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THE ROLE OF DBPA AND B ADHESINS IN THE PATHOGENESIS OF LYME BORRELIOSIS

Jemiina Salo

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To my family

ABSTRACT

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The Role of DbpA and B adhesins in the pathogenesis of Lyme borreliosis

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Lyme borreliosis is a tick-borne infectious disease common in the Northern hemisphere. It is most commonly caused by *B. burgdorferi* sensu stricto*, B. garinii* or *B. afzelii*. The *Borrelia* bacteria have several surface proteins, or adhesins, which mediate attachment to different tissues and molecules in the host. Two of these adhesins, Decorin binding protein A and B (DbpA and B) facilitate bacterial adhesion to extracellular proteoglycan decorin. The aim, therefore, of this study is to characterize and compare the biological activities of DbpA and B of the three genospecies and elucidate the role of these interactions in the pathogenesis of Lyme borreliosis.

This study shows that Dbps have different binding properties to decorin and decorin expressing cells. In addition to decorin, the Dbps facilitate bacterial adhesion to another proteoglycan, biglycan, and biglycan expressing cells. Biglycan as a molecule is highly similar to decorin, but its tissue distributions in humans and other mammals vary significantly. *Borrelia* is able to attach to human vascular endothelial cells through an interaction with biglycan, and the Dbps of *B. garinii* facilitate the flow tolerant bacterial adhesion to endothelial cells. Furthermore, DbpA and B adhesins are both needed for full arthritis development in mice, and they enable the bacteria to persist as DNA after the antibiotic treatment in mice.

This study demonstrates the biological differences of DbpA and B of *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*, and reveals a greater role of the adhesins in the pathogenesis of Lyme borreliosis than was previously assumed.

Keywords: *Borrelia burgdorferi* sensu lato, adhesion, DbpA, DbpB, decorin, biglycan, endothelial cells, mouse model, persistence

TIIVISTELMÄ

JEMIINA SALO

DbpA- ja B-adhesiiniproteiinien merkitys Lymen borrelioosin patogeneesissä

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Lymen borrelioosi on *Ixodes*-puutiaisten (kansankielellä punkkien) välityksellä leviävä infektiotauti, jota tavataan pohjoisella pallonpuoliskolla etenkin Pohjois- ja Keski-Euroopassa sekä Pohjois-Amerikassa. Viimeisen vuosikymmenen aikana taudin levinneisyysalue on laajentunut merkittävästi rannikkoalueilta Etelä- ja Keski-Suomeen. Ihmiselle tautia aiheuttaa pääasiassa kolme eri *Borrelia burgdorferi* sensu lato -alalajia: *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* ja *Borrelia azfelii*, joiden kudoshakuisuus ja taudinkuva eroavat toisistaan. Molekyylitason mekanismeja, jotka selittäisivät *Borrelia*-alalajien erilaisen käyttäytymisen ihmisessä, ei vielä täysin tunneta. *Borrelia*-bakteerin kyky sitoutua soluväliaineen dekoriiniin DbpA- ja DbpB-adhesiiniproteiinien (engl. Decorin binding protein) välityksellä on osoitettu tärkeäksi infektion kannalta. Dekoriinia ilmennetään sidekudoksissa, erityisesti ihossa ja nivelissä. Tutkimuksen tavoitteena oli kuvata eri *Borrelia*-alalajien Dbp-proteiinien sitoutuminen dekoriiniin ja muihin mahdollisiin ligandeihin, sekä selvittää tämän sitoutumisaktiivisuuden merkitys Lymen borrelioosin patogeneesissä.

Tutkimuksessa havaittiin eri *Borrelia*-alalajien Dbp-proteiineilla olevan erilainen kyky sitoutua dekoriiniin ja dekoriinia ilmentäviin soluihin. Lisäksi Dbp-proteiinien havaittiin sitoutuvaan biglykaani-nimiseen molekyyliin, ja sitä ilmentäviin soluihin. Biglykaani on dekoriinin kaltainen proteoglykaani, mutta sen kudosjakauma elimistössä eroaa merkittävästi dekoriinin jakaumasta. *Borrelia*-bakteerien osoitettiin sitoutuvan verisuonten endoteelisoluihin biglykaanin välityksellä. Keskeinen havainto oli *B. garinii*n-alalajin DbpA- ja B-proteiinien merkitys endoteelisitoutumisessa myös virtauksen alla verenkiertoa simuloivassa koeasetemassa. Tutkimuksessa havaittiin Dbp-proteiineilla olevan tärkeä merkitys myös bakteerin leviämisessä, nivelturvotuksen synnyssä ja antibioottihoidon jälkeisessä DNA-persistoinnissa Lymen borrelioosin hiirimallissa.

Nämä tutkimustulokset tuovat merkittävästi lisää tietoa eri *Borrelia*-alalajien Dbp-proteiinien sitoutumisominaisuuksista, sekä näiden sitoutumisominaisuuksien merkityksestä *Borrelia*-infektion leviämisessä, niveltulehduksen synnyssä ja antibioottihoidon jälkeisessä bakteeri-DNA:n persistoinnissa.

Avainsanat: *Borrelia burgdorferi* sensu lato, sitoutuminen, DbpA, DbpB, dekoriini, biglykaani, endoteelisolut, hiirimalli, persistointi

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ABBREVIATIONS

ACA	acrodermatitis chronica atropicans
AEC	3-amino-9-ethylcarbazole
Ba	*Borrelia afzelii*
BCA	Bicinchoninic acid
BSA	bovine serum albumin
BSK	Barbour-Stoenner-Kelly
*Bb*sl	*Borrelia burgdorferi* sensu lato
*Bb*ss	*Borrelia burgdorferi* sensu stricto
Bg	*Borrelia garinii*
CFSE	carboxyfluorescein diacetate succinimidyl ester
Dbp	decorin binding protein
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
ELISA	enzyme linked immunosorbent assay
EM	erythema migrans
EPS	electroporation solution
FBS	fetal bovine serum
FCS	fetal calf serum
FHL	factor H-like protein
Fla	flagellin
GAG	glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HE	hematoxyline-eosin
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HFF	human foreskin fibroblast
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell

Ig	immunoglobulin
kD	kilo dalton
LA	Lyme arthritis
LB	Lyme borreliosis
LNB	Lyme neuroborreliosis
NTA	nitrilotriacetic acid
ORF	open reading frame
Osp	outer surface protein
PBS	phosphate buffered saline
PBS-T	Phosphate buffered saline containing tween
PCR	polymerase chain reaction
PMSF	phenylmethanesulphonylfluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLRP	small leucine-rich protein
SOC	super optimal broth
TBS	Tris buffered saline
TNF	tumor necrosis factor
wt	wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications.

I Jemiina Salo, Vuokko Loimaranta, Pekka Lahdenne, Matti K. Viljanen and Jukka Hytönen; Decorin binding by DbpA and B of *Borrelia garinii*, *Borrelia afzelii*, and *Borrelia burgdorferi* sensu stricto; *Journal of Infectious Diseases*; 2011 Jul 1;204(1):65-73

II Jemiina Salo*, Annukka Pietikäinen*, Mirva Söderström, Kaisa Auvinen, Marko Salmi, Rhodaba Ebady, Tara J. Moriarty, Matti K. Viljanen and Jukka Hytönen; Flow tolerant adhesion of a bacterial pathogen to human endothelial cells through interaction with biglycan; *Journal of Infectious Diseases;* 2016 May 15; 213(10): 1623-31
* Equal contribution

III Jemiina Salo, Annukka Jaatinen, Mirva Söderström, Matti K. Viljanen and Jukka Hytönen; Decorin binding proteins of *Borrelia burgdorferi* promote arthritis development and joint specific post-treatment DNA persistence in mice; *PLoS One*; 2015 Mar 27:10 (3)

1 INTRODUCTION

Lyme borreliosis (LB) is a multisystemic infectious disease caused by *Borrelia burgdorferi* sensu lato (*Bb*sl). The bacteria are transmitted by infected *Ixodes* ticks into the skin of the new host. In early infection, a skin rash called *erythema migrans* can be seen around the tick bite. If *Bb*sl succeed in evading from innate and adaptive immune responses, the bacteria can disseminate to distant organs and cause a persistent infection. Late manifestations of the disease are usually associated with symptoms in the joints, skin, heart and central nervous system. Different *Bb*sl genospecies are known to favor specific tissues or organs, but the mechanisms behind this phenomenon are still unknown.

For bacteria to disseminate, colonize tissues, and cause persistent infection they need surface localized proteins called adhesins to mediate the bacterial attachment to the host molecules. *Bb*sl have a wide variety of surface proteins that mediate attachment to different tissues and molecules in the tick or mammalian host. Decorin binding proteins A and B (DbpA and B) are two well characterized borrelial adhesins. DbpA and B are expressed during mammalian infection, and they mediate bacterial attachment to proteoglycan decorin, which is closely associated with collagen fibers in the extracellular matrix of the connective tissues. Even though no single factor has been shown to be crucial for the pathogenicity of *Bb*sl, DbpA and B are needed for the overall infectivity of the bacteria. The genes encoding DbpA and the B molecules of different *Borrelia* genospecies are sequenced, but little attention has been paid as yet on the possible differences in their biological activities. This study focuses on characterizing and comparing the biological activities of the DbpA and B of different *Bb*sl genospecies and elucidating the role of these interactions in the pathogenesis of Lyme borreliosis.

2 REVIEW OF THE LITERATURE

2.1 Lyme borreliosis

2.1.1 Epidemiology

Lyme borreliosis (LB) is the most common tick-borne infectious disease in the Northern hemisphere, and it is caused by *Borrelia burgdorferi* sensu lato bacteria (*Bb*sl, later in this thesis referred as to "*Borrelia*"). Three of the *Borrelia* genospecies, *Borrelia garinii* (*Bg*), *Borrelia afzelii* (*Ba*) and *Borrelia burgdorferi* sensu stricto (*Bb*ss), are primarily responsible for human infections, and they all have varying geographic distribution. All three genospecies are common in Europe, while *Bg* and *Ba* are encountered in Asia and *Bb*ss is found in North America. (Stanek et al., 2012; Steere, 2001)

In nature, *Borrelia* lives in an enzootic cycle involving vertebrate hosts and arthropod vectors (Barbour and Fish, 1993). The predominant reservoir species for the bacteria in Europe are the yellow-necked mouse (*Apodemus flavicollis*), the long-tailed field mouse (*Apodemus sylvaticus*) and vole (*Clethrionomys glareolus*) (Lane et al., 1991); the main reservoir host in the North America is the white-footed mouse (*Peromyscus leucopus*) (Spielman et al., 1984). In addition to small mammals, birds are reservoir animals for *Borrelia*; they can carry the infected ticks for long distances during seasonal migration (Comstedt et al., 2006; Kurtenbach et al., 1998; Richter et al., 2000). Humans, on the other hand, are regarded as incidental or dead-end hosts in the life cycle of this bacteria.

Four species of hard-bodied ticks within the *Ixodidae* family are primarily responsible for transmitting *Borrelia*. The castor bean tick (*Ixodes ricinus*) is the main vector in Europe, the taiga tick (*I. persulcatus*) in Asia, and the deer tick (*I. scapularis*) and the Western black-legged tick (*I. pacificus*) in North America (Barbour and Fish, 1993; Lane et al., 1991). The *Ixodes* ticks have three stages, larval, nymphal and adult, and they require three blood meals during their life cycle. Each feeding takes between two and eleven days (Balashov YuS, 1984). The engorged tick then falls off its host and seeks humid vegetation to moult into its next development stage. Transovarian transmission has not been shown to exist with *Bb*sl (Sormunen et al., 2016a; Sormunen et al., 2016b) and, therefore, the nymphs and adult females are mainly responsible for infecting new vertebrate hosts. Adult males feed rarely and adhere to the hosts mainly for mating (Oliver, 1989). Animal experiments have demonstrated that for *Borrelia* to infect a new host *I. ricinus* ticks need to feed for at least 17 hours (Kahl et al., 1998) and *I. scapularis* and *I. pacificus* ticks must feed for about 48 hours (des Vignes et al., 2001; Peavey and Lane, 1995). The natural cycle of *Borrelia* is presented in Figure 1.

In Finland, the distribution of ticks has expanded from the Åland archipelago and South west coast toward Central Finland during the last decade. Approximately, 10

to 30 % of the ticks in Finland are infected with *Borrelia* (Junttila et al., 1999; Sormunen et al., 2016a; Sormunen et al., 2016b). In 2015, there were 1, 874 laboratory confirmed Lyme borreliosis cases registered with the National institute of Health and Welfare in Finland, but the actual number of cases is estimated to be two or three times greater.

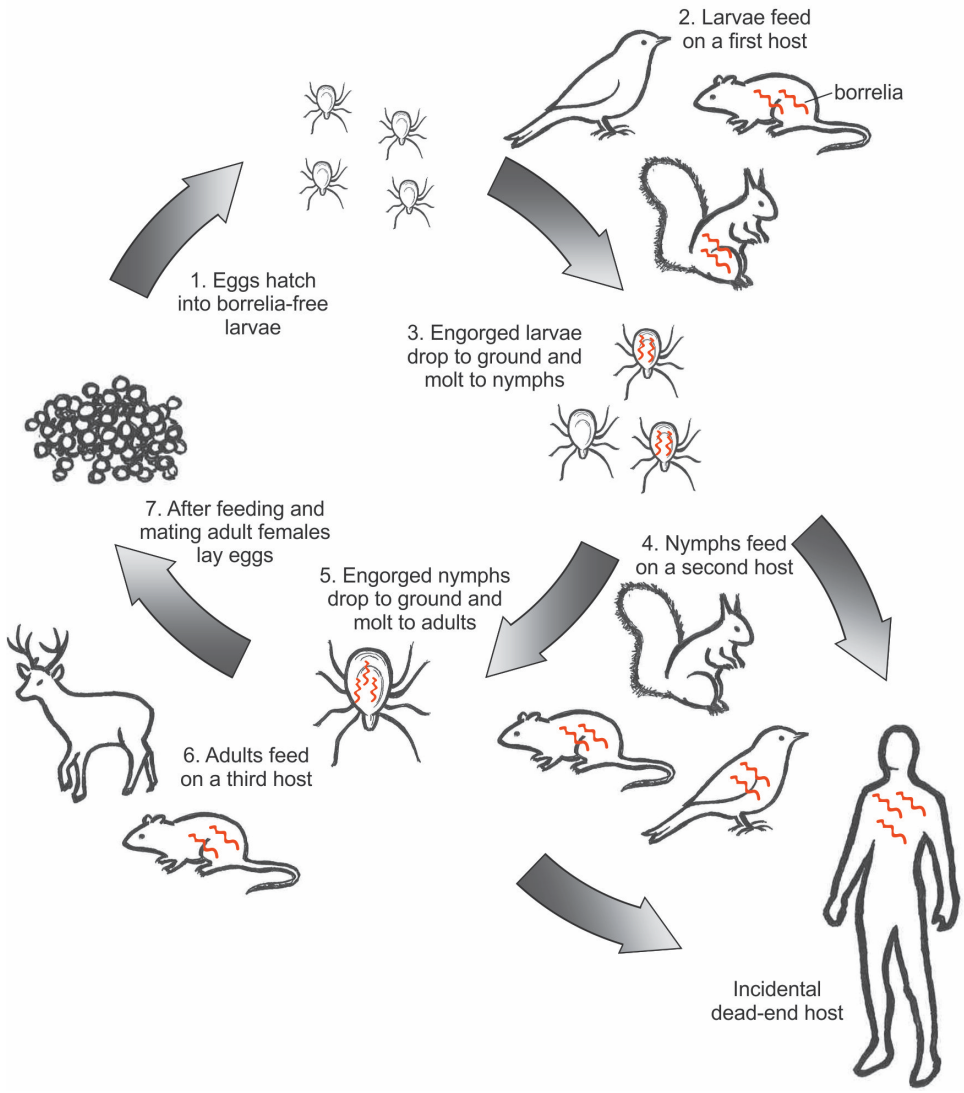


Figure 1. The natural cycle of *Borrelia*. After hatching from eggs *Borrelia*-free larvae feed on the first host, which is usually a rodent. If the host is infected by *Bb*sl, the bacteria can disseminate to the midgut of the feeding tick and infect the vector. The engorged larvae drop to the ground and molt to nymphs. Nymphs feed on a second host, and infected vectors can carry *Bb*sl to a new host. The engorged nymphs drop to the ground and molt to adults. Adult females feed on the third host, and after feeding and mating they lay eggs into the ground. Lyme disease is transmitted to new hosts by infected nymphs or adults, and humans are regarded incidental dead-end hosts for *Bb*sl. Modified from (Radolf et al., 2012).

2.1.2 Pathogenesis

Borrelia does not secrete toxins, and its principal virulence factors are outer surface proteins, which enable the bacteria to invade and adhere to multiple different tissues in the host. For *Borrelia* to colonize a tick the bacterium migrates into the tick during feeding where it needs to attach to a tick receptor called TROSPA (tick receptor for OspA) in the midgut with its outer surface protein A (OspA) (Pal et al., 2004a). When the tick feeds again OspA expression on *Borrelia* is down regulated and another outer surface protein, OspC, expression is up regulated (Schwan and Piesman, 2000; Schwan et al., 1995). The increased expression of OspC on borrelial surface in the tick midgut is crucial for the bacteria to migrate to the salivary glands and infect a new host (Pal et al., 2004b).

In addition to outer surface molecules of *Borrelia*, tick salivary protein Salp15 has been demonstrated to have a significant role in the early events of the LB infection (Ramamoorthi et al., 2005). Salp 15 is bound on *Borrelia* by OspC, and it protects *Borrelia* from complement-mediated killing (Schuijt et al., 2008) and inhibits adaptive immune responses by suppressing dendritic cell functions (Hovius et al., 2008).

After being transmitted to the skin of a vertebrate host, *Borrelia* adheres to host molecules and tissues and begins disseminating further in the skin and to distant organs. Several outer surface molecules are known to facilitate adherence of the bacteria to different host molecules and cells (Brissette and Gaultney, 2014; Coburn et al., 2013). The specific roles of these proteins are discussed later in this thesis.

2.1.3 Clinical manifestations

The clinical manifestations of LB are traditionally separated into three different stages: local skin infection, early disseminated infection and late disseminated infection (Steere, 2001). However, it is not always easy to distinguish the stages from each other because they can overlap and the manifestations can vary among patients.

In a local infection, a skin rash called erythema migrans (EM) appears around the tick bite usually in 5 to 18 days (ranging between 1 to 129 days) after detachment of the tick (Logar et al., 2004; Nadelman et al., 1996). Approximately half of the European patients develop noticeable EM (Singh and Girschick, 2004) while the number in North-American patients is around 80 % (Steere, 2001). EM is a red, circular or oval in shape, expanding skin rash which can be either homogenous or fade in the middle, thus producing a ring-like appearance. Usually EM is a single skin lesion; however multiple EM lesions, as a sign of disseminated infection, can also be seen (Eriksson et al., 2013). The skin lesion is commonly accompanied by flu-like symptoms, such as fever, headache, malaise, fatigue, arthralgia and myalgia. *Borrelia* lymphocytoma is a rare cutaneous manifestation that is more common in children than it is in adults (Stanek and Strle, 2008; Strle et al., 1992). A bluish-red, tumor-like skin infiltrate is usually seen in the ear lobe,

on the nipple or on the scrotum. Reported lymphocytoma cases are exclusively seen in Europe, and they are predominantly caused by *Ba* (Arnež and Ružić-Sabljić, 2015).

