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Structural analysis of the infectious phenotype associated outer surface lipoproteins from *Borrelia burgdorferi* paralogous gene family PFam54

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Abstract

Lyme disease is a tick-borne infection caused by the spirochete *Borrelia burgdorferi*. The bacterium is transferred from infected *Ixodes* ticks to the mammalian host organism during the tick feeding process. During the blood meal of an infected *Ixodes* tick *B. burgdorferi* switches the expression of several genes in response to the mammalian host-specific signals and the occurring changes in temperature, cell density, pH and nutrient level. Many of these upregulated genes from *B. burgdorferi* are coding for the outer surface proteins, thought to be associated with the pathogenesis of Lyme disease. Potentially those proteins may assist the bacteria to transfer and to adapt to the new environment as the bacteria should be able to attach to the specific receptors/ligands in the new host organism, as well as be able to spread, proliferate and resist the host's immune response. Outer surface proteins belonging to the paralogous gene family PFam54 have showed to exhibit the highest upregulation rates in conditions mimicking the transfer process of bacteria from infected *Ixodes* ticks to the mammalian host organisms, pointing to the potential importance of these proteins in the pathogenesis of Lyme disease. Paralogous gene family PFam54 consists of nine members located on linear plasmid 54 (lp54) – BBA65, BBA64, BBA66, BBA68, BBA69, BBA70, BBA71, BBA72, BBA73 (BBA70, BBA71 and BBA72 does not produce functional proteins). Besides having the highest upregulation rates between the proteins with altered expression, it has been showed that the PFam54 family member proteins have an essential or even a vital role in the pathogenesis of Lyme disease. So for example the mutant strain lacking the expression of BBA64 could not cause the infection after a tick bite. Except BBA68, which binds complement factor H and factor H like protein 1, the exact receptors or ligands for the members of the PFam54 family are not known and the three dimensional structures of the proteins could gain a better insight into their functions and the pathogenesis of Lyme disease. Taking this into account and to promote the development of novel therapeutic and prophylactic strategies against Lyme disease, the crystal structures for BBA64, BBA66 and BBA73 proteins from the PFam54 family have been determined. The crystal structures were each analyzed to reveal the conservation of the relevant residues in the PFam54 family and to appreciate about the potential ligand/receptor binding sites.

Kopsavilkums

Laima slimība ir ērču pārnēsāta infekcijas slimība, kuras izraisītājs ir spiroheta *Borrelia burgdorferi*. Inficētu *Ixodes* ērču barošanās laikā baktērija var nonākt zīdītāja organismā. Ērcei uzsākot asins sūkšanas procesu, *B. burgdorferi* spēj izmainīt vairāku gēnu ekspresijas līmeni kā atbildes reakciju uz temperatūras, šūnu blīvuma, pH un barības vielu pieejamības izmaiņām. Daudzi no *B. burgdorferi* gēniem, kuru ekspresijā tiek novērotas izteiktas izmaiņas kodē dažādus ārējās virsmas proteīnus, savukārt šie ārējās virmas proteīni tiek saistīti ar Laima slimības patoģenēzi. Tieši ārējās virsmas proteīni var potenciāli nodrošināt baktērijas pārnesi no ērces uz zīdītāja organismu, kā arī pielāgošanos krasi atšķirīgajiem apstākļiem. Tas ir tādēļ, ka tie ir nepieciešami, lai baktērija spētu saistīties pie potenciālajiem ligandiem vai receptoriem jaunajā saimniekorganismā, spētu izplatīties, vairoties un cīnīties ar siltasiņu organisma imūnās sistēmas reakciju. Vislielāko ekspresijas pieaugumu tādos *in vitro* apstākļos, kas līdzinās inficētas ērces piesūkšanās procesam zīdītāja organismam, ir uzrādījuši paralogās gēnu ģimenes PFam54 locekļi, norādot uz šo proteīnu potenciāli svarīgo lomu Laima slimības patoģenēzē. Paralogā gēnu ģimene PFam54 sastāv no 9 locekļiem, kas novietoti uz lineārās plazmīdas 54 (lp54) - BBA65, BBA64, BBA66, BBA68, BBA69, BBA70, BBA71, BBA72, BBA73 (BBA70, BBA71 un BBA72 neveido funkcionālus proteīnus). Bez tā, ka PFam54 locekļi uzrāda visaugstāko ekspresijas līmeni starp visiem proteīniem ar izmainītu ekspresijas līmeni, vairākos pētījumos ir parādīts, ka šie proteīni ir svarīgi pilnvērtīgai Laima slimības norisei zīdītāju organismā vai pat vitāli svarīgi, lai *B. burgdorferi* spētu izraisīt saslimšanu. Tā piemēram, izmainīts *B. burgdorferi* celms, kas neekspresēja BBA64 proteīnu, nespēja izraisīt Laima slimību pēc inficētas ērces kodiena. Izņemot BBA68, kurš saista komplementa faktoru H un faktoram H līdzīgo proteīnu 1, receptori vai ligandi pie kuriem saistās PFam54 ģimenei piederošie proteīni nav vēl zināmi, tādēļ šo proteīnu trīs-dimensionālās struktūras spētu sniegt labāku ieskatu Laima slimības patoģenēzes procesos. Ņemot vērā iepriekšminēto, kā arī lai veicinātu jaunu zāļu izstrādi pret Laima slimību, tika noteiktas PFam54 ģimenes locekļu BBA64, BBA66 un BBA73 proteīnu telpiskās struktūras. Visas proteīnu telpiskās struktūras tika analizētas, lai noteiktu iespējamos konservatīvos rajonus starp PFam54 biedriem un lai izzinātu potenciālo ligandu/receptoru saistīšanās vietas.

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ABBREVIATIONS

3D – three dimensional
α-MGalDAG – α-monogalactosyl diacylglycerol
aa– amino acid
ACA – acrodermatitis chronica atrophicans
B. burgdorferi – Borrelia burgdorferi
bp – base pair
BSA – Bovine serum albumin
cDNA – complimentary DNA
CFH – complement regulator factor H
CFHL-1 – factor H-like protein 1
cp – circular plasmid
CRASPs – complement regulator-acquiring surface proteins
DNA – dezoxiribonucleic acid
ECL – enhanced chemiluminescence
EDTA – ethylenediamine tetraacetic acid
EM – erythema migrans
LB – Lyme borreliosis
LB medium – lysogeny broth
LD – Lyme disease
lp – linear plasmid
LPS – lipopolysaccharide
Ig – immunoglobulin
IPTG – isopropyl-β-D-thiogalactopyranoside
kb – kilobase
kDa – kilodalton
mRNA – messenger RNA
MAC – membrane attack complex
MAD – multi-wavelength anomalous dispersion (diffraction)
MALDI-TOF – matrix assisted laser desorption/ionization-time-of-flight mass spectrometer
MW – molecular weight
Ni-NTA – nickel-nitrilotriacetic acid
NMR – nuclear magnetic resonance
Nt – nucleotide
OD_{600} – optical density measured at a wavelength of 600 nm
ORF – open reading frame
Osp – outer surface protein
PAGE – polyacrylamide gel electrophoresis
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PDB – Protein Data Bank
PMSF – phenylmethylsulfonyl fluoride
rOspA – recombinant outer surface protein A
rpm – revolutions per minute
RNA – ribonucleic acid
RT-PCR – reverse-transcriptase polymerase chain reaction
SAD – single wavelength anomalous diffraction (dispersion)
SDS – Sodium dodecyl sulfate
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel
TAE – Tris-acetate-EDTA
TEMED – tetramethylethylenediamine
TEV – *Tobacco etch virus*
TFA – trifluoroacetic acid
WT – wild type

INTRODUCTION

Borrelia burgdorferi is the causative agent of Lyme disease that is transmitted from infected *Ixodes* ticks to a mammalian host organism during the blood meal. During the enzootic life cycle spirochete *B. burgdorferi* resides in different host organisms – *Ixodes* ticks and various mammalian organisms.

B. burgdorferi contains unusual amount of lipoproteins on the outer surface as about 8% or 150 of the coding genes from *B. burgdorferi* genome expresses lipoproteins; this is significantly higher frequency than in any other bacterial genome that is sequenced to date. Theoretically, the lipoproteins of *B. burgdorferi* can be localized in three different positions – the periplasmic leaflet of the cytoplasmic membrane, the periplasmic leaflet of the outer membrane or the outer leaflet of the outer membrane and thus located on the cell surface. As the surface-localized *B. burgdorferi* lipoproteins interact with the environment and are thought to be the major players in the pathogenesis of Lyme disease, understanding of their role, functions and structure could be invaluable step to advance the understanding of the pathogenesis of Lyme disease. Taking into account that only 6% of the *B. burgdorferi* lipoproteins have some similarities with the proteins from other organisms, and for most of them the functions are not known, the determination of the crystal structures for those *B. burgdorferi* outer surface lipoproteins could be very valuable. From all those approximately 150 lipoproteins found in *B. burgdorferi* the crystal structures have been determined only for 15 proteins. Several studies have shown that *B. burgdorferi* has managed to switch the expression of different genes in response to a mammalian host-specific signals, pH and temperature shift or cell density changes.

B. burgdorferi genes coding for the outer surface lipoproteins BBA64, BBA66 and BBA73 are the members of the paralogous gene family PFam54 located on the 54 kb linear plasmid (lp54) which is one of the twelve linear plasmids of *B. burgdorferi*. It has been showed by several studies that the respective proteins expressed by the genes located on lp54 play an important role in the host adaptation of *B. burgdorferi* and are associated with LD pathogenesis. The important role of these proteins can be further explained by the fact that during the temperature shift *in vitro* from 23°C to 35°C resembling the temperature change during the transfer of spirochete from ticks to a warm blooded animal and in a response to the mammalian host-specific signals, the proteins produce the highest upregulation rates among all the borrelial proteins.

Recent studies have revealed that BBA64 has a vital role in the transfer of *B. burgdorferi* from infected *Ixodes* ticks to the host organism after the tick bite. Also the outer surface protein BBA66 is important in the pathogenesis of Lyme disease as the inactivation of the gene has showed that the protein is necessary for the transmission of *B. burgdorferi* from infected *Ixodes* ticks to the mammalian host organism as the transmission of altered bacteria was significantly impaired in comparison to WT bacteria.

The aim of this study was to solve the 3D structures of the outer surface proteins from PFam54 family as that could gain more understanding of how exactly the paralogous gene family PFam54 coded proteins ensure the transfer of *B. burgdorferi* from ticks to the mammalian host and the structures could be potentially used for drug design to stop the transmission of the spirochete. The three-dimensional structures have been solved for BBA64 at 2.4 Å, BBA66 at 2.25 Å and BBA73 at 2.09 Å (also for a truncated version of BBA73 at 1.88 Å). The crystal structures have been compared to each other to reveal the overall structural similarities between the homologous proteins, the essential residues necessary for the overall fold conservation and the electrostatic properties of the molecules.

Work that needs to be done to achieve the goal:

1. Choosing of the optimal expression vector and expression system and PCR amplification of the genes coding for the outer surface proteins;
2. Cloning and expression of the recombinant outer surface proteins in the bacterial expression system;
3. Protein purification by immobilized metal ion affinity and ion-exchange chromatography and optimization of the purification to obtain >90% pure protein for crystallization trials;
4. Crystallization of the purified proteins, optimization of the favourable condition variants and collection of the protein crystal diffraction data by using an X-ray source;
5. Determination of protein 3D structures;
6. Comprehensive analysis of the protein tertiary structures.

1. LITERATURE REVIEW

1.1. Lyme disease

Lyme borreliosis as a disease was first recognized in 1975 by Dr. Allen Steere in area of Lyme, Connecticut, USA where a number of children had developed symptoms thought to be related with juvenile rheumatoid arthritis (Steere et al., 1977b). The investigation for the reasons of numerous occasions of juvenile rheumatoid arthritis in the area of Lyme was started after two mothers reported about the symptoms to the State Health Department of Connecticut. The investigation was started by Dr. Allen Steere together with Dr. Stephen Malawista and Dr. David Snydman and the study revealed an epidemic of oligoarticular arthritis in the area of Lyme. Because the prevalence of juvenile rheumatoid arthritis was almost 100 times greater than the expected value and serologic tests were not positive for agents known to cause arthritis the disease was categorized as previously unrecognized clinical entity and it was named Lyme arthritis (Steere et al., 1977b). Already at that time it was suggested that in the transmission of the disease some arthropod vector may be involved. Further investigation revealed that there is some connection between Lyme arthritis and erythema chronicum migrans (Steere et al., 1977a). The skin lesion was characterized as a papule that expanded to form a large ring with central clearing and usually was observed for about 3 weeks. More than half of the patients with erythema migrans developed a monoarticular or oligoarticular arthritis after 4 days to 22 weeks after onset of the skin lesion (Steere et al., 1977a). Erythema migrans was recommended as a diagnostic marker for Lyme arthritis. By studying Lyme arthritis it was recognized that in Connecticut there was almost 30-fold difference in cases between the communities in the east side and west side of the Connecticut river (Steere et al., 1978). At that time it was also showed that *Ixodes scapularis* ticks were much more common on the east side of the Connecticut river where the incidence of Lyme arthritis was much higher that on the west side (Wallis et al., 1978). Also the ticks were found more frequently on humans as well as on dogs and cats in the eastern communities. Relationship between the *Ixodes* ticks and Lyme disease was revealed although the identification of the exact aetiological agent was unsuccessful at this stage.

1.1.1. Epidemiology and tick vectors of Lyme disease

B. burgdorferi is maintained in nature in a complex enzootic life cycle involving *Ixodes* ticks and different mammalian organisms (Anguita et al., 2003; Pal and Fikrig, 2003) **(Figure 1.)**.

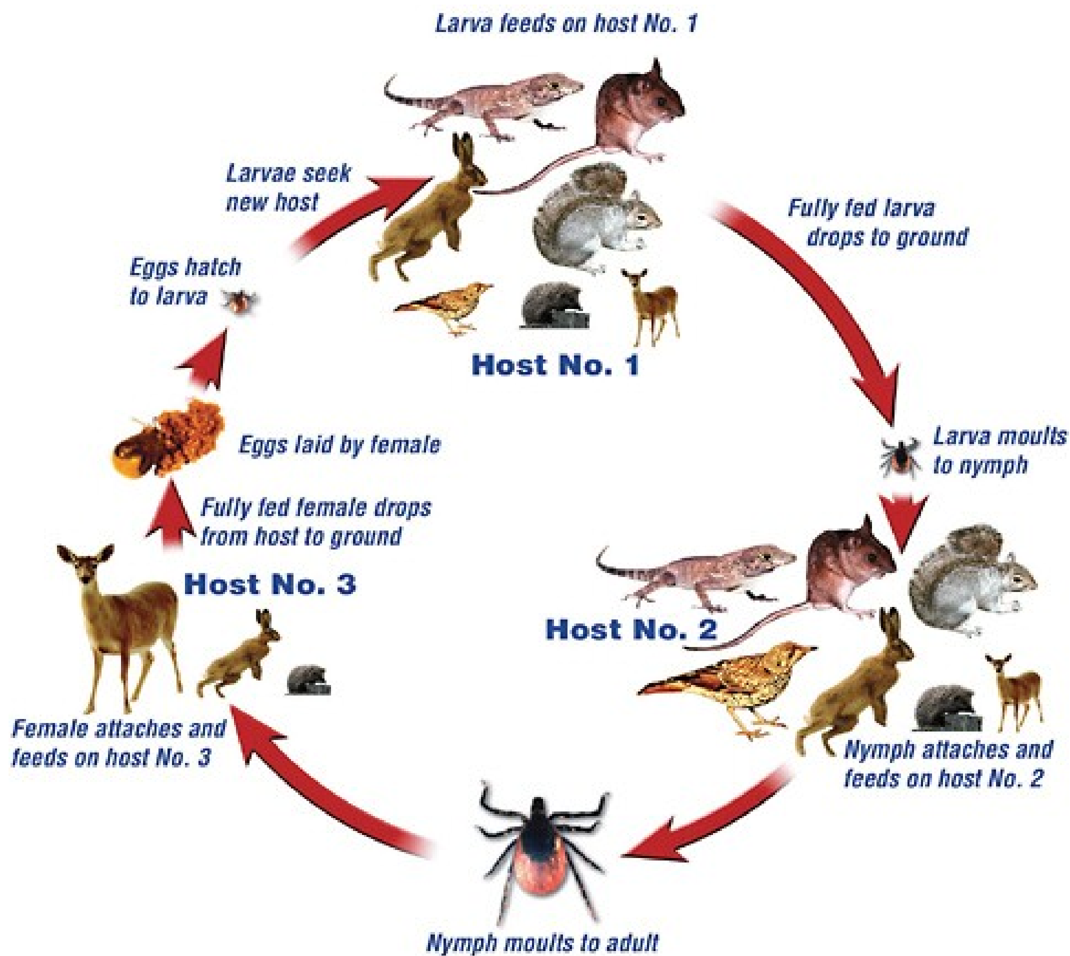

Figure 1. Ticks life cycle. The picture has been adapted from www.shelter-island.org.

The main vectors that transmit the causative agent of Lyme disease are *Ixodes ricinus* ticks in Europe, *I. persulcatus* in Asia and *I. scapularis* and *I. pacificus* in North America. The ticks have three different stages – larvae, nymphs and adults and at each stage the ticks should have a blood meal **(Figure 2.)**.

Figure 2. *Ixodes scapularis* ticks showing the three stages of their lifetime – larva, nymph and adult (male and female). The picture has been adapted from Tick encounter Resource Center (tickencounter.org).

Larvae and nymphs feed mostly in spring and summer on small or medium-sized animals like rodents and birds, while adults feed mostly in autumn on larger host animals like deer until the daytime temperature remains above freezing (Anderson, 1989a; Anderson et al., 1987; Matuschka et al., 1991). It should be mentioned that adult males generally do not take a blood meal but are necessary to fertilize the adult females. Adult females usually can be found questing about knee-high on the tips of the branches of low growing shrubs and can readily attack humans and pets. Once they have finished the blood meal they drop off the host and can over-winter by hiding under the old grass or leaves (Anderson, 1991). Next year they lay a mass of eggs and then die. In the late summer larvae appears from the eggs. *B. burgdorferi* cannot be transferred transovary by *Ixodes* ticks, therefore the ticks obtain the spirochete from mammals during the blood meal which can be transferred to other mammalian, including humans, during the following blood meal (Rollend et al., 2013). As larvae can be found usually on grass, they typically attaches to small mammals, such as mice (Anderson, 1989b). Once larvae has finished the blood meal on some mammalian host organism it detaches and molt, re-emerging the following spring as nymphs. After nymphs attach and blood feed on animals in spring and summer, they drop off and emerges as adults in the autumn (Anderson, 1989b). The seasonal character of Lyme borreliosis in the accordance with the enzootic life cycle of *Ixodes* ticks is showed in **Figure 3.**

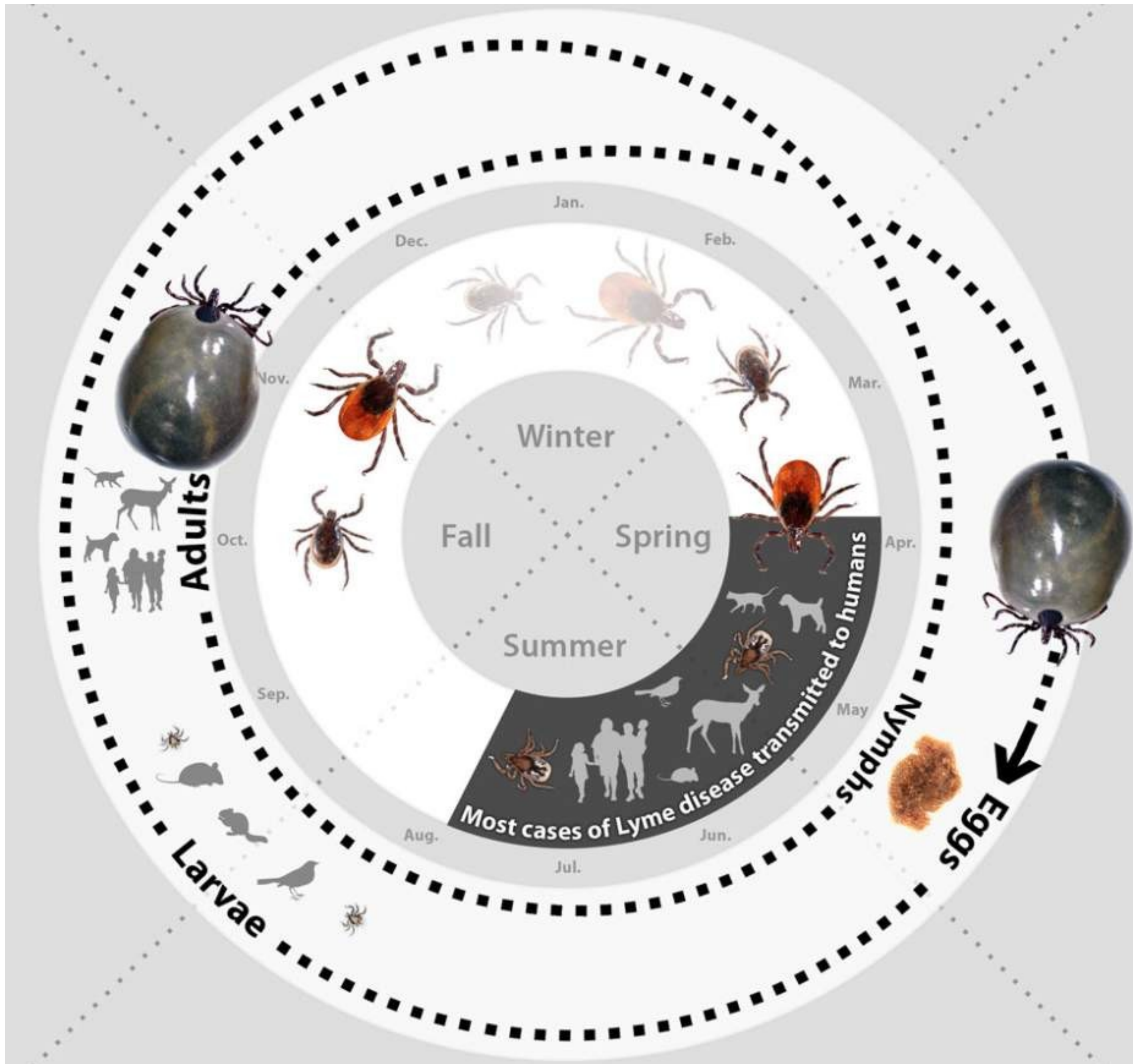

Figure 3. Seasonal character of Lyme disease accordance to the enzootic life cycle. Picture adapted from Tick encounter Resource Center (tickentouner.org).

The proportion of infected *Ixodes* ticks that carries Lyme disease agent *B. burgdorferi* varies between 15% and 40% and the value can be different between the countries (Wang et al., 2003). When *Ixodes* tick has attached to the host organism it should stay attached for at least 24 h to transfer the spirochetes to the new host organism and with the highest chance to get transferred at the third day (des Vignes et al., 2001; Piesman et al., 1987). Therefore, when an *Ixodes* tick infected with Lyme disease spirochetes bite some animals, like humans and possibly escape detection by the victim and feed long enough so that the infection can be transmitted, the Lyme disease can be acquired.

Lyme disease is common infection in European countries, especially in central Europe, Scandinavia and Eastern Europe were forests and grassland dominates (Barbour and Fish, 1993; Steere et al., 2004). In Latvia approximately 400-800 new cases of Lyme disease have been identified each year while the incidence varies between the countries **(Table 1.)**.

Country	2008		2009		2010		2011		2012	
	Total No	Per 100 000	Total No	Per 100 000	Total No	Per 100 000	Total No	Per 100 000	Total no	Per 100 000
Latvia	488	21.5	720	31.8	829	36.9	866	38.8	724	35.4
Estonia	1423	106.0	1787	133.3	1721	128.4	2303	171.8	1546	115.4
Lithuania	1153	34.0	3600	106.6	2558	76.6	2429	73.9	2440	81.5
Finland	1277	24.1	1466	27.5	1444	27.0	1662	30.9	1588	29.4
Norway	345	7.2	273	5.6	288	5.8	247	4.9	255	5.1
Poland	8248	21.6	10 329	27.1	9003	23.6	9170	23.8	8791	22.8

Table 1. Total number of cases and incidence per 100 000 inhabitants of Lyme disease in the Baltic States and other European countries from 2008 to 2012. The epidemiological data are taken from EpiNorth network project for communicable disease control in Northern Europe (http://www.epinorth.org/).

1.1.2. Pathogenesis of Lyme borreliosis

B. burgdorferi can be transmitted to the mammalian host organism only after a tick bite. When an *Ixodes* tick after attachment to the host organism starts the blood meal it causes the changes in the pH, temperature and nutrient levels in the tick midgut which in turn switches the expression of different genes in *B. burgdorferi* (Brooks et al., 2003; Ojaimi et al., 2003; Revel et al., 2002; Tokarz et al., 2004). *B. burgdorferi* starts to replicate rapidly in the ticks midgut during the blood meal by increasing the number of bacteria by more that 300-fold after 72 h of feeding (De Silva and Fikrig, 1995; Piesman et al., 1990). The changes in the gene expression profiles upregulates different outer surface proteins of *B. burgdorferi* thought to play an important role in the transmission process, dissemination, chemotaxis and fight against the host's immune response (Ojaimi et al., 2003; Tokarz et al., 2004). After about 36 h of feeding,

spirochetes have migrated from the ticks midgut to the salivary glands also due to the changes in the expression of several outer surface proteins including OspA and OspC (de Silva et al., 1999; Pal et al., 2004b; Tilly et al., 2013). Upregulated OspC protein binds to a tick saliva protein Salp15 and the binding may not only drag the bacteria from the midgut to the salivary glands but also may protect against anti-OspC immunoglobulin binding or interfering with $CD4^+$ T-cell activation (Hovius et al., 2007; Hovius et al., 2008; Ramamoorthi et al., 2005). OspC protein is also important for initial colonization of the host organism in the mouse model (Radolf and Caimano, 2008). Besides OspC many other proteins are necessary for the transmission process including BBA64 and BBA66 (Gilmore et al., 2010; Patton et al., 2013a).

After the transmission of *B. burgdorferi* from ticks to mammalian host organism, the spirochetes continue to proliferate in the dermis reaching high numbers (Liveris et al., 2002). Further adhesion to different host cells and extracellular matrix by *B. burgdorferi* is thought to be very important to initiate the infection and has been showed that the spirochete can bind a variety of tissues and disseminate through the organism (Coburn et al., 2005; Patton et al., 2011; Steere et al., 2004).

1.1.3. Diagnosis of Lyme disease

Diagnosis of Lyme disease is based on a history of tick bite, clinical signs and erythema migrans (Wormser et al., 2006). Although diagnostic tests and laboratory diagnosis can be applied to confirm the infection, it is thought that serological testing can be too insensitive in the first two weeks of infection to be helpful diagnostically (Aguero-Rosenfeld et al., 2005; Nowakowski et al., 2001; Tugwell et al., 1997). In cases for which there is uncertainty serum samples should be tested following a two-step procedure: a sensitive ELISA (enzyme-linked immunosorbent analysis) followed by Western-blot (IgM and IgG) analysis (Wilske et al., 2007) **(Table 2)**. In the first month of Lyme disease infection IgM response gives highest sensitivity but at later stage detection of IgG should be conducted. Two to four weeks after infection, immunoglobulin of the IgM class are found, reaching a peak at six weeks, and their titer drops after 4-6 months. While IgG antibodies occur at six to eight weeks after the infection and remains for many years after the infection displaying a slow decay in titer as determined by ELISA or Western-blot method. False-positive results are found mainly with IgM antibodies, which appear in persons suffering from other bacterial diseases (Porwancher et al., 2011).

ELISA IgM antibody	ELISA IgG antibody	Western blot	Likely Interpretation
Positive	Positive	Positive	Likely Lyme disease
Positive	Negative	Negative	Early infection or false positive
Negative	Positive	Positive	Late or previous infection
Negative	Negative	(usually not performed if IgM and IgG are negative)	No infection present; symptoms may be due to another cause or antibody levels too low to detect

Table 2. Strategy to interpret the serodiagnostic results obtained from ELISA and Western blot analysis for Lyme disease.

A wide range of other methods have been developed for the direct detection of *B. burgdorferi* in the clinical samples, such as PCR, microscopy, detection of different *B. burgdorferi* proteins and nucleic acids and cultivation (Barbour, 1984; Berger et al., 1995; Goodman et al., 1995).

Dark-field microscopy in general can be used only when there are a large number of spirochetes expected, like in the suspensions of tick midguts or spirochetes grown in culture media (De Silva and Fikrig, 1995; Piesman et al., 1990). The diagnostic value of microscopy in the clinical laboratory is limited as the density of the bacteria cells in the clinical samples is very low.

To grow *B. burgdorferi* in culture, a specific medium in necessary that was developed over time gaining different modifications and according to the exact composition is called Barbour-Stoenner-Kelly II medium (BSKII), BSK-H or MKP (Barbour, 1984; Kelly, 1971; Pollack et al., 1993; Preac-Mursic et al., 1986). Disadvantage for research and diagnosis purposes is that the cultures should be incubated for up to 12 weeks which is much longer period that for most other bacterial pathogens (Barbour, 1984).

Different kind of PCR techniques, like standard PCR, nested PCR and real-time PCR can be used for diagnostic purposes, although amplification of *B. burgdorferi* DNA by PCR is not usually applied in a routine clinical care because of the nature and expenses

of those methods and the accuracy is not as high as for ELISA and Western blot method (Aguero-Rosenfeld et al., 2005; Dumler, 2001).

1.1.4. Clinical manifestations

Lyme disease is a multi-systemic infectious disease with a diverse clinical picture and can be divided into three stages regarding the clinical observations (Steere, 2001). The first stage is characterized as erythema migrans, the second stage as neurological or cardiac manifestation and the third stage as arthritis (Steere, 2001). Although it should be kept in mind that the progression of the disease can be variable between the patients, the common thing is that the disease affects mainly the skin, nervous system, heart and joints (Wormser et al., 2006).

It is concluded that erythema migrans is the most common dermatological manifestation of Lyme disease (Dandache and Nadelman, 2008; Steere, 2006; Wormser et al., 2006). Erythema migrans is usually described as an expanding, annular, erythematous skin lesion with central clearing, also called a "bull's eye rush" (Steere, 2001) **(Figure 4a)**. For the majority of patients erythema migrans develops at the site of tick inoculation within 7 to 14 days after detachment and approximately 80% of patients with Lyme disease develops erythema migrans (Aucott et al., 2012; Steere and Sikand, 2003). To distinguish erythema migrans from some other dermatological manifestation, like some inflammatory reactions that usually develops at the site where an insect bites, it has been set that the minimal diameter should be 5 cm, although the average size for erythema migrans is 15 cm (Wormser et al., 2006). While the "traditional" erythema migrans lesion was described as having a central clearing, many studies in Europe and USA have showed that with *B. burgdorferi* (most commonly found in USA) the central clearing can be found only in 9% cases (Smith et al., 2002) **(Figure 4b)**. The ability to cause erythema migrans at all is also different between the borrelial strains (Tijsse-Klasen et al., 2013). Erythema migrans is usually accompanied by a variety of symptoms such as arthralgia, malaise, fatigue, headache and flu-like symptoms (Nadelman et al., 1996; Wormser et al., 2006). Although in those approximately 20% cases when erythema migrans does not develop the other symptoms can still persist (Steere and Sikand, 2003).

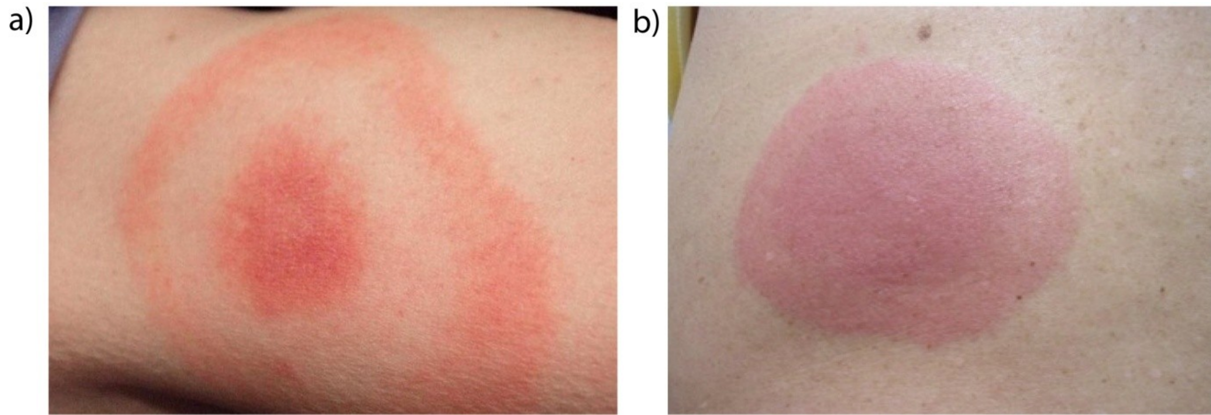

Figure 4. Erythema migrans. (a) Erythema migrans with a typical “bull’s eye rush” and (b) with a continuous surface. Pictures adapted from Lymepedia.org.

Besides erythema migrans two other dermatological manifestations that are less common can be found between Lyme disease patients – borrelial lymphocytoma and acrodermatitis chronicum atrophicans. Borrelial lymphocytoma is the least common of the three dermatological manifestations (Picken et al., 1997). Lesions typically occur 20 to 40 days after a tick bite but may appear also months later (Asbrink and Hovmark, 1988). It appears like a solitary bluish-red nodules varying from one to few centimetres in diameter and occurrs most frequently on the earlobes in children and the nipple or areolar regions in adults while not so frequently may be found in genital regions, trunk and extremities (Colli et al., 2004; Gissler and Heininger, 2002; Strle et al., 1992) **(Figure 5)**. Borrelial lymphocytoma may be difficult to differentiate from lymphoma (Colli et al., 2004).

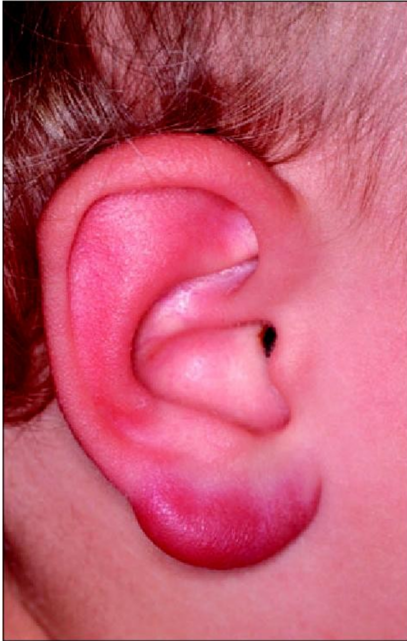

Figure 5. Borrelial lymphocytoma. Picture adapted from (Mullegger, 2004).

Acrodermatitis chronicum atrophicans (ACA) is a late cutaneous manifestation of Lyme disease occurring months to years after inoculation of untreated infection (Asbrink and Hovmark, 1988; Duray et al., 1989). ACA in appearance is similar to acute oedematous phase of scleroderma with a characteristic swelling of soft tissue and ACA is primary associated with *B. afzelii* infection in Europe (Ohlenbusch et al., 1996).

Weeks to years after the untreated infection different neurological complications may occur affecting different parts of the central and peripheral nervous system (Halperin, 2008). The first symptoms are usually observed several weeks after a tick bite and are associated with meningitis, cranial neuritis, encephalomyelitis, encephalopathy and axonal polyneuropathy (Halperin, 1989; Halperin, 1995). Neuroborreliosis is thought to be associated with *B. garinii* and *B. afzelii* strains that are common in Europe while in USA *B. burgdorferi* sensu stricto is the main strain associated with Lyme disease and therefore neurological symptoms are common solely in Europe (Strle and Stanek, 2009; Strle et al., 2006). It should be mentioned that neuroborreliosis in Europe is found in less than 10% untreated patients (Strle et al., 2006).

Cardiac manifestations are also found in untreated patients but as in the case with neuroborreliosis the number of patients showing the respective symptoms is below 10% (Sigal, 1995). The symptoms can occur weeks to years after the tick bite and result in heart block and muscle dysfunction that can be cured by an antibiotic course (Sigal,

1995). Possibly because nowadays the Lyme disease is recognized and cured more effective, the cardiac manifestations have become quite rare and only individual cases have been reported (Karmacharya and Aryal, 2013; Koene et al., 2012).

Lyme arthritis is a common outcome for patients with untreated Lyme disease (Steere, 2001). As in the case with cardiac manifestations and neuroborreliosis, Lyme arthritis is a complication of untreated Lyme disease in less than 10% (Sigal, 1998). Lyme arthritis, in contrary to neuroborreliosis is found mainly between the patients in USA because the symptoms are associated with *B. burgdorferi* sensu stricto that is the strain found mainly in USA but less than 10% in Europe (Strle and Stanek, 2009; Vogerl et al., 2012). Lyme arthritis usually affects the joints, especially the knees and is characterized by joint inflammation with swelling large effusions and little pain (Puius and Kalish, 2008).

1.1.5. Treatment of Lyme disease

For treatment of Lyme disease doxycycline (100 mg twice per day), amoxicillin or cefuroxime axetil (500 mg 3 times per day) for 14-21 days is recommended for the treatment of adult patients with early localized or early disseminated Lyme disease associated with erythema migrans, in the absence of specific neurologic manifestations (Loewen et al., 1999; Perry and Brogden, 1996; Puius and Kalish, 2008). Doxycycline is relatively contraindicated during pregnancy or lactation period and in children under 8 years of age. Antibiotics recommended for children are amoxicillin (50 mg/kg per day in 3 divided doses with maximum of 500 mg per dose) and cefuroxime (30 mg/kg per day in 2 divided doses with maximum of 500 mg per dose) (Arnez, 2007; Fingerle and Huppertz, 2007).

Macrolide antibiotics are not recommended for early Lyme disease, because in clinical trials have been found to be less effective (Donta, 2012; Hunfeld et al., 2004). When used, they should be reserved for patients who are intolerant of, or should not take amoxicillin, doxycycline or cefuroxime. For adults with these limitations, recommended dosage for azithromycin is 500 mg orally per day for 7–10 days and for clarithromycin, 500 mg orally twice per day for 14–21 days (if the patient is not pregnant), or erythromycin, 500 mg orally 4 times per day for 14–21 days (Donta, 2003). Patients treated with macrolides should be closely observed to ensure resolution of the clinical manifestations (Hunfeld et al., 2004).

