *B. afzelii* have different ligands and biological functions compared to the *B. garinii* and *B. burgdorferi* Dbps [132].

A different approach used to investigate DbpAB binding with certain ligands was used by Strnad and colleagues [119]. Single-molecule force spectroscopy was utilized to measure bond dissociation forces by mechanically pulling on the bound molecules. DbpA and DbpB of *B. afzelii* A91 were examined individually for decorin and laminin binding. Both proteins were found to exhibit stronger interactions with decroin than laminin. Dynamic force spectroscopy experiments with varying pulling speed demonstrated that bond lifetimes of DbpA and decorin are by far the longest, while DbpB and laminin show the shortest lifetime. Binding probabilities were largest for DbpA and DbpB interacting with decorin compared to lammin. Also, it was observed that the DbpA-decorin bond becomes highly stabilized under force load. Apparently, the complexes formed between Dbps and ECM components do not have the same dissociation behavior in solution and when force is applied [119].

Several different studies have demonstrated that DbpAB have an impact on the virulence and infectious phenotype in a mammalian host. Already in 2001 it was shown that *B. burgdorferi* experiences colonization defects in most tissues of decorin-deficient mice at low doses. At higher inocula, less spirochetes were detected specifically in joint tissue and decreases arthritis severity was observed [133]. The infectivity of *B. burgdorferi* mutants deficient in DbpAB was shown to be reduced [47–49,51,134,135], what correlates with the decreased ability to colonize various tissues [48,49,135] and to cause a persistent infection [48]. Upon complementation of DbpA [51] or DbpAB [47] into the knock-out mutant, the infectivity of the wild type was restored. Also, it was found that either DbpA or DbpB is reducing the infectivity significantly, demonstrating the importance of both proteins in mammalian infection. In comparison, though, DbpA is more essential for infectivity than DbpB [49].

As a fact, most genetic manipulations are carried out in *B. burgdorferi*. Since it has not been achieved yet to create a knock-out in European strains, comparative studies are only conducted using a gain of function approach in *B. burgdorferi*, lacking respective genes. Still there is only a minor amount of *in-vivo* studies exist that demonstrate differences of allelic variants of virulence genes in an isogenic strain background. To gain a better understanding of the variant Dbps, Lin and colleagues created isogenic *B. burgdorferi* mutants expressing DbpA of either *B. burgdorferi*, *B. garinii* or *B. afzelii* [81]. The mutants were tested in murine model to assess differences in tissue tropism. Also, recombinant DbpA protein of each species was produced and examined for binding activity against decorin and dermatan sulfate. It was found that DbpA of *B. garinii* causes the greatest colonization at the inoculation site and heart, leading

also to most severe carditis. Also, the greatest binding activity for both tested substrates was observed. In contrast, *B. burgdorferi* DbpA showed the weakest binding activities, however leading to the most stable joint colonization, probably due to other ligands (dermatan epimers, biglycan) present in mouse joints [52]. The group of Salo also demonstrated that not only DbpA, but also DbpA of *B. burgdorferi* is essential for early and late arthritis development [132].

1.7.2. Dissemination and Motility

Dissemination from the initial site of infection (site of the tick bite) to secondary colonization sites in the vertebrate host is an important step for the spirochetes to cause a persistent infection [107]. In mammals the migration occurs via hematogenous and non-hematogenous routes, like through the lymphatic system or directly through tissues [5,136]. Even though migration through the vasculature is the best understood pathway, the mechanism is not perfectly clarified due to difficulties to evaluate the spirochetes under shear stress conditions of the blood stream. However, in 2008, Moriarty and colleagues visualized disseminating *B. burgdorferi* within the vasculature of a mammalian host [137]. They engineered an infectious, green-fluorescing strain of *B. burgdorferi* and monitored it in real time using conventional and spinning disc confocal intravital microscopy (IVM). It was found that dissemination by the hematogenous route is a multi-stage process. First, the spirochetes transiently tether to the endothelium, followed by dragging along the vessel wall. Stationary adhesion, mostly observed at endothelial junctions, is the last step before transmigration through the vessel wall happens [137].

It was found that the fibronectin- and GAG-binding protein BBK32 plays a major role in the initiation events of vascular dissemination, namely transient and dragging interactions. Also, BBK32 might contribute to stationary adhesion, but additional adhesion proteins are likely necessary too [106]. Later, the group of Moriarty showed that BBK32-mediated adhesion is a two-step process: the initial fibronectin interactions during tethering permit the formation of more stable GAG interactions in the dragging phase [107]. Additionally, BBK32-deletion mutants do not exhibit a decrease in transmigration through the vessel wall *in vivo*, indicating that BBK32 is not required for extravasation. However, in absence of the integrin-binding protein P66 almost no transmigration occurred [109]. Also using IVM, OspC-mediated extravasation was observed facilitated by interactions with dermatan sulfate [110]. In contrast, the ability of DbpAB to aid in vascular adhesion was not yet observed *in vivo*. Salo and colleagues used an *in vitro* flow chamber to mimic shear stress of the blood stream. The Dbps

of *B. burgdorferi*, *B. garinii* and *B. afzelii* were each complemented into non-infectious *B. burgdorferi* B313 that is missing multiple plasmids. Apparently, only DbpAB of *B. garinii* mediate tethering interactions and DbpA was even found to be flow dependent. The Dbps of the different species were binding to biglycan, expressed by human endothelial cells, with different affinities [108]. Furthermore, DbpAB-deficient *B. burgdorferi* was observed to be attenuated in early hematogenous dissemination, leading to a colonization defect [47,48].

Similarly, the force dependence of DbpA of *B. afzelii* A91 interacting with decorin was demonstrated using atomic force microscopy. The bond between DbpA and decorin was shown to become greatly stabilized under force load, indicating the importance of DbpA to resist the shear stresses when adhering to blood vessels. In contrast, other bonds between Dbps and ECM components, especially DbpB and laminin, seem to facilitate long-term stationary adhesions in equilibrium and short term-binding under force, which allows *Borrelia* to detach under flow [119].

The same group also created recombinant *B. burgdorferi* 313, expressing either DbpAB, BBK32 or RevA of *B. afzelii* A91, to assess the motility in a near natural environment. A feeding set-up was created which should mimic the natural tick feeding on an infected host. First, *I. ricinus* ticks were put on fresh rabbit blood for 24 hours to induce feeding. This was followed by 4 hours of feeding on rabbit serum covering an ECM analog, wherein the *Borrelia* were embedded. Every hour rabbit serum samples were collected, and quantitative PCR was conducted to assess spirochete numbers. A significant increase in numbers and hence in motility was shown for DbpAB and BBK32 [119].

Seemingly, the initial adherence followed by dissemination and invasion of tissues of *Borrelia* in the mammalian host is not a straightforward process with many different adhesion proteins participating. Additionally, some of the binding activities may overlap partially or are correlated [92]. Also, considering *Borrelia* as a challenging experimental system with limited genetic tools, it is difficult to systemically characterize all adhesion pathways with respect to their biochemical functions, their impacts on host cell biology and their roles during infection [105].

The following passage ranging from page 14-43 contain classified information and is contained in the full version of the thesis only, which is stored at Faculty of Science, USB.

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