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SUMMARY

Lyme borreliosis (LB) is the most prevalent tick-borne zoonosis and an important emerging infectious disease in Europe, North America and Far Eastern countries. The causative agents of LB are pathogenic *Borrelia* species. *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s. are responsible for the vast majority of cases and can cause different disease manifestations. Due to changes in ecological conditions that favor tick propagation the number of infections increases steadily. Incidence rates show great geographical variations and range from 0.3 to 130 cases per 100,000 inhabitants per year. The first and only vaccine against LB, which was based on the recombinant OspA protein and provided protection against *B. burgdorferi* s.s. infections, was withdrawn from the market in 2002.

Our aim was to identify novel highly conserved vaccine candidates that could protect against the three major pathogenic *Borrelia* species causing human disease. In search for such antigens human sera obtained from patients with borreliosis and containing high levels of anti-borrelial antibodies were used in conjunction with bacterial surface display libraries that express *Borrelia* peptides on the surface of *E. coli*. Approximately 150 candidate antigens were selected using this antigen identification approach, among which were also the antigens previously reported as antigenic and protective, such as OspC and DbpA. For further selection of the most promising candidates for vaccine development, all antigens were subjected to an *in vitro* validation procedure consisting of gene conservation analysis with a collection of pathogenic *B. burgdorferi* sensu lato (s.l.) strains, surface expression analysis, and immunogenicity studies to determine the reactivity with individual human sera. Subsequently, 25 antigens selected with this validation procedure were expressed in recombinant form and tested for protection in a murine challenge model. The animal studies resulted in the selection of two protective vaccine candidates that are considered for further studies.

In parallel with this antigen validation approach, proteomics analyses were applied in order to determine the surface localization of *Borrelia* proteins. Membrane fractions of three *Borrelia* genospecies, *B. burgdorferi* (strain B31), *B. afzelii* (strain K78) and *B. garinii*

(strain PBi) were prepared and analyzed by nano-HPLC MS/MS to identify the composition of the membranome. In addition, we also performed partial trypsin digest and biotin labeling of intact bacteria followed by protein extraction and peptide analysis by mass spectroscopy. This combined approach allowed a comprehensive identification of conserved surface proteins of the three main human pathogenic *Borrelia* genospecies.

ZUSAMMENFASSUNG

Die Lyme-Borreliose (LB) ist eine durch Zecken übertragene Zoonose, die in Europa, Nord Amerika und dem Fernen Osten weit verbreitet ist. Die Krankheit wird durch pathogene *Borrelia* sp. ausgelöst, wobei für die überwiegende Mehrheit der Fälle *B. afzelii*, *B. garinii* und *B. burgdorferi* s.s. verantwortlich sind. Durch Umweltveränderungen, welche die Verbreitung von Zecken fördern, steigt die Anzahl der Infektionen stetig. Die Inzidenzrate beläuft sich derzeit, abhängig von der geographischen Region, auf 0.3 bis 130 Fälle pro 100.000 Einwohner pro Jahr. Der einzige jemals zugelassene Impfstoff enthielt rekombinantes OspA und schützte vor *B. burgdorferi* s.s. Infektionen. Dieser Impfstoff wurde allerdings 2002 vom Markt genommen.

Ziel der Arbeit war die Identifizierung von hochkonservierten Impfstoffkandidaten, welche vor Infektionen mit *B. afzelii*, *B. garinii* und *B. burgdorferi* s.s. schützen sollen. Dazu wurden in *E. coli* Genombibliotheken hergestellt, die an der Oberfläche *Borrelia* Peptide exprimieren, und mit hochtitrigen Seren von Borreliose Patienten gescreent wurden. Ungefähr 150 Impfstoffkandidaten wurden so identifiziert, wobei darunter auch die aus der Literatur bekannten Antigene OspC und DbpA zu finden waren. Die weitere Selektion und *in vitro* Validierung der identifizierten Antigene umfasste die Untersuchung der Genkonservierung in einer Sammlung pathogener *B. burgdorferi* sensu lato (s.l.) Stämme, die Untersuchung der Expression der Antigene an der Oberfläche mit Hilfe von Durchflusszytometrie und Immunogenizitätsstudien, wobei dazu einzelne Humansenen verwendet wurden. 25 vielversprechende Antigene wurden anschließend in rekombinanter Form hergestellt und im Mausmodell getestet, wobei zwei dieser Antigene eine Schutzwirkung zeigten und weiter charakterisiert werden.

Parallel zur beschriebenen Antigenidentifizierung (AIP) wurde die Oberflächenexpression der *Borrelia* Proteine mit Hilfe von Proteomics untersucht. Zu diesem Zweck wurden Membranfraktionen der drei *Borrelia* Genospezies *B. burgdorferi* (Stamm B31), *B. afzelii* (Stamm K78) und *B. garinii* (Stamm PBi) hergestellt und deren Zusammensetzung mit Hilfe von nano-HPLC MS/MS analysiert. Darüber hinaus wurden

Proteinextrakte von intakten Bakterien, die mit Trypsin behandelt (partieller Oberflächenverdau) und mit Hilfe von Biotin gefärbt wurden, hergestellt und anschließend mit Massenspektroskopie untersucht. Mit Hilfe beider Methoden (AIP® und Proteomics) konnten somit konservierte Oberflächenproteine der drei pathogenen *Borrelia* Genospezies identifiziert werden.

1 INTRODUCTION

1.1 History of Lyme borreliosis

Lyme borreliosis was described as a new clinical entity in the mid 70's. Allen C. Steere investigated a group of young people with rashes and swollen joints in Old Lyme, Connecticut with the initial diagnosis of juvenile rheumatoid arthritis. Some of the patients presented with an expanding red skin lesion, and cases showed dramatic increase in spring and early fall, therefore it was suggested that the disease had an infectious cause and a vector borne pathogen was involved (Steere et al., 1977). The disease has, however, been known in Europe under a variety of names since 1880's. In the year 1883, Buchwald described a chronic skin change, which received the name *acrodermatitis chronica atrophicans* (ACA). In the year 1910 the Swedish physician Afzelius discovered a circular moving red rash after a tick bite, that he named *erythema migrans* (EM). Neurological symptoms in connection with tick bites were described already in 1922 by the French researchers Garin and Bujadoux. In 1951 the Swedish clinician Hollström successfully treated EM patients with penicillin providing proof for infectious cause. The causative agent of Lyme borreliosis was finally discovered in 1982 by W. Burgdorfer and colleagues, who isolated a previously unidentified bacterium from the hard tick *Ixodes scapularis* that was named *Borrelia burgdorferi* (Burgdorfer et al., 1982). In 1984 *Borrelia* cells were also observed in skin biopsies (Steere et al., 1984).

1.2 Causative agents of Lyme borreliosis

Lyme borreliosis, or Lyme disease, is the most commonly reported tick-borne disease in Europe, North America and Asia. The disease is caused by the arthropod-borne spirochete *Borrelia burgdorferi* sensu lato (s.l.) that can be divided into at least 15 genospecies. These *Borrelia* genospecies occur in different geographic regions, and live in nature in enzootic cycles involving hard body ticks and a wide range of animal hosts (Table 1.1). Among these

genospecies at least three are pathogenic for humans: *B. burgdorferi* sensu stricto (s.s.), *B. afzelii* and *B. garinii*.

Three pathogenic species	Principal tick vector	Location
<i>Borrelia burgdorferi</i> s.s.	<i>Ixodes scapularis</i>	Northeastern and north central US
	<i>Ixodes pacificus</i>	Western US
	<i>Ixodes ricinus</i>	Europe
<i>Borrelia garinii</i>	<i>Ixodes ricinus</i>	Europe
	<i>Ixodes persulcatus</i>	Asia
<i>Borrelia afzelii</i>	<i>Ixodes ricinus</i>	Europe
	<i>Ixodes persulcatus</i>	Asia
Minimally pathogenic or nonpathogenic species	Principal tick vector	Location
<i>Borrelia bissettii</i>	<i>Ixodes spinipalpis</i>	Western US
	<i>Ixodes pacificus</i>	
<i>Borrelia californiensis</i>	<i>Ixodes pacificus</i>	Western US
	<i>Ixodes spinipalpis</i>	
	<i>Ixodes jellisonii</i>	
<i>Borrelia andersonii</i>	<i>Ixodes dentatus</i>	Eastern US
<i>Borrelia carolinensis</i>	<i>Ixodes scapularis</i>	Southeastern US
	<i>Ixodes minor</i>	
<i>Borrelia americana</i>	<i>Ixodes minor</i>	Southeastern US
<i>Borrelia spielmanii</i>	<i>Ixodes ricinus</i>	Europe
<i>Borrelia lusitaniae</i>	<i>Ixodes ricinus</i>	Europe
<i>Borrelia valaisiana</i>	<i>Ixodes ricinus</i>	Europe and Asia
<i>Borrelia japonica</i>	<i>Ixodes ovatus</i>	Japan
<i>Borrelia tanukii</i>	<i>Ixodes tanukii</i>	Japan
<i>Borrelia turdae</i>	<i>Ixodes turdus</i>	Japan
<i>Borrelia sinica</i>	<i>Ixodes persulcatus</i>	China

Table 1.1: The *B. burgdorferi* s.l. genospecies, their tick vectors and geographic location.

1.3 Clinical manifestations of Lyme borreliosis

Lyme borreliosis is a multi-systemic infection that can involve multiple organs and tissues, resulting in skin, cardiac, neurological and musculoskeletal disorders. The primary characteristics of Lyme borreliosis are similar worldwide, but there are regional variations in the prevalence of certain symptoms. This is related to the different species causing disease and their occurrence in different geographical regions: *B. burgdorferi* s.s. in North America, and *B. afzelii* and *B. garinii* in Europe (Baranton et al., 2001). With each of the *Borrelia* spp., the infection usually begins with erythema migrans (EM), the red skin rash caused by localised infection of the skin. In general, Lyme borreliosis occurs in stages, with remission and exacerbations with different clinical manifestations (Figure 1.1) at each stage (Steere, 1989). Early infection (stage 1) consists of localised infection of the skin, followed within days or weeks by disseminated infection (stage 2), and months to years later by persistent infection (stage 3). However, disease manifestation shows individual variations; some patients have only localised infection of the skin, while others display only later manifestations of the illness, such as arthritis. The appearance of the different clinical syndromes of Lyme borreliosis are also related to diverse pathogenesis by different *Borrelia* spp.

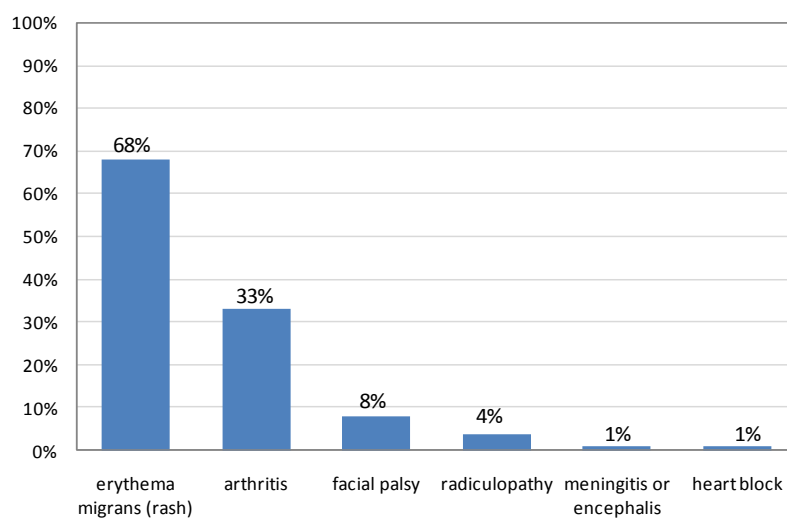


Figure 1.1: Reported clinical findings among Lyme disease patients in United States, 1992-2004. Percent of clinical findings among 119,965 patients for whom at least one symptom was reported. These total more than 100% because more than one clinical finding was reported for some patients. Source: Centers for Disease Control (www.cdc.gov)

The most common symptom of stage 1 of LB is EM, which occurs in 70-80% of infected people (Nadelman et al., 1996; Steere and Sikand, 2003). This skin lesion is often followed by flu like symptoms, such as myalgia, arthralgia, headache and fever. These non-specific symptoms occur in 50% of patients with EM.

Within several days or weeks after the onset of initial EM lesion, bacteria move into the blood stream from the site of infection and to more distant tissues and organs. Neurological, cardiovascular and arthritic symptoms that occur in this stage include meningitis, cranial neuropathy and intermittent inflammatory arthritis.

Stage 3 of infection is chronic and occurs from months to years after the tick bite. The most common symptom in North America is rheumatoid arthritis, caused by *B. burgdorferi* s.s. Persistent infection of the central nervous system with *B. garinii* causes more severe neurological symptoms during stage 3, and a persistent infection of the skin with *B. afzelii* results in acrodermatitis chronica atrophicans (ACA) (Wilske, 2003).

1.4 Enzootic cycles of *B. burgdorferi* s.l. and the tick ecology

B. burgdorferi s.l. species live in nature in enzootic cycle involving ticks and a wide range of animal hosts. As listed in Table 1.1, the main tick vector in Europe is *Ixodes ricinus*, while *Ixodes persulcatus* is primarily responsible for transmission in Asia. In the northeastern and upper midwestern United States *Ixodes scapularis* (syn. *dammini*) is the main vector, and *Ixodes pacificus* is the main vector in the western part of the USA.

During the two-year life cycle, the tick passes through four major stages of life: egg, larvae, nymph, and adult (Figure 1.2). The life cycle begins with eggs being laid (app. 2000 eggs) in early spring. In summer, the larvae hatch out and take their first blood meal, usually on small mammals. This is when they often acquire the first spirochete infection. During fall and winter larvae are dormant and molt into nymphs during the spring. In the following summer nymphs take their blood meal and can transmit infection to host. The spirochetes are typically transmitted to humans by nymphs, after the tick has been feeding for more than 24 hours. These nymphs molt into adults in fall, feed during next spring on large mammals on which they also mate, and then drop off and lay eggs for the next generation's life cycle to

begin. Humans are an incidental (dead end) host and are not involved at all in the life cycle of the spirochetes.

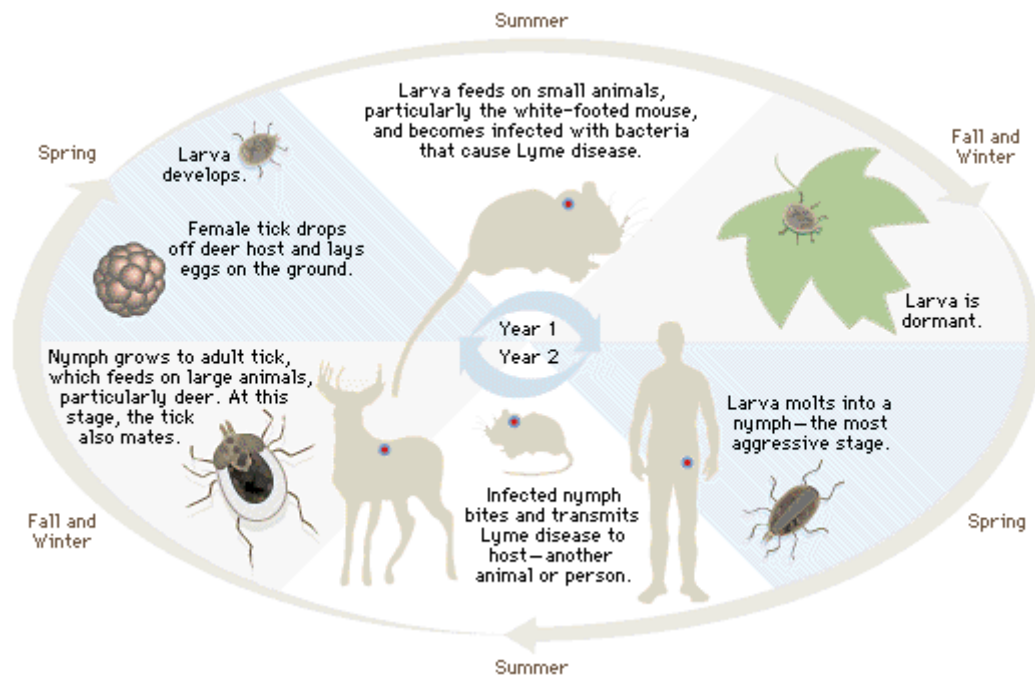


Figure 1.2: The two-year *Ixodes scapularis* life cycle. As an incidental host, humans can be infected mostly during second spring of the two-year ticks' life cycle, or rather by the infected nymphs. Nymph is smaller than adult tick and therefore harder to spot on the skin. (Source: www.brown.edu)

1.5 Diagnosis and treatment

Lyme borreliosis can be a difficult condition to diagnose. The diagnosis of LB is based on symptoms, objective physical findings (such as EM, facial palsy, or arthritis), and a history of possible exposure to infected ticks. The characteristic EM lesion is the best parameter for diagnosis of early Lyme borreliosis. Unfortunately, 20-30 percent of patients do not develop this rash, and the usual symptoms of early disease, such as fatigue, fever, or headache, are too non-specific to be diagnostic. Lyme borreliosis in a symptomatic patient can be diagnosed using a variety of serological tests in order to test presence of anti-borrelial antibodies. Anti-borrelial antibodies can typically be found between two and four weeks after contracting the disease, but sometimes the antibodies do not appear for up to eight weeks. This means that people may have LB even if antibodies are not present at the very early stage. On the other hand, a positive antibody test does not necessarily mean that

Borrelia has recently been contracted. The serum antibodies may be found several years after an infection is over. Moreover, false-positive tests in patients with non-specific findings can lead to confusions. All these facts contribute to the uncertain diagnosis for LB, which is the great medical problem. In order to avoid wrong diagnosis and to minimize false positive results, CDC recommends the use of already established two-step approach. The two-step approach is the most common diagnostic test for active disease and also for previous infection using a sensitive ELISA followed by an immunoblot analysis (Centers for Disease Controls, www.cdc.gov), which is performed only if previous ELISA showed positive results. If a person has been truly infected with *Borrelia*, the immunoblot analysis will be likely to be positive. Otherwise negative immunoblot results suggest false positive ELISA test.

Antimicrobial agents are the principal method of treatment of Lyme borreliosis infection. The antibiotic used and duration of treatment depend on the stage of disease and severity of symptoms. Early Lyme borreliosis is typically treated with oral tetracyclines, such as doxycycline, and semi-synthetic penicillins, such as amoxicillin or penicillin V. Arthritic and neurological disease entities are treated with high-dose intravenous penicillin G or ceftriaxone (EUCALB, meduni09.edis.at/eucalb/cms/index.php).

1.6 Prevention

In the year 1998, the first vaccine against Lyme borreliosis, LYMERix (Glaxo Smith-Kline), was launched on the market in the US and was shown to be safe and effective for prevention of Lyme borreliosis (Van Hoesche et al., 1996). The LYMERix vaccine contained recombinant lipidated OspA adsorbed to the adjuvant aluminium hydroxide (Steere et al., 1998). OspA is mainly expressed in the gut of the tick and human immune responses to *Borrelia* do not usually include humoral or cellular adaptive immunity directed against OspA. Thus, these OspA antibodies produced by vaccination are not used to fight infection in the body. Instead, the antibodies enter the gut of the tick when it takes a blood meal (Fikrig et al., 1992). There, they neutralize the spirochetes and block migration from the midgut to the salivary glands that allows *B. burgdorferi* s.s. to enter the vertebrate host. Thus, the vaccine prevents transmission of the spirochete from the tick to the human host. Three

doses of LYMERix were needed for optimal protection in the period of one year. After the first two doses vaccine efficacy against Lyme borreliosis was 49%, and after all three doses 76% (Steere et al., 1998; Van Hoeck et al., Clin Infect Dis, 1999). However, shortly after LYMERix was commercially available, it was withdrawn from the market in 2002. One of the reasons was the short lived protection due to fast declining OspA antibody levels requiring booster injections every year. It seems that the main reason for low vaccine sales was the perception that the vaccine induced arthritis in certain vaccines, however, this could not be proven scientifically and epidemiologically (Abbott, *Nature* 2006).

1.7 Epidemiology of Lyme borreliosis

LB is the most common tick-borne zoonosis in Europe and North America. In countries with lower incidence of the disease it is still not a notifiable disease and no exact data are available. In the US, state health departments reported 28,921 confirmed cases and 6,277 probable cases of Lyme disease to CDC in 2008 (Figure 1.3). This represents more than 100% increase in confirmed cases compared to 1994 (www.cdc.gov/ncidod/dvbid/lyme).

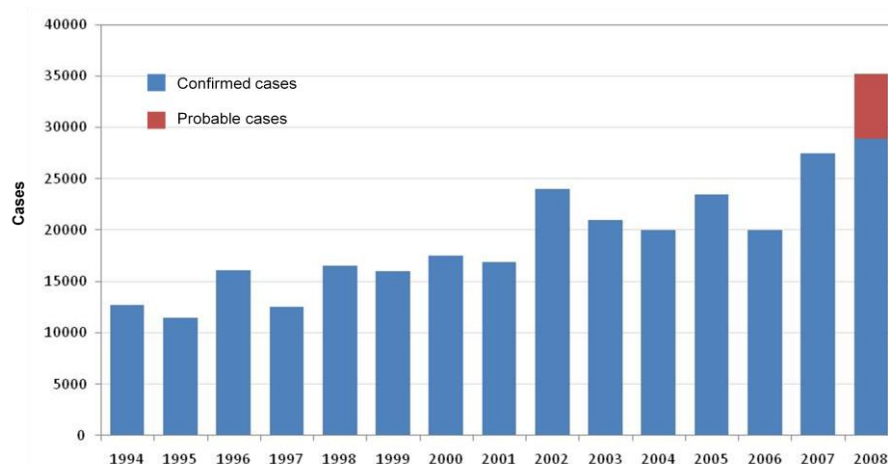


Figure 1.3: Reported cases of Lyme borreliosis by year, USA, from 1994 to 2008. Source: Center for Disease Control (www.cdc.gov).

In Europe the incidence of Lyme borreliosis differs greatly among countries, with an overall increased prevalence from west to east. Several studies show that reported incidence in several European countries increased markedly over the previous 10-20 years, for example in Germany, the Netherlands and the United Kingdom (Smith et al., 2000; Hofhuis et al., 2006; Fülöp and Poggensee, 2008; www.hpa.org.uk). In Austria, the incidence of Lyme borreliosis is 135, and in Slovenia 206 cases per 100,000 population per year (www.EUCALB.com). In some risk groups, such as farmers, forestry workers, hikers, runners or vacationers, seroprevalence and disease incidence rates have increased, also in children <15 years and adults between 39 and 59 years, without gender preference.

The high incidence of Lyme disease is linked to changes in the forest habitats as well as social factors (Barbour and Fish, 1993). An environmental change such as forest fragmentation has led to a loss of rodent predators, such as wolves and birds of prey and consequently to an increase in the mouse population that is the major reservoir for tick propagation. More recently patchy reforestation has increased the deer population and thus the number of ticks. Middle class suburban sprawl and the increasing use of woodland areas for recreation such as camping and hiking has brought humans into closer contact with the large number of infected ticks. All these factors contribute to a wider distribution of Lyme borreliosis (Ostfeld and Keesing, 2000; Brownstein et al., 2005; Ogden et al., 2009).