1.1.6. Prevention of Lyme disease

Since the recognition of Lyme disease in 1980's many studies have been made to improve our understanding about the etiological agent of Lyme disease, its tick vector and how the disease is maintained in nature and initiated in mammalian host organisms (Murray and Shapiro, 2010). Prevention of Lyme disease is an important part of management, especially for rural residents.

Although vaccination could be the best way for prevention of Lyme disease in population, there is no vaccine on the market at the moment. *B. burgdorferi* outer surface protein OspA based vaccine developed and released on the market by SmithKline Beecham in 1998 was voluntarily withdrawn from the market in 2002 due to poor sales and several reports questioning the safety of the vaccine (Clark and Hu, 2008).

Avoiding areas thought to be at high risk, like wooden and grassy areas would be the best preventive measures (Clark and Hu, 2008; Connally et al., 2009; Murray and Shapiro, 2010). For persons living close to endemic areas additional recommendations could include to wear ligh-colored protective clothing and to perform frequent body checks for ticks (Connally et al., 2009). Use of different tick and insect repellents can be recommended, especially repellents containing DEET (diethyl-3-methylbenzamide) followed by picaridin (KBR 3023) for which it has been showed to reduce the insect bites by 85% at 1 h after application (Costantini et al., 2004; Pretorius et al., 2003) **(Figure 6.)**.

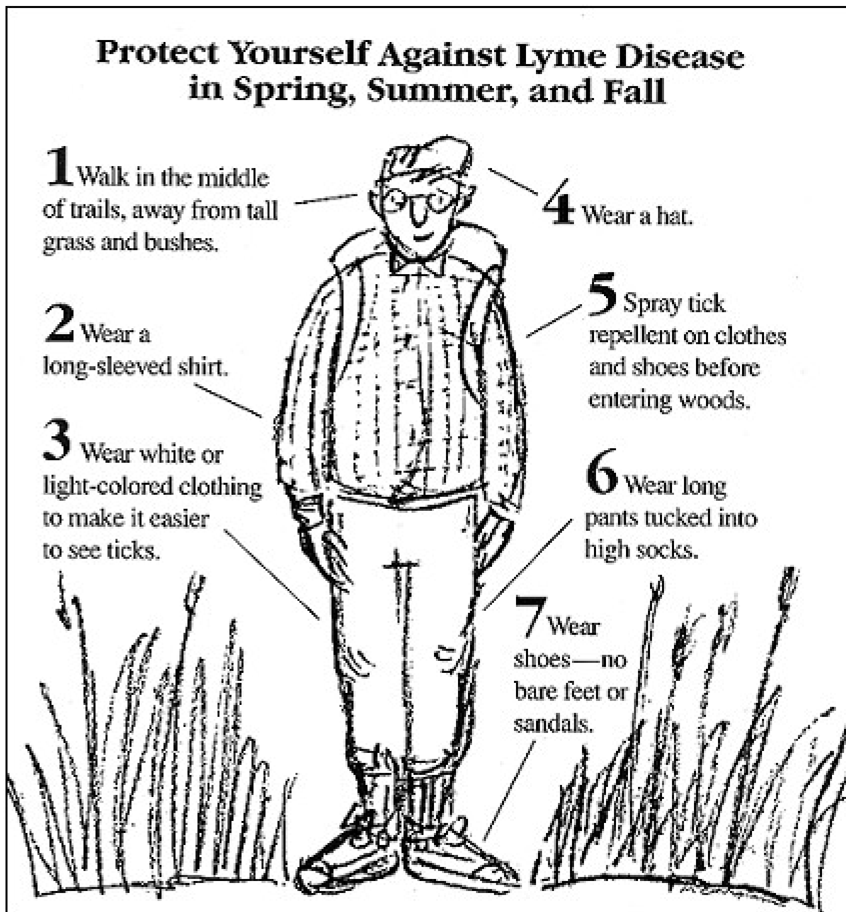

Figure 6. Protective measures to use to prevent Lyme disease. Picture adapted from Centers for Disease Control and Prevention (www.cdc.gov) bulletin.

Removal of ticks within 24 h of attachment can usually prevent acquisition of Lyme disease (Piesman et al., 1987). To remove already attached tick fine-tipped forceps are recommended for tick removal by grasping the tick as close as possible to the skin without compressing the ticks body too much (Duscher et al., 2012; Roupakias et al., 2011).

If the tick has been attached already for 36 h or longer, in areas with high rates of tick infection (more than 20%), a single dose of 200 mg doxycycline during the next 72 h after tick removal may be considered as a recommendation as it was showed to decrease the incidence (Bratton et al., 2008; Nadelman et al., 2001; Piesman and Hojgaard, 2012). This kind of prophylaxis is not recommended for persons who are contraindicated to doxycycline, for example, pregnant women and children younger than eight years (Nadelman et al., 2001).

Removal of leaf litter and mowing can result in significant reduction in tick numbers from 70% to almost 100% (Schulze et al., 1995). Also controlled burning of last year's vegetation can reduce the ticks population that is quite common thing in Latvia although assigned as an illegal activity (Stafford et al., 1998).

1.1.7. Vaccine development against Lyme disease

Vaccination against the infection is a highly effective way to control the spread of the disease in the population. However, at the moment there are no commercially available vaccines to prevent Lyme disease in humans. Although in 1998 there was a vaccine developed for the USA market by SmithKline Beecham (now called GlaxoSmithKline) based on the outer surface protein A (OspA) from *B. burgdorferi* and named as LYMErix. The vaccine contained lipidated OspA absorbed on aluminium hydroxide adjuvant in phosphate-buffered saline with phenoxyethanol as a preservative (Embers and Narasimhan, 2013). OspA protein was obtained by recombinant techniques in *Escherichia coli* cells and after the expression and purification the protein was attached with the N-terminus to the lipid moiety. Three doses of 0.5 ml of rOspA vaccine was administered intramuscularly at 0, 1 and 12 months for optimal protection and the vaccine was intended for persons 15-70 years of age. With time there appeared several reports to the Vaccine Adverse Event Reporting System (VAERS) that monitors vaccine safety, suggesting that the vaccine could cause arthritis, arthrosis, arthralgia, myalgia and autoimmune responses (Lathrop et al., 2002; Rose et al., 2001). Increasing number of reports led to anti-vaccine attitude, several lawsuits and decreased support from physicians therefore decreasing the turnover of the company that resulted in withdrawn of the vaccine from the market in 2002 (Poland, 2011).

Interesting fact about the OspA vaccine is the manner how the vaccine protects from the infection. It was known that the patients with Lyme disease does not produce antibodies against OspA and also it became evident that OspA protein is downregulated as the tick begins to feed on the host organism (Schwan et al., 1995). This paradox was explained by showing that the vaccine prevents the transmission of the bacteria from ticks to mammalian host as the antibodies against OspA produced after the vaccination eliminates the spirochete already in the feeding tick (de Silva et al., 1996; Fikrig et al., 1992).

Several new vaccine candidates have been identified in the recent years (Floden et al., 2013; Livey et al., 2011; Poljak et al., 2012; Probst et al., 2012). Different approaches can be used to make an effective vaccine and to prevent Lyme disease **(Figure 7.)**. A goal for an effective vaccine would be to block the transmission of the pathogen from the infected *Ixodes* ticks to the mammalian host, therefore an appropriate protein from *B. burgdorferi* that plays a significant role in the transfer process and could be potentially blocked should be found. Besides this approach to control the infection rates also other possibilities exist. It is also possible to vaccinate the reservoir host, for example, white-footed mouse, to decrease the acquisition of *B. burgdorferi* by *Ixodes* ticks (Anderson, 1989a; Levine et al., 1985). It was showed that bait containing rOspA protein that was given to *Peromyscus leucopus* mice produced an antibody response and therefore significantly reduced the prevalence of *B. burgdorferi* in nymphs (Meirelles Richer et al., 2011).

Another way would be to affect the tick vector to stop the transmission of several tick-related pathogens, including *B. burgdorferi*. A potent approach could be to use chemicals that are toxic to the vector (Carroll et al., 2009; Raghavendra et al., 2011; Rosario-Cruz et al., 2009). Para-transgenic approaches could be used to modify the vectors ability to carry the pathogens or to reproduce (Aksoy et al., 2008; Hurwitz et al., 2011). Also vaccines that are developed to target different vector antigens that are critical for the vector could be used (de la Fuente et al., 2011; Mathias et al., 2012; Parizi et al., 2012).

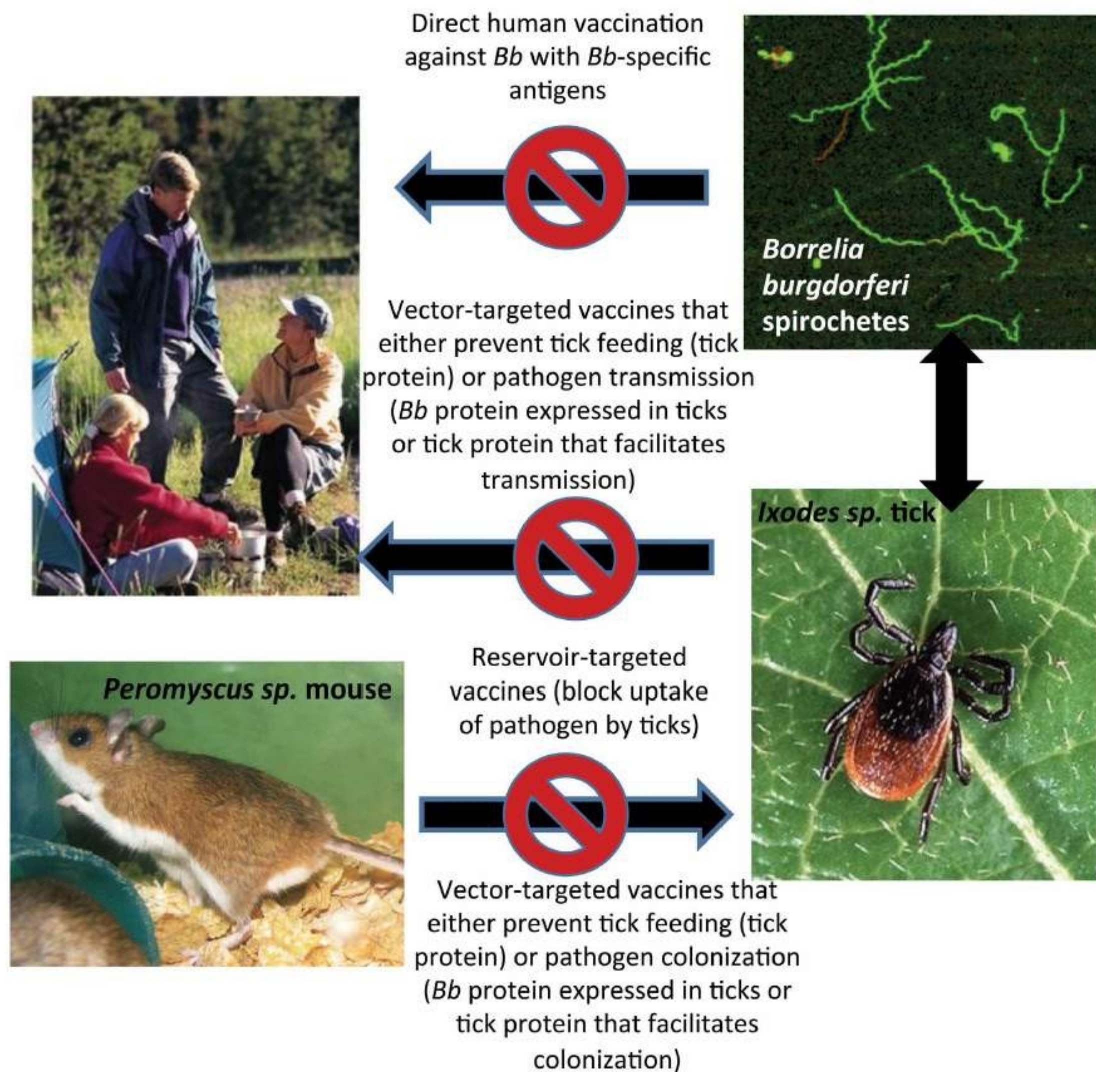

Figure 7. Steps at which interruption of *B. burgdorferi* transmission to humans can be achieved through vaccination. Figure adapted from Embers and Narasimham, 2013.

1.2. Borrelia burgdorferi

B. burgdorferi is a spirochete discovered in 1982 by Willy Burgdorfer (Burgdorfer et al., 1982). Before the discovery of *B. burgdorferi* much work was done by Dr. Steere and Dr. Malawista in the area of Lyme, Connecticut, USA, to describe the symptoms of Lyme disease, including the erythema migrans (Steere et al., 1977a; Steere et al., 1977b). Although the exact etiological agent of Lyme disease was not known at that time, many studies were conducted to exclude one by one the different agents, including toxins, viruses and rickettsiae that could be responsible for the disease (Burgdorfer, 1984). Viruses and toxins were excluded from the list as possible etiological agents of Lyme disease when it became evident that Lyme arthritis can be cured by antibiotics

(Steere et al., 1980). But since it was already established that erythema migrans (the dermatological manifestation of Lyme disease) is related with a bite of an *Ixodes* tick (Steere and Malawista, 1979) the search for the agent was done by dissecting the *Ixodes* ticks to try to discover the potential agent (Burgdorfer, 1984). The ticks for the studies done by Dr. Burgdorfer were provided by Dr. Benach from Shelter Island, New York, where Lyme disease was known to be endemic (Burgdorfer, 1984).
Finally, the spirochetes were isolated from the midguts of *Ixodes* ticks and observed on dark-field microscopy and scanning electron microscopy **(Figure 8)**.

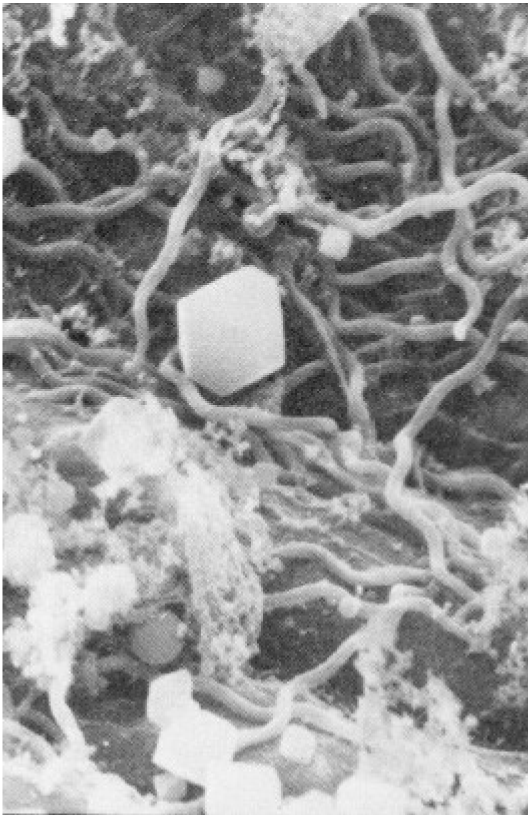

Figure 8. One of the first scanning electron microscopy pictures of Lyme disease agent *B. burgdorferi* in the midgut of *Ixodes dammini* from Shelter Island, New York. Picture adapted from Burgdorfer, 1984.

In 1983, at the first international meeting on Lyme disease at Yale university, USA, a group of researchers that were involved in the discovery and characterization of the Lyme disease spirochete agreed that the new infectious agent would be named *Borrelia burgdorferi*, after Willy Burgdorfer.

Later investigation revealed various mammalian hosts that are involved in the complex enzootic cycle to maintain the spirochetes in the nature (Anderson et al., 1983; Burgdorfer, 1984; Levine et al., 1985).

B. burgdorferi spirochete is approximately 0.33 μm in diameter and 10 to 20 μm in length, thus being 30-60 times longer than they are wide (Goldstein et al., 1996). Spirochete *B. burgdorferi* is composed from a cytoplasmic or inner membrane that encloses the cytoplasmic contents and from a fragile outer membrane whereas between the two membranes there is a peptidoglycan layer and flagella (Barbour et al., 1986; Kudryashev et al., 2009; Yanagihara et al., 1984) **(Figure 9.)**.

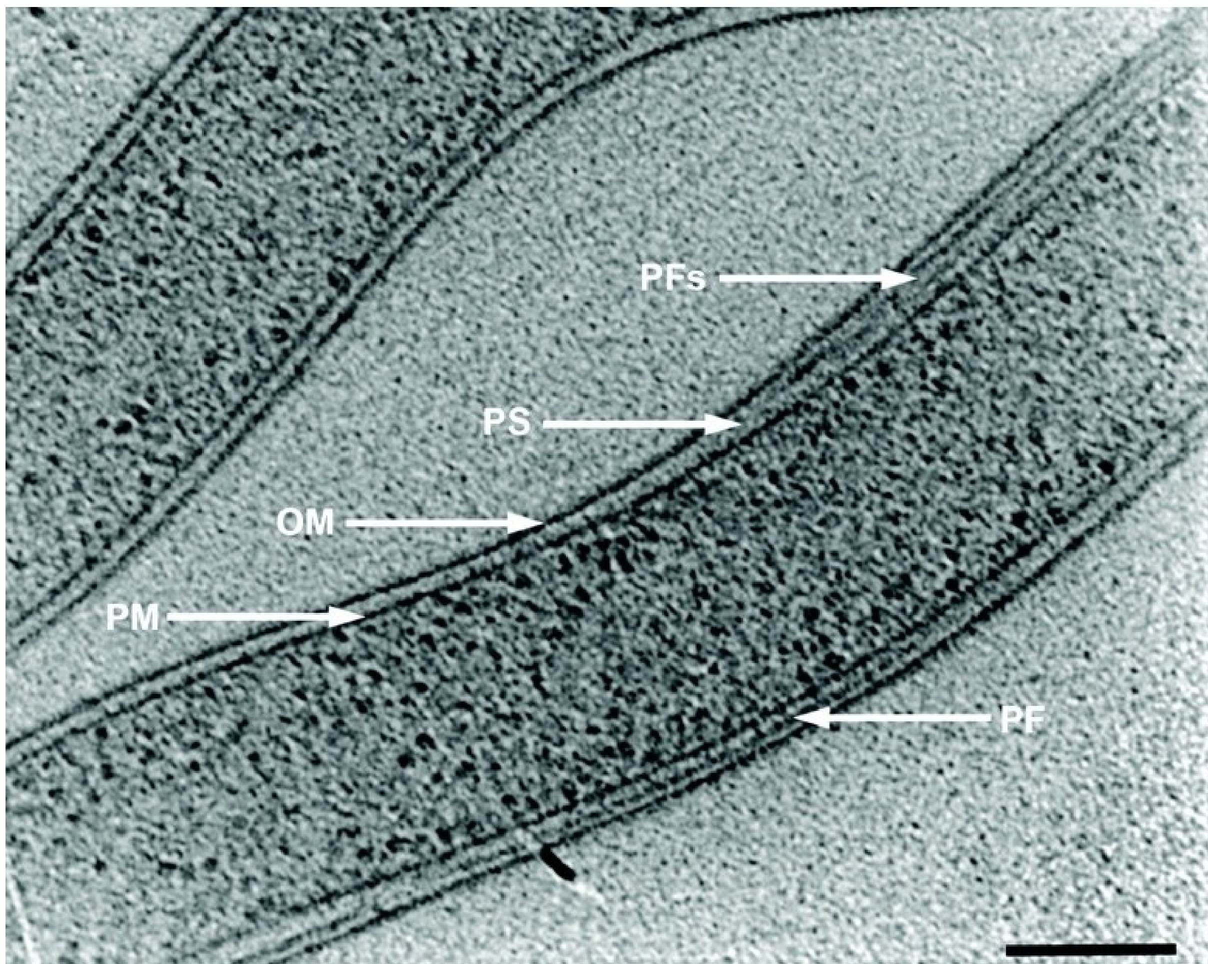

Figure 9. Electron cryotomography picture of *B. burgdorferi*. a 1.8 nm thick longitudinal slice of a cell representing the outer membrane (OM), plasma membrane (PM), periplasmic flagella (PFs), and periplasmic space (PS). Picture adapted from (Charon et al., 2009).

It should be stressed here, that the flagella, necessary for the motility of the cell is common between all species of spirochetes, but the difference between some other

bacteria that also has flagella is that in spirochetes the flagella is located in the periplasmic space between the inner and outer membranes (Charon and Goldstein, 2002; Charon et al., 2009). Flagella has a role not only in the motility of *B. burgdorferi* but also has a skeletal function as it was concluded from the studies where mutants lacking the flagella gained a straight rod like shape while normally the bacteria has flat-wave morphology (Motaleb et al., 2000; Sartakova et al., 2001). Periplasmic flagella in *B. burgdorferi* is made from 7-11 flagellas grouped together in a bundle of approximately 0.67 nm in diameter that extends from one end of the cell to the other (Charon et al., 2009; Goldstein et al., 1996). Periplasmic flagella is made similar in structure to flagella from other bacteria having a basal body, hook and filament containing single major flagellin FlaA and minor flagellin FlaB (Ge et al., 1998; Sal et al., 2008). Periplasmic flagella wraps around the axis of the cell cylinder in a right-handed sense and during the rotation can move the bacteria, for example, if two groups of 7 to 11 periplasmic flagella rotate in opposite directions, it generates a backward-moving waves along the cell body that propel the cell forward (Charon and Goldstein, 2002; Goldstein et al., 1994) **(Figure 10.)**.

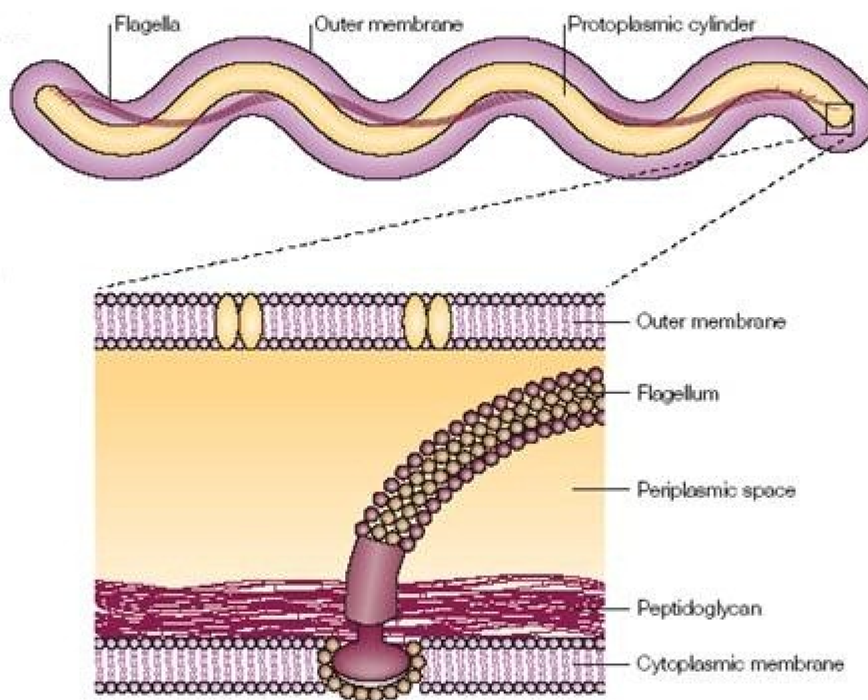

Figure 10. Wrapping of the flagella around the spirochetes rod shapes body. Adapted from (Rosa et al., 2005).

B. burgdorferi has two lipid bilayers (called outer membrane and inner membrane) as in Gram-negative bacteria like *E. coli* although there are some differences which however leads to believe that *B. burgdorferi* is distinct from a typical Gram-negative bacteria (Kudryashev et al., 2009).

The first difference to mention between a typical Gram-negative bacteria and *B. burgdorferi* is that the outer membrane of spirochete is fragile as already mentioned, and can be disrupted with different detergents, centrifugation or resuspension. The increased fragility could be explained because *B. burgdorferi* has relatively low density of transmembrane-spanning proteins and therefore the two membranes are not possibly held together so tightly as, for example, in *E. coli* (Jones et al., 1995; Radolf, 1994; Walker et al., 1991).

The second difference that distinguishes *B. burgdorferi* from typical Gram-negative bacteria is the composition of the borrelial outer cell membrane. The outer membrane of *Borrelia* is fluid and consists of 45-62% protein, 23-50% lipid and 3-4% carbohydrate (Barbour et al., 1986). The phospholipid content of *B. burgdorferi* has been identified to contain approximately 36% α-monogalactosyl diacylglycerol (α-MGalDAG), 12% phosphatidylcholine and 11% phosphatidylglycerol as the major lipids (Hossain et al., 2001). The difference here is that *B. burgdorferi* does not have phosphatidylethanolamine and lipopolysaccharide (LPS), but in opposite it has non-LPS glycolipid antigens (Belisle et al., 1994; Eiffert et al., 1991; Takayama et al., 1987; Wheeler et al., 1993). Non-LPS glycolipids represent about 50% of the total lipids and contain only galactose as the monosaccharide constituent (Hossain et al., 2001). Interestingly, that diglyceride-based glycolipids, like MGalDAG that is found in *B. burgdorferi* are more common in Gram-positive bacteria and mycoplasmas, but essentially absent from Gram-negative bacteria although found in some *Pseudomonas* species (Shaw, 1970).

The third difference is that in contrast to typical Gram-negative bacteria the outer membrane of *B. burgdorferi* contains a large number of lipoproteins facing the surroundings of the bacteria (Brandt et al., 1990).

1.2.1. Classification

The phylum Spirochetes is composed of class Spirochetes and order Spirochetales. The order is composed of three families - Brachyspiraceae, Leptospiraceae, Spirochaetaceae

(Paster and Dewhirst, 2000). Within the family Spirochaetaceae is the genus *Borrelia* **(Figure 11.)**.

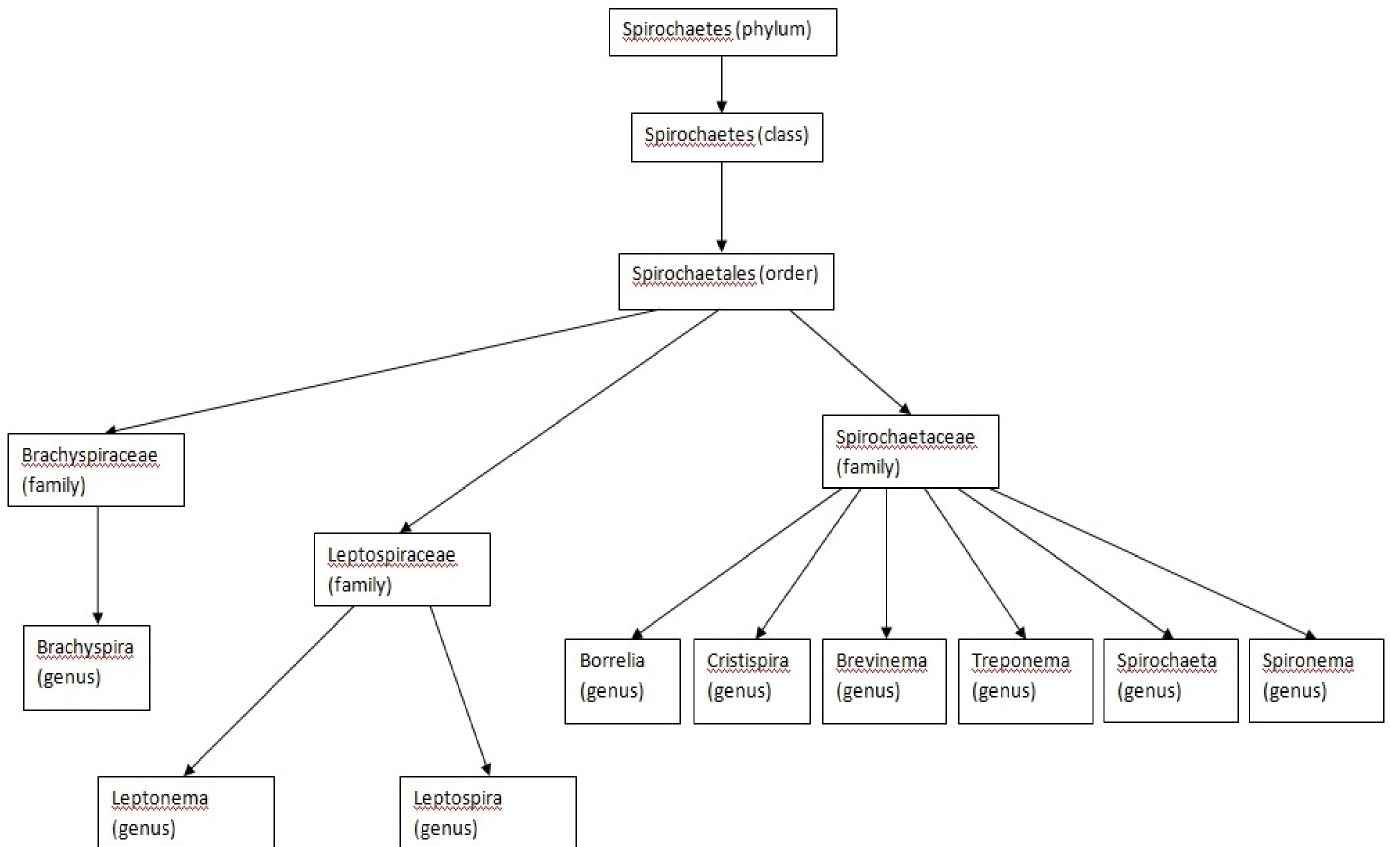

Figure 11. Phylogeny of spirochetes. The tree is made based on the data found in (Rosa et al., 2005).

Since *B. burgdorferi* was first isolated in 1982 and identified as a new species of genus *Borrelia* taxonomists determined that Lyme disease spirochetes are quite different and should be categorized as a separate species (Belfaiza et al., 1993). The original isolate was named *B. burgdorferi* sensu stricto, while the entire complex was named *B. burgdorferi* sensu lato (Baranton et al., 1992).

B. burgdorferi sensu lato complex is composed of 18 named spirochete genospecies and still not named group proposed as genospecies 2, while the current number of described species is thought not to be final (Postic et al., 2007; Rudenko et al., 2011) **(Table 3)**.

Borrelia species	Vector	Hosts/reservoirs	Geographical distribution	Reference
B. afzelii	*I. ricinus I. persulcatus*	Rodents	Asia, Europe	(Canica et al., 1993)
B. americana	*I. pacificus, I. minor*	Birds	United States	(Rudenko et al., 2009b)
B. andersonii	*I. dentatus*	Cotton tail rabbit	United States	(Marconi et al., 1995)
B. bavariensis	*I. ricinus*	Rodents	Europe	(Margos et al., 2008)
B. bissettii	*I. ricinus, I. scapularis, I. pacificus, I. minor*	Rodents	Europe, United States	(Postic et al., 1998)
B. burgdorferi sensu stricto	*I. ricinus, I. scapularis, I. pacificus*	Rodents, birds, lizards, big mammals	Europe, United States	(Baranton et al., 1992)
B. californiensis	*I. pacificus, I. jellisonii, I. spinipalpis*	Kangaroo rat, mule deer	United States	(Postic et al., 2007)
B. carolinensis	*I. minor*	Rodents, birds	United States	(Rudenko et al., 2009a)
B. garinii	*I. ricinus, I. persulcatus, I. hexagonus, I. nipponensis*	Birds, lizards, rodents	Asia, Europe	(Baranton et al., 1992)
B. japonica	*I. ovatus*	Rodents	Japan	(Kawabata et al., 1993)
B. kurtenbachii	*I. scapularis*	Rodents	Europe, United States	(Margos et al., 2010)
B. lusitaniae	*I. ricinus*	Rodents, lizards	Europe, North Africa	(Le Fleche et al., 1997)
B. sinica	*I. ovatus*	Rodents	China	(Masuzawa et al., 2001)
B. tanukii	*I. tanuki*	Unknown (possibly dogs and cats)	Japan	(Fukunaga et al., 1996)
B. turdi	*I. turdus*	Birds	Japan	(Fukunaga et al., 1996)
B. spielmanii	*I. ricinus*	Rodents	Europe	(Richter et al., 2006)
B. valaisiana	*I. ricinus, I. granulatus*	Birds, lizards	Asia, Europe	(Wang et al., 1997)
B. yangtze	*Haemaphysalis longicornis, I. granulatus*	Rodents	China	(Chu et al., 2008)
Genomospecies 2	*I. pacificus*	Unknown	United States	(Postic et al., 2007)

Table 3. Currently known spirochete species from *B. burgdorferi* sensu lato complex. Table adapted from (Rudenko et al., 2011)

All of *B. burgdorferi* sensu lato genospecies are transmitted by *Ixodes* ticks between different mammalian hosts while only *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* are the species that are the common cause of Lyme disease (van Dam et al., 1993;

Wilske, 2005). The other genospecies can also infect humans and other mammalian but only in rare cases it has been described to cause Lyme disease (Derdakova and Lencakova, 2005). The individual cases that are described to cause Lyme disease includes *B. bavariensis*, *B. bissettii*, *B. spielmanii*, *B. lusitaniae* and *B. valaisiana* (Margos et al., 2009; Richter et al., 2004; Rudenko et al., 2009c; Rudenko et al., 2008).

1.2.2. Genome

B. burgdorferi sensu stricto strain B31 has been studied in the most detail as it was the first strain for which the whole genome sequence was obtained (Fraser et al., 1997). Nowadays the complete genome sequence has been obtained also for other borrelial strains like *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. spielmanii* and *B. bissettii* (Casjens et al., 2011; Schutzer et al., 2012).

B. burgdorferi B31 contains a linear 910 725 bp chromosome and 12 linear and 9 circular plasmids with the total size of approximately 610 000 bp (Casjens et al., 2000; Fraser et al., 1997) **(Figure 12.)**. On the chromosome of the *B. burgdorferi* B31 there are 815 predicted genes (Casjens et al., 2012) and about 60% of them have some similarities with the genes in other organisms and for them the function or role is partly understood. For 10% of genes there are similarities between other organisms but the function or role is not known. For the remaining 30% of the genes no similarities can be found and therefore the functions are unknown (Fraser et al., 1997). Another story is about the plasmids where out from the 535 predicted protein genes, only 6% shows any similarities with the genes from any other organisms (Casjens et al., 2000). The vast majority of genes located on the plasmids are thought to code for the outer surface lipoproteins that possibly play some role in the pathogenesis of Lyme disease as the loss of the plasmids in culture results in loss of the infectivity in mice (Norris et al., 1992; Palmer et al., 2000).

	B31	N40	JD1	297
Linear plasmids	12	8	11	9
Circular plasmids	9	8	9	10
Total plasmids	21	16	20	19
lp5	+	−	−	−
lp17	+	+	+	+
lp21	+	−	−	−
lp25	+	+	+	(+)[a]
lp28-1	+	−	+	+
lp28-2	+	+	−	−
lp28-3	+	(+)[a]	+	+
lp28-4	+	+	+	+
lp28-5	−	+	+	+
lp28-6	−	−	+	+
lp28-7	−	−	+	−
lp36	+	+	+	+
lp38	+	+	+	+
lp54	+	+	+	+
lp56	+	−	−	−
cp9-1	+	+	−	−
cp9-2	(+)[a]	−	−	−
cp26	+	+	+	+
cp32-1	+	−	+ (fused)[b]	+
cp32-3	+	−	+	+
cp32-4	+	+ (trunc)[b]	−	+
cp32-5	(+)[a]	+	+ (fused)[b]	+
cp32-6	+	−	+	+
cp32-7[c]	+	+ (trunc)[b]	−	+
cp32-8	+	−	+	−
cp32-9	+	+	+	+ (trunc)[b]
cp32-10	+ (int)[b]	+	+	−
cp32-11	−	−	+	+
cp32-12	−	+	+	+

Figure 12. *B. burgdorferi* sensu stricto plasmids present in four different strains. Plasmids are named according to their DNA topology – linear "lp" and circular "cp" and approximate size in Kbp. [a]Plasmids known to be in some cultures of the indicated strain, but which were not present in the sequenced culture. Their sequences remain

undetermined. [b]Structural differences from otherwise organizationally similar plasmids are indicated as follows: trunc, truncated compared to other homologous plasmids; int, B31 cp32-10 is integrated into plasmid lp56; fused, JD1 cp32-1 and cp32-5 are fused into one large circular ‘‘cp32-1+5’’ plasmid. Figure adapted from (Casjens et al., 2012).

The chromosome carries the minimal set of genes that are necessary for cell maintenance and replication including DNA, RNA and protein biosynthesis, DNA repair, glycolysis, motility and chemotaxis (Fraser et al., 1997). Although the plasmids mostly code for different putative outer surface proteins, a few metabolic or housekeeping genes can be found there while in the *B. burgdorferi* genome no genes coding for proteins involved in respiration, amino acid metabolism, nucleotide and lipid biosynthesis can be identified, which could explain the specific requirements for growth in culture and the highly specific host environments (Bono et al., 1998; Margolis et al., 1994; Nowalk et al., 2006; Pollack et al., 1993).

B. burgdorferi 12 linear and 9 circular plasmids are low copy number plasmids having one or two copies per cell (Casjens and Huang, 1993; Kitten and Barbour, 1992). The linear plasmids have unusually high number of pseudogenes and paralogous genes while several circular plasmids are homologous nearly throughout the entire length (Casjens et al., 1997; Casjens et al., 2000). The large number of paralogous genes on the plasmids suggests that vast duplicative rearrangements have occurred on the plasmids resulting in genes that were susceptible to further evolutional selection giving rise to those 107 gene families found today (Casjens et al., 2000).

As most of the plasmids, probably all but cp26, can be lost without affecting the growth in the culture, the total size of the chromosome, carrying the housekeeping genes of bacteria is around 900 000 bp and that ranks the *B. burgdorferi* among the smallest bacterial genomes known to date (580 070 bp for the smallest bacterial genome *Mycoplasma genitalium*) (Fraser et al., 1995; Fraser et al., 1997).