Early disseminated LB typically involves the central and peripheral nervous system or the heart. Lyme neuroborreliosis (LNB) emerges within weeks to few months after the tick bite. In Europe, LNB is mainly caused by *Bg*, and the early clinical symptoms include peripheral pareses (particularly facial nerve palsy), lymphocytic meningitis, and meningoradiculitis (also known as Bannwarth's syndrome). In North America, LNB is not as common manifestation as it is in Europe and it usually appears as subacute meningitis caused by *Bb*ss. In contrast, acute cardiac symptoms, such as atrioventricular (I-III) conduction disturbances, rhythm disturbances, myocarditis, or pericarditis, are more often seen in North-America than they are in Europe (Hytönen et al., 2008; Stanek et al., 2012; Steere, 2001).

Several months or years after the onset of the disease symptoms of Lyme arthritis (LA), chronic skin disorders called acrodermatitis chronica atropicans (ACA) may appear. LA usually involves the large joints, especially the knee, and it is characterized by intermittent mono- or oligo arthritis attacks of joint swelling and pain (Steere, 2001). It was thought that LA cases were more common in North America than in Europe, but a nationwide study in Germany revealed that joint manifestations are quite common in Europe as well (Priem et al., 2003). The chronic skin manifestations usually involve extensor sides of the extremities, and appear as red or bluish-red expanding lesions. ACA is predominantly caused by *Ba*, and thus, it does not occur in North America (Hytönen et al., 2008; Stanek and Strle, 2009).

2.1.4 Diagnosis

Diagnosis of LB is based on clinical symptoms and signs in the patient. Laboratory tests are used to confirm the disseminated infections. Patients suffering from general and non-specific symptoms should not be tested for LB because of the possibility of false-positive test results, when then lead to over diagnosis. Several laboratory methods have been developed to diagnose LB patients. Polymerase chain reaction (PCR) and culture are the most commonly used direct detection methods, but the low concentration of the bacteria in the infected tissues and body fluids limits the sensitivity of these particular applications. The best results with PCR have been obtained prior to antibiotic treatment from skin biopsy samples of EM and ACA patients and the synovial fluid of LA patients. In addition to relatively low sensitivity, *Borrelia* cultures are very laborious and time consuming (2-6 weeks), and therefore the method of choice is the detection of *Borrelia* specific antibodies from sera or cerebrospinal fluid.

Antibodies against borrelial structures appear approximately at two weeks of infection. A two-tier approach is commonly used in *Borrelia* serology. First, a sensitive

screening assay, usually based on an enzyme-linked immunosorbent assay (ELISA) methodology, is performed, followed by a more specific immunoblot assay (Wilske et al., 2007). Serology has two major limitations in LB diagnostics. First, the slow antibody response in the serum of an infected patient makes this method unreliable for the diagnosis of early infection; therefore a diagnosis of EM is usually clinical. Secondly, the persistent nature of *Borrelia* specific antibodies in the serum of a treated patient complicates making a distinction between an active infection and an old immunity.

To confirm a suspected LNB patient, *Borrelia* specific intrathecal antibody production should be demonstrated (Wilske et al., 2007). In addition to serology, new laboratory applications in LNB diagnostics are under development. CXCL13, a B-cell-attracting chemokine, is a potential diagnostic biomarker in the cerebrospinal fluid of untreated LNB patients (Hytönen et al., 2014; Ljøstad and Mygland, 2008; Schmidt et al., 2011; Tjernberg et al., 2011). CXCL13 concentration in the CFS is highly elevated in untreated LNB patients compared to many other neuroinflammatory conditions and it decreases rapidly after treatment, thus making it a good indicator of an active infection (Hytönen et al., 2014).

2.1.5 Treatment and prevention

Borrelia is susceptible to tetracyclines, most penicillins and several cephalosporins and macrolides (Hunfeld et al., 2005; Morgenstern et al., 2009; Wormser et al., 2006). Acquired anti-microbial resistance against commonly used antibiotics has not been reported (Hunfeld and Brade, 2006). The Finnish guidelines and practices for treating LB recommend 2-3 weeks of oral amoxicillin treatment for EM and 2-3 weeks of intravenous ceftriaxone treatment for disseminated diseases (Hytönen et al., 2008). Recently, retrospective studies have been published that show that oral doxycycline is as effective as intravenous ceftriaxone for treating LNB with peripheral or central nervous system symptoms (Bremell and Dotevall, 2014; Kowalski et al., 2011). However, double blind randomized prospective studies are needed to verify these results and to include oral doxycycline in the guidelines for the treatment of disseminated LB.

There is no human vaccine available against LB at the moment. In 1998, the Food and Drug Administration in the United States approved an OspA based vaccine, LYMErix™, but it was withdrawn from the market four years later due to safety concerns and decreased demand (Nigrovic and Thompson, 2007). Other antigens and molecular combinations have been tested as vaccine candidates in animal models, but so far only one multivalent OspA vaccine containing protecting epitopes of six different OspA serotypes has reached phase 1/2 of human studies (Wressnigg et al., 2014; Wressnigg et al., 2013). Before new vaccines are launched, therefore, the only way to prevent LB in endemic regions is the use of protective clothing, regular skin inspections and prompt removal of attached ticks.

2.2 *Borrelia burgdorferi* sensu lato

2.2.1 Genospecies

The genus *Borrelia* belongs to the phylum *Spirochaetes* along with four other genera, *Leptospira*, *Brachyspira*, *Spirochaeta* and *Treponema*, based on the 16S rRNA sequences (Paster et al., 1991). There are two major branches of bacteria within the *Borrelia* genus, namely the causative agents of LB and relapsing fever. A proposal for dividing the genus has been put forward (Gupta et al., 2013) and that subject has produced an intensive debate within the scientific community. The causative agents of LB are generally referred to as *Borrelia burgdorferi* sensu lato (*Bb*sl).

The *Bb*sl complex includes 20 different genospecies (Casjens et al., 2011a; Pritt et al., 2016; Rudenko et al., 2011). Three of these genospecies, *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*, commonly infect humans, while seven, B. *bavariensis*, *B. bissettii*, *B. kurtenbachii*, *B. lusitaniae*, *B. spielmanii*, *B. valaisiana*, and *B. mayonii* are potentially pathogenic.

2.2.2 Structure

Borrelia is a motile spiral-shaped bacterium, the length of which varies between 10 to 20 μm and a diameter 0.18-0.25 μm (Barbour, 1984). Based on phylogenetic analyses and 16S rRNA gene sequences, spirochetes form a distinct entity unrelated to the other main bacterial groups (Paster et al., 1991; Woese, 1987). The cell wall of spirochetes consists of inner and outer membranes, which enclose periplasmic flagellae. *Borrelia* has seven to ten flagellae, and the number vary among the genospecies (Barbour and Hayes, 1986). The flagellae are attached to the cytoplasmic inner membrane at one end near the terminus of the bacterium; the other ends of the flagellae are twisted together in the center of the bacterium thus enabling the spirochete to take its characteristic form. While rotating the multi-component flagella structures, the spirochete can effectively penetrate through viscous tissues (Zhao et al., 2014).

Borrelia has similarities to Gram-negative bacteria, such as a double membrane-envelope. However, analyses of the borrelial cell membrane have revealed that it does not contain lipopolysaccharide (Takayama et al., 1987) or phosphatidylethanolamine (Belisle et al., 1994) which are common components of Gram-negative bacteria. The outer membrane of *Borrelia* contains transmembrane proteins in low density while the number of lipoproteins is remarkably high compared to other bacteria. It is estimated that two thirds of the over 120 lipoproteins expressed by *Borrelia* are located on the outer surface (Zückert, 2014). The schematic structure of *Borrelia*'s double membrane is presented in Figure 2.

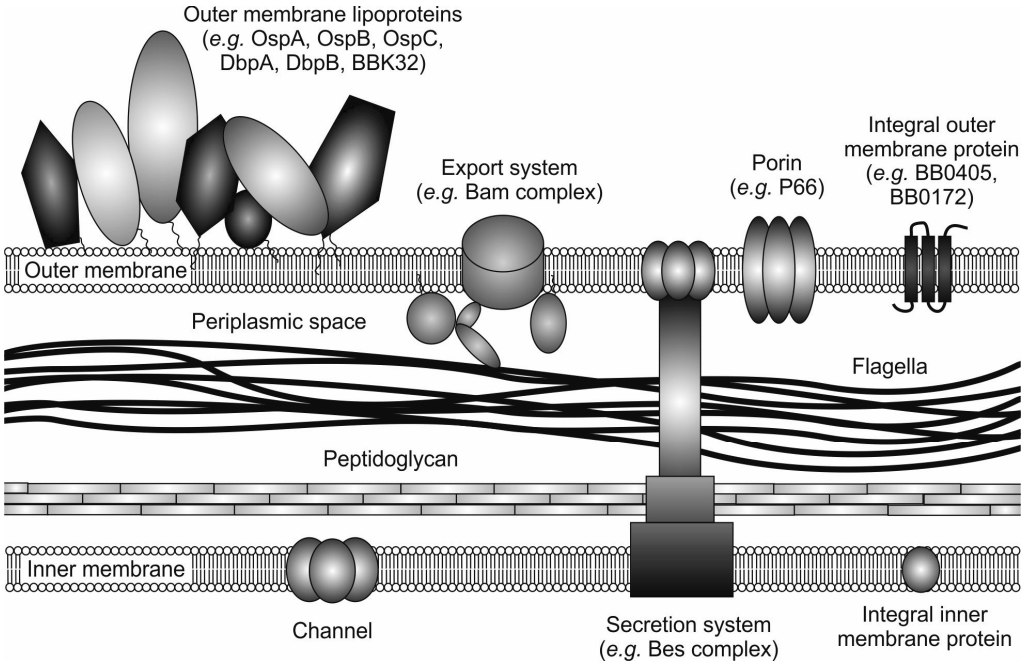
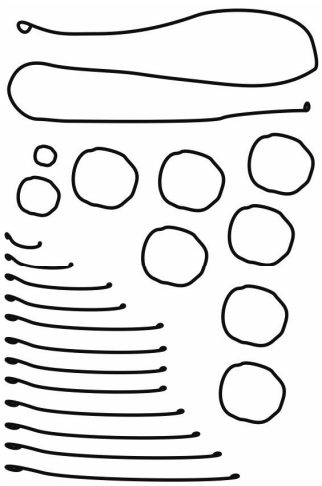


Figure 2. A schematic presentation of the membrane structure of *Borrelia*. The cell wall of *Borrelia* consists of two lipid bilayers, outer and inner membranes, which enclose periplasmic flagellae and peptidoglycan layer. Modified from (Pinne et al., 2006; Radolf et al., 2012)

2.2.3 Genome

The genome of *Borrelia* is unique among bacteria. It consists of one linear chromosome, approximately 900 000 base pairs in size, and 11 to 21 linear and circular plasmids, the number of which can vary among the *Borrelia* genospecies and strains (Casjens et al., 2011b; Schutzer et al., 2011) (Figure 3). The chromosome and linear plasmids have covalently closed hairpin telomeres (Barbour and Garon, 1987; Casjens et al., 1997), and the linear plasmids are referred to as "minichromosomes" due to their shape and low copy number in a bacterial cell (Barbour, 1993). *Bb*ss B31 was the first *Borrelia* strain that was completely sequenced (Fraser et al., 1997). Altogether, sequences of five *Bg* (Brenner et al., 2012; Casjens et al., 2011b; Jiang et al., 2012a; Tatusova et al., 2014), five *Ba* (Casjens et al., 2011b; Jiang et al., 2012b; Kurilshikov et al., 2014; Schüler et al., 2015) and 14 *Bb*ss (Fraser et al., 1997; Schutzer et al., 2011) isolates have been determined according to the genome database of the National Center for Biotechnology Information (NCBI).

The whole genome sequence of *Bb*ss B31 has revealed that more than 40 % of its genome, including genes essential for the bacteria to survive in the natural cycle, is located in the linear and circular plasmids, which make *Borrelia* unusual among prokaryotes. The plasmids can easily be lost during *in vitro* cultivation, which may then affect the infectivity of the bacteria. The *Bb*ss B31 chromosome contains 853 and the plasmids 430 predicted coding sequences (open reading frames), and the G + C content of the chromosome and the plasmids is relatively low at approximately 28 %. (Fraser et al., 1997)



Bbss B31	Bbss N40	Bg PBr	Ba Pko
Cromosome:	Cromosome:	Cromosome:	Cromosome:
910 724 bp	902 191 bp	903 870 bp	903 609 bp
Plasmids (kbp):	Plasmids (kbp):	Plasmids (kbp):	Plasmids (kbp):
lp-5 (5)	lp-17 (21)	lp-17 (21)	lp-17 (25)
lp-17 (17)	lp-25 (23)	lp-25 (33)	lp-28-2 (27)
lp-21 (19)	lp-28-2 (30)	lp-28-1 (38)	lp-28-3 (23)
lp-25 (24)	lp-28-4 (29)	lp-28-3 (18)	lp-28-4 (22)
lp-28-1 (28)	lp-28-5 (27)	lp-28-4 (40)	lp-28-7 (27)
lp-28-2 (30)	lp-36 (29)	lp-28-7 (27)	lp-28-8 (21)
lp-28-3 (29)	lp-38 (38)	lp-36 (37)	lp-32-10 (30)
lp-28-4 (27)	lp-54 (54)	lp-54 (57)	lp-38 (34)
lp-36 (37)	cp-9 (9)	cp-26 (27)	lp-54 (58)
lp-38 (39)	cp-26 (26)	cp-32-5 (30)	cp-26 (27)
lp-54 (54)	cp-32-4 (16)	cp-32-10 (35)	cp-32-1 (30)
lp-56 (53)	cp-32-5 (30)		cp-32-3 (31)
cp-9 (9)	cp-32-7 (17)		cp-32-5 (31)
cp-26 (26)	cp-32-9 (31)		cp-32-7 (25)
cp-32-1 (31)	cp-32-10 (30)		cp-32-9 (29)
cp-32-3 (30)	cp-32-12 (28)		cp-32-11 (31)
cp-32-4 (30)			cp-32-12 (30)
cp-32-6 (30)			
cp-32-7 (31)			
cp-32-8 (31)			
cp-32-9 (31)			

Figure 3. Schematic structure of the *Bb*sl genome. The plasmids harbored by *B. burgdorferi* sensu stricto B31 and N40, *B. garinii* PBr and *B. afzelii* Pko strains are listed. The name of the plasmid indicates the structure of the molecule, linear (lp) or circular (cp), and the approximated size. The actual sizes of the plasmids are in brackets.

2.2.4 Adhesins

Adhesion to different host cells and surfaces is crucial in the pathogenesis of a bacterial infection. The molecular interactions between a pathogen and its host are facilitated by surface-localized bacterial proteins, adhesins, and various host molecules. Adhesins enable the bacteria to adhere and breach through either mucosal or epithelial cells, disseminate via either the vascular or the lymphatic systems, evade immune responses, and colonize tissues and organs. Without the ability to bind with a sufficient enough strength to host tissues, the bacteria will be cleared by innate immune mechanisms, such as the flow of body fluids. On the other hand, the adhesion of the pathogen to host tissues has to be transient enough to enable dissemination from the initial site of entry deeper into the host.

One of the most studied phenomena of bacterial adherence is the attachment mediated by bacterial cell surface structures called pili or fimbriae. Pili are long and flexible heteropolymeric structures containing adhesins at the distal end. For example, FimH, which is expressed by most *Enterobactericaceae* (including uropathogenic *E. coli*), is a well-characterized pilus associated adhesin (Hanson and Brinton, 1988; Maurer and Orndorff, 1987). For example, FimH forms shear-induced catch-bonds with D-mannose on the cell surface, which enables the bacteria to adhere on bladder epithelium (Hung et al., 2002; Sauer et al., 2016; Thomas et al., 2002). Furthermore, several pilus associated adhesin of *Neisseria meningitides* have been identified as mediating bacterial adhesion under flow. PilE, PilV and PilX, all components of type IV pili, play an essential role in the endothelial cell adhesion, which enables *N. meningitides* to breach through the blood brain barrier and cause meningitis (Brissac et al., 2012; Takahashi et al., 2012). In addition to

fimbria-associated proteins, various outer surface localized proteins mediate a bacterial adhesion. For example, YadA is a well-characterized *Yersinia* adhesin, which binds to collagen, fibronectin, and laminin (El Tahir and Skurnik, 2001).

Borrelia does not have fimbriae on its surface. However, the outer membrane of *Borrelia* carries a wide variety of transmembrane and surface conjugated proteins that mediate the bacterial adherence to tick and mammalian structures (Brissette and Gaultney, 2014; Coburn et al., 2005). Several borrelial adhesins bind to specific macromolecules in serum or extracellular fluids, on vascular endothelium, or in the extracellular matrix (ECM) of connective tissues. All these interactions are essential for the bacteria to cause disseminated and persistent infection.

During the dissemination process, *Borrelia* has to evade the innate immune responses to survive in the circulation. The spirochete expresses several adhesins, CspA, CspZ, ErpP, ErpC and ErpA, also known as the Complement regulator acquiring surface proteins (CRASPs), which are able to bind factor H and factor H-like proteins (Hellwage et al., 2001; Kraiczy et al., 2001a; Kraiczy et al., 2001b; Kraiczy and Stevenson, 2013). Factor H regulates the complement system by binding to the C3b molecule in the alternative pathway (Rodríguez de Córdoba et al., 2004). By binding to factor H, the bacterium is able to evade complement-mediated killing (Alitalo et al., 2001; Kraiczy et al., 2001a).

The adhesion of *Borrelia* to the vascular endothelium has been studied with intravital microscopy in the LB mouse model (Moriarty et al., 2008; Norman et al., 2008). This sophisticated method revealed that the escape from the vasculature is a multi-step process that includes transient tethering associations, short-term dragging interactions, stationary adhesion and extravasation. The initiation of this cascade is mediated by a borrelial adhesin, BBK32 (Norman et al., 2008). BBK32 is a 47 kD lipoprotein that is encoded in lp-36 (Probert and Johnson, 1998). It binds to the host fibronectin and glycosaminoglycans (GAGs), especially heparin and dermatan sulphate, with independent adhesion sites (Fischer et al., 2006). Fibronectin is a complex glycoprotein that is present in plasma and ECM, and it contains distinct motifs that interact with integrins, collagen, heparin and gelatin (Romberger, 1997). In addition to BBK32, five other *Borrelia* adhesins, BB0347, CspA, CspZ, RevA and RevB, are known to bind fibronectin (Brissette et al., 2009a; Gaultney et al., 2013; Hallström et al., 2010).

Adherence to ECM plays an important role in tissue colonization. Apart from fibronectin, various macromolecules are recognized by borrelial adhesins. Two well-characterized adhesins are Decorin binding protein A and B (DbpA, DbpB) (Guo et al., 1998; Guo et al., 1995), which facilitate bacterial adherence to a proteoglycan called decorin and GAGs, mainly dermatan sulphate. Dbps and their role in LB are discussed more closely later in this thesis. In addition to DbpA and B, a few other GAG-binding adhesins are identified, namely, Bgp, BBK32, and Lmp-1 (Fischer et al., 2006; Parveen and Leong, 2000; Yang et al., 2016). Further, *Borrelia* has outer surface proteins mediating the adhesion to collagen, integrins, laminin, plasminogen and aggrecan. A list of known adhesins and their functions is offered in Table 1.