1.8 Biology of *B. burgdorferi* s.l.

B. burgdorferi s.l. belongs to the family Spirochaetaceae, which is subdivided into the medically important genera Treponema, Leptospira and Borrelia. *B. burgdorferi* s.l. is a spiral shaped (Figure 1.4) vigorously motile bacterium that grows under microaerophilic conditions. The Borreliae are longer (10-30 µm long, 0.2-0.5 µm wide) and more loosely coiled than the other spirochetes (Hovind-Hougen et al., 1986). The Borrelia spirochetes have the cell wall morphology of a Gram-negative bacterium consisting of a cytoplasmic membrane surrounded by peptidoglycan and loosely associated outer membrane (Figure 1.4 B). The outer membrane is fluid, lacks lipopolysaccharides (Takayama et al., 1987) and consists of phospholipids and outer membrane proteins (Barbour and Hayes, 1986). It has a very low density of integral, membrane-spanning proteins (Radolf et al., 1994). Outer

membrane proteins (Omp) of these bacteria are of great interest because of their location at the cell surface, which may facilitate *Borrelia*-host interactions. *Borrelial* Omps include outer surface (lipo)proteins (Osps), other lipoproteins, and integral membrane spanning proteins. Outer surface lipoproteins (Osps) are the most abundant proteins (Haake, 2000) and the best studied Omps in *Borrelia*. They play a major role in host inflammatory response activation and tissue inflammation. They also play an important role in adaptive responses and *Borrelia* pathogenicity, which include antigenic variation, complement evasion, and adherence mechanisms.

Borrelia also has some characteristics of Gram-positive bacterium, such as the low G+C content of the genome (28.6%), and the close association of the inner membrane with the peptidoglycan cell wall.



Figure 1.4: Structure and morphology of *Borrelia*. (Source: Rosa, Tilly, Stuart, 2005, Structure and morphology of *Borrelia*).

The periplasmic flagella are located between the two membranes, not extracellularly as for most bacteria. It appears to be a unique structure and the most complex of bacterial flagella. They are composed of the FlaA sheath proteins, and multiple FlaB core proteins (Ge et al., 1998). Regarding different species, *Borrelia* has a bundle of 7–11 helically shaped periplasmic flagella attached at each end of the cell. They form a tight ribbon as they wrap around the cell cylinder generating a backward-mowing wave (Charon et al., 2009), which enables these bacteria to swim in both low and high viscosity, almost gel-like media. Beside

their motile function, flagella have also structural function (Motaleb et al., 2000). Unlike other bacteria where the shape is regulated by the peptidoglycan layer of the cell wall, the flat-wave morphology of the *Borrelia* cell is conferred by periplasmic flagella.

The *Borrelia* genome

The genome of *Borrelia* has a very unusual structure and is perhaps the most structurally complex among bacteria. It is a segmented genome that is composed of one linear chromosome and a varying number of both linear and circular plasmids (Xu et al., 1995) ranging in size from 5 to 56 kb (Samuels et al., 1993). In 1997 the genome of *B. burgdorferi* s.s. strain B31 was completely sequenced by TIGR (Fraser et al., 1997), and the subsequent *in silico* analysis showed that the 1.5 Mb genome sequence is encoded in a single chromosome plus a highly dynamic complement of 12 linear and 9 circular plasmids (Figure 1.5) (Casjens et al., 2000). About 90% of the 0.91 Mb chromosomal genome comprises coding regions and most of the encoded genes are homologous to the genes of known function. They code for proteins involved in DNA replication, transcription and translation; DNA repair system and recombination; transport, nutrient uptake and energy metabolism; motility and chemotaxis, and the regulation of gene expression. By contrast, the plasmids have a higher content of non-coding sequences and pseudogenes, and only less than 10% of 419 plasmid carried genes have predicted functions. Genes related to pathogenicity are primarily located on this extra-chromosomal genome. Interestingly, none of the plasmid genes of *Borrelia* have similarity to known bacterial virulence genes. This favored the hypothesis that *B. burgdorferi* s.l. plasmids encode functions that are specific to the spirochaete infectious cycle, and that the *Borrelia* host-pathogen interactions are dissimilar from the other well-studied bacterial pathogens. *B. burgdorferi* s.l. plasmids vary in stability, some frequently lost after only a few generations of *in vitro* growth and others stably maintained with continuous passages (Schwan et al., 1988). The consequences of plasmid loss for growth of *B. burgdorferi* s.l. in vivo also vary. The two linear plasmids, lp25 and lp28-1 are absolutely necessary for persistent infection in mammals (Labandeira-Ray et al., 2001; Grimm et al., 2004). Lp25 contains the gene *pncA*, which encodes a nicotinamidase that is involved in the biosynthesis of NAD, which is crucial for growth within a host (Purser et al., 2000). Lp28-1 contains the vmp-like sequence (*vls*) locus, which is responsible for antigenic

variation important for immune evasion. The genes coding for biosynthetic reactions, such as the synthesis of amino acids, fatty acids, cofactors and nucleotides, are absent from the borrelial genome. Therefore *Borrelia* requires a complex medium supplemented with mammalian serum, such as BSKII medium, for *in vitro* growth (Barbour, 1986; Zückert, 2007).

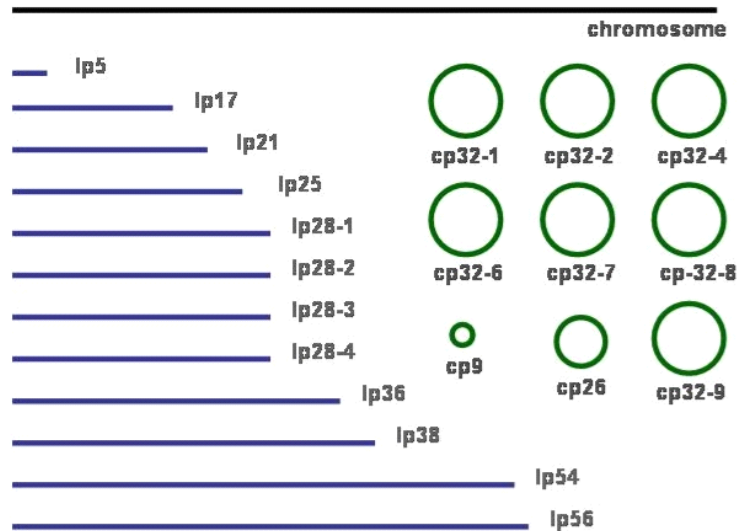


Figure 1.5: Schematic representation of the *B. burgdorferi* B31 genome complexity.

Using comparative genomics *B. garinii* PBi and *B. afzelii* PKo genomes were analyzed (Glöckner et al., 2004; Glöckner et al., 2006). Studies confirmed closer relationship between *B. garinii* and *B. afzelii*, than either of them to *B. burgdorferi* s.s. Furthermore, studies showed clearly that three genomic elements, the chromosome and the plasmids cp26 and lp54, are common to all analyzed *Borrelia* species. These three elements are relatively well conserved and colinear. Based on its ubiquitous nature, cp26 seems to provide functions required for bacterial variability (Byram et al., 2004). While the B31 genome has plasmid content of 47%, *B. garinii* PBi and *B. afzelii* PKo strains contain only 29 and 36% plasmid sequences of their genomes, respectively. *B. garinii* PBi contains three circular and eight linear plasmids, and *B. afzelii* PKo nine circular and seven linear plasmids (determined in still infectious low passage strains).

1.9 Typing of *B. burgdorferi* s.l. – phenotypic and genotypic methods

Serotyping of *B. burgdorferi* s.l. based on the heterogeneity of OspA represents the most commonly used method for this genospecies. Based on the differential reactivity of European and North American *B. burgdorferi* s.l. isolates with eight OspA-specific monoclonal antibodies, seven different OspA serotypes (serotype 1 to 7) were defined by Wilske et al. in 1993. These seven OspA serotypes correlate well with the current classification of *B. burgdorferi* s.l. OspA serotype 1 corresponds to *B. burgdorferi* s.s., OspA serotype 2 to *B. afzelii*, and OspA serotypes 3-7 to *B. garinii*. OspA serotyping provides a simple approach for the analysis of phenotypic characteristics among *Borrelia* species.

However, molecular typing based on genetic characteristics of *Borrelia* provides more precise information about diversity among *Borrelia* species. During the past several years, a number of genotyping methods have been used to assess the genetic relationships among *Borrelia* species, such as PCR and restriction fragment length polymorphism analysis (RFLP). PCR amplification with species-specific primers, in which the conserved 16S rRNA gene loci (Marconi et al., 1992; Liebisch et al., 1998) are targeted, can be used directly for species identification of the *Borrelia* isolates, or rather for differentiation of the three human pathogenic species: *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*.

Using the PCR-based RFLP approach, Livey et al. (1995) classified *ospC* locus into 35 major groups. This high *ospC* diversity was enhanced by Seinost et al. (1999) and Baranton et al. (2001). They defined 58 *ospC* groups within the three pathogenic species analyzing 149 sequences from data banks. Beside sequence variability, these 58 *ospC* groups differ also in their ability to cause dissemination. Based on this, one can distinguish between invasive and non-invasive *ospC* types (see Figure A1, Appendixess), meaning that only restricted *ospC* groups belong to invasive strains, underlining the role of OspC in *Borrelia* pathogenicity (Lagal et al., 2003; Earnhart et al., 2005).

1.10 Surface plasticity of *Borrelia*

During its complex enzootic cycle, *Borrelia* has to adapt to diverse environments and must interact with many different host and vector tissues. To do so, *Borrelia* senses its environment and coordinates the synthesis of numerous proteins. This enables *Borrelia* to rest in the tick midgut for a year waiting for the next tick bite. Upon contact with blood and sensing changes in pH and temperature, *Borrelia* alters its protein expression pattern (Schwan et al., 1995). The best characterized changes in expression are linked to the OspA and OspC proteins (Schwan and Piesman, 2000; Ohnishi et al., 2001) (Figure 1.6).

Outer surface protein A (OspA)

OspA is one of the best studied proteins of *B. burgdorferi* s.l. species. It is encoded on the *B. burgdorferi* B31 linear plasmid lp54, and its molecular mass ranges from 31 to 34 kDa among different *Borrelia* species. OspA is expressed on spirochetes in unfed nymphs and adult ticks, as well as in culture. It serves as an anchor for *Borrelia* cells in the midgut of the unfed tick (Pal et al., 2000) and allows spirochetes to avoid endocytosis by the tick gut cells during digestion of the blood meal. In the midgut of the tick, OspA binds TROSPA (a Tick Receptor for OspA), and during tick feeding expression of both OspA and TROSPA is decreased (Schwan et al., 1995; Pal et al., 2004). Upon down-regulation of OspA expression, *Borrelia* is able to move from the tick midgut to the salivary glands and then further to the bloodstream of the host. Expression of other outer surface proteins such as OspC is then up-regulated.

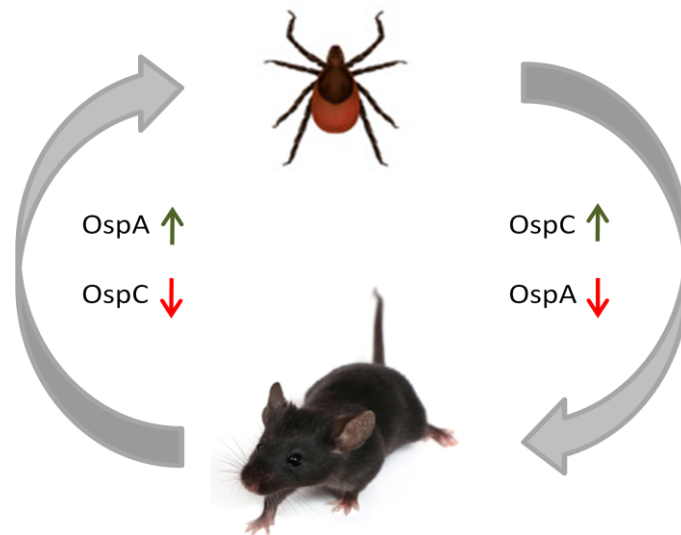


Figure 1.6: Reciprocal expression of OspA and OspC. According to the changes in temperature, pH, or presence of blood, *Borrelia* change the expression of a number of its proteins. The best characterized alteration is the reciprocal expression of the OspA and OspC proteins. High OspA expression occurs during colonization of the tick's midgut, where the temperature is below 32°C and the pH value is basic. During migration *Borrelia* enters an environment with temperatures between 32 and 37°C and with lower pH, which stimulates OspC expression.

Outer surface protein C (OspC)

OspC is one of the first and most heavily targeted borrelial antigens by vertebrate immune system. *ospC* is a highly polymorphic single-copy gene localized on the *B. burgdorferi* B31 circular plasmid cp26. The molecular mass of OspC varies from 20 to 25 kDa among different *Borrelia* species. Its expression is turned on during tick feeding (Schwan et al., 1995) and bacterial migration from the midgut to the salivary glands of the tick where OspC binds a tick protein Salp 15, which protects the spirochete from antibody-mediated killing upon entry of host tissues. Once the infection is established in a host, OspC production is not required for persistence (Tilly et al., 2006).

Another important OspC function during spirochete dissemination is their affinity to plasminogen (Lagal et al., 2006). Since *Borrelia* lacks surface protease activities (Fuchs et al., 1994), plasminogen binding can facilitate spreading of *Borrelia* through the skin and other tissue matrices and is required for efficient dissemination within the tick. *Borrelia* first encounters plasminogen during the blood meal of the tick. In the presence of host plasminogen activator, bound plasminogen can be rapidly converted into active plasmin (Coleman et al., 2000). Surface-associated plasmin on *B. burgdorferi* can directly degrade

fibronectin, a major component of the extracellular matrix, as well as laminin and vitronectin (Fuchs et al., 1994). In addition to OspC, OspA and some Erp proteins are also reported to bind plasminogen (Fuchs et al., 1994; Lagal et al., 2006; Brissette et al., 2009). Although not strictly required for infection, plasminogen is important for efficient dissemination, evidenced by the decreased spirochetemia in plasminogen-deficient mice (Coleman et al., 1997).

1.11 Adhesins as virulence factors and their role in disease pathogenicity

During dissemination and homing to specific sites, *Borrelia* interacts with cells of the connective tissues, including macrophages, dendritic cells, fibroblasts, and the associated ECM. *Borrelia* expresses cell surface proteins that interact specifically with different components of the ECM. Among the *Borrelia* proteins that bind ECM components are decorin-binding proteins DbpA and DbpB (Guo et al., 1998), GAG-binding protein Bgp (Parveen et al., 2006), fibronectin adhesin BBK32 (Probert et al., 1998), and integrin-binding receptors like P66 (Coburn et al., 2003) (Figure 1.7). These interactions with the ECM allow colonization and expulsion of the bacteria.

Decorin-binding proteins

Borrelia binds specifically to the collagen-associated proteoglycan decorin that is a common component of the different tissues (Guo et al., 1995). Decorin consist of a 36 kDa core protein, modified with as many as three N-linked oligosaccharides and usually linked to glycosaminoglycans (GAGs). In mammalian host, *Borrelia* expresses Decorin binding protein A and B (20 kDa and 19 kDa respectively), which are encoded on the linear plasmid lp54 together with the *ospA* and *ospB* locus. DbpA and DbpB are surface exposed lipoproteins and show 56% sequence similarity (Hagman et al., 1998). Soluble DbpA, added to *Borrelia* is capable of inhibiting the attachment of *Borrelia* to immobilized decorin (Guo et al., 1998), and is highly polymorphic compared to DbpB (Roberts et al., 1998). Recent studies showed that besides similar weight, the same operon, and the binding to the host decorin and glycosaminoglycans, DbpA and DbpB also show antigenic cross-reactivity (Shi et al.,

2008). When produced at an increased level, these two virulence factors are interchangeable for the protection of *Borrelia* against elimination by immune defenses. However, their contribution to dissemination and tissue colonization differ.

Glycosaminoglycan-binding proteins

In addition to recognizing the proteoglycan decorin, *B. burgdorferi* s.l. can bind to non-decorin GAG chains produced by a wide variety of cells (Isaacs, 1994). *Borrelia* expresses multiple GAG-binding proteins; a unique repertoire of adhesins produced by a particular strain is responsible for its specific GAG-binding specificity. One of the borrelial GAG-binding proteins is Bgp (*Borrelia* GAG-binding protein). Bgp is 26 kDa protein which is both, secreted into the medium and expressed on the surface of *B. burgdorferi* s.l. (Parveen and Leong, 2000; Cluss et al., 2004). Recombinant Bgp partially inhibits *Borrelia* to attach to purified GAGs and to cultured mammalian cells.

Fibronectin-binding protein

Another differentially expressed protein of *Borrelia* is the 47 kDa fibronectin binding protein (BBK32) (Probert and Johnson, 1998). Fibronectin is a complex glycoprotein, present both in plasma (soluble form) and in extracellular matrix (insoluble form), which contains distinct domains for interaction with heparin, collagen, gelatin, integrins and other plasma and tissue components. The borrelial fibronectin binding protein consists of two domains, the largely unstructured N-terminal domain being responsible for fibronectin binding (Probert et al., 2001). The Fn-binding sequence in this domain is conserved among *B. burgdorferi* s.l. strains and homolog with Fn-binding proteins of the phylogenetically distinct bacteria *Staphylococcus aureus* and *Streptococcus pyogenes*. All these proteins recognize the same segment of fibronectin by a mechanism that involves the formation of a new beta strand (Raibaud et al., 2005). The expression of BBK32 is upregulated already during tick feeding and is also produced in mammalian host. Studies showed that antibodies to this protein can partially protect against tick-borne infection with *B. burgdorferi* s.s. (Fikrig et al., 2000). *In vitro* cultivation showed that the expression of BBK32 varies with the strain and/or culture conditions.

Integrin-binding proteins

Borrelia binds also to different types of integrins; at least three members of the integrin family of cell surface receptors including the fibrinogen receptor, the vitronectin receptor, and the fibronectin receptor (Coburn et al., 1993 and 1998). These heterodimeric receptors mediate a variety of cell-cell and cell-ECM interactions and transduce signals between extracellular and intracellular environment (Hynes, 2002). The ability of spirochetes to bind different integrin receptors promote initial colonization of the blood vessel wall by two ways: by promoting binding directly to endothelial cells (adhesion to vitronectin receptor), or indirectly, via activated platelets (adhesion to fibrinogen receptor). One of the better characterized proteins that bind to integrin is *B. burgdorferi* s.l. protein P66. P66 is localized on the surface of *B. burgdorferi* s.l. (Bunikis et al., 1995) and act as a porin (Skare et al., 1997). Recombinant P66 binds specifically to both β_3 -chain integrins and inhibits attachment of intact *Borrelia* to these receptors (Coburn et al., 1999). Studies showed that antibodies against P66 are bactericidal (Exner et al., 2000) and elicit protection.

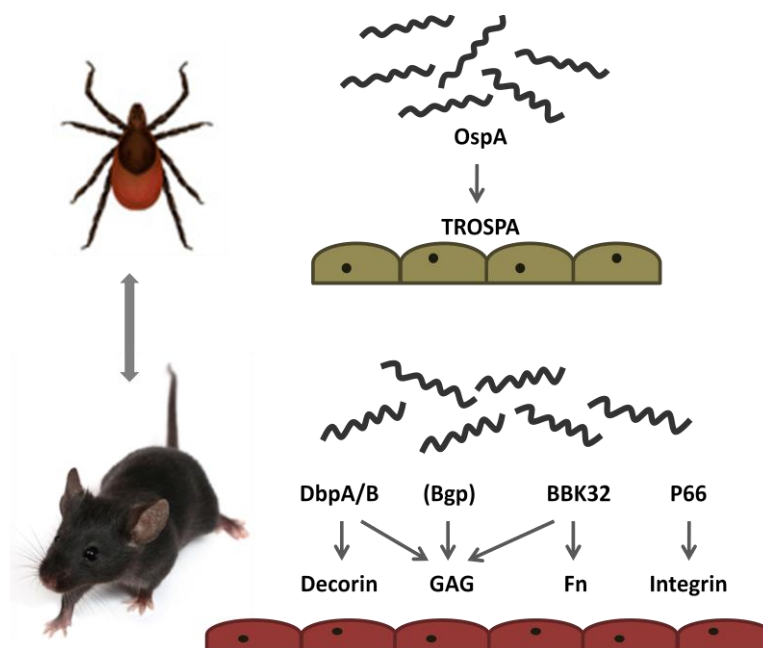


Figure 1.7: Interactions between *Borrelia* and host cells. In the tick's midgut OspA binds TROSPA which enable *Borrelia* attachment to the endothelial cells. After transmission to mammals, *Borrelia* interacts with different components of the extracellular matrix (ECM). For this purpose *Borrelia* expresses several adhesins: DbpA/B that bind decorin and GAG; Bgp which recognize GAG; fibronectin binding protein BBK32; and P66 which bind integrins. (Figure adopted from Coburn, Fischer and Leong, 2005).

Borrelia is able to migrate actively from dermis into the blood stream and from the blood to mammalian tissues, such as joints, skin or heart, where the spirochetes persist and may not be accessible to circulating antibodies. The expression of several distinct proteins that promote bacterial attachment to cell surfaces or to components of the ECM, underline the fact, that adhesion is critical to the biology of *Borrelia* and the pathogenesis of Lyme borreliosis.

1.12 Host immunity to *B. burgdorferi* s.l.

Borrelia being an extracellular pathogen is mainly attacked by the innate immune system, as well as by the humoral arm of the adaptive immunity. Immune cells first encounter *Borrelia* at the site of the tick bite. In order to control and eradicate *Borrelia*, the host mounts both innate and adaptive immune responses, resulting in macrophage- and antibody-mediated killing of the spirochete (Steere et al., 2004).

Complement mediated lysis of bacteria may be the first line of host defense. However, some strains are able to resist killing by complement (see next chapter) and persist in skin. The lipoproteins and other components of these spirochetes act as PAMPS (pathogen-associated molecular pattern) and activate macrophages through Toll-like receptor 2 (Wooten et al., 2002), which release potent pro-inflammatory cytokines, including TNF- α and IFN- γ . Macrophages engulf and kill spirochetes. Together with dendritic cells, macrophages present lipid antigens to NKT cells via CD-1 receptor (Kinjo et al., 2006). By this way, innate and adaptive immune responses against *Borrelia* are bridged.

Borrelial lipoproteins play an important role in adaptive immune response, since they induce the production of specific antibodies. As B-cell mitogens, spirochetal lipoproteins, which are major PAMPS and pro-inflammatory agonist, cross-link B-cell receptors directly leading to T-cell independent B-cell response (Ma and Weis, 1993; Whitmire and Garon, 1993). Humoral immune responses to nonlipidated spirochetal proteins are more likely to be T-cell dependent. Macrophages and dendritic cells present spirochetal peptides to CD4+ and CD8+ T-cells (Steere and Glickstein, 2004). The primary role of CD4+ T-cells is to prime T-cell dependent B-cell response, and CD8+ T-cells appear to be an important source of IFN- γ

(Dong et al., 1997), a critically important cytokine for control of infection. Spirochetes react to this barrage of immune reactants by down-regulating certain outer-surface proteins.

The combination of all these responses leads to production of specific antibodies, which bridge the innate and acquired immune systems by opsonizing borreliae by phagocytes via the Fc receptors (Talkington and Nickell, 2001). Considering that *Borrelia* can evade complement deposition, opsonization by antibodies followed by binding to Fc receptors might be the most efficient in phagocytic removal of *Borrelia*.