1.2.3. Outer surface lipoproteins

As mentioned already in the general section about *B. burgdorferi*, regarding the main differences between a typical Gram-negative bacteria and *B. burgdorferi*, one of them was that *Borrelia* contains unusual amount of lipoproteins on the outer surface (Haake, 2000). From the genome of *B. burgdorferi* 8% or about 150 of the coding genes

expresses lipoproteins and this is significantly higher frequency than in any other bacterial genome that is sequenced to date (for comparison – in *Helicobacter pylori* 1.3% genes coding for lipoproteins) (Casjens et al., 2000; Fraser et al., 1997). The unique membrane architecture as discussed in the general section about *B. burgdorferi* suggests that the export, structure and function of spirochetal lipoproteins have features that are unique for these organisms (Haake, 2000). The secretion of the lipoproteins in *B. burgdorferi* starts when the signal recognition particle binds the newly synthesized protein and carries it to the preprotein translocase complex in the cytoplasmic membrane. The protein is transported through the membrane by assisting the SecYE transmembrane complex made from SecA and SecDF. SecA binds the signal sequence and pushes it through the transmembrane channel, then gets released and binds again achieving that the entire protein is translocated through the membrane. In the periplasmic space the proteins are lipidated and the process occurs in three steps, similar as in other Gram-negative bacteria (Haake, 2000). After the lipidation in the periplasmic space the lipoproteins can be transported to the outer membrane and two proteins in Gram-negative *E. coli* have been identified responsible for localization of the proteins to the outer membrane (Matsuyama et al., 1997). Homologous proteins have not been found in *B. burgdorferi* for those proteins suggesting that the proteins in *B. burgdorferi* may be transported to the outer membrane by a unique pathway again pointing to the difference between a typical Gram-negative bacteria (Haake, 2000).

Theoretically, the lipoproteins of *B. burgdorferi* can be localized in three different positions – the periplasmic leaflet of the cytoplasmic membrane, the periplasmic leaflet of the outer membrane or the outer leaflet of the outer membrane and thus located on the cell surface. Since it is not possible to determine the exact location of the protein from the primary structure yet, experimental determination of surface exposure is critical to identify the proteins that are located on the surface and involved in the pathogenesis of Lyme disease. Triton X-114 extraction and phase partitioning has been applied to isolation of the outer membrane of a variety of different spirochetes and the localization on the outer leaflet of the outer membrane has been confirmed for several proteins (Brooks et al., 2006; Bryksin et al., 2010; Hughes et al., 2008).

1.2.4. Tick-host interactions

Larval ticks obtain the Lyme disease agent while feeding on various infected host organisms, moults into a nymph and then transmit the spirochete to a new host organism during the next blood meal (Steere, 2001). It has been suggested that the interactions between *B. burgdorferi* and *Ixodes* ticks are highly specific and that infection associated *Borrelia* can infect only a specific tick species (from more than 700 species) that belong to *Ixodidea* family, for example, *I. ricinus*, *I. scapularis*, *I. persulcatus*, *I. pacificus*, *I. hexagonus* or *I. nipponensis* (Fikrig and Narasimhan, 2006). The basis for those observations could be that there have been identified several tick proteins, for example, Salp25D and Salp20 that are necessary for acquisition of *B. burgdorferi* by *Ixodes* ticks and that protects *B. burgdorferi* from lysis resulting from alternative complement pathway activation (Narasimhan et al., 2007; Ribeiro et al., 2006; Tyson et al., 2007). In nature, *B. burgdorferi* can persist in *Ixodes* ticks for months mostly located in the gut of unfed ticks. Although little is known about the mechanisms how the spirochetes persists in *Ixodes* ticks the conditions that are encountered there can be severe (de la Fuente et al., 2008). Because of the seasonal changes the temperature difference can be significant; also the gut of an unfed tick is poor in nutrients primarily because gut epithelia store nutrients in cellular endosomes and during this period *B. burgdorferi* expresses only the genes to maintain the basic cell processes and to simply persist in the arthropod environment (Ojaimi et al., 2002; Pal and Fikrig, 2003). When the tick starts to feed on mammalian organism it has been showed that the spirochete manages to switch the expression of different genes in response to a mammalian host-specific signals, pH and temperature shift or cell density changes (Angel et al., 2010; Brooks et al., 2003; Carroll et al., 2000; Embers et al., 2004; Indest et al., 1997; Ojaimi et al., 2003; Ramamoorthy and Scholl-Meeker, 2001; Revel et al., 2002; Tokarz et al., 2004). While in *Ixodes* tick the bacteria expressed the minimal amount of genes necessary to persist, when the tick starts to feed the above-mentioned changes induces the expression of various genes thought to assist the bacteria to travel from ticks midgut to the salivary glands, to transfer to the new host organism, to fight against the immune response, to proliferate and to disseminate to various tissues (Hughes et al., 2008; Narasimhan et al., 2002; Ojaimi et al., 2003). Among the best studied proteins that are involved still in the tick environment and experiences changes in the expression profiles when the tick starts to feed are OspA and OspC (Pal et al., 2004a; Yang et al., 2004). OspA binds the

receptor TROSPA found in the ticks midgut and this interaction is thought to hold the spirochetes in the ticks midgut (Pal et al., 2004b). When the tick start the blood meal, the OspA protein is downregulated but another protein – OspC is upregulated (Pal et al., 2004a; Schwan et al., 1995). *B. burgdorferi* OspC protein binds tick salivary protein Salp15 and thus helps the spirochetes to transfer from the midgut, where they are not anymore bound to OspA because of the downregulation of the protein, to ticks salivary glands (Pal et al., 2004a). Spirochetes migrate from the infected ticks midgut to the salivary glands for several hours after the initiation of feeding, which lasts for 3-7 days (Fikrig and Narasimhan, 2006; Piesman, 1993). Different outer surface proteins are upregulated during the tick feeding process and much remains to be studied, however it has been suggested that the proteins expressed by the genes residing exactly on lp54 play an important role in the adaptation of *Borrelia* and are associated with borrelial pathogenesis (Hughes et al., 2008; Ojaimi et al., 2003; Tokarz et al., 2004). Recent studies have revealed that BBA64 and BBA66 have an important role in the transfer of *B. burgdorferi* from infected *Ixodes* ticks to the host organism after the tick bite (Gilmore et al., 2010; Patton et al., 2013b).

1.2.5. PFam54 family

The paralogous gene family PFam54 members located on lp54 consists of nine open reading frames – *bba64*, *bba65*, *bba66*, *bba68*, *bba69*, *bba70*, *bba71*, *bba72*, *bba73* – although *bba71* and *bba72* are truncated genes and *bba70* is missing in most of *B. burgdorferi* strains (Casjens et al., 2012). The amino acid sequence identity among the 9 PFam54 paralogous gene family members located on lp54 of *B. burgdorferi* varies from 17% to 60%. It has been showed that the paralogous gene family PFam54 members that are located on 54 kb linear plasmid (lp54) exhibit the highest upregulation rates between those more than a hundred proteins and are therefore thought to be important in the pathogenesis of Lyme disease (Angel et al., 2010; Ojaimi et al., 2003; Tokarz et al., 2004). The functions of the paralogous protein family members are thought to be different, as reported by several studies indicating that the expression level, timing and the target receptors/ligands differ substantially among the PFam54 family members (Gilmore et al., 2008; Gilmore et al., 2007). Although all of these proteins are thought to be related to the pathogenesis of *B.burgdorferi,* the exact function has been established only for one protein, CspA or BBA68, from the PFam54 family. CspA is a

complement regulator factor H and factor H-like protein-1 (FHL-1) binding protein, and thus, it assists the bacteria to resist the host immune response (Kraiczy et al., 2004). CspA is the only PFam54 member that is known to bind complement regulators, as verified by several studies (Kraiczy et al., 2001b; Kraiczy et al., 2006; Kraiczy et al., 2009; Wallich et al., 2005). Another PFam54 family member, BBA64, plays an essential role in the transfer of *B.burgdorferi* from infected *Ixodes* ticks to a mammalian host organism after a tick bite, although the exact ligand or receptor is not known (Cordes et al., 2004; Gilmore et al., 2010). Recently it has been showed that BBA66 is important for the transmission of *B. burgdorferi* from infected *Ixodes* ticks to the mammalian host organism (Patton et al., 2013a).

1.2.6. Complement regulator-acquiring surface proteins or CRASPs

To resist the host's immune response *B. burgdorferi* has managed to bind the major alternative-pathway complement regulators factor H (CFH) and factor H-like protein 1 (CFHL-1) (Zipfel et al., 2002). The proteins that bind complement factors H and FHL-1 in *Borrelia* are termed as complement regulator-acquiring surface proteins or CRASPs (Kraiczy et al., 2001a; Kraiczy et al., 2001b). There have been identified five CRASP proteins in *B. burgdorferi* belonging to three different gene families (Kraiczy and Stevenson, 2013). In *B. burgdorferi* the five CRASP proteins are known as CspA, CspZ, ErpA, ErpC and ErpP belonging to three different gene families (Hellwage et al., 2001; Kraiczy and Stevenson, 2013).

By binding the hosts complement regulators factor H and FHL-1 the bacteria mimics the action of the host's own cells that binds complement regulators by using glycosaminoglycans and heparin located on the cells surface and thus avoiding the hosts complement attack (Kuhn et al., 1995; Schneider et al., 2009).

CRASPs are not only different in the primary and tertiary protein structures, but also produce different binding affinities for the complement regulators CFH, CFHL-1 and CFHRs. CspA (also known as BbCRASP-1 or BBA68) is a member of the PFam54 gene family and shows the ability to bind both CFH and CFHL-1 (Kraiczy et al., 2004). CspZ (also known as BbCRASP-2) is genetically distinct from the other CFH and CFHL-1 binding proteins and does not share any sequence similarity with other CFH/CFHL-1 binding proteins and has showed the ability to bind CFH and CFHL-1 (Hartmann et al., 2006; Rogers and Marconi, 2007). ErpP, ErpC and ErpA (also known

as BbCRASP-3, BbCRASP-4 and BbCRASP-5) belongs to OspE/F-related (Erp) protein family have showed the ability to bind CFH and CFHR1, CFHR2 and CFHR5 proteins (Hammerschmidt et al., 2012; Hellwage et al., 2001; Siegel et al., 2010).

1.2.7. Crystal structures of borrelial outer surface proteins

As the surface-localized *B. burgdorferi* lipoproteins interact with the environment and are thought to be the major players in the pathogenesis of Lyme disease, understanding of their role, functions and structure could be invaluable step to advance the understanding of the pathogenesis of Lyme disease.

Just for a small fraction from those more than a hundred lipoproteins expressed by *B. burgdorferi* the function has been established and they are involved in the transmission of the bacteria from ticks to mammalian, evasion of complement killing, antigen variation and adherence mechanisms (Coburn et al., 2005; Gilmore et al., 2010; Kraiczy and Stevenson, 2013; Patton et al., 2013a; Zhang et al., 1997). Taking into account that only 6% of the *B. burgdorferi* lipoproteins have some similarities with the proteins from other organisms, and for most of them the functions are not known, the determination of the crystal structures for those *B. burgdorferi* outer surface lipoproteins could be also very valuable. From all those approximately 150 lipoproteins found in *B. burgdorferi* the crystal structures have been determined only for 15 lipoproteins (including the three crystal structures described in the PhD thesis) and they are – BBA64 (PDB entry 4ALY, (Brangulis et al., 2013c)), BBA73 (PDB entry 4AXZ, (Brangulis et al., 2013b)), BBA66 (PDB entry 2YN7 (Brangulis et al., 2013a)), BBA65 (PDB entry 4BG5), BBA68 (PDB entry 1W33, (Cordes et al., 2005)), ErpC (PDB entry 4BF3 (Caesar et al., 2013a) and PDB entry 4BXM), OspE (PDB entry 4J38, (Bhattacharjee et al., 2013)), DbpA (solution NMR structure, PDB entry 2LQU, (Wang, 2012)), OspA (PDB entry 2FKG, (Li et al., 1997)), BB0167 (PDB entry 3OON), OspB (PDB entry 1RJL, (Becker et al., 2005)), VlsE (PDB entry 1L8W, (Eicken et al., 2002)), OspC (PDB entry 1GGQ, (Kumaran et al., 2001)), ErpP (PDB entry 4BOB), BBH06 (PDB entry 4BG0).

2. Materials and Methods

2.1. *E.coli* strains

BL21 (DE3): *F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])* (Stratagene, USA)

JM110: *rpsL* (Strr) *thr leu thi*-1 *lacY galK galT ara tonA tsx dam dcm supE44 Δ*(*lac-proAB*) [F' *traD36 proAB lacIqZΔM15*] (Stratagene, USA)

DH5α: *F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17$(r_K^- m_K^+)$, λ–* (Invitrogen, USA)

B834: *F^- ompT $hsdS_B(r_B^- m_B^-)$ gal dcm met [DE3]*

2.2. Expression vectors

pETm-11 – expression vector with T7/lac promoter, coding for kanamycin drug resistance gene, N-terminal 6xHis tag and TEV protease cleavage site. pETm-11 is a modified version of pET-24d expression vector containing a MAD insert (EMBL, Germany).

2.3. Polymerase chain reaction (PCR) and primers

PCR reaction mix was made as described by the manufacturer (Thermo Fisher Scientific, USA). Proof-reading polymerase was used. PCR reaction was carried out in Eppendorf Mastercycler Thermal Cycler (Eppendorf, Germany) using 30 cycles and the following conditions:

- Initialization + 95°C 2 min
- Denaturation + 95°C 40 sec
- Annealing + 54°C 30 sec
- Extension/elongation + 72°C 1,5 min
- Final elongation +72°C 5 min

Primers used in the PCR are summarized in table 4.

Name	Sequence (from 5' to 3')	Description
KB-15 KB-16	F* – CAT GCC ATG GGC AAA GAC AGC AAT GAA AGC R* – GCT TGC GGC CGC TTA CTG AAT TGG AGC AAG AAT	Amplification of *Bba64* (aa 28-302)
KB-7 KB-8	F – CAT GCC ATG GGC AAT CTA AAC GAA GAT TAT AAA R – GCT TGC GGC CGC TTA CAT TAT ACT AAT GTA TGC	Amplification of full-length *Bba66* (aa 24-310)
KB-54 KB-8	F – CAT GCC ATG GGC TCT GGC AGT AAA TTA CAA R – GCT TGC GGC CGC TTA CAT TAT ACT AAT GTA TGC	Amplification of truncated *Bba66* (aa 191-310)
KB-1 KB-2	F – CAT GCC ATG GGC AAC AAC ACA GAA GCG ATA R – GCT TGC GGC CGC TTA GTA GTG TAT GTG GTC ACA	Amplification of *Bba73* (aa 28-296)
KB-81 KB-2	F – CAT GCC ATG GGC GAG AAT GAA AAA TTG ATT R – GCT TGC GGC CGC TTA GTA GTG TAT GTG GTC ACA	Amplification of truncated *Bba73* (aa 87-296)

*F – Forward; *R – Reverse;

2.4. DNA agarose gel electrophoresis

Electrophoresis was performed using 1 % agarose (Thermo Fisher Scientific) gels containing 1 µg/ml ethidium bromide in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.0).

2.5. DNA fragment purification from agarose gels

DNA fragments were purified from excised gel fragments by using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA) following the product manual procedure provided by the manufacturer.

2.6. Restriction of DNA fragments by endonucleases

Restriction enzymes *NcoI* and *NotI* (Thermo Fisher Scientific, USA) and 10x Tango buffer (Thermo Fisher Scientific, USA) was used to cleave the DNA fragments and the expression vector. Restriction mix was made following the manufacturer's instructions except that the reaction mix was incubated at 37°C for 4 h.

2.7. Ligation reaction of PCR products and the expression vector

The ligation of PCR products and the expression vector was done by preparing the following reaction mix:

Expression vector DNA – 50 ng

Insert DNA – 5:1 – concentration ratio over the expression vector

10X T4 DNA Ligase buffer (Thermo Fisher Scientific) – 2 µl

T4 DNA Ligase (Thermo Fisher Scientific) – 1 µl

Water – up to 20 µl

Ligation mix was incubated for 1 h at room temperature.

2.8. Transformation of *E.coli*

Transformation was done immediately after the incubation of ligation mix for 1 h. All of the ligation mix (20 µl) was added to the competent cells. The competent cells with the ligation mix were incubated for 20 min on ice and then the samples were subjected to a heat shock at 42°C for 40 s. After the heat shock the cells were placed back on ice for 2 min and 1 ml of pre-warmed LB medium (10 g/l Bacto tryptone (Difco Laboratories), 5 g/l Bacto yeast extract (Difco Laboratories) and 10 g/l sodium chloride (Stanlab, Poland)) was added and incubated for 1 h at 37°C with aeration at ~ 200 rpm. Cells were pelleted at 5000 rpm for 1 min and almost all of the supernatant was removed. The cell pellet was resuspended in the remaining LB medium and applied on pre-warmed LB agar plates (10 g/l Bacto tryptone (Difco Laboratories), 5 g/l Bacto yeast extract (Difco Laboratories), 10 g/l sodium chloride (Stanlab, Poland), 15 g/l Bacto agar) containing canamycin (10 mg/ml). Plates were incubated at 37°C overnight.

2.9. Inoculation in liquid LB medium

Resulting colonies from LB agar plates were inoculated into liquid LB medium containing canamycin (10 mg/ml) and incubated for 24 h at 37°C in a shaker CH-4103 (Infors AG, Switzerland).

2.10. Plasmid DNA isolation from recombinant *E.coli* culture

Plasmid DNA was isolated by using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scienitfic) according to the manufacturer’s instructions.

2.11. Analysis of recombinant clones

The isolated plasmid DNA was analysed for the presence of the ligated DNA fragment by PCR using the respective primers corresponding to the inserted DNA fragment.

2.12. Plasmid sequencing to approve the accuracy

Isolated plasmid DNA was sequenced using the primers corresponding to the analysed gene fragment and the following reaction mix was made:

Big Dye (Applied Biosystems) – 1 µl
Big Dye 5x reaction buffer – 2 µl
Primer – 1 µl
Plasmid DNA – 2 µl
Water – 4 µl

PCR conditions for the sequencing reaction (25 cycles):
96°C – 30 s
50°C – 15 s
60°C – 4 min

Precipitation of DNA after the sequencing reaction – 35 µl of 96 % ethanol and 1.5 µl 7.5 M ammonium acetate (Sigma-Aldrich) was added and the mixture was left for 15 min at -20°C. Then centrifugated at 13 000 rpm for 15 min by using centrifuge 5417C (Eppendorf, Germany). The supernatant was completely removed and 200 µl of 70 % ethanol was added and centrifugated for 12 min at 13 000 rpm. The supernatant was again completely removed and the tubes were left open to air dry.
Sequencing of the samples was carried out on 3130xl Genetic analyzer (Applied Biosystems). The sequencing data were analyzed by using VectorNTI software (Invitrogen, USA).

2.13. Recombinant protein expression

The recombinant plasmid was transformed into *E.coli* BL21(DE3) cells following the transformation protocol as described previously. The cells were grown in modified

2xTYP media (supplemented with kanamycin (10 mg/ml), 133 mM phosphate buffer, pH 7.4, and glucose (4 g/l)) with vigorous agitation at 25°C until an OD_{600} of 0.8–1.0, induced with 0.2 mM IPTG and grown for an additional 16–20 h. Cells were harvested by centrifugation and frozen at -20°C for storage.

2.14. Se-Met labeled protein expression

The recombinant plasmid was transformed into *E.coli* B834 (DE3) cells following the transformation protocol as described previously. The cells were grown in modified 2xTYP media (supplemented with kanamycin (10 mg/ml), 133 mM phosphate buffer, pH 7.4, and glucose (4 g/l)) with vigorous agitation at 25°C until an OD_{600} of 0.8 –1.0 and then centrifugated. The pellet was resuspended in 0501 medium (without methionine) (Seleno-Met medium base from Athena Enzyme Systems) supplemented with 0502 medium (Seleno-Met Nutrient mix from Athena Enzyme Systems) and glucose (5 g/l) and grown for a further 2 h. IPTG (0.5 mM) and a mixture of selenomethionine and methionine (5:1) was added and cultivation was continued for 20 h.

2.15. Cell lysis with ultrasonic disintegrator

Harvested cell pellet was resuspended in lysis buffer consisting of 20 mM NaH_2PO_4, 200 mM NaCl and 1 mM PMSF. Cells were sonicated by using Ultrasonic Disintegrator type UD-20 (Techpan, Poland) and then centrifugated for 30 min at 10 000 rpm, 4°C.

2.16. Protein purification by using Ni-NTA chromatography

Supernatant that was formed after the centrifugation of lysed cells was applied to Ni-NTA agarose (Qiagen, Germany) column. Column was washed twice with a wash buffer containing 20 mM NaH_2PO_4 (Reahim, Russia), 200 mM NaCl (Reahim, Russia) and 15 mM imidazole (AppliChem GmbH, Germany). 6xHis tagged protein was eluted with the elution buffer containing 20 mM NaH_2PO_4 (Reahim, Russia), 200 mM NaCl (Reahim, Russia) and 300 mM imidazole (AppliChem GmbH, Germany).

2.17. 6xHis tag cleavage

TEV protease (2 mg/ml) in 1:20 volume ratio was added to the eluted protein fraction to remove the N-terminal 6xHis tag from the protein of interest. The reaction was incubated overnight at room temperature. The cleaved protein was applied to Ni-NTA agarose (Qiagen, Germany) column to remove the protease, the cleaved 6xHis tag and the remnants of the uncleaved protein.

2.18. Protein purification by using ion-exchange chromatography

Proteins were further purified by ion-exchange chromatography on a Mono Q 5/50 GL column (GE Healthcare, UK) linked to an ÄKTA chromatography system (Amersham Biosciences) at a flow rate of 1ml/min.

2.19. Protein concentration

Fractions containing the protein after the purification were combined and concentrated by using Amicon filter units (Millipore corporation, Germany).

2.20. SDS-PAGE analysis

Proteins were analysed on SDS-PAGE gels consisting of upper layer (2 %) and lower layer (10 %) on Mighty small II system (Amersham Biosciences, USA). The composition of SDS-PAGE:

Upper gel (2 %)

0.6 M TRIS-HCl, pH 6.8 – 0.5 ml
Water – 3.75 ml
30 % acrylamide solution – 0.7 ml
10 % SDS – 0.05 ml
10 % ammonium persulfate – 0.07 ml
TEMED – 0.007 ml

Lower gel (10 %)

0.6 M TRIS-HCl, pH 8.8 – 2 ml
Water – 4.5 ml
30 % acrylamide solution – 3.3 ml
10 % SDS – 0.1 ml
10 % ammonium persulfate – 0.1 ml
TEMED – 0.01 ml

2.21. SDS-PAGE gel staining with Coomassie Brilliant blue G-250

SDS-PAGE gels were washed with 50% ethanol solution for 10 min on a shaker (Luckham model R100, Luckham Ltd., England). Then the wash solution was removed and the Coomassie blue solution added so that the gel is completely immersed and left to stand for 10 min. The Commassie blue solution was removed and water added to decolour the gel.

2.22. Western blot analysis

SDS-PAGE gel was placed on a hybridization nitrocellulose membrane Hybond-C Extra (Amersham Biosciences, UK) between four sheets of filter paper (Amersham Biosciences, UK) soaked previously in the transfer buffer. The parameters were set to 25 V and 100 mA for one gel for 1 h in semi-dry transfer cell Trans-blot SD (Bio-Rad, USA).

Membrane was blocked for 45 min by adding 5% skimmed milk solution in 1xPBS. The membrane was washed twice for 5 min with PBS wash buffer. The membrane was incubated for 45 min with penta-His antibody (Qiagen, Germany) in a 1xPBS buffer at a dilution level of 1:1000. The membrane was washed twice for 5 min with PBS wash buffer with agitation. The membrane was incubated with secondary antibodies (anti-mouse antibodies) diluted in 1xPBS at the level of 1:5000. Finally, the membrane was washed twice with PBS wash buffer. Chemiluminescence was used as a detection system. Addition of ECL reagents (Amersham Biosciences, UK) resulted in chemiluminescence and Hyperfilm ECL (Amersham Biosciences, UK) was used to

record the emmited signals from the Hybond-C Extra membrane (Amersham Biosciences, UK).

2.23. Protein analysis by mass spectrometer MALDI-TOF

To verify the purity of the proteins and Se-Met incorporation in case of Se-Met derivatives, MALDI-TOF mass spectrometry was performed. 1 µl of protein (4 mg/ml in 20 mM Tris-HCl pH 8.0) was mixed with 1 µl 0.1% TFA and 1 µl matrix solution, containing 15 mg/ml 2.5-dihydroxyacetophenone in 20 mM ammonium citrate and 75 % ethanol. 1 µl of obtained mix was loaded on target plate, dried and analyzed by Bruker Daltonics Autoflex mass spectrometer (Bruker Biosciences, USA).

2.24. Estimation of multimeric state by gel filtration chromatography

Protein sample was loaded onto a pre-packed Superdex 200 10/300 GL column, connected to an ÄKTA chromatography system (Amersham Biosciences). The column was pre-equilibrated with the same buffer as the relevant protein and run at a flow rate of 0.7 ml/min. Bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa) were used as MW reference standards.

2.25. Protein crystallization

Crystallization was performed by the sitting drop vapour-diffusion technique by mixing 1µl of protein with an equal volume of precipitant solution. Initial screening was performed using several 96 reagent sparse-matrix screens from Molecular Dimensions (Molecular Dimensions Ltd, UK). In case of a crystal hit in the crystallization screen, optimization was performed regarding the actual conditions. The crystals were flash-frozen in liquid nitrogen before the data collection.

2.26. Diffraction data collection and structure determination

Diffraction data for the native and Se-Met labeled proteins were collected at Max-lab beamline I911-3 and I911-2 (Lund university, Sweden) using Marmosaic 225 detector (Marresearch GmbH, Germany).

Phases for the crystal structures were determined by using SAD, MAD or also molecular replacement method.
Reflections were indexed and scaled using MOSFLM and SCALA programs from the CCP4 program suite (Battye et al., 2011; Evans, 2006; Winn et al., 2011).
In case of MAD, data were merged using CAD and Se atom positions and initial phases were determined using SHELX C/D/E (Sheldrick, 2008). The initial protein model was built automatically in BUCCANEER and minor re-building of the model was done manually in COOT (Cowtan, 2006; Emsley and Cowtan, 2004). Water molecules were picked automatically in COOT and inspected manually. Crystallographic refinement was carried out with REFMAC5 (Murshudov et al., 1997).
In case of molecular replacement, initial phases were obtained by using Phaser (McCoy et al., 2007) and the crystal structure of a paralogous protein as a search model.

3. RESULTS

3.1. Crystal structure of an outer surface lipoprotein BBA64 from Lyme disease agent *Borrelia burgdorferi* which is critical to ensure the infection after a tick bite

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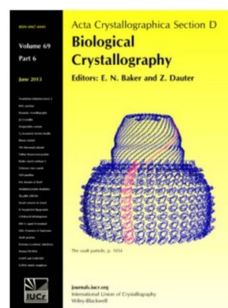

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Structure of an outer surface lipoprotein BBA64 from the Lyme disease agent *Borrelia burgdorferi* which is critical to ensure infection after a tick bite

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Lyme disease is a tick-borne infection caused by the transmission of *Borrelia burgdorferi* from infected *Ixodes* ticks to a mammalian host during the blood meal. Previous studies have shown that the expression of *B. burgdorferi* surface-localized lipoproteins, which include BBA64, is up-regulated during the process of tick feeding. Although the exact function of BBA64 is not known, this lipoprotein is critical for the transmission of the spirochete from the tick salivary glands to the mammalian organism after a tick bite. Since the mechanism of development of the disease and the functions of the surface lipoproteins associated with borreliosis are still poorly understood, the crystal structure of the *B. burgdorferi* outer surface lipoprotein BBA64 was solved at 2.4 Å resolution in order to obtain a better insight into the pathogenesis of *B. burgdorferi* and to promote the discovery of novel potential preventive drugs against Lyme disease. In this study, the crystal structure of BBA64 was also compared with that of the paralogous protein CspA (also referred to as BbCRASP-1, CRASP-1 or BBA68). CspA is the complement regulator-acquiring surface protein-1 of *B. burgdorferi*; its structure is known, but its function apparently differs from that of BBA64. It is demonstrated that unlike the homologous CspA, BBA64 does not form a homodimer. Their differences in function could be explained by divergence in their amino-acid sequences, electrostatic surface potentials and overall tertiary structures. The C-terminal part of BBA64 has a different conformation to that of CspA; the conformation of this region is essential for the proper function of CspA.

1. Introduction

Borrelia burgdorferi, the causative agent of Lyme disease, is transmitted from infected *Ixodes* ticks to a mammalian host organism during the blood meal (Burgdorfer *et al.*, 1982; Steere *et al.*, 2004).

The change in the host organism on transfer from *Ixodes* ticks to mammals and the necessity to proliferate and to resist the immune response of the host has forced *Borrelia* to adapt to changing environments. Studies have shown that *B. burgdorferi* switches the expression of different genes in response to mammalian host-specific signals, pH and temperature shifts or cell-density changes (Angel *et al.*, 2010; Brooks *et al.*, 2003; Carroll *et al.*, 2000; Embers *et al.*, 2004; Indest *et al.*, 1997; Ojaimi *et al.*, 2003; Ramamoorthy & Scholl-Meeker, 2001; Revel *et al.*, 2002; Tokarz *et al.*, 2004).

The *B. burgdorferi* genes coding for the outer surface lipoproteins BBA64 and BBA68 are members of the paralogous gene family Pfam54 located on the 54 kb linear plasmid

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(lp54), which is one of the 12 linear plasmids of *B. burgdorferi* (Casjens *et al.*, 2000, 2012; Fraser *et al.*, 1997). It has been suggested that the proteins expressed by the genes residing on lp54 play an important role in the adaptation of *Borrelia* and are associated with borrelial pathogenesis (Hughes *et al.*, 2008; Ojaimi *et al.*, 2003; Tokarz *et al.*, 2004). During a temperature shift *in vitro* from 296 to 308 K, which resembles the temperature change during the transfer of the spirochete from ticks to a warm-blooded animal, and in response to mammalian host-specific signals, the 54 kb linear plasmid had the highest number of differentially expressed genes of all of the borrelial plasmids and the members of the paralogous gene family Pfam54 particularly stood out (Brooks *et al.*, 2003; Ojaimi *et al.*, 2003; Tokarz *et al.*, 2004).

Recent studies have revealed that BBA64 plays a vital role in the transfer of *B. burgdorferi* from *Ixodes* ticks to the host organism after the tick bite (Gilmore *et al.*, 2010). A mutant borrelial strain with an inactivated BBA64 gene is incapable of ensuring the transmission of *B. burgdorferi* and infection is therefore not initiated (Anguita *et al.*, 2000; Gilmore *et al.*, 2007, 2010; Schmit *et al.*, 2011; Patton *et al.*, 2011). However, the exact ligand or receptor for BBA64 is still under investigation.

CspA has 25% sequence identity to BBA64 and is the only member of the Pfam54 proteins for which both structure and function are known. CspA binds complement regulator factor H and factor-H-like protein-1 (FHL-1; Kraiczy, Skerka, Kirschfink, Brade *et al.*, 2001, Wallich *et al.*, 2005) and plays an essential role in decreasing the immune response of the host by inactivating the alternative complement pathway (Kraiczy, Skerka, Kirschfink, Zipfel *et al.*, 2001).

To gain further understanding of exactly how BBA64 ensures the transfer of *B. burgdorferi* from ticks to the mammalian host and to potentially use the protein as a novel drug target to stop the transmission of the spirochete, we have solved the three-dimensional structure of recombinant BBA64 at 2.4 Å resolution and have compared the structure with the previously determined crystal structure of the homologous protein CspA (PDB entry 1w33; Cordes *et al.*, 2004, 2005). The structure of BBA64 revealed an overall fold similar to that of CspA except for the C-terminal α-helix, which has a different conformation compared with that of CspA. In CspA the C-terminal α-helix is involved in the formation of a homodimer and is essential for the proper function of CspA (Cordes *et al.*, 2006; Kraiczy *et al.*, 2009). Although it was originally considered that the cleft between the monomers in CspA could be an ideal place for ligand binding and thus might be conserved in homologous proteins, it is also reasonable to believe that the potential ligand-binding sites may vary between the homologues. This is supported by the facts that dimer formation was not observed in the case of BBA64 and the crystal structure revealed a different orientation of the C-terminal α-helix, which is necessary for dimer formation of CspA, and also revealed the proper binding of complement regulator factor H and FHL-1 in a monomeric state of CspA (Cordes *et al.*, 2006; Kraiczy *et al.*, 2006).

2. Materials and methods

2.1. Cloning and expression of native and SeMet-labelled proteins

The gene for recombinant BBA64 protein was amplified from *B. burgdorferi* strain B31 genomic DNA by PCR so that the signal sequence (amino acids 1–32) of the protein as predicted by *SignalP* 3.0 and *LipoP* 1.0 was excluded (Bendtsen *et al.*, 2004; Juncker *et al.*, 2003). The PCR product with primer-introduced restriction sites for *Nco*I and *Not*I endonucleases was cleaved with the respective enzymes and ligated into the pETm_11 expression vector (EMBL, Heidelberg) encoding a TEV (*Tobacco etch virus*) protease cleavage site followed by a His tag. The obtained plasmid was transformed into *Escherichia coli* strain RR1 and the cells were grown overnight at 310 K on LB agar plates containing kanamycin. Colonies were inoculated into liquid LB medium containing kanamycin at 310 K for a further 24 h. Plasmid DNA was isolated from the resulting culture and DNA sequencing was carried out for all of the obtained clones to ensure that no errors had occurred. For the overexpression of hexahistidine-tagged fusion protein, the plasmid of the correct construct was transformed into *E. coli* BL21 (DE3) cells, which were grown in modified 2×TYP medium [2TY medium containing $16\ \mathrm{g\ l^{-1}}$ bacto-tryptone (Difco), $10\ \mathrm{g\ l^{-1}}$ bacto yeast extract (Difco) and $5\ \mathrm{g\ l^{-1}}$ NaCl, supplemented with $10\ \mathrm{mg\ ml^{-1}}$ kanamycin, 133 m*M* phosphate buffer pH 7.4 and $4\ \mathrm{g\ l^{-1}}$ glucose] with vigorous agitation at 298 K until an OD of 0.8–1.0 was reached and were then induced with 0.2 m*M* IPTG and cultivated for a further 16–20 h.

For the expression of SeMet-labelled protein, the plasmid was transformed into *E. coli* B834 (DE3) cells. The cells were grown in modified 2×TYP medium (supplemented with 133 m*M* phosphate buffer pH 7.4 and $2\ \mathrm{g\ l^{-1}}$ glucose) until an OD of 1.0 was reached and then centrifuged; the pellet was resuspended in 0501 medium (without methionine) (SelenoMet medium base from Athena Enzyme Systems) supplemented with 0502 medium (SelenoMet Nutrient mix from Athena Enzyme Systems) and glucose ($5\ \mathrm{g\ l^{-1}}$) and grown for a further 2 h. IPTG (0.5 m*M*) and a mixture of selenomethionine and methionine (5:1) was added and cultivation was continued for 20 h.

2.2. Protein purification and His-tag cleavage

The cells were harvested by centrifugation and lysed by sonication. The cell debris was removed by centrifugation and the recombinant protein with a six-histidine tag was purified from the lysate using affinity chromatography on an Ni–NTA agarose column (Qiagen). The recombinant protein was eluted with a high imidazole concentration followed by buffer exchange into 20 m*M* Tris–HCl pH 8.0 using an Amicon centrifugal filter unit (Millipore).

The hexahistidine tag was removed from BBA64 by addition of recombinant TEV protease to the protein ($5\ \mathrm{mg\ ml^{-1}}$ in 20 m*M* Tris–HCl pH 8.0) and incubation for 12 h at room temperature. After cleavage, the protease, digested His tag and remaining uncleaved protein were removed by passage

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through an Ni–NTA column (Qiagen). The BBA64 protein was further purified by ion-exchange chromatography on a Mono Q 5/50 GL column (GE Healthcare) linked to an ÄKTA chromatography system (Amersham Biosciences) at a flow rate of 1 ml min^{-1}. The fractions containing pure protein were buffer-exchanged into 20 m*M* Tris–HCl pH 8.0 and concentrated to a concentration of 8 mg ml^{-1} using an Amicon centrifugal filter unit (Millipore).

2.3. Mass spectrometry

To verify SeMet incorporation, MALDI–TOF mass spectrometry was performed for both SeMet-labelled and native protein. 1 µl protein (4 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0) was mixed with 1 µl 0.1% TFA and 1 µl matrix solution consisting of 15 mg ml^{-1} 2,5-dihydroxyacetophenone in 20 m*M* ammonium citrate, 75% ethanol. 1 µl of the obtained mixture was loaded onto the target plate, dried and analyzed using a Bruker Daltonics Autoflex mass spectrometer. The results indicated that the SeMet-labelled protein had a molecular weight that was about 200 Da larger than that of the native protein, which is consistent with the incorporation of all four possible SeMet residues.

The protein state in the crystals was tested using MALDI–TOF mass spectrometry essentially as described above. The obtained protein crystals were dissolved in 20 m*M* Tris–HCl pH 8.0 and compared with the protein batch used for crystallization.

2.4. Estimation of the multimeric state by gel-filtration chromatography

Purified protein sample (5 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 0.5 *M* NaCl) was loaded into a pre-packed Superdex 200 10/300 GL column connected to an ÄKTA chromatography system (Amersham Biosciences). The column was pre-equilibrated with the same buffer and run at a flow rate of 0.7 ml min^{-1}. Bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa) were used as molecular-weight reference standards.

2.5. Crystallization of native and SeMet-labelled protein

Crystallization was performed by the sitting-drop vapour-diffusion technique by mixing 1 µl protein solution with an equal volume of precipitant solution. Initial screening was performed using several 96-reagent sparse-matrix screens and a prospective crystal hit was obtained from Structure Screen I + II from Molecular Dimensions. Further optimization of the favourable condition was performed by examining about 300 variant conditions. Needle-shaped crystals with approximate dimensions of 800 × 60 × 20 µm were obtained using a precipitant solution consisting of 20% PEG 2000 MME, 0.05 *M* ammonium sulfate, 15% glycerol. SeMet-labelled protein crystals were obtained under the same conditions. Because of the relatively high concentration of PEG 2000 MME and glycerol in the crystallization mixture, no additional cryoprotection was used before the crystals were flash-cooled in liquid nitrogen.