Table 1. The *Borrelia* adhesins

Adhesin	Location in the genome	Characteristics / Functions		Reference(s)
		In vitro	*In vivo*	
BBA70	lp54	Binds plasminogen	Not determined	(Koenigs et al., 2013)
BBK32	lp36	Binds fibronectin, heparin sulphate, and dermatan sulphate	Involved in vascular adhesion and joint colonization	(Fischer et al., 2006; Lin et al., 2015; Moriarty et al., 2012; Probert and Johnson, 1998)
BBB07	cp26	Binds $\alpha_3\beta_1$-integrins	Not determined	(Behera et al., 2008)
BB0172	Chromosome	Binds $\alpha_3\beta_1$-integrins	Immunization with a BB0172-derived peptide protects mice against LB	(Small et al., 2014; Wood et al., 2013)
BB0337 (enolase)	Chromosome	Binds plasminogen and induces fibrinolysis	Immunization with recombinant protein does not protect mice against LB	(Nogueira et al., 2012)
BB0347	Chromosome	Binds fibronectin	Not determined	(Gaultney et al., 2013; Moriarty et al., 2012)
BbHtrA	Chromosome	Binds and degrades aggrecan, neurocan, brevican, versican, biglycan, decorin, and fibronectin	Immunization with recombinant protein does not protect mice against LB	(Russell et al., 2013; Russell and Johnson, 2013; Ullmann et al., 2015)
Bgp	Chromosome	*Borrelia* glycosaminoglycan-binding protein; binds heparan sulphate, dermatan sulphate, and aggrecan, and has nucleosidase activity	Not essential for the infection of immunodeficient mice	(Parveen et al., 2006; Parveen and Leong, 2000; Russell and Johnson, 2013)
Bmp family	Chromosome	Basic membrane proteins; BmpA binds laminin	BmpA and BmpB play a role in joint colonization and joint swelling	(Pal et al., 2008; Verma et al., 2009)
CspA (CRASP-1)	lp54	Binds factor H, factor H-like protein 1 (FHL1), fibronectin, laminin, plasminogen and collagen Types I, III, and IV	Expressed during tick-to-mammal and mammal-to-tick transmission, but not during the established infection involved in the immune evasion	(Bykowski et al., 2007; Hallström et al., 2010; Kraiczy et al., 2001b)

CspZ (CRASP-2)	lp 28-3	Binds factor H, FHL1, fibronectin, laminin, plasminogen, and collagen	Expressed during mammal infection (≥ 2 weeks of infection), involved in the immune evasion	(Bykowski et al., 2007; Hallström et al., 2010)
ErpP (CRASP-3) **ErpC** (CRASP-4) **ErpA** (CRASP-5)	cp32	Collectively called as OspE; Bind factor H, FHL1, FHL2, FHL5, and plasminogen	Expressed in feeding ticks and during mammalian infection	(Hellwage et al., 2001)
DbpA **DbpB**	lp54	Decorin binding proteins: bind decorin, dermatan sulphate, and heparin sulphate	Needed in the early dissemination and play a role in the colonization of joints and heart	(Fischer et al., 2003; Guo et al., 1995; Parveen et al., 2003; Shi et al., 2008a; Weening et al., 2008)
ErpX	lp56	Binds laminin	Not determined	(Brissette et al., 2009b)
Lmp1	Chromosome	Binds chondroitin-6-sulphate and has a role in epithelial, endothelial, synovial, and glial cell adhesion	Involved in the colonization of skin, joint, heart, and bladder	(Yang et al., 2009; Yang et al., 2016)
OspA **OspB**	lp54	Outer surface proteins; bind TROSPA-receptor in tick midgut; OspA binds plasminogen; OspB inhibits neutrophil functions	Needed for tick colonization	(Fuchs et al., 1994; Hartiala et al., 2008; Howe et al., 1986; Pal et al., 2004a)
OspC	cp26	Outer surface protein; binds tick salivary protein Salp15 and plasminogen	Protects *Borrelia* from complement mediated killing and suppresses denritic cell functions by binding Salp15	(Hovius et al., 2008; Lagal et al., 2006; Marconi et al., 1993; Schuijt et al., 2008)
P66	Chromosome	Binds $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrins and acts as a porin	Facilitates dissemination	(Bunikis et al., 1995; Coburn et al., 1999; Ristow et al., 2015; Skare et al., 1997)
RevA	cp32	Binds fibronectin	Plays a role in dissemination and heart colonization	(Brissette et al., 2009a; Byram et al., 2015; Gilmore and Mbow, 1998; Pal et al., 2008)
RevB	cp9	Binds fibronectin	Not determined	(Brissette et al., 2009a; Pal et al., 2008)

2.2.5 Genetic manipulations

The deletion and insertion of specific genes or genetic segments in living cells or organisms have enabled the study of the role and function of specific molecules in different experimental settings. The unusual segmented genome and challenging *in vitro* growth requirements, among others, make *Borrelia* difficult to transform. Despite all these limiting factors, a variety of genetically manipulated *Borrelia* strains are described.

A complex liquid culture medium is required for cultivation of *Borrelia in vitro* due to the incapability of the bacterium to synthesize essential macromolecules, *e.g.* amino acids, nucleotides, and fatty acids for its growth (Fraser et al., 1997). Over the years, several modifications have been made in the original culture medium formulated by Kelly for cultivation of *B. hermsii*, a relapsing fever *Borrelia* species (Kelly, 1971). Three versions of this culture medium, Barbour-Stone-Kelly medium II (BSK II) (Barbour, 1984), BSK-H (Pollack et al., 1993) and a modified Kelly-Pettenkofer medium (MKP) (Preac-Mursic et al., 1986) are currently used to cultivate *Bb*sl strains. *Borrelia* cultures are incubated in microaerophilic conditions. The average generation time of the bacteria is 12 hours (Barbour and Hayes, 1986).

Electroporation is a method of choice when transforming *Borrelia*. The first description of genetic manipulation of *Borrelia* is by Samuels and co-workers where they introduce coumermycin resistance gene, *gyrB*, into a high passage non-infectious *Bb*ss B31 strain by electroporation (Samuels et al., 1994). Due to the low transformation efficiency and long generation time, it takes 3 to 6 weeks for the bacteria to fully recover after the electrotransformation.

The first described shuttle vector, which could autonomously replicate in *Borrelia*, was derived from *Lactococcus lactis*, but it was unstable in *Borrelia* (Sartakova et al., 2000). Since then, more stable shuttle vectors have been developed by combining replication regions from *E. coli* plasmids and sequences from endogenous linear and circular *Bb*ss plasmids (Byram et al., 2004; Eggers et al., 2002; Stewart et al., 2003; Stewart et al., 2001).

Borrelia has complex mechanisms to eliminate exogenous DNA, and genes encoding putative restriction enzymes are carried in lp25 and lp56 (Lawrenz et al., 2002). The first transformations were performed on high-passage, non-infectious, laboratory strains, which were lacking several circular and linear plasmids. Gain-of-function mutant derivatives of these laboratory *Borrelia* strains have made it possible to study the specific roles of individual proteins on the bacterial surface (Fischer et al., 2003). Further, infective genetically manipulated *Borrelia* strains have also been successfully developed, which has made it possible to study the role of borrelial adhesins and other proteins in a murine LB model. One approach has been to inactivate the putative restriction enzyme gene, *bbe02*, in lp25 in a *Bb*ss strain lacking lp56 (Kawabata et al., 2004). Resulting clones are transformable by shuttle vectors and infectious in ticks and mice (Jacobs et al., 2006).

Another approach is to complement a *Bb*ss strain lacking lp25 and lp56 with shuttle vector carrying *bbe22* (*pncA*) gene of lp25 (Purser et al., 2003; Xu et al., 2005). The lp25 and lp56 deficient strain is highly transformable, and the complementation

with the *bbe22* gene restores the infectivity of the bacteria in mice. Most of the genetic studies have been performed using the *Bb*ss B31 strain; however, genetic manipulations are reported to be successfully utilized at least into four other *Bb*ss strains, one *Bg* and one *Ba* strain (Eggers et al., 2001; Fingerle et al., 2007; Hübner et al., 2001; Samuels and Garon, 1997; Siegel et al., 2008; Stewart et al., 2001).

Homologous recombination is inefficient in *Borrelia*, and the construction of infective knock-out *Borrelia* strains is laborious and time consuming. A new efficient transformation technology using the Clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR associated protein 9 (Cas9) -system (Peters et al., 2015) could be a potential for *Borrelia* transformations. CRISPR/Cas9 machinery is present in approximately 40 % of bacteria. However, it remains to be studied whether it is found in spirochetes and can that methodology be utilized in *Borrelia* mutagenesis.

2.3 Decorin binding proteins A and B

2.3.1 Structure

Borrelia has two decorin binding proteins, DbpA and DbpB, which are localized on the outer membrane of the bacteria (Guo et al., 1998; Guo et al., 1995). Dbps are approximately 20 kD in size, but their size and amino acid sequence vary among the *Bb*sl strains. The amino acid sequences of DbpA are approximately 60 % similar in the *Bb*ss and *Bg* strains, while the DbpA sequences of *Ba* strains share 40-50 % similarity when compared with the *Bb*ss and *Bg* strains (Heikkilä et al., 2002b). On the other hand, the amino acid sequences of DbpA within each genospecies are 67-100 % identical (Heikkilä et al., 2002b). The *dbpB* sequence is more conserved than the *dbpA* (Roberts et al., 1998). The amino acid sequences of DbpB are 60-70 % identical among the three genospecies, and the intraspecies similarity is between 99-100 % (Heikkilä et al., 2002a).

The three dimensional structures of DbpA and B of *Bb*ss are solved (Feng and Wang, 2015; Fortune et al., 2014; Wang, 2012). The structures of DbpA and B are highly similar. Both of the adhesins function as monomers and consist of five α-helixes. Four of the α-helixes form a hydrophobic core, while the fifth helix is packed against helixes 2, 3 and 4 (Fortune et al., 2014). In DbpB, the fifth α-helix is shorter leaving the unstructured C-terminal end longer than in DbpA (Feng and Wang, 2015). Three lysine residues, K82, K162 and K170, are shown to be critical in the decorin binding of DbpA (Brown et al., 1999), and the synthetic EAKVRA-peptide containing one of the critical lysines (K82) is able to inhibit the DbpA-decorin interaction (Pikas et al., 2003). The structural analyses of DbpA and B confirmed these findings and reveal a positively charged cleft formed around these three lysines, which are responsible for the binding of the GAG side chain of decorin.

In addition to these three lysines, DbpA of *Bb*ss has another epitope responsible for the GAG binding (Benoit et al., 2011). In the C-terminus of the adhesin, two lysines, K187 and K189, appear to facilitate the GAG binding, especially for small

molecular weight heparin (Morgan and Wang, 2013). However, the additional GAG-binding motif is not present in all the *Bb*sl strains.

2.3.2 Expression

The *dbpA* and *dbpB* genes are encoded within one operon in the linear plasmid 54 (Hagman et al., 1998). The expression of the *dbpAB* operon is regulated by RpoS and RpoN sigma factors (Hübner et al., 2001), and a schematic presentation of the expression of DbpA and B adhesins is presented in Figure 4. The RpoS and RpoN sigma factors are typically associated with general stress responses in bacteria, *e.g.*, changes in temperature, availability of nutrients, or changes in the pH. The consumed blood meal of the tick activates the RpoN-pathway in the bacteria, which in turn upregulates the expression of RpoS. In addition to the RpoN-pathway, the expression of RpoS is regulated by another pathway, which involves the RpoD sigma factor and sRNA called $DsrA_{Bb}$ (Samuels, 2011). RpoD induces a longer form of the RpoS mRNA than the RpoN-pathway. $DsrA_{Bb}$, the expression of which is elevated by an increased temperature in *Borrelia*, post-transciptionally regulates the RpoS expression in low bacteria density. However, the molecular mechanisms in these regulatory pathways are still not completely understood. The expression of Dbps, similar to other outer surface proteins like OspC, is upregulated by RpoS.

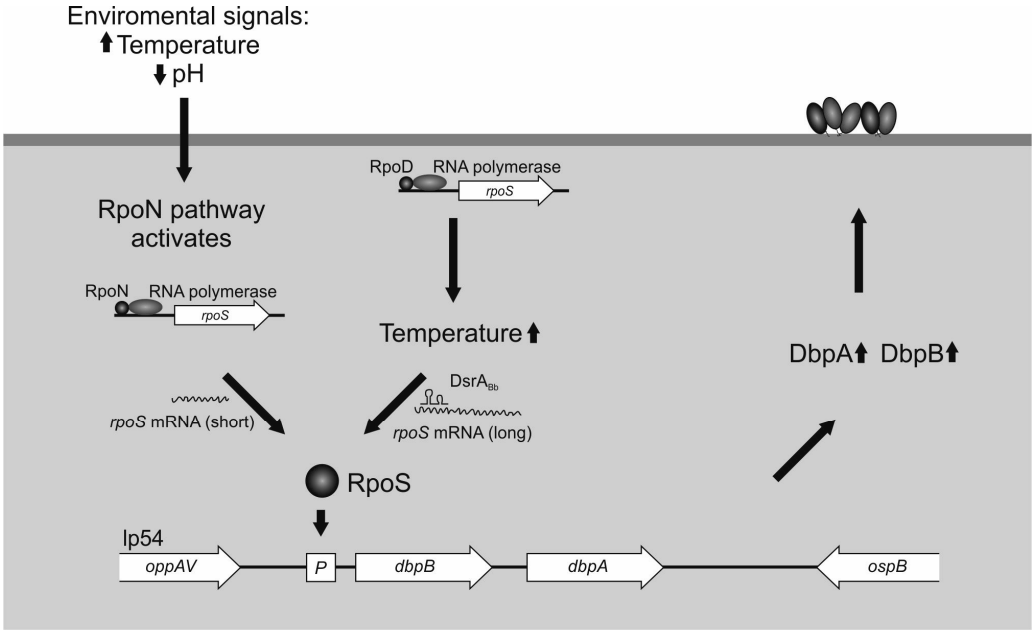


Figure 4. The expression of DbpA and B adhesins of *Borrelia*. Environmental signals, e.g. increased temperature and decreased pH, upregulates the expression of the RpoN sigma factor, which upregulates the expression of another sigma factor, RpoS. Alternatively, the expression of RpoS is regulated by the RpoD sigma factor and $DsrA_{Bb}$.The expression of DbpA and B is upregulated by RpoS. The *dbpA* and *dbpB* genes are located in the linear plasmid 54 (lp54), and their transcription is regulated by a single promotor (P). Modified from (Samuels, 2011)

2.3.3 The role of DbpA and B in the pathogenesis of Lyme borreliosis

The role of Dbps in the pathogenesis of LB is studied widely in mouse models. Decorin deficient mice are resistant to *Bb*ss infection when using a small inoculum (10^2 bacteria via needle inoculation or ≤ 2 infected ticks per mouse) (Brown et al., 2001). The dissemination and joint colonization of *Borrelia* is decreased in decorin deficient mice compared with the wild type mice. In the chronic stage of the disease, *dbpA* expression of the bacteria is increased, and the *Borrelia* is protected better from humoral immunity in the decorin-rich tissues, *i.e.*, the joints and skin, than it is in the heart and bladder (Liang et al., 2004).

Various *dbpA* and/or *dbpB* deficient infective *Borrelia* strains have been constructed (Blevins et al., 2008; Fortune et al., 2014; Lin et al., 2014a; Shi et al., 2008a; Shi et al., 2006; Shi et al., 2008b; Weening et al., 2008; Xu et al., 2007). The *dbpAB* operon appears not to be essential for *Bb*ss to infect mice (Blevins et al., 2008; Shi et al., 2006; Weening et al., 2008). *Borrelia* strains that are lacking either DbpA or DbpB or both of the adhesins are able to infect mice. However, 130-58 000 fold bigger infective doses (ID_{50} values) are reported to be required when the mice are infected with a *dbpA*, *dbpB* or *dbpAB* deficient strain compared with the *dbpAB* expressing strain (Blevins et al., 2008; Shi et al., 2008a; Weening et al., 2008). Additionally, a *dbpAB* knock-out *Borrelia* strain is able to colonize the tick midgut and infect naïve mice (Blevins et al., 2008).

DbpA and B adhesins have a role to play in the early dissemination of *Borrelia*. The dissemination of the *dbpAB* deficient bacteria to the distant organs, especially to the heart and joints, is delayed (Imai et al., 2013; Shi et al., 2008a; Weening et al., 2008). The complementation of the knock-out strain with a *dbpA* or *dbpB* gene does not restore the infectivity of the bacteria to the same level as with the *dbpAB* complemented strain, and the dissemination to the heart is severely impaired with the strain expressing only DbpA or DbpB adhesin (Shi et al., 2008a). The joint tissues were effectively colonized, but they harbored a lower amount of bacteria than the controls joints. Furthermore, the overexpression of DbpA or B in the *dbpAB* deficient bacteria does not compensate for the missing adhesin, and dissemination remains impaired when compared with the DbpA and B expressing strain (Shi et al., 2008b). However, differences in the bacterial load in the heart and joint tissues between *dbpAB* deficient and *dbpAB* expressing strains are abolished in the chronic stage of the infection (Imai et al., 2013).

The role of critical decorin/GAG binding structures in DbpA has been studied in the LB mouse model (Fortune et al., 2014; Lin et al., 2014a). Mutating one of the three critical lysines in DbpA to alanine in the genetically manipulated *Bb*ss strain expressing only DbpA (not DbpB) and impaired the infectivity of the bacteria to the same level as it was with the *dbpAB* deficient strain (Fortune et al., 2014). Moreover, the deletion of 11 C-terminal amino acids of DbpA of *Ba* VS461 in the *Bb*ss background strain significantly reduced the bacterial load in the skin, ear, and bladder samples (Lin et al., 2014a).

Taken together, the DbpA and B adhesins are essential in the overall pathogenicity of the *Borrelia* even though redundant binding activities are shown for other borrelial adhesins. The deletion of either one or both of the adhesins or inactivating the decorin/GAG binding activity in the adhesin severely attenuates the infectivity of the bacteria. The role of Dbp adhesins in the pathogenesis of LB is summarized in the Figure 5.

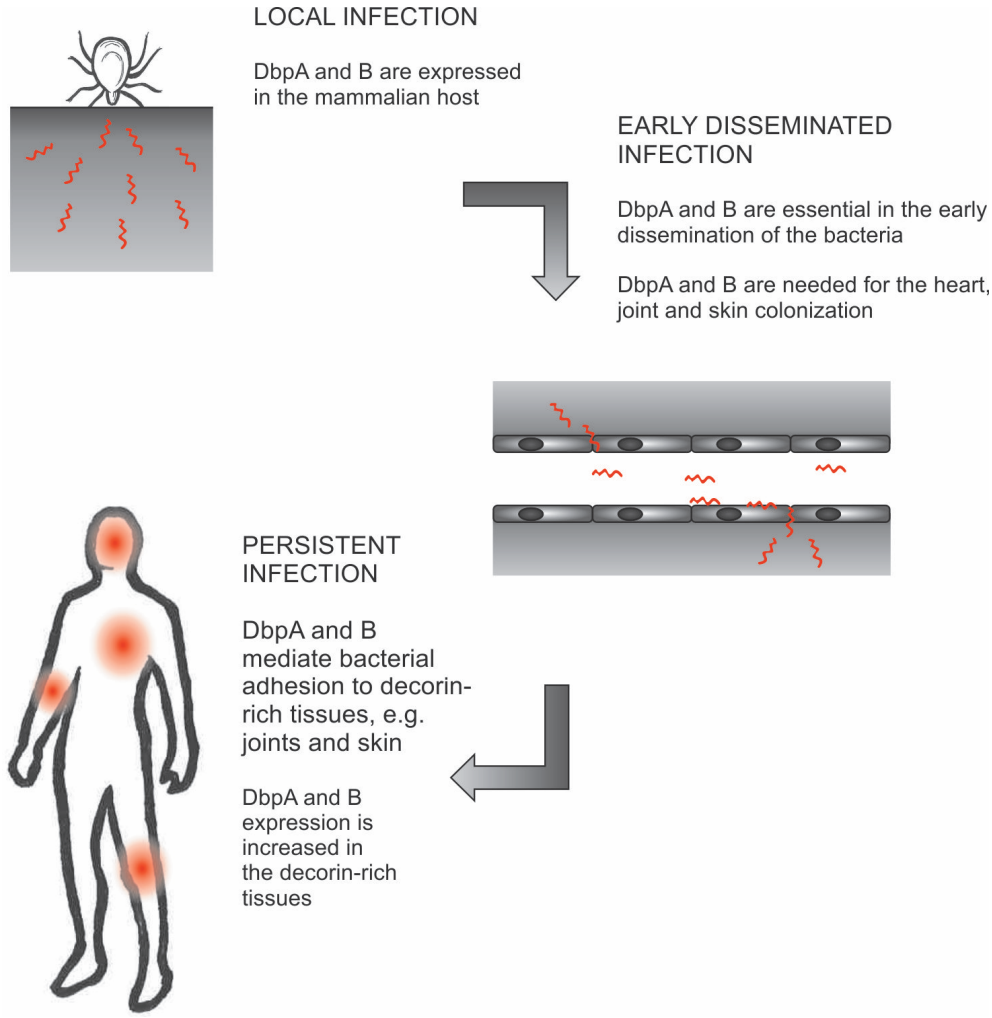


Figure 5. The role of DbpA and B adhesins in the pathogenesis of Lyme borreliosis. The putative steps where DbpA and B contribute to the pathogenesis of LB are based on mouse studies. Modified from (Coburn et al., 2013)

2.4 Proteoglycans in the host tissues

2.4.1 Extracellular matrix

The extracellular matrix (ECM) is composed of fiber-forming and interfibrillar macromolecules that include collagens, elastin, microfibrillar proteins, proteoglycans, and glycoproteins (Järveläinen et al., 2009). In addition to providing mechanical strength to tissues and organs, ECM is a dynamic structure that influences the behavior of cells (Daley et al., 2008). Minor changes in the ECM structure or its components can lead to altered physiological properties of a tissue and, eventually, to development of a disease.