Many studies underlined the critical importance of specific antibodies in the host response to tick-borne *Borrelia* species that cause Lyme borreliosis. If present before the bacteria are introduced, these specific antibodies can prevent infection and dissemination into organs from the skin.

Certain antibodies against *Borrelia* have unique characteristics. They can be inducers of antigenic variation of *Borrelia*. Upon immune pressure imposed by host antibodies, *Borrelia* can change its Osp expression allowing new serotypes to appear (Xu et al., 2006). The most important antigenic variation is recombination of the *vls* gene locus which helps *Borrelia* to escape host immune system (the *vls* antigenic variation is more detailed explained in Chapter 1.13 Immune evasion).

Another unique characteristic of certain *Borrelia* specific antibodies is the bactericidal effect in the absence of complement (Bockenstedt et al., 1993). The first reported *Borrelia*-specific complement-independent mAb was specific to OspB (CB2, IgG1), and shown to exert bactericidal effects *in vitro* (Coleman et al., 1992). Binding of CB2 to *Borrelia* caused destabilization and destruction of the outer membrane by creating openings in the membrane, and resulting in osmotic lysis. (LaRocca et al., 2009). This bactericidal effect is very specific to *Borrelia*. This direct bactericidal activity was also reported for anti-OspA antibodies (Scriba et al., 1993; Aydintug et al., 1994; Ma et al., 1995). Since complement is inactivated in the tick, this mechanism of action of borrelcidal antibodies together with the blocking of transmission in the tick (de Silva et al., 1996 and 1999) is the likely mechanism of action of the LYMERix vaccine.

1.13 Immune evasion

To avoid clearance by the host's immune system, *Borrelia* developed several mechanisms to inhibit both innate and adaptive immune response.

Inactivation of complement

Borrelia develops resistance to certain forms of complement-dependent killing by expression of complement regulator-acquiring surface proteins (CRASPs) and proteins that belong to OspE/F Erp family. Presented on the bacterial surface these proteins are able to bind complement Factor H and Factor H-like protein-1 (FHL-1) and thus to prevent the formation of cytolytic complement activation products on the borrelial surface (Kraiczy et al., 2001). The CRASPs bind to the C-terminus of Factor H or FHL-1, orienting them in such a way as to allow for the continuation of their regulatory function, which is the cleavage of C3b by Factor I and prevention of complement activation (Hartmann et al., 2006). Proteins of the OspE/F Erp family also function in similar manner (Alitalo et al., 2004). *Borrelia* expresses a wide repertoire of Erp proteins that can bind Factor H molecules from a wide variety of mammalian hosts, which allows the bacterium to resist complement-mediated killing in any of the potential hosts that it might encounter (Hovis et al., 2006). Regarding complement evasion, different *Borrelia* strains can be divided into serum-resistant and serum-sensitive strains. *B. afzelii* isolates are mainly serum-resistant, whereas the majority of *B. burgdorferi* s.s. isolates display an intermediate serum-sensitive phenotype. In contrast, *B. garinii* isolates are killed effectively by complement and therefore are classified as serum-sensitive (van Burgel et al., 2010). *B. garinii* possesses only a small number of the circular plasmids which carry genes for the Erp proteins. This could explain the serum-sensitivity of the *B. garinii* strains, and the reduced number of possible hosts.

Recombination of the *vls* gene locus

The *vls* antigenic variation system plays a major role in *B. burgdorferi* s.l. immune evasion. The *vls* locus is localized on the linear plasmid lp28-1 and is comprised of an ORF (*vlsE*) encoding the 35 kDa lipoprotein VlsE, and a contiguous array of 11-15 (depending on

the strain) silent cassettes. The cassettes show strong homology to the central region of VlsE, and contain six variable regions flanked by highly conserved sequences (Zhang et al., 1998). VlsE expression begins around the time that OspC production ceases (Crother et al., 2004). While present on bacterial surface, VlsE is targeted by the adaptive immune response of the mammalian host that induces *vlsE* sequence-changes through the exchange of DNA silent cassettes by recombination. New variants of the 35 kDa lipoprotein are continuously expressed on the bacterial surface. This antigenic variation helps *Borrelia* to escape antibody-mediated defense directed against the VlsE and to establish persistent infection for which VlsE antigenic variation seems to be crucial (Bankhead et al., 2007). The function of VlsE independent of its antigenic variation properties is not known yet. However, *vlsE* antigenic switching in *Borrelia* occurs only during mammalian infections, suggesting that host factor or factors are required to activate the antigenic variation process.

Lateral gene transfer by bacteriophages

The cp32 circular plasmids encode a bacteriophage that participates in transfer of genetic information from one strain of *B. burgdorferi* s.l. to another. This genetic transfer is thought to play a role in the generation of new antigenic variants and could be an important mechanism of pathogenesis. To escape selective immunological pressure in the host, *Borrelia* can change their *ospC* sequences through lateral gene transfer. The transfer can occur between *B. burgdorferi* s.l. strains during coinfection, and in the same *Borrelia* species (Livey et al., 1995; Wang et al., 1999). This high frequency of gene exchange could enable the spirochetes to react effectively to environmental selection pressures.

1.14 ANTIGENome technology

To identify vaccine candidates naturally recognized by the human immune system, a new approach based on genomic peptide libraries in combination with human sera was developed by Intercell AG (Etz et al., 2001; Etz et al., 2002; Henics et al., 2003). Besides identification of antigenic proteins, this technology comprises also an *in vitro* validation

process in order to select vaccine candidate antigens that are then expressed in recombinant forms and tested for protection in animal models.

The technology is based on the use of human immune sera and generation of pathogen specific bacterial surface display libraries. IgGs purified from human sera that are selected from individuals with disease manifestations are used for screening of bacterial surface libraries generated from genomic DNA of the pathogen. The libraries are screened by magnetic cell sorting that allows selection of *E. coli* clones carrying antigenic epitopes that induce an immune response in humans. Further validation and selection of vaccine candidates includes (i) gene conservation analysis with a panel of clinical isolates, (ii) serological analysis of human immune response to selected epitopes within identified antigens, (iii) determination of surface expression, and finally (iv) animal studies to test for protection. Figure 1.8 summarizes the steps included in this ANTIGENome technology. This approach was successfully applied for several human pathogens, such as *Streptococcus pneumoniae* (Giefing et al., 2008), *Helicobacter pylori* (Meinke et al., 2009), and also other pathogenic *Streptococcus* and *Staphylococcus* species (Etz et al., PNAS, 2002; Meinke et al., 2005, Fritzer et al., 2010).

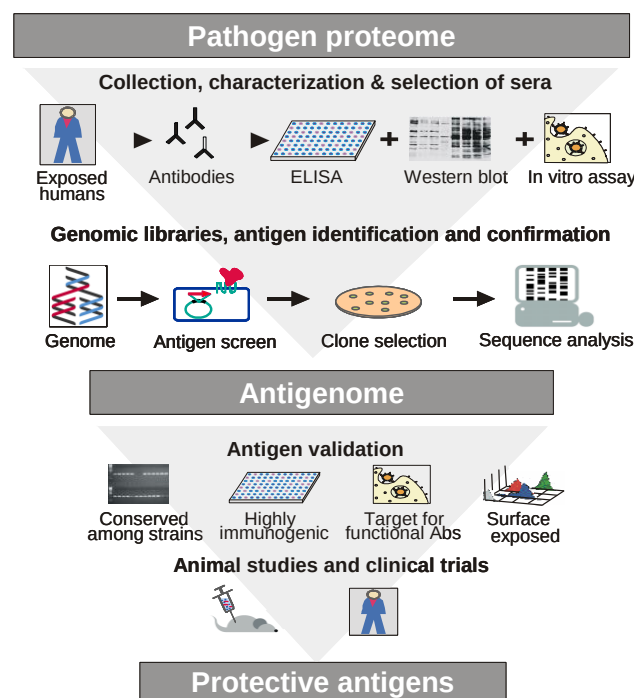


Figure 1.8: Scheme of the antigen identification and validation approach (Nagy et al., 2004).

2 STUDY OBJECTIVES

The major aim of my work was to identify highly conserved protein antigens from *Borrelia* species using Intercell's ANTIGENome technology and select vaccine candidates based on in vitro and in vivo assays.

1. Collection of human sera and *Borrelia* strains
2. Selection of sera for screening
3. Generation of genomic libraries with sufficient coverage
4. Identification of candidates by genomic library screen
5. Bioinformatic analysis of selected candidates
6. In vitro validation of candidates for prioritization
7. Expression of recombinant proteins
8. Selection of vaccine candidates in animal model

An additional aim was a comprehensive proteomic analysis and identification of surface exposed proteins of the three major pathogenic *Borrelia* species.

1. Extract preparation from three *Borrelia* strains
2. Partial surface digest
3. Biotinylation of intact bacteria
4. Fractionation of outer membrane proteins
5. Protein identification by mass spectrometry in collaboration with Andreas Kungl, ProtAffin AG, Graz.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Laboratory equipment

Blockheater QBT2	Grant Instruments Ltd, England
Centrifuges	HERAEUS Megafuge 2.0, Kendro Lab. Products, UK HERAEUS Multifuge 3S-R, Serial No. 40168704, Kendro Lab. Products, UK HERAEUS Biofuge Fresco, Serial No. 40421492, Kendro Lab. Products, UK EPPENDORF Centrifuge 5415 R, Serial No. 542601063, Eppendorf
CO2 Incubator	HERA Safe, Serial No. 99107680, Kendro Lab. Products, UK
Colonycounter	Counterstat Flash, Serial No. 045/4000, IUL Instruments
E. coli Pulser	Serial No. 942-BRO-865, Bio-Rad Laboratories
Film Processor	Optimax X-Ray Film Processor; Serial No. 117010-0102-6332, Protec Medizintechnik GmbH & Co KG, Germany
FACS	Beckman Cytomics FC 500, Beckman Coulter, USA
FACS tubes	5ml, Falcon
Heater	Thermomixer compact, Eppendorf
Blockheater	QBT2, Grant Instruments Ltd, UK
Laminar flow	HERA Safe, Serial No. 40029531, Kendro Lab. Products, UK HERA Safe, Serial No. 40083460, Kendro Lab. Products, UK
Microplate Reader	Tecan Sunrise, Austria
Microscope	Dark Field Microscope, ZEISS,
Needles (sterile)	30G ^{1/2} 0.3x13 mm, Microlance 3, Becton Dickinson
pH-meter	Mettler Toledo Seven-easy, Germany

Pipettes & tips	Eppendorf
Pipette boy	Inegra Bioscience
Polypropylene tubes	1.5, 2, 15 and 50 ml, Sarstedt
Powersupply Power Pac	Bio-Rad, USA
Power washer PW96	Tecan, Austria
Shaker	GFL Rocking Shaker 3013, Gesellschaft für Labortechnik
Sonication	Sonoplus HD2070, UW2070, Bandelin
Syringes (sterile)	Omnifix-F 1 ml, Braun
Thermocycler	Biometra T3000, Germany Biometra T3, Germany Biometra T-Gradient, Germany
UV-VIS Photometer	Spectrophotometer U-2001, Serial No. 9836-037, Hitachi Instruments Inc., USA
UV Transilluminator	TFX-35M, Life Technologies, USA
Vacuum pump	Neuberger, KNF Comfort
Waterbath	Merck Eurolab GmbH

3.1.2 Cell culture equipment

Micro Well Plates (sterile)	24 well flat bottom, NUNC, Germany 96 well Maxisorp, NUNC, Germany
Serum Vacutainer SST tubes	2.5 and 9.5 ml, Becton Dickinson, Austria
Serum Microtainer tubes	Becton Dickinson, Austria
Surgical instruments	Scissors, tweezers, forceps

3.1.3 Chemicals and Reagents

2,2-Acino-bis-3 (Ethylbenzthiazoline-6-sulfonic acid) liquid substrate system (ABTS), Sigma
Acetic acid, Fluka

Aceton, Sigma-Aldrich

Acrilamide, Sigma-Aldrich

Albumin bovine serum (BSA), Biomol

Albumin bovine serum (BSA), Serologicals

Agarose, electrophoresis grade, Invitrogen

Amidosulfo betaine-14,3-[N,N-Dimethyl(3-myristoylamino propyl)ammonio]propanesulfonate (ASB-14), Sigma-Aldrich

Ammonium Chloride, Sigma-Aldrich

Ammonium Hydroxide, Sigma-Aldrich

Ampicillin, Sigma-Aldrich

APS (Ammoniumperdisulfat), Sigma-Aldrich

BenchMark™ Prestained Protein Ladder, Invitrogen

Bromophenol blue, Sigma-Aldrich

BugBuster® Protein Extraction Reagent, Novagen

Calcium Carbonate, Sigma-Aldrich

Citric Acid Trisodium Salt Dihydrate, Sigma-Aldrich

CMRL-1066 Medium, Gibco BRL

Coomassie® Brilliant Blue R-250, Bio-Rad

1 Kb DNA Ladder, Gibco™

100 bp DNA Ladder, Invitrogen ECL detection kit, Amersham

1,4-Dithiothreitol (DTT), Biosynth

ECL detection kit, Amersham

Ethanol, Merck

Ethidium Bromide, Sigma-Aldrich

Ethylenediaminetetraacetic acid disodium salt dihydrate 99+% (EDTA), Sigma-Aldrich

Ficoll, BD

Formaldehyde Solution, 37%, Neolab Migge Laborbedarf Vertriebs GmbH

Glucose, Sigma-Aldrich

Glutaraldehyde, Sigma-Aldrich

Glycerol, Sigma Ultra, approx 99% GC, Sigma-Aldrich

Glycine, Fluka

Hydrochloric acid, 37%, A.C.S. reagent, Aldrich

HEPES, Fluka

Imidazole, Sigma-Aldrich

Isopropyl β -D-Thiogalacto-Pyranoside (IPTG), Sigma

Kanamycin Sulfate, GibcoTM

Magnesium chloride, Invitrogen

2-Mercaptoethanol, Sigma Methanol, Fluka

Methanol, Fluka

N-Acetyl-D-Glucosamine, Sigma-Aldrich

Neopeptone, Difco BD

Octyl-B-D-glucopyranoside, Sigma-Aldrich

Orange G, Sigma-Aldrich

Phenol:Chloroform:Isoamyl Alcohol 25:24:1, Invitrogen

Phosphomycin, Sigma-Aldrich

Ponceau Solution, Sigma-Aldrich

2-Propanol (Isopropanol), Fluka

Pyruvic Acid Sodium Salt Sigma Ultra, Sigma-Aldrich

Rabbit Serum, Sigma-Aldrich

Sea Kem® LE Agarose, Cambrex Bioscience

Silver Nitrate, Sigma-Aldrich

Simply Blue Safe Stane Solution, Invitrogen

Skim Milk Powder, Fluka

Sodium Acetate, Merck

Sodium Azide, Sigma-Aldrich

Sodium Bicarbonate, Sigma-Aldrich

Sodium Carbonate, Sigma-Aldrich

Sodium Chloride, Sigma-Aldrich

Sodium Dodecyl Sulfate (SDS), Gibco™

Sodium Hydroxide Phosphate, Merck

Sodium Hydroxide, Sigma-Aldrich

Sucrose >99.5% (GC), Sigma-Aldrich

Sulfamethoxazole, Sigma-Aldrich

Sulfo-NHS-SS-Biotin, Pierce

TEMED (N,N,N,N-Tetra-methyl-ethylendiamine), Bio-Rad

TC Yeastolate, Difco BD

TRIZMA®base, minimum 99.9%, Sigma-Aldrich

Trypsine, Sigma-Aldrich

Tryptone, Oxoid

Tween® 20, Sigma-Aldrich

Xylene cyanol FF, Aldrich Chem.

3.1.4 Solutions

LB Medium

10 g Tryptone, 5 g yeast extract, 10 g NaCl, ddH₂O to 1 l, adjust pH to 7.0 and autoclave to sterilize.

BSK II Medium (pH 7.6)

900 ml sterile water (superMQ), 50 g BSA, 5 g Neopeptone, 6 g Hepes, 0.7 g Citric Acid Trisodium Salt Dihydrate, 5 g Glucose, 2 g TC Yeastolate, 0.8 g Pyruvic Acid Sodium Salt Sigma Ultra, 0.4 g N-Acetyl-D-Glucosamine, 2.2 g Sodium Bicarbonate, 100 ml CMRL-1066 Medium(10x), filter sterile and add rabbit serum to 6%.

PBS

10x stock solution (pH 7.3): 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄ × 7 H₂O, 2 g KH₂PO₄, add ddH₂O to end volume of 1 l.

	1x PBS: 10x PBS 1:10 diluted with dd H ₂ O.
PBS-T	1x PBS with 0.1% Tween®20 (Sigma, Germany).
TE-Buffer	10 ml 1 M Tris-HCl pH 8.0, 2 ml 0.5 M EDTA, add ddH ₂ O to end volume of 1 l.

Construction of genomic libraries

Stop-Buffer	100 mM EDTA, 30% glycerol, 0.5% Orange G, 0.074% xylene cyanol FF.
10x TAE-Buffer	48.4 g Tris-base, 10.9 g glacial acetic acid, 2.29 g EDTA, add ddH ₂ O to end volume of 1 l.
2% Tris-Acetate-EDTA-agarose gel	2 g agarose dissolved in 100 ml 1x TAE-Buffer.
10x Agarose gel loading dye	15% Ficoll, 0.2% bromphenol blue, 0.2% xylene cyanol FF.

SDS-PAGE and Western blot

Electrophoresis buffer	5x stock solution: 15.1 g Tris-base, 72 g Glycine, 50 ml 10% SDS, add ddH ₂ O to end volume of 1 l. Working solution: 1x.
5x reducing sample buffer	60 mM Tris-HCl (pH 6.8), 25% Glycerol, 2% SDS, 14.4 mM 2-Mercaptoethanol, 0.1% Bromphenolblau, add dd H ₂ O to end volume of 1 l.
Transfer buffer	39 mM Glycine, 48 mM Tris-base, 0.037% SDS, 20% Methanol, add ddH ₂ O to end volume of 1 l.
Membrane stripping buffer	12 ml 0.5 M Tris-base, 20 ml 10% SDS, 2 ml 2-Mercaptoethanol, add ddH ₂ O to end volume of 100 ml.

Separation of outer membrane

TSM buffer	1 ml 1 M Tris pH 7.5, 3 ml 5 M NaCl, 0.5 ml 1 M MgCl ₂ 95.5 ml ddH ₂ O
TSEA buffer	1 ml 1M Tris pH 7.5, 3 ml 5 M NaCl, 2 ml 0.5 M EDTA

0.05 g Natrium azid, 94 ml ddH₂O

Partial trypsin digest

PBS-Mg	1x PBS with 5 mM Mg
15% running gel	5.8 ml Acrylamide (40%), 5 ml 3 M Tris-HCl, 4.2 ml ddH ₂ O, 5 µl TEMED, 50 µl APS
4% stocking gel	0.6 ml Acrylamide (40%), 2 ml 3 M Tris-HCl, 3.2 ml ddH ₂ O, 4.5 µl TEMED, 45 µl APS
Silver reagent	0.8 g AgNO ₃ dissolved in 4 ml H ₂ O and dropwise added to Staining buffer
Staining buffer	20 ml ddH ₂ O, 0.4 ml 5 M NaOH, 1.3 ml 25% NH ₄ OH Add ddH ₂ O to end volume of 100 ml
Developer	0.4 g citric acid, 0.4 ml formaldehyde, 800 ml ddH ₂ O

Biotinylation

1 mM biotin	6 g biotin (Sulfo-NHS-SS-Biotin, Pierce) diluted in 1 ml ice cold PBS
Binding buffer	1x PBS with 0.1 % ASB-14
Elution buffer	10 mM DTT in 1x PBS with 0.1% ASB-14

3.2 Antigen identification

Antigen identification was performed as previously described by Etz et al., 2002 .

3.2.1 Characterization of human sera

Preparation of bacterial antigen extracts

To generate bacterial lysates, *B. burgdorferi* strain B31 and *B. afzelii* strain K78 was grown for 1 week in BSK-II medium and bacteria collected by centrifugation (3,000 rpm, 10 min, 4°C). Cells were washed twice with PBS, and after re-suspension lysed by sonication (2 min, pulse 5, 100% power, on ice, sonicator type). The supernatant was collected by

centrifugation (3,500 rpm, 15 min, 4°C). The protein concentration was determined by Bradford assay using dye reagent concentrate (Bio-Rad Laboratories, Austria).

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA plates (Maxisorb, Milipore) were coated with 5-10 mg/ml total protein diluted in coating buffer (0.1 M sodium carbonate pH 9.2). Two dilutions of human sera (10,000x, 50,000x) were made in PBS containing 1% BSA. Highly specific Horse Radish Peroxidase (HRP)-conjugated anti-human IgG secondary antibodies (Southern Biotech, USA) were used according to the manufacturer's recommendations (at 1,000X dilution). Antigen-antibody complexes were quantified by measuring the conversion of the substrate (ABTS) to coloured product based on OD_{405nm} readings by automatic ELISA reader (TECAN SUNRISE, Austria).

IgG isolation from human sera

Human sera were heat inactivated at 56°C for 30 min. After removing the precipitated proteins by centrifugation, the supernatant was filtered through a 0.22 µm Spin-x® filter (Costar, U.S.A.). Antibodies against *E. coli* proteins were removed by incubating the heat inactivated sera with whole *E. coli* cells (DH5alpha, transformed with pHIE11). *E. coli* was inoculated in 10 ml LB-medium supplemented with 50 µg/ml Kanamycin (LB-Kan) and incubated overnight at 37°C. 10 ml overnight culture was transferred to 500 ml LB-Kan and incubated at 37°C until OD_{600nm}=0.6 was achieved. *E. coli* cells were collected by centrifugation (4,500 rpm, 4°C, 10 min), washed twice with 500 ml 2x PBS and re-suspended in 1x PBS to a final volume of 100 µl. One ml serum and 100 µl of bacterial cell suspension were mixed and incubated for 6 to 15 hours at 4°C under continuous agitation (Rotator Drive STR4, Stuart Scientific, U.K.). Cells were centrifuged at 8,000 rpm at 4°C for 10 min, and supernatant was transferred into a new tube. Adsorption was repeated twice using the same volume of *E. coli* cell suspension. After finishing depletion, cells were centrifuged, and the serum sterile filtered through a 0.22 µm Spin-x® filter.

Finally, sera were diluted 1:3 with Binding Buffer pH 8.0 (Pierce, U.S.A.) and applied to Ultralink Immobilised Protein G columns (Pierce, U.S.A.). Diluted sera were applied twice and bound IgGs were washed with 20x bed volume of Binding Buffer. IgGs were eluted by

adding 5x bed volume of Elution Buffer pH 2.8 (Pierce, U.S.A.) and collected in 1x bed volume aliquots. To avoid denaturation of immunoglobulins the pH was immediately adjusted by adding 1/10 of the volume of Neutralisation Buffer (1 M Tris, pH 8.0). The elution of IgGs was monitored by measuring the adsorbance at 280 nm. The most concentrated fractions were pooled and dialysed against PBS. After sterile filtration the IgG concentration was determined by 12% SDS-PAGE, in comparison to a BSA standard. Pooled IgG fractions were biotinylated according to the manufacturer's instructions (EZ-Link Sulfo-NHS-LC-Biotin, Pierce, U.S.A.). Biotinylation was controlled by qualitative Western blot analysis.