2.6. Data collection and structure determination

The crystal structure of BBA64 was determined using the multiple-wavelength anomalous diffraction (MAD) technique. Diffraction data for the native and SeMet-labelled proteins were collected on beamline I911-3 at Max-lab (Lund University, Sweden) using a MAR Mosaic 225 detector (MAR Research GmbH). Native crystals of BBA64 diffracted to 2.4 Å resolution and SeMet-labelled crystals diffracted to 2.8 Å resolution. The space group was $P2_12_12_1$ in both cases, with nearly identical unit-cell parameters.

Reflections were indexed and scaled using the *MOSFLM* and *SCALA* programs from the *CCP*4 program suite (Battye *et al.*, 2011; Evans, 2006; Winn *et al.*, 2011). Data were merged using *CAD* (Dodson *et al.*, 1997) and Se-atom positions and initial phases were determined using *SHELXC*/*D*/*E* (Sheldrick, 2008). The initial protein model was built automatically in *Buccaneer* (Cowtan, 2006) and minor rebuilding of the model was performed manually in *Coot* (Emsley & Cowtan, 2004). Water molecules were picked automatically in *Coot* and inspected manually. Crystallographic refinement was carried out with *REFMAC*5 (Murshudov *et al.*, 2011).

3. Results and discussion

3.1. Quality of the model and overall structure

The asymmetric unit contained two protein molecules designated chains *A* and *B*. The protein model of chain *A* was built for residues 92–302 and that of chain *B* for residues 102–297. The signal sequence (residues 1–32) had already been excluded at the expression stage and residues 33–91 could not be seen in the electron-density map. In order to determine whether residues 33–91 were disordered or were absent in the crystal, we performed mass spectrometry of the crystallized material. The results revealed that although present in the purified protein, residues 33–49 were absent in the crystallized material, probably owing to a proteolytic susceptibility as reported for CspA (Cordes *et al.*, 2005). Meanwhile, residues 50–91 could not be seen in the electron-density map probably owing to the flexible nature of the N-terminal part of the protein molecule, which might form an unstructured region as indicated by the secondary-structure prediction software *Jpred* 3 (Cole *et al.*, 2008) and which is likely to serve as a linker between the structured region of the protein and the cell surface.

Residues Asp92–Lys101, Asp216–Pro224 and Leu298–Gln302 in chain *B* were not included in the model owing to weak electron density, although the same regions were well defined in chain *A* and thus no information on protein structure was lost.

The crystal structure of BBA64 reveals the same fold as originally detected in CspA (Cordes *et al.*, 2005) and consists of seven α-helices (from ten to 28 residues in length and named *A*–*G*) crossing at different angles, which are connected by loops of different lengths (Fig. 1*a*). The model also includes five sulfate ions and 114 water molecules. A summary of the

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data-collection, refinement and validation statistics is given in Table 1.

3.2. Oligomerization state and C-terminal α-helix

The crystal structure of BBA64 revealed essentially the same overall protein fold as that of CspA, except for the C-terminal part, which has different conformations in the two homologous proteins. In CspA the C-terminal α-helix (α-helix E) forms a stalk-like extension that protrudes outwards from the protein molecule and serves as a basis for dimer formation. The C-terminal α-helix F in BBA64, which corresponds to α-helix E in CspA, instead forms a loop that is not present in CspA and switches backwards, becoming part of a compact α-helical domain. Therefore, the crystal structure of BBA64 clearly indicates that the protein does not form a stable homodimer by interaction of the C-terminal α-helices as in the case of CspA. Since dimerization appears to be essential for the proper function of CspA, as the proposed ligand-binding site was located in the cleft between the two monomers, we performed an additional analysis of the oligomerization state of BBA64. To exclude loss of oligomerization owing to proteolysis, we first confirmed the molecular mass of the BBA64 monomer (peak at 28.7 kDa) by mass spectrometry. We then applied the purified BBA64 protein onto a calibrated gel-filtration column and observed that the protein eluted at between 43 and 25 kDa, indicating that BBA64 (31.6 kDa) is most likely to be a monomer and does not form a stable dimer in a solution.

Furthermore, the interaction of the monomers in the CspA protein leads to the formation of a homodimer in a crystal containing two molecules per asymmetric unit which interact

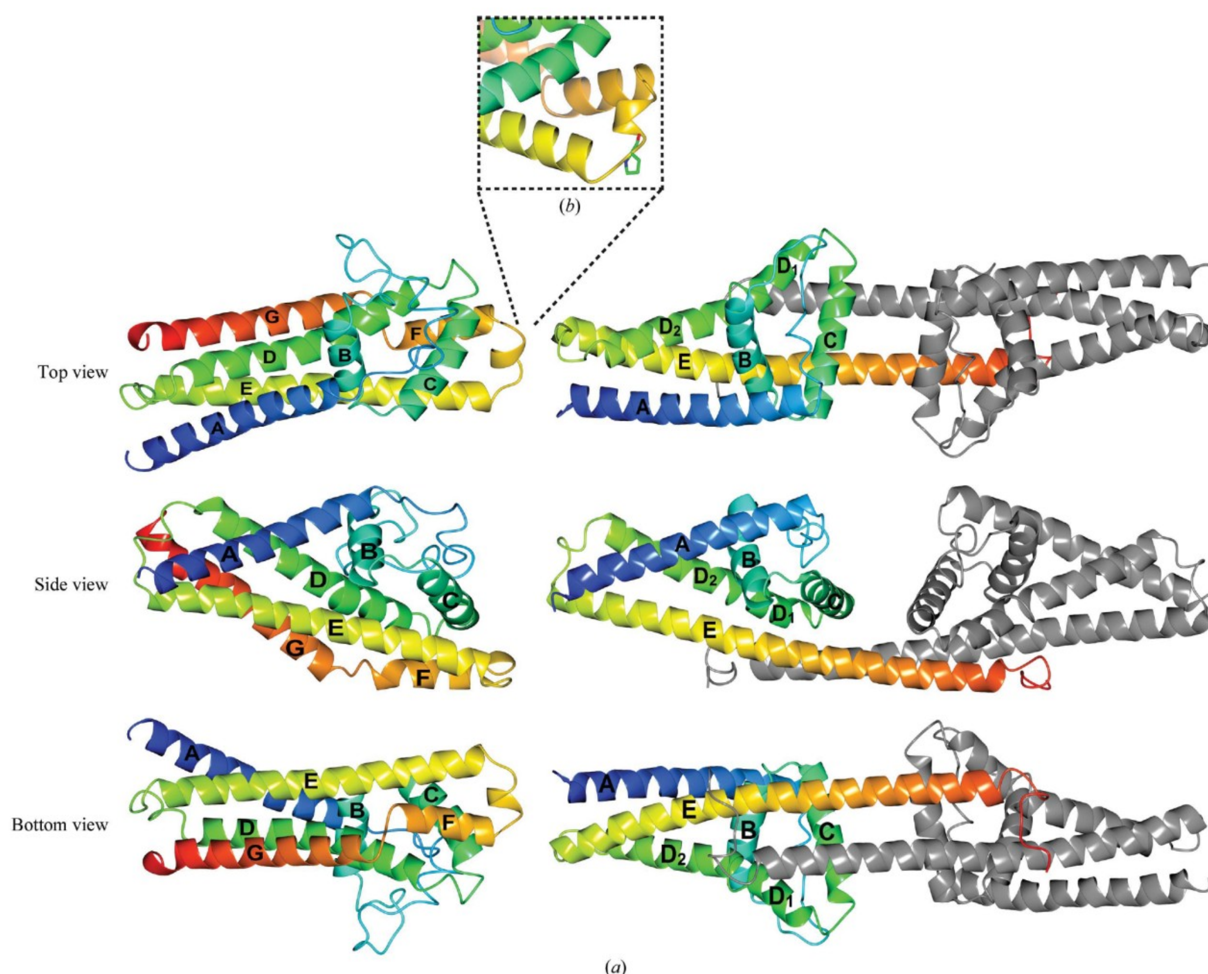

Figure 1
Crystal structures of BBA64 and the orthologous protein CspA. (*a*) A cartoon representation of three different views of BBA64 (left column) and CspA (right column) rotated by 90° in a horizontal plane and coloured using a rainbow colour scheme starting from a blue colour at the N-terminal part and gradually changing to a red colour approaching the C-terminal part of the model. The seven α-helices that form BBA64 are labelled from A to G starting from the N-terminal part; in CspA the α-helices are named from A to E starting from the N-terminal part. CspA is represented as a homodimer by overlapping the C-terminal α-helices, forming an extensive 2240.9 Å^2 contact area. (*b*) A loop between α-helices E and F in BBA64 contains a proline residue and thus limits the flexibility of the loop region.

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with their C-terminal α-helices and bury an extensive surface area of 2240.9 Å^2 in the interface site (Fig. 1*b*). This clearly demonstrates that dimer formation is highly probable for CspA. In contrast, although the BBA64 crystal structure also contains two protein molecules in the asymmetric unit, the interface between the two monomers buries a surface area of only 534 Å^2; other possible interfaces in the crystal as defined by the protein interface prediction software *PISA* (Krissinel & Henrick, 2007) bury surface areas of 188–370 Å^2, indicating that stable dimer formation is unlikely for BBA64.

It could be speculated that structural rearrangements in the C-terminal part of BBA64 could occur under certain conditions (*e.g.* at different pH values or temperatures) by switching the folded-back helix into an extended conformation and therefore promoting dimer formation. However, we consider this to be unlikely since a proline residue (Pro258) is located at the end of α-helix *E*, promoting the formation of a turn and limiting the flexibility of the respective helices in relation to each other (Fig. 2*c*).

Table 1
Data and structure-quality statistics.

Values in parentheses are for the highest resolution bin.

		SeMet		
Data set	Native	Peak	Remote	Inflection
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters				
a (Å)	49.15	49.38	49.35	49.11
b (Å)	70.14	69.97	70.09	69.91
c (Å)	186.19	186.50	186.70	186.12
Wavelength (Å)	1.0000	0.9791	0.9686	0.9793
Resolution (Å)	40.00–2.40 (2.46–2.53)	40–2.77 (2.94–2.77)	40–2.74 (2.91–2.74)	40–3.04 (3.23–3.04)
No. of reflections	92002	278029	287525	75123
No. of unique reflections	25348	19973	20587	14969
Completeness (%)	98.2 (99.4)	99.6 (100.0)	99.5 (100.0)	99.2 (100.0)
R_{merge}†	0.10 (0.39)	0.18 (0.76)	0.19 (0.83)	0.13 (0.38)
$\langle I/\sigma(I)\rangle$	8.70 (3.1)	12.5 (4.1)	11.8 (3.6)	8.9 (3.7)
Average multiplicity	3.6 (3.6)	14.0 (14.4)	14.0 (14.5)	5.0 (5.2)
Refinement				
R_{work}	0.215 (0.255)			
R_{free}	0.261 (0.355)			
No. of reflections	24057			
Average *B* factor (Å^2)				
Overall	28.5			
From Wilson plot	33.7			
No. of atoms				
Protein	3231			
Ligand	25			
Water	114			
R.m.s. deviations‡				
Bond lengths (Å)	0.018			
Bond angles (°)	2.060			
Protein geometry§				
Bad rotamers (%)	5.6			
Residues with bad bonds (%)	0.0			
Residues with bad angles (%)	0.0			
Clashscore, all atoms	5.2			
Ramachandran statistics				
Residues in most favoured regions (%)	96.4			
Residues in allowed regions (%)	3.6			
Outliers (%)	0.0			

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity. ‡ R.m.s. deviations from ideal values (Engh & Huber, 1991). § Calculated using *MolProbity* (Chen *et al.*, 2010).

3.3. Sequence comparison

Although dimer formation in CspA may be essential for the proper function of the protein, as has been proposed previously (Cordes *et al.*, 2005, 2006; Kraiczy *et al.*, 2009), there appears to be a contradiction as it has been shown that CspA is able to bind complement factor H and also FHL-1 under denatured conditions after Tris–Tricine SDS–PAGE separation (Kraiczy *et al.*, 2004) and formation of the homodimer has only been observed at very high protein concentrations (Cordes *et al.*, 2005). Therefore, the different orientation of the C-terminal α-helix of BBA64 and its inability to form a dimer may not be the only explanation for the diverse functions of the proteins. Analysis of BBA69, which is another member of the homologous Pfam54 protein family and has the highest sequence identity to CspA among the family members (61% identity), shows that it is thought to form homodimers in the same way as CspA but does not bind either complement factor H or FHL-1. The C-terminal α-helix amino-acid sequence of BBA69 was even made identical to that of CspA using site-directed mutagenesis and indicated that the C-terminal part of CspA is not sufficient to provide binding of complement regulators and that different parts of the protein cooperate to ensure the proper function of CspA (Kraiczy *et al.*, 2009; Wywial *et al.*, 2009). C-terminal deletion mutants of CspA also produced protein which did not bind complement regulators, suggesting that the C-terminal part of the protein could be directly involved in complement factor H and FHL-1 binding or could be responsible for the stability of the overall protein fold, as the functional assays for CspA were performed under reduced conditions (Wallich *et al.*, 2005). Therefore, to obtain further understanding about the differences in the structures and functions of the respective proteins, we performed a structure-based sequence alignment to highlight their differences and to indicate their importance in relation to the different appearances of the proteins (Fig. 2*a*).

Three potential sites on CspA which are essential for the function of the protein have been predicted (Wallich *et al.*, 2005; Kraiczy *et al.*, 2004, 2009; McDowell *et al.*, 2005). One of the sites, which is proposed to be involved in binding complement factor H and factor-H-like protein-1 (FHL-1), resides on α-helix *C* (residues 145–154) located at the cleft between the two molecules if a homodimer is formed. The

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second and third sites are located at the C-terminal α-helix *E* (residues 204–213 and 233–242) which constitutes the required surface area for dimer formation; interruption of this region could disrupt the formation of the dimers or affect the correct folding of a molecule and thus affect the function of the protein (Fig. 2*d*). By using mutational analysis, a number of specific residues located at these sites on CspA helices *C* and *E* have been identified which are relevant for dimer formation or interaction with FHL-1 and factor H binding proteins (Kraiczy *et al.*, 2009). Therefore, we searched for the respective residue locations in BBA64 using structure-based sequence alignment in order to justify the differences in protein function.

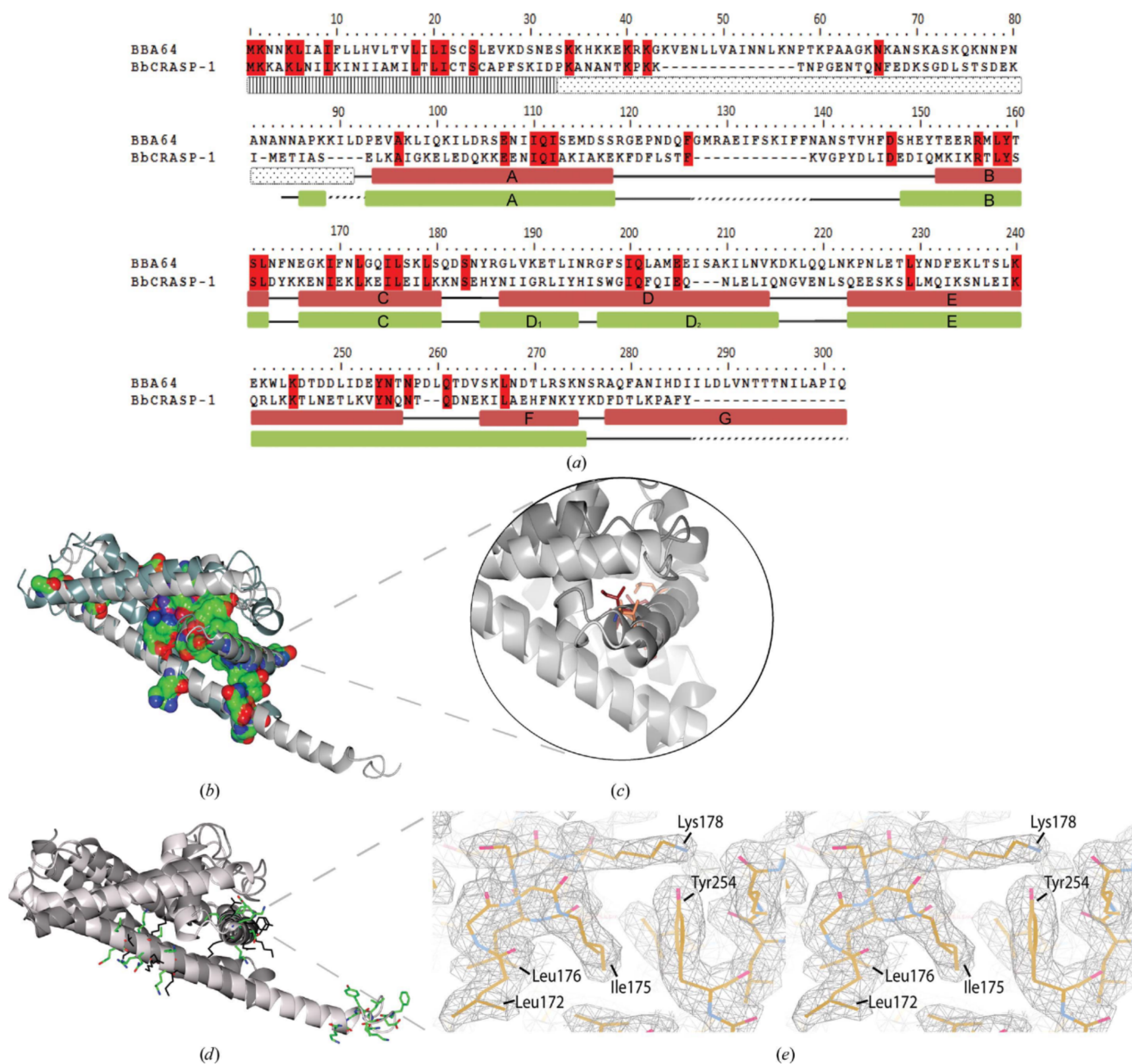

Figure 2
Structure-based sequence alignment of the homologous proteins BBA64 and CspA. (*a*) The initial alignment was obtained using the *PRALINE* multiple sequence-alignment tool and was adjusted manually based on the three-dimensional structures. Conserved residues are highlighted in red. The signal sequence region for BBA64 is shown as a rectangle filled with vertical stripes and the N-terminal part of BBA64 which cannot be seen in the electron-density map is shown as a dotted rectangle. Secondary-structure elements as deduced from the crystal structures of the BBA64 and CspA proteins are represented as rectangles for α-helices and as lines for loop regions and are shown below the sequence alignment. (*b*) Location of the conserved residues in superimposed crystal structures of the two homologous proteins BBA64 (dark grey) and CspA (light grey). Conserved amino acids are shown using a surface-representation model of the residues. (*c*) Conserved hydrophobic residues are directed inwards towards the protein core and are not exposed on the surface, suggesting that they are necessary for correct folding of the molecule and are relevant for α-helix orientation. (*d*) Superimposed crystal structures of BBA64 (dark grey with residues in black) and CspA (light grey with residues in green) showing the residues in three regions identified in CspA as being necessary for correct binding of complement factor H and FHL-1. (*e*) Stereoview of the $2mF_o - DF_c$ electron-density map contoured at 1.0σ located on α-helix *C* of BBA64 and representing protein residues as stick models.

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In the first region, which is thought to be involved in the binding of complement regulators, residues Lys136–Glu147 in α-helix *C* which forms the cleft in the dimer were systematically mutated, leading to only a slightly lower complement binding except for the residue Leu146, the mutation of which to a histidine led to a significant decrease in the binding of both complement factor H and FHL-1. Comparison of the amino-acid sequence of the potential binding site located at α-helix *C* of CspA with the same region of BBA64 revealed that Leu146 is conserved in the two homologues, together with other hydrophobic residues including Ile139, Leu142, Ile145 and Leu149. Structural analysis of the respective region of BBA64 and CspA reveals that all five conserved amino acids are located in the hydrophobic core of the protein monomer at the interface of α-helices *C* and *D*, and are not exposed at the surface of a monomer (Fig. 2*c*). Considering that all of these residues are hydrophobic, it can be assumed that they are necessary for the stability of the core and that the mutation of any of these residues could dislocate α-helix *C* or simply lead to incorrectly folded nonfunctional protein. Therefore, the difference in the nonconserved residues located on the surface of the two protein molecules, in this case on α-helix *C*, could explain the divergent characters and functions of the homologous proteins.

The second region of CspA, which is involved in the formation of the dimer interface located in the C-terminal part of the protein, was also exposed to mutational analysis. The crystal structure of BBA64 reveals a different conformation to that of CspA in that the C-terminal α-helix does not form a stalk-like extension running outwards from the central part of the protein and thus does not support dimer formation by itself, and the residues that were detected to be critical for dimer formation (Tyr240, Asp242 and Leu246) at the end of the C-terminal α-helix of CspA are substituted by other residues in BBA64, which is consistent with its monomeric state. In the C-terminal α-helix of BBA64, in addition to the

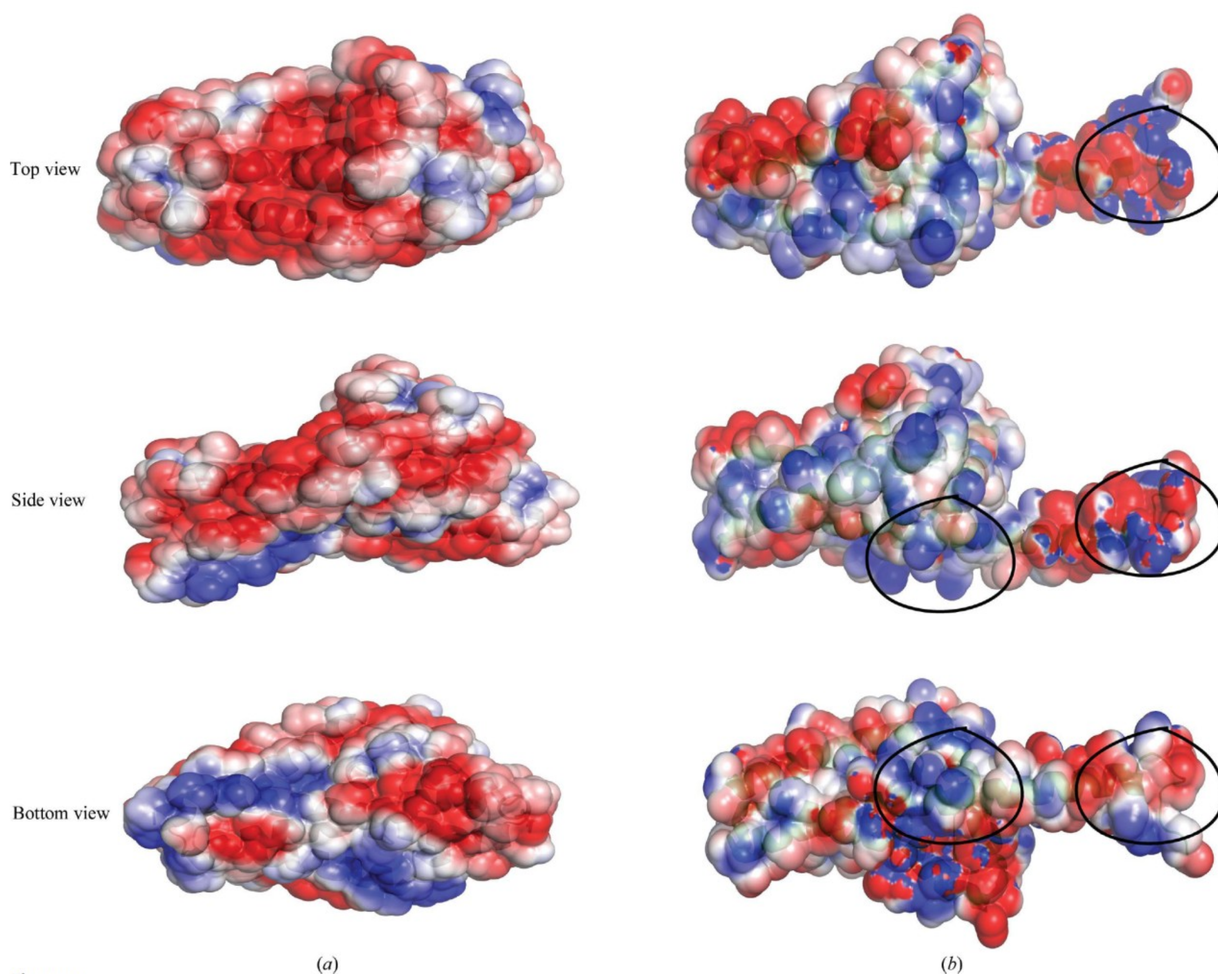

Figure 3
Electrostatic potential surface of BBA64 and CspA. The BBA64 monomer (*a*) is shown in the same orientation and on the same scale as the CspA monomer (*b*). Top, side and bottom views are shown in the same orientation as in Fig. 1. The electrostatic potential (red, negative; blue, positive) was calculated using *APBS* (Baker *et al.*, 2001).

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substitution of the residues relevant to dimer formation, there is an extension of 16 residues in the C-terminal α-helix *G*. The importance of this extension can be simply understood from the crystal structure: the α-helix interacts with the other α-helices *D* and *F* in BBA64 covering the whole length and forming a compact structure; in addition, it buries several hydrophobic residues at the interface between the helices, forming a more stable folded-back conformation. By analyzing the three different parts of CspA important for binding factor H and FHL-1 and comparing them with the same regions in BBA64, it becomes clear that although CspA can bind complement regulators as a monomer (as judged from its ability to bind complement factor H and FHL-1 after SDS–PAGE separation), the three regions that are necessary for proper binding exhibit considerable sequence diversity between the two homologous proteins and thus could also explain the inability of BBA64 to bind complement regulators in addition to the different fold of the C-terminal α-helix.

Interestingly, one general difference that can be noticed on analyzing the sequences and superimposed crystal structures is located in the loop region between α-helices *A* and *B* of BBA64 and CspA (Fig. 1). The loop in CspA consists of 17 residues and is remarkably shorter than that in BBA64, where the loop is formed of 29 residues, making this region of the protein more flexible. In addition, structure analysis using the *PISA* (*Protein Interfaces, Surfaces and Assemblies*) prediction tool (Krissinel & Henrick, 2007) reveals that the largest possible interface region in BBA64 that could interact with other molecules buries 534 $Å^2$ of surface area and also involves a large portion of the loop region between α-helices *A* and *B*. Therefore, we speculate that the loop region in BBA64 is likely to be related to the potential protein function and could be involved in formation of the protein binding site.

3.4. Overall electrostatic properties of BBA64 and CspA

In addition to comparison of the overall protein fold and amino-acid sequence, we also performed a comparison of the electrostatic properties of the two homologous proteins. The charge distribution of a molecule can influence the binding of a ligand or receptor, especially if the contact area between the two molecules buries a large surface area, as could be the case in CspA if we assume that all three of the regions described above are indeed necessary for binding complement factor H and FHL-1. From the electrostatic potential surface of BBA64 it can be deduced that the dominant charge of the molecule is negative and that only a small region at the bottom of the molecule has a positive surface potential (Fig. 3). The surface potentials of BBA64 and CspA could also explain the difference in their respective binding partners, as the surface potential of the segments in the CspA molecule which are thought to be necessary for the binding of complement factor H and FHL-1 (marked by circles in Fig. 3) are mainly positively charged, which is the opposite of the same segments in BBA64.

3.5. Potential function of BBA64

With regard to the potential ligand or receptor for BBA64, it has been shown that after treatment of *B. burgdorferi* with anti-BBA64 (an antibody for the outer surface protein BBA64) the binding of the bacteria to HUVEC (human umbilical vein endothelial cells) or H4 (human neuroglial cells) cells is significantly reduced, indicating that the potential binding partner that provides adherence and invasion of the Lyme disease agent could be located on the cell surface of the mammalian host organism (Schmit *et al.*, 2011). Considering that lipoprotein BBA64 is necessary to provide the transfer of *Borrelia* from tick salivary glands to the host organism, the binding of *Borrelia* to mammalian cells described above could reduce the range of the possible ligand/receptor search, leading to the exclusion of, for example, tick salivary gland proteins, which could otherwise be potential ligand candidates for consideration in assisting in the transport of *B. burgdorferi* to the host organism. The location of the receptors on the surface of the host cells could also explain the vital role of the protein in the transfer of *B. burgdorferi* from ticks to mammals and the observation that *Borrelia* lacking BBA64 synthesis was unable to infect mice *via* tick feeding but that infection could be initiated after needle inoculation of *B. burgdorferi* (Gilmore *et al.*, 2010; Patton *et al.*, 2011). It should be noted that the expression of the outer surface lipoprotein BBA64 is also highly upregulated in tissues during chronic murine infection throughout the infection period, indicating that the protein not only contributes to the transfer of *Borrelia* from ticks to the host organism and the establishment of the infection but also to the maintenance and persistence of Lyme disease (Gilmore *et al.*, 2008).

4. Conclusions

The crystal structure of *B. burgdorferi* outer surface lipoprotein BBA64, which plays a vital role in the pathogenesis of Lyme disease, has been solved at 2.4 Å resolution. There are several differences between BBA64 and the related homologous CspA protein, such as surface-exposed residue divergence and dissimilar electrostatic surface potentials, that could explain the distinct functions of these proteins. However, the main difference is the orientation of the C-terminal α-helix, which in BBA64 does not form a stalk-like extension running outwards from the protein and does not promote the formation of a homodimer, while in CspA the C-terminal part has been shown to be essential for the correct function of the protein and is involved in dimer formation. Although the exact function of BBA64 is still unclear, taking into account that the protein is a relevant component of *B. burgdorferi* pathogenesis the determination of the crystal structure is an important step forward to help to clarify the exact binding partner of the protein and to contribute to drug development against Lyme disease.

Coordinates and structure factors have been deposited in the Protein Data Bank (Berman *et al.*, 2000) with accession code 4aly.

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References

Angel, T. E., Luft, B. J., Yang, X., Nicora, C. D., Camp, D. G. II, Jacobs, J. M. & Smith, R. D. (2010). *PLoS One*, **5**, e13800.

Anguita, J., Samanta, S., Revilla, B., Suk, K., Das, S., Barthold, S. W. & Fikrig, E. (2000). *Infect. Immun.* **68**, 1222–1230.

Baker, N. A., Sept, D., Joseph, S., Holst, M. J. & McCammon, J. A. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 10037–10041.

Battye, T. G. G., Kontogiannis, L., Johnson, O., Powell, H. R. & Leslie, A. G. W. (2011). *Acta Cryst.* D**67**, 271–281.

Bendtsen, J. D., Nielsen, H., von Heijne, G. & Brunak, S. (2004). *J. Mol. Biol.* **340**, 783–795.

Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. & Bourne, P. E. (2000). *Nucleic Acids Res.* **28**, 235–242.

Brooks, C. S., Hefty, P. S., Jolliff, S. E. & Akins, D. R. (2003). *Infect. Immun.* **71**, 3371–3383.

Burgdorfer, W., Barbour, A. G., Hayes, S. F., Benach, J. L., Grunwaldt, E. & Davis, J. P. (1982). *Science*, **216**, 1317–1319.

Carroll, J. A., Cordova, R. M. & Garon, C. F. (2000). *Infect. Immun.* **68**, 6677–6684.

Casjens, S. R. *et al.* (2012). *PLoS One*, **7**, e33280.

Casjens, S., Palmer, N., van Vugt, R., Huang, W. M., Stevenson, B., Rosa, P., Lathigra, R., Sutton, G., Peterson, J., Dodson, R. J., Haft, D., Hickey, E., Gwinn, M., White, O. & Fraser, C. M. (2000). *Mol. Microbiol.* **35**, 490–516.

Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. & Richardson, D. C. (2010). *Acta Cryst.* D**66**, 12–21.

Cole, C., Barber, J. D. & Barton, G. J. (2008). *Nucleic Acids Res.* **36**, W197–W201.

Cordes, F. S., Kraiczy, P., Roversi, P., Simon, M. M., Brade, V., Jahraus, O., Wallis, R., Goodstadt, L., Ponting, C. P., Skerka, C., Zipfel, P. F., Wallich, R. & Lea, S. M. (2006). *Int. J. Med. Microbiol.* **296**, Suppl. 40, 177–184.

Cordes, F. S., Kraiczy, P., Roversi, P., Skerka, C., Kirschfink, M., Simon, M. M., Brade, V., Lowe, E. D., Zipfel, P., Wallich, R. & Lea, S. M. (2004). *Acta Cryst.* D**60**, 929–932.

Cordes, F. S., Roversi, P., Kraiczy, P., Simon, M. M., Brade, V., Jahraus, O., Wallis, R., Skerka, C., Zipfel, P. F., Wallich, R. & Lea, S. M. (2005). *Nature Struct. Mol. Biol.* **12**, 276–277.

Cowtan, K. (2006). *Acta Cryst.* D**62**, 1002–1011.

Dodson, E. J., Winn, M. & Ralph, A. (1997). *Methods Enzymol.* **277**, 620–633.

Embers, M. E., Ramamoorthy, R. & Philipp, M. T. (2004). *Microbes Infect.* **6**, 312–318.

Emsley, P. & Cowtan, K. (2004). *Acta Cryst.* D**60**, 2126–2132.

Engh, R. A. & Huber, R. (1991). *Acta Cryst.* A**47**, 392–400.

Evans, P. (2006). *Acta Cryst.* D**62**, 72–82.

Fraser, C. M. *et al.* (1997). *Nature (London)*, **390**, 580–586.

Gilmore, R. D., Howison, R. R., Dietrich, G., Patton, T. G., Clifton, D. R. & Carroll, J. A. (2010). *Proc. Natl Acad. Sci. USA*, **107**, 7515–7520.

Gilmore, R. D., Howison, R. R., Schmit, V. L. & Carroll, J. A. (2008). *Microb. Pathog.* **45**, 355–360.

Gilmore, R. D., Howison, R. R., Schmit, V. L., Nowalk, A. J., Clifton, D. R., Nolder, C., Hughes, J. L. & Carroll, J. A. (2007). *Infect. Immun.* **75**, 2753–2764.

Hughes, J. L., Nolder, C. L., Nowalk, A. J., Clifton, D. R., Howison, R. R., Schmit, V. L., Gilmore, R. D. & Carroll, J. A. (2008). *Infect. Immun.* **76**, 2498–2511.

Indest, K. J., Ramamoorthy, R., Solé, M., Gilmore, R. D., Johnson, B. J. & Philipp, M. T. (1997). *Infect. Immun.* **65**, 1165–1171.

Juncker, A. S., Willenbrock, H., Von Heijne, G., Brunak, S., Nielsen, H. & Krogh, A. (2003). *Protein Sci.* **12**, 1652–1662.

Kraiczy, P., Hanssen-Hübner, C., Kitiratschky, V., Brenner, C., Besier, S., Brade, V., Simon, M. M., Skerka, C., Roversi, P., Lea, S. M., Stevenson, B., Wallich, R. & Zipfel, P. F. (2009). *Int. J. Med. Microbiol.* **299**, 255–268.

Kraiczy, P., Hellwage, J., Skerka, C., Becker, H., Kirschfink, M., Simon, M. M., Brade, V., Zipfel, P. F. & Wallich, R. (2004). *J. Biol. Chem.* **279**, 2421–2429.

Kraiczy, P., Rossmann, E., Brade, V., Simon, M. M., Skerka, C., Zipfel, P. F. & Wallich, R. (2006). *Wien. Klin. Wochenschr.* **118**, 669–676.

Kraiczy, P., Skerka, C., Kirschfink, M., Brade, V. & Zipfel, P. F. (2001). *Eur. J. Immunol.* **31**, 1674–1684.

Kraiczy, P., Skerka, C., Kirschfink, M., Zipfel, P. F. & Brade, V. (2001). *Int. Immunopharmacol.* **1**, 393–401.

Krissinel, E. & Henrick, K. (2007). *J. Mol. Biol.* **372**, 774–797.

McDowell, J. V., Harlin, M. E., Rogers, E. A. & Marconi, R. T. (2005). *J. Bacteriol.* **187**, 1317–1323.

Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* D**67**, 355–367.

Ojaimi, C., Brooks, C., Casjens, S., Rosa, P., Elias, A., Barbour, A., Jasinskas, A., Benach, J., Katona, L., Radolf, J., Caimano, M., Skare, J., Swingle, K., Akins, D. & Schwartz, I. (2003). *Infect. Immun.* **71**, 1689–1705.

Patton, T. G., Dietrich, G., Dolan, M. C., Piesman, J., Carroll, J. A. & Gilmore, R. D. (2011). *PLoS One*, **6**, e19536.

Ramamoorthy, R. & Scholl-Meeker, D. (2001). *Infect. Immun.* **69**, 2739–2742.

Revel, A. T., Talaat, A. M. & Norgard, M. V. (2002). *Proc. Natl Acad. Sci. USA*, **99**, 1562–1567.

Schmit, V. L., Patton, T. G. & Gilmore, R. D. (2011). *Front. Microbiol.* **2**, 141.

Sheldrick, G. M. (2008). *Acta Cryst.* A**64**, 112–122.

Steere, A. C., Coburn, J. & Glickstein, L. (2004). *J. Clin. Invest.* **113**, 1093–1101.

Tokarz, R., Anderton, J. M., Katona, L. I. & Benach, J. L. (2004). *Infect. Immun.* **72**, 5419–5432.

Wallich, R., Pattathu, J., Kitiratschky, V., Brenner, C., Zipfel, P. F., Brade, V., Simon, M. M. & Kraiczy, P. (2005). *Infect. Immun.* **73**, 2351–2359.

Winn, M. D. *et al.* (2011). *Acta Cryst.* D**67**, 235–242.

Wywial, E., Haven, J., Casjens, S. R., Hernandez, Y. A., Singh, S., Mongodin, E. F., Fraser-Liggett, C. M., Luft, B. J., Schutzer, S. E. & Qiu, W.-G. (2009). *Gene*, **445**, 26–37.

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3.2. Structural characterization of the *Borrelia burgdorferi* outer surface protein BBA73 implicates dimerization as a functional mechanism

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Structural characterization of the *Borrelia burgdorferi* outer surface protein BBA73 implicates dimerization as a functional mechanism

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ABSTRACT

Borrelia burgdorferi, which is the causative agent of Lyme disease, is transmitted from infected *Ixodes* ticks to a mammalian host following a tick bite. Upon changing the host organism from an *Ixodes* tick to a warm-blooded mammal, the spirochete must adapt to very different conditions, which is achieved by altering the expression of several genes in response to a changing environment. Recently, considerable attention has been devoted to several outer surface proteins, including BBA73, that undergo dramatic upregulation during the transmission of *B. burgdorferi* from infected *Ixodes* ticks to mammals and that are thought to be important for the establishment and maintenance of the infection. These upregulated proteins could reveal the mechanism of pathogenesis and potentially serve as novel drug targets to prevent the transmission of the pathogenic bacteria.