Proteoglycans consist of a core protein and a glycosaminoglycan (GAG) side chain(s). The number and size of the side chains and the chemical structure of the GAGs vary greatly among the proteoglycans. Based on the cellular/subcellular location, gene/protein homology and the presence of specific features in the protein core, these macromolecules are divided into four major classes: Intracellular, pericellular, cell-surface, and extracellular proteoglycans (Iozzo and Schaefer, 2015). Furthermore, extracellular proteoglycans are divided into three subclasses, namely, small leucine-rich proteoglycans (SLRPs), hyalectans and the testican/SPOCK family, all of which have distinct functions in the ECM.

SLRPs are the largest family of proteoglycans. They are characterized by a relatively small core protein that varies between 36 and 42 kDa in size and contains leucine-rich repeats (Iozzo and Schaefer, 2015; Schaefer and Iozzo, 2008). The covalently attached GAG side chains of the SLRPs are composed of chondroitin sulphate, dermatan sulphate, or keratin sulphate. SLRPs are the structural components of various tissues, *e.g.*, articular cartilage, the vascular wall, skin, and cornea, and they are involved in the complex processes in development and homeostasis, for example in fibrillogenesis, angiogenesis, and inflammation (Järveläinen et al., 2009). There are 18 different SLRPs, of which decorin and biglycan are the two most studied.

2.4.2 Vascular endothelium

The endothelial cells in the blood vasculature are covered by glycocalyx, a jelly-like layer that consists of proteoglycans, GAGs, glycoproteins and absorbed plasma proteins (Masola et al., 2014). The negatively charged glycocalyx is a dynamic structure, the thickness and composition of which is influenced by the underneath endothelial cells as well as the shear stress caused by the circulating blood. The expression of proteoglycans and glycoproteins varies among different endothelial cell lines. Additionally, the shedding of the proteoglycans and glycoproteins as well the degree of GAG depolymerization changes in different vascular beds.

2.4.3 Decorin

Decorin consists of an approximately 36 kD core protein and a single GAG chain that can either be dermatan sulphate or chondroitin sulphate (Brennan et al., 1984; Krusius and Ruoslahti, 1986). The GAG side chain is attached covalently to the first serine (S4) in the N-terminus of the mature core protein (Krusius and Ruoslahti, 1986), and the proportion of dermatan and chondroitin sulphate residues in the GAG chain varies among the decorin expressing tissues (Brennan et al., 1984). The crystal structure of the core protein reveals that decorin contains 12 leucine-rich repeats and the protein is folded to a more open conformation than it was previously predicted (Scott et al., 2004). In the leucine-rich repeat domain, there are three possible sites for N-linked oligosaccharides that are thought to prevent self-aggregation and promote interactions between decorin and other ECM molecules (Iozzo, 1997). The schematic structure of decorin is presented in Figure 6.

In humans, the gene that encodes decorin is located on chromosome 12 (Danielson et al., 1993; Vetter et al., 1993). Decorin is expressed widely in the connective tissues, especially in the skin and joints. With its core protein, decorin binds to Type I and II collagen (Fleischmajer et al., 1991), and is a structural component of the skin and vascular wall (Reed and Iozzo, 2002; Williams, 2001). In addition to providing mechanical strength to tissues, decorin is also involved in fibrillogenesis (Zhang et al., 2006) and angiogenesis (Järveläinen et al., 2006; Järveläinen et al., 1992), and acts as a signal molecule in various biological processes, such as cell differentiation, proliferation, migration, and apoptosis (Moreth et al., 2012). Moreover, decorin is shown to be released from the ECM to act as a damage-associated molecular pattern (DAMP) upon tissue stress or injury (Moreth et al., 2012). Decorin acts as a pro-inflammatory signal molecule, and it interacts with the transforming growth factor β1, and Toll-like receptors (TLR) 2 and 4. Thus, decorin acts as a crucial regulator of inflammatory responses.

2.4.4 Biglycan

Along with decorin and asporin biglycan belongs to class I in the SLRP family (Iozzo and Schaefer, 2015). The crystal structures of biglycan and decorin are highly similar (Scott et al., 2006), while their amino acid sequences are only 57 % similar (Iozzo, 1997). Contrary to decorin, biglycan contains two GAG side chains in the N-terminus of the core protein (Fisher et al., 1989) (see Figure 6). However, in both of the proteoglycans, the GAGs are composed of dermatan and chondroitin sulphate.

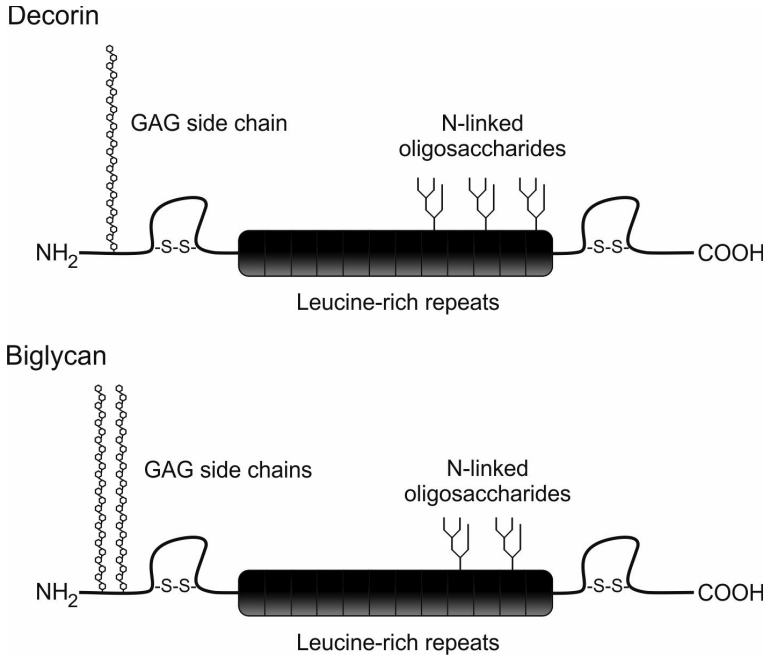


Figure 6. The schematic structure of decorin and biglycan. Both of the proteoglycans consist of core protein of which the N-terminal end is covalently attached to one or two glycosaminoglycan (GAG) side chains. The GAG side chains are composed of chondroitin and dermatan sulphate disaccharide subunits. The core proteins contain 12 leucine-rich repeats, and two or three possible sites for N-linked oligosaccharides. The disulphide bonds (S-S) on both sides of the leucine-rich repeats stabilize the conformation of the core protein. Modified from (Edwards, 2012)

The biglycan gene is located in the X-chromosome (Fisher et al., 1991). Despite the similar structures, the expression and functions vary significantly between biglycan and decorin. Biglycan is expressed in bone and vascular endothelium (Bianco et al., 1990) and it regulates postnatal skeletal growth (Xu et al., 1998). Additionally, biglycan is produced and secreted by circulating macrophages (Schaefer et al., 2005), and it acts as DAMP in sterile inflammation (Moreth et al., 2012). Soluble biglycan is shown to interact with TLR2 and 4 in pathogen-associated and sterile inflammation, which in turn stimulates the expression of various chemokines and cytokines, *e.g.*, TNF-alpha and CXCL13. Furthermore, biglycan interacts with the purinenergic $P2X_7$/$P2X_4$ receptors. The multi-receptor crosstalk by biglycan signaling provides an important link between the innate and the adaptive immunity.

2.5 Animal models for Lyme borreliosis

2.5.1 General

Various animal models have been developed to study Lyme borreliosis. In general, many animal species are susceptible to *Borrelia* infection, but they are also resistant to the symptoms seen in the human disease. However, young animals with

immature immune responses, as well as some mutated strains, do develop the disease and can be used as animal models to study various symptoms of LB. (Barthold et al., 2010a)

The *Bb*ss infected laboratory mouse (*Mus musculus*) is the most studied animal model for LB. The murine model for LB is discussed more closely in the next chapter. Other rodents susceptible to *Borrelia* infection include laboratory rats (*Rattus norvegicus*) (Barthold et al., 1988), hispid cotton rats (*Sigmodon hispidus*) (Burgdorfer and Gage, 1987), Mongolian gerbils (*Meriones unguiculatus*) (Preac Mursic et al., 1990), white-footed mice (*Peromyscus leucopus*) (Piesman et al., 1987), grass-hopper mice (*Onychomys leucogaster*) (Czub et al., 1992), guinea pigs (*Cavia porcellus*) (Krinsky et al., 1982), and Syrian hamsters (*Mesocricetus auratus*) (Piesman et al., 1987).

Laboratory rabbits (*Oryctolagus cuniculus*) are also susceptible to *Borrelia* infection. Rabbits develop erythema migrans similar to that seen in human patients (Foley et al., 1995; Krinsky et al., 1982), and they have been used in several vaccine studies (Exner et al., 2000; Schutzer and Luan, 2003; Shang et al., 2000). New Zealand White (NZW) rabbits develop EM lesions within three to eight days after needle inoculation or a tick challenge (Foley et al., 1995). In addition to skin manifestations, rabbits develop a systemic infection and *Borrelia* can thus be cultured from multiple tissues, *e.g.*, the skin, joints, and spinal cord. However, the NZW rabbits clear the infection spontaneously by 12 weeks post-inoculation. Most rabbits seem to have an effective and unique sterilizing immune response to *Borrelia*, the understanding of which can offer new insights into the pathogenesis of LB.

Larger LB animal models include dogs and non-human primates. In endemic areas, many dogs are naturally infected by *Borrelia* and are seropositive, but they do not show clinical signs of the disease (Levy and Magnarelli, 1992). However, some dogs develop a disseminated infection that resembles the human LB or even a fatal kidney disease (Littman et al., 2006). Lameness is the most prevalent symptom in canine LB, and it is usually transient, although it can occur in multiple episodes. The canine model is mainly used to study the pathogenesis of Lyme arthritis or used in vaccine studies. Of the non-human primates, rhesus macaques (*Macaca mulatta*) are studied extensively for the pathogenesis of LB, especially neuroborreliosis (Pachner et al., 1995; Philipp et al., 1993; Roberts et al., 1995). Rhesus macaques develop a multiorgan *Borrelia* infection that is similar to the human disease. Signs and symptoms of local and early disseminated infection, *i.e.*, primary and secondary EM, splenomegaly, fever, conjunctivitis and CSF pleocytosis, are seen in *Bb*ss infected macaques. Additionally, arthritis, carditis, and peripheral and central nervous system inflammations are described in this model. Apart from humans and macaques, the signs of neuroborreliosis have been seen in horses (Imai et al., 2011).

2.5.2 Murine models for Lyme borreliosis

Lyme borreliosis is widely studied in mouse models, and indeed, various inbred and outbred mouse strains are susceptible to *Borrelia* infection. The disease outcome is influenced by the age and genotype of the infected mouse, the route and site of inoculation, as well as the used *Borrelia* strain and the infective dose (Barthold et al., 1990; de Souza et al., 1993). Arthritis is the most commonly studied manifestation in the murine model. However, *Borrelia* infected mice can develop carditis, vasculitis, myositis and peripheral neuritis, as well as persistent infection. On the other hand, EM and neuroborreliosis are not manifested in the LB mouse model.

C3H/He mice are widely used in experimental *Borrelia* infections. C3H/He mice develop severe polyarthritis that affects multiple joints when the mice are infected with *Bb*ss at three weeks of age (Barthold et al., 1990). The joint swelling is most obvious at two to three weeks of infection, especially in the tibiotarsal joints. In addition to joint manifestations, *Bb*ss infected C3H/He mice develop carditis, vasculitis, and a high *Borrelia* specific serum IgG antibody response. Older C3H/He mice, infected at the age of 12 weeks, are susceptible to LB and develop carditis, but the severity and extensity of the arthritis are decreased when compared to younger animals (Barthold et al., 1993). BALB/c mice are regarded as a LA resistant genotype (Barthold, 1991). Nevertheless, BALB/c mice are susceptible to *Borrelia*, and they develop carditis and chronic infection, but none or only mild joint manifestations can be detected.

Various strains of *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. bavariensis*, *B. japonica*, *B. bissettii*, *B. spielmanii* and *B. lusitanitae* can infect laboratory mice (Anderson et al., 1990; Kaneda et al., 1998; Masuzawa et al., 1994; Pachner et al., 2004; Tuomi et al., 2002; Yrjänäinen et al., 2006; Zeidner et al., 2001). However, most of the experimental infections are done using different *Bb*ss strains. *Borrelia* easily loses its plasmids during *in vitro* cultivation, which affects the infectivity of the bacteria. Previously, low-passage wild type *Borrelia* strains were used in experimental infections, but new knowledge and advanced methods have enabled the construction and use of genetically manipulated *Borrelia* strains in various animal models.

The site of inoculation influences the disease outcome. The direct inoculation of *Borrelia* to the footpad of a mouse has been used to induce a local infection (Christopherson et al., 2003; Masuzawa et al., 1994). Severe arthritis in the rear legs and mild cardiac symptoms are predominant after subcutaneous inoculation at the base of the tail (Keane-Myers et al., 1996), while inoculation in the dorsal thoracic region favors development of carditis (Motameni et al., 2005). In needle inoculations, the lowest infective dose is achieved by using a intradermal route (Barthold, 1991). In general, the use of infected ticks induces the most natural course of infection.

2.5.3 Antibiotic treatment of experimental Lyme borreliosis

The effectiveness of antimicrobial agents is studied widely in the murine model of LB. Ceftriaxone, penicillin G, amoxicillin-clavulanic acid, vancomycin, evermicin and tigecycline are able to eradicate cultivable *Borrelia* from the tissue samples of infected

mice, whereas chloramphenicol, oxytetracycline, doxycycline, erythromycin, azithromycin, and clarythomycin are ineffective in LB murine model (Barthold et al., 2010b; Kazragis et al., 1996; Konishi and Nakao, 1997; Malawista et al., 1994; Moody et al., 1994; Pavia et al., 2001). In more recent studies, it has been shown that borrelial remnants, *e.g.* DNA, antigens or RNA, persist in mice for months after the antibiotic treatment (Bockenstedt et al., 2012; Bockenstedt et al., 2002; Hodzic et al., 2008; Hodzic et al., 2014; Yrjänäinen et al., 2010). Further, cultivable *Borrelia* are detected after ceftriaxone treatment when the mice were given immunosuppressant treatment using anti-TNF-alpha simultaneously or four weeks after the antibiotic treatment (Yrjänäinen et al., 2007). In addition to mice, *Bb*ss DNA, RNA, and antigens were detected in tissue samples of *Borrelia* infected rhesus macaques after antibiotic therapy (Embers et al., 2012). The interpretations of these findings have raised an intense debate in the scientific community.

3 AIMS OF THE STUDY

The purposes of the present study were:

- To characterize and compare the binding of DbpA and B of *Bb*ss, *Bg* and *Ba* binding to decorin and another class I SLRP family proteoglycan, biglycan,
- To evaluate the role of DbpA and B of *Bb*ss, *Bg* and *Ba* in bacterial adhesion to proteoglycan expressing cells,
- To study the role of DbpA and B adhesins of *Bb*ss in bacterial dissemination, in the development of joint manifestations and in the post-treatment persistence in mice.

4 MATERIALS AND METHODS

4.1 Bacteria

4.1.1 *Borrelia burgdorferi* sensu lato

In this thesis low passage wild type *Bb*ss (strains N40, B31, HB-19, and SH-2-82), *Bg* (strains SBK40, SBK46, Å218, and 387), *Ba* (strains A91, 570, bo23, and pKo), and high passage *Bb*ss B313 and *Ba* 1082 were used. All *Bg* and *Ba* strains were isolated from skin biopsy samples taken from Finnish patients except for *Bg* Å218 which is a tick-isolate and *Ba* pKo which was kindly provided by Volker Fingerle (National Reference Centre for *Borrelia*, Bavarian Food and Health Safety Authority, Munich, Germany). *Bb*ss wild type strains were kindly provided by Sven Bergström (University of Umeå, Sweden), and B313 was supplied by Thomas Kamradt (Deutches Rheuma-Forschungszentrum, Berlin, Germany). The spirochetes were cultivated in Barbour-Stoenner-Kelly II (BSK II) medium (Barbour, 1984) at 33 °C and passaged once a week. All used bacteria strains are listed in Table 2.

Table 2. Bacteria strains

***Borrelia burgdorferi* sensu lato strains**	
Borrelia afzelii A91	*Ba* A91
Borrelia afzelii 570	*Ba* 570
Borrelia afzelii bo23	*Ba* bo23
Borrelia afzelii pKo	*Ba* pKo
Borrelia afzelii 1082	*Ba* 1082
Borrelia burgdorferi sensu stricto N40	*Bb*ss N40
Borrelia burgdorferi sensu stricto B31	*Bb*ss B31
Borrelia burgdorferi sensu stricto B313	*Bb*ss B313
Borrelia burgdorferi sensu stricto HB-19	*Bb*ss HB-19
Borrelia burgdorferi sensu stricto SH-2-82	*Bb*ss SH-2-82
Borrelia garinii SBK40	*Bg* SBK40
Borrelia garinii SBK46	*Bg* SBK46
Borrelia garinii Å218	*Bg* Å218
Borrelia garinii 387	*Bg* 387
***Bb*ss B313 derivative strains**	
Borrelia burgdorferi sensu stricto B313 + pBSV2	B313/*dbpAB*/pBSV2
Borrelia burgdorferi sensu stricto B313 + pBSV2/*dbpAB*/*B. afzelii* A91	B313/*dbpAB*/*Ba*
Borrelia burgdorferi sensu stricto B313 + pBSV2/*dbpAB*/*B. burgdorferi* s.s. N40	B313/*dbpAB*/*Bbss*
Borrelia burgdorferi sensu stricto B313 + pBSV2/*dbpAB*/*B. garinii* SBK40	B313/*dbpAB*/*Bg*
Genetically manipulated *Bb*ss B31 strains	
Borrelia burgdorferi sensu stricto B31 5A13/Δ*dbpAB*/E22/1	*ΔdbpAB*
Borrelia burgdorferi sensu stricto B31 5A13/Δ*dbpAB*/*dbpAB*/2	*ΔdbpAB/dbpAB*
Borrelia burgdorferi sensu stricto B31 5A13/Δ*dbpAB*/*dbpA*/1	*ΔdbpAB/dbpA*
Borrelia burgdorferi sensu stricto B31 5A13/Δ*dbpAB*/*dbpB*/1	*ΔdbpAB/dbpB*

***Escherichia coli* strains**
Escherichia coli INFαF
Escherichia coli Novablue Giga Singles
Escherichia coli M15
Escherichia coli BL21(DE3)pLysS
Escherichia coli DH5α

4.1.2 Generation and characterization of *Bb*ss B313 derivative strains

*Bb*ss B313 is a high passage laboratory strain that is missing several plasmids including lp36 and lp54 (Sadziene et al., 1993) which carry a wide variety of outer surface protein encoding genes, *e.g., dbpAB* (Fraser et al., 1997). B313 derivative strains expressing DbpA and B of *Bbss*, *Bg* and *Ba* were constructed by PCR amplifying *dbpAB* operons containing the promoter sequence from the genomic DNA of *Bbss* N40, *Bg* SBK40 and *Ba* A91 using the specific primers listed in Table 3. Purified PCR products were ligated into the pBSV2 shuttle vector (a generous gift from Patricia Rosa, The National Institute of Health, MT, USA) and transformed into *E. coli* DH5α strain to get an adequate plasmid yield. The constructed pBSV2 plasmids were purified from the *E. coli* using the Plasmid midi kit (Qiagen) according to manufactorer's instructions. 30 ng of the purified plasmid was precipitated with 0.1 M sodium acetate (pH 5.2) and 67 % EtOH at -20 °C for 2 hours, washed twice with 70 % EtOH and air-dried. Before the transformation into B313 the dry plasmid pellet was dissolved in 5 µl of H_2O.