3.2.2 Generation of bacterial surface display libraries in *E. Coli*

Preparation of genomic DNA

Cells from a 400 ml bacterial culture (*B. afzelii* K78) were harvested (5,000 rpm, 20 min, room temperature), washed with 80 ml 50 mM Tris pH 7.4 and re-suspended in 10 ml 50 mM Tris pH 7.4 / 25% Sucrose / 50 mM EDTA. The suspension was transferred to a fresh glass tube, Lysozyme (final conc.: 1.5 mg/ml) and SDS (final conc.: 2%) were added. To lyse cells, the tube was incubated on ice for 1h. Proteinase K (final conc.: 0.1 mg/ml) was added for 10 min at 37°C, followed by Phenol/Chloroform (1:1) extraction, that was repeated several times. The final extraction step was performed with Chloroform/Isoamylalcohol (1:24) to remove traces of Phenol. The sample was treated with RNase A (final conc.: 10 µg/ml) for 1 h at room temperature and Phenol/Chloroform and Chloroform/Isoamylalcohol extractions were performed as described above. DNA in the remaining supernatant was precipitated by addition of 1/10th of the starting volume of 3 M NaAc (pH 5.3) and 2.5x of the volume of 99.5% Ethanol. After 1 h incubation at -20°C, the mixture was centrifuged (20,000 rpm, 15 min) and the pellet washed with 70% Ethanol. Finally, the pellet was dissolved in 500 to 1,000 µl TE-Buffer.

Vectors

The **pMAL4.31** (Figure 3.1) was designed to allow frame selection of randomly generated genomic DNA fragments, and was constructed on a pASK-IBA backbone with the beta-lactamase (*bla*) gene exchanged with the Kanamycin resistance gene. The *bla* gene was cloned into the multiple cloning site as described for pMAL4.1 (Etz et al., 2002).

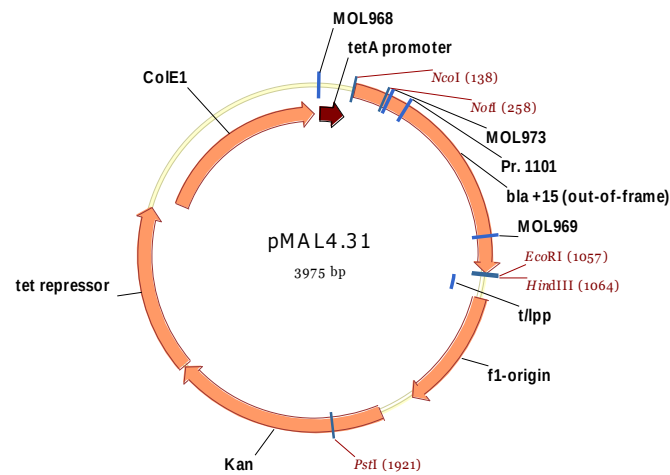


Figure 3.1: Frame selection vector pMAL4.31.

The **pHIE11** plasmid (Figure 3.2) encodes FhuA with unique *FseI* and *NotI* restriction sites in the DNA encoding loop 5. FhuA protein expression is under the control of the IPTG-inducible *lacUV5* promoter.

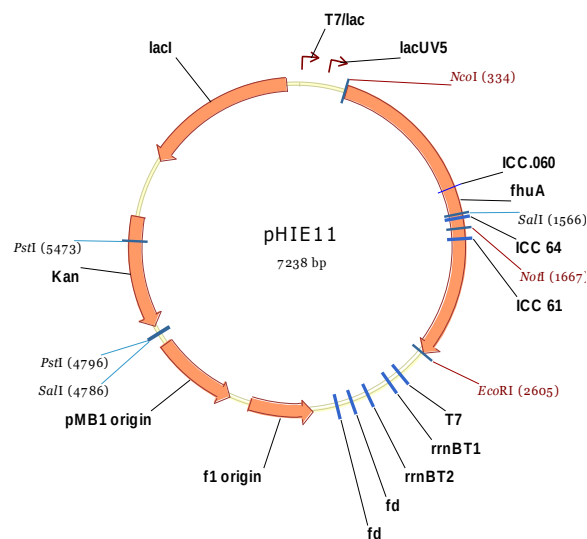


Figure 3.2: FhuA display vector pHIE11.

The **pMAL9.1** plasmid (Figure 3.3) encodes LamB with unique *FseI* and *NotI* restriction sites. The expression of LamB is under the control of the IPTG-inducible *lacUV5* promoter.

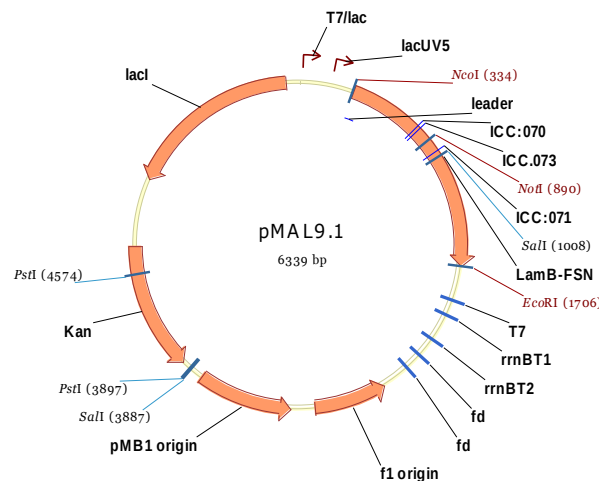


Figure 3.3: LamB display vector pMAL9.1.

Construction of genomic libraries

B. afzelii K78 genomic DNA was mechanically sheared into fragments ranging in size between 150 and 300 bp using a cup-horn sonicator (Bandelin Sonoplus UV 2200 sonicator equipped with a BB5 cup horn, 10 sec. pulses at 100% power output) or into fragments with sizes between 50 and 70 bp by mild DNase I treatment (Novagen, U.S.A.). Genomic DNA digestion was performed in the presence of $MnCl_2$ in a 10 μ l volume at 20°C for 10 min to ensure double-stranded cleavage by the enzyme. The reaction was stopped with 2 μ l of “Stop-Buffer” and fragmentation efficiency was evaluated on a 2% Tris-Acetate-EDTA-agarose gel. Fragments were blunt-ended twice by using T4 DNA polymerase in the presence of 100 μ M dNTPs (Fermentas, Germany). Fragments were used immediately for ligation reactions into *SmaI*-digested pMAL4.31, or were frozen at -20°C for subsequent use. Recombinant DNA was transformed into DH10B electrocompetent *E. coli* cells (Invitrogen, Germany). Transformed DH10B cells were plated on LB-agar containing 50 μ g/ml each of Kanamycin and Ampicillin. Plates were incubated overnight at 37°C and colonies collected for large scale DNA extraction. A representative plate was stored and saved for collecting

colonies for colony PCR analysis and large scale sequencing. Colony PCR assay was used to initially determine the approximate fragment size distribution as well as insertion efficiency. From sequencing data the precise fragment size, the junction intactness at the insertion site as well as the frame selection accuracy ($3n+1$ rule) was evaluated.

DNA sequencing and sequence analysis of pMAL4.31 clones

Bacterial library clones were sent to the company MWG Biotech AG, Germany, for sequencing. DNA sequencing was performed with purified plasmid DNA and with the oligonucleotide Pneu (GCACTGGCTGGTTTCGCTACCG). The obtained DNA sequences were blasted against available genomic sequence from *B. burgdorferi* s.s. B31 in order to identify the source of the DNA insert.

Construction of bacterial surface display libraries

The pMAL4.31 vector containing the *Borrelia* library was digested with restriction enzymes *FseI* and *NotI* to excise genomic DNA fragments. The entire population of fragments was then transferred into vectors pHIE11 (FhuA) or pMAL9.1 (LamB), which were also digested with restriction enzymes *FseI* and *NotI* that recognise an 8 bp GC sequence. This enabled that reading frames selected in the pMAL4.31 vector were maintained in each of the platforms vectors.

The plasmid library was then transformed into *E. coli* DH5alpha cells by electroporation. Transformants were plated on LB-agar plates supplemented with Kanamycin (50 µg/ml), and grown overnight at 37°C at a density yielding individual colonies. Cells were then scraped off the surface of the plates, washed with fresh LB-medium and stored in aliquots for library screening at -80°C.

3.2.3 Magnetic cell sorting

Approximately 1.0×10^8 cells of the respective *E. coli* library were used for individual experiments. Cells were grown in 5 ml LB-medium supplemented with 50 µg/ml Kanamycin (LB-Kan) for 50 min at 37°C. Protein expression was induced with 1 mM IPTG (Sigma, Austria) for 40 min. Cells were harvested at 3,000 rpm for 10 min at 4°C and washed twice

with 5 ml fresh LB-Kan. An aliquot containing approximately 8×10^6 cells was transferred to an Eppendorf tube. Cells were harvested at 8,000 rpm for 10 min at 4°C and re-suspended in 100 µl LB-Kan. 15 µl MACS microbeads coupled to streptavidin (Miltenyi Biotec, Germany) were added and the mixture was incubated for 45 min at 4°C.

To remove cells non-specifically adhering to a MACS column (Miltenyi Biotec, Germany), a column was prepared, fixed on magnet and equilibrated with 4 ml LB-Kan. The MACS microbead cell suspension was mixed with 1 ml LB-Kan and loaded onto the MACS column. The flow-through was collected and loaded once more onto the column. The second flow-through was collected and cells were harvested at 8,000 rpm for 10 min at 4°C. The cells were re-suspended in 100 µl LB-Kan and incubated overnight at 4°C with 10 and/or 20 µg of biotinylated IgG pools.

On the next day cells were collected at 8,000 rpm for 10 min at 4°C, washed once with 1 ml LB-Kan to remove unbound IgGs and re-suspended in 100 µl LB-Kan. 15 µl MACS microbeads coupled to streptavidin were added and incubated for 45 min at 4°C. *E. coli* cells were removed by centrifugation at 8,000 rpm for 10 min at 4°C and were washed three times with 1 ml LB-Kan and subsequently re-suspended in 1 ml LB-Kan. A portion of the cells (10 µl) was diluted and plated on LB-agar plates supplemented with Kanamycin (50 µg/ml) and grown overnight at 37°C for determination of input CFU. The rest of the MACS microbead cell suspension was loaded onto a MACS column equilibrated with 4 ml LB-Kan, which was attached to a magnet. After binding to the column, the column was washed three times with 3 ml LB-Kan. The elution was performed by removing the magnet and eluting with 2 ml of LB-Kan medium. After the column was washed (3x with 3 ml LB-Kan) the eluate was loaded again and the elution process repeated. Selected cells were plated onto LB-agar plates supplemented with Kanamycin (50 µg/ml) and grown overnight at 37°C. To test the efficiency of the MACS screening procedure, selected clones were analysed by Western blot analysis with the corresponding human IgG pool used for the MACS selection.

3.2.4 Western blot analysis

Lysate preparation from selected *E. coli* clones

Bacteria were grown overnight at 37°C in 1 ml LB-Kan. Overnight cultures were re-inoculated (1:10) in 1 ml LB-Kan and incubated for 2 h at 37°C. Protein expression was induced with 1 mM IPTG and incubation was continued for 2 h at 37°C.

SDS-PAGE

PAGEr Duramide® Precast Gels (Cambrex Bio Science, U.S.A.) were used according to the instruction manual. Cell pellets were mixed with 5x reducing sample buffer and heated for 10 min at 95°C. BenchMark™ prestained protein ladder (MW 6-175 kDa) served as indicator for protein size. Proteins were separated with a constant current of 25 mA / gel and gels were further used for Western blotting.

Western Blot

Separated proteins were transferred onto a nitrocellulose membrane (Hybond™; 0.45 Micron, Amersham Bioscience, England) using a semi-dry transfer system (Trans-Blot®SD; BioRad, USA). Gels and membranes were assembled in a blot sandwich and blotted in Transfer buffer at 80 mA for 1 h at room temperature. Blotted proteins were visualised by Ponceau S (Sigma, Austria) staining. Non-specific binding sites were blocked by immersing membranes in 5% milk-PBS-T for one hour at room temperature. Membranes were washed three times for 15 min with PBS-T solution and subsequently incubated with first antibody solution (human serum pool diluted 1:2,000 with 5% milk powder in PBS-T) overnight at 4°C. After washing steps were repeated, membranes were incubated with secondary antibody solution (goat anti-human IgG-HRP from Southern Biotechnology Organisation, USA.; 1:1,000 diluted with 5% milk-PBS-T) for one hour at room temperature. Prior to detection, membranes were washed three times for 15 min with PBS-T. Signals were visualised using an ECL detection kit (Amersham Pharmacia Biotech, England), mixing equal volumes of the solutions (0.125 ml/cm² membrane) and incubating then with a membrane for one minute at room temperature. Membranes were wrapped into a fresh

plastic bag, and exposed to a HyperfilmTMECL (Amersham Bioscience, England) in an X-ray film cassette. Exposure times varied from 3 min to one hour.

DNA sequencing

96 to 800 single clones/ MACS screen, which were grown on LB-agar plates supplemented with Kanamycin (50 µg/ml) (see MACS screening), were picked and added to 96 well plates and sent to MWG Biotech AG (Germany) for DNA sequencing. The DNA sequencing was performed with the oligonucleotide Fneu (CGTATGCGTAATGACATCAAC) for FhuA-clones, and with the oligonucleotide Lprimer (GCCGGTCTGGAAAACATC) for LamB-clones.

Bioinformatics

DNA sequences analysis was performed at Intercell AG by the Bioinformatic department using an in-house database (ARIS). Sequences were trimmed by recognition of the restriction site sequences of the *FseI* and *NotI* sites surrounding the insert (*FseI*: GGCCGGCC; *NotI*: GCGGCCGC). Trimmed sequences were blasted against the *B. burgdorferi* s.s. B31 genomic sequence. Sequences not recognised by this initial blast were individually blasted against both the *B. burgdorferi* s.s. B31 and *B. garinii* PBi genomes. All further analyses, such as Hits per ORF, Clone mapping etc., were performed manually.

3.3 Antigen validation

3.3.1 Gene conservation analysis

Borrelia strain collection

Borrelia strains isolated mainly from patients with different symptoms of Lyme borreliosis were collected from different sources (Table 3.1). The genospecies of the strains were determined by species specific PCR (Marconi and Garon, 1992) and by sequencing the *ospA* and *ospC* genes.

No.	Strain	Species	OspA	OspC	Isolation	Disease*
1	M76	<i>B. garinii</i>	nd	020	CSF	H Inv
2	W	<i>B. garinii</i>	5	013	CSF	H Inv
3	P	<i>B. afzelii</i>	2	008	Skin	H Inv
4	K6	<i>B. garinii</i>	nd	019	Skin	H inv
5	K67	<i>B. burgdorferi</i>	1	047	Skin	H
6	N40	<i>B. burgdorferi</i>	1	026	<i>I. scapularis</i>	na
7	Sh-2-82	<i>B. burgdorferi</i>	1	030	<i>I. scapularis</i>	H Inv
8	K86	<i>B. burgdorferi</i>	1	028	Skin	H
9	LAS	<i>B. burgdorferi</i>	1	002	Skin	H Inv
10	HB19	<i>B. burgdorferi</i>	1	004	Blood	H Inv
11	Lenz	<i>B. burgdorferi</i>	1	002	Myocardium	H Inv
12	10/2002	<i>B. afzelii</i>	2	037	Skin	H
13	BITS-B	<i>B. garinii</i>	6	017	<i>I. ricinus</i>	H Inv
14	57	<i>B. garinii</i>	5	013	CSF	H Inv
15	60	<i>B. garinii</i>	6	012	CSF	H Inv
16	130/91	<i>B. afzelii</i>	2	010	Skin	H
17	H13	<i>B. garinii</i>	6	025	Heart	H Inv
18	K78	<i>B. afzelii</i>	2	007	Skin	H
19	VS461	<i>B. afzelii</i>	2	022	<i>I. ricinus</i>	H Inv
20	B31	<i>B. burgdorferi</i>	1	002	<i>I. scapularis</i>	H Inv
21	PKo	<i>B. afzelii</i>	2	009	Skin	H Inv
22	Z10	<i>B. garinii</i>	2	034	<i>I. ricinus</i>	H Inv
23	Bol10	<i>B. garinii</i>	3	023	CSF	H Inv
24	ZS7	<i>B. burgdorferi</i>	1	001	<i>I. ricinus</i>	H Inv
25	NBS16	<i>B. garinii</i>	3	033	<i>I. ricinus</i>	H
26	KL11	<i>B. garinii</i>	6	019	<i>I. ricinus</i>	H Inv
27	H9	<i>B. afzelii</i>	2	005	Skin	H
28	123/91	<i>B. afzelii</i>	2	011	Skin	H
29	Simon	<i>B. afzelii</i>	2	011	Skin	H
30	ACA1	<i>B. afzelii</i>	2	008	Skin	H Inv

Table 3.1: List of *Borrelia* strains used in gene distribution analysis. OspA, OspA serotypes (Wilske et al., 1993), OspC, OspC type according to Livey et al. (1995). *; Type of disease associated with the corresponding OspC type, H; pathogenic for human, H Inv; pathogenic for human and causing invasive disease (e.g. neuroborreliosis, Lyme arthritis), nd; not determined, na; not associated with disease in humans.

The OspA types were classified according to the OspA serotypes defined by Wilske et al. (1993), the nomenclature of the OspC types was used according to Livey et al. (1995). *Borrelia* strains with the most prevalent OspC types associated with human disease, invasive and non-invasive disease were selected for the gene distribution analysis (Table 3.1).

Preparation of genomic DNA

A frozen vial containing 1 ml of *Borrelia* cells was thawed and inoculated into 9 ml of fresh BSK-II medium and incubated at 34°C with 5% CO₂. Growth was monitored by counting *Borrelia* cells using dark-field microscopy. The culture was harvested by centrifugation at 4,500 g for 15 minutes after cell density of >10⁷ cells/ml was reached. Bacteria were collected and frozen until DNA extraction. The DNA was extracted using Wizar® Genomic DNA Purification kit (Promega) following the manufacturer's instruction. The Wizard® Genomic DNA Purification Kit is based on a four-step process. The first step in the purification procedure lyses the cells and the nuclei following an RNase digestion step. The cellular proteins are then removed by a salt-precipitation step, which precipitates the proteins but leaves the high molecular weight genomic DNA in solution. Finally, the genomic DNA is concentrated and desalted by isopropanol precipitation.

Gene amplification by PCR

PCR was performed with a collection of *Borrelia burgdorferi* s.l. isolates with primers specific for the genes of interest. Primers were designed for all identified ORFs using the public program Primer3 (<http://frodo.wi.mit.edu>). In order to facilitate PCR amplification, the fragment size was set to max. 1,000 bp. The oligonucleotides were designed to incorporate all regions, which were identified as antigenic (listed in Table A1, Appendices). The genomic sequence from both *B. burgdorferi* s.s. (B31) and *B. garinii* (PBi) was used for the oligonucleotide design when possible.. PCR was performed in reaction volume of 25 µl using Taq polymerase (1U), 5 nM dNTPs, 10 pMol of each oligonucleotide, and a kit according to the manufacturer's instructions (Invitrogen, the Netherlands). A standard set up with 30 cycles (1x: 5min. 95°C; 30x: 30 sec. 95°C, 30 sec. 56°C, 30 sec. 72°C; 1x: 4 min. 72°C) was performed, first then conditions were adapted for individual primer pairs in case standard

procedure failed. The PCR amplification was performed in a Biometra T3 Thermocycler. All negative PCR reactions in the first amplification round were repeated applying the following conditions:

5 µl genomic DNA (diluted 1:100 in ddH₂O)
2.5 µl 10x buffer (Invitrogen Taq Polymerase I)
1.0 µl 50 mM MgCl₂
2.5 µl 2 mM dNTPs
Each 1 µl 10 pM oligonucleotides
0.2 µl Invitrogen Taq Polymerase I (5 U/µl)
11.8 µl ddH₂O

The DNA samples were subsequently visualised by electrophoresis on a 1.6% agarose gel and stained with EtBr.

3.3.2 Peptide ELISA

Peptide synthesis

Peptides were synthesized in small scale (4 mg resin; up to 288 in parallel) using standard F-moc chemistry on a Rink amide resin (PepChem, Tübingen, Germany) using a SyroII synthesizer (Multisynthech, Witten, Germany). After the sequence was assembled, peptides were elongated with Fmoc-epsilon-amino-hexanoic acid (as a linker) and biotin (Sigma, St. Louis, MO; activated like a normal amino acid). Peptides were cleaved off the resin with 93% TFA, 5% triethylsilane, and 2% water for one hour, dried under vacuum and freeze dried three times from acetonitrile/water (1:1). The presence of the correct mass was verified by mass spectrometry on a Reflex III MALDI-TOF (Bruker, Bremen, Germany). The peptides were used without further purification.

Enzyme linked immune assay (ELISA)

Biotin-labelled peptides (at the N-terminus) were coated on Streptavidin ELISA plates (EXICON) at 10 µg/ml concentration according to the manufacturer's instructions. Highly specific Horse Radish Peroxidase (HRP)-conjugated anti-human IgG secondary antibodies (Southern Biotech) were used according to the manufacturer's recommendations (dilution: 1:1,000x). Sera were tested at two serum dilutions, 200x and 1,000x. Following manual

coating, peptide coated plates were processed by the Gemini 160 ELISA robot (TECAN) with a built-in ELISA reader (GENIOS, TECAN).

3.3.3 Surface staining

Generation of immune sera

E. coli clones selected by MACS screen and verified by Western blotting to harbour immunogenic epitopes were grown in LB medium supplemented with 50 µg/ml Kanamycin at 37°C. Overnight cultures were diluted 1:10, grown until an OD₆₀₀ of 0.5 and induced with 0.2 mM IPTG for 2 hours. Pelleted bacterial cells were resuspended in PBS buffer and disrupted by sonication on ice, generating a crude cell extract. An aliquot corresponding to 5x10⁷ cells (based on OD₆₀₀ measurement) was injected into NMRI mice intravenously (i.v.), followed by a booster injection after 2 weeks. Serum was generated from blood taken 1 week after the second injection. Epitope specific antibody levels were measured by peptide ELISA.

Surface staining by flow cytometry

The *B. afzelii* strain K78 was cultivated at 34°C in BSK-II medium. The number of spirochetes was determined by dark-field microscopy and bacterial suspension diluted with fresh BSK-II medium to a concentration of 10⁶ cell/ml. Mouse sera were heat inactivated for 30 min at 56°C and diluted 1:25 with fresh BSK-II medium. 100 µl of this diluted sera was added to 100 µl (10⁵ cells) of the *Borrelia* suspension (finally serum dilution 1:50). The assay suspension was gently mixed and incubated at room temperature for 1 hour. After incubation, the samples were centrifuged at 5,000 rpm for 3 min, and washed once with 1 ml PBS containing 0.5% BSA. Secondary staining was done with PE conjugated anti-mouse antibodies which were used according to the manufacturer's recommendations (diluted in 100 µl) and performed on ice for 45 min. The samples were centrifuged at 5,000 rpm for 3 min, washed with 1 ml PBS containing 0.5% BSA, and resuspended in 1 ml of PBS-1% paraformaldehyde. Samples were vortexed and analyzed by flow cytometry with Beckman Cytomics FC 500.