To promote effective treatments for Lyme disease and to gain better insight into *B. burgdorferi* pathogenesis, we have determined the crystal structure of the upregulated outer surface protein BBA73 at 2.0 Å resolution.

We observed that the BBA73 protein exists as a homodimer both in the crystal and in solution. The monomers interact with their N-terminal α-helices and form a cleft that could potentially serve as a ligand or receptor binding site. To confirm that the protein dimerizes through the interaction of the N-terminal regions, we produced an N-terminal deletion mutant of BBA73 to disrupt dimerization, and we determined the crystal structure of the truncated BBA73 protein at 1.9 Å resolution. The truncated protein did not form a homodimer, and the crystal structure confirmed that the overall fold is identical to that of the native BBA73 protein. Notably, a paralogous protein CspA from *B. burgdorferi* with known crystal structure also forms a homodimer, albeit through an entirely different interaction between the monomers.

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1. Introduction

Lyme disease is the most common tick-borne infection caused by the transfer of *Borrelia burgdorferi* from infected *Ixodes* ticks to a mammalian host organism. If not treated by antibiotics or in the case of an inadequate treatment at early stages of the infection, the disease can cause severe neurological symptoms (neuroborreliosis), dermatitis (acrodermatitis chronica atrophicans) or joint inflammation (Lyme arthritis) [1–4].

When an infected *Ixodes* tick bites a mammalian organism and begins to feed, *B. burgdorferi* responds to the mammalian host-specific signals and the occurring changes in temperature, cell density, pH and nutrient level by upregulating the expression of various predominantly surface-exposed proteins, including BBA73, that are thought to be associated with the pathogenesis of *B. burgdorferi* [5–11]. The upregulated proteins are considered to be essential for *B. burgdorferi*, first, to migrate from the midgut of the tick to the salivary glands; secondly, to enter the mammalian host organism; and thirdly, to help the bacteria to disseminate, target to specific tissues and resist the immune response of the vertebrate host organism [12].

Much attention has been directed to the genes residing on the linear plasmid 54 (lp54). lp54 is one of the twelve linear plasmids of *B. burgdorferi* in addition to 9 circular plasmids and the chromosome. Nine genes belonging to the paralogous gene family PFam54 are among the most differentially expressed borrelial genes [6,13] and thus are of particular interest. The *B. burgdorferi* outer surface protein BBA73 is one of the members of the gene family PFam54 and has been shown by several studies to respond to different environmental changes associated with the transfer of the bacteria from *Ixodes* ticks to a mammalian organism. *bba73* has been identified as the second highest temperature-responsive gene through

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the analysis of all the putative ORFs of *B. burgdorferi* demonstrating increased expression at 35 °C relative to 25 °C [6]. BBA73 is also one of the most upregulated proteins identified after the addition of blood to a spirochete culture at 35 °C for 48 h, thus indicating the potentially important role of BBA73 in the transfer from ticks to the vertebrate host organism [5]. The expression of BBA73 is also increased by a decrease in the pH of the medium from 8.0 to 7.0, which simulates the pH change upon transfer from a tick to a mammalian host [7]. Moreover, BBA73 has been characterized as a potential vaccine candidate based on its antigenicity and conservation among the major Lyme disease-causing borrelial species [14,15]. These observations clearly suggest that the expression of BBA73 is regulated by pH, temperature and host-specific signals, and the protein can be reasonably considered an important constituent of *B. burgdorferi* pathogenesis.

To facilitate the determination of the function of this apparently important protein involved in the pathogenesis of *B. burgdorferi* and to promote the development of novel potential drugs against Lyme disease, we have determined the crystal structure of the outer surface protein BBA73 at 2.0 Å resolution.

2. Materials and methods

2.1. Cloning and expression of BBA73 and BBA73S

The gene encoding the outer surface protein BBA73 of *B. burgdorferi* strain B31 was amplified by PCR from genomic DNA excluding the signal sequence (residues 1–27). The amplified product was ligated into the pETm_11 expression vector (EMBL) encoding an N-terminal 6×His tag followed by a TEV (Tobacco Etch Virus) protease cleavage site. The plasmid was transformed into the *E. coli* strain RR1, and the cells were grown overnight at 37 °C on LB agar plates containing kanamycin. Colonies were inoculated into LB medium containing kanamycin at 37 °C for another 24 h. Plasmid DNA was isolated from the resulting culture and verified by DNA sequencing. For the overexpression of the 6×His-tagged target protein, the plasmid was transformed into *E. coli* BL21(DE3) cells. The cells were grown in modified 2× TYP media (supplemented with kanamycin (10 mg/ml), 133 mM phosphate buffer, pH 7.4, and glucose (4 g/l)) with vigorous agitation at 25 °C until an OD_{600} of 0.8–1.0, induced with 0.2 mM IPTG and grown for an additional 16–20 h. For the truncated protein (BBA73S), the procedure was identical except that the construct was designed to begin at residue Glu87.

2.2. Protein purification and 6×His tag cleavage

E. coli cells were lysed by sonication, and the cell debris was removed by centrifugation. The recombinant proteins BBA73 and BBA73S containing 6×His tags were purified using affinity chromatography on a Ni–NTA agarose (Qiagen, Germany) column followed by a buffer exchange to 10 mM Tris–HCl, pH 8.0, using an Amicon centrifugal filter unit (Millipore).

A recombinant TEV protease was added to BBA73 and BBA73S to remove the 6×His tag, and the reaction was incubated overnight at room temperature. The protease, the digested 6×His tag and the remnants of uncleaved protein were removed using an additional round of Ni–NTA chromatography. Both proteins were further purified using ion-exchange chromatography on a Mono Q 5/50 GL column (GE Healthcare).

2.3. Mass spectrometry

The state of the protein in the crystals was analyzed using MALDI-TOF mass spectrometry and compared with an identical protein batch used for crystallization. The obtained protein crystals were dissolved in 10 mM Tris–HCl, pH 8.0, and 1 μl of protein (11 mg/ml in 10 mM Tris–HCl, pH 8.0) was mixed with 1 μl of 0.1% TFA and 1 μl of matrix solution containing 15 mg/ml 2,5-dihydroxyacetophenone in 20 mM ammonium citrate and 75% ethanol. An identical procedure was performed on the batch of protein used for crystallization, and 1 μl of the obtained mixture was loaded on the target plate, dried and analyzed using a Bruker Daltonics Autoflex mass spectrometer.

2.4. Estimation of the multimeric state using size exclusion chromatography

The purified protein sample at a concentration of 4 mg/ml in 10 mM Tris–HCl, pH 8.0, and 0.2 M, 0.5 M or 1 M NaCl was loaded onto a prepacked Superdex 200 10/300 GL column (Amersham Biosciences). The column was preequilibrated with an identical buffer and run at a flow rate of 0.7 ml/min. Bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa) were used as MW reference standards.

2.5. Crystallization of the native and truncated protein

Crystallization was performed using the sitting drop vapor diffusion method in 96-well plates by mixing 1 μl of protein with an equal volume of precipitant solution. Sparse-matrix screens consisting of 96 reagents were used for the initial screening of crystallization conditions for BBA73, and protein crystals were obtained in 12% PEG 20,000 and 0.1 M MES, pH 6.5, from the Structure Screen 1 & 2 (Molecular Dimensions Ltd., UK). The conditions were further optimized, and needle-shaped crystals of BBA73 were obtained in 14% PEG 3350, 0.1 M HEPES, pH 7.0, and 0.05 M NaH_2PO_4. The BBA73S protein was crystallized in 18% PEG 3350 and 0.1 M MES, pH 6.5, resulting in a different, tetragonal crystal form. As a cryoprotectant, the mother liquor with 25% glycerol was used in both cases, and crystals were flash-frozen in liquid nitrogen.

2.6. Data collection and structure determination

Diffraction data for the native and truncated proteins were collected at the MAX-lab beamlines I911-3 and I911-2 (Lund, Sweden). Reflections were indexed and scaled using MOSFLM [16] and SCALA [17] from the CCP4 suite [18]. Initial phases for BBA73 were obtained by molecular replacement using Phaser [19] and the crystal structure of the paralogous protein BBA64 as a search model (22% sequence identity, PDB entry 4ALY). For BBA73S, the crystal structure of BBA73 was used as a search model. For BBA73, molecular replacement was followed by automatic model building using BUCCANEER [20], and for both structures, the models were improved by manual rebuilding in COOT [21]. Crystallographic refinement was performed using REFMAC5 [22]. Water molecules were picked automatically in COOT and inspected manually.

A summary of the data collection, refinement and validation statistics for BBA73 and BBA73S is provided in Table 1.

3. Results and discussion

3.1. Overall crystal structure of BBA73 and BBA73S

The protein model for full-length BBA73 was built for residues 71–287 and for residues 87–287 of the truncated protein (BBA73S). The signal sequence of BBA73 (residues 1–27) was already excluded from the expressed protein, and residues 28–70 at the N-terminus of BBA73 were not observed in the electron density map. The electron density for the C-terminal residues 288–296 in

Table 1
Statistics for data and structure quality.

Dataset	Native BBA73	Truncated BBA73
Space group	P 2_12_12	P 4_32_12
Unit cell dimensions		
A (Å)	153.63	74.34
B (Å)	47.10	74.34
C (Å)	34.50	95.62
Wavelength (Å)	1.0000	1.0408
Resolution (Å)	40.15–2.09	23.03–1.88
Highest resolution bin (Å)	2.20–2.09	1.98–1.88
No. of reflections	216455	106455
No. of unique reflections	15642	21966
Completeness (%)	99.9 (99.8)	98.6 (99.7)
R_{merge}	0.07 (0.36)	0.06 (0.49)
I/σ (*I*)	22.8 (6.8)	17.0 (3.1)
Multiplicity	13.8 (12.2)	4.8 (4.8)
Refinement		
R_{work}	0.215 (0.231)	0.204 (0.271)
R_{free}	0.244 (0.298)	0.235 (0.340)
Average B-factor (Å^2)		
Overall	32.0	23.9
From Wilson plot	29.1	29
No. of atoms		
Protein	1795	1667
Ligand	0	0
Water	87	84
RMS deviations from ideal		
Bond lengths (Å)	0.011	0.009
Bond angles (°)	1.382	1.124
Ramachandran outliers (%)		
Residues in most favored regions (%)	98.15	98.02
Residues in allowed regions (%)	1.85	1.98
Outliers (%)	0.00	0.00

*Values in parentheses are for the highest resolution bin.

both BBA73 and BBA73S were largely uninterpretable, and these residues were therefore excluded from the final model. To determine whether the N-terminal residues 28–70 in BBA73 are disordered or absent in the crystal, we analyzed the crystallized material using mass spectrometry. The results revealed that residues 28–49, although present in the purified protein, are absent in the crystallized material likely due to a proteolytic susceptibility similar to that reported for the homologous proteins BBA64 and CspA [23]. In contrast, residues 50–70, although present in the crystal, could not be observed in the electron density map likely due to the flexible nature of the N-terminus of the protein, which is most likely unstructured and serves as a linker between the structured domain and the cell surface.

The crystal structure of the BBA73 monomer consists of seven α-helices, which are from 5 to 28 residues long, that are connected by loops of different lengths, and the overall fold is similar to the homologous proteins BBA64 and CspA (Fig. 1A).

3.2. PFam54 family members and the dimerization of BBA73

The amino acid sequence identity among the 11 members (9 members located on lp54) of the PFam54 paralogous gene family of *B. burgdorferi* varies from 17% to 60%. The functions of the paralogous protein family members are thought to be different, as reported by several studies indicating that the expression level, timing and the target receptors/ligands differ substantially among the PFam54 family members [24,25]. Although all of these proteins are thought to be related to the pathogenesis of *B. burgdorferi*, the exact function has been established only for one protein, CspA, from the PFam54 family. CspA is a complement regulator factor H and factor H-like protein-1 (FHL-1) binding protein, and thus, it assists the bacteria to resist the host immune response. CspA is the only PFam54 member that is known to bind complement regulators, as verified by several studies [26–29]. Previously, crystal structures have been determined for two members of the PFam54 family: the aforementioned CspA and an outer surface protein, BBA64. BBA64 plays an essential role in the transfer of *B. burgdorferi* from infected *Ixodes* ticks to a mammalian host organism after a tick bite, although the exact ligand or receptor is not known [30,31]. The major difference between CspA and BBA64 as observed from the crystal structures of both proteins is the different orientation of the C-terminal α-helix, which, in the case of BBA64, does not form a stalk-like extension protruding outwards from the globular protein fold but instead bends backwards to form a compact globular domain [23]. The studies on C-terminal deletion mutants of CspA have indicated that the C-terminal α-helix is essential for the binding of the complement regulators, although additional regions have also been determined to be associated with the binding of the complement factor H and FHL-1 [29,32,33]. The importance of the C-terminal region became evident when it was shown that the C-terminal α-helix of CspA is involved in dimerization by burying an extensive 2240.9 Å^2 surface area at the dimer interface. Dimerization of CspA has been proposed to be important for its function, and it is thought that the complement factor H and FHL-1 bind at the cleft between the monomers (Fig. 1B) [23,29,34].

The crystal structure of BBA73 revealed that the overall protein fold is more similar to the homologous protein BBA64 because the C-terminal α-helix does not form a stalk-like extension as was observed for CspA (Fig. 1C). However, using size exclusion chromatography, we determined that BBA73 forms a stable homodimer in solution. A closer inspection of the BBA73 crystal structure and the prediction of possible interfaces using the PISA software (Protein Interfaces, Surfaces and Assemblies prediction tool) revealed that the N-terminal α-helices of two monomers interact with each other and form a 600 Å^2 contact area in which several large hydrophobic residues (2 phenylalanines and 3 isoleucines from each subunit) are buried. Because a moderate 600 Å^2 contact area cannot be regarded as a reliable dimerization interface, we wished to confirm our assumption that the N-terminal α-helix is indeed sufficient and necessary for dimerization in solution and that the observed interaction is not merely a crystal contact. We produced an N-terminal deletion mutant of BBA73 (named BBA73S in this study) excluding the first 16 residues from α-helix A (residues 71–86) that were observed to be involved in the dimerization. The crystal structure of the truncated protein BBA73S revealed that the overall protein structure is identical to BBA73, demonstrating that the deletion of the N-terminal residues from α-helix A does not affect the overall fold of the molecule. Size exclusion chromatography indicated that BBA73S eluted as a monomer, confirming that the N-terminal α-helix is indeed necessary for dimerization (Fig. 1A). As previously mentioned, BBA73 appeared to contain a slightly longer N-terminal region than that observed for the homologous proteins CspA and BBA64, and approximately 20 residues from α-helix A are found in the dimerization interface (residues 71–90). Additionally, the secondary structure prediction software PSIPRED v3.0 [35] predicted with a high confidence that the N-terminal α-helix could be approximately 11 residues longer than that in the solved crystal structure. Therefore, the actual interaction surface between the monomers in solution could be even larger and could already begin from residue Leu61. This prediction would be consistent with the crystal structure of BBA73, although electron density was not observed for this region likely due to the flexible nature of the N-terminal region of the molecule.

The exact reason for BBA73 dimerization remains unknown, but the cleft between the monomers might serve as a binding surface for a potential ligand/receptor.

To further explore the characteristics of the BBA73 dimer, the electrostatic surface potential for BBA73 was analyzed. The

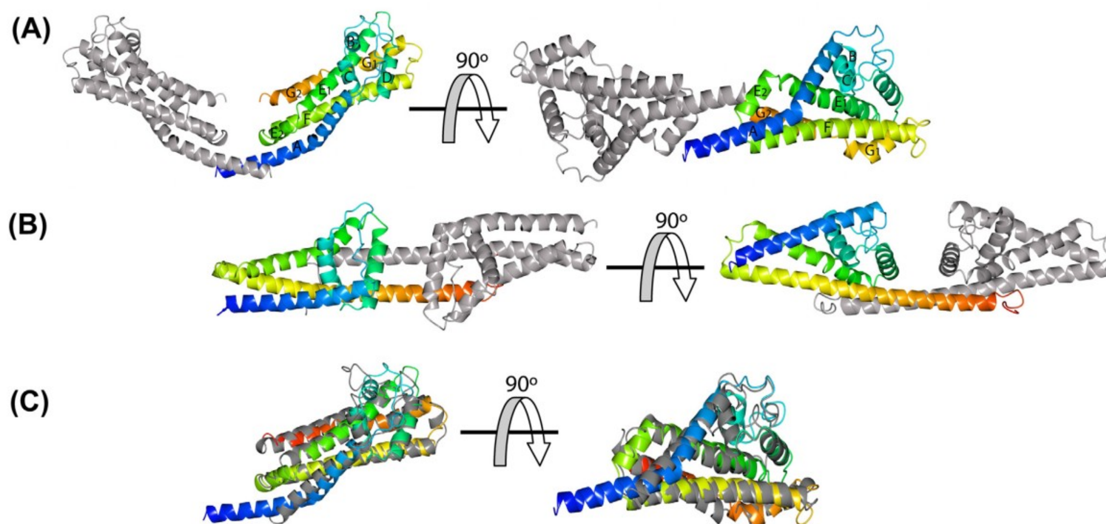

Fig. 1. Differences in the crystal structures of the homologous proteins BBA73, CspA and BBA64. (A) Homodimer of BBA73 formed by the interaction of the N-terminal α-helices as viewed from two different angles rotated by 90 degrees in the horizontal plane. (B) Homodimer of the homologous protein CspA formed by the interaction of the C-terminal α-helices viewed in an identical orientation as for BBA73. (C) Superimposed crystal structures of BBA73 (colored molecule) and BBA64 (gray) monomers oriented at different angles, as shown for the BBA73 dimers. In the dimers, one molecule of the dimer is shown in rainbow representation colored in blue at the N-terminus and gradually switching to red at the C-terminus, whereas the other monomer is colored gray. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

analysis of the dimer revealed that the cleft between the monomers is predominately negatively charged, suggesting that this negative charge could be necessary for the binding of a positively charged ligand/receptor (Fig. 2). In contrast, the bottom of the dimer molecule is predominately positively charged, which could possibly contribute to the protein function as well.

4. Residue conservation in the homologous proteins

A structure-based sequence alignment comparing BBA73 with other members of the homologous protein family PFam54 with known crystal structures is shown in Fig. 3. The sequence identity among the homologous proteins ranges from 18% between BBA73 and CspA to 22% between BBA73 and BBA64. Structure-based sequence comparison revealed several segments in the crystal structures of the three homologous proteins that were conserved among all of the members. One of the most conserved regions among all three members is located in α-helices C and D (as defined in BBA73). Structural comparison reveals that the conserved residues (residues Arg145, Tyr148, Ser150, Leu151, Ile158, Leu161, Ile164 and Leu165, as numbered in BBA73) are facing the hydrophobic core of the proteins and are apparently necessary for the stabilization of the fold in this region. One partially buried conserved salt bridge, which links Arg145 and Glu194, is observed in all of the structures. Presumably, the salt bridge plays an important role in the stabilization of the fold and the positioning of α-helices C and E (as designated in BBA73). Although this region of the molecule is associated with the potential binding site in CspA and the sequence alignment indicates high conservation among the three homologous proteins, actually the surface-exposed residues that could be involved in ligand/receptor binding differ among the homologous proteins, which may reflect the diverse functions of the three proteins. There are also conserved residues in α-helices E and G of BBA73 that correspond to α-helices D and E, respectively, in BBA64 and CspA (residues Ile189, Gln190, Glu194 and Tyr245, as numbered in BBA73) all of which also point toward the protein core and are located in nearly identical positions in all of the protein structures, which suggests their role in the preservation of the overall fold.

There are only a few residues (Glu100, Gln104, Lys236 and Asn248, as numbered in BBA73) that are exposed to the surface of the protein molecules and are conserved among the homologs. None of the four conserved residues are located near the cleft between the monomers that is suggested as a potential binding site.

5. Accession numbers

The coordinates and structure factors for BBA73 and the N-terminal truncation mutant of BBA73 have been deposited in the Protein Data Bank with the accession numbers 4AXZ and 4B2F.

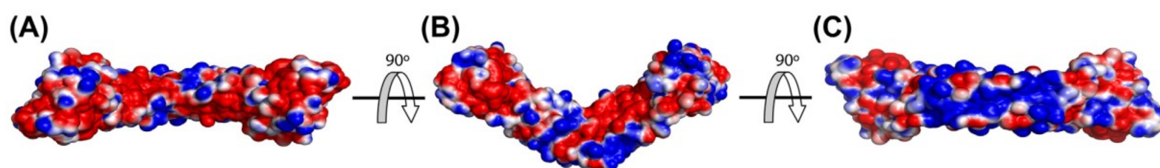

Fig. 2. Electrostatic surface potential of the BBA73 dimer. The homodimer is shown in three different orientations, each rotated by 90 degrees. The electrostatic potential (red, negative; blue, positive) was calculated using APBS [41], and the surface contour level is set to −1 kT/e (red) and +1 kT/e (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

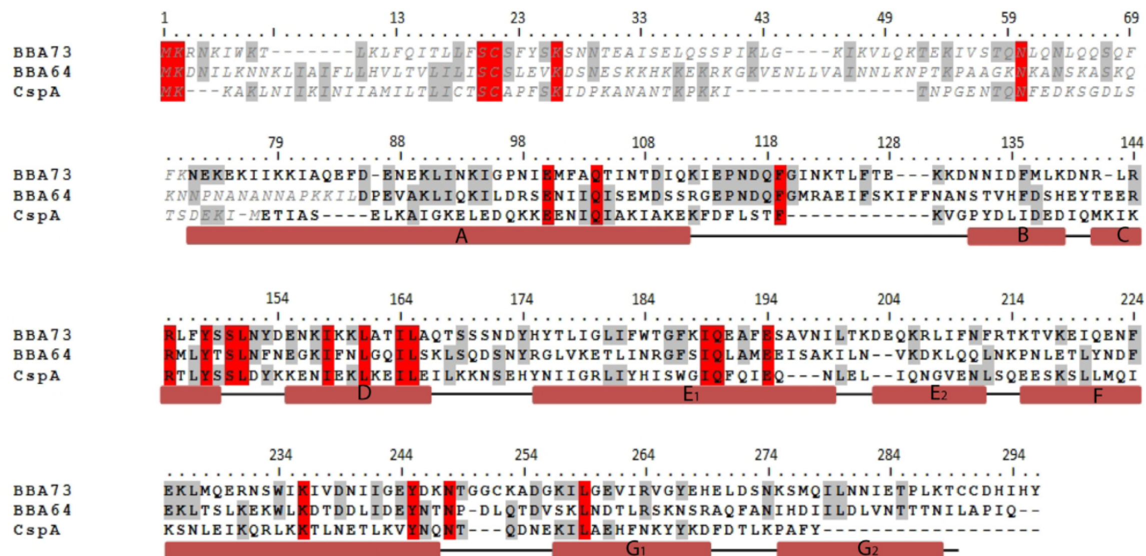

Fig. 3. Structure-based sequence alignment of BBA73 and the homologous proteins BBA64 and CspA. Residues not observed in the electron density map for BBA73, BBA64 and CspA are indicated in italics and colored gray. Conserved residues of all three homologous proteins are colored red, but residues conserved between any two members are colored in gray. The secondary structure is represented for BBA73 below the alignment sequence as cylinders for α-helices and as lines for loop regions. The numbering above the alignment is shown for BBA73. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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References

[1] R.R. Mullegger, M. Glatz, Skin manifestations of Lyme borreliosis: diagnosis and management, Am. J. Clin. Dermatol. 9 (2008) 355–368.

[2] A.R. Pachner, D. Cadavid, G. Shu, D. Dail, S. Pachner, E. Hodzic, S.W. Barthold, Central and peripheral nervous system infection, immunity, and inflammation in the NHP model of Lyme borreliosis, Ann. Neurol. 50 (2001) 330–338.

[3] A.C. Steere, L. Glickstein, Elucidation of Lyme arthritis, Nat. Rev. Immunol. 4 (2004) 143–152.

[4] J.J. Halperin, Neurologic manifestations of Lyme disease, Curr. Infect. Dis. Rep. 13 (2011) 360–366.

[5] R. Tokarz, J.M. Anderton, L.I. Katona, J.L. Benach, Combined effects of blood and temperature shift on Borrelia burgdorferi gene expression as determined by whole genome DNA array, Infect. Immun. 72 (2004) 5419–5432.

[6] C. Ojaimi, C. Brooks, S. Casjens, P. Rosa, A. Elias, A. Barbour, A. Jasinskas, J. Benach, L. Katona, J. Radolf, M. Caimano, J. Skare, K. Swingle, D. Akins, I. Schwartz, Profiling of temperature-induced changes in Borrelia burgdorferi gene expression by using whole genome arrays, Infect. Immun. 71 (2003) 1689–1705.

[7] J.A. Carroll, R.M. Cordova, C.F. Garon, Identification of 11 pH-regulated genes in Borrelia burgdorferi localizing to linear plasmids, Infect. Immun. 68 (2000) 6677–6684.

[8] T.E. Angel, B.J. Luft, X. Yang, C.D. Nicora, D.G. Camp 2nd, J.M. Jacobs, R.D. Smith, Proteome analysis of Borrelia burgdorferi response to environmental change, PLoS One 5 (2010) e13800.

[9] C.S. Brooks, P.S. Hefty, S.E. Jolliff, D.R. Akins, Global analysis of Borrelia burgdorferi genes regulated by mammalian host-specific signals, Infect. Immun. 71 (2003) 3371–3383.

[10] K.J. Indest, R. Ramamoorthy, M. Sole, R.D. Gilmore, B.J. Johnson, M.T. Philipp, Cell-density-dependent expression of Borrelia burgdorferi lipoproteins in vitro, Infect. Immun. 65 (1997) 1165–1171.

[11] A.T. Revel, A.M. Talaat, M.V. Norgard, DNA microarray analysis of differential gene expression in Borrelia burgdorferi, the Lyme disease spirochete, Proc. Natl. Acad. Sci. USA 99 (2002) 1562–1567.

[12] M.R. Kenedy, T.R. Lenhart, D.R. Akins, The role of Borrelia burgdorferi outer surface proteins, FEMS Immunol. Med. Microbiol. 66 (2012) 1–19.

[13] S. Casjens, N. Palmer, R. van Vugt, W.M. Huang, B. Stevenson, P. Rosa, R. Lathigra, G. Sutton, J. Peterson, R.J. Dodson, D. Haft, E. Hickey, M. Gwinn, O. White, C.M. Fraser, A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete Borrelia burgdorferi, Mol. Microbiol. 35 (2000) 490–516.

[14] A. Poljak, P. Comstedt, M. Hanner, W. Schuler, A. Meinke, B. Wizel, U. Lundberg, Identification and characterization of Borrelia antigens as potential vaccine candidates against Lyme borreliosis, Vaccine 30 (2012) 4398–4406.

[15] E. Wywial, J. Haven, S.R. Casjens, Y.A. Hernandez, S. Singh, E.F. Mongodin, C.M. Fraser-Liggett, B.J. Luft, S.E. Schutzer, W.G. Qiu, Fast, adaptive evolution at a bacterial host-resistance locus: the PFam54 gene array in Borrelia burgdorferi, Gene 445 (2009) 26–37.

[16] A.G. Leslie, The integration of macromolecular diffraction data, Acta Crystallogr. D Biol. Crystallogr. 62 (2006) 48–57.

[17] P. Evans, Scaling and assessment of data quality, Acta Crystallogr. D Biol. Crystallogr. 62 (2006) 72–82.

[18] M.D. Winn, C.C. Ballard, K.D. Cowtan, E.J. Dodson, P. Emsley, P.R. Evans, R.M. Keegan, E.B. Krissinel, A.G. Leslie, A. McCoy, S.J. McNicholas, G.N. Murshudov, N.S. Pannu, E.A. Potterton, H.R. Powell, R.J. Read, A. Vagin, K.S. Wilson, Overview of the CCP4 suite and current developments, Acta Crystallogr. D Biol. Crystallogr. 67 (2011) 235–242.

[19] A.J. McCoy, R.W. Grosse-Kunstleve, P.D. Adams, M.D. Winn, L.C. Storoni, R.J. Read, Phaser crystallographic software, J. Appl. Crystallogr. 40 (2007) 658–674.

[20] K. Cowtan, The Buccaneer software for automated model building. 1. Tracing protein chains, Acta Crystallogr. D Biol. Crystallogr. 62 (2006) 1002–1011.

[21] P. Emsley, K. Cowtan, Coot: model-building tools for molecular graphics, Acta Crystallogr. D Biol. Crystallogr. 60 (2004) 2126–2132.

[22] G.N. Murshudov, A.A. Vagin, E.J. Dodson, Refinement of macromolecular structures by the maximum-likelihood method, Acta Crystallogr. D Biol. Crystallogr. 53 (1997) 240–255.

[23] F.S. Cordes, P. Roversi, P. Kraiczy, M.M. Simon, V. Brade, O. Jahraus, R. Wallis, C. Skerka, P.F. Zipfel, R. Wallich, S.M. Lea, A novel fold for the factor H-binding protein BbCRASP-1 of Borrelia burgdorferi, Nat. Struct. Mol. Biol. 12 (2005) 276–277.

[24] R.D. Gilmore Jr., R.R. Howison, V.L. Schmit, A.J. Nowalk, D.R. Clifton, C. Nolder, J.L. Hughes, J.A. Carroll, Temporal expression analysis of the Borrelia burgdorferi paralogous gene family 54 genes BBA64, BBA65, and BBA66 during persistent infection in mice, Infect. Immun. 75 (2007) 2753–2764.

[25] R.D. Gilmore Jr., R.R. Howison, V.L. Schmit, J.A. Carroll, Borrelia burgdorferi expression of the bba64, bba65, bba66, and bba73 genes in tissues during persistent infection in mice, Microb. Pathog. 45 (2008) 355–360.

[26] P. Kraiczy, E. Rossmann, V. Brade, M.M. Simon, C. Skerka, P.F. Zipfel, R. Wallich, Binding of human complement regulators FHL-1 and factor H to CRASP-1 orthologs of Borrelia burgdorferi, Wien Klin Wochenschr. 118 (2006) 669–676.

[27] P. Kraiczy, C. Skerka, M. Kirschfink, V. Brade, P.F. Zipfel, Immune evasion of Borrelia burgdorferi by acquisition of human complement regulators FHL-1/reconectin and Factor H, Eur. J. Immunol. 31 (2001) 1674–1684.

[28] R. Wallich, J. Pattathu, V. Kitiratschky, C. Brenner, P.F. Zipfel, V. Brade, M.M. Simon, P. Kraiczy, Identification and functional characterization of

complement regulator-acquiring surface protein 1 of the Lyme disease spirochetes Borrelia afzelii and Borrelia garinii, Infect. Immun. 73 (2005) 2351–2359.
[29] P. Kraiczy, C. Hanssen-Hubner, V. Kitiratschky, C. Brenner, S. Besier, V. Brade, M.M. Simon, C. Skerka, P. Roversi, S.M. Lea, B. Stevenson, R. Wallich, P.F. Zipfel, Mutational analyses of the BbCRASP-1 protein of Borrelia burgdorferi identify residues relevant for the architecture and binding of host complement regulators FHL-1 and factor H, Int. J. Med. Microbiol. 299 (2009) 255–268.
[30] F.S. Cordes, P. Kraiczy, P. Roversi, C. Skerka, M. Kirschfink, M.M. Simon, V. Brade, E.D. Lowe, P. Zipfel, R. Wallich, S.M. Lea, Crystallization and preliminary crystallographic analysis of BbCRASP-1, a complement regulator-acquiring surface protein of Borrelia burgdorferi, Acta Crystallogr. D Biol. Crystallogr. 60 (2004) 929–932.
[31] R.D. Gilmore Jr., R.R. Howison, G. Dietrich, T.G. Patton, D.R. Clifton, J.A. Carroll, The bba64 gene of Borrelia burgdorferi, the Lyme disease agent, is critical for mammalian infection via tick bite transmission, Proc. Natl. Acad. Sci. USA 107 (2010) 7515–7520.
[32] J.V. McDowell, M.E. Harlin, E.A. Rogers, R.T. Marconi, Putative coiled-coil structural elements of the BBA68 protein of Lyme disease spirochetes are required for formation of its factor H binding site, J. Bacteriol. 187 (2005) 1317–1323.
[33] P. Herzberger, C. Siegel, C. Skerka, V. Fingerle, U. Schulte-Spechtel, B. Wilske, V. Brade, P.F. Zipfel, R. Wallich, P. Kraiczy, Identification and characterization of the factor H and FHL-1 binding complement regulator-acquiring surface protein 1 of the Lyme disease spirochete Borrelia spielmanii sp. nov, Int. J. Med. Microbiol. 299 (2009) 141–154.
[34] F.S. Cordes, P. Kraiczy, P. Roversi, M.M. Simon, V. Brade, O. Jahraus, R. Wallis, L. Goodstadt, C.P. Ponting, C. Skerka, P.F. Zipfel, R. Wallich, S.M. Lea, Structure-function mapping of BbCRASP-1, the key complement factor H and FHL-1 binding protein of Borrelia burgdorferi, Int. J. Med. Microbiol. 296 (Suppl. 40) (2006) 177–184.
[35] D.T. Jones, Protein secondary structure prediction based on position-specific scoring matrices, J. Mol. Biol. 292 (1999) 195–202.

3.3. Crystal structure of the infectious phenotype associated outer surface protein BBA66 from Lyme disease agent *Borrelia burgdorferi*

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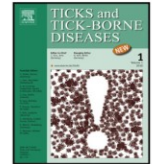

Original article

Crystal structure of the infectious phenotype-associated outer surface protein BBA66 from the Lyme disease agent *Borrelia burgdorferi*

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ABSTRACT

Borrelia burgdorferi, the causative agent of Lyme disease is transmitted to the mammalian host organisms by infected *Ixodes* ticks. Transfer of the spirochaetal bacteria from *Ixodes* ticks to the warm-blooded mammalian organism provides a challenge for the bacteria to adapt and survive in the different environmental conditions. *B. burgdorferi* has managed to differentially express genes in response to the encountered changes such as temperature and pH variance or metabolic rate to survive in both environments. In recent years, much interest has been turned on genes that are upregulated during the borrelial transfer to mammalian organisms as this could reveal the proteins important in the pathogenesis of Lyme disease. BBA66 is one of the upregulated outer surface proteins thought to be important in the pathogenesis of *B. burgdorferi* as it has been found out that BBA66 is necessary during the transmission and propagation phase to initiate Lyme disease. As there is still little known about the pathogenesis of *B. burgdorferi*, we have solved the crystal structure of the outer surface protein BBA66 at 2.25 Å resolution. A monomer of BBA66 consists of 6 α-helices packed in a globular domain, and the overall folding is similar to the homologous proteins BBA64, BBA73, and CspA. Structure-based sequence alignment with the homologous protein BBA64 revealed that the conserved residues are mainly located inwards the core region of the protein and thus may be required to maintain the overall fold of the protein.

Unlike the other homologous proteins, BBA66 has an atypically long disordered region at the N terminus thought to act as a "tether" between the structural domain and the cell surface.

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Introduction

Lyme disease is a common tick-borne infection caused by the transfer of the spirochete *Borrelia burgdorferi* from infected *Ixodes* ticks to vertebrate host organisms (Burgdorfer et al., 1982; Steere et al., 1983). During the enzootic life cycle, *B. burgdorferi* presents the ability to change the expression profile of proteins in order to adapt to the actual environment, whether it is a tick or a vertebrate host. The environmental signals that cause *B. burgdorferi* to change the expression profile of the proteins are rather complex and involve changes in temperature, pH, cell density, nutrient level, and mammalian-specific factors contained in the blood meal as well as depend on the bacterial replication rate and metabolic status as found out recently (Angel et al., 2010; Brooks et al., 2003; Caimano et al., 2007; Carroll et al., 1999, 2000; Jutras et al., 2013; Ojaimi et al., 2003; Tokarz et al., 2004). Observed differential gene expression in the conditions resembling unfed ticks and fed ticks have revealed changes in the expression profiles in a number of *B. burgdorferi* proteins associated with the cell membrane, cell metabolism, biosynthesis, and other basic functions which are thought to enable *B. burgdorferi* to adapt to the changing environment (Angel et al., 2010; Hefty et al., 2001; Ojaimi et al., 2003). Outer surface proteins could be the most related with the pathogenesis of Lyme disease as the transfer of bacteria from ticks to mammalian organisms requires attachment, dissemination, colonization, and resistance against the host's immune response. The current knowledge about the pathogenesis of *B. burgdorferi* has clearly pointed out on several leading candidates among the outer surface proteins that potentially have an important role in the pathogenesis of Lyme disease. Between those candidates, the outer surface proteins belonging to the paralogous gene family PFam54 that are located on the 54-kb linear plasmid (lp54) of *B. burgdorferi* strain B31 clearly stand out exhibiting the highest upregulation (Casjens et al., 2012; Fraser et al., 1997; Wywial et al., 2009). There is a cluster of 9 genes (*bba64*, *bba65*, *bba66*, *bba68*, *bba69*, *bba70*, *baa71*, *bba72*, *bba73*) on lp54 belonging to the PFam54 family. Genes *bba64*, *bba65*, *bba66*, *bba71*, and *bba73* have shown the highest upregulation rates

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during tick feeding and spirochaete transmission process, as presented by studies either in vivo or in vitro comparing the gene expression in conditions encountered in ticks and in mammalian organisms (Angel et al., 2010; Casjens et al., 2012; Clifton et al., 2006; Hughes et al., 2008; Ojaimi et al., 2003). The function has already been determined for the outer surface protein CspA (BBA68 or BbCrasp-1) from the PFam54 family. CspA binds complement regulators factor H and factor-H like protein-1 (FHL-1) and helps the bacteria to resist the host's immune response (Kraiczy et al., 2001a, b). Another member of the PFam54 family, BBA64, has been shown to play an essential role in the transfer of bacteria from the tick to the mammalian organism as a mutant strain deficient in the synthesis of BBA64 was unable to cause infection after tick bite (Gilmore et al., 2010; Patton et al., 2011).