Table 3. Primers used in the different PCR applications.

Purpose	Primer	Vector	Sequence (5'-3')
Construction of B313/*dbpAB*/*Bb*ss	*dbpAB/Bbss* FW *dbpAB/Bbss* REV	pBSV2	AATA **GCA TGC** ACA AGC CAG ATT GCA TAG C AATA **GGA TCC** TTG ATT ATC GGG CGA AGA G
Construction of B313/*dbpAB*/*Bg*	*dbpAB/Bg* FW *dbpAB/Bg* REV	pBSV2	AATA **GCA TGC** ACA TTA TTT GGC AAA CTG GC AATA **GGA TCC** GGT ACT TTA CGA CAG TCT TG
Construction of B313/*dbpAB*/*Ba*	*dbpAB/Ba* FW *dbpAB/Ba* REV	pBSV2	AATA **GCA TGC** CCC CTG GCA AAA TAA AAT TC AATA **GGA TCC** TTA TTT TTG ATT TTT AGT TTG TTT TTC
Expression of recombinant DbpA	*dbpA/Bbss* FW *dbpA/Bbss* REV	pET-30	**GAC GAC GAC AAG** ATG AAT AAA TAT CAA AAA ACT TTC A **GAG GAG AAG CCC GGT** TTA GTT ATT TTT GCA TTT TTC ATC
Expression of recombinant DbpB	*dbpB/Bbss* FW *dbpB/Bbss* REV	pET-30	**GAC GAC GAC AAG** ATG GTC TTG TTT TTT GAT CTA TT **GAG GAG AAG CCC GGT** TTA TTT CTT TTT TTT GCT TTT ATT AT
Nested PCR	*flaB* OUTER FW *flaB* OUTER REV *flaB* INNER REV *flaB* INNER FW		CACACCAGCATCACTTTCAGGGTCTC CAACCTCATCTGTCATTGTAGCATCTTTTATTT GCATTTTCAATTTTAGCAAGTGATG TTTCAGGGTCTCAGGCGTCTT
RT-PCR	*ospA* FW *ospA* REV *ospA* PROBE		AATATTTATTGGGAATAGGTCTAA CACCAGGCAAATCTACTGA 6FAM-TTAATAGCATGTAAGCAAAATGTTAGCA-XT-PH

Note: Restriction enzyme sites in the primers are bold type.

B313 bacteria were cultured to a final concentration of 5×10^7 to 10^8/ml, washed twice with an ice cold electroporation solution (EPS; 0.27 M sucrose, 15 % (v/v) glycerol), and re-suspended in EPS to a final concentration of 10^9 *Borrelia* /ml. 30 µg of plasmid DNA were electroporated (2.5 kV, 25 µF, 200 Ω) into 50 µl of electrocompetent B313. The bacteria were allowed to recover at 33°C for 24 h before kanamycin (Sigma-Aldrich, St. Louis, MO, USA) was added (final concentration 200 µg/ml) and the suspension was aliquoted into 96-well plates. The plates were incubated in a CO_2 incubator at 35°C for 2-3 weeks. The resulting genetically manipulated *Borrelia* strains containing *dbpAB* operon of the three different genospecies were named B313/*dbpAB/Bbss*, B313/*dbpAB/Bg* and B313/*dbpAB/Ba*, and the control strain containing only pBSV2 was named B313/pBSV2.

The expression of DbpA and B was studied in Western blot assay. Five million bacteria were lysed in a Nupage sample buffer (Invitrogen, Carlsbad, CA, USA), and the proteins were separated in 10 % SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose membranes (Optitran Ba-S 83, Schleicher & Schuell, Dassel, Germany). The membranes were blocked with 5% non-fat milk in phosphate buffer saline (PBS) and probed with polyclonal anti-Dbp rabbit serum and an anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-flagellin B (FlaB) antibody (H9724; a gift from S. Bergström) with anti-mouse secondary antibody (Santa Cruz Biotechnology) was used as a control. ECL chemiluminescent reagents (GE healthcare, Little Chalfont, UK) were used in the detection. The results were imaged using a AGFA Curix60 developing machine and autoradiography films (Santa Cruz Biotechnology).

Surface localization of the adhesins was demonstrated with proteinase K-treatment. The enzyme degrades proteins by cleaving the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups (Ebeling et al., 1974). The bacteria were washed twice with 5 mM $MgCl_2$ PBS and diluted to 10^8 bacteria/ml. Proteinase-K (Promega, Madison, WI, USA) was added to the cell suspension at a final concentration of 100 µg/ml, and the bacteria were incubated at room temperature for 30 min. Enzyme activity was stopped with 5 mM phenylmethanesulphonylfluoride (PMSF), and the cells were washed twice with PBS containing 5 mM $MgCl_2$ before preparing the samples for Western blot analyses as mentioned above.

4.1.3 Genetically manipulated *Bb*ss B31 strains

The genetically manipulated infective *Bb*ss B31 5A13 strains were kindly provided by Fang-Ting Liang (Louisiana State University, Baton Rouge, LA, USA). In this thesis, the *dbpAB* knock out strain, ΔdbpAB/E22/1 (*ΔdbpAB*), the DbpA and B expressing strain, ΔdbpAB/dbpAB/2 (*ΔdbpAB/dbpAB*), the DbpA expressing strain, ΔdbpAB/dbpA/1 (*ΔdbpAB/dbpA*), and the DbpB expressing strain, ΔdbpAB/dbpB/1 (*ΔdbpAB/dbpB*) were used (Shi et al., 2008a). All these strains are identical to each other based on their genetic composition except for their ability to express DbpA and/or DbpB.

4.1.4 *Escherichia coli*

The *E. coli* host cell strains used in cloning and the expression of recombinant proteins were INFαF (Invitrogen, Leek, The Netherlands), Novablue Giga Singles (Novagen, Darmstadt, Germany), M15 (Qiagen, Hilden, Germany), BL21(DE3)pLysS (Novagen), and DH5α. The *E.coli* strains were cultured in a liquid or solid Luria-Bertani medium or after transformation in a liquid super optimal broth with catabolite repression (SOC, Novagen) at 37°C. When culturing the bacteria in the liquid medium rapid shaking (250 rpm) was provided.

4.2 Cell lines

4.2.1 Human foreskin fibroblasts

Human foreskin fibroblasts (HFF) were kindly provided by Tytti Vuorinen (Department of Virology, University of Turku). HFF cells were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen) that contained 7 % fetal calf serum (FCS, Hyclone, UT, USA) and gentamycin (Nalgene, Israel) in plastic culture flasks in a CO_2 incubator at 37 °C, and passaged once a week. Before the cell adhesion studies the cells were passaged to a Lab-TekTM 8 well chamber slide (Nunc, Rockhester, NY, USA), and cultured for two days in a CO_2 incubator at 37 °C.

4.2.2 Human umbilical vein endothelial cells

The human umbilical endothelial cells (HUVEC) were kindly provided by Marko Salmi (Department of Medical Microbiology and Immunology, University of Turku). HUVECs were cultured in Endothelial cell growth medium (Promocell, Heidelberg, Germany) that contained penicillin and streptomycin (Sigma, St Louis, USA) in CO_2 incubator at 37 °C, and passaged twice a week. Before the cell adhesion studies under static conditions, the cells were passaged to Lab-TekTM 8 well chamber slide, and cultured for three days in a CO_2 incubator at 37 °C. HUVECs used in flow experiments were commercial primary cells pooled from multiple donors (Lonza, Basel, Switzerland) and cultured in a EGM™ medium (Lonza, Basel, Switzerland).

4.3 Recombinant decorin binding proteins

E. coli BL21(DE3)pLysS cell lines expressing DbpA and B of *Bbss* N40 were constructed with a pET-30 Ek/LIC vector system (Novagen) according to manufacturer's instructions and specifically designed primers (see Table 2). The cell lines expressing DbpA and B of *Bg* 40 and *Ba* A91 strains were kindly provided by Pekka Lahdenne (University of Helsinki). These strains were constructed using a pQE-30 vector system in *E. coli* M15 host cells (Invitrogen).

The proteins were expressed by culturing *E. coli* in the liquid Luria Bertani medium. Overnight cultures of the bacteria were diluted 1:50 in LB media and grown in an orbital shaker (250 rpm) at 37 °C until OD_{600} was between 0.5 and 1.0. IPTG (Sigma) was added to the cultures (final concentration 1 mM), and the bacteria were grown for 3 hours before they were harvested by centrifugation (6 000 × g, 15 min, 4 °C).

The proteins were purified under native conditions using Ni-NTA agarose beads according to manufacturer's instructions (Qiagen) and dialyzed against PBS. The protein concentration was determined with a BCA™ protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

4.4 Methods to study the interactions between recombinant proteins and proteoglycans

4.4.1 Western blot analysis with biotinylated proteoglycans

Decorin (from bovine cartilage, Sigma) was biotinylated with EZ-Link™ NHS-LC-Biotin (Pierce) according to manufacturer's instructions. Biotinylated decorin (1 µg/ml) was used as the primary reagent and HRP-conjugated streptavidin (0.1 µg/ml, Pierce) was the secondary reagent in the Western analysis. The assay was otherwise performed as described above.

4.4.2 Microtiter plate assays

Enhanced binding microtiter plates (ThermoFicher Scientific, Vantaa, Finland) were coated with purified recombinant Dbps (10 µg/ml) in PBS and blocked with PBS containing 1% BSA, both for 1h at 37 °C. After the blocking the plates were shaken dry and stored in -70 °C.

The Dbp coated wells were incubated with either biotinylated decorin (1 µg/ml) or biglycan (10 mM) in PBS for 1h at 37 °C, washed three times with PBS-T (PBS containing 0.05% Tween 20) and incubated with 1 µg/ml alkaline phosphatase-conjugated streptavidin (Pierce) in PBS for 1h at 37 °C. After the washings, the alkaline phosphatase was allowed to react with the p-NPP-Na2 substrate (1 mg/ml, Reagen, Toivala, Finland) for 25 min before the reaction was stopped with 1M NaOH and the absorbancies (OD_{405}) were measured with a Multiskan EX spectrophotometer (ThermoFisher Scientific). Results are expressed as OD_{405} values subtracted from the background absorbance.

In the inhibition experiments, the inhibitors were pre-incubated on the plates or with biotinylated decorin/biglycan for one hour at 37 °C. The assay was otherwise done as described above. When inhibiting the interaction between DbpB/*Bg* and decorin, LALREAKQAIVETG-peptide (based on the DbpB sequence of *Bg* 40; 10 µg/ml; hereafter EAKQA-peptide; Haartman Institute, Helsinki, Finland), dermatan sulphate (50 µg/ml; from shark cartilage, Sigma), chondroitin-6-sulphate (50 µg/ml; from shark cartilage, Sigma) and chondroitin-4-sulphate (50 µg/ml; from bovine trachea, Sigma) were used as inhibitors, and unlabelled decorin (10 µg/ml) was used as

a positive and BSA fraction V (100 µg/ml; Serological Proteins Inc., Kankakee, IL, USA) as a negative control. The adhesion of DbpB/*Bg* and DbpB/*Bb*ss to biglycan was inhibited by dermatan sulphate (50 µg/ml), chondroitin-4-suphate (50 µg/ml), and chondroitin-6-sulphate (50 µg/ml), while unlabeled biglycan (0.1 µM) and BSA fraction V (1 µM) were used as a positive and a negative control, respectively.

4.4.3 Surface plasmon resonance assay

Surface Plasmon resonance (SPR) assays were done using Biacore X machine (Biacore AB, Uppsala, Sweden) and CM5 chips (GE Healthcare, Uppsala, Sweden). Flow cells of the chips were coated with the recombinant DbpA or B adhesins (50 µg/ml) in the coating buffer (10 mM sodium acetate, pH 4.5 or 5.0) using the amine coupling kit according to manufacturer's instructions. The reference cell was coated with the plain coating buffer. The changes in resonance units (RUs) caused by the binding of decorin or biglycan to Dbps on the chip were measured. Decorin was dissolved in PBS (1mg/ml) and further diluted in a HBS-P running buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 0.005 % (v/v) Surfactant P20), while biglycan was diluted in the HBS-P running buffer. Final decorin concentrations of 10 µg/ml, 50 µg/ml and 100 µg/ml and biglycan concentrations of 0.1 µM, 0.5 µM, and 1 µM were used in these assays. All binding experiments were run at room temperature using a flow rate of 10 µl/min. The background signal from the buffer coated reference cell was subtracted from the Dbp signal. The BIAevaluation program (versions 3.0 and 4.1, Biacore) was utilized for the affinity curve calculations.

4.5 Bacterial adhesion assays

4.5.1 Dot blot adhesion assay

Wild type *Borrelia* strains and B313 were grown to logarithmic growth phase (5-10 × 10^7 bacteria/ml), washed with PBS and counted in a Neubauer counting chamber. The bacteria were resuspended in PBS (final concentration 5 x 10^8/ml), and 1 µl of the bacterial suspension was dotted onto a nitrocellulose membrane (Protran BA 85/20 0.45µm, Schleicher & Schuell). The dots were allowed to dry before the membrane was blocked with 3% non-fat dry milk in TBS-T (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20). The membranes were incubated with biotinylated decorin (1 µg/ml) in TBS-T in orbital shaker for 1h at room temperature. The binding of biotinylated decorin was detected with streptavidin-HRP (0.1 µg/ml) in TBS-T. Detection was done as in the Western blot assay. The intensities of the dots were quantified with a UVP BioSpectrum® AC Imaging System. Value 1 was given to B313 which was used as a negative control.

4.5.2 Cell adhesion assays under static conditions

The HFF or HUVEC **c**ells were fixed with -20 °C acetone for 15 min and washed twice with PBS-T. Wild type or genetically modified B313 strains were stained with

CFSE as described above and re-suspended in 0.25 % BSA/PBS at a concentration of 5 × 10^7/ml. B313 and B313/pBSV2 were used as negative controls. One million CFSE stained bacteria were allowed to adhere to the cells in slide chambers for 1 h at 37 °C. The slides were washed twice with PBS, and the adhered *Borrelia* were visualized with a confocal microscope (Zeiss LSM510 META/Zeiss 780). Images of ten random fields per chamber were analysed with the Image J analysing program and the number of individual bacteria was counted from every field.

Inhibition assays were carried out using B313/*dbpAB/Bg* and B313/*dbpAB/Bb*ss strains. Decorin (100 µg/ml), EAKVQA-peptide (10 mg/ml), dermatan sulphate (50 µg/ml), chondroitin-6-sulphate (50 µg/ml), and chondroitin-4-sulphate (50 µg/ml) were used as inhibitors in the adhesion assay to HFF cells. Biglycan (1 µM), decorin, (1µM), dermatan sulphate (50 µg/ml), and chondroitin-6-sulphate (50 µg/ml) were used as inhibitors in the adhesion assay to HUVECs. BSA (100 µg/ml or 10 µM) was used as a negative control. The inhibitors were pre-incubated with the CFSE stained bacteria at 37 °C for one hour, except for the EAKVQA-peptide, which was pre-incubated with the cells. The assay was otherwise performed as described above.

To further characterize the role of GAGs in the interaction between HUVECs and B313/*dbpAB/Bg* or B313/*dbpAB/Bb*ss the cells were treated with chondroitinase ABC enzyme (from *Proteus vulgaris*, Sigma) which catalyses the degradation of chondroitin sulphate, dermatan sulphate and hyaluronate. Cells were grown for two days on the chamber slide before they were washed twice with PBS, and incubated with 10 mM chondroitinase ABC and 1 × chondroitinase ABC buffer (50 mM tris base, 5 mM sodium acetate, 0.01 % BSA) in RPMI in a CO_2 incubator at 37 °C for 2 hours. After washing the cells with PBS, 10^7 CFSE stained B313/pBSV2, B313/*dbpAB/Bg* and B313/*dbpAB/Bb*ss were applied on the cells, and the cell adhesion experiment was carried out as described above.

4.5.3 Cell adhesion assay under flow conditions

HUVECs were seeded on Ibidi 1µ-Slide$VI^{0.4}$ ibiTreat flow chamber slides (Ibidi, Verona, WI, USA) and cultured for three days. Just before the experiment the cell layer was dyed with CellMask™ Deep Red plasma membrane stain (Molecular Probes, Eugene, OR, USA) to verify the confluency of the cell layer.

Genetically manipulated B313 strains in an exponential growth phase were washed once with PBS and stained with fluorescent CellMask™ Orange plasma membrane stain (Molecular Probes) 1:2000 in PBS for 5 minutes at 33 °C. Unbound dye was removed by washing the bacteria with PBS. Fluorescently labelled *Borrelia* were adjusted to 10^8/ml in Hank's balanced salt solution and transferred to 10 ml syringes. These bacteria were perfused through the HUVEC flow chamber channels at 1 dyne/cm^2. Interactions between the *Borrelia* and HUVECs were monitored by recording two minutes videos with a Zeiss Axiovert 200M confocal microscope. The data were analyzed using Volocity software (PerkinElmer, Waltham, MA, USA) by counting brightly fluorescent bacteria from specified 100 µm × 30 µm areas.

4.6 Detection of proteoglycan expression by endothelial and epithelial cells

4.6.1 Decorin and biglycan staining of the cell cultures

The presence of proteoglycans was confirmed by staining the acetone fixed cell cultures with monoclonal anti-decorin (clone 9xx) or anti-biglycan (clone 3E2) antibodies. The primary antibody was diluted in 5 µg/ml in PBS and the secondary antibody, HRP-conjugated goat-anti-mouse IgG (Santa Cruz Biotechnology), 0.4 µg/ml in PBS. Both incubations were done at 37 °C for one hour, and the cells were washed after the incubation twice with PBS-T and once with PBS. After the washes, HRP was allowed to react with an AEC substrate (1 mM 3-amino-9-ethylcarbazole, 5 % (v/v) dimethylformamide, 0.1 % (v/v) hydrogen peroxidise in an acetate buffer (pH 5.5)) at room temperature for 30 min, and the slides were imaged with a light microscope.

4.6.2 Western blot analysis

Proteoglycans were detected from a concentrated HFF starvation medium and HUVEC lysate using Western blot. The culture medium of nearly confluent HFF cells was changed to a starvation medium (growth medium without serum) to induce decorin expression and incubated overnight. The starvation medium was concentrated approximately ten fold with centrifugal filters (Amicon Ultra-15 centrifugal filter unit 10000NMWL, Millipore, Cork, Ireland) by sentrifugation at 4000 × g at 4 ˚C for 25 minutes.