3.4 Protein expression and purification

Cloning of *Borrelia* genes for recombinant expression

The gene of interest was amplified from genomic DNA of *B. afzelii* strain K78 by PCR using gene specific primers. The primers were designed not to have restriction sites present in the respective gene that aid directional cloning of the amplified PCR product. The length of the gene specific part of the primer ranged between 15 and 30 nucleotides. The obtained PCR product was digested with the appropriate restriction enzymes, cloned into the pET28b (+) vector (Novagen) and transformed into *E. coli* DH5 α cell. The gene was cloned into pET28b (+) to allow expression of the proteins with His-tag at the C-terminal end. The resulting plasmid was checked by sequencing the insert. Once the recombinant plasmids were confirmed to contain the gene of interest, *E. coli* BL21 CodonPlus (DE3)-RIPL (Invitrogen) cells were transformed with the plasmid for protein expression.

Expression and purification of recombinant proteins

E. coli BL21 CodonPlus (DE3)-RIPL cells harbouring the recombinant plasmid were grown till log phase in the required culture volume. Once an OD_{600nm} of 0.6 was reached, the expression was induced by addition of IPTG (at final concentration of 0.5 mM) and cells were further incubated for 3 hours at 37°C. The cells were harvested by centrifugation, lysed by a combination of freeze-thaw method and mechanically disrupted with Bug-buster® (Novagen). The lysate was separated by centrifugation into soluble (supernatant) and insoluble (pellet) fractions. Depending on the location of the protein, different purification strategies were applied. In case the His-tagged protein was expressed in the soluble fraction, protein purification was done by incubating the supernatant with Ni-Sepharose beads (Ni-Sepharose™ 6 Fast Flow, GE Healthcare). Since the placement of the hexa Histidine (6xHis) tag at the C-terminus of the expressed protein, only full length proteins were purified by the Ni-Sepharose column.

The column was washed successively (with (1.) three column volumens (CV) of Washing buffer I (WB I: 50 mM Tris-HCl pH 8.0, 1% Triton X-114), (2.) ten CV with 20 mM Imidazole in WB I, (3.) followed by ten CV with 40 mM Imidazole in WB I, (4.) then with 3

CV 50 mM Tris-HCl pH 8.0; (5.) wash with 3 CV of WB II (500 mM Imidazole, 20 mM NaH_2PO_4 , 0.5 mM NaCl,) with 50 mM Tris-HCl pH 8.0; (6.) wash with 3 CV of WB II with PBS pH 7.4; (7.) wash with 3 CV of WB II and HEPES pH 7.0; (8.) wash with 3 CV of WB II with HEPES pH 6.2; (9.) wash with 500 mM Imidazole, 8 M urea, 0.5 mM NaCl, Tris-HCl pH 8.0) and proteins eluted. The elution fractions were collected and concentrated and the protein concentration was determined by the Bradford method. The purity and identity of the protein solutions were checked by SDS-PAGE and Western blot, respectively. In case the protein was present in the insoluble fraction, the pellet was solubilised in a buffer containing 8 M urea and applied onto the Ni-NTA column under denaturing conditions. The column was washed and proteins eluted. Prior to concentration and protein determination, protein solutions containing 8 M urea were dialysed against PBS to remove traces of urea. Removal of urea was accomplished in several steps and in the presence of β -mercaptoethanol.

3.5 Animal studies

Cultivation of challenge strain

The virulent *Borrelia burgdorferi* strain N40 was used as challenge strain. The N40 strain is an *I. scapularis* isolate and was obtained from Sven Bergström, Department of Molecular Biology, Umeå Universitet, Umeå, Sweden. Strain was grown in BSKII medium.

A frozen stock of bacteria was prepared in the following way: bacteria were grown at 34°C until concentration of 10^5 bacteria/ml. Approximately 1 ml culture was placed into CRYO tubes and frozen at -80°C until further use.

For each challenge experiment one frozen stock was thawed and incubated in 10 ml BSK-II medium for two days. The bacterial cell number was determined via microscopic evaluation with a dark-field microscope with a 40x objective and a 10x eyepiece. 4.5 μl of *Borrelia* culture was applied on a microscopic slide and covered with 18x18 mm cover-slip (without air bubbles). At least 20-30 spirochetes were counted in at least two viewing field. To determine the *Borrelia* concentration, the number of cells counted was divided with the number of fields viewed. The obtained number was multiplied with the factor 3.6×10^5 that

gave the number of *Borrelia* per ml. The factor 3.6×10^5 is the number of viewing fields on a cover-slip, ZEISS microscope: 1620, divided by the volume under it in millilitre, 4.5×10^{-3} .

Determination of the actual challenge dose was performed by limiting dilution and determined by the most probable number method (a method of getting quantitative data on concentration commonly employed by performing serial dilutions of a bacterial culture into media and then further dividing the serial dilutions into aliquots). After the culture was adjusted as needed for the animal experiments (10^4 - 10^5 bacteria/ml), three 10 ml cultures were prepared with following concentrations: 10 bacteria/ml, 1 bacteria/ml, and 0.1 bacteria/ml. For each dilution 8 wells in 24 well plates were filled with 1 ml culture and 1 ml fresh BSK-II medium. The plates were sealed with adhesive tape and incubated at 34°C and 5% ($\pm 1\%$) CO₂ for 2-3 weeks. After incubation the presence of *Borrelia* in each well was determined by viewing up to 40 viewing fields in a dark-field microscope. The solution was considered bacterium free when no *Borrelia* was detected in 40 viewing fields. The *Borrelia* concentration in the first dilution (10 bacteria/ml) was read from the most probable number table (Table A3 in Appendices) with 95% confidence interval.

Immunisation

6-8 week old female C3H/HeN mice (Harlan Winkelmann, Borcheln, Germany) were used, 10 mice/group. Mice were immunized subcutaneously (flank), three times with a biweekly interval. Positive control groups were vaccinated with 1 µg lipidated OspA (kindly provided by Sven Bergström). Test antigens were used at 50 µg of each recombinant antigen or PBS (negative control) mixed with either CFA/IFA (Complete/Incomplete Freud's Adjuvant), ALUM (Aluminium hydroxide), or IC31® high (100 nmol L-KLK; 4 nmol ODN1a). One week after the last booster immunization, mice were bled from the tail vein to obtain hyper-immune sera.

Animal challenge

Mice were challenged with 100 µl *Borrelia* culture (10^4 or 10^3 bacteria/mouse) injected subcutaneously.

Organ cultures

Four weeks post challenge mice were anaesthetised by a mixture of Ketalar and Dormicum and sacrificed by cervical dislocation. The heart, knee joint and ear was put in BSK-II medium supplemented with phosphomycin and sulfamethoxazole for detection of live *Borrelia* bacteria. The cultures were screened by microscopy for living bacteria starting after one week. Cultures where no bacteria were observed after four weeks were evaluated as negative.

3.6 Proteomics

3.6.1 Outer membrane preparation

Cell fractionation to obtain outer membranes was done as described by Magnarelli et al., 1989, with slight modification of the protocol. 1 l *Borrelia* culture (10^8 - 10^9 cell/ml) was harvested by centrifugation at 9,000 rpm for 20 min. To remove growth medium, the pellet was washed with 50 ml TSM buffer, centrifuged again at 9,000 rpm for 15 min (4°C), and resuspended in 5 ml TSM buffer and kept on ice for 15. For solubilisation of membrane proteins, 1.2 ml 10% octyl-B-D-glucopyranoside (OGP) in TSEA buffer was added and sample further incubated for 60 min at 37°C. The cell lysate was centrifuged at 20,000 rpm for 30 min at 25°C. The supernatant (solubilised membrane proteins) was transferred into a fresh tube and further incubated for 30 min at 56°C. The supernatant was centrifuged at 20,000 rpm for 30 min and at 37°C and then filtered through a 0.45 µm nitrocellulose filter and dialysed against distilled water at 4°C for 3-4 days. Water was changed every day. Dialysis cassette with 3,500 Da cut off was used. Finally the lysate was centrifuged at 20,000 rpm for 30 min and at 25°C. The pellet was dissolved in water.

3.6.2 Partial trypsin digest

Borrelia cells were harvested by centrifugation at 4,500 rpm for 10 min, and washed twice with PBS-Mg (5 mM). Cells were resuspended in the same buffer at a final concentration of 2×10^9 cells/ml. 25 µl Trypsin in 0.0001 N HCl was added to 0.5 ml bacterial suspension. Only diluted HCl was added to the negative control samples (without Trypsin).

Samples were incubated at 20°C, and aliquots were taken after 1, 2, 3 hours, or overnight incubation. Cells were harvested by centrifugation at 4,500 rpm for 10 min, and supernatant (partial digested outer surface proteins) was stored at -20°C till analysis. The supernatants were analyzed by 15% Schagger gel. Harvested cells were washed twice with PBS-Mg and used for Western blot analysis.

For Schagger gel 15% running gel (5,8 ml Acrylamide (40%), 5 ml 3M Tris-HCl, 4.2 ml H₂O, 5 µl TEMED, 50 µl APS) and 4% stacking gel (0.6 ml Acrylamide (40%), 2 ml 3M Tris-HCl, 3.4 ml H₂O, 4.5 µl TEMED, 45 µl APS) were prepared. 10 µl supernatant was loaded on the gels and separated at 100V. Subsequently gel was silver stained according to Oakley et al. (1980) with modifications in protocol (see Table 3.2).

Step	Reagent	Duration
1st Fixing	50% methanol/ 10% acetic acid	30 min
2nd Fixing	20% methanol	10 min
Wash	0.5x PBS	10 min
Sensitizing	5% glutaraldehyde / 1mM DTT	20 min
Wash	ddH ₂ O, 4°C	4 x 10 min
Silver	Silver reagent	10 min
Wash	ddH ₂ O, 4°C	2 x 1 min
Development	Developer	until brown stain visible
Stop	50 mM EDTA	

Table 3.2: Silver staining protocol. All steps were performed by room temperature and on shaker.

3.6.3 Biotin-labelling of *Borrelia* surface proteins

Biotinylation of whole cells

Bacteria were washed three times with PBS-Mg, and resuspended in the same buffer at a final concentration of 1x10⁹ cells/ml (end volume 10 ml). Half of the culture was used as a negative control, and to the other half 500 µl of 10 mM Sulfo-NHS-SS-Biotin (6g Biotin in 1 ml ice-cold PBS) (PIERCE, USA) prepared immediately before use was added. Reaction was incubated at 4°C for 30 min. The cells were washed with 3x with ice-cold PBS to remove non-reacted biotinylation reagent.

Cell lysis of biotin-labelled bacteria

Bacteria were solubilized in 1 ml PBS-1% ASB-14 at 4°C (without vortex!). ASB-14 or amidosulfobetaine-14 is zwitterionic detergent used for solubilisation of membrane proteins. After 1 hour incubation, the soluble fraction (solubilised membrane proteins) was separated from insoluble pellet by centrifugation at 20,000 rpm for 20 min. Biotin-labelled outer membrane proteins were isolated from soluble fraction (see next paragraph).

Isolation of labelled proteins

The Streptavidin column (Thermo Fischer Scientific Inc.) was equilibrated with 3 column volumes of PBS. The biotinylated samples were loaded to the column and allowed to enter the resin bed. The negative control sample was used with another column. Samples were incubated for 10 min and washed with 10 column volumes of Binding buffer (see chapter 3.1.4 Solutions). All washing steps were separately collected. The bound biotinylated proteins were eluted with 5-10 column volumes of Elution buffer (see chapter 3.1 Solutions) and stored at -20°C till analysis.

4 RESULTS

4.1 Antigen identification

4.1.1 Selection of human serum samples for genomic antigen screens

A large collection of human sera was obtained from approximately 1400 patients with medical diagnosis of borreliosis as well as from individuals with occupational exposure to ticks (forest workers) (Table 4.1). These serum samples were obtained from existing collections from Dr. D. Hulinska (NIPH, Prague, Czech Republic), Prof. Dr. G. Stanek (MUV, Vienna, Austria) and Prof. Dr. R. Stauber (Medizinische Universitätsklinik, Graz, Austria). The disease symptoms recorded and medical diagnosis of these patients included acute borreliosis (erythema migrans, EM), neuroborreliosis (NB), Lyme arthritis (LA), cardiological and dermatological symptoms (acrodermatitis chronica atrophica, ACA). Sera from healthy individuals served as negative controls. In order to select rich sources of *Borrelia*-specific antibodies, the human serum samples were analyzed by ELISA using whole bacterial extracts from *B. burgdorferi* strain B31, as described under Materials and Methods, and two dilutions of sera, 1:10,000 and 1:50,000.

	Healthy	Cardiological	Dermatologic	Neurologic	Arthritic	not	Σ
MUV	200	8	121	40	49	317	735
NIPH	50	77	117	134	52	43	473
Graz	-	-	-	-	-	203	203
							1411

Table 4.1: Number of donors diagnosed with borreliosis with different symptoms.

We detected a broad range of reactivities with the sera. The anti-borrelia antibody titer was considered high, if the ELISA reading at the 10,000-fold dilution was higher than 600 units OD_{405nm} (examples shown on Figure 4.1). The ELISA analysis showed that many

of the sera obtained from the healthy exposed individuals possessed only low levels of *Borrelia* specific antibodies. Therefore, most of the sera with the highest titers were identified among the sera obtained from patients. The disease symptoms recorded from these patients ranged from erythema migrans, neuroborreliosis, arthritis, to cardiological and other dermatological manifestations.

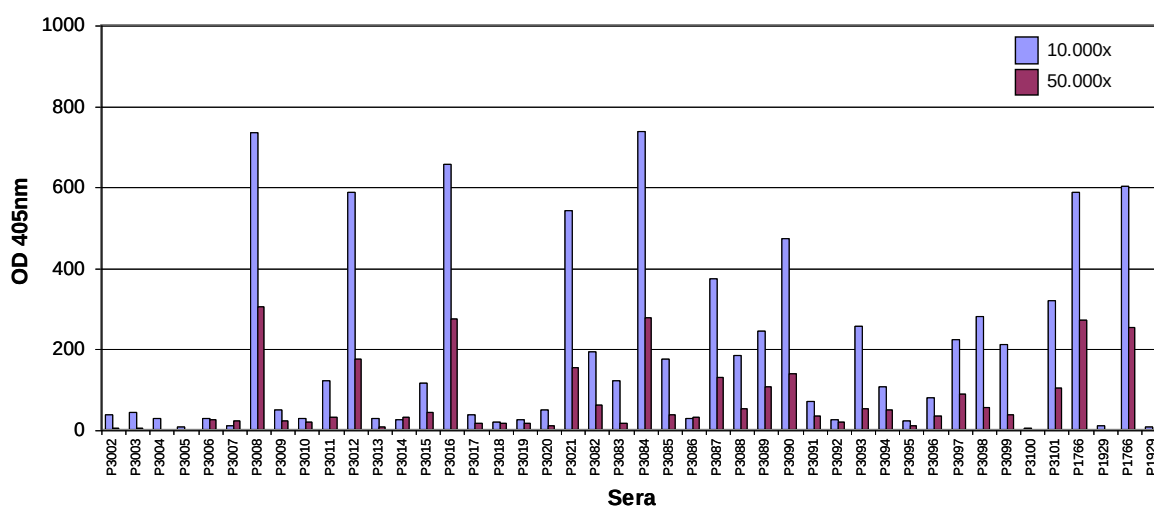


Figure 4.1: IgG levels of human sera with total *Borrelia* lysate. Human serum samples were tested at 10.000x and 50.000X dilution for immune reactivity against *B. burgdorferi* B31 whole cell lysate by ELISA. A representative experiment is shown among many.

For antigen identification screening, individual sera were pooled according to diagnosis of the patient. Six different serum pools were prepared, encompassing 25 high titer sera specific for distinct patient groups (Table 4.2). For example, the NB pool consists of five sera isolated from patients suffering from neuroborreliosis. Two serum pools, LB I and LB II, were compiled with sera from patients with multiple disease manifestations or without information on specific symptoms. Sera used for these two groups are delivered from patients with diagnosed Lyme borreliosis, which include all possible symptoms.

To prepare the sera for library screening, an absorption step with *E. coli* DH5alpha whole cells was necessary to remove antibodies recognizing the surface of *E. coli* in order to prevent non-specific selection of clones instead of antibody binding to *Borrelia* derived epitopes.

Serum pool	Sera in pool	Disease/Source
LBI	P1755, P1766, P1772, P1806, P1811	Lyme borreliosis I
EM	P3055, P3144, P3186, P3219	Erythema migrans
NB	P3077, P3084, P3286, P3288, P3337	Neuroborreliosis
LBII	P3016, P3150, P3301, P3344, P3394	Lyme borreliosis II
ACA	P3183, P3209, P3336	Acrodermatitis chronica atropicans
LA	P3120, P3239, P3251	Lyme arthritis

Table 4.2: Serum pools for antigen identification. Pools were assembled based on the disease manifestation of the donors. Concentrations of IgGs in serum pools were determined by SDS-PAGE analysis (Figure 4.2).

After adsorption, IgGs were purified from pooled sera by protein G affinity chromatography and biotinylated. IgGs were analyzed by SDS-PAGE for purity and quantity (Figure 4.2).

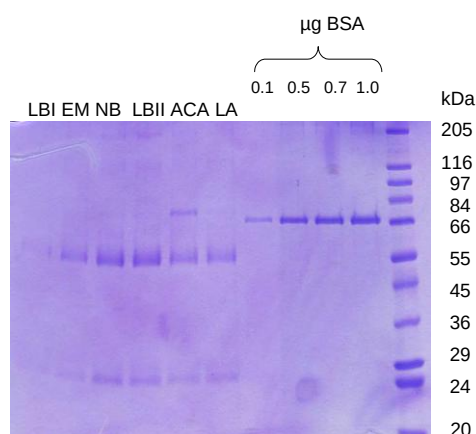


Figure 4.2: SDS-PAGE analysis of IgG pools. Purified IgGs of the six serum pools were analysed for purity and IgG quantity by SDS-PAGE. As a standard for determining the protein concentration, BSA was included in the analysis. 5 µl IgG solution/lane was loaded.

The pooled IgG preparations were tested for immune reactivity with *Borrelia* proteins using whole bacterial extracts in Western blot analysis. We confirmed that the selected IgG pools recognized multiple protein bands in extracts of two different *Borrelia* species, *B. burgdorferi* B31 and *B. afzelii* K78, as showed in Figure 4.3. Patterns of recognized protein bands were overall comparable between the two *Borrelia* extracts. Five major bands were

visible with all six serum pools (~90 kDa, ~60 kDa, ~40 kDa, ~32 kDa and ~20 kDa). The NB serum pool showed some differences in strength, and the ACA serum pool revealed some extra bands with strong signal (84 kDa) with B31 cell extract but less bands with K78 cell extract (84 kDa, ~30 kDa).

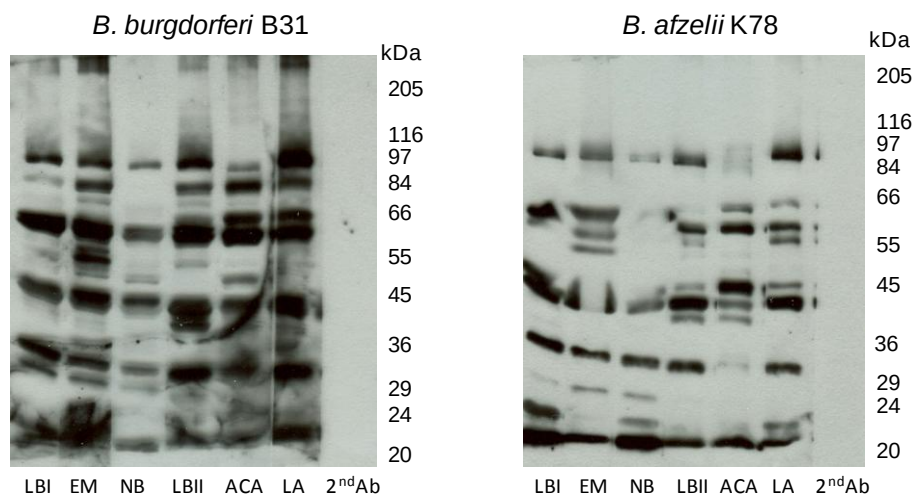


Figure 4.3: Western blot analysis of *Borrelia* extracts with biotinylated IgG pools. Total bacterial lysates from *B. burgdorferi* B31 and *B. afzelii* K78 were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were cut into slices and incubated with the biotinylated IgG pools indicated. Protein bands were visualized by chemiluminescence on X-ray films.

4.1.2 Generation of bacterial surface display libraries with sufficient coverage of the *Borrelia* genome

The ANTIGENome technology relies on the generation of genomic libraries encoding peptides derived from genomic DNA (Etz et al, 2001; Etz et al 2002; Henics et al, 2003; Meinke et al, 2005). To express *Borrelia* derived peptide epitopes, two different genomic libraries were generated from *B. afzelii* strain K78 with two platform proteins for surface expression, LamB and FhuA. Our choice of strain was stimulated by the fact, that *B. afzelii* represents the major pathogen causing human borreliosis in Europe. The K78 strain was isolated from a borreliosis patient by Prof. Gerold Stanek at the Medical University of Vienna.

Genomic DNA of the strain K78 was digested into approximately 50 bp long fragments in order to generate LamB library, or sonicated into 150-400 bp long fragments which were used for generation of FhuA library. Fragments were first cloned into the frame

selection vector pMAL4.31, which was transformed into *E. coli* DH10Bb cells. Transformants were plated out on selective agar plates where only clones carrying inserts in frame with the coding region of the β -lactamase gene can grow. The total number of clones obtained after frame selection for the two libraries is listed in Table 4.3.

Library	Borrelia strain	Platform	Genome coverage	pMAL4.31 (CFU)	Platform (CFU)
K78-LamB	<i>B. afzelii</i> K78	LamB	7x	200,000	800,000
K78-FhuA	<i>B. afzelii</i> K78	FhuA	9x	80,000	200,000

Table 4.3: Genomic libraries generated for antigen identification.

To assess the quality of the libraries, approximately 500 randomly chosen clones were sequenced from each of the frame selection libraries. The bioinformatic analysis showed that the actual insert size was close to the intended ones and that the majority of clones fell within a narrow size range (Figure 4.4).

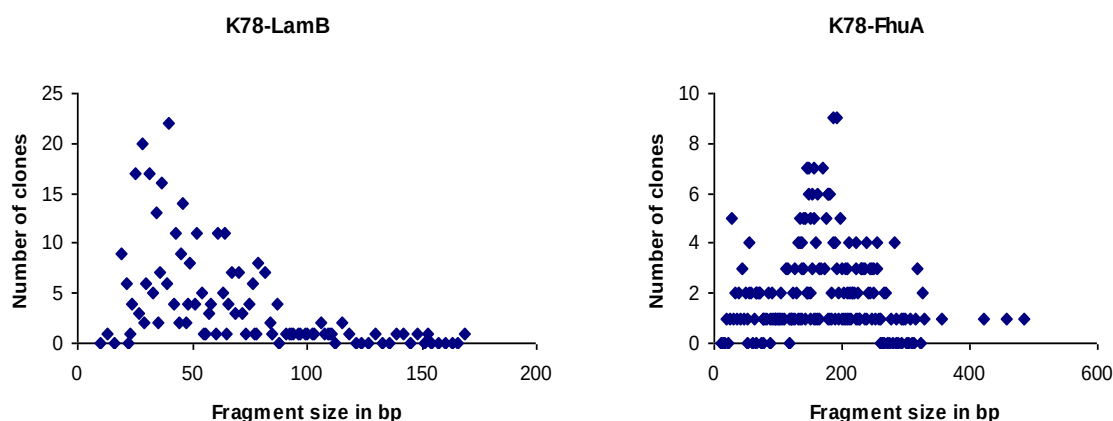


Figure 4.4: Size distribution of genomic *Borrelia* libraries. Intended fragment size for K78-LamB library (display vector pMAL9.1) was ~50 bp, and for K78-FhuA library (display vector pHIE11) between 150 and 300 bp.