Although the exact receptors/ligands for BBA66 are not known, it was recently shown that the protein is important in dissemination and transmission of *B. burgdorferi* from infected *Ixodes* ticks to the mammalian host organism therefore boosting the interest to detect the exact function of the protein (Patton et al., 2013). As the protein function is directly related to its three-dimensional structure, we have solved the crystal structure of BBA66 at 2.25 Å resolution. The crystal structure could promote the understanding of the pathogenesis of Lyme disease and hopefully give some advancement in the development of novel drugs against Lyme disease.

Materials and methods

Cloning, expression, and purification

The gene encoding the BBA66 protein was PCR-amplified from genomic DNA of *Borrelia burgdorferi* strain B31. Two fragments of different length were amplified corresponding to amino acid residues 24–411 and 191–411. In the first case (residues 24–411) excluding the signal sequence of the protein (residues 1–23) as predicted by SignalP 4.1 (Petersen et al., 2011) and in the second case (residues 191–411) excluding also the N-terminal disordered region as predicted by the programmes DISOPRED2 and POODLE (Hirose et al., 2007; Ward et al., 2004). The fragments were inserted into the pETm_11 expression vector (EMBL, Heidelberg), and the construct containing 6xHis tag and TEV protease cleavage site at the N terminus was transformed into *E. coli* strain RR1, and the cells were grown overnight at 37 °C on LB agar plates containing kanamycin. Colonies were inoculated in liquid LB medium containing kanamycin at 37 °C for another 24 h. Plasmid DNA was isolated from the resulting culture, and DNA sequencing was carried out for all of the obtained clones to make sure that no errors have occurred. For overexpression of the recombinant protein, the plasmid was transformed into *E. coli* BL21(DE3), and the cells were grown in a modified 2xTYP media (supplemented with kanamycin (10 mg/ml), 133 mM phosphate buffer, pH 7.4, and glucose (4 g/l) with vigorous agitation at 25 °C until an OD_{600} 0.8–1.0. Overexpression was induced by the addition of 0.2 mM IPTG, and the cells were cultivated for additional 16–20 h. The cells were harvested by centrifugation and lysed by sonication, and the homogenate was clarified by centrifugation. The target protein was purified by immobilized metal affinity chromatography on a Ni-NTA agarose (Qiagen, Germany) column. The 6xHis tag was cleaved from the BBA66 by Tobacco Etch Virus (TEV) protease at 293 K for 16 h. The protein was further purified using Ni-affinity chromatography to remove the TEV protease and the cleaved 6xHis tag. The final target protein contained only 4 extra residues (GAMG) at the N terminus.

Gel filtration chromatography

For estimation of the multimeric state, the purified protein (9 mg/ml in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl) was loaded onto a pre-packed Superdex 200 10/300 GL column, connected to an ÄKTA chromatography system (Amersham Biosciences). The column was pre-equilibrated with the same buffer and run at a flow rate of 0.7 ml/min. Bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa) were used as MW reference standards.

Protein crystallization

For crystallization, the native protein was concentrated to 9 mg/ml in 20 mM Tris-HCl, pH 8, using an Amicon Ultra 10 (Millipore). Crystallization of the BBA66 was carried out by the sitting-drop vapour diffusion method in 96-well crystallization plates using sparse-matrix screens from Molecular Dimensions (Molecular Dimensions Ltd, UK). One microlitre of protein solution was mixed with an equal amount of precipitant solution. The optimized conditions consisted of 18% polyethylene glycol 2000 monomethyl ether (PEG 2000 MME) and 0.1 M Tris-HCl, pH 9.0. Cryoprotection was performed by adding 30% glycerol to the precipitation mix, and the crystals were flash-frozen in liquid nitrogen.

Data collection and processing

X-ray diffraction data from the BBA66 protein crystals were collected at the MAX-lab beamline I911-3 (Sweden, Lund University). The crystals diffracted to 2.25 Å resolution and the crystal space group was determined to be P1 with 2 molecules per asymmetric unit. The data were indexed, integrated, and scaled using the MOSFLM and SCALA programmes from the CCP4 programme suite (Battye et al., 2011; Evans, 2006; Winn et al., 2011).

Structure determination

The crystal structure of BBA66 was solved by molecular replacement using *Phaser* (McCoy et al., 2007) and as a search model, the crystal structure of the homologous protein BBA73 (PDB entry 4AXZ) was used (Brangulis et al., 2013a). CHAINSAW (Schwarzenbacher et al., 2004) was used to remove the residues from the search model that were different between the 2 protein sequences. For the completion and improvement of the crystal structure, BUCCANEER software (Cowtan, 2006) was used to automatically build the model. Refinement was performed using REFMAC5 (Murshudov et al., 1997) and manual model rebuilding and addition of water molecules were done by using COOT (Emsley and Cowtan, 2004). Data collection and refinement statistics are given in Table 1.

Results

The crystal structure of BBA66

Crystallization of 2 different BBA66 truncation variants was attempted, as 2 different expression constructs were made (residues 24–411 and residues 191–411). At first, the expression construct of full-length BBA66 protein (residues 24–411) excluding the signal sequence of the protein (residues 1–23) was produced. The protein was expressed, however after purification by using Ni-affinity chromatography and ion-exchange chromatography, the protein showed a slight degradation as judged by SDS-PAGE. Besides that, the crystallization trials did not succeed. It encouraged the creation of a new expression construct for BBA66 by altering the length of the recombinant protein, a strategy applied also for another *B. burgdorferi* protein (Eicken et al., 2001). The new construct was made for residues 191–411, the crystals were obtained, and the structure was solved. The crystals of the outer surface

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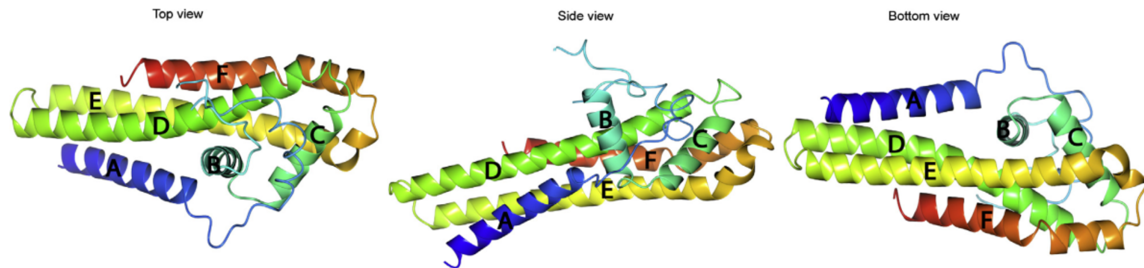

Fig. 1. An overall view of *Borrelia burgdorferi* outer surface protein BBA66. The gap between α-helices B and C in the crystal structure of BBA66 is a consequence of poor electron density for residues 241–245. Top, side, and bottom view of the molecule obtained by rotating the protein by 90°. α-helices are named from A to F starting from the N-terminal part. Protein structure coloured using a rainbow colour scheme starting from blue at the N-terminal part and gradually switching to red as approaching the C-terminal part of the molecule. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

protein BBA66 from *B. burgdorferi* belonged to space group P1 and contained 2 BBA66 protein molecules in the asymmetric unit (designated as chain A and chain B) and about 57% solvent as detected by MATTHEWS (Kantardjieff and Rupp, 2003). BBA66 monomer contains a globular domain consisting of 6 α-helices (A–F) connected by loops of different length (Fig. 1). The crystal structure was not built for residues 241–245 in both chains A and B because the respective residues were residing at the loop region and exhibited poor electron density. Also at the N terminus, the first 2 residues (residues 191–192) and the remnants from the expression tag consisting of 4 residues (GAMG) of the recombinant protein produced weak electron density. The overall crystal structure, except the flexible loop regions and α-helix F (in comparison to CspA), is similar to the crystal structures of the homologous proteins BBA64 [PDB entry 4ALY; C^{α} root-mean-square deviation (r.m.s.d.) 2.48 Å; identity of 18% over 219 residues (Brangulis et al., 2013b)], BBA73 [PDB entry 4AXZ; C^{α} root-mean-square deviation (r.m.s.d.) 1.87 Å; identity of 16% over 225 residues (Brangulis et al., 2013a)] and CspA [PDB code 1W33; C^{α} root-mean-square deviation (r.m.s.d.) 2.94 Å; identity of 14% over 182 residues (Cordes et al., 2005)]. The C-terminal α-helix F in BBA66 bents inwards in a similar way as observed in BBA64 and BBA73 and does not form a stalk-like extension as in CspA (Cordes et al., 2005). Recently, it was shown that in BBA73 the prolongated N-terminal α-helix in comparison to other homologous proteins from the PFam54 family is necessary to form a homodimer (Brangulis et al., 2013a). Because the stalk-like extension in CspA and N-terminally located prolongated α-helix in BBA73 is necessary for dimerization and this in turn may be related to the protein functions, we determined the oligomerization state for BBA66. Using gel filtration chromatography, we determined that the protein (exploiting the globular domain of BBA66 corresponding to residues 191–411) is a monomer in solution and the results are consistent with those obtained for the homologous protein BBA64 which is also a monomer in a solution.

Table 1
Statistics of data and structure quality.

Dataset	Native
Space group	P 1
Unit cell dimensions	
a (Å)	40.91
b (Å)	51.12
c (Å)	58.88
Wavelength (Å)	1.0000
Resolution (Å)	29.78–2.25
Highest resolution bin (Å)	2.25–2.36
No. of reflections	41,556
No. of unique reflections	21,363
Completeness (%)	94.3 (85.2)
R_{merge}	0.06 (0.29)
I/σ (I)	8.7 (2.2)
Multiplicity	1.9 (1.8)
Refinement	
R_{work}	0.190 (0.298)
R_{free}	0.232 (0.343)
Average B-factor (Å^2)	
Overall	32.0
From Wilson plot	33.8
No. of atoms	
Protein	3491
Water	61
RMS deviations from ideal	
Bond lengths (Å)	0.018
Bond angles (°)	1.712
Ramachandran outliers (%)	
Residues in most favoured regions (%)	96.19
Residues in allowed regions (%)	3.10
Outliers (%)	0.71

Values in parentheses are for the highest resolution bin.

Disordered N-terminal region

By analyzing the primary sequence of the protein using the secondary structure and disordered region prediction software PSIPRED, DISOPRED, and POODLE (Hirose et al., 2007; Jones, 1999; Ward et al., 2004), it was evident that the first 190–200 residues in BBA66 do not form any secondary structure elements, but with high confidence form a disordered region (Fig. 2). Even more, as the crystal structures for the homologous proteins BBA64 (PDB code

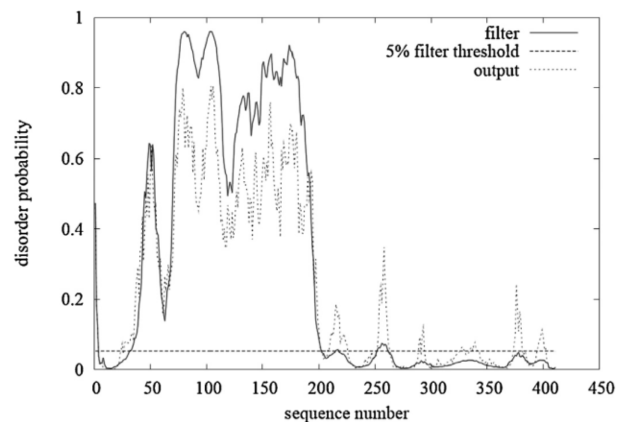

Fig. 2. Prediction of disordered regions in BBA66 from *Borrelia burgdorferi*. Disordered regions have been predicted by software DISOPRED2 (Ward et al., 2004) with high confidence to cover the N-terminal part of the protein.

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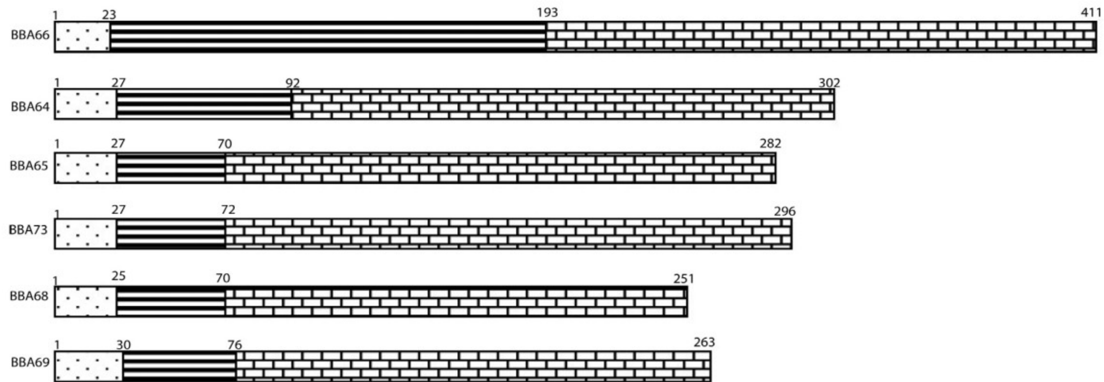

Fig. 3. Comparison of the structural elements between the homologous proteins BBA66, BBA64, BBA65, BBA73, and BBA68 (CspA). The structural elements (signal sequences, disordered regions, and structural domains) have been predicted using SignalP 4.0, PSIPRED, DISOPRED, and POODLE. For BBA66, BBA64, and CspA, the location of the structural elements have been confirmed by their crystal structures. The Signal sequence regions have been shown using a dotted pattern; disordered loop regions are represented as the horizontal lines and the globular functional domains as bricks.

4ALY), BBA73 (PDB code 4AXZ), and CspA (PDB code 1W33) have been determined, the sequence alignment showed that if the overall fold for BBA66 would be the same, then the structured domain in BBA66 should start approximately from residues 190 to 200. The results were consistent with the data obtained from the secondary structure and disordered region prediction for BBA66, and therefore the second expression construct for the recombinant protein BBA66 was made starting from residue Ser191 (residues 191–411).

Interestingly, the N-terminal disordered region in BBA66 (approximate number of residues 30–200) does not show any significant homology to the homologous proteins BBA64, BBA65, BBA73, BBA69, CspA (8–14% similarity from the sequence alignment of the N terminus), although also the homologous proteins BBA64, BBA65, BBA73, BBA69, and CspA have a disordered N-terminal region as predicted by PSIPRED, DISOPRED, and POODLE and concluded from the crystal structures (Brangulis et al., 2013b; Cordes et al., 2005; Hirose et al., 2007; Jones, 1999; Ward et al., 2004). The disordered region has not been observed in the crystal structures of BBA64, BBA73, and CspA, although the region was included in the crystallized protein, verifying that the region is probably flexible and disordered. In favour of this sequence diversity at the N-terminal part might be that the disordered region does not play any direct role in the protein function as it likely serves only as a flexible connection between the cell surface and the structured protein domain. Therefore it is probable that over time the non-conservative mutations have changed significantly the primary structure between the homologous proteins at the N terminus. The N-terminal disordered regions of outer surface proteins in *B. burgdorferi* are thought to be a common feature, and in several publications the region has been named a "tether" region (Chen et al., 2011; Kumru et al., 2011; Zückert, 2013). The different length of the "tether" region has been described to play some role in providing a space between different outer surface proteins and thus creating a multi-storey model on the surface of bacteria and also ensuring the shielding of certain proteins from the environment and antibody response (Bunikis and Barbour, 1999; Zückert, 2013).

It should be stressed that the disordered N-terminal region in BBA66 is much longer than in any other homologous proteins from *B. burgdorferi* (Fig. 3). The disordered region in BBA66 is unusually long covering almost 200 residues.

Also interesting fact is that a search for the conserved domains in the outer surface protein BBA66 using the programme BLAST (The Basic Local Alignment Search Tool) (Altschul et al., 1990) recognizes only one domain that covers residues 75–197. The conserved domain belongs to the Herpes virus major outer envelope glycoprotein (BLLF1) family – the most abundantly expressed glycoprotein

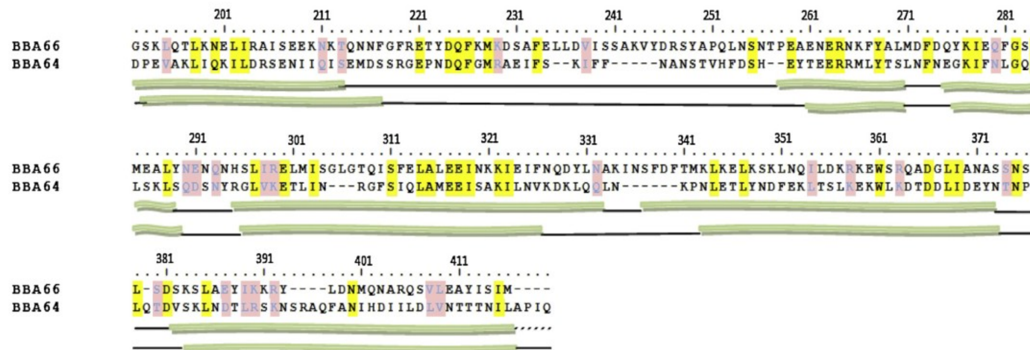

Fig. 4. Structure-based sequence alignment of BBA66 (PDB code 2NY7) and BBA64 (PDB code 4ALY). Secondary structure assignments below the alignment are shown for both BBA66 and BBA64, but numbering is for BBA66. Helices are indicated as green cylinders and loop regions as black lines. Conserved residues are highlighted in red colour with white fonts. Conservative substitutions are shown in blue font with pink background. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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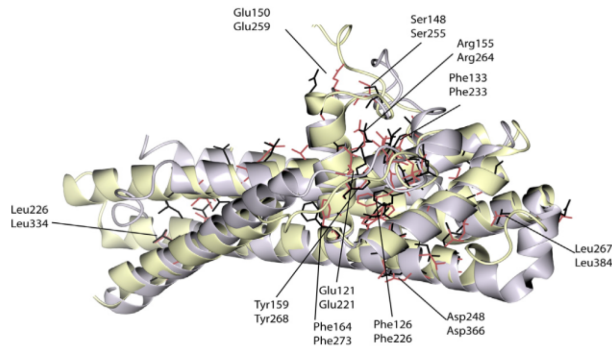

Fig. 5. Conserved residues between the homologous proteins BBA66 (light grey) and BBA64 (dark grey) represented on both structures which are superimposed. Side chains are shown as ribbons.

in the viral envelope of the Herpes viruses (E-value 8.99×10^{-4}). The crystal structure for the major envelope glycoprotein has been determined, and the region exerting the similarity with BBA66 is also disordered in the major envelope glycoprotein, and the segment is not included in the final model of the protein structure (Szakonyi et al., 2006).

Structural comparison of the homologous proteins

Comparison of the crystal structures between the homologous proteins BBA66, BBA64, BBA73, and CspA reveals that the core secondary structure elements are conserved between the members, although the primary sequence similarity is low between them, ranging from 16 to 24%. In order to gain a better insight into the differences between the tertiary structures of the homologous proteins, we have compared in more detail the crystal structure of BBA66 and BBA64. Both protein structures can be superimposed very well and the conserved residues have been revealed using the structure-based sequence alignment (Fig. 4). The 2 protein molecules likely have different functions in the pathogenesis of *B. burgdorferi* as the regulation and expression profile for both proteins appears to be different (Clifton et al., 2006; Gilmore et al., 2007, 2008). The conserved residues as can be seen from the superimposed structure alignment are mainly located inwards the protein core and are thought to stabilize the tertiary structure (Fig. 5). As mentioned previously, although the primary sequence conservation is low between the 2 proteins, the overall fold is similar. From the superimposed structures, it is obvious that there are just a few surface-exposed conserved residues indicating that the evolution has retained the tertiary structure of the proteins while changing their surface-exposed residues and thus the protein functions.

Discussion

Although the exact function for the outer surface lipoprotein BBA66 is not known, it was recently shown that BBA66 is important for transmission of *B. burgdorferi* from infected *Ixodes* ticks to the mammalian host organism (Patton et al., 2013). BBA66 along with the homologous proteins BBA64, BBA65, and BBA73 belonging to the paralogous gene family PFam54 are highly upregulated in response to mammalian host environment mimicking conditions as detected by several studies (Brooks et al., 2003; Caimano et al., 2007; Carroll et al., 1999, 2000; Ojaimi et al., 2003; Tokarz et al., 2004). The gene expression profile for BBA66 as detected from total RNA by quantitative reverse transcription PCR from *B. burgdorferi*-infected mouse ear tissues indicates that BBA66 is expressed early in the infection and the expression increases during the infection period and reaches even 16-fold upregulation on day 100 p.i. (Gilmore et al., 2007, 2008). Regarding the expression of the protein during the infection period at early- and late-infection period, development of antibody response to BBA66 has been detected from sera collected from a Lyme disease patient and infected mice, indicating immunogenicity of the protein (Clifton et al., 2006; Nowalk et al., 2006). The protein also seems to be conserved among different *B. burgdorferi* sensu lato isolates and the relapsing fever *Borrelia* species (Hughes et al., 2008). Taken together, these data indicate an essential role for BBA66 in the pathogenesis in early stages of infection and also during persistent infection.

Regarding the functional differences between the homologous proteins BBA66, BBA73, BBA64, and CspA belonging to the PFam54 family, it has been suggested that the genes from the PFam54 family have passed through repeated gene duplications, gene losses, and sequence diversification over time (Wywial et al., 2009). It was also suggested that besides the driven genetic variability between the members also structural diversification has occurred leading to functional differences (Wywial et al., 2009). From the crystal structures of BBA66, BBA64, BBA73, and CspA, it is possible to see that the proteins gain almost identical tertiary protein structures, and this observation might suggest that there could be still some functional similarities between the members. However, there are several things that suggest that the functions are different between the respective PFam54 members. Firstly, the primary sequence diversity is high between the proteins ranging from 16 to 24%, and the conserved residues are mainly located inwards the protein core and might be necessary for stabilization of the fold. While if the proteins gained some functional similarities, we would expect to identify some conservation at the potential binding site. Secondly, it has been demonstrated that only CspA shows the ability to bind complement regulators factor H and factor H-like protein 1 (McDowell et al., 2005). And thirdly, a recent study about the functional nature of BBA66 revealed that BBA66 is important in the transmission of bacteria from infected *Ixodes* ticks to mammalian organisms (Patton et al., 2013). Meanwhile, it has been shown that the homologous protein BBA64 is even vital for the transmission process, which might be due to the different receptors/ligands for both proteins (Gilmore et al., 2010).

In this study, we have solved the crystal structure of an upregulated outer surface protein BBA66 at 2.25 Å resolution. We have shown that the overall fold of BBA66 is similar to the homologous proteins with already solved crystal structures, although the primary sequence diversity is high between the members.

The protein is upregulated during the early and late infection period and plays an important role in the transmission process of *B. burgdorferi* from infected ticks to mammalian hosts indicating the potentially relevant role in the pathogenesis of Lyme disease. Therefore, the atomic structure of BBA66 could help to gain more understanding about the pathogenesis of Lyme disease and could promote the development of novel drugs against Lyme disease.

Accession numbers

The coordinates and structure factors have been deposited in the Protein Data Bank with the accession number 2YN7.

Acknowledgments

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Angel, T.E., Luft, B.J., Yang, X., Nicora, C.D., Camp 2nd, D.G., Jacobs, J.M., Smith, R.D., 2010. Proteome analysis of *Borrelia burgdorferi* response to environmental change. PLoS ONE 5, e13800.

Battye, T.G., Kontogiannis, L., Johnson, O., Powell, H.R., Leslie, A.G., 2011. iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. Acta Crystallogr. D Biol. Crystallogr. 67, 271–281.

Brangulis, K., Petrovskis, I., Kazaks, A., Baumanis, V., Tars, K., 2013a. Structural characterization of the *Borrelia burgdorferi* outer surface protein BBA73 implicates dimerization as a functional mechanism. Biochem. Biophys. Res. Commun. 434, 848–853.

Brangulis, K., Tars, K., Petrovskis, I., Kazaks, A., Ranka, R., Baumanis, V., 2013b. Structure of an outer surface lipoprotein BBA64 from the Lyme disease agent *Borrelia burgdorferi* which is critical to ensure infection after a tick bite. Acta Crystallogr. D Biol. Crystallogr. 69, 1099–1107.

Brooks, C.S., Hefty, P.S., Jolliff, S.E., Akins, D.R., 2003. Global analysis of *Borrelia burgdorferi* genes regulated by mammalian host-specific signals. Infect. Immun. 71, 3371–3383.

Bunikis, J., Barbour, A.G., 1999. Access of antibody or trypsin to an integral outer membrane protein (P66) of *Borrelia burgdorferi* is hindered by Osp lipoproteins. Infect. Immun. 67, 2874–2883.

Burgdorfer, W., Barbour, A.G., Hayes, S.F., Benach, J.L., Grunwaldt, E., Davis, J.P., 1982. Lyme disease – a tick-borne spirochetosis? Science 216, 1317–1319.

Caimano, M.J., Iyer, R., Eggers, C.H., Gonzalez, C., Morton, E.A., Gilbert, M.A., Schwartz, I., Radolf, J.D., 2007. Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals provides insight into RpoS function during the enzootic cycle. Mol. Microbiol. 65, 1193–1217.

Carroll, J.A., Garon, C.F., Schwan, T.G., 1999. Effects of environmental pH on membrane proteins in *Borrelia burgdorferi*. Infect. Immun. 67, 3181–3187.

Carroll, J.A., Cordova, R.M., Garon, C.F., 2000. Identification of 11 pH-regulated genes in *Borrelia burgdorferi* localizing to linear plasmids. Infect. Immun. 68, 6677–6684.

Casjens, S.R., Mongodin, E.F., Qiu, W.G., Luft, B.J., Schutzer, S.E., Gilcrease, E.B., Huang, W.M., Vujadinovic, M., Aron, J.K., Vargas, L.C., Freeman, S., Radune, D., Weidman, J.F., Dimitrov, G.I., Khouri, H.M., Sosa, J.E., Halpin, R.A., Dunn, J.J., Fraser, C.M., 2012. Genome stability of Lyme disease spirochetes: comparative genomics of *Borrelia burgdorferi* plasmids. PLoS ONE 7, e33280.

Chen, S., Kumru, O.S., Zückert, W.R., 2011. Determination of *Borrelia* surface lipoprotein anchor topology by surface proteolysis. J. Bacteriol. 193, 6379–6383.

Clifton, D.R., Nolder, C.L., Hughes, J.L., Nowalk, A.J., Carroll, J.A., 2006. Regulation and expression of bba66 encoding an immunogenic infection-associated lipoprotein in *Borrelia burgdorferi*. Mol. Microbiol. 61, 243–258.

Cordes, F.S., Roversi, P., Kraiczy, P., Simon, M.M., Brade, V., Jahraus, O., Wallis, R., Skerka, C., Zipfel, P.F., Wallich, R., Lea, S.M., 2005. A novel fold for the factor H-binding protein BbCRASP-1 of *Borrelia burgdorferi*. Nat. Struct. Mol. Biol. 12, 276–277.

Cowtan, K., 2006. The Buccaneer software for automated model building. 1. Tracing protein chains. Acta Crystallogr. D Biol. Crystallogr. 62, 1002–1011.

Eicken, C., Sharma, V., Klabunde, T., Owens, R.T., Pikas, D.S., Hook, M., Sacchettini, J.C., 2001. Crystal structure of Lyme disease antigen outer surface protein C from *Borrelia burgdorferi*. J. Biol. Chem. 276, 10010–10015.

Emsley, P., Cowtan, K., 2004. Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132.

Evans, P., 2006. Scaling and assessment of data quality. Acta Crystallogr. D Biol. Crystallogr. 62, 72–82.

Fraser, C.M., Casjens, S., Huang, W.M., Sutton, G.G., Clayton, R., Lathigra, R., White, O., Ketchum, K.A., Dodson, R., Hickey, E.K., Gwinn, M., Dougherty, B., Tomb, J.F., Fleischmann, R.D., Richardson, D., Peterson, J., Kerlavage, A.R., Quackenbush, J., Salzberg, S., Hanson, M., van Vugt, R., Palmer, N., Adams, M.D., Gocayne, J., Weidman, J., Utterback, T., Watthey, L., McDonald, L., Artiach, P., Bowman, C., Garland, S., Fuji, C., Cotton, M.D., Horst, K., Roberts, K., Hatch, B., Smith, H.O., Venter, J.C., 1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature 390, 580–586.

Gilmore Jr., R.D., Howison, R.R., Schmit, V.L., Nowalk, A.J., Clifton, D.R., Nolder, C., Hughes, J.L., Carroll, J.A., 2007. Temporal expression analysis of the *Borrelia burgdorferi* paralogous gene family 54 genes BBA64, BBA65, and BBA66 during persistent infection in mice. Infect. Immun. 75, 2753–2764.

Gilmore Jr., R.D., Howison, R.R., Schmit, V.L., Carroll, J.A., 2008. Borrelia burgdorferi expression of the bba64, bba65, bba66, and bba73 genes in tissues during persistent infection in mice. Microb. Pathog. 45, 355–360.

Gilmore Jr., R.D., Howison, R.R., Dietrich, G., Patton, T.G., Clifton, D.R., Carroll, J.A., 2010. The bba64 gene of *Borrelia burgdorferi*, the Lyme disease agent, is critical for mammalian infection via tick bite transmission. Proc. Natl. Acad. Sci. U. S. A. 107, 7515–7520.

Hefty, P.S., Jolliff, S.E., Caimano, M.J., Wikel, S.K., Radolf, J.D., Akins, D.R., 2001. Regulation of OspE-related, OspF-related, and Elp lipoproteins of *Borrelia burgdorferi* strain 297 by mammalian host-specific signals. Infect. Immun. 69, 3618–3627.

Hirose, S., Shimizu, K., Kanai, S., Kuroda, Y., Noguchi, T., 2007. POODLE-L: a two-level SVM prediction system for reliably predicting long disordered regions. Bioinformatics 23, 2046–2053.

Hughes, J.L., Nolder, C.L., Nowalk, A.J., Clifton, D.R., Howison, R.R., Schmit, V.L., Gilmore Jr., R.D., Carroll, J.A., 2008. Borrelia burgdorferi surface-localized proteins expressed during persistent murine infection are conserved among diverse *Borrelia* spp. Infect. Immun. 76, 2498–2511.

Jones, D.T., 1999. Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. 292, 195–202.

Jutras, B.L., Chenail, A.M., Stevenson, B., 2013. Changes in bacterial growth rate govern expression of the *Borrelia burgdorferi* OspC and Erp infection-associated surface proteins. J. Bacteriol. 195, 757–764.

Kantardjieff, K.A., Rupp, B., 2003. Matthews coefficient probabilities: Improved estimates for unit cell contents of proteins, DNA, and protein-nucleic acid complex crystals. Protein Sci. 12, 1865–1871.

Kraiczy, P., Skerka, C., Brade, V., Zipfel, P.F., 2001a. Further characterization of complement regulator-acquiring surface proteins of *Borrelia burgdorferi*. Infect. Immun. 69, 7800–7809.

Kraiczy, P., Skerka, C., Kirschfink, M., Zipfel, P.F., Brade, V., 2001b. Mechanism of complement resistance of pathogenic *Borrelia burgdorferi* isolates. Int. Immunopharmacol. 1, 393–401.

Kumru, O.S., Schulze, R.J., Rodnin, M.V., Ladokhin, A.S., Zückert, W.R., 2011. Surface localization determinants of *Borrelia* OspC/Vsp family lipoproteins. J. Bacteriol. 193, 2814–2825.

McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., Read, R.J., 2007. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674.

McDowell, J.V., Harlin, M.E., Rogers, E.A., Marconi, R.T., 2005. Putative coiled-coil structural elements of the BBA68 protein of Lyme disease spirochetes are required for formation of its factor H binding site. J. Bacteriol. 187, 1317–1323.

Murshudov, G.N., Vagin, A.A., Dodson, E.J., 1997. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D Biol. Crystallogr. 53, 240–255.

Nowalk, A.J., Gilmore Jr., R.D., Carroll, J.A., 2006. Serologic proteome analysis of *Borrelia burgdorferi* membrane-associated proteins. Infect. Immun. 74, 3864–3873.

Ojaimi, C., Brooks, C., Casjens, S., Rosa, P., Elias, A., Barbour, A., Jasinskas, A., Benach, J., Katona, L., Radolf, J., Caimano, M., Skare, J., Swingle, K., Akins, D., Schwartz, I., 2003. Profiling of temperature-induced changes in *Borrelia burgdorferi* gene expression by using whole genome arrays. Infect. Immun. 71, 1689–1705.

Patton, T.G., Dietrich, G., Dolan, M.C., Piesman, J., Carroll, J.A., Gilmore Jr., R.D., 2011. Functional analysis of the *Borrelia burgdorferi* bba64 gene product in murine infection via tick infestation. PLoS ONE 6, e19536.

Patton, T.G., Brandt, K.S., Nolder, C., Clifton, D.R., Carroll, J.A., Gilmore, R.D., 2013. Borrelia burgdorferi bba66 gene inactivation results in attenuated mouse infection by tick transmission. Infect. Immun. 81, 2488–2498.

Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.

Schwarzenbacher, R., Godzik, A., Grzechnik, S.K., Jaroszewski, L., 2004. The importance of alignment accuracy for molecular replacement. Acta Crystallogr. D Biol. Crystallogr. 60, 1229–1236.

Steere, A.C., Grodzicki, R.L., Kornblatt, A.N., Craft, J.E., Barbour, A.G., Burgdorfer, W., Schmid, G.P., Johnson, E., Malawista, S.E., 1983. The spirochetal etiology of Lyme disease. N. Engl. J. Med. 308, 733–740.

Szakonyi, G., Klein, M.G., Hannan, J.P., Young, K.A., Ma, R.Z., Asokan, R., Holers, V.M., Chen, X.S., 2006. Structure of the Epstein-Barr virus major envelope glycoprotein. Nat. Struct. Mol. Biol. 13, 996–1001.

Tokarz, R., Anderton, J.M., Katona, L.I., Benach, J.L., 2004. Combined effects of blood and temperature shift on *Borrelia burgdorferi* gene expression as determined by whole genome DNA array. Infect. Immun. 72, 5419–5432.

Ward, J.J., Sodhi, J.S., McGuffin, L.J., Buxton, B.F., Jones, D.T., 2004. Prediction and functional analysis of native disorder in proteins from the three kingdoms of life. J. Mol. Biol. 337, 635–645.

Winn, M.D., Ballard, C.C., Cowtan, K.D., Dodson, E.J., Emsley, P., Evans, P.R., Keegan, R.M., Krissinel, E.B., Leslie, A.G., McCoy, A., McNicholas, S.J., Murshudov, G.N., Pannu, N.S., Potterton, E.A., Powell, H.R., Read, R.J., Vagin, A., Wilson, K.S., 2011. Overview of the CCP4 suite and current developments. Acta Crystallogr. D Biol. Crystallogr. 67, 235–242.

Wywial, E., Haven, J., Casjens, S.R., Hernandez, Y.A., Singh, S., Mongodin, E.F., Fraser-Liggett, C.M., Luft, B.J., Schutzer, S.E., Qiu, W.G., 2009. Fast, adaptive evolution at a bacterial host-resistance locus: the PFam54 gene array in *Borrelia burgdorferi*. Gene 445, 26–37.

Zückert, W.R., 2013. A call to order at the spirochaetal host-pathogen interface. Mol. Microbiol. 89, 207–211.

4. DISCUSSION

Lyme disease is an infectious disease caused by the spirochete that is transmitted from infected *Ixodes* ticks to the mammalian host organism during the blood meal (Steere, 2001). In this PhD work the outer surface lipoproteins of *B. burgdorferi* from the PFam54 paralogous gene family were studied, thought to be important in the pathogenesis of Lyme disease, to reveal the structural properties of those proteins.

Lipoproteins may play an important role in the pathogenesis of Lyme disease as they constitute 8% of the genes coded by the *B. burgdorferi* genome (Casjens et al., 2000; Fraser et al., 1997). Not only the large partition of *B. burgdorferi* genome devoted for the expression of lipoproteins could explain the importance in pathogenesis, but also the fact that the lipoproteins are located on the surface of bacteria (Haake, 2000). Because of their specific location the lipoproteins are thought to be involved in the transfer of *B. burgdorferi* from ticks to mammalian, fight against the hosts immune response, attachment to specific receptors/ligands in the new host organism, spread in the organism, and proliferation – the main tasks necessary for *B. burgdorferi* to establish the Lyme disease (Steere, 2001). For most of the lipoproteins found in *B. burgdorferi* the potential function or role has not been established yet and the respective genes does not show any sequence similarities with the genes from any other organisms (Casjens et al., 2000; Fraser et al., 1997). Because of their uniqueness and potential importance in the pathogenesis of Lyme disease, the lipoproteins have been extensively studied in many different laboratories.

One of the things that have been showed to be the same among the different laboratories is that over 100 different proteins exhibit altered expression in conditions mimicking the transfer of *B. burgdorferi* from infected *Ixodes* ticks to mammalian organism (Ojaimi et al., 2003; Tokarz et al., 2004). From all those proteins with the altered expression pattern it has been showed that the paralogous gene family PFam54 members that are located on 54 kb linear plasmid (lp54) exhibit the highest upregulation rates and therefore are thought to be important in the pathogenesis of Lyme disease (Angel et al., 2010; Ojaimi et al., 2003; Tokarz et al., 2004). The paralogous gene family PFam54 located on lp54 consists of nine open reading frames – *bba64*, *bba65*, *bba66*, *bba68*, *bba69*, *bba70*, *bba71*, *bba72*, *bba73* – although *bba71* and *bba72* are truncated genes and *bba70* is missing in most of *B. burgdorferi* strains (Casjens et al., 2012).