HUVECs were washed twice with PBS before they were removed from the culture flask with trypsin and washed (370 × g, 10 min, 22 °C) with RPMI containing 10 % fetal bovine serum (FBS). Washed cells were diluted to a lysis buffer (3×10^7 cells/ml; 20 % Triton X-100, 1.5 M NaCl, 0.1 M Tris-base, 15 mM $MgCl_2$) and lysed for one hour on ice. The cell debris was removed by centrifugation (10 000 × g, 20 min, 4°C) and the supernatant was collected for the Western blot analysis.

Before the Western blot analysis, the concentrated HFF medium and HUVEC lysate were treated with chondroitinase ABC to cleave the GAG side chains from the proteoglycans. The samples were incubated for 3 h at 37 °C with the chondroitinase ABC enzyme (5 u/ml) and 1 × chondroitinase ABC buffer. The enzyme treated samples were loaded on a 10 % Bis-Tris gel and transferred to nitrocellulose membrane. The membrane was blocked with 5 % non-fat milk in PBS overnight at 4 ˚C. Anti-decorin (clone 9xx) and anti-biglycan (clone 3E2) were used as primary antibodies and HRP-conjugated goat α-mouse IgG as the secondary antibody. ECL reagents were used for detection.

4.6.3 Immunohistochemical analysis of the human tissue sections

The presence of decorin and biglycan in the human skin, heart, and brain tissues was studied using immunohistochemistry. The tissue specimens were formalin fixed for 24 hours, dehydrated, paraffin-embedded, and sectioned. The proteoglycans were stained using monoclonal decorin (dilution 1:300; clone 9xx) or biglycan antibody (dilution 1:1000; clone 4E1-1G7, Abnova, Taipei, Taiwan) and EnVision detection kit (Dako,

Hamburg, Germany). After the primary and HRP-labeled secondary antibody incubations, epitope retrieval was performed in a microwave oven in Tris-EDTA-buffer (pH 9). Detection with diaminobenzidine was performed using an automatic staining machine (LabVision Corporation; Fremont, CA, USA).

4.6.4 siRNA silencing of biglycan expression on HUVECs

The messenger RNA expression of biglycan was silenced by ON-TARGET plus human biglycan smart pool siRNA (Thermo Scientific Dharmacon). The HUVECs were removed to 24 well plates (60 000 cells / well) or Lab-Tek^{TM} 8-well chamber slides (30 000 cells / well), and grown with EBM-2 culture media without antibiotics for 24 hours. Biglycan siRNA or control siRNA were transfected into the cells using Lipofectamine® RNAiMAX reagents (Invitrogen) according to manufacturer's instructions. The siRNA was allowed to silence the biglycan expression for 72 hours before the cells were used in the adhesion experiments.

The presence of biglycan was monitored by flow cytometry and Western blot analyses after the siRNA treatment. For the flow cytometry analysis, HUVECs were detached using 5 mM EDTA. The expression of biglycan was detected using monoclonal anti-biglycan (clone 4E1-1G7) as the primary antibody and R-phycoerytrin-conjugated goat anti-mouse IgG (Southern biotech, Birmingham, AL, USA) as the secondary antibody. The AK-1 primary antibody (In vivo Biotech Services, Henningsdorf, Germany) was used as a negative control. Fluorescence was detected using a FACSCalibur instrument (Becton-Dickinson, Franklin Lakes, NJ, USA), and data were analyzed using a FlowJo software (Tree Star, Ashland, OR, USA). The percentage of positive cells was calculated by subtracting the values obtained with the negative control antibody from the values obtained with the biglycan antibody. The geometric mean fluorescence intensities (MFIs) were calculated without subtracting the negative control antibody values.

Western blot analysis on the siRNA treated cells was done as described above. GAPDH detection (clone 6C5, Hytest, Turku, Finland) was used as a control to demonstrate the equal loading of cell lysate on the gel.

4.7 *In vivo* model for Lyme borreliosis

4.7.1 Mice

Female C3H/HeNhsd (C3H/He) mice were ordered from Harlan (Netherlands). All animal experiments were carried out in strict accordance with the recommendations of the Finnish Act on the Use of Animals for Experimental Purposes of the Ministry of Agriculture and Forestry in Finland. The protocol was approved by the National Animal Experiment Board in Finland (Permission Numbers STH619A, PH518A/ESAVI/3043/04.10.03/2011 and ESAVI/5507/04.10.07/2014). All efforts were made to minimize suffering of the animals.

4.7.2 Experimental design

Four different mouse experiments were done to study the role of DbpA and B in the dissemination, the arthritis development, and the persistence of *Bbss* infection in mice. Four-week-old C3H/He mice were infected with 10^6 Δ*dbpAB/dbpAB* (40 mice), Δ*dbpAB/dbpA* (8 mice), Δ*dbpAB/dbpB* (8 mice) or Δ*dbpAB* (38 mice) bacteria by intradermal syringe inoculation in the lower back. Twelve control animals were injected with an equal volume of PBS (see Figure 7).

In experiment I, the mice were infected with Δ*dbpAB/dbpAB* (4 mice), Δ*dbpAB/dbpA* (8 mice), Δ*dbpAB/dbpB* (8 mice), or Δ*dbpAB* (2 mice) bacteria to study the role of DbpA and B adhesins in the development of joint manifestations. The medio-lateral diameter of the hind tibiotarsal joints was measured once a week, and the measurer was blinded to the group's identity. The mice were killed at seven weeks of infection, and tissue samples from ear, bladder and tibiotarsal joint were collected for *Borrelia* culture.

In experiment II, the role of Dbps in post treatment persistence was studied. The mice were infected with Δ*dbpAB/dbpAB* (20 mice) or Δ*dbpAB* (20 mice). Subgroups of the mice were treated with ceftriaxone (Rocephalin®, Roche, Mannheim, Germany) or with ceftriaxone and anti-TNF-alpha (rat murine chimeric TNF-alpha antibody of IgG2ak isotype; Centocor, Malvern, PA, USA). Ceftriaxone was administered at two weeks of infection twice a day 25 mg/kg intraperitoneally for five days. Anti-TNF-alpha was administered at seven weeks of infection once a week 10 mg/kg intraperitoneally for four weeks. The development of joint manifestations was monitored as described above. At six and nine weeks of infection ear biopsy samples were collected for *Borrelia* culture to determine the infection status of the animals. The mice were killed at 15 weeks of infection. At the end of the experiment, tissue samples from ear, bladder, and the tibiotarsal joint were collected for *Borrelia* culture and PCR analyses. Blood was collected for serology and one tibiotarsal joint for histology.

In experiment III, the experiment read was set up to determine the infection status and bacterial load in joint tissues at two weeks of infection, a time point before any treatment. The mice were infected with Δ*dbpAB/dbpAB* (8 mice) or Δ*dbpAB* (8 mice), and killed at two weeks of infection. Tissue samples from ear, bladder, and the tibiotarsal joint were collected for *Borrelia* culture, one tibiotarsal joint was collected for quantitative PCR analysis, and blood was collected for serology.

In experiment IV, the mice were infected with Δ*dbpAB/dbpAB* (12) or Δ*dbpAB* (12) and treated with ceftriaxone at two and six weeks of infection to study the effect of a later treatment time point to the development of joint manifestations and post treatment persistence. The development of joint manifestations was monitored as explained above. The animals were killed at 15 weeks of infection. At the end of the experiment, tissue samples from ear, bladder and the tibiotarsal joint were collected for culture and PCR analyzes, joints for antigen detection and blood for serology.

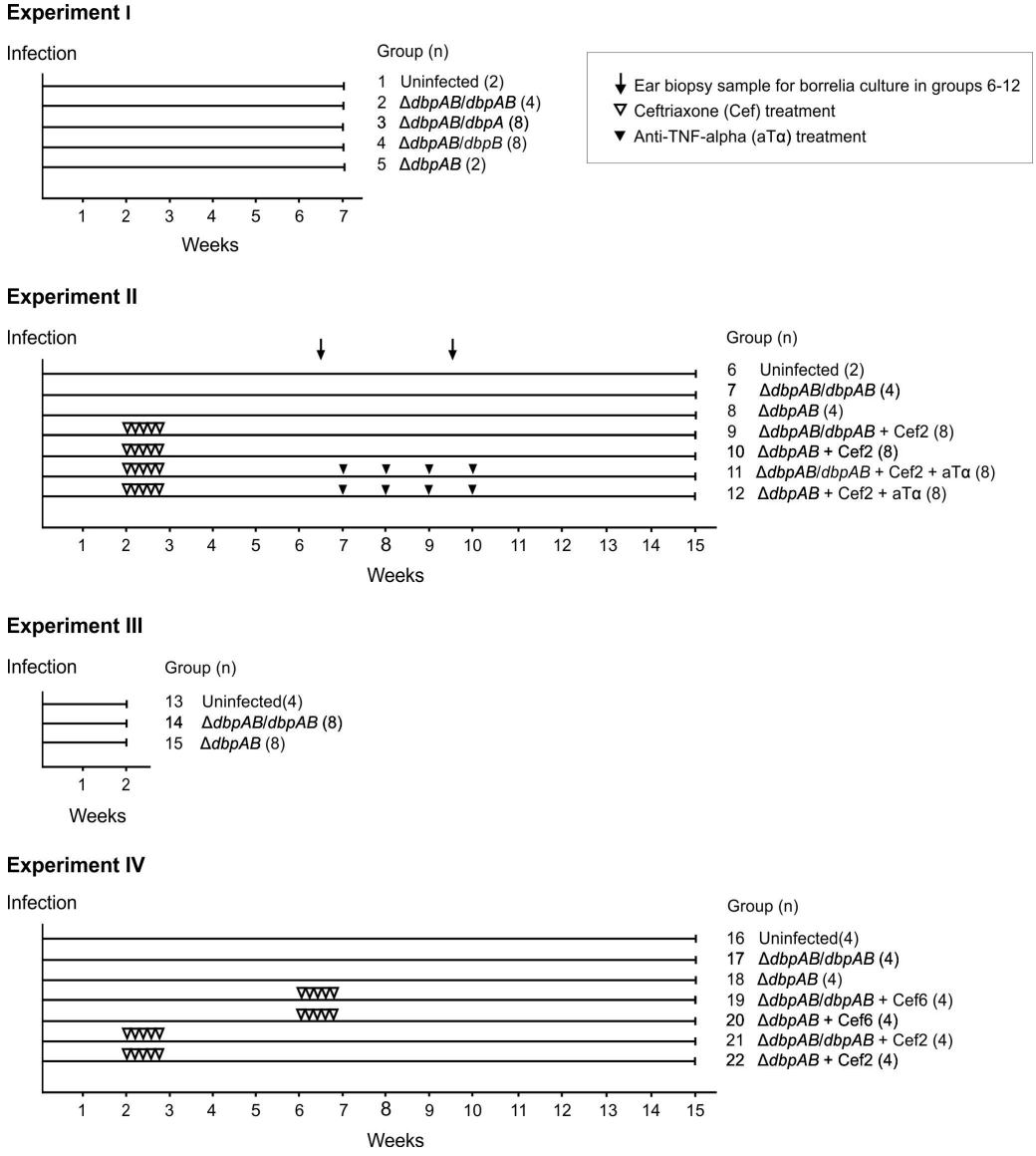


Figure 7. The design of the mouse experiments. In Experiment I, four Δ*dbpAB/dbpAB*, eight Δ*dbpAB/dbpA*, eight Δ*dbpAB/dbpB*, two Δ*dbpAB* infected animals and two uninfected control animals were killed at seven weeks of infection. In Experiment II, 16 infected animals were treated with ceftriaxone and 16 with ceftriaxone and anti-TNF-alpha. The ceftriaxone treatment was started at two weeks (25 mg/kg twice a day for 5 days) and the anti-TNF-alpha treatment was started at seven weeks of infection (10 mg/kg once a week for 4 weeks). Ear biopsy samples were collected at 6 and 9 weeks of infection to monitor the dissemination of the infection. In experiment III, the mice were killed at two weeks to study infection kinetics and bacterial load in the joints. In experiment IV, eight infected animals were treated with ceftriaxone at six weeks of infection and eight animals at two weeks of infection. In experiments I, II, and IV the diameter of the tibiotarsal joints was measured weekly. Modified from the original publication III.

4.7.3 The analysis of the mouse samples

All tissue samples for the *Borrelia* culture, PCR analyses, and the histology were collected with sterile instruments, which were disinfected in ethanol between the dissections of the different samples. The effectiveness of ethanol disinfection was validated by processing the tissue samples of uninfected control animals at the end. For the *Borrelia* culture the ear, bladder and joint tissue samples were grown in BSK II medium containing phosphomycin (50 µg/ml; Sigma-Aldrich) and rifampin (100 µg/ml; Sigma-Aldrich) at 33 °C for a maximum of 6 weeks. The presence of viable *Borrelia* was monitored every two weeks using a dark-field microscope.

Before the DNA extraction, the ear, bladder and joint tissue samples were stored at -20 °C. Tissue samples were incubated with proteinase-K (275 µg/ml) at 56 °C overnight before the DNA was extracted using NucliSENS easyMAG kit (Biomérieux, Marcy l'Etoile, France) according to manufacturer's instructions. PCR analyses were performed using a nested PCR and real-time PCR methods, and all runs included a positive and a negative control. In the nested PCR, the choromosomal flagellin gene (*flaB*) was amplified according to the method described by Yrjänäinen and co-workers (Yrjänäinen et al., 2010) using two sets of primers. The PCR products were analyzed on agarose gels. In the real-time PCR, a 102 bp product of *ospA* gene was amplified using the method described by Ivacic and co-workers (Ivacic et al., 2007), a LightCycler 480 Probes master kit and LightCycler 480 II equipment (Roche). All used primers are listed in Table 2. To analyze the bacterial load in the joint samples, the *ospA* PCR was performed quantitatively. The actual bacterial load in each sample was calculated from the standard curve, and the PCR was repeated three times. Data were expressed as the number of *Borrelia* genomes per 100 ng of extracted DNA.

IgG specific antibodies against whole cell *Bb*ss extract, C6 peptide, and DbpA and DbpB were measured using in house ELISA. *Bb*ss B31 (ATCC 35210) whole cell lysate, biotinylated C6 peptide (Biotin-MKKDDQIAAAIALRGMAKDGKFAVK), and recombinant DbpA and DbpB of *Bb*ss were used as antigens in the assays. The detailed protocol is explained in Study III. All samples were analysed as duplicates and the results are expressed as OD_{492} values.

To study histological changes in tibiotarsal joints (III: experiment II, groups 6-12), the tissue samples were formalin-fixed, demineralized, embedded in paraffin, sectioned at 5 µm, and stained with hematoxyline-eosin (HE) using routine histology techniques. Findings of joint disease were evaluated in the sagittal joint sections by an experienced pathologist blinded to the experimental protocol.

4.7.4 Antigen detection

To detect persisting antigens from the tibio-tarsal joint samples of antibiotic treated mice proteins were extracted from the tissue samples and used as antigens to immunize the mice (see Figure 8).

Proteins were extracted from joint samples (III: experiment III, groups 11-17) using a total protein extraction kit (Millipore) according to manufacturer's instructions. The samples were homogenized using Tissue Lyser LT (50 Hz, 2 x 5 min) and 5 mm metal beads (Qiagen). Protein concentrations were analyzed with BCA™ Protein Assay Kit according to the manufacturer's instructions. The extracted proteins were used to immunize naive C3H/He mice. Each mouse was immunized with a protein extract from one joint sample.

Antigen-adjuvant emulsions were prepared by mixing TiterMax® Gold Adjuvant (Sigma-Aldrich) and protein solution 1:1 in a syringe immediately before immunization. Twenty eight C3H/He mice were immunized by subcutaneous injection of 100 µl of adjuvant-antigen emulsion, and two mice with adjuvant-buffer (negative control) emulsion in the lower back. A booster immunization was performed two weeks later. The mice received 100 µg of the antigen in the primary immunization, and 50 µg in the booster immunization. Sera were collected two weeks after the second immunization.

The presence of *Borrelia* specific antibodies was analysed with a Western blot assay. 3.75 x 10^6 Δ*dbpAB/dbpAB* bacteria were lysed in the Nupage sample buffer, the proteins were separated in 10 % SDS-PAGE gels, and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in PBS and incubated with mouse sera from immunized animals (1:100 dilution). Sera from Δ*dbpAB/dbpAB* and Δ*dbpAB* infected (1:1000 dilution) animals were used as positive controls. The anti-mouse IgG antibody was used as a secondary reagent (1:4000 dilution) and ECL in detection.

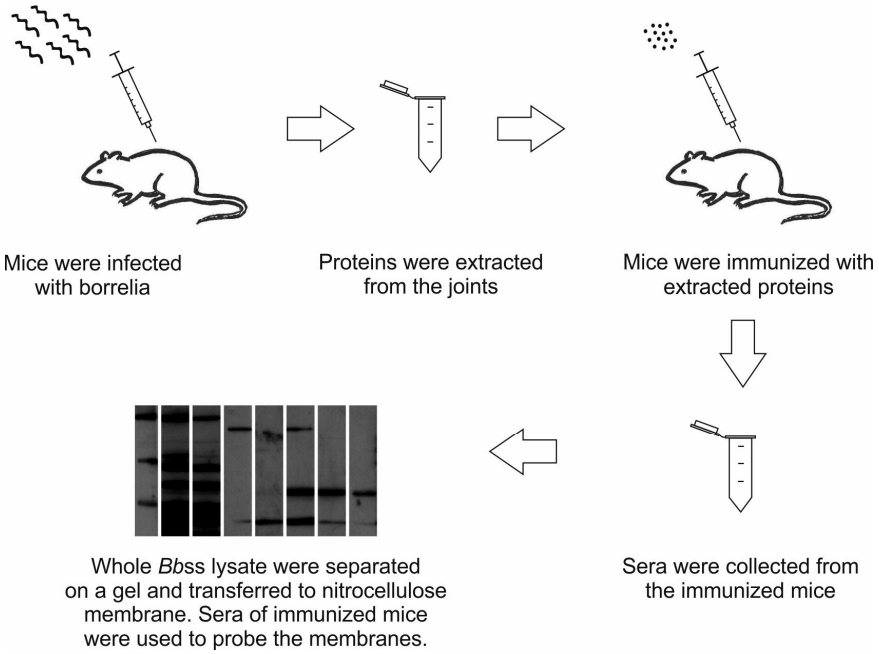


Figure 8. A schematic presentation of the borrelial antigen detection protocol.

4.8 Statistical analysis

Statistical analyses were done using IBM SPSS Statistics (Version 22). The statistical significance of differences in the microtiter plate assays, dot adhesion assay, cell adhesion assays, joint diameter measurements, serum antibody levels and bacterial load in joint samples, when there was more than two groups, was determined by Analysis of Variance (ANOVA). In all other assays except the joint diameter measurements, Post Hoc comparisons between the means were done with Dunnett t-test if there was a clear control, otherwise Tukey's honestly significant difference test was used. In the joint diameter measurements, cage-specific means were used as independent observations. Statistical significance was determined as $p \leq 0.05$. When comparing *Borrelia* infected mice to non-infected controls, Bonferroni correction was used to adjust the critical p-value. Statistical analysis of the bacterial load, when there were two groups (III: Experiment III), was done using the independent samples T-test.

5 RESULTS AND DISCUSSION

5.1 The adhesion of DbpA and B of *B. burgdorferi* sensu lato to decorin and biglycan

5.1.1 The DbpB of *Bg* and *Bb*ss bind to decorin and biglycan under static condition

The cloned *dbp* genes in the constructed *E. coli* expression strains were sequenced (data not shown), and the recombinant His-tagged DbpA and B proteins of correct sizes were identified in the SDS gel electrophoresis (I: Figure 1A). The comparison of amino acid sequences of the six Dbp molecules revealed that they all contained the three critical lysines needed for the decorin/GAG binding activity (III: Supplement Figure. 1).