After quality control of the frame selection libraries, inserts were transferred from the pMAL4.31 to the display plasmids pMAL9.1 (LamB) or pHIE11 (FhuA), creating bacterial surface display libraries that present peptide epitopes encoded by *Borrelia* on the surface of *E. coli*.

4.1.3 MACS screening of *E. coli* libraries with human IgG preparations from patients' sera

In order to identify antigenic *Borrelia* proteins, both LamB and FhuA bacterial surface display genomic libraries were incubated with the IgG fractions of six human serum pools depleted from *E. coli* antibodies and biotinylated as described previously (Etz et al., 2002).

Altogether 12 screens were performed with the two display platforms and 6 IgG pools. To assess the performance of the individual bacterial surface display screens, each library was screened with six IgG pools and without the selective human IgG pool in parallel. The number of obtained *E. coli* clones carrying *B. afzelii* inserts selected after screening with and without the addition of human IgGs are listed in Table 4.4. Screening with the K78-FhuA platform resulted in higher background selection of clones relative to the K78-LamB screens.

Selected *E. coli* clones were analyzed by Western blotting to confirm their antigenicity and therefore to estimate screening efficiency. For this purpose, 20 clones were randomly picked from each screen and analyzed by Western blot using the same human IgG pools used for the MACS screen. If more than 30% of selected clones were positive in the Western blot analysis, the screen was considered to be successful, and 600 to 800 clones were picked and sequenced. Western blots from two FhuA screens, NB and ACA, could not be evaluated because the signal was too weak and no differences between selected clones and negative control (FhuA clone without insert) were detectable. Despite the calculated low enrichment for the K78-LamB screens, Western blot analyses confirmed stronger immune reactivity of selected clones compared to K78-FhuA screens (Table 4.4). An example of Western blot analysis is shown in Figure 4.5. Altogether approximately 8000 clones were picked from all 12 screens and their inserts sequenced.

Library	Serum pool	Input CFU	Eluted CFU with IgGs	Eluted CFU without IgGs	Enrichment	Percentage of positive clones
K78-FhuA	LBI	1.2x10 ⁷	64,000	12,000	5x	40%
K78-FhuA	EM	1.1x10 ⁷	104,000		9x	55%
K78-FhuA	NB	1x10 ⁷	84,000		7x	not evaluable
K78-FhuA	LBII	1.3x10 ⁷	160,000		13x	50%
K78-FhuA	ACA	1.3x10 ⁷	88,000		7x	not evaluable
K78-FhuA	LA	2x10 ⁷	128,000		11x	65%
K78-LamB	LBI	3.8x10 ⁷	16,800	7,600	2.2x	70%
K78-LamB	EM	2.6 x10 ⁷	9,600		1.2x	40%
K78-LamB	NB	3.8 x10 ⁷	23,600		3.1x	50%
K78-LamB	LBII	1.9 x10 ⁷	12,800		1.7x	65%
K78-LamB	ACA	2.0 x10 ⁷	14,400		1.9x	40%
K78-LamB	LA	3.8 x10 ⁷	8,400		1.1x	50%

Table 4.4: Number of cells selected by K78 screens in comparison to negative control. Enrichment, difference between CFU selected with and without IgGs. Percentage of positive clones, percentage of clones that showed positive reaction with human IgGs in Western blot analysis.

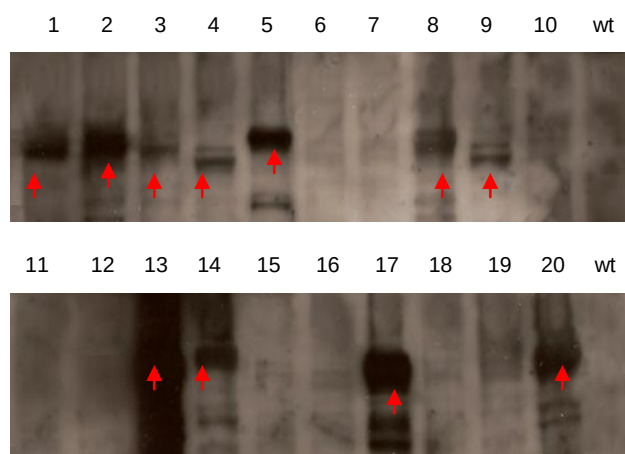


Figure 4.5: Western blot analysis of the clones selected by screening the K78-FhuA library with EM IgG pool. 20 clones selected by MACS screen with EM IgG pool were analyzed by Western blot. 11 clones, marked with arrows, were identified as positive, which is equivalent to 55%. Wt, FhuA clone without insert (negative control).

Bioinformatic analysis

Insert sequences of 70% of all K78-FhuA and 83% of all K78-LamB clones sent for sequencing could be mapped to the published genomic sequence (*B. burgdorferi* B31 and *B. garinii* PBi; Fraser et al., 1997, and Glöckner et al., 2004, respectively). Altogether, 5510 successfully sequenced clones were analyzed using an in-house developed algorithm. First, sequences were trimmed based on restriction site sequences surrounding the inserts in display vectors, and then blasted against the available *B. burgdorferi* B31 genome. Since *B. afzelii* gDNA was used for constructing the libraries, clone sequences and the genome sequence were from two different *Borrelia* species. This explains that only about 50% of sequences matched the B31 genome. Therefore, the remaining sequences were manually blasted against the two publicly available genomes, *B. burgdorferi* B31 and *B. garinii* PBi. Combining the results of the automatic and manual blasts 90% of insert sequences could be identified. For further Western blot analysis only those clones were selected, where at least 2 insert sequences matched the same region (epitope) of an individual ORF, or when the selected clone was part of an ORF annotated as putative surface or secreted protein, independent from the number of hits. This resulted in selection of 267 and 92 ORFs with the K78-FhuA and K78-LamB libraries, respectively.

Confirmation of the immune reactivity of selected *E. coli* clones

After the bioinformatic analysis, selected clones were analyzed by Western blot to confirm their reactivity with antibodies used for the screening procedure. 623 total cell extracts of selected clones were prepared and proteins were separated by SDS-PAGE. The proteins were blotted onto nitrocellulose membranes and probed with the same serum pools as used for the screening. To evaluate expression of the FhuA / LamB fusion protein and prevent false negative results, membranes were stripped after Western blot analysis with human IgGs and incubated with an anti-FhuA / anti-LamB antibody. Besides selected clones from the MACS screens, an FhuA / LamB clone without insert was also loaded onto the SDS gel. When the membranes were incubated with IgG pools, the FhuA / LamB clone without insert served as a negative control, and when the membranes were incubated with an anti-

FhuA / anti-LamB antibody as a positive control. An example of the Western blot analysis with selected bacterial clones is shown in Figure 4.6.

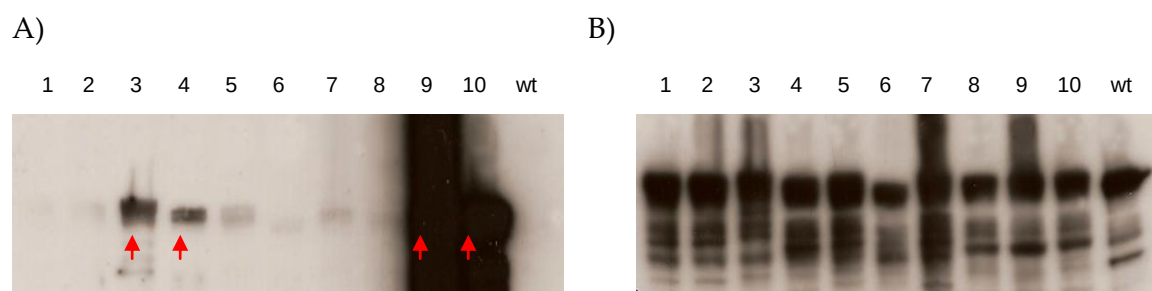


Figure 4.6: Western blot analysis of the K78-FhuA clones selected after DNA sequencing. A) X-ray film from the membrane incubated with ACA serum IgG pool. Clones identified as positive are marked with arrows. B) X-ray film from the same membrane after stripping and incubation with anti-FhuA antibodies. Wt, the FhuA clone without insert.

The Western blot analysis confirmed 115 and 55 ORFs of the K78-FhuA and K78-LamB screen selected clones (43 and 60%), respectively. I found that there was a very good correlation between the selection frequency of a given ORF and the likelihood of positive Western blot result (Table 4.5).

Platform	Hits	No of tested ORFs	Number of confirmed ORFs	% (positive / tested ORFs)
FhuA	≥10	37	32	86
	5-9	73	45	62
	4	36	9	25
	3	63	20	32
	2	58	9	16
	total	267	115	43
LamB	≥10	17	14	82
	5-9	22	17	77
	4	8	4	50
	3	20	9	45
	2	25	11	44
	total	92	55	60

Table 4.5: Correlation between selection frequency of antigens and Western blot positivity of corresponding clones. Hits: selection frequency. Tested ORFs, number of ORFs selected for Western blot analysis represented by all *E. coli* clones matching the ORF. Positive ORFs, ORFs that showed positive reaction with human IgGs based on Western blot with corresponding *E. coli* clones.

Selection of antigenic proteins from *B. afzelii*

All proteins for which at least one clone was positive by Western blot analysis are listed in Table 4.6. The antigens are listed according to the selection frequency (number of hits) based on all screens.

Ba ORF	Bb ORF	Bg ORF	Common name	FhuA		LamB		Summary
				Hits	WB	Hits	WB	Hits
BAP001	BB0844	BGP096	hypothetical protein	84	+	229	+	313
BAP024	BBK32	BGP100	immunogenic protein P35	99	+	48	+	147
BA0149	BB0147	BG0147	flagellar filament 41 kDa core protein (<i>flaB</i>)	44	+	27	+	71
BAP015	BBG10	BGP027	hypothetical protein	44	+	17	+	61
BA0737	BB0744	BG0765	antigen, p83/100	43	+	11	+	54
BAP035	-	BGP001	hypothetical protein	50	+	6	nt	56
BAA18	BBQ37	BGP292	hypothetical protein	1	nt	51	+	52
BAP036	-	BGP036	hypothetical protein	46	+	0	nt	46
BAP031	BBS41	-	outer surface protein G	19	+	24	+	43
BA0327	BB0329	BG0330	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (<i>oppA-2</i>)	40	+	0	nt	40
BA0210	BB0210	BG0212	surface-located membrane protein 1 (<i>lmp1</i>)	35	+	4	nt	39
BAP008	BBA57	BGA58	hypothetical protein	35	+	1	nt	36
BAP026	BBL27	BGP132	hypothetical protein	31	+	0	nt	31
BA0538	BB0545	BG0555	xylulokinase (<i>xylB</i>)	1	nt	29	+	30
BAP028	BBM15	BGP069	hypothetical protein	11	+	18	+	29
BAP029	BBN39	-	ErpB2 protein	12	+	17	+	29
BAP007	BBA36	BGA30	Lipoprotein	24	+	2	+	26
BAP017	BBG21	BGP116	predicted coding region	12	+	13	+	25
BA0513	BB0518	BG0529	heat shock protein 70 (<i>dnaK-2</i>)	20	+	3	+	23
BAP025	BBQ34	BGP110	hypothetical protein	18	+	5	+	23
BAP021	BBH13	-	hypothetical protein	22	+	0	nt	22
BAP038	BBS36	BGP301	hypothetical protein	18	+	3	+	21
BAP029	-	BGP049	hypothetical protein	15	+	5	-	20
BAP019	BBG24	BGP012	hypothetical protein	12	+	6	+	18
BA0237	BB0238	BG0241	hypothetical protein	15	+	2	+	17
BAP020	BBG30	BGP003	hypothetical protein	0	nt	17	+	17
BAD16	BBG20	BGP017	hypothetical protein	7	-	9	+	16
BAP005	BBA24	BGA21	decorin binding protein A (<i>dbpA</i>)	10	+	6	+	16
BA0675	BB0680	BG0703	methyl-accepting chemotaxis protein (<i>mcp-4</i>)	6	+	6	+	12
BA0454	BB0457	BG0466	excinuclease ABC, subunit C (<i>uvrC</i>)	2	-	10	+	12
BA0553	BB0560	BG0570	heat shock protein 90 (<i>hspG</i>)	10	+	2	-	12
BA0759	BB0765	BG0788	hypothetical protein	7	+	5	+	12
BAP013	BBB19	BGB18	outer surface protein C (<i>ospC</i>)	12	+	0	nt	12
BAP018	BBG23	BGP013	hypothetical protein	11	+	1	nt	12
BA0150	BB0149	BG0148	flagellar hook-associated protein 2 (<i>fliD</i>)	10	+	1	nt	11
BA0221	BB0221	BG0224	flagellar motor switch protein (<i>fliG-1</i>)	10	+	1	nt	11

Ba ORF	Bb ORF	Bg ORF	Common name	FhuA		LamB		Summary
				Hits	WB	Hits	WB	Hits
BA0627	BB0633	BG0655	exodeoxyribonuclease V, beta chain (<i>recB</i>)	7	-	4	+	11
BAD29	BBF25	BGP030	hypothetical protein	0	nt	11	+	11
BAP039	-	BGA26	hypothetical protein	11	+	0	nt	11
BA0135	BB0136	BG0138	penicillin-binding protein (<i>pbp-1</i>)	10	+	0	nt	10
BA0384	BB0384	BG0385	basic membrane protein C (<i>bmpC</i>)	1	nt	9	+	10
BAP002	BBA03	BGA02	outer membrane protein	10	+	0	nt	10
BAP006	BBA34	BGA28	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (<i>oppAV</i>)	10	+	0	nt	10
BAP036	BBR35	-	hypothetical protein	10	+	0	nt	10
BA0056	BB0058	BG0057	predicted coding region	9	+	0	nt	9
BA0192	BB0193	BG0191	predicted coding region	1	nt	8	+	9
BA0329	BB0330	BG0331	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (<i>oppA-3</i>)	7	+	2	nt	9
BA0356	BB0359	BG0359	carboxyl-terminal protease (<i>ctp</i>)	9	+	0	nt	9
BA0619	BB0625	BG0644	N-acetylmuramoyl-L-alanine amidase, putative	9	+	0	nt	9
BA0733	BB0740	BG0761	hypothetical protein	1	nt	8	+	9
BAP021	BBG33	-	hypothetical protein	8	+	1	nt	9
BAP022	BBH09	BGP199	hypothetical protein	8	+	1	nt	9
BAB12	BBM26	BGP191	hypothetical protein	0	nt	9	+	9
BAK08	BBQ32	BGP190	hypothetical protein	8	+	1	nt	9
BA0429	BB0434	BG0441	stage 0 sporulation protein J (<i>spo0J</i>)	2	+	7	+	9
BA0055	BB0057	BG0056	glyceraldehyde 3-phosphate dehydrogenase (<i>gap</i>)	1	nt	7	+	8
BA0507	BB0512	BG0523	hypothetical protein	6	+	2	nt	8
BA0514	BB0519	BG0530	GrpE protein (<i>grpE</i>)	5	+	3	-	8
BA0596	BB0603	BG0616	membrane-associated protein p66	8	+	0	nt	8
BA0605	BB0612	BG0628	ATP-dependent Clp protease, subunit X (<i>clpX</i>)	6	+	2	nt	8
BA0790	BB0794	BG0820	hypothetical protein	8	+	0	nt	8
BAP029	BBO40	BGP138	erpM protein	0	nt	8	+	8
BAP033	BBQ03	BGP310	outer membrane protein, putative	7	+	1	nt	8
BAP025	BBQ13	BGP040	hypothetical protein	6	+	2	nt	8
BAP040	-	BGP022	hypothetical protein	7	+	1	nt	8
BA0215	BB0215	BG0218	phosphate ABC transporter, periplasmic phosphate-binding protein (<i>pstS</i>)	5	+	2	-	7
BA0321	BB0323	BG0324	hypothetical protein	6	+	1	nt	7
BA0345	BB0347	BG0348	fibronectin/fibrinogen-binding protein, putative	0	nt	7	+	7
BA0352	BB0353	BG0354	hypothetical protein	7	+	0	nt	7
BA0364	BB0366	BG0365	aminopeptidase I (<i>yscI</i>)	5	+	2	nt	7
BA0419	BB0420	BG0423	sensory transduction histidine kinase, putative	7	+	0	nt	7
BA0643	BB0648	BG0671	serine/threonine kinase, putative	3	nt	4	+	7
BA0644	BB0649	BG0672	heat shock protein (<i>groEL</i>)	7	+	0	nt	7
BA0663	BB0668	BG0691	flagellar filament outer layer protein (<i>flaA</i>)	6	+	1	nt	7
BAP031	BBR42	BGP260	outer surface protein F	5	+	2	-	7

Ba ORF	Bb ORF	Bg ORF	Common name	FhuA		LamB		Summary
				Hits	WB	Hits	WB	Hits
BAP042	-	BGP085	hypothetical protein	6	+	1	nt	7
BAP044	-	BGP321	hypothetical protein	7	+	0	nt	7
BA0152	BB0151	BG0149	N-acetylglucosamine-6-phosphate deacetylase (<i>nagA</i>)	5	+	1	nt	6
BA0181	BB0181	BG0180	flagellar hook-associated protein (<i>flgK</i>)	6	+	0	nt	6
BA0251	BB0252	BG0255	hypothetical integral membrane protein	3	nt	3	+	6
BA0283	BB0283	BG0286	flagellar hook protein (<i>flgE</i>)	6	+	0	nt	6
BA0295	BB0295	BG0298	heat shock protein (<i>hslU</i>)	6	+	0	nt	6
BA0300	BB0300	BG0304	cell division protein (<i>ftsA</i>)	0	nt	6	+	6
BA0380	BB0382	BG0381	basic membrane protein B (<i>bmpB</i>)	4	+	2	-	6
BA0389	BB0389	BG0390	DNA-directed RNA polymerase (<i>rpoB</i>)	5	+	1	nt	6
BA0606	BB0613	BG0629	ATP-dependent protease LA (<i>lon-2</i>)	6	+	0	nt	6
BA0653	BB0658	BG0681	phosphoglycerate mutase (<i>gpmA</i>)	6	+	0	nt	6
BA0778	BB0783	BG0807	hypothetical protein	6	+	0	nt	6
BA0781	BB0786	BG0811	general stress protein (<i>ctc</i>)	0	nt	6	+	6
BA0792	BB0796	BG0822	hypothetical protein	6	+	0	nt	6
BAP009	BBA73	BGA73	antigen, P35, putative	6	+	0	nt	6
BAP011	BBB16	BGB15	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (<i>oppAIV</i>)	5	+	1	nt	6
BAP025	BBL06	BGP173	hypothetical protein	5	+	1	nt	6
BAP031	BBO39	BGP260	erpL protein	6	+	0	nt	6
BA0582	BB0589	BG0602	phosphate acetyltransferase	0	nt	6	+	6
BA0745	BB0751	BG0773	hypothetical protein	3	+	2	+	5
BA0015	BB0015	BG0015	uridine kinase (<i>udk</i>)	2	-	3	+	5
BA0072	BB0074	BG0073	peptide chain release factor 2 (<i>prfB</i>)	5	+	0	nt	5
BA0078	BB0081	BG0079	hypothetical protein	4	+	1	nt	5
BA0235	BB0236	BG0229	hypothetical protein	5	+	0	nt	5
BA0388	BB0388	BG0389	DNA-directed RNA polymerase (<i>rpoC</i>)	3	+	2	nt	5
BA0417	BB0418	BG0421	hypothetical protein	4	+	1	nt	5
BA0572	BB0579	BG0591	DNA polymerase III, subunit alpha (<i>dnaE</i>)	4	+	1	nt	5
BAP023	BBJ41	BGA66	antigen, P35, putative	4	+	1	nt	5
BAP030	BBO34	BGP314	hypothetical protein	5	+	0	nt	5
BAP032	BBP28	BGP028	Lipoprotein	5	+	0	nt	5
BAB21	BBQ41	BGP313	hypothetical protein	1	nt	4	+	5
BA0075	BB0077	BG0076	hypothetical protein	4	+	0	nt	4
BA0087	BB0090	BG0091	V-type ATPase, subunit K, putative	1	nt	3	+	4
BA0126	BB0127	BG0129	ribosomal protein S1 (<i>rpsA</i>)	3	+	1	nt	4
BA0314	BB0315	BG0318	hypothetical protein	3	+	1	nt	4
BA0539	BB0546	BG0556	hypothetical protein	3	+	1	nt	4
BA0546	BB0553	BG0563	hypothetical protein	4	+	0	nt	4
BA0564	BB0571	BG0582	uridylate kinase (<i>smbA</i>)	2	-	2	+	4
BA0581	BB0588	BG0601	pfs protein (<i>pfs-2</i>)	3	+	1	nt	4
BA0665	BB0670	BG0693	purine-binding chemotaxis protein (<i>cheW-3</i>)	0	nt	4	+	4
BA0748	BB0754	BG0776	ABC transporter, ATP-binding protein	3	+	1	nt	4

Ba ORF	Bb ORF	Bg ORF	Common name	FhuA		LamB		Summary
				Hits	WB	Hits	WB	Hits
BA0784	BB0789	BG0814	cell division protein (<i>ftsH</i>)	3	+	1	nt	4
BAP003	BBA04	BGA03	antigen, S2	4	+	0	nt	4
BAP012	BBB17	BGB16	IMP dehydrogenase (<i>guaB</i>)	4	+	0	nt	4
BAP014	BBC01	BGP297	hypothetical protein	2	-	2	+	4
BAB34	BBN01	BGP242	hypothetical protein	2	-	2	+	4
BAP045	BBS42	-	associated protein A	2	+	2	nt	4
BA0091	BB0094	BG0095	V-type ATPase, subunit A (<i>atpA</i>)	3	+	0	nt	3
BA0106	BB0109	BG0110	acetyl-CoA C-acetyltransferase (<i>fadA</i>)	3	+	0	nt	3
BA0198	BB0199	BG0197	hypothetical protein	0	nt	3	+	3
BA0200	BB0200	BG0199	D-alanine--D-alanine ligase (<i>ddlA</i>)	0	nt	3	+	3
BA0231	BB0232	BG0235	HbbU protein	0	nt	3	+	3
BA0343	BB0345	BG0346	hypothetical protein	3	+	0	nt	3
BA0431	BB0436	BG0443	DNA gyrase, subunit B (<i>gyrB</i>)	3	+	0	nt	3
BA0442	BB0446	BG0454	aspartyl-tRNA synthetase (<i>aspS</i>)	3	+	0	nt	3
BA0469	BB0472	BG0484	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (<i>murA</i>)	3	+	0	nt	3
BA0475	BB0479	BG0490	ribosomal protein L4 (<i>rplD</i>)	2	+	1	nt	3
BA0601	BB0608	BG0621	aminoacyl-histidine dipeptidase (<i>pepD</i>)	3	+	0	nt	3
BA0609	BB0615	BG0632	ribosomal protein S4 (<i>rpsD</i>)	1	nt	2	+	3
BA0767	BB0772	BG0796	flagellar P-ring protein (<i>flgI</i>)	3	+	0	nt	3
BA0829	BB0832	BG0857	hypothetical protein	3	+	0	nt	3
BA0841	BB0843	-	hypothetical integral membrane protein	0	nt	3	+	3
BAP004	BBA21	BGA19	hypothetical protein	1	nt	2	+	3
BAB14	BBL28	BGP273	Lipoprotein	3	+	0	nt	3
BAP027	BBM03	BGP043	hypothetical protein	3	+	0	nt	3
BAP026	BBR27	-	hypothetical protein	3	+	0	nt	3
BA0062	BB0064	BG0063	methionyl-tRNA formyltransferase (<i>fnt</i>)	2	+	0	nt	2
BA0240	BB0241	BG0244	glycerol kinase (<i>glpK</i>)	0	nt	2	+	2
BA0464	BB0467	BG0480	hypothetical protein	2	+	0	nt	2
BA0754	BB0760	BG0784	hypothetical protein	0	nt	2	+	2
BA0682	BB0687	BG0710	phosphomevalonate kinase, putative	2	+	0	nt	2
BA0703	BB0710	BG0733	DNA primase (<i>dnaG</i>)	2	+	0	nt	2
BAP016	BBG12	BGP025	hypothetical protein	2	+	0	nt	2
BA0582	BB0589	BG0061	phosphate acetyltransferase	0	nt	2	+	2
BAP043	-	BGP267	hypothetical protein	2	+	0	nt	2

Table 4.6: List of antigens selected in all 12 K78-FhuA and K78-LamB screens. Selected antigens are listed according to total number of hits. +, antigen tested positive in Western blot analysis. -, ORF was tested by Western blot analysis and showed no reaction with human IgGs. nt, not tested. Ba, *B. afzelii*; Bb, *B. burgdorferi*; Bg, *B. garinii*.