Some studies have tried to reveal the exact functions for some of the PFam54 family lipoproteins (Gilmore et al., 2008; Gilmore et al., 2010; Patton et al., 2013a). For outer surface lipoprotein BBA64 it has been showed that it is vital for the transfer of *B. burgdorferi* from infected *Ixodes* ticks to the mammalian organism (Gilmore et al., 2010; Patton et al., 2011). Although the exact receptors/ligands for BBA64 have not been determined. Therefore we determined the crystal structure of BBA64 at 2.4 Å resolution in order to gain more understanding about the pathogenesis of Lyme disease. The crystal structure of BBA64 revealed a similar fold to the homologous protein CspA (also known as BBA68) that is also the member of the PFam54 family and for which the crystal structure has been determined previously (Cordes et al., 2005). The only difference between the two crystal structures was that the C-terminal part forms a different conformation between the two homologous proteins. In CspA the C-terminal α-helix forms a stalk-like extension protruding outwards from the protein molecule and serves as a basis for the dimer formation while in BBA64 the C-terminal α-helix forms a loop not present in CspA and switches backwards, becoming a part of a compact α-helical domain. Although because of the high sequence diversity between the two homologous proteins (17% sequence identity) the results could be treated as expected, but previous studies about the CspA protein showed that a proper orientation of the C-terminal α-helix is necessary for the functioning of CspA (Cordes et al., 2006). The C-terminal α-helix in CspA has an indirect role in the functioning of the protein because the C-terminal α-helix form an extensive dimerization interface of 2240.9 A^2 and forms a homodimer (Cordes et al., 2006). It was postulated that the binding pocket in CspA formed between the two monomers could be advantageous as the movements of the monomers could regulate the access to the binding site and the binding pocket is too small for the antibodies to approach (Caesar et al., 2013b; Cordes et al., 2006). From those studies it was also assumed that the binding cleft probably could be conserved between the homologous proteins from the PFam54 family (Wywial et al., 2009). As we observed that the C-terminal α-helix in BBA64 has a different conformation to that in CspA and this could prohibit the protein to form a stable homodimer by interaction with the C-terminal α-helices as in the case of CspA, we analyzed the oligomerization state of BBA64. Gel filtration chromatography revealed that BBA64 is a monomer and thus indicating that the proteins belonging to the PFam54 family does not form a similar binding site that is located between the monomers.

By using the sequence comparison of BBA64 and CspA we analyzed the residues found in CspA to be important for complement regulators factor H and FHL-1 binding to explain their functional differences (Kraiczy et al., 2004; Kraiczy et al., 2009; McDowell et al., 2005; Wallich et al., 2005). By analyzing the three different parts in CspA important for factor H and FHL-1 binding and comparing that with the same regions in BBA64 it become clear that although CspA could bind complement regulators as a monomer (as judged from the ability to bind complement factor H and FHL-1 after SDS-PAGE separation) the three regions necessary for a proper binding exhibit a considerable sequence diversity between the two homologous proteins and thus could also explain the inability to bind complement regulators by BBA64 besides the different fold of the C-terminal α-helix.

Although the potential ligand or receptor for BBA64 has not been determined, it has been shown that after the treatment of *B. burgdorferi* with Anti-BBA64 (antibodies for the outer surface protein BBA64), the binding of bacteria to HUVEC (human umbilical vein endothelial cells) or H4 (human neuroglial cells) cells is significantly reduced indicating that the potential binding partner could be located on the cell surface of the mammalian host organism and provide the adherence and invasion of the Lyme disease agent (Schmit et al., 2011). Suggesting that BBA64 is necessary to provide the transfer of *Borrelia* from tick salivary glands to the host organism, the binding of *Borrelia* to mammalian cells as mentioned above could reduce the range for the possible ligand/receptor search, excluding for example tick salivary gland proteins which otherwise could be potential ligand candidates for consideration to assist in transport of *B. burgdorferi* to the host organism. The location of the receptors on the surface of the host cells could also explain the vital role of the protein in the transfer of *B. burgdorferi* from ticks to mammalian and the observation that *Borrelia* lacking BBA64 synthesis was unable to infect mice via tick feeding but the infection was initiated after needle inoculation of *B. burgdorferi* (Gilmore et al., 2010; Patton et al., 2011). The expression of BBA64 is highly upregulated also in tissues during chronic murine infection throughout the infection period indicating that the protein contributes not only to the transfer of *Borrelia* from ticks to the host organism and establishment of the infection, but also to the maintenance and persistence of Lyme disease (Gilmore et al., 2008).

We have determined the crystal structure also for another member from the Pfam54 family, named BBA73. The crystal structure of BBA73 revealed that the overall fold is similar to BBA64 and does not form a stalk-like extension as was observed for CspA.

The interesting fact that we showed is that BBA73 could still form a stable homodimer as determined by gel-filtration chromatography. The results implied to think that there is a possible dimerization interface in BBA73 that is absent in the homologous proteins with already solved crystal structures, like BBA64 and CspA (while CspA can form dimers by interacting with the C-terminal α-helix that forms a stalk-like extension that is missing in BBA73 and BBA64). First, by analyzing the crystal structure we identified the potential site involved in the dimerization covering the N-terminal α-helix. Then we made a truncation mutant of BBA73 excluding a part of the N-terminal α-helix. The truncated protein proved the importance of the N-terminal α-helix in the dimerization of BBA73 as the truncated protein did not form a homodimer and the overall structure was preserved as observed from the crystal structure of the truncated protein. BBA73 has been described as an important protein in the pathogenesis of Lyme disease as it has been identified as the second highest temperature-responsive gene through the analysis of all the putative ORFs of *B. burgdorferi* demonstrating increased expression at 35°C relative to 25°C (Ojaimi et al., 2003). Determination of the crystal structure could facilitate the determination of the function of the protein as well as the crystal structure has revealed the potential binding site for ligands/receptors in BBA73 that could be located in the cleft between the two monomers.

Recently it was showed that BBA66 that is another member of the PFam54 family is important in the pathogenesis of Lyme disease (Patton et al., 2013a). We determined the crystal structure of BBA66 at 2.25 Å resolution using the molecular replacement method with the structure of BBA73 as a search model. The overall fold for BBA66 generally proved to be the same as for the homologous proteins. While the overall fold is the same, the sequence differences are remarkable between the homologous proteins, ranging from 16% to 24%. This could clearly explain the functional differences between the homologous proteins as it has been suggested that in *B. burgdorferi* there have been events of genome duplication that gave the appearance of more than a hundred paralogous gene families (Casjens et al., 2012). This could be exactly was has happened with the PFam54 family members as the analysis of the conserved residues between the members have revealed that the residues are located inwards the proteins core. This means that the residues are most probably conserved so that the overall fold of the proteins is conserved while the surface localized residues have changed over time giving rise to proteins exhibiting different functions.

In summary, the thesis has provided a comprehensive work about the proteins playing an important or even vital role in the pathogenesis of Lyme disease. While knowing the great importance of those proteins in the pathogenesis of Lyme disease the exact receptors/ligands are not known for those proteins. Taking into account that the tertiary protein structure is directly related with the protein function we determined the crystal structures of several outer surface lipoproteins for which the specific role in the pathogenesis was determined previously. Therefore the crystal structures of those proteins could help to gain more understanding about the pathogenesis of Lyme disease and could promote the development of novel drugs against Lyme disease.

5. CONCLUSIONS

1. Removing the signal peptide coding sequence from the genes that were under the study and cloning them into the expression vector pETm_11 was showed to be a perfect strategy to express the proteins in sufficient amount to use for crystallization.
2. The crystal structure of recombinant BBA64 protein from *B. burgdorferi* that is vital for the bacteria to establish Lyme disease has been determined at 2.4 Å resolution.
3. The crystal structure of BBA64 has revealed that the protein structure is similar to other homologous proteins from the PFam54 family, although in comparison to the homologous CspA, BBA64 does not form a homodimer and the C-terminal part has a different conformation.
4. The crystal structure of *B. burgdorferi* outer surface protein BBA73, which is upregulated during the infection period and possibly plays some important role in the pathogenesis of Lyme disease, has been determined at 2.09 Å resolution.
5. BBA73 has a similar overall fold to the homologous proteins and BBA73 exists as a homodimer both in the crystal and in solution.
6. The BBA73 protein dimerizes through the interaction of the N-terminal regions that is different from the homologous proteins CspA which also forms a homodimer but by interacting with the C-terminal regions. The assumption that the N-terminal part is involved in the dimerization was successfully proven by producing an N-terminal deletion mutant of BBA73. The truncated protein was crystallized and the structure was solved at 1.88 Å resolution to make sure that the overall fold is not affected by the truncation and the shortened protein did not form a homodimer indeed.
7. The dimerization in BBA73 forms a cleft between the two monomers and that could potentially serve as a ligand or receptor binding site in the protein.
8. The crystal structure for an outer surface protein BBA66, which is important in the transfer of *B. burgdorferi* from an infected tick to the mammalian organism during the tick feeding, has been determined at 2.25 Å resolution.
9. Despite the low primary sequence similarity with the other homologous proteins, BBA66 has the same overall fold which is common between the PFam54 family members, and BBA66 has an atypically long disordered N-terminal region.

6. MAIN THESIS FOR DEFENCE

1. The *Escherichia coli* expression system in combination with the expression vector pETm_11 can be used for optimal production of *B. burgdorferi* outer surface lipoproteins.
2. The determination of the crystal structure for *B. burgdorferi* outer surface lipoprotein BBA64 is critical to ensure that we find out more details about the protein that is vital for the pathogenesis of Lyme disease.
3. Crystal structure of *B. burgdorferi* outer surface protein BBA66 that plays an important role in the transfer of spirochete from ticks to mammalian does show structural similarities with other PFam54 family members, while the conserved residues are located inwards the protein core.
4. Crystal structure of BBA73 protein from *B. burgdorferi*, that is one of the most upregulated proteins during the transfer of bacteria from ticks to mammalian, is different from the other PFam54 family members because it forms a homodimer by interacting with the N-terminal α-helices.

7. LIST OF ORIGINAL PUBLICATIONS

1. Brangulis, K., I. Petrovskis, A. Kazaks, V. Baumanis, K. Tars, 2013a. Structural characterization of the Borrelia burgdorferi outer surface protein BBA73 implicates dimerization as a functional mechanism. Biochem Biophys Res Commun 434, 848-53.

2. Brangulis, K., K. Tars, I. Petrovskis, A. Kazaks, R. Ranka, V. Baumanis, 2013b. Structure of an outer surface lipoprotein BBA64 from the Lyme disease agent Borrelia burgdorferi which is critical to ensure infection after a tick bite. Acta Crystallogr D Biol Crystallogr 69, 1099-107.

3. Brangulis, K., I. Petrovskis, A. Kazaks, K. Tars, R. Ranka, 2013c. Crystal structure of the infectious phenotype associated outer surface protein BBA66 from Lyme disease agent *Borrelia burgdorferi*. Ticks and Tick-borne Diseases, 5, 63-8.

8. APPROBATION OF THE RESEARCH (THESIS)

1. K. Brangulis, K. Tars, I. Petrovskis, A. Kazaks, R. Ranka, V. Baumanis. Laima slimības ierosinātāja *Borrelia burgdorferi* ārējā virsmas proteīna BBA69 struktūras analīze. Latvijas Universitātes 71. konference, mutisks referāts, 2013.
2. K. Brangulis, K. Tars, I. Petrovskis, R. Ranka, V. Baumanis. Crystallization and data analysis of *B. burgdorferi* outer surface proteins (Abstact). FEBS practical and lecture course on modern methods of protein crystallography “BioCrys 2012”, Portugal, Oeiras, 2012.
3. K. Brangulis, K. Tars, I. Petrovskis, R. Ranka, V. Baumanis. Crystal structure of *B. burgdorferi* outer surface protein (Abstact). Oulu university practical course on protein crystallography “From data to structures”, Finland, Oulu, 2012.
4. K. Brangulis, K. Tars, I. Petrovskis, A. Kazaks, R. Ranka, V. Baumanis. *B. burgdorferi* patoģenēzē nozīmīgo ārējo virsmas proteīnu kristalizācija un struktūras analīze. Latvijas Universitātes 70. konference, mutisks referāts, 2012.
5. K. Brangulis, K. Tars, I. Petrovskis, R. Ranka, V. Baumanis. Crystal structure of an outer surface protein BBA64 from *Borrelia burgdorferi* in comparison to BbCRASP-1. The FEBS Journal, 2012. Vol. 279, supplement 1:196, Seville, Spain.

6. K. Brangulis, K. Tars, I. Petrovskis, R. Ranka, V. Baumanis. Comparison of X-ray structures of two homologous *B. burgdorferi* outer surface proteins (Abstact). EMBO practical course "Computational structural biology – from data to structure to function", UK, Hinxton, 2011.
7. K. Brangulis, R. Ranka, I. Petrovskis, K. Tars, V. Capligina, V. Baumanis. Cloning, expression, purification and crystallisation of *B.burgdorferi* outer surface protein *BbA69* for structural and functional studies. XI International Jena Symposium on tick-borne diseases. 2011. p.78, Germany, Weimar.
8. K. Brangulis, R. Ranka, K. Tārs, I. Petrovskis, A. Kazāks, V. Baumanis. *B.burgdorferi* ārējo virsmas proteīnu ekspresija, attīrīšana, kristalizēšana un strukturālie pētījumi. Latvijas Universitātes 69. konference, mutisks referāts, 2011.
9. Kalvis Brangulis, Renāte Ranka, Ivars Petrovskis, Kaspars Tārs, Renāte Medne, Dace Bandere, Viesturs Baumanis. *B. burgdorferi* ārējās virsmas proteīna BbA69 klonēšana, ekspresija, attīrīšana un kristalizācija. RSU 2011. gada Zinātniskā konference, 2011. 145 lpp
10. K. Brangulis, R. Ranka, I. Petrovskis, K. Tars, V. Baumanis. Expression and crystallization of Bb0689, a mammalian host-specific up-regulated outer surface protein of *B.burgdorferi.* The FEBS Journal, 2011. Vol. 278, supplement 1:275, Torino, Italy.
11. K. Brangulis, R. Ranka, I. Petrovskis, K. Tars, V. Baumanis. Expression, purification and crystallization of various mammalian host-specific up-regulated *B.burgdorferi* outer surface proteins (Abstact). EMBO practical course "High-throughput methods for Protein Production and Crystallization", France, Marseille, 2011.
12. K. Brangulis, R. Ranka, V. Čapligina, V. Sondore, V. Baumanis. *B. burgdorferi* imunogēno proteīnu ekspresijas optimizācija un klonēšanas stratēģijas izvēle. Latvijas Universitātes 68. konference, mutisks referāts, 2010
13. K. Brangulis, R. Ranka, V. Capligina, V. Baumanis. Strategy of recombinant BBK32 protein cloning and expression for structural studies. The FEBS Journal, 2010. Vol. 277, supplement 1:275, Sweden, Goteborg.
14. V. Capligina, R. Ranka, K. Brangulis, A. Bormane, V. Baumanis. Application of real-time TaqMan multiplex PCR in epidemiological study of *B.burgdorferi* and

A.phagocytophilum in tick samples. The FEBS Journal, 2010, Vol. 277, supplement 1:102

15. K. Brangulis, R. Ranka, V. Čapligina, V. Sondore, R. Medne, V. Baumanis. Laima slimības ierosinātāja rekombinantā proteīna BBK32 īpašību izpēte. RSU 2010. Gada Zinātniskā konference, 2010, 202lpp
16. K. Brangulis. Laima slimības ierosinātāja rekombinantā proteīna BBK32 īpašību izpēte. RSU 59. Medicīnas nozares studentu zinātniskā konference, 2010, 51 lpp
17. K. Brangulis, R. Ranka, I. Petrovskis, V. Baumanis. Cloning and expression of different *B.burgdorferi* outer surface proteins for structural and functional studies (Abstract). EMBO/EMBL practical course Protein expression, purification and crystallisation, Germany, Hamburg, 2010.
18. R. Ranka, K. Brangulis, V. Sondore, V. Baumanis. Antigenicity of recombinant fibronectin binding protein fragments in Lyme disease. The Febs Journal. 2009. Volume 276, supplement 1: 162
19. V. Capligina, R. Ranka, A. Bormane, K. Brangulis, V. Baumanis. Pathogens in ticks: Prevalence of Borrelia sp., Anaplasma sp., Ehrlichia sp., Babesia sp., Rickettsia sp. and tick-borne encephalytis virusi n Latvia. VIII Nordic-Baltic Congress on Infectious Diseases. 2009.
20. R. Ranka**,** K. Brangulis, V. Sondore, V. Baumanis. Recombinant BBK32 protein fragment in serodiagnosis of Lyme disease. The Febs Journal. 2008. Volume 275, supplement 1: 275.

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10. REFERENCES

Aguero-Rosenfeld, M.E., G. Wang, I. Schwartz, G.P. Wormser, 2005. Diagnosis of lyme borreliosis. Clin Microbiol Rev 18, 484-509.

Aksoy, S., B. Weiss, G. Attardo, 2008. Paratransgenesis applied for control of tsetse transmitted sleeping sickness. Adv Exp Med Biol 627, 35-48.

Anderson, J.F., 1989a. Epizootiology of Borrelia in Ixodes tick vectors and reservoir hosts. Rev Infect Dis 11 Suppl 6, S1451-9.

Anderson, J.F., 1989b. Ecology of Lyme disease. Conn Med 53, 343-6.

Anderson, J.F., 1991. Epizootiology of Lyme borreliosis. Scand J Infect Dis Suppl 77, 23-34.

Anderson, J.F., R.C. Johnson, L.A. Magnarelli, 1987. Seasonal prevalence of Borrelia burgdorferi in natural populations of white-footed mice, Peromyscus leucopus. J Clin Microbiol 25, 1564-6.

Anderson, J.F., L.A. Magnarelli, W. Burgdorfer, A.G. Barbour, 1983. Spirochetes in Ixodes dammini and mammals from Connecticut. Am J Trop Med Hyg 32, 818-24.

Angel, T.E., B.J. Luft, X. Yang, C.D. Nicora, D.G. Camp, 2nd, J.M. Jacobs, R.D. Smith, 2010. Proteome analysis of Borrelia burgdorferi response to environmental change. PLoS One 5, e13800.

Anguita, J., M.N. Hedrick, E. Fikrig, 2003. Adaptation of Borrelia burgdorferi in the tick and the mammalian host. FEMS Microbiol Rev 27, 493-504.

Arnez, M., 2007. Antibiotic treatment of children with erythema migrans. Clin Infect Dis 44, 1133-4; author reply 1137-9.

Asbrink, E., A. Hovmark, 1988. Early and late cutaneous manifestations in Ixodes-borne borreliosis (erythema migrans borreliosis, Lyme borreliosis). Ann N Y Acad Sci 539, 4-15.

Aucott, J.N., L.A. Crowder, V. Yedlin, K.B. Kortte, 2012. Bull's-Eye and Nontarget Skin Lesions of Lyme Disease: An Internet Survey of Identification of Erythema Migrans. Dermatol Res Pract 2012, 451727.

Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J.C. Piffaretti, M. Assous, P.A. Grimont, 1992. Delineation of Borrelia burgdorferi sensu stricto, Borrelia garinii sp. nov., and group VS461 associated with Lyme borreliosis. Int J Syst Bacteriol 42, 378-83.

Barbour, A.G., 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J Biol Med 57, 521-5.

Barbour, A.G., D. Fish, 1993. The biological and social phenomenon of Lyme disease. Science 260, 1610-6.

Barbour, A.G., S.F. Hayes, R.A. Heiland, M.E. Schrumpf, S.L. Tessier, 1986. A Borrelia-specific monoclonal antibody binds to a flagellar epitope. Infect Immun 52, 549-54.

Battye, T.G., L. Kontogiannis, O. Johnson, H.R. Powell, A.G. Leslie, 2011. iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. Acta Crystallogr D Biol Crystallogr 67, 271-81.

Becker, M., J. Bunikis, B.D. Lade, J.J. Dunn, A.G. Barbour, C.L. Lawson, 2005. Structural investigation of Borrelia burgdorferi OspB, a bactericidal Fab target. J Biol Chem 280, 17363-70.

Belfaiza, J., D. Postic, E. Bellenger, G. Baranton, I.S. Girons, 1993. Genomic fingerprinting of Borrelia burgdorferi sensu lato by pulsed-field gel electrophoresis. J Clin Microbiol 31, 2873-7.

Belisle, J.T., M.E. Brandt, J.D. Radolf, M.V. Norgard, 1994. Fatty acids of Treponema pallidum and Borrelia burgdorferi lipoproteins. J Bacteriol 176, 2151-7.

Berger, B.W., R.C. Johnson, C. Kodner, L. Coleman, 1995. Cultivation of Borrelia burgdorferi from human tick bite sites: a guide to the risk of infection. J Am Acad Dermatol 32, 184-7.

Bhattacharjee, A., J.S. Oeemig, R. Kolodziejczyk, T. Meri, T. Kajander, M.J. Lehtinen, H. Iwai, T.S. Jokiranta, A. Goldman, 2013. Structural basis for complement evasion by Lyme disease pathogen Borrelia burgdorferi. J Biol Chem.

Bono, J.L., K. Tilly, B. Stevenson, D. Hogan, P. Rosa, 1998. Oligopeptide permease in Borrelia burgdorferi: putative peptide-binding components encoded by both chromosomal and plasmid loci. Microbiology 144 (Pt 4), 1033-44.

Brandt, M.E., B.S. Riley, J.D. Radolf, M.V. Norgard, 1990. Immunogenic integral membrane proteins of Borrelia burgdorferi are lipoproteins. Infect Immun 58, 983-91.

Brangulis, K., I. Petrovskis, A. Kazaks, K. Tars, R. Ranka, 2013a. Crystal structure of the infectious phenotype-associated outer surface protein BBA66 from the Lyme disease agent Borrelia burgdorferi. Ticks Tick Borne Dis.

Brangulis, K., I. Petrovskis, A. Kazaks, V. Baumanis, K. Tars, 2013b. Structural characterization of the Borrelia burgdorferi outer surface protein BBA73 implicates dimerization as a functional mechanism. Biochem Biophys Res Commun 434, 848-53.

Brangulis, K., K. Tars, I. Petrovskis, A. Kazaks, R. Ranka, V. Baumanis, 2013c. Structure of an outer surface lipoprotein BBA64 from the Lyme disease agent Borrelia burgdorferi which is critical to ensure infection after a tick bite. Acta Crystallogr D Biol Crystallogr 69, 1099-107.

Bratton, R.L., J.W. Whiteside, M.J. Hovan, R.L. Engle, F.D. Edwards, 2008. Diagnosis and treatment of Lyme disease. Mayo Clin Proc 83, 566-71.

Brooks, C.S., P.S. Hefty, S.E. Jolliff, D.R. Akins, 2003. Global analysis of Borrelia burgdorferi genes regulated by mammalian host-specific signals. Infect Immun 71, 3371-83.

Brooks, C.S., S.R. Vuppala, A.M. Jett, D.R. Akins, 2006. Identification of Borrelia burgdorferi outer surface proteins. Infect Immun 74, 296-304.

Bryksin, A.V., A. Tomova, H.P. Godfrey, F.C. Cabello, 2010. BmpA is a surface-exposed outer-membrane protein of Borrelia burgdorferi. FEMS Microbiol Lett 309, 77-83.

Burgdorfer, W., 1984. Discovery of the Lyme disease spirochete and its relation to tick vectors. Yale J Biol Med 57, 515-20.

Burgdorfer, W., A.G. Barbour, S.F. Hayes, J.L. Benach, E. Grunwaldt, J.P. Davis, 1982. Lyme disease-a tick-borne spirochetosis? Science 216, 1317-9.

Caesar, J.J., S. Johnson, P. Kraiczy, S.M. Lea, 2013a. ErpC, a member of the complement regulator-acquiring family of surface proteins from Borrelia burgdorferi, possesses an architecture previously unseen in this protein family. Acta Crystallogr Sect F Struct Biol Cryst Commun 69, 624-8.

Caesar, J.J., R. Wallich, P. Kraiczy, P.F. Zipfel, S.M. Lea, 2013b. Further structural insights into the binding of complement factor H by complement regulator-acquiring surface protein 1 (CspA) of Borrelia burgdorferi. Acta Crystallogr Sect F Struct Biol Cryst Commun 69, 629-33.

Canica, M.M., F. Nato, L. du Merle, J.C. Mazie, G. Baranton, D. Postic, 1993. Monoclonal antibodies for identification of Borrelia afzelii sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. Scand J Infect Dis 25, 441-8.

Carroll, J.A., R.M. Cordova, C.F. Garon, 2000. Identification of 11 pH-regulated genes in Borrelia burgdorferi localizing to linear plasmids. Infect Immun 68, 6677-84.

Carroll, J.F., D.E. Hill, P.C. Allen, K.W. Young, E. Miramontes, M. Kramer, J.M. Pound, J.A. Miller, J.E. George, 2009. The impact of 4-Poster deer self-treatment devices at three locations in Maryland. Vector Borne Zoonotic Dis 9, 407-16.

Casjens, S., W.M. Huang, 1993. Linear chromosomal physical and genetic map of Borrelia burgdorferi, the Lyme disease agent. Mol Microbiol 8, 967-80.

Casjens, S., R. van Vugt, K. Tilly, P.A. Rosa, B. Stevenson, 1997. Homology throughout the multiple 32-kilobase circular plasmids present in Lyme disease spirochetes. J Bacteriol 179, 217-27.

Casjens, S., N. Palmer, R. van Vugt, W.M. Huang, B. Stevenson, P. Rosa, R. Lathigra, G. Sutton, J. Peterson, R.J. Dodson, D. Haft, E. Hickey, M. Gwinn, O. White, C.M. Fraser, 2000. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete Borrelia burgdorferi. Mol Microbiol 35, 490-516.

Casjens, S.R., E.F. Mongodin, W.G. Qiu, J.J. Dunn, B.J. Luft, C.M. Fraser-Liggett, S.E. Schutzer, 2011. Whole-genome sequences of two Borrelia afzelii and two Borrelia garinii Lyme disease agent isolates. J Bacteriol 193, 6995-6.

Casjens, S.R., E.F. Mongodin, W.G. Qiu, B.J. Luft, S.E. Schutzer, E.B. Gilcrease, W.M. Huang, M. Vujadinovic, J.K. Aron, L.C. Vargas, S. Freeman, D. Radune, J.F. Weidman, G.I. Dimitrov, H.M. Khouri, J.E. Sosa, R.A. Halpin, J.J. Dunn, C.M. Fraser, 2012. Genome stability of Lyme disease spirochetes: comparative genomics of Borrelia burgdorferi plasmids. PLoS One 7, e33280.

Charon, N.W., S.F. Goldstein, 2002. Genetics of motility and chemotaxis of a fascinating group of bacteria: the spirochetes. Annu Rev Genet 36, 47-73.

Charon, N.W., S.F. Goldstein, M. Marko, C. Hsieh, L.L. Gebhardt, M.A. Motaleb, C.W. Wolgemuth, R.J. Limberger, N. Rowe, 2009. The flat-ribbon configuration of the periplasmic flagella of Borrelia burgdorferi and its relationship to motility and morphology. J Bacteriol 191, 600-7.

Chu, C.Y., W. Liu, B.G. Jiang, D.M. Wang, W.J. Jiang, Q.M. Zhao, P.H. Zhang, Z.X. Wang, G.P. Tang, H. Yang, W.C. Cao, 2008. Novel genospecies of Borrelia burgdorferi sensu lato from rodents and ticks in southwestern China. J Clin Microbiol 46, 3130-3.

Clark, R.P., L.T. Hu, 2008. Prevention of lyme disease and other tick-borne infections. Infect Dis Clin North Am 22, 381-96, vii.

Coburn, J., J.R. Fischer, J.M. Leong, 2005. Solving a sticky problem: new genetic approaches to host cell adhesion by the Lyme disease spirochete. Mol Microbiol 57, 1182-95.

Colli, C., B. Leinweber, R. Mullegger, A. Chott, H. Kerl, L. Cerroni, 2004. Borrelia burgdorferi-associated lymphocytoma cutis: clinicopathologic, immunophenotypic, and molecular study of 106 cases. J Cutan Pathol 31, 232-40.

Connally, N.P., A.J. Durante, K.M. Yousey-Hindes, J.I. Meek, R.S. Nelson, R. Heimer, 2009. Peridomestic Lyme disease prevention: results of a population-based case-control study. Am J Prev Med 37, 201-6.

Cordes, F.S., P. Kraiczy, P. Roversi, C. Skerka, M. Kirschfink, M.M. Simon, V. Brade, E.D. Lowe, P. Zipfel, R. Wallich, S.M. Lea, 2004. Crystallization and preliminary crystallographic analysis of BbCRASP-1, a complement regulator-acquiring surface protein of Borrelia burgdorferi. Acta Crystallogr D Biol Crystallogr 60, 929-32.

Cordes, F.S., P. Roversi, P. Kraiczy, M.M. Simon, V. Brade, O. Jahraus, R. Wallis, C. Skerka, P.F. Zipfel, R. Wallich, S.M. Lea, 2005. A novel fold for the factor H-binding protein BbCRASP-1 of Borrelia burgdorferi. Nat Struct Mol Biol 12, 276-7.

Cordes, F.S., P. Kraiczy, P. Roversi, M.M. Simon, V. Brade, O. Jahraus, R. Wallis, L. Goodstadt, C.P. Ponting, C. Skerka, P.F. Zipfel, R. Wallich, S.M. Lea, 2006. Structure-function mapping of BbCRASP-1, the key complement factor H and FHL-1 binding protein of Borrelia burgdorferi. Int J Med Microbiol 296 Suppl 40, 177-84.

Costantini, C., A. Badolo, E. Ilboudo-Sanogo, 2004. Field evaluation of the efficacy and persistence of insect repellents DEET, IR3535, and KBR 3023 against Anopheles gambiae complex and other Afrotropical vector mosquitoes. Trans R Soc Trop Med Hyg 98, 644-52.

Cowtan, K., 2006. The Buccaneer software for automated model building. 1. Tracing protein chains. Acta Crystallogr D Biol Crystallogr 62, 1002-11.

Dandache, P., R.B. Nadelman, 2008. Erythema migrans. Infect Dis Clin North Am 22, 235-60, vi.

de la Fuente, J., A. Estrada-Pena, J.M. Venzal, K.M. Kocan, D.E. Sonenshine, 2008. Overview: Ticks as vectors of pathogens that cause disease in humans and animals. Front Biosci 13, 6938-46.
de la Fuente, J., J.A. Moreno-Cid, M. Canales, M. Villar, J.M. de la Lastra, K.M. Kocan, R.C. Galindo, C. Almazan, E.F. Blouin, 2011. Targeting arthropod subolesin/akirin for the development of a universal vaccine for control of vector infestations and pathogen transmission. Vet Parasitol 181, 17-22.
De Silva, A.M., E. Fikrig, 1995. Growth and migration of Borrelia burgdorferi in Ixodes ticks during blood feeding. Am J Trop Med Hyg 53, 397-404.
de Silva, A.M., S.R. Telford, 3rd, L.R. Brunet, S.W. Barthold, E. Fikrig, 1996. Borrelia burgdorferi OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. J Exp Med 183, 271-5.
de Silva, A.M., N.S. Zeidner, Y. Zhang, M.C. Dolan, J. Piesman, E. Fikrig, 1999. Influence of outer surface protein A antibody on Borrelia burgdorferi within feeding ticks. Infect Immun 67, 30-5.
Derdakova, M., D. Lencakova, 2005. Association of genetic variability within the Borrelia burgdorferi sensu lato with the ecology, epidemiology of Lyme borreliosis in Europe. Ann Agric Environ Med 12, 165-72.
des Vignes, F., J. Piesman, R. Heffernan, T.L. Schulze, K.C. Stafford, 3rd, D. Fish, 2001. Effect of tick removal on transmission of Borrelia burgdorferi and Ehrlichia phagocytophila by Ixodes scapularis nymphs. J Infect Dis 183, 773-8.
Donta, S.T., 2003. Macrolide therapy of chronic Lyme Disease. Med Sci Monit 9, PI136-42.
Donta, S.T., 2012. Issues in the diagnosis and treatment of lyme disease. Open Neurol J 6, 140-5.
Dumler, J.S., 2001. Molecular diagnosis of Lyme disease: review and meta-analysis. Mol Diagn 6, 1-11.
Duray, P.H., E. Asbrink, K. Weber, 1989. The cutaneous manifestations of human Lyme disease: a widening spectrum. Adv Dermatol 4, 255-75; discussion 276.
Duscher, G.G., R. Peschke, A. Tichy, 2012. Mechanical tools for the removal of Ixodes ricinus female ticks--differences of instruments and pulling or twisting? Parasitol Res 111, 1505-11.
Eicken, C., V. Sharma, T. Klabunde, M.B. Lawrenz, J.M. Hardham, S.J. Norris, J.C. Sacchettini, 2002. Crystal structure of Lyme disease variable surface antigen VlsE of Borrelia burgdorferi. J Biol Chem 277, 21691-6.
Eiffert, H., H. Lotter, K. Jarecki-Khan, R. Thomssen, 1991. Identification of an immunoreactive non-proteinaleous component in Borrelia burgdorferi. Med Microbiol Immunol 180, 229-37.
Embers, M.E., S. Narasimhan, 2013. Vaccination against Lyme disease: past, present, and future. Front Cell Infect Microbiol 3, 6.
Embers, M.E., R. Ramamoorthy, M.T. Philipp, 2004. Survival strategies of Borrelia burgdorferi, the etiologic agent of Lyme disease. Microbes Infect 6, 312-8.
Emsley, P., K. Cowtan, 2004. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr 60, 2126-32.
Evans, P., 2006. Scaling and assessment of data quality. Acta Crystallogr D Biol Crystallogr 62, 72-82.
Fikrig, E., S. Narasimhan, 2006. Borrelia burgdorferi--traveling incognito? Microbes Infect 8, 1390-9.
Fikrig, E., S.W. Barthold, F.S. Kantor, R.A. Flavell, 1992. Long-term protection of mice from Lyme disease by vaccination with OspA. Infect Immun 60, 773-7.
Fingerle, V., H.I. Huppertz, 2007. [Lyme borreliosis in children. Epidemiology, diagnosis, clinical treatment, and therapy]. Hautarzt 58, 541-50, quiz 551-2.

Floden, A.M., T. Gonzalez, R.A. Gaultney, C.A. Brissette, 2013. Evaluation of RevA, a fibronectin-binding protein of Borrelia burgdorferi, as a potential vaccine candidate for lyme disease. Clin Vaccine Immunol 20, 892-9.

Fraser, C.M., J.D. Gocayne, O. White, M.D. Adams, R.A. Clayton, R.D. Fleischmann, C.J. Bult, A.R. Kerlavage, G. Sutton, J.M. Kelley, R.D. Fritchman, J.F. Weidman, K.V. Small, M. Sandusky, J. Fuhrmann, D. Nguyen, T.R. Utterback, D.M. Saudek, C.A. Phillips, J.M. Merrick, J.F. Tomb, B.A. Dougherty, K.F. Bott, P.C. Hu, T.S. Lucier, S.N. Peterson, H.O. Smith, C.A. Hutchison, 3rd, J.C. Venter, 1995. The minimal gene complement of Mycoplasma genitalium. Science 270, 397-403.

Fraser, C.M., S. Casjens, W.M. Huang, G.G. Sutton, R. Clayton, R. Lathigra, O. White, K.A. Ketchum, R. Dodson, E.K. Hickey, M. Gwinn, B. Dougherty, J.F. Tomb, R.D. Fleischmann, D. Richardson, J. Peterson, A.R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M.D. Adams, J. Gocayne, J. Weidman, T. Utterback, L. Watthey, L. McDonald, P. Artiach, C. Bowman, S. Garland, C. Fuji, M.D. Cotton, K. Horst, K. Roberts, B. Hatch, H.O. Smith, J.C. Venter, 1997. Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi. Nature 390, 580-6.

Fukunaga, M., A. Hamase, K. Okada, M. Nakao, 1996. Borrelia tanukii sp. nov. and Borrelia turdae sp. nov. found from ixodid ticks in Japan: rapid species identification by 16S rRNA gene-targeted PCR analysis. Microbiol Immunol 40, 877-81.

Ge, Y., C. Li, L. Corum, C.A. Slaughter, N.W. Charon, 1998. Structure and expression of the FlaA periplasmic flagellar protein of Borrelia burgdorferi. J Bacteriol 180, 2418-25.

Gilmore, R.D., Jr., R.R. Howison, V.L. Schmit, J.A. Carroll, 2008. Borrelia burgdorferi expression of the bba64, bba65, bba66, and bba73 genes in tissues during persistent infection in mice. Microb Pathog 45, 355-60.

Gilmore, R.D., Jr., R.R. Howison, G. Dietrich, T.G. Patton, D.R. Clifton, J.A. Carroll, 2010. The bba64 gene of Borrelia burgdorferi, the Lyme disease agent, is critical for mammalian infection via tick bite transmission. Proc Natl Acad Sci U S A 107, 7515-20.

Gilmore, R.D., Jr., R.R. Howison, V.L. Schmit, A.J. Nowalk, D.R. Clifton, C. Nolder, J.L. Hughes, J.A. Carroll, 2007. Temporal expression analysis of the Borrelia burgdorferi paralogous gene family 54 genes BBA64, BBA65, and BBA66 during persistent infection in mice. Infect Immun 75, 2753-64.

Gissler, S., U. Heininger, 2002. Borrelia lymphocytoma ("lymphadenosis benigna cutis"). Arch Dis Child 87, 12.

Goldstein, S.F., N.W. Charon, J.A. Kreiling, 1994. Borrelia burgdorferi swims with a planar waveform similar to that of eukaryotic flagella. Proc Natl Acad Sci U S A 91, 3433-7.

Goldstein, S.F., K.F. Buttle, N.W. Charon, 1996. Structural analysis of the Leptospiraceae and Borrelia burgdorferi by high-voltage electron microscopy. J Bacteriol 178, 6539-45.

Goodman, J.L., J.F. Bradley, A.E. Ross, P. Goellner, A. Lagus, B. Vitale, B.W. Berger, S. Luger, R.C. Johnson, 1995. Bloodstream invasion in early Lyme disease: results from a prospective, controlled, blinded study using the polymerase chain reaction. Am J Med 99, 6-12.

Haake, D.A., 2000. Spirochaetal lipoproteins and pathogenesis. Microbiology 146 (Pt 7), 1491-504.

Halperin, J.J., 1989. Nervous system manifestations of Lyme disease. Rheum Dis Clin North Am 15, 635-47.

Halperin, J.J., 1995. Neuroborreliosis. Am J Med 98, 52S-56S; discussion 56S-59S.

Halperin, J.J., 2008. Nervous system Lyme disease. Infect Dis Clin North Am 22, 261-74, vi.

Hammerschmidt, C., T. Hallstrom, C. Skerka, R. Wallich, B. Stevenson, P.F. Zipfel, P. Kraiczy, 2012. Contribution of the infection-associated complement regulator-acquiring surface protein 4 (ErpC) to complement resistance of Borrelia burgdorferi. Clin Dev Immunol 2012, 349657.

Hartmann, K., C. Corvey, C. Skerka, M. Kirschfink, M. Karas, V. Brade, J.C. Miller, B. Stevenson, R. Wallich, P.F. Zipfel, P. Kraiczy, 2006. Functional characterization of BbCRASP-2, a distinct outer membrane protein of Borrelia burgdorferi that binds host complement regulators factor H and FHL-1. Mol Microbiol 61, 1220-36.