First, the adhesion of recombinant DbpA and B molecules to decorin was analyzed using a Western blot assay. Under denaturing conditions, only DbpB/*Bg* and DbpB/*Bb*ss showed detectable binding to the biotinylated decorin (I: Figure 1A). Then, the interactions between the individual adhesins and proteoglycans were studied under native conditions with a microtiter plate assay. In addition to decorin binding, the adhesion of individual DbpA and B molecules to another Class I proteoglycan in the SLRP family, biglycan, was studied. Under static conditions DbpB/*Bg* and DbpB/*Bb*ss showed clear and statistically significantly higher binding to decorin and biglycan when compared to other tested Dbps (I: Figure 1B and II: Figure 4A). DbpB/*Bg* was the strongest decorin binder, while DbpB/*Bb*ss bound to biglycan with the strongest affinity. All other four adhesins showed only negligible binding to both studied proteoglycans, even though the binding of DbpB/*Ba* to decorin and biglycan was slightly stronger than with all DbpA adhesins.

5.1.2 The DbpA and B of *Bg* have the highest affinity to decorin and biglycan under flow conditions

After characterizing the adhesion of Dbps to proteoglycans under static conditions, the interactions between these molecules were studied in more detail using a Surface Plasmon Resonance (SPR) assay. Under flow, DbpB/*Bg*, DbpA/*Bg* and DbpB/*Bb*ss interacted with decorin and biglycan clearly more than the other Dbps (I: Figure 2B and II: Figure 5A and B). The binding of Dbps to these proteoglycans was dose dependent (I: Figure 2A and II: Supplementary Figure 7B). DbpB/*Bg* exhibited the highest affinity to decorin as well as to biglycan. Surprisingly, DbpA/*Bg*, which showed only negligible binding in the microtiter plate assay, interacted strongly to decorin and biglycan under a constant flow. DbpB/*Bb*ss showed moderate binding to both proteoglycans. Under the tested conditions the binding of DbpA/*Bb*ss and DbpB/*Ba* to decorin and biglycan was modest, while no interactions could be detected

between DbpA/*Ba* and the proteoglycans. BSA was used as a negative control, and no interactions were observed between BSA and Dbps in the SPR assay (II: Supplementary Figure 7A).

5.1.3 The DbpB of *Bb*ss and *Bg* bind to the glycosaminoglycan side chain of decorin and biglycan

It has been previously demonstrated with the DbpA and B of *Bb*ss that the decorin binding ability can be inhibited by glycosaminoglycans (Fischer et al., 2003) or peptides containing the EAKVRA motif (Pikas et al., 2003). To dissect more closely the Dbp-decorin and Dbp-biglycan interactions, a set of inhibitors was introduced to the microtiter plate assay.

Dermatan sulphate inhibited the binding of DbpB/*Bg* to decorin and biglycan most efficiently ($P \leq 0.001$), similarly to unlabeled decorin or biglycan, which were used as positive controls (I: Figure 5A and II: Supplementary Figure 6A). Other tested GAGs, chondroitin-6-sulphate and chondroitin-4-sulphate, also inhibited the interactions statistically and significantly ($P \leq 0.001$). In addition to the GAGs, the EAKVQA-peptide (which was designed based on the DbpB/*Bg* sequence) inhibited the DbpB/*Bg* and the decorin interaction significantly ($P \leq 0.001$) (I: Figure 5A). In addition, dermatan sulphate, chondroitin-6-sulphate and chondroitin-4-sulpahte inhibited significantly ($P \leq 0.001$) the interaction between DbpB/*Bb*ss and biglycan (II: Supplementary Figure 6B). BSA was used as a negative control in all the experiments, and it did not have any effect on the interactions.

Taken together, the above results demonstrate that Dbps bind to decorin with different affinities, and in addition to decorin-binding, Dbps adhere to another proteoglycan called biglycan. Among the tested adhesins, the DbpB molecules of each genospecies interacted more with the proteoglycans than did the DbpA adhesins in stationary or flow conditions. DbpB/*Bg* and DbpB/*Bb*ss showed clear binding to decorin and biglycan in all tested methods. Interestingly, DbpA/*Bg* interacted with decorin and biglycan only under flow conditions, thereby suggesting the adhesin is flow dependent.

Previously, the biological activities of Dbps have been studied exclusively using *Bb*ss derived adhesins. Our results reveal that there are differences in the ligand binding activities among DbpA and B adhesins of *Bb*ss, *Bg* and *Ba*, even though, they all contain the three critical lysines shown to be needed for decorin/GAG binding. Recently, similar results were obtained when the DbpA-decorin interaction of different *Bb*sl isolates was studied (Benoit et al., 2011; Lin et al., 2014a). In contrast to our results, these results showed that the DbpA of *Bb*ss and *Ba* exhibited moderate decorin-binding ability. The discrepancy between these two studies and ours is most likely explained by the use of different *Borrelia* isolates.

Bbss N40 strain is a tick isolate, which is known to be heterogeneous in different laboratories. Our *Bbss* N40 strain is different than the *Bbss* strain N40-D10/E9 used by Benoit, Lin, and their co-workers. In addition, we have derived the adhesins from *Ba*

strain A91 while Benoit, Lin, and their co-workers have used the DbpA of *Ba* strain VS461. DbpA of *Ba* VS461 contains an additional C-terminal domain responsible for GAG/heparin binding, which is lacking in DbpA of *Ba* A91. Thus by deleting 11 C-terminal amino acids, the decorin-binding activity of DbpA/*Ba* VS461 is greatly diminished. More recently, Morgan and co-workers have shown that a flexible linker in DbpA can modulate the GAG binding activity, so thus the primary amino acid sequence does not alone determine the ligand binding properties of the adhesin (Morgan et al., 2015). They demonstrated by using NMR structures that DbpA of *Bb*ss N40 contains a flexible linker between helixes 1 and 2. It is able to block the binding of GAGs to the positively charged cleft formed around the three critical lysines. The flexible linker is not found in the DbpA of *Bg* PBr (Morgan and Wang, 2015), which has been shown to be a robust decorin/GAG binder (Lin et al., 2014a). These recent studies support our findings, namely that Dbps of different genospecies or strains indeed do have different ligand binding properties.

Surprisingly, one of the studied adhesins, DbpA of *Bg,* appears to be flow dependent. So far, no other borrelial adhesin has been described to mediate adherence only under flow conditions. However, bacterial adhesins of other pathogens are shown to have shear-induced adhesion mechanisms, such as FimH of *E.coli*. Recently, the catch-bond mechanism of FimH was revealed by crystallizing three separate structures of the adhesin (Sauer et al., 2016). The two-domain adhesin binds to the D-mannose of epithelial glycoproteins, and mechanical stress reinforces the adhesin-ligand interaction by changing the FimH structure. The mechanism for how DbpA/*Bg* binds to decorin, biglycan or dermatan sulphate still needs further study.

As previously described with DbpA of *Bb*ss, our data confirms that Dbps bind to the GAG side chain, especially to the dermatan sulphate of the proteoglycan (Fischer et al., 2003). However, in the study done by Benoit and co-workers, the DbpAs of the different *Bb*ss, *Bg* and *Ba* strains bind exclusively to heparin and dermatan sulphate, but not to chondroitin-6-sulphate (Benoit et al., 2011). In our studies, both dermatan sulphate and chondroitin-6-sulphate inhibited the decorin/biglycan binding. The controversy between these studies can be explained by their different methodological approach. We have used only an inhibition assay to identify the critical ligand structure of the proteoglycan, while Benoit and co-workers set up an assay where they could study the adhesion of DbpA to different ligand structures directly. In conclusion, the Dbps bind to the dermatan sulphate side chain of proteoglycan, regardless of the core protein sequence.

5.2 Borrelial adhesion to proteoglycans and proteoglycan expressing cells

5.2.1 Decorin and biglycan are expressed by different cell types

The presence of decorin and biglycan on human cell cultures and tissue sections was confirmed before the bacterial adhesion assays. The expression of proteoglycans on

fibroblasts and endothelial cells was studied using AEC staining and Western blot analysis (I: Figure 4B, and II: Figure 2A and B). The staining of the fixed cell cultures revealed that HFF cells express decorin, while biglycan is expressed by HUVECs. The Western blot analyses of the cell lysate or, in the case of HFF cells, of the concentrated culture medium, confirm the same finding. The HFF cells express decorin, but not biglycan, while the expression profile of these proteoglycans is opposite to the HUVECs (II: Figure 2A).

The presence of decorin and biglycan was also studied in human skin, heart and brain tissues by immunohistochemical stainings. Decorin was detected in dermal fibroblasts, while epidermis, blood vessel wall and vascular endothelial cells did not contain the proteoglycan (III: Supplementary Figure 3). On the other hand, biglycan was detected in the vascular endothelial cells of brain, skin and heart tissues (III: Figure 2C and Supplementary Figure 2).

To conclude, these results confirm that decorin and biglycan have different tissue distributions as described before (Bianco et al., 1990). Indeed, the tissue section and cell culture stainings confirm that decorin is expressed by epithelial cells, while biglycan is expressed by endothelial cells of different vascular beds.

5.2.2 Wild type *Borrelia* strains bind to the human umbilical vein endothelial cells

The adhesion of the three representative wild type *Borrelia* strains, *Bg* SBK40, *Ba* A91 and *Bb*ss N40, to HUVECs was studied using fluorescently labeled bacteria. When compared to the surface deficient B313 strain all three wild type strains showed clear adhesion to the cells (II: Figure 1A). Variation was observed among the experiments, which could be explained by the heterogenic nature of primary cell cultures. However, in two out of three experiments *Bg* SBK40 and *Bb*ss N40 bound clearly more to the HUVECs than to the *Ba* A91 strain.

These results indicate that wild type *Bb*ss and *Bg* strains have more potent adhesins on the outer surface than *Ba*. The adhesion studies with individual DbpA and B recombinant proteins demonstrated that the *Bb*ss and *Bg* derived Dbps bound to biglycan, which is expressed on HUVECs, more efficiently than the Dbps of *Ba*. Thus, the differences in the HUVEC binding of wild type *Borrelia* strains could be explained by the different biglycan binding ability of Dbps. However, wild type *Borrelia* strains have several other outer surface proteins that facilitate bacterial adhesion to endothelial cells. For example, endothelial cells express fibronectin, which is recognized by at least six different borrelial adhesins, namely BBK32, BB0347, CspA, CspZ, RevA and RevB.

5.2.3 The DbpA and B adhesins of *Bg* and *Bb*ss promote borrelial adhesion to human foreskin fibroblasts and human umbilical vein endothelial cells

To study the specific role of DbpA and B adhesins in the cell adhesion knock-in *Bb*ss B313 strains were generated. B313 is a laboratory *Borrelia* strain that lacks several

plasmids carrying outer surface protein genes, including *dbpA* and *dbpB*. The *dbpAB* operon of *Bb*ss, *Bg* and *Ba* was PCR amplified and cloned to the pBSV2 shuttle vector, which then was taken into B313. Complemented B313 strains were characterized by PCR (data not shown) and a Western blot assay (I: Figure 3A). The outer surface localization of DbpA and B of B313 derivative strains was shown with proteinase-K treatment (I: Figure 3B).

After characterization, the binding of the B313 derivative strains to human fibroblasts and endothelial cells was studied. Both B313/*dbpAB/Bg* and B313/*dbpAB/Bbss* strains exhibited statistically significantly ($P \leq 0.01$) higher binding to HFFs and HUVECs than did the vector control B313/pBSV2 (I: Figure 4C and II: Figure 1B). Slightly more interactions between the B313/*dbpAB/Bg* and the cells were seen compared with the B313/*dbpAB/Bb*ss strain in both of the assays. On the other hand, the adhesion of B313/*dbpAB/Ba* to both cell lines appeared to be at the background level.

These findings are consistent with the previous results of recombinant Dbps. The strains that expressed Dbps with high decorin or biglycan binding ability as individual molecules also mediated the bacterial adhesion to cells that were expressing these proteoglycans. Therefore, the differences already obtained from the HUVEC binding of wild type *Borrelia* strains could at least in part be explained by the proteoglycan binding ability of individual Dbps. The study by Benoit and others further confirm our finding that individual Dbp-proteins have an effect on the cell adhesion property of the bacteria (Benoit et al., 2011). Indeed, the complementation of the non-adherent, non-infectious *Borrelia* strain with only DbpA enables the bacteria to adhere to fibroblasts. Similar to our results, these results indicate that the Dbp(s) of *Bg* facilitate the highest binding to decorin expressing cells, while the adhesin(s) of *Ba* had no or only minor effects on the fibroblast or endothelial cell binding.

5.2.4 The adhesion of *Borrelia* to fibroblasts and endothelial cells is mediated by the Dbp-proteoglycan interaction

To confirm the role of decorin and biglycan in the cell adhesion, a set of soluble proteoglycans, glycosaminoglycans and a peptide were introduced to the cell adhesion assay. Decorin, EAKVQA-peptide and dermatan sulphate significantly inhibited ($P \leq 0.001$) the adherence of B313/*dbpAB/Bg* and B313/*dbpAB/Bb*ss to HFFs (I: Figure 5B and C), while other tested GAGs, chondroitin-6-sulphate, and chondroitin-4-sulphate, had only little, statistically not significant, effect on the interaction. In the case of HUVECs, the binding of B313/*dbpAB/Bg* and B313/*dbpAB/Bb*ss was inhibited significantly by biglycan and dermatan sulphate ($P \leq 0.001$) (II: Figure 3A and B). Additionally, chondroitin-6-sulphate inhibited the binding of B313/*dbpAB/Bb*ss to HUVECs significantly ($P \leq 0.01$), although not as efficiently as biglycan and dermatan sulphate. BSA, which was used as a negative control, did not have effect on any of the experiments.

To further confirm the role of biglycan in the *Borrelia*-HUVEC-interaction, the expression of the proteoglycan was silenced by specific siRNAs. Flow cytometry and Western blot analyses showed that the siRNA treatment diminishes biglycan expression on HUVECs (II: Figure 3C and Supplementary Figure 5). Importantly, the biglycan siRNA treatment significantly reduced the binding of B313/*dbpAB/Bg* and B313/*dbpAB/Bb*ss to HUVECs when compared to the control siRNA treated cells ($P \leq 0.01$) (II: Figure 3D).

Additionally, the role of GAGs in the Dbp-endothelial cell interaction was studied by degrading the dermatan and chondroitin sulphates on the cultured cells with chondroitinase ABC. The enzyme treatment on the cells significantly impaired the adhesion of B313/*dbpAB/Bg* and B313/*dbpAB/Bb*ss to HUVECs compared to the untreated cells ($P \leq 0.05$) (II: Supplementary Figure 4).

In conclusion, the above results confirm that the Dbps of *Bg* and *Bb*ss interact through decorin and biglycan with the human fibroblasts and endothelial cells, respectively. The ligand structures in the proteoglycans appear to be the dermatan sulphate side chain. On the other hand, based on these results, it can be concluded that Dbps interact with dermatan sulphate and dermatan sulphate containing macromolecules, regardless of the core protein, to which the carbohydrate residue is attached.

Previously, it has been shown that Dbps are essential in the later stages of the infection when the bacteria are colonized in the decorin rich tissues, especially the joints (Liang et al., 2004). However, our results suggest that Dbps could also play a role in the hematogenous dissemination of *Borrelia* and interact with the endothelial cells in the blood vasculature.

5.2.5 The DbpA and B adhesins of *Bg* promote borrelial adhesion to human umbilical cord endothelial cells under flow

After analyzing in various set-ups the role of Dbps in borrelial adhesion to cells under static conditions, the adhesion of B313 derivative strains to endothelial cells was studied in a flow chamber model. This *in vitro* method mimics the physiological conditions in the blood vasculature and enables to study the role of individual molecules participating in the vascular escape of disseminating bacteria. In line with the SPR results, which demonstrated that both DbpA and B of *Bg* bind clearly to biglycan under flow, these results show that B313/*dbpAB/Bg* interacted significantly more with HUVECs compared to the background strain, B313/pBSV2 ($P \leq 0.01$) (II: Figure 6). The interactions between B313/*dbpAB/Bg* and HUVECs were both rapid and transient (< 1 second) classifying them as tethering interactions (Moriarty et al., 2012; Norman et al., 2008). Interestingly, the binding of B313/*dbpAB/Ba* and B313/*dbpAB/Bb*ss to HUVECs under flow did not differ from B313/pBSV2.

In conclusion, this experiment shows that *Borrelia* needs both of the DbpA and B adhesins to be flow tolerant in order to support the bacterial adhesion to endothelial cells under flow. *In vivo* mouse studies using intravital microcopy have

revealed that another borrelial adhesin, BBK32, is essential in the bacterial adhesion to vascular endothelium (Moriarty et al., 2012). The adhesion of the spirochetes to the vascular wall is suggested to be a two-step process. First, the cascade is initiated by high-affinity binding of BBK32 to fibronectin, which leads to tethering of the bacteria on the vascular bed. After the tethering step, lower affinity interactions can take place, leading to dragging of the bacteria. In addition to fibronectin binding, BBK32 binds to GAGs with lower affinity (Lin et al., 2014b; Moriarty et al., 2012). However, BBK32 is not alone responsible for the bacterial adherence to the vascular endothelium, and other fibronectin-binding proteins, RevA, RevB and BB0347, cannot restore the vascular interactions when expressed on a non-adhesive *Borrelia* strain (Moriarty et al., 2012). The results of the present study indicate that DbpA and B of *Bg* mediate the tethering interactions of the bacteria to endothelial cells under flow, and therefore Dbps are likely to have a role in the vascular adhesion cascade also *in vivo*.

5.3 The role of Dbps in dissemination, arthritis development, and persistence of *Bb*ss infection in mice

5.3.1 DbpA and B are essential in early dissemination

To study the role of Dbps of *Bb*ss in the pathogenesis of LB, mice were infected with DbpA and B deficient *Borrelia* strain (*ΔdbpAB*) or strains expressing DbpA (*ΔdbpAB/dbpA*), DbpB (*ΔdbpAB/dbpB*) or both of the adhesins (*ΔdbpAB/dbpAB*). Four different mouse experiments were performed to study the role of these adhesins in the dissemination, arthritis development and post-treatment persistence in Lyme borreliosis (see Figure 8). To study the impact of DbpA and B in the dissemination, C3H/He mice were infected with *ΔdbpAB/dbpAB* and *ΔdbpAB* (Figure 8, experiments I, II, III and IV).

At two weeks of infection, there was a clear difference between the mice infected with *ΔdbpAB/dbpAB* and *ΔdbpAB* (III: Table 4). All eight mice infected with *ΔdbpAB/dbpAB* had culture positive bladder and tibiotarsal joint tissues samples, and seven out of eight animals had culture positive ear samples. In contrast, none of the ear and bladder samples of the *ΔdbpAB* infected mice harbored cultivable *Borrelia*, and only three out of eight joint samples were culture positive. The presence of borrelial DNA was tested using two separate PCR methods; a nested PCR, which amplifies *flaB* gene, and a quantitative real-time PCR amplifying the *ospA* gene. The PCR analyses of the tibiotarsal joint samples revealed that four out of the eight *ΔdbpAB* infected mice carried borrelial DNA (one culture negative sample was tested positive with both PCR methods), while all joint samples of the *ΔdbpAB/dbpAB* infected animals were PCR positive. Furthermore, the bacterial load in the PCR positive joint samples was significantly higher ($P = 0.003$) in the *ΔdbpAB/dbpAB* infected mice than in the *ΔdbpAB* infected mice (III: Figure 4, experiment III).