Altogether, 151 candidates showed positive reaction with human IgGs by *E. coli* lysate Western analysis. 18 antigens were selected by both libraries, 10 with the K78-LamB and 123 with the K78-FhuA libraries. Interesting observation was that most of the candidates

selected with screening of display libraries with LB IgGs pools were also selected when screened with LA pool. The three pools, EM, NB and ACA, showed much more heterogeneity especially for candidates selected with lower number of hits.

The most immunogenic among the 151 selected candidates was the hypothetical protein BAP001 (BB0844), selected 313 times by screening with libraries. Second among the most immunogenic candidates with 147 hits was immunogenic protein P35 (BAP024 or BBK32). As a fibronectin binding protein this candidate has a very important function in disease pathogenicity (Probert et al., 2001). Another very interesting and highly immunogenic candidate was flagellar filament core protein (BA0149 or BB0147). Since *Borrelia* has periplasmic flagella this is another proof for fluidity of borrelial outer membrane. In general, hypothetical proteins were frequent among the most immunogenic candidates, and with 64 hypothetical proteins selected altogether they form the largest group of the ANTIGENome. Percentage of hypothetical proteins identified (42%) doesn't differ from the percentage in the borrelial genome (41%). Among selected antigens with known function borrelial lipoproteins or outer surface proteins were selected (e.g. Outer surface protein C) forming another important group. Many proteins with function involved in dissemination or immune evasion of *Borrelia* were also selected, like immunogenic fibronectin binding protein BAP024 (BBK32), decorin binding protein A, and membrane associated protein P66.

The percentage and the number of hits of selected proteins involved in DNA metabolism and transcription was low (12%). Since these are unlikely to be interesting for vaccine development, these proteins were not included in further validation assays.

4.2 Antigen validation

In order to select the most promising antigens as vaccine candidates for animal testing, 132 selected antigens were subjected to *in vitro* validation. The major aspect of the *in vitro* validation was the investigation of the presence of the genes encoding the selected antigens in a collection of pathogenic *Borrelia* spp. strains causing Lyme borreliosis. Further, selected antigens were tested for immune responses in humans using peptides covering the

selected epitopes. Finally, surface localization of selected antigens on *in vitro* cultivated cells was determined by immune staining and detected by flow cytometry.

4.2.1 Gene distribution studies with different isolates of *Borrelia* spp.

The 132 antigens, identified in the bacterial surface display screens, were tested by PCR for the presence of the encoding gene in 30 different isolates (Table 3.1), which belonged to 24 different *ospC* types. Genes with high prevalence among clinically relevant strains were considered for further studies. Oligonucleotides were designed and synthesized that enabled the amplification of the genes corresponding to the identified antigens. Conserved regions of genes flanking the region of the selected clone, when possible, were used for design of the primers. The PCR experiments were performed first with 20 strains (strains 1-20), then repeated with the additional 10 strains (strains 21-30). This procedure was selected, because not all strains were available at the start of the gene distribution analysis. Figure 4.7 shows example of the PCR analysis for two genes, BBA21 and BBG10 with strains selected for analysis during my study. The BBA21 gene was present in all strains, while BBG10 was missing in 50% of strains, in addition it showed variation in size. From a total of 132 genes analyzed, 42 were present in 100% of the strains tested, 36 in > 85%, while 54 genes were absent in more than 25% of the strains tested, as listed in Table 4.7. Despite several trials, eight plasmid and one chromosome encoded genes couldn't be amplified from any of tested strains, probably due to either high sequence variability or plasmid loss.

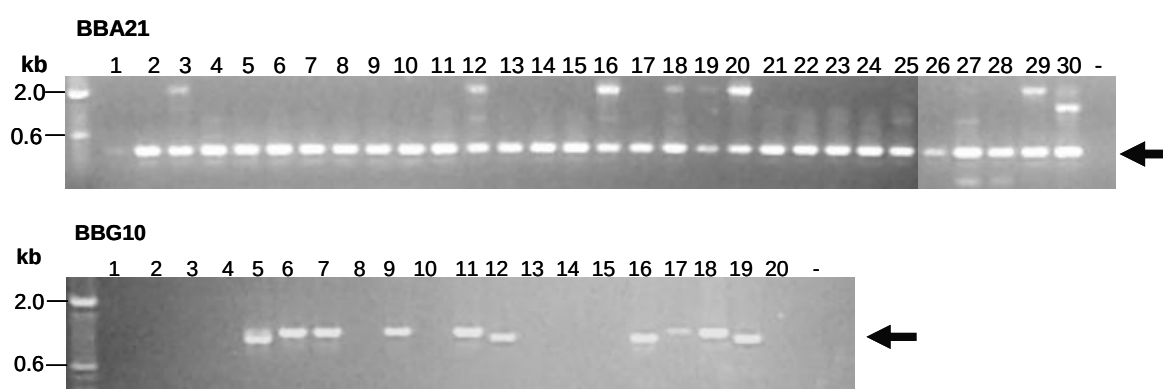


Figure 4.7: Gene distribution example. (A) PCR analysis of BBA21. Predicted size of the amplified fragment was 420 bp. (B) PCR analysis of BBG10. Predicted size of the amplified fragment was 1100 bp. The arrow indicates the position of the specific amplicon. 1-30, *Borrelia burgdorferi* s.l. strains listed in Table 3.1; -, no genomic DNA added. Oligonucleotides used for the gene distribution analysis are listed in Table A1, Appendices.

Ba ORF	Bb ORF	Bg ORF	Common name	PCR1	PCR2	positive tested
BA0056	BB0058	BG0057	hypothetical protein	17/20	9/10	26/30
BA0060	BB0062	BG0061	hypothetical protein	20/20	10/10	30/30
BA0062	BB0064	BG0063	methionyl-tRNA formyltransferase (fmt)	19/20	nd	19/20
BA0072	BB0074	BG0073	peptide chain release factor 2 (prfB)	20/20	nd	20/20
BA0075	BB0077	BG0076	hypothetical protein	6/20	nd	6/20
BA0078	BB0081	BG0079	hypothetical protein	20/20	10/10	30/30
BA0087	BB0090	BG0091	V-type ATPase, subunit K, putative	20/20	10/10	30/30
BA0091	BB0094	BG0095	V-type ATPase, subunit A (atpA)	20/20	10/10	30/30
BA0106	BB0109	BG0110	acetyl-CoA C-acetyltransferase (fadA)	20/20	10/10	30/30
BA0126	BB0127	BG0129	ribosomal protein S1 (rpsA)	20/20	nd	20/20
BA0135	BB0136	BG0138	penicillin-binding protein (pbp-1)	20/20	10/10	30/30
BA0149	BB0147	BG0147	flagellar filament 41 kDa core protein (flaB)	20/20	nd	20/20
BA0150	BB0149	BG0148	flagellar hook-associated protein 2 (fliD)	20/20	nd	20/20
BA0152	BB0151	BG0149	N-acetylglucosamine-6-phosphate deacetylase (nagA)	20/20	nd	20/20
BA0181	BB0181	BG0180	flagellar hook-associated protein (flgK)	20/20	nd	20/20
BA0192	BB0193	BG0191	hypothetical protein	16/20	8/10	24/30
BA0200	BB0200	BG0199	D-alanine--D-alanine ligase (ddlA)	17/20	10/10	27/30
BA0210	BB0210	BG0212	surface-located membrane protein 1 (lmp1)	18/20	10/10	28/30
BA0215	BB0215	BG0218	phosphate ABC transporter, periplasmic phosphate-binding protein (pstS)	19/20	10/10	29/30
BA0221	BB0221	BG0224	flagellar motor switch protein (fliG-1)	20/20	nd	20/20
BA0235	BB0236	BG0229	hypothetical protein	20/20	10/10	30/30
BA0237	BB0238	BG0241	hypothetical protein	10/20	nd	10/20
BA0283	BB0283	BG0286	flagellar hook protein (flgE)	20/20	nd	20/20
BA0295	BB0295	BG0298	heat shock protein (hslU)	20/20	nd	20/20
BA0314	BB0315	BG0318	hypothetical protein	8/20	nd	8/20
BA0321	BB0323	BG0324	hypothetical protein	20/20	10/10	30/30
BA0327	BB0329	BG0330	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-2)	13/20	nd	13/20
BA0329	BB0330	BG0331	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-3)	20/20	10/10	30/30
BA0343	BB0345	BG0346	hypothetical protein	19/20	nd	19/20
BA0345	BB0347	BG0348	fibronectin/fibrinogen-binding protein, putative	14/20	5/10	19/30
BA0352	BB0353	BG0354	hypothetical protein	12/20	nd	12/20
BA0356	BB0359	BG0359	carboxyl-terminal protease (ctp)	18/20	10/10	28/30
BA0364	BB0366	BG0365	aminopeptidase I (yscI)	20/20	10/10	30/30
BA0380	BB0382	BG0381	basic membrane protein B (bmpB)	19/20	10/10	29/30
BA0388	BB0388	BG0389	DNA-directed RNA polymerase (rpoC)	17/20	nd	17/20
BA0389	BB0389	BG0390	DNA-directed RNA polymerase (rpoB)	20/20	nd	20/20
BA0417	BB0418	BG0421	hypothetical protein	20/20	10/10	30/30
BA0419	BB0420	BG0423	sensory transduction histidine kinase, putative	0/20	nd	0/20
BA0429	BB0434	BG0441	stage 0 sporulation protein J (spoJ)	9/20	nd	9/20
BA0431	BB0436	BG0443	DNA gyrase, subunit B (gyrB)	20/20	nd	20/20
BA0442	BB0446	BG0454	aspartyl-tRNA synthetase (aspS)	20/20	10/10	30/30
BA0454	BB0457	BG0466	excinuclease ABC, subunit C (uvrC)	19/20	10/10	29/30

Ba ORF	Bb ORF	Bg ORF	Common name	PCR1	PCR2	positive tested
BA0464	BB0467	BG0480	conserved hypothetical protein	20/20	10/10	30/30
BA0469	BB0472	BG0484	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (murA)	18/20	10/10	28/30
BA0475	BB0479	BG0490	ribosomal protein L4 (rplD)	16/20	nd	16/20
BA0507	BB0512	BG0523	hypothetical protein	20/20	10/10	30/30
BA0513	BB0518	BG0529	heat shock protein 70 (dnaK-2)	16/20	nd	16/20
BA0514	BB0519	BG0530	GrpE protein (grpE)	4/20	nd	4/20
BA0539	BB0546	BG0556	hypothetical protein	20/20	nd	20/20
BA0546	BB0553	BG0563	hypothetical protein	11/20	nd	11/20
BA0553	BB0560	BG0570	heat shock protein 90 (htpG)	19/20	nd	19/20
BA0564	BB0571	BG0582	uridylate kinase (smbA)	20/20	10/10	30/30
BA0572	BB0579	BG0591	DNA polymerase III, subunit alpha (dnaE)	20/20	nd	20/20
BA0581	BB0588	BG0601	pfs protein (pfs-2)	20/20	10/10	30/30
BA0596	BB0603	BG0616	membrane-associated protein p66	20/20	10/10	30/30
BA0601	BB0608	BG0621	aminoacyl-histidine dipeptidase (pepD)	16/20	nd	16/20
BA0605	BB0612	BG0628	ATP-dependent Clp protease, subunit X (clpX)	18/20	10/10	28/30
BA0606	BB0613	BG0629	ATP-dependent protease LA (lon-2)	13/20	nd	13/20
BA0619	BB0625	BG0644	N-acetylmuramoyl-L-alanine amidase, putative	13/20	nd	13/20
BA0643	BB0648	BG0671	serine/threonine kinase, putative	20/20	10/10	30/30
BA0644	BB0649	BG0672	heat shock protein (groEL)	19/20	nd	19/20
BA0653	BB0658	BG0681	phosphoglycerate mutase (gpmA)	20/20	10/10	30/30
BA0663	BB0668	BG0691	flagellar filament outer layer protein (flaA)	20/20	nd	20/20
BA0665	BB0670	BG0693	purine-binding chemotaxis protein (cheW-3)	20/20	10/10	30/30
BA0675	BB0680	BG0703	methyl-accepting chemotaxis protein (mcp-4)	20/20	10/10	30/30
BA0682	BB0687	BG0710	phosphomevalonate kinase, putative	20/20	10/10	30/30
BA0703	BB0710	BG0733	DNA primase (dnaG)	19/20	nd	19/20
BA0733	BB0740	BG0761	hypothetical protein	19/20	10/10	29/30
BA0737	BB0744	BG0765	antigen, p83/100	16/20	10/10	26/30
BA0745	BB0751	BG0773	hypothetical protein	20/20	10/10	30/30
BA0748	BB0754	BG0776	ABC transporter, ATP-binding protein	20/20	10/10	30/30
BA0754	BB0760	BG0784	hypothetical protein	20/20	10/10	30/30
BA0759	BB0765	BG0788	hypothetical protein	20/20	10/10	30/30
BA0767	BB0772	BG0796	flagellar P-ring protein (flgI)	19/20	nd	19/20
BA0778	BB0783	BG0807	hypothetical protein	1/20	nd	1/20
BA0784	BB0789	BG0814	cell division protein (ftsH)	20/20	10/10	30/30
BA0790	BB0794	BG0820	hypothetical protein	16/20	10/10	26/30
BA0792	BB0796	BG0822	hypothetical protein	20/20	10/10	30/30
BA0829	BB0832	BG0857	hypothetical protein	18/20	10/10	28/30
BAP001	BB0844	BGP096	hypothetical protein	5/20	nd	5/20
BAP002	BBA03	BGA02	outer membrane protein	8/20	nd	8/20
BAP003	BBA04	BGA03	antigen, S2	14/20*	8/10*	22/30
BAP004	BBA21	BGA19	hypothetical protein	20/20	10/10	30/30
BAP005	BBA24	BGA21	decorin binding protein A (dbpA)	0/20	nd	0/20
BAP006	BBA34	BGA28	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppAV)	19/20	10/10	29/30

Ba ORF	Bb ORF	Bg ORF	Common name	PCR1	PCR2	positive tested
BAP007	BBA36	BGA30	lipoprotein	20/20	10/10	30/30
BAP008	BBA57	BGA58	hypothetical protein	8/20	nd	8/20
BAP009	BBA73	BGA73	antigen, P35, putative	11/20	nd	11/20
BAP011	BBB16	BGB15	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppAIV)	4/20	nd	4/20
BAP012	BBB17	BGB16	IMP dehydrogenase (guaB)	20/20	nd	20/20
BAP013	BBB19	BGB18	outer surface protein C (ospC)	20/20	10/10	30/30
BAP014	BBC01	BGP297	conserved hypothetical protein	9/20	3/10	12/30
BAP015	BBG10	BGP027	hypothetical protein	11/20	nd	11/20
BAP016	BBG12	BGP025	hypothetical protein	13/20	nd	13/20
BAP017	BBG21	BGP116	hypothetical protein	12/20	nd	12/20
BAP018	BBG23	BGP013	hypothetical protein	2/20	nd	2/20
BAP019	BBG24	BGP012	hypothetical protein	11/20	nd	11/20
BAP020	BBG30	BGP003	hypothetical protein	0/20	0/10	0/30
BAP021	BBG33	-	hypothetical protein	18/20	4/10	22/30
BAP022	BBH09	BGP199	hypothetical protein	16/20	10/10	26/30
BAP021	BBH13	-	hypothetical protein	4/20	nd	4/20
BAP023	BBJ41	BGA66	antigen, P35, putative	17/20	9/10	26/30
BAP024	BBK32	BGP100	immunogenic protein P35	13/20	5/10	18/30
BAP025	BBL06	BGP173	hypothetical protein	8/20	nd	8/20
BAP026	BBL27	BGP132	hypothetical protein	8/20	nd	8/20
BAB14	BBL28	BGP273	lipoprotein	6/20	nd	6/20
BAP027	BBM03	BGP043	hypothetical protein	16/20	10/10	26/30
BAP028	BBM15	BGP069	hypothetical protein	8/20	nd	8/20
BAA18	BBM29	BGP292	hypothetical protein	8/20	nd	8/20
BAP029	BBN39	-	ErpB2 protein	5/20	nd	5/20
BAP030	BBO34	BGP314	hypothetical protein	11/20	nd	11/20
BAP031	BBO39	BGP260	erpL protein	4/20	nd	4/20
BAP029	BBO40	BGP138	erpM protein	4/20	2/10	6/30
BAP032	BBP28	BGP028	lipoprotein	0/20	nd	0/20
BAP033	BBQ03	BGP310	outer membrane protein, putative	12/20	nd	12/20
BAP025	BBQ13	BGP040	hypothetical protein	20/20	10/10	30/30
BAP024	BBQ32	BGP190	hypothetical protein	10/20	nd	10/20
BAP025	BBQ34	BGP110	hypothetical protein	20/20	8/10	28/30*
BAP026	BBR27	-	hypothetical protein	8/20	nd	8/20
BAP026	BBR35	-	hypothetical protein	10/20	nd	10/20
BAP031	BBR42	BGP260	outer surface protein F	8/20	nd	8/20
BAP038	BBS36	BGP301	hypothetical protein	4/20	nd	4/20
BAP031	BBS41	-	outer surface protein G	4/20	nd	4/20
BAP045	BBS42	-	associated protein A	9/20	nd	9/20
BAP039	-	BGA26	hypothetical protein	0/20	nd	0/20
BAP035	-	BGP001	hypothetical protein	9/20	nd	9/20
BAP040	-	BGP022	hypothetical protein	0/20	nd	0/20
BAP041	-	BGP036	hypothetical protein	0/20	nd	0/20
BAP029	-	BGP049	hypothetical protein	0/20	nd	0/20

Ba ORF	Bb ORF	Bg ORF	Common name	PCR1	PCR2	<u>positive tested</u>
BAP042	-	BGP085	hypothetical protein	3/20	nd	3/20
BAP043	-	BGP267	hypothetical protein	0/20	nd	0/20
BAP044	-	BGP321	hypothetical protein	1/20	nd	1/20

Table 4.7: Gene conservation analysis. Ba, *B. afzelii*; Bb, *B. burgdorferi* s.s.; Bg, *B. garinii*; PCR1, polymerase chain reaction analysis with strains 1 to 20 (Table 3.1); PCR2, polymerase chain reaction analysis with strains 21 to 30 (Table 3.1), * Oligonucleotides cross-hybridise with paralogous genes.

4.2.2 Serological analysis of selected epitopes of identified *Borrelia* antigens

In order to assess the broadness of immunogenicity, the presence of antibodies in individual (not pooled!) human sera specific for the identified *Borrelia* epitopes was determined by peptide ELISA. The human sera used for this analysis (listed in Table A3, Appendices) corresponded to those that were included in the various serum pools applied for the identification of antigens by the bacterial surface display screens. For those clone sequences that encompassed more than 30 amino acids (most of the K78-FhuA clones), multiple peptides of 25 amino acids were designed with an overlap of 5 to 6 amino acids. For K78-LamB clones one peptide per clone was enough to cover immunogenic region. In total, 538 synthetic peptides representing epitopes (clone regions) identified in the 120 ORFs from *B. afzelii* K78 were designed and synthesized. Many of these peptides were shown to be immunogenic in humans and the results for the 100 most immunogenic peptides are shown in Figure 4.8. Peptides were ranked according to the score that was calculated based on signal intensity and number of reactive sera. It is evident that some of the selected peptides were highly reactive with many of the tested human sera (e.g. BAOba0565.02 or BAOba0077.01), while others showed intermediate reactivity (Figure 4.8). For some of the antigens, it was observed that multiple peptides from the same antigens showed different reactivity, further delineating the immunogenic region of the respective antigen (e.g. BA0314 or BA0546). The number of antigens selected only by screening with the K78-LamB library was low, thus the number of peptides covering these epitopes was also low. From 18 peptides covering K78-LamB clones, only 4 showed reaction with human sera, and only one with significant reactivity. Peptides that cover K78-FhuA epitopes are more numerous (529 peptides) and more immunogenic in particular. All peptides analyzed for reactivity with 22 human sera are listed in Table A4, Appendices.