Hellwage, J., T. Meri, T. Heikkila, A. Alitalo, J. Panelius, P. Lahdenne, I.J. Seppala, S. Meri, 2001. The complement regulator factor H binds to the surface protein OspE of Borrelia burgdorferi. J Biol Chem 276, 8427-35.

Hossain, H., H.J. Wellensiek, R. Geyer, G. Lochnit, 2001. Structural analysis of glycolipids from Borrelia burgdorferi. Biochimie 83, 683-92.

Hovius, J.W., N. Ramamoorthi, C. Van't Veer, K.A. de Groot, A.M. Nijhof, F. Jongejan, A.P. van Dam, E. Fikrig, 2007. Identification of Salp15 homologues in Ixodes ricinus ticks. Vector Borne Zoonotic Dis 7, 296-303.

Hovius, J.W., T.J. Schuijt, K.A. de Groot, J.J. Roelofs, G.A. Oei, J.A. Marquart, R. de Beer, C. van 't Veer, T. van der Poll, N. Ramamoorthi, E. Fikrig, A.P. van Dam, 2008. Preferential protection of Borrelia burgdorferi sensu stricto by a Salp15 homologue in Ixodes ricinus saliva. J Infect Dis 198, 1189-97.

Hughes, J.L., C.L. Nolder, A.J. Nowalk, D.R. Clifton, R.R. Howison, V.L. Schmit, R.D. Gilmore, Jr., J.A. Carroll, 2008. Borrelia burgdorferi surface-localized proteins expressed during persistent murine infection are conserved among diverse Borrelia spp. Infect Immun 76, 2498-511.

Hunfeld, K.P., T.A. Wichelhaus, R. Rodel, G. Acker, V. Brade, P. Kraiczy, 2004. Comparison of in vitro activities of ketolides, macrolides, and an azalide against the spirochete Borrelia burgdorferi. Antimicrob Agents Chemother 48, 344-7.

Hurwitz, I., A. Fieck, A. Read, H. Hillesland, N. Klein, A. Kang, R. Durvasula, 2011. Paratransgenic control of vector borne diseases. Int J Biol Sci 7, 1334-44.

Indest, K.J., R. Ramamoorthy, M. Sole, R.D. Gilmore, B.J. Johnson, M.T. Philipp, 1997. Cell-density-dependent expression of Borrelia burgdorferi lipoproteins in vitro. Infect Immun 65, 1165-71.

Jones, J.D., K.W. Bourell, M.V. Norgard, J.D. Radolf, 1995. Membrane topology of Borrelia burgdorferi and Treponema pallidum lipoproteins. Infect Immun 63, 2424-34.

Karmacharya, P., M.R. Aryal, 2013. Heart stopping tick. World J Cardiol 5, 148-50.

Kawabata, H., T. Masuzawa, Y. Yanagihara, 1993. Genomic analysis of Borrelia japonica sp. nov. isolated from Ixodes ovatus in Japan. Microbiol Immunol 37, 843-8.

Kelly, R., 1971. Cultivation of Borrelia hermsi. Science 173, 443-4.

Kitten, T., A.G. Barbour, 1992. The relapsing fever agent Borrelia hermsii has multiple copies of its chromosome and linear plasmids. Genetics 132, 311-24.

Koene, R., D.R. Boulware, M. Kemperman, S.H. Konety, M. Groth, J. Jessurun, P.M. Eckman, 2012. Acute heart failure from lyme carditis. Circ Heart Fail 5, e24-6.

Kraiczy, P., B. Stevenson, 2013. Complement regulator-acquiring surface proteins of Borrelia burgdorferi: Structure, function and regulation of gene expression. Ticks Tick Borne Dis 4, 26-34.

Kraiczy, P., C. Skerka, V. Brade, P.F. Zipfel, 2001a. Further characterization of complement regulator-acquiring surface proteins of Borrelia burgdorferi. Infect Immun 69, 7800-9.

Kraiczy, P., C. Skerka, M. Kirschfink, V. Brade, P.F. Zipfel, 2001b. Immune evasion of Borrelia burgdorferi by acquisition of human complement regulators FHL-1/reconectin and Factor H. Eur J Immunol 31, 1674-84.

Kraiczy, P., E. Rossmann, V. Brade, M.M. Simon, C. Skerka, P.F. Zipfel, R. Wallich, 2006. Binding of human complement regulators FHL-1 and factor H to CRASP-1 orthologs of Borrelia burgdorferi. Wien Klin Wochenschr 118, 669-76.

Kraiczy, P., J. Hellwage, C. Skerka, H. Becker, M. Kirschfink, M.M. Simon, V. Brade, P.F. Zipfel, R. Wallich, 2004. Complement resistance of Borrelia burgdorferi correlates with the

expression of BbCRASP-1, a novel linear plasmid-encoded surface protein that interacts with human factor H and FHL-1 and is unrelated to Erp proteins. J Biol Chem 279, 2421-9.

Kraiczy, P., C. Hanssen-Hubner, V. Kitiratschky, C. Brenner, S. Besier, V. Brade, M.M. Simon, C. Skerka, P. Roversi, S.M. Lea, B. Stevenson, R. Wallich, P.F. Zipfel, 2009. Mutational analyses of the BbCRASP-1 protein of Borrelia burgdorferi identify residues relevant for the architecture and binding of host complement regulators FHL-1 and factor H. Int J Med Microbiol 299, 255-68.

Kudryashev, M., M. Cyrklaff, W. Baumeister, M.M. Simon, R. Wallich, F. Frischknecht, 2009. Comparative cryo-electron tomography of pathogenic Lyme disease spirochetes. Mol Microbiol 71, 1415-34.

Kuhn, S., C. Skerka, P.F. Zipfel, 1995. Mapping of the complement regulatory domains in the human factor H-like protein 1 and in factor H1. J Immunol 155, 5663-70.

Kumaran, D., S. Eswaramoorthy, B.J. Luft, S. Koide, J.J. Dunn, C.L. Lawson, S. Swaminathan, 2001. Crystal structure of outer surface protein C (OspC) from the Lyme disease spirochete, Borrelia burgdorferi. EMBO J 20, 971-8.

Lathrop, S.L., R. Ball, P. Haber, G.T. Mootrey, M.M. Braun, S.V. Shadomy, S.S. Ellenberg, R.T. Chen, E.B. Hayes, 2002. Adverse event reports following vaccination for Lyme disease: December 1998-July 2000. Vaccine 20, 1603-8.

Le Fleche, A., D. Postic, K. Girardet, O. Peter, G. Baranton, 1997. Characterization of Borrelia lusitaniae sp. nov. by 16S ribosomal DNA sequence analysis. Int J Syst Bacteriol 47, 921-5.

Levine, J.F., M.L. Wilson, A. Spielman, 1985. Mice as reservoirs of the Lyme disease spirochete. Am J Trop Med Hyg 34, 355-60.

Li, H., J.J. Dunn, B.J. Luft, C.L. Lawson, 1997. Crystal structure of Lyme disease antigen outer surface protein A complexed with an Fab. Proc Natl Acad Sci U S A 94, 3584-9.

Liveris, D., G. Wang, G. Girao, D.W. Byrne, J. Nowakowski, D. McKenna, R. Nadelman, G.P. Wormser, I. Schwartz, 2002. Quantitative detection of Borrelia burgdorferi in 2-millimeter skin samples of erythema migrans lesions: correlation of results with clinical and laboratory findings. J Clin Microbiol 40, 1249-53.

Livey, I., M. O'Rourke, A. Traweger, H. Savidis-Dacho, B.A. Crowe, P.N. Barrett, X. Yang, J.J. Dunn, B.J. Luft, 2011. A new approach to a Lyme disease vaccine. Clin Infect Dis 52 Suppl 3, s266-70.

Loewen, P.S., C.A. Marra, F. Marra, 1999. Systematic review of the treatment of early Lyme disease. Drugs 57, 157-73.

Marconi, R.T., D. Liveris, I. Schwartz, 1995. Identification of novel insertion elements, restriction fragment length polymorphism patterns, and discontinuous 23S rRNA in Lyme disease spirochetes: phylogenetic analyses of rRNA genes and their intergenic spacers in Borrelia japonica sp. nov. and genomic group 21038 (Borrelia andersonii sp. nov.) isolates. J Clin Microbiol 33, 2427-34.

Margolis, N., D. Hogan, K. Tilly, P.A. Rosa, 1994. Plasmid location of Borrelia purine biosynthesis gene homologs. J Bacteriol 176, 6427-32.

Margos, G., A. Hojgaard, R.S. Lane, M. Cornet, V. Fingerle, N. Rudenko, N. Ogden, D.M. Aanensen, D. Fish, J. Piesman, 2010. Multilocus sequence analysis of Borrelia bissettii strains from North America reveals a new Borrelia species, Borrelia kurtenbachii. Ticks Tick Borne Dis 1, 151-8.

Margos, G., S.A. Vollmer, M. Cornet, M. Garnier, V. Fingerle, B. Wilske, A. Bormane, L. Vitorino, M. Collares-Pereira, M. Drancourt, K. Kurtenbach, 2009. A new Borrelia species defined by multilocus sequence analysis of housekeeping genes. Appl Environ Microbiol 75, 5410-6.

Margos, G., A.G. Gatewood, D.M. Aanensen, K. Hanincova, D. Terekhova, S.A. Vollmer, M. Cornet, J. Piesman, M. Donaghy, A. Bormane, M.A. Hurn, E.J. Feil, D. Fish, S. Casjens, G.P. Wormser, I. Schwartz, K. Kurtenbach, 2008. MLST of housekeeping genes captures geographic population structure and suggests a European origin of Borrelia burgdorferi. Proc Natl Acad Sci U S A 105, 8730-5.

Masuzawa, T., N. Takada, M. Kudeken, T. Fukui, Y. Yano, F. Ishiguro, Y. Kawamura, Y. Imai, T. Ezaki, 2001. Borrelia sinica sp. nov., a lyme disease-related Borrelia species isolated in China. Int J Syst Evol Microbiol 51, 1817-24.

Mathias, D.K., J.L. Plieskatt, J.S. Armistead, J.M. Bethony, K.B. Abdul-Majid, A. McMillan, E. Angov, M.J. Aryee, B. Zhan, P. Gillespie, B. Keegan, A.R. Jariwala, W. Rezende, M.E. Bottazzi, D.G. Scorpio, P.J. Hotez, R.R. Dinglasan, 2012. Expression, immunogenicity, histopathology, and potency of a mosquito-based malaria transmission-blocking recombinant vaccine. Infect Immun 80, 1606-14.

Matsuyama, S., N. Yokota, H. Tokuda, 1997. A novel outer membrane lipoprotein, LolB (HemM), involved in the LolA (p20)-dependent localization of lipoproteins to the outer membrane of Escherichia coli. EMBO J 16, 6947-55.

Matuschka, F.R., P. Fischer, K. Musgrave, D. Richter, A. Spielman, 1991. Hosts on which nymphal Ixodes ricinus most abundantly feed. Am J Trop Med Hyg 44, 100-7.

McCoy, A.J., R.W. Grosse-Kunstleve, P.D. Adams, M.D. Winn, L.C. Storoni, R.J. Read, 2007. Phaser crystallographic software. J Appl Crystallogr 40, 658-674.

McDowell, J.V., M.E. Harlin, E.A. Rogers, R.T. Marconi, 2005. Putative coiled-coil structural elements of the BBA68 protein of Lyme disease spirochetes are required for formation of its factor H binding site. J Bacteriol 187, 1317-23.

Meirelles Richer, L., M. Aroso, T. Contente-Cuomo, L. Ivanova, M. Gomes-Solecki, 2011. Reservoir targeted vaccine for lyme borreliosis induces a yearlong, neutralizing antibody response to OspA in white-footed mice. Clin Vaccine Immunol 18, 1809-16.

Motaleb, M.A., L. Corum, J.L. Bono, A.F. Elias, P. Rosa, D.S. Samuels, N.W. Charon, 2000. Borrelia burgdorferi periplasmic flagella have both skeletal and motility functions. Proc Natl Acad Sci U S A 97, 10899-904.

Mullegger, R.R., 2004. Dermatological manifestations of Lyme borreliosis. Eur J Dermatol 14, 296-309.

Murray, T.S., E.D. Shapiro, 2010. Lyme disease. Clin Lab Med 30, 311-28.

Murshudov, G.N., A.A. Vagin, E.J. Dodson, 1997. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr D Biol Crystallogr 53, 240-55.

Nadelman, R.B., J. Nowakowski, G. Forseter, N.S. Goldberg, S. Bittker, D. Cooper, M. Aguero-Rosenfeld, G.P. Wormser, 1996. The clinical spectrum of early Lyme borreliosis in patients with culture-confirmed erythema migrans. Am J Med 100, 502-8.

Nadelman, R.B., J. Nowakowski, D. Fish, R.C. Falco, K. Freeman, D. McKenna, P. Welch, R. Marcus, M.E. Aguero-Rosenfeld, D.T. Dennis, G.P. Wormser, 2001. Prophylaxis with single-dose doxycycline for the prevention of Lyme disease after an Ixodes scapularis tick bite. N Engl J Med 345, 79-84.

Narasimhan, S., F. Santiago, R.A. Koski, B. Brei, J.F. Anderson, D. Fish, E. Fikrig, 2002. Examination of the Borrelia burgdorferi transcriptome in Ixodes scapularis during feeding. J Bacteriol 184, 3122-5.

Narasimhan, S., B. Sukumaran, U. Bozdogan, V. Thomas, X. Liang, K. DePonte, N. Marcantonio, R.A. Koski, J.F. Anderson, F. Kantor, E. Fikrig, 2007. A tick antioxidant facilitates the Lyme disease agent's successful migration from the mammalian host to the arthropod vector. Cell Host Microbe 2, 7-18.

Norris, S.J., C.J. Carter, J.K. Howell, A.G. Barbour, 1992. Low-passage-associated proteins of Borrelia burgdorferi B31: characterization and molecular cloning of OspD, a surface-exposed, plasmid-encoded lipoprotein. Infect Immun 60, 4662-72.

Nowakowski, J., I. Schwartz, D. Liveris, G. Wang, M.E. Aguero-Rosenfeld, G. Girao, D. McKenna, R.B. Nadelman, L.F. Cavaliere, G.P. Wormser, 2001. Laboratory diagnostic techniques for patients with early Lyme disease associated with erythema migrans: a comparison of different techniques. Clin Infect Dis 33, 2023-7.

Nowalk, A.J., C. Nolder, D.R. Clifton, J.A. Carroll, 2006. Comparative proteome analysis of subcellular fractions from Borrelia burgdorferi by NEPHGE and IPG. Proteomics 6, 2121-34.

Ohlenbusch, A., F.R. Matuschka, D. Richter, H.J. Christen, R. Thomssen, A. Spielman, H. Eiffert, 1996. Etiology of the acrodermatitis chronica atrophicans lesion in Lyme disease. J Infect Dis 174, 421-3.

Ojaimi, C., C. Brooks, S. Casjens, P. Rosa, A. Elias, A. Barbour, A. Jasinskas, J. Benach, L. Katona, J. Radolf, M. Caimano, J. Skare, K. Swingle, D. Akins, I. Schwartz, 2003. Profiling of temperature-induced changes in Borrelia burgdorferi gene expression by using whole genome arrays. Infect Immun 71, 1689-705.

Ojaimi, C., C. Brooks, D. Akins, S. Casjens, P. Rosa, A. Elias, A. Barbour, A. Jasinskas, J. Benach, L. Katonah, J. Radolf, M. Caimano, J. Skare, K. Swingle, S. Sims, I. Schwartz, 2002. Borrelia burgdorferi gene expression profiling with membrane-based arrays. Methods Enzymol 358, 165-77.

Pal, U., E. Fikrig, 2003. Adaptation of Borrelia burgdorferi in the vector and vertebrate host. Microbes Infect 5, 659-66.

Pal, U., X. Yang, M. Chen, L.K. Bockenstedt, J.F. Anderson, R.A. Flavell, M.V. Norgard, E. Fikrig, 2004a. OspC facilitates Borrelia burgdorferi invasion of Ixodes scapularis salivary glands. J Clin Invest 113, 220-30.

Pal, U., X. Li, T. Wang, R.R. Montgomery, N. Ramamoorthi, A.M. Desilva, F. Bao, X. Yang, M. Pypaert, D. Pradhan, F.S. Kantor, S. Telford, J.F. Anderson, E. Fikrig, 2004b. TROSPA, an Ixodes scapularis receptor for Borrelia burgdorferi. Cell 119, 457-68.

Palmer, N., C. Fraser, S. Casjens, 2000. Distribution of twelve linear extrachromosomal DNAs in natural isolates of Lyme disease spirochetes. J Bacteriol 182, 2476-80.

Parizi, L.F., N.W. Githaka, C. Logullo, S. Konnai, A. Masuda, K. Ohashi, I. da Silva Vaz, Jr., 2012. The quest for a universal vaccine against ticks: cross-immunity insights. Vet J 194, 158-65.

Paster, B.J., F.E. Dewhirst, 2000. Phylogenetic foundation of spirochetes. J Mol Microbiol Biotechnol 2, 341-4.

Patton, T.G., G. Dietrich, M.C. Dolan, J. Piesman, J.A. Carroll, R.D. Gilmore, Jr., 2011. Functional analysis of the Borrelia burgdorferi bba64 gene product in murine infection via tick infestation. PLoS One 6, e19536.

Patton, T.G., K.S. Brandt, C. Nolder, D.R. Clifton, J.A. Carroll, R.D. Gilmore, 2013a. Borrelia burgdorferi bba66 gene inactivation results in attenuated mouse infection by tick transmission. Infect Immun.

Patton, T.G., K.S. Brandt, C. Nolder, D.R. Clifton, J.A. Carroll, R.D. Gilmore, 2013b. Borrelia burgdorferi bba66 gene inactivation results in attenuated mouse infection by tick transmission. Infect Immun 81, 2488-98.

Perry, C.M., R.N. Brogden, 1996. Cefuroxime axetil. A review of its antibacterial activity, pharmacokinetic properties and therapeutic efficacy. Drugs 52, 125-58.

Picken, R.N., F. Strle, E. Ruzic-Sabljic, V. Maraspin, S. Lotric-Furlan, J. Cimperman, Y. Cheng, M.M. Picken, 1997. Molecular subtyping of Borrelia burgdorferi sensu lato isolates from five patients with solitary lymphocytoma. J Invest Dermatol 108, 92-7.

Piesman, J., 1993. Standard system for infecting ticks (Acari: Ixodidae) with the Lyme disease spirochete, Borrelia burgdorferi. J Med Entomol 30, 199-203.

Piesman, J., A. Hojgaard, 2012. Protective value of prophylactic antibiotic treatment of tick bite for Lyme disease prevention: an animal model. Ticks Tick Borne Dis 3, 193-6.

Piesman, J., J.R. Oliver, R.J. Sinsky, 1990. Growth kinetics of the Lyme disease spirochete (Borrelia burgdorferi) in vector ticks (Ixodes dammini). Am J Trop Med Hyg 42, 352-7.
Piesman, J., T.N. Mather, R.J. Sinsky, A. Spielman, 1987. Duration of tick attachment and Borrelia burgdorferi transmission. J Clin Microbiol 25, 557-8.
Poland, G.A., 2011. Vaccines against Lyme disease: What happened and what lessons can we learn? Clin Infect Dis 52 Suppl 3, s253-8.
Poljak, A., P. Comstedt, M. Hanner, W. Schuler, A. Meinke, B. Wizel, U. Lundberg, 2012. Identification and characterization of Borrelia antigens as potential vaccine candidates against Lyme borreliosis. Vaccine 30, 4398-406.
Pollack, R.J., S.R. Telford, 3rd, A. Spielman, 1993. Standardization of medium for culturing Lyme disease spirochetes. J Clin Microbiol 31, 1251-5.
Porwancher, R.B., C.G. Hagerty, J. Fan, L. Landsberg, B.J. Johnson, M. Kopnitsky, A.C. Steere, K. Kulas, S.J. Wong, 2011. Multiplex immunoassay for Lyme disease using VlsE1-IgG and pepC10-IgM antibodies: improving test performance through bioinformatics. Clin Vaccine Immunol 18, 851-9.
Postic, D., M. Garnier, G. Baranton, 2007. Multilocus sequence analysis of atypical Borrelia burgdorferi sensu lato isolates--description of Borrelia californiensis sp. nov., and genomospecies 1 and 2. Int J Med Microbiol 297, 263-71.
Postic, D., N.M. Ras, R.S. Lane, M. Hendson, G. Baranton, 1998. Expanded diversity among Californian borrelia isolates and description of Borrelia bissettii sp. nov. (formerly Borrelia group DN127). J Clin Microbiol 36, 3497-504.
Preac-Mursic, V., B. Wilske, G. Schierz, 1986. European Borrelia burgdorferi isolated from humans and ticks culture conditions and antibiotic susceptibility. Zentralbl Bakteriol Mikrobiol Hyg A 263, 112-8.
Pretorius, A.M., M. Jensenius, F. Clarke, S.H. Ringertz, 2003. Repellent efficacy of DEET and KBR 3023 against Amblyomma hebraeum (Acari: Ixodidae). J Med Entomol 40, 245-8.
Probst, C., A. Ott, T. Scheper, W. Meyer, W. Stocker, L. Komorowski, 2012. N-terminal disulfide-bridging of Borrelia outer surface protein C increases its diagnostic and vaccine potentials. Ticks Tick Borne Dis 3, 1-7.
Puius, Y.A., R.A. Kalish, 2008. Lyme arthritis: pathogenesis, clinical presentation, and management. Infect Dis Clin North Am 22, 289-300, vi-vii.
Radolf, J.D., 1994. Role of outer membrane architecture in immune evasion by Treponema pallidum and Borrelia burgdorferi. Trends Microbiol 2, 307-11.
Radolf, J.D., M.J. Caimano, 2008. The long strange trip of Borrelia burgdorferi outer-surface protein C. Mol Microbiol 69, 1-4.
Raghavendra, K., T.K. Barik, B.P. Reddy, P. Sharma, A.P. Dash, 2011. Malaria vector control: from past to future. Parasitol Res 108, 757-79.
Ramamoorthi, N., S. Narasimhan, U. Pal, F. Bao, X.F. Yang, D. Fish, J. Anguita, M.V. Norgard, F.S. Kantor, J.F. Anderson, R.A. Koski, E. Fikrig, 2005. The Lyme disease agent exploits a tick protein to infect the mammalian host. Nature 436, 573-7.
Ramamoorthy, R., D. Scholl-Meeker, 2001. Borrelia burgdorferi proteins whose expression is similarly affected by culture temperature and pH. Infect Immun 69, 2739-42.
Revel, A.T., A.M. Talaat, M.V. Norgard, 2002. DNA microarray analysis of differential gene expression in Borrelia burgdorferi, the Lyme disease spirochete. Proc Natl Acad Sci U S A 99, 1562-7.
Ribeiro, J.M., F. Alarcon-Chaidez, I.M. Francischetti, B.J. Mans, T.N. Mather, J.G. Valenzuela, S.K. Wikel, 2006. An annotated catalog of salivary gland transcripts from Ixodes scapularis ticks. Insect Biochem Mol Biol 36, 111-29.
Richter, D., D.B. Schlee, R. Allgower, F.R. Matuschka, 2004. Relationships of a novel Lyme disease spirochete, Borrelia spielmani sp. nov., with its hosts in Central Europe. Appl Environ Microbiol 70, 6414-9.

Richter, D., D. Postic, N. Sertour, I. Livey, F.R. Matuschka, G. Baranton, 2006. Delineation of Borrelia burgdorferi sensu lato species by multilocus sequence analysis and confirmation of the delineation of Borrelia spielmanii sp. nov. Int J Syst Evol Microbiol 56, 873-81.

Rogers, E.A., R.T. Marconi, 2007. Delineation of species-specific binding properties of the CspZ protein (BBH06) of Lyme disease spirochetes: evidence for new contributions to the pathogenesis of Borrelia spp. Infect Immun 75, 5272-81.

Rollend, L., D. Fish, J.E. Childs, 2013. Transovarial transmission of Borrelia spirochetes by Ixodes scapularis: a summary of the literature and recent observations. Ticks Tick Borne Dis 4, 46-51.

Rosa, P.A., K. Tilly, P.E. Stewart, 2005. The burgeoning molecular genetics of the Lyme disease spirochaete. Nat Rev Microbiol 3, 129-43.

Rosario-Cruz, R., C. Almazan, R.J. Miller, D.I. Dominguez-Garcia, R. Hernandez-Ortiz, J. de la Fuente, 2009. Genetic basis and impact of tick acaricide resistance. Front Biosci (Landmark Ed) 14, 2657-65.

Rose, C.D., P.T. Fawcett, K.M. Gibney, 2001. Arthritis following recombinant outer surface protein A vaccination for Lyme disease. J Rheumatol 28, 2555-7.

Roupakias, S., P. Mitsakou, A.A. Nimer, 2011. Tick removal. J Prev Med Hyg 52, 40-4.

Rudenko, N., M. Golovchenko, L. Grubhoffer, J.H. Oliver, Jr., 2009a. Borrelia carolinensis sp. nov., a new (14th) member of the Borrelia burgdorferi Sensu Lato complex from the southeastern region of the United States. J Clin Microbiol 47, 134-41.

Rudenko, N., M. Golovchenko, L. Grubhoffer, J.H. Oliver, Jr., 2011. Updates on Borrelia burgdorferi sensu lato complex with respect to public health. Ticks Tick Borne Dis 2, 123-8.

Rudenko, N., M. Golovchenko, T. Lin, L. Gao, L. Grubhoffer, J.H. Oliver, Jr., 2009b. Delineation of a new species of the Borrelia burgdorferi Sensu Lato Complex, Borrelia americana sp. nov. J Clin Microbiol 47, 3875-80.

Rudenko, N., M. Golovchenko, D. Ruzek, N. Piskunova, N. Mallatova, L. Grubhoffer, 2009c. Molecular detection of Borrelia bissettii DNA in serum samples from patients in the Czech Republic with suspected borreliosis. FEMS Microbiol Lett 292, 274-81.

Rudenko, N., M. Golovchenko, A. Mokracek, N. Piskunova, D. Ruzek, N. Mallatova, L. Grubhoffer, 2008. Detection of Borrelia bissettii in cardiac valve tissue of a patient with endocarditis and aortic valve stenosis in the Czech Republic. J Clin Microbiol 46, 3540-3.

Sal, M.S., C. Li, M.A. Motalab, S. Shibata, S. Aizawa, N.W. Charon, 2008. Borrelia burgdorferi uniquely regulates its motility genes and has an intricate flagellar hook-basal body structure. J Bacteriol 190, 1912-21.

Sartakova, M.L., E.Y. Dobrikova, M.A. Motaleb, H.P. Godfrey, N.W. Charon, F.C. Cabello, 2001. Complementation of a nonmotile flaB mutant of Borrelia burgdorferi by chromosomal integration of a plasmid containing a wild-type flaB allele. J Bacteriol 183, 6558-64.

Schmit, V.L., T.G. Patton, R.D. Gilmore, Jr., 2011. Analysis of Borrelia burgdorferi Surface Proteins as Determinants in Establishing Host Cell Interactions. Front Microbiol 2, 141.

Schneider, M.C., B.E. Prosser, J.J. Caesar, E. Kugelberg, S. Li, Q. Zhang, S. Quoraishi, J.E. Lovett, J.E. Deane, R.B. Sim, P. Roversi, S. Johnson, C.M. Tang, S.M. Lea, 2009. Neisseria meningitidis recruits factor H using protein mimicry of host carbohydrates. Nature 458, 890-3.

Schulze, T.L., R.A. Jordan, R.W. Hung, 1995. Suppression of subadult Ixodes scapularis (Acari: Ixodidae) following removal of leaf litter. J Med Entomol 32, 730-3.

Schutzer, S.E., C.M. Fraser-Liggett, W.G. Qiu, P. Kraiczy, E.F. Mongodin, J.J. Dunn, B.J. Luft, S.R. Casjens, 2012. Whole-genome sequences of Borrelia bissettii, Borrelia valaisiana, and Borrelia spielmanii. J Bacteriol 194, 545-6.

Schwan, T.G., J. Piesman, W.T. Golde, M.C. Dolan, P.A. Rosa, 1995. Induction of an outer surface protein on Borrelia burgdorferi during tick feeding. Proc Natl Acad Sci U S A 92, 2909-13.

Shaw, N., 1970. Bacterial glycolipids. Bacteriol Rev 34, 365-77.

Sheldrick, G.M., 2008. A short history of SHELX. Acta Crystallogr A 64, 112-22.

Siegel, C., T. Hallstrom, C. Skerka, H. Eberhardt, B. Uzonyi, T. Beckhaus, M. Karas, R. Wallich, B. Stevenson, P.F. Zipfel, P. Kraiczy, 2010. Complement factor H-related proteins CFHR2 and CFHR5 represent novel ligands for the infection-associated CRASP proteins of Borrelia burgdorferi. PLoS One 5, e13519.

Sigal, L.H., 1995. Early disseminated Lyme disease: cardiac manifestations. Am J Med 98, 25S-28S; discussion 28S-29S.

Sigal, L.H., 1998. Musculoskeletal manifestations of Lyme arthritis. Rheum Dis Clin North Am 24, 323-51.

Smith, R.P., R.T. Schoen, D.W. Rahn, V.K. Sikand, J. Nowakowski, D.L. Parenti, M.S. Holman, D.H. Persing, A.C. Steere, 2002. Clinical characteristics and treatment outcome of early Lyme disease in patients with microbiologically confirmed erythema migrans. Ann Intern Med 136, 421-8.

Stafford, K.C., 3rd, J.S. Ward, L.A. Magnarelli, 1998. Impact of controlled burns on the abundance of Ixodes scapularis (Acari: Ixodidae). J Med Entomol 35, 510-3.

Steere, A.C., 2001. Lyme disease. N Engl J Med 345, 115-25.

Steere, A.C., 2006. Lyme borreliosis in 2005, 30 years after initial observations in Lyme Connecticut. Wien Klin Wochenschr 118, 625-33.

Steere, A.C., S.E. Malawista, 1979. Cases of Lyme disease in the United States: locations correlated with distribution of Ixodes dammini. Ann Intern Med 91, 730-3.

Steere, A.C., V.K. Sikand, 2003. The presenting manifestations of Lyme disease and the outcomes of treatment. N Engl J Med 348, 2472-4.

Steere, A.C., T.F. Broderick, S.E. Malawista, 1978. Erythema chronicum migrans and Lyme arthritis: epidemiologic evidence for a tick vector. Am J Epidemiol 108, 312-21.

Steere, A.C., J. Coburn, L. Glickstein, 2004. The emergence of Lyme disease. J Clin Invest 113, 1093-101.

Steere, A.C., S.E. Malawista, J.H. Newman, P.N. Spieler, N.H. Bartenhagen, 1980. Antibiotic therapy in Lyme disease. Ann Intern Med 93, 1-8.

Steere, A.C., S.E. Malawista, J.A. Hardin, S. Ruddy, W. Askenase, W.A. Andiman, 1977a. Erythema chronicum migrans and Lyme arthritis. The enlarging clinical spectrum. Ann Intern Med 86, 685-98.

Steere, A.C., S.E. Malawista, D.R. Snydman, R.E. Shope, W.A. Andiman, M.R. Ross, F.M. Steele, 1977b. Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities. Arthritis Rheum 20, 7-17.

Strle, F., G. Stanek, 2009. Clinical manifestations and diagnosis of lyme borreliosis. Curr Probl Dermatol 37, 51-110.

Strle, F., E. Ruzic-Sabljic, J. Cimperman, S. Lotric-Furlan, V. Maraspin, 2006. Comparison of findings for patients with Borrelia garinii and Borrelia afzelii isolated from cerebrospinal fluid. Clin Infect Dis 43, 704-10.

Strle, F., D. Pleterski-Rigler, G. Stanek, A. Pejovnik-Pustinek, E. Ruzic, J. Cimperman, 1992. Solitary borrelial lymphocytoma: report of 36 cases. Infection 20, 201-6.

Takayama, K., R.J. Rothenberg, A.G. Barbour, 1987. Absence of lipopolysaccharide in the Lyme disease spirochete, Borrelia burgdorferi. Infect Immun 55, 2311-3.

Tijsse-Klasen, E., N. Pandak, P. Hengeveld, K. Takumi, M.P. Koopmans, H. Sprong, 2013. Ability to cause erythema migrans differs between Borrelia burgdorferi sensu lato isolates. Parasit Vectors 6, 23.

Tilly, K., A. Bestor, P.A. Rosa, 2013. Lipoprotein succession in Borrelia burgdorferi: similar but distinct roles for OspC and VlsE at different stages of mammalian infection. Mol Microbiol 89, 216-27.

Tokarz, R., J.M. Anderton, L.I. Katona, J.L. Benach, 2004. Combined effects of blood and temperature shift on Borrelia burgdorferi gene expression as determined by whole genome DNA array. Infect Immun 72, 5419-32.

Tugwell, P., D.T. Dennis, A. Weinstein, G. Wells, B. Shea, G. Nichol, R. Hayward, R. Lightfoot, P. Baker, A.C. Steere, 1997. Laboratory evaluation in the diagnosis of Lyme disease. Ann Intern Med 127, 1109-23.

Tyson, K., C. Elkins, H. Patterson, E. Fikrig, A. de Silva, 2007. Biochemical and functional characterization of Salp20, an Ixodes scapularis tick salivary protein that inhibits the complement pathway. Insect Mol Biol 16, 469-79.

van Dam, A.P., H. Kuiper, K. Vos, A. Widjojokusumo, B.M. de Jongh, L. Spanjaard, A.C. Ramselaar, M.D. Kramer, J. Dankert, 1993. Different genospecies of Borrelia burgdorferi are associated with distinct clinical manifestations of Lyme borreliosis. Clin Infect Dis 17, 708-17.

Vogerl, M., D. Zubrikova, K. Pfister, 2012. Prevalence of Borrelia burgdorferi s. l. in Ixodes ricinus ticks from four localities in Bavaria, Germany. Berl Munch Tierarztl Wochenschr 125, 401-6.

Walker, E.M., L.A. Borenstein, D.R. Blanco, J.N. Miller, M.A. Lovett, 1991. Analysis of outer membrane ultrastructure of pathogenic Treponema and Borrelia species by freeze-fracture electron microscopy. J Bacteriol 173, 5585-8.

Wallich, R., J. Pattathu, V. Kitiratschky, C. Brenner, P.F. Zipfel, V. Brade, M.M. Simon, P. Kraiczy, 2005. Identification and functional characterization of complement regulator-acquiring surface protein 1 of the Lyme disease spirochetes Borrelia afzelii and Borrelia garinii. Infect Immun 73, 2351-9.

Wallis, R.C., S.E. Brown, K.O. Kloter, A.J. Main, Jr., 1978. Erythema chronicum migrans and lyme arthritis: field study of ticks. Am J Epidemiol 108, 322-7.

Wang, G., D. Liveris, B. Brei, H. Wu, R.C. Falco, D. Fish, I. Schwartz, 2003. Real-time PCR for simultaneous detection and quantification of Borrelia burgdorferi in field-collected Ixodes scapularis ticks from the Northeastern United States. Appl Environ Microbiol 69, 4561-5.

Wang, G., A.P. van Dam, A. Le Fleche, D. Postic, O. Peter, G. Baranton, R. de Boer, L. Spanjaard, J. Dankert, 1997. Genetic and phenotypic analysis of Borrelia valaisiana sp. nov. (Borrelia genomic groups VS116 and M19). Int J Syst Bacteriol 47, 926-32.

Wang, X., 2012. Solution structure of decorin-binding protein A from Borrelia burgdorferi. Biochemistry 51, 8353-62.

Wheeler, C.M., J.C. Garcia Monco, J.L. Benach, M.G. Golightly, G.S. Habicht, A.C. Steere, 1993. Nonprotein antigens of Borrelia burgdorferi. J Infect Dis 167, 665-74.

Wilske, B., 2005. Epidemiology and diagnosis of Lyme borreliosis. Ann Med 37, 568-79.

Wilske, B., V. Fingerle, U. Schulte-Spechtel, 2007. Microbiological and serological diagnosis of Lyme borreliosis. FEMS Immunol Med Microbiol 49, 13-21.

Winn, M.D., C.C. Ballard, K.D. Cowtan, E.J. Dodson, P. Emsley, P.R. Evans, R.M. Keegan, E.B. Krissinel, A.G. Leslie, A. McCoy, S.J. McNicholas, G.N. Murshudov, N.S. Pannu, E.A. Potterton, H.R. Powell, R.J. Read, A. Vagin, K.S. Wilson, 2011. Overview of the CCP4 suite and current developments. Acta Crystallogr D Biol Crystallogr 67, 235-42.

Wormser, G.P., R.J. Dattwyler, E.D. Shapiro, J.J. Halperin, A.C. Steere, M.S. Klempner, P.J. Krause, J.S. Bakken, F. Strle, G. Stanek, L. Bockenstedt, D. Fish, J.S. Dumler, R.B. Nadelman, 2006. The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis 43, 1089-134.

Wywial, E., J. Haven, S.R. Casjens, Y.A. Hernandez, S. Singh, E.F. Mongodin, C.M. Fraser-Liggett, B.J. Luft, S.E. Schutzer, W.G. Qiu, 2009. Fast, adaptive evolution at a bacterial host-resistance locus: the PFam54 gene array in Borrelia burgdorferi. Gene 445, 26-37.

Yanagihara, Y., K. Kamisango, S. Yasuda, S. Kobayashi, I. Mifuchi, I. Azuma, Y. Yamamura, R.C. Johnson, 1984. Chemical compositions of cell walls and polysaccharide fractions of spirochetes. Microbiol Immunol 28, 535-44.

Yang, X.F., U. Pal, S.M. Alani, E. Fikrig, M.V. Norgard, 2004. Essential role for OspA/B in the life cycle of the Lyme disease spirochete. J Exp Med 199, 641-8.

Zhang, J.R., J.M. Hardham, A.G. Barbour, S.J. Norris, 1997. Antigenic variation in Lyme disease borreliae by promiscuous recombination of VMP-like sequence cassettes. Cell 89, 275-85.

Zipfel, P.F., C. Skerka, J. Hellwage, S.T. Jokiranta, S. Meri, V. Brade, P. Kraiczy, M. Noris, G. Remuzzi, 2002. Factor H family proteins: on complement, microbes and human diseases. Biochem Soc Trans 30, 971-8.