At later time points, the difference in the culture and PCR results between the *ΔdbpAB/dbpAB* and *ΔdbpAB* infected mice decreased and eventually, at 15 weeks of infection, was abolished. At seven weeks of infection, the only difference was seen in the ear samples (III: Table 1). Neither of the *ΔdbpAB* infected mice had culture positive ear samples, while all the bladder and joint samples *of ΔdbpAB* infected mice and all tissue samples of the *ΔdbpAB/dbpAB* infected mice harbored cultivable *Borrelia*. At 15 weeks of infection, all eight *ΔdbpAB/dbpAB* infected mice and seven out of eight *ΔdbpAB* infected mice (transient infection in one mouse) had culture or PCR positive ear, bladder, and joint samples (III: Tables 2, 3 and 5). There was no difference in the bacterial loads of the tibiotarsal joint samples between the *ΔdbpAB/dbpAB* and *ΔdbpAB* infected animals at 15 weeks of infection (III: Figure 4, experiments II and IV).

To conclude, these results demonstrate that the *dbpAB* deficient *Borrelia* strain is able to infect immunocompetent mice when using high infection load (10^6 bacteria). However, the early dissemination of *ΔdbpAB* in mice is attenuated when compared with the *ΔdbpAB/dbpAB* infected mice. Later in the infection the early survival defect is abolished, and *ΔdbpAB* colonize tibiotarsal joints similarly as *ΔdbpAB/dbpAB*. Our results are in line with the previously published studies on the role of DbpA and B adhesins in the dissemination of *Borrelia* (Blevins et al., 2008; Imai et al., 2013; Shi et al., 2006; Weening et al., 2008).

5.3.2 Both the DbpA and B of *B. burgdorferi* sensu stricto are needed for full arthritis development in murine LB model

To study the role of Dbps in the development of joint manifestations mice were infected with *ΔdbpAB/dbpAB, ΔdbpAB/dbpA, ΔdbpAB/dbpB* and *ΔdbpAB*, and the diameter of the tibiotarsal joints were measured weekly (Figure 8, experiments I, II and IV). In all these experiments, only the strain expressing both DbpA and B adhesins was able to cause clear and prominent joint swelling with the highest values at four weeks of infection (III: Figure 2A, B and C). By 7 weeks of infection, no measureable changes in tibiotarsal joint diameter of the *ΔdbpAB/dbpA, ΔdbpAB/dbpB* or *ΔdbpAB* infected mice were detected compared to uninfected mice (III: Figure 2A), while the post-mortem joint samples (except in one *ΔdbpAB/dbpA* infected mouse) were *Borrelia* culture positive (III: Table 1).

During the longer follow-up, until week 15, Δ*dbpAB/dbpAB* caused two statistically significant ($P \leq 0.05$) joint swelling episodes, at 3-5 and 9-11 weeks of infection (III: Figure 2B and C). The joint swelling caused by Δ*dbpAB* was mild and emerged at 10 weeks of infection (III: Figure 2B). The histological evaluations of the tibiotarsal joint samples at 15 weeks of infection revealed that the mice infected with Δ*dbpAB/dbpAB* had thickened synovial membranes with proliferation of synovial lining cells, fibroblast and capillary proliferation as well as a mild chronic inflammation containing mainly lymphocytes (III: Figure 2D). In addition, the articular cartilage surface showed mild degenerative changes. In *ΔdbpAB* infected mice the findings were minor and showed minimal thickening of the synovium, consisting

mainly of synovial fibroblasts, while no inflammatory cells, capillary proliferation, or articular cartilage surface damage were seen (III: Figure 2E).

Taken together, these results demonstrate that both DbpA and B of *Bb*ss are needed to cause a clear and prominent joint swelling with histologically evident arthritic lesions in C3H mice. However, the results by Lin and co-workers demonstrate that the DbpA of *Bb*ss alone (without DbpB) is able to cause histologically evident arthritis (Lin et al., 2014a). The discrepant results can be explained by the use of different *Borrelia* strains, and a different method to detect joint symptoms. Lin et al used histological scoring to measure the severity of arthritis, while we measured the diameter of tibiotarsal joints. Unfortunately, we performed histological stainings on the joint samples of the Δ*dbpAB/dbpAB* and Δ*dbpAB* infected mice, but not on the joint samples of the Δ*dbpAB/dbpA* or Δ*dbpAB/dbpB* infected mice.

In addition to Dbps, it has been shown that several borrelial outer surface proteins, *e.g.*, BmpA and B, and BBA57, participate in the development of murine LA (Pal et al., 2008; Yang et al., 2013). Recently, two additional borrelial adhesins, BBK32 and Lmp1, were shown to be involved in the joint colonization of the bacteria (Lin et al., 2015; Yang et al., 2016). However, the role of these adhesins in the genesis of LA remains still to be studied.

5.3.3 The DbpA and B of *Bb*ss promote post-treatment DNA persistence in the murine LB model

To study the effect of antibiotic treatment in Δ*dbpAB* and Δ*dbpAB/dbpAB* infected mice the animals were treated with ceftriaxone either two or six weeks of the infection and followed until week 15 (Figure 7, Experiments II and IV). In the Δ*dbpAB/dbpAB* infected animals, the ceftriaxone treatment at two weeks of infection prevented the development of joint swelling (III: Figure 2B), and at six weeks of infection, abolished the symptoms close to the background level of the uninfected control mice (III: Figure 2C).

The *Borrelia* cultures of all infected and ceftriaxone treated mice were negative, while all samples of the infected and untreated animals (except one transiently Δ*dbpAB* infected mouse) were *Borrelia* culture positive (III: Tables 2 and 5). Interestingly, the PCR results revealed that all the Δ*dbpAB/dbpAB* infected and ceftriaxone treated mice retained borrelial DNA in their joint samples regardless of the treatment time point, whereas only one out of twelve Δ*dbpAB* infected and treated mice contained borrelial DNA in their joints samples (III: Tables 3 and 5). The qPCR results showed that the ceftriaxone treatment did not have a statistically significant effect on the bacterial load in the joint samples of the Δ*dbpAB/dbpAB* infected and treated animals compared to the untreated infected mice (III: Figure 4). The ear and bladder samples of all the infected and treated mice were *Borrelia* PCR negative regardless of the infective strain or treatment time point (III: Tables 2 and 5).

A sub-group of the Δ*dbpAB/dbpAB* and Δ*dbpAB* infected and ceftriaxone treated mice (at two weeks of infection) were immunosuppressed by anti-TNF-aplha to

characterize the nature of the persisting material after antibiotic treatment. Previously, our group has shown that a part of *Bb*ss infected and ceftriaxone treated C3H mice become culture positive after immunosuppression by anti-TNF-alpha (Yrjänäinen et al., 2007). However, in this current study, the immunosuppression did not turn the PCR positive joint samples of the Δ*dbpAB/dbpAB* infected and ceftriaxone treated mice to *Bb*ss culture positive. In the present study, two daily doses of ceftriaxone appeared to be more optimal for the treatment of LB than the single daily dose used in the previous study. In conclusion, these results suggest that the persisting remnants in the joints of Δ*dbpAB/dbpAB* infected and ceftriaxone treated mice are DNA or DNA containing structures rather than live bacteria.

In addition to retaining viable bacteria or borrelial DNA, the presence of antigens was studied after the antibiotic treatment. Joint samples of Δ*dbpAB/dbpAB* and Δ*dbpAB* infected and ceftriaxone treated mice (at two or six weeks of infection) were investigated, but no *Borrelia* specific antigens were detected (Figure 9). However, Bockenstedt and co-workers have elegantly shown, with a similar assay, that *Borrelia* specific antigens persist in mouse joints after antibiotic treatment. One possible explanation for these discrepant results can be the difference in the sample preparation. Bockenstedt and co-workers prepared the antigen from the mouse patella for the immunizations, while we used the whole tibiotarsal joints for the antigen preparation.

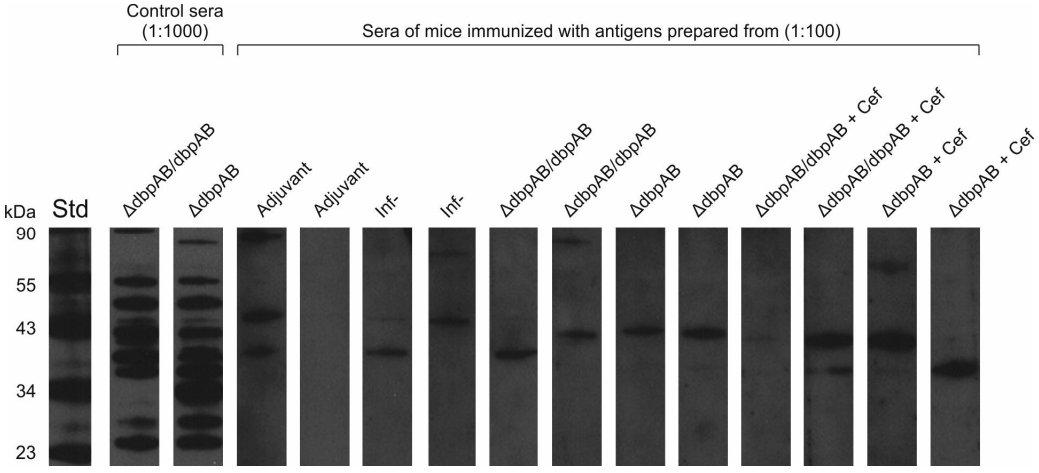


Figure 9. *B. burgdorferi* sensu stricto immunoblot. Whole cell lysate prepared from the strain *ΔdbpAB/dbpAB* was separated in SDS-PAGE and transferred on nitrocellulose membranes. The membranes were probed with the sera of *ΔdbpAB/dbpAB* and *ΔdbpAB* infected mice (1:1000 dilution) as positive controls. Sera (1:100 dilution) of mice immunized (see materials and methods for details) with the joint proteins prepared from uninfected (inf-), *ΔdbpAB/dbpAB* infected, *ΔdbpAB* infected, *ΔdbpAB/dbpAB* infected and ceftriaxone treated, and *ΔdbpAB* infected and ceftriaxone treated mice were used to probe the membranes. The serum of the mouse receiving adjuvant only was used as a negative control.

The antibiotic treatment at two or six weeks of the infection did not inhibit the development of IgG antibodies against the whole *Bb*ss antigen in the *ΔdbpAB/dbpAB* infected mice (III: Figure 3A and B). There was a statistically significant increase in the antibody levels from two to 15 weeks in both the untreated and treated mice (at two week time point) ($P \leq 0.001$) (III: Figure 3C). To the contrary, there was a difference in the antibody levels in the sera of *ΔdbpAB* infected and untreated mice compared to the treated mice (III: Figure 3A and B). The antibody levels remained the same in the sera of the *ΔdbpAB* infected mice at two weeks of infection and in the sera of infected and treated mice at 15 weeks of infection if the mice had received ceftriaxone at the two week time point (III: Figure 3D). In contrast, the antibody levels *ΔdbpAB* infected mice increased significantly from two to 15 weeks if the mice were not treated ($P = 0.027$) (III: Figure 3D).

Taken together, these results show that Dbps are needed to promote *Bb*ss DNA persistence, specifically in mouse joints after antibiotic treatment. However, antibiotic treatment appears to abolish the joint swelling and spirochetemia of *ΔdbpAB/dbpAB* infected mice. The relevance of borrelial DNA persistence after antibiotic treatment in the mice has raised an intense discussion in the scientific community. Alan Barbour, a pioneer in the LB field, has proposed a scheme for how the different forms of *Borrelia* persist after antibiotic treatment (Barbour, 2012). In this scheme, there are five different forms of the bacteria or its remnants, and in only two of them can the bacteria replicate and be cultured from the tissue samples. In the last three forms, the bacteria or their remnants are no longer viable, but borrelial DNA and/or antigens still persist in the infected mammal. In addition to persisting borrelial DNA and antigens, the mRNA of multiple *Bb*ss genes of non-cultivable bacteria was detected 12 months after ceftriaxone treatment in the mice (Hodzic et al., 2014). However, the results from these animal studies should not be translated directly to clinical management of human LB, and more studies are still needed to reveal the molecular mechanisms of this phenomenon.

In conclusion, the results of the mouse experiments show that DbpA and B of *Bb*ss promote a fast and large-scale dissemination of Lyme borreliosis in mice. This, in turn, induces arthritis development and leads to post-treatment persistence of bacterial DNA in mouse joints.

5.4 Limitations of the study

The adhesion properties of DbpA and B molecules of three different *Bb*sl genospecies were studied using different experimental set ups, and the role of these adhesins in the pathogenesis of LB was studied in a murine model. This study has two main limitations. First, more DbpA and B adhesins of different *Bb*sl isolates could have been used in the adhesion assays. In Study I, the binding of 13 different wild type *Borrelia* strains to decorin was studied (I: Figure 6), and three *Borrelia* isolates, *Bb*ss N40, *Bg* 40 and *Ba* A91, were chosen to be the representative strains of the three different

Borrelia genospecies. The adhesion assays with individual Dbps revealed that the DbpA of *Bb*ss N40, which we had in our lab, exhibited only weak interactions with decorin. However, the DbpA of *Bb*ss strain N40-D10/E9 or B31 bind decorin and GAGs clearly (Benoit et al., 2011; Lin et al., 2014a). The use of these particular DbpA molecules in the flow chamber assay may have demonstrated flow resistant adhesion properties of these adhesins as well.

Second, the presence of borrelial mRNA could have been studied in the joint samples of *Bb*ss infected and antibiotic treated mice. As a matter of fact, we collected tissue samples for RNA isolation in the last mouse experiment. The samples were stored in RNA later® solution (Qiagen), which is standard protocol for the isolation of RNA from mouse tissue samples. However, the protocol appeared not be optimal for joint samples. The mRNA isolation was successful for the softer tissues, bladder, and ear samples, but not for the joint samples, which contained tendons and bone. The demonstration of borrelial mRNA in post-treatment mouse joint samples would have helped with the characterization of the nature of the persisting material, whether they were live bacterial cells or dead cell remnants.

6 CONCLUSIONS

Borrelia has a wide range of surface localized adhesins that mediate the bacterial adhesion to numerous host molecules and tissues. None of these molecules alone has been shown to be responsible for the infectivity of the bacteria, and many of them have redundant functions that make it challenging to study the role of individual adhesins in the pathogenesis of Lyme borreliosis. However, different knock-in and knock-out strategies in borrelial transformation enabled the identification of crucial molecules for the infectivity of the bacteria. In this study, the role of DbpA and B surface proteins in the pathogenesis of LB was assessed using recombinant Dbp molecules and genetically manipulated *Bb*ss B313 strains in a wide variety of *in vitro* adhesion assays and by infecting mice with *dbpA* and/or *dbpB* deficient *Borrelia* strains.

The adhesion studies with individual DbpA and B demonstrated that Dbps of different genospecies bind to decorin with different affinities, and in addition to decorin-binding, Dbps adhere to another proteoglycan called biglycan. Among the tested adhesins, the DbpB molecules of each genospecies interacted more with the proteoglycans than did the DbpA adhesins under stationary or flow conditions. DbpB/*Bg* appeared to facilitate most robust binding to decorin and biglycan in all of the tested experimental set-ups. Interestingly, DbpA/*Bg* interacted with decorin and biglycan only under flow conditions, thus suggesting the adhesin to be flow dependent.

The cell adhesion studies on *Bb*ss B313 derivative strains showed that the Dbps of *Bb*ss and *Bg* are able to mediate borrelial adhesion to human fibroblasts and endothelial cells. The borrelial adhesion to fibroblasts is facilitated by a Dbp-decorin interaction, while the ligand structure on the endothelial adhesion is biglycan. Interestingly, the interaction between Dbps of *Bg* and biglycan appeared to be flow tolerant.

In the mouse experiments, *dbpAB* deficient *Borrelia* showed an early dissemination defect, which can be explained by the inability of the knock-out bacteria to bind to the biglycan on the endothelial cells. In addition, DbpA and B adhesins are both needed for full arthritis development and post-treatment DNA persistence in the mice.

In conclusion, this study demonstrates the biological differences of DbpA and B of *B. burgdorferi* sensu stricto, *B. garinii* and B. *afzelii*, and reveals a greater role for the adhesins in the pathogenesis of Lyme borreliosis than previously assumed. The results of this study regarding the role of DbpA and B in the pathogenesis Lyme borreliosis are highlighted in red in Figure 10.

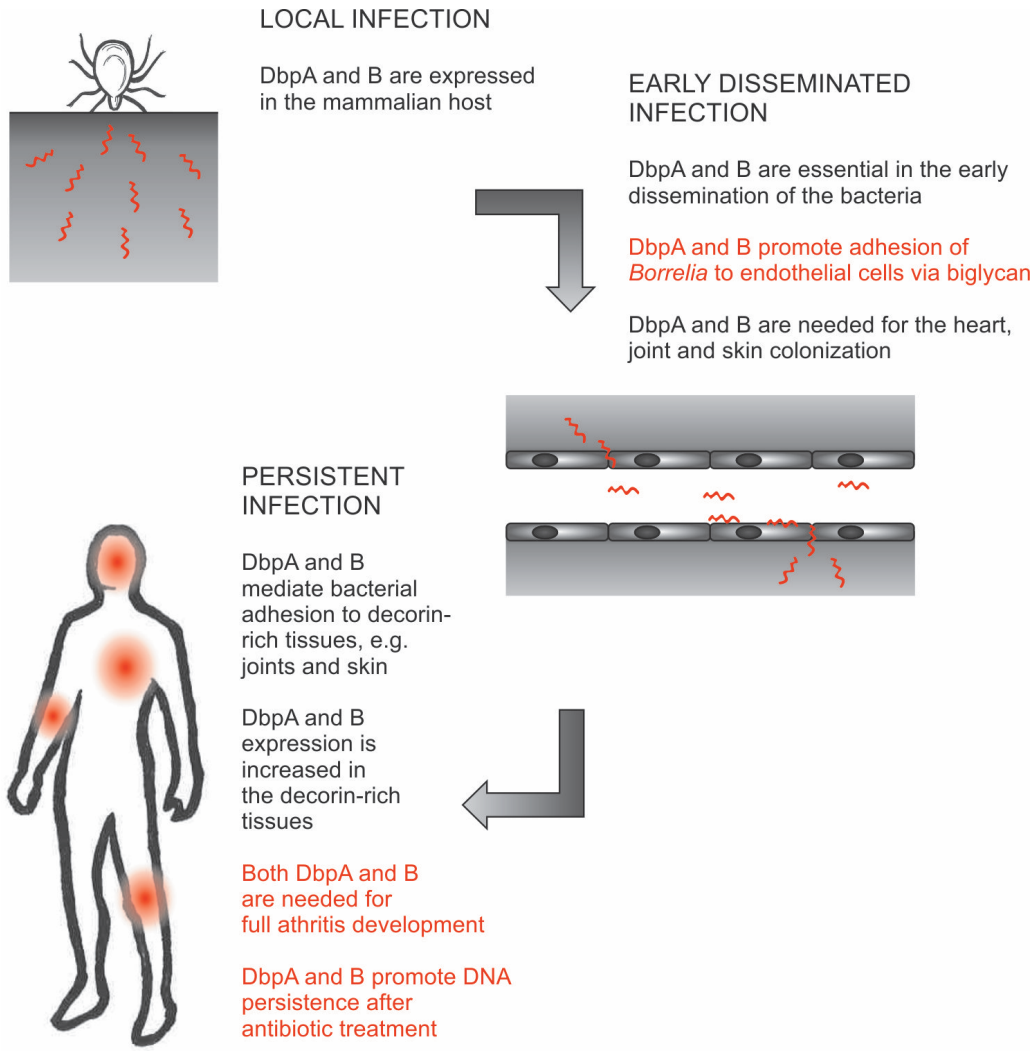


Figure 10. The role of DbpA and B adhesins in the pathogenesis of Lyme borreliosis. The putative steps where DbpA and B contribute to the pathogenesis of LB are based on *in vitro* and *in vivo* studies. The red text indicates the findings of the present study. Modified from (Coburn et al., 2013)

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