Peptide	Ba ORF	P1755	P1766	P1772	P1806	P1811	P3055	P3144	P3186	P3219	P3077	P3084	P3288	P3150	P3301	P3344	P3394	P3183	P3336	P1789	P1796	P1938	P1941	Score
BAObbb19.03	BAP013																							50
BAObbs42.01	BAP045																							47
BAObgp096.01	BAP001																							46
BAOba0565.02	BA0546																							45
BAOba0378.04	BA0364																							42
BAObb0612.04	BA0605																							42
BAObb0238.02	BA0237																							42
BAOba0077.01	BA0075																							39
BAOamds.11	BA0619																							39
BAObgp096.03	BAP001																							39
BAOba0323.03	BA0314																							37
BAObb0181.06	BA0181																							37
BAObb0359.01	BA0356																							37
BAObgp036.01	BAP041																							37
BAObb0359.03	BA0356																							36
BAOba0128.04	BA0126																							36
BAObb0512.06	BA0507																							36
BAObb0680.03	BA0675																							36
BAObbq34.02	BAP043																							36
BAObgp43.03	BAP044																							35
BAOpbp-1.08	BA0135																							35
BAObb0359.04	BA0356																							35
BAObbb19.04	BAP013																							35
BAObgp321.02	BAP044																							34
BAObb0796.01	BA0792																							34
BAOoppA-4.01	BAP006																							34
BAOba0128.02	BA0126																							33
BAObgp027.22	BAP015																							33
BAObb0058.01	BA0056																							33
BAObb0512.03	BA0507																							33
BAObb106.04	BAP025																							33
BAObbr35.01	BAP036																							32
BAOba0242.03	BA0235																							31
BAOba0403.02	BA0389																							31
BAObb0512.04	BA0507																							31
BAObbr35.02	BAP036																							31
BAObbs36.06	BAP037/8																							31
BAObb0215.04	BA0215																							31
BAObb0418.01	BA0417																							31
BAObgp321.03	BAP044																							30
BAOba0819.02	BA0784																							30
BAObb0353.04	BA0351																							30

Peptide	Ba ORF	P1755	P1766	P1772	P1806	P1811	P3055	P3144	P3186	P3219	P3077	P3084	P3288	P3150	P3301	P3344	P3394	P3183	P3336	P1789	P1796	P1938	P1941	Score
BAObb0658.02	BA0653																							30
BAObb0658.03	BA0653																							30
BAObb106.02	BAP025																							29
BAObb0612.03	BA0605																							29
BAOba0077.02	BA0075																							29
BAOba0378.02	BA0364																							29
BAOf1aB.13	BA0149																							29
BAOba0358.01	BA0345																							29
BAOf1aA.01	BA0663																							28
BAOppA-3.06	BA0329																							28
BAOba0485.02	BA0469																							28
BAObb0612.01	BA0605																							28
BAOf1iG-1.04	BA0221																							28
BAObgp096.06	BAP001																							28
BAOba0403.05	BA0389																							27
BAOba0779.01	BA0745																							27
BAObb0353.03	BA0351																							27
BAObb0612.05	BA0605																							27
BAOamds.07	BA0619																							27
BAObb0613.02	BA0606																							27
BAOba0670.01	BA0643																							26
BAObb0058.08	BA0056																							26
BAObb0680.02	BA0675																							26
BAOba0710.02	BA0682																							26
BAOppA-2.16	BA0327																							25
BAObb19.02	BAP013																							25
BAObgp085.01	BAP042																							25
BAObb0512.01	BA0507																							24
BAObb0512.05	BA0507																							24
BAObb0353.02	BA0351																							24
BAObb0680.05	BA0675																							24
BAOf1aA.05	BA0663																							23
BAOamds.08	BA0619																							23
BAOba0128.01	BA0126																							23
BAObb0512.02	BA0507																							23
BAObbg24.02	BAP019																							23
BAOba0623.02	BA0601																							23
BAOba0356.01	BA0343																							23
BAOba0403.03	BA0389																							22
BAObb0658.01	BA0653																							22
BAOba0832.03	BA0784																							22
BAOppA-2.08	BA0327																							22

Peptide	Ba ORF	P1755	P1766	P1772	P1806	P1811	P3055	P3144	P3186	P3219	P3077	P3084	P3288	P3150	P3301	P3344	P3394	P3183	P3336	P1789	P1796	P1938	P1941	Score
BAOba0323.02	BA0314																							22
BAObgp321.01	BAP044																							22
BAObb0612.02	BA0605																							22
BAObbb19.01	BAP013																							22
BAObb0359.02	BA0356																							21
BAOp66.04	BA0596																							21
BAOoppA-2.15	BA0327																							21
BAObb0353.01	BA0351																							21
BAOba0303.01	BA0295																							21
BAOfiaB.04	BA0149																							20
BAObb0649.03	BA0644																							20
BAObbg24.05	BAP019																							20
BAOba0089.01	BA0087																							20
BAOba0565.01	BA0546																							19
BAOba0356.03	BA0343																							19
BAOba0491.01	BA0475																							19

Figure 4.8: Peptide ELISA with human sera. Immune reactivities with human sera are shown for the 100 most immunogenic peptides which cover selected antigenic regions. Peptides are named as follows: BAO, *B. afzelii* sequence as template; ORF name (e.g. bb0058) or gene name (e.g. oppA-3); .01-.22, peptide number. Blue shaded cell, < 50 ELISA units; yellow shaded cell, between 50 and 100 ELISA units; orange shaded cell, between 100 and 200 ELISA units; red shaded cell, values > 200 ELISA units. The total reactivity score is calculated with 22 human sera analyzed.

4.2.3 Determination of the surface location of antigens

Antigens were selected for analysis of surface exposure on live *Borrelia* by flow cytometry. Epitope specific immune sera were generated in mice with immunization of total bacterial lysates prepared from MACS screen selected clones. Selected clones were previously stored at -80°C (with glycerol). However, some of the clones couldn't be re-inoculated and growth after storage, which caused decrease in the number of candidate antigens. Mice were immunized with 169 bacterial surface display clones, representing 113 antigens. These 169 clones were distributed in 130 groups containing one to three clones corresponding to the same antigens. Antigens with large epitope identified were represented by more than one hyper-immune sera.

Mice were immunized twice with 100 µg bacterial cell lysate with two weeks interval. One week after the second immunization blood samples were taken for generation

of hyper-immune sera. Sera were characterized for epitope reactivity by Western blot with respective library clones and in flow cytometry (FC) with library strain K78. Prior to FC analysis sera were diluted 1:50 with BSK II medium, heat inactivated and incubated with *B. afzelii* strain K78, as described by Materials and Methods. Three representative FC graphs are shown in Figure 4.9, and the complete results listed in Table 4.8. When compared to the buffer control, we could see a shift of at least 20% of the cells with 40 hyper-immune sera. These 40 sera represent 32 antigens, from which one antigen is represented by two related orthologs, BAP037 and BAP038, with 61% amino acid identity. 11 antigens showed prominent shift of over 70%. Among these are some hypothetical proteins (e.g. BA0056, BAP019, BAP037), flagellar proteins (e.g. BA0221), and immunogenic proteins (e.g. BAP024, BA0737).

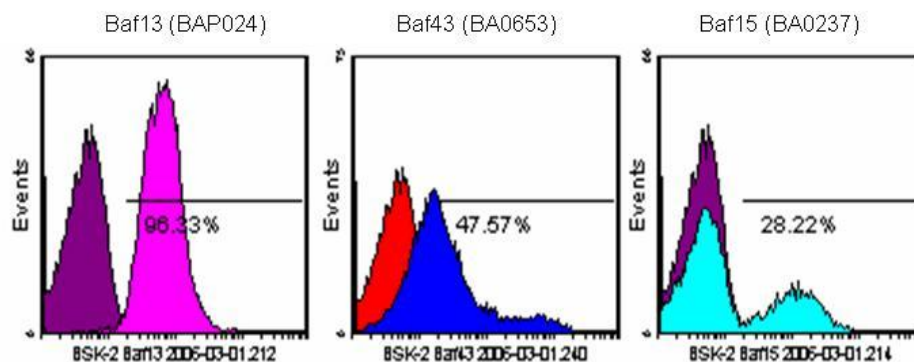


Figure 4.9: Surface staining with epitope specific immune sera. Three representative graphs showing sera with high (Baf13), medium (Baf43) and low (Baf15) staining intensity by flow cytometry.

BA ORF	Serum ID	FC pos %
BA0056	Baf28	91
	Baf29	90
BA0060	Baf137	6
BA0062	Baf89	2
BA0072	Baf70	3
BA0075	Baf104	16
BA0078	Baf77	10
BA0087	Baf127	4
BA0091	Baf82	11
BA0106	Baf83	9
BA0126	Baf113	8
BA0135	Baf22	75
BA0149	Baf11	60
	Baf12	46
BA0150	Baf23	23
	Baf24	43

BA ORF	Serum ID	FC pos %
BA0665	Baf128	8
BA0675	Baf45	19
BA0682	Baf100	15
BA0703	Baf101	8
BA0733	Baf135	15
BA0737	Baf16	83
BA0745	Baf110	5
BA0748	Baf87	16
BA0754	Baf141	16
BA0759	Baf46	9
BA0767	Baf121	27
BA0778	Baf47	14
BA0784	Baf96	17
BA0790	Baf48	67
BA0792	Baf49	13
BA0829	Baf111	8

BA ORF	Serum ID	FC pos %
BA0152	Baf114	9
BA0181	Baf35	18
	Baf36	62
BA0192	Baf134	7
BA0200	Baf132	10
BA0210	Baf1	36
	Baf2	80
BA0215	Baf71	7
BA0221	Baf26	87
BA0235	Baf105	19
BA0237	Baf15	28
BA0283	Baf37	62
BA0321	Baf58	0
BA0327	Baf18	77
	Baf19	58
	Baf20	18
BA0329	Baf34	25
BA0345	Baf131	7
BA0351	Baf38	12
BA0356	Baf55	12
BA0364	Baf93	14
BA0388	Baf107	10
BA0389	Baf118	10
BA0417	Baf78	9
BA0419	Baf59	3
BA0429	Baf97	16
BA0431	Baf84	10
BA0442	Baf85	8
BA0464	Baf98	13
BA0469	Baf108	20
BA0475	Baf99	10
BA0507	Baf39	21
BA0513	Baf64	3
BA0514	Baf60	6
BA0539	Baf119	7
BA0546	Baf109	13
BA0553	Baf90	8
BA0564	Baf133	0
BA0581	Baf86	13
BA0596	Baf40	30
	Baf41	27
BA0601	Baf120	8
BA0605	Baf42	45
BA0619	Baf30	24
	Baf31	15
BA0643	Baf129	7
BA0644	Baf72	2

BA ORF	Serum ID	FC pos %
BAP001	Baf69	2
BAP002	Baf65	2
BAP003	Baf80	16
BAP004	Baf140	9
BAP005	Baf66	4
BAP006	Baf25	30
BAP007	Baf67	6
BAP008	Baf91	6
BAP012	Baf122	14
BAP013	Baf94	10
BAP014	Baf139	7
BAP015	Baf6	76
	Baf7	8
	Baf8	21
BAP016	Baf123	7
BAP017	Baf3	2
BAP018	Baf63	3
BAP019	Baf4	64
	Baf5	86
BAP020	Baf136	13
BAP021	Baf73	2
	Baf143	26
BAP022	Baf50	28
BAP023	Baf81	19
BAP024	Baf13	96
	Baf14	13
BAP025	Baf51	11
	Baf75	22
BAP026	Baf88	12
BAP027	Baf103	8
BAP028	Baf33	12
	Baf57	4
BAP029	Baf138	9
BAP030	Baf52	22
BAP031	Baf92	9
	Baf54	13
BAP032	Baf74	2
BAP033	Baf32	74
BAP034	Baf53	39
BAP035	Baf125	20
BAP036	Baf112	6
BAP037 / BAP038	Baf10	86
BAP039	Baf124	5
BAP040	Baf126	12
BAP041	Baf142	10
BAP042	Baf76	1
BAP043	Baf68	2

BA ORF	Serum ID	FC pos %	BA ORF	Serum ID	FC pos %
BA0653	Baf43	48	BAP044	Baf95	14
BA0663	Baf44	14	BAP045 / BBS42	Baf102	11

Table 4.8: Results of the surface staining of *Borrelia* by flow cytometric detection. Data are expressed as the percentage of cells with increased fluorescence relative to the buffer control.

4.2.4 Selection of the most promising vaccine candidates – ranking based on the *in vitro* validation results

In order to reduce the number of vaccine candidates and select the most promising ones, several validation steps were applied: (i) gene conservation among diverse *Borrelia* strains, (ii) immunogenicity in humans and (iii) surface location, considering number of hits and analysis of the (iv) selection frequency and (v) novelty (intellectual property analysis). This resulted in the selection of 25 antigens. All 25 antigens together with their ORF names, results of different validation assays and protein sequence homology between three most prevalent human pathogenic *Borrelia* genospecies are listed in Table 4.9. 22 candidates showed beside good gene distribution among tested strains (87-100%) also high reaction with human sera and/or were confirmed as surface located. The other three candidates - BA0581, BA0380, BA0345 - were selected also based on their predicted function. BA0581 is the pfs protein (pfs-2; Schauder et al., 2001) and play a role in quorum sensing. In addition, this candidate was present in all tested *Borrelia* strains. BA0380 showed also a high prevalence among tested strains (97%) and it is a basic membrane protein B (bmp B; Pal et al., 2008). The percentage of strains positive for BA0345 wasn't that high, only 63%, however its role as a fibronectin/fibrinogen-binding protein evoked our interest.

All antigens showed >70% aa sequence homology at the protein level, which is very important since we want to find those that will protect against all three pathogenic species. 16 from 25 selected candidates were identified as surface located, and 17 showed high immune reactivity with human sera in peptide ELISA. Regarding gene prevalence, majority of the candidates (19 of the 25) were detected in more than 80% of tested *Borrelia* strains.

Ba ORF	Bb ORF	Bg ORF	Common names	Mw Bb (kDa)	Hits	GC %	Surface staining	Peptide ELISA (score)	Protein homology Ba-Bb (%)*	Protein homology Ba-Bg (%)*
BA0364	BB0366	BG0365	aminopeptidase I (yscI)	51,6	7	100	-	42	96	95
BA0507	BB0512	BG0523	hypothetical protein	254,2	8	100	21	36	93	94
BA0675	BB0680	BG0703	methyl-accepting chemotaxis protein (mcp-4)	84,7	12	100	-	36	92	95
BA0135	BB0136	BG0138	penicillin-binding protein (pbp-1)	71,9	10	100	75	35	94	94
BA0792	BB0796	BG0822	hypothetical protein	21,6	6	100	-	34	91	96
BA0235	BB0236	BG0239	hypothetical protein	75,2	5	100	-	31	95	96
BA0417	BB0418	BG0421	hypothetical protein	38,9	5	100	-	31	86	91
BA0653	BB0658	BG0681	phosphoglycerate mutase (gpmA)	29,1	6	100	48	30	95	92
BA0221	BB0221	BG0224	flagellar motor switch protein (fliG-1)	47,9	11	100	87	28	91	91
BA0329	BB0330	BG0331	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-3)	62,5	9	100	25	28	93	94
BA0581	BB0588	BG0601	pfs protein (pfs-2)	29,2	4	100	-	-	87	86
BAP006	BBA34	BGA28	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppAV)	61,1	10	97	30	34	83	85
BA0380	BB0382	BG0381	basic membrane protein B (bmpB)	38,2	6	97	-	-	84	86
BA0356	BB0359	BG0359	carboxyl-terminal protease (ctp)	53,6	9	93	-	37	96	95
BA0605	BB0612	BG0628	ATP-dependent Clp protease, subunit X (clpX)	47,7	8	93	45	29	97	96
BA0210	BB0210	BG0212	surface-located membrane protein 1 (Imp1)	128,2	39	93	80	-	84	74
BA0056	BB0058	BG0057	hypothetical protein	76,5	9	87	91	33	94	94
BA0737	BB0744	BG0765	antigen, p83/100	80,0	54	87	83	-	84	87
BA0790	BB0794	BG0820	hypothetical protein	168,9	8	87	67	-	91	92
BA0327	BB0329	BG0330	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-2)	60,8	40	65	77	25	91	97
BA0345	BB0347	BG0348	fibronectin/fibrinogen-binding protein, putative	55,5	7	63	-	-	91	92
BAP024	BBK32	BGP100	immunogenic protein P35	40,9	147	60	96	-	71	71
BA0237	BB0238	BG0241	hypothetical protein	30,5	17	50	77	42	91	92
BAP034	BBQ32	BGP190	hypothetical protein	12,9	9	50	39	-	90	95
BAP038	BBS36	BGP301	hypothetical protein	21,6	18	20	86	31	86	98

Table 4.9: List of the 25 vaccine candidates selected based on *in vitro* validation assays. Antigens are listed with indication of all three ORF names in the three analyzed genomes together with gene name. Hits represent the number of selected clones by MACS; GC, gene distribution, percentage of *Borrelia* strains positive for tested antigen; surface staining, determination of surface location, percentage present a shift of the cells comparing to the buffer control; Peptide ELISA, immunogenicity test; Protein homology, percentage of aa identity among *B. afzelii* K78, *B. garinii* PBi and *B. burgdorferi* B31 sequences. -, negative assay results. *, alignments generated with clustalw2 (version 2.0.10).

Among these 25 antigens, we selected those that were found with a high number of hits (e.g. BAP024 which was detected 147 times), but also those that were selected only with few clones in the library screening (e.g. BA0507 with 8 hits) and scores positive in different validation assays. The most immunogenic candidate, hypothetical protein BAP001 (313 hits), didn't show positive result in different validation assays and wasn't selected for further animal studies. Although the ELISA results were very promising for some of the BAP001 peptides (Figure 4.8), the other two assay results were negative. BAP001 was present in 25% of tested strains (5/20 *Borrelia* strains), and couldn't be detected as surface exposed in flow cytometry. The major reason not to select this protein for further studies was its low homology among different *Borrelia* strains (60% homology between B31 and PBi).

To further validate selected vaccine candidates, recombinant bacterial proteins were generated and tested for protection in animal model.

4.3 Generation of recombinant proteins representing the selected vaccine candidates

Of the 25 selected antigens (listed in Table 4.10), 24 could be amplified and inserted into the pET28b vector. The BA0380 gene couldn't be cloned after several attempts. The genes were cloned without signal peptide and larger ones were expressed with two non-overlapping fragments, resulting in 28 constructs. The genes inserted into pET28b vector were under transcriptional control of a T7 promoter, and inducible by the addition of IPTG. Expression of the recombinant proteins was performed in the host strain BL21 CodonPlus (DE3)-RIPL. The unique feature of this strain is that tRNAs for some rare codons in *E. coli* can be efficiently expressed. This is especially important in the case of *Borrelia*, since its genome is very AT-rich (~75%), compared to *E. coli* genome with less than 50% AT-regions. Expression of most proteins was higher in BL21 CodonPlus (DE3)-RIPL than in BL21 star (the same *E. coli* strain without rare codon optimized tRNA expression).

The purification of the *B. afzelii* protein BA0210-1 is shown as a prototype procedure in Figure 4.10. Proteins identified in the insoluble fraction after lysis of the bacterial cells were solubilised by the addition of 8 M Urea before loading on the Ni-NTA column. All proteins were eluted following the same scheme. From 28 constructs, only two were

expressed and purified from soluble fraction, BA0210-1 and BA0737. The other 26 proteins were purified from insoluble fractions and subsequently solubilised as described by Materials and Methods.

Ba ORF	Bb ORF	Bg ORF	Common names	Mw Bb (kDa)	Protein constructs (aa from-to)	Mw construct (kDa)
BA0056	BB0058	BG0057	hypothetical protein	76,5	BA0056-1 (91-415)	39,3
BA0135	BB0136	BG0138	penicillin-binding protein (pbp-1)	71,9	BA0135-1 (37-246); BA0135-2 (241-628)	25 45,5
BA0210	BB0210	BG0212	surface-located membrane protein 1 (lmp1)	128,2	BA0210-1 (27-709); BA0210-2 (701-1067)	79,4 43,4
BA0221	BB0221	BG0224	flagellar motor switch protein (fliG-1)	47,9	BA0221 (1-408)	49
BA0235	BB0236	BG0239	hypothetical protein	75,2	BA0235-1 (20-460)	51,1
BA0237	BB0238	BG0241	hypothetical protein	30,5	BA0237 (21-257)	28,5
BA0327	BB0329	BG0330	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-2)	60,8	BA0327 (22-527)	59
BA0329	BB0330	BG0331	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-3)	62,5	BA0329-1 (1-339); BA0329-2 (333-541)	39,8 25,7
BA0345	BB0347	BG0348	fibronectin/fibrinogen-binding protein, putative	55,5	BA0345 (1-472)	56,4
BA0356	BB0359	BG0359	carboxyl-terminal protease (ctp)	53,6	BA0356 (29-476)	51,4
BA0364	BB0366	BG0365	aminopeptidase I (yscI)	51,6	BA0364 (7-458)	52
BA0380	BB0382	BG0381	basic membrane protein B (bmpB)	38,2	-	
BA0417	BB0418	BG0421	hypothetical protein	38,9	BA0417 (28-349)	37,8
BA0507	BB0512	BG0523	hypothetical protein	254,2	BA0507-2 (399-870)	56,3
BA0581	BB0588	BG0601	pfs protein (pfs-2)	29,2	BA0581 (22-264)	27,8
BA0605	BB0612	BG0628	ATP-dependent Clp protease, subunit X (clpX)	47,7	BA0605 (1-430)	48,6
BA0653	BB0658	BG0681	phosphoglycerate mutase (gpmA)	29,1	BA0653 (2-253)	30,2
BA0675	BB0680	BG0703	methyl-accepting chemotaxis protein (mcp-4)	84,7	BA0675 (35-753)	81,9
BA0737	BB0744	BG0765	antigen, p83/100	80	BA0737 (23-663)	74,7
BA0790	BB0794	BG0820	hypothetical protein	168,9	BA0790-2 (470-983); BA0790-3 (979-1467)	61,5 56,4
BA0792	BB0796	BG0822	hypothetical protein	21,6	BA0792 (23-181)	19,6
BAP006	BBA34	BGA28	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppAV)	61,1	BAP006-1 (251-528)	33,7
BAP024	BBK32	BGP100	immunogenic protein P35	40,9	BAP024-2 (116-352)	28,9
BAP034	BBQ32	BGP190	hypothetical protein	12,9	BAP034 (21-111)	12
BAP038	BBS36	BGP301	hypothetical protein	21,6	BAP038 (2-180)	22,4

Table 4.10: Recombinant borrelia antigens generated. The numbers refer to the cloned region (aa from-to). aa, amino acids. Mw: calculated size in kDa based on construct length (aa number).

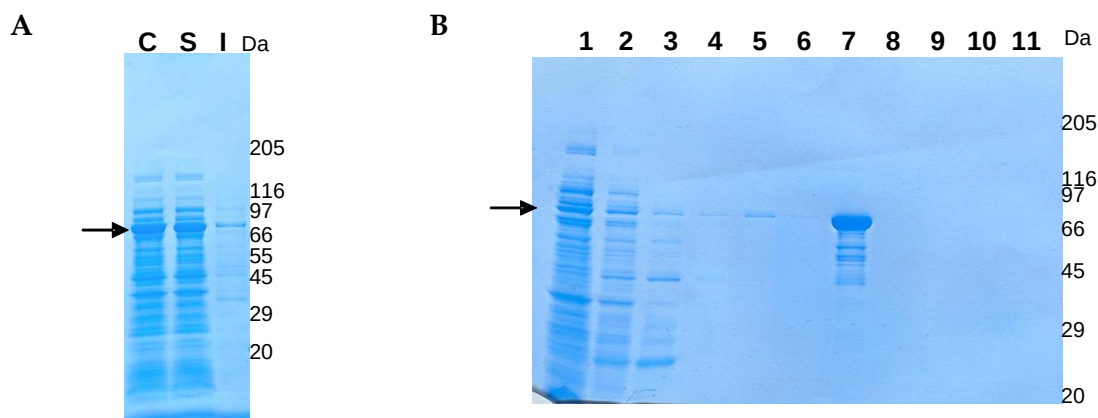


Figure 4.10: Purification of BA0210-1 antigen. **A;** Over expression of BA0210-1 (C, crude extract) and distribution in soluble (S) and insoluble (I) fraction. **B;** purification of BA0210-1 over a Ni-Sepharose column. Numbers 1 through 11 represent different washing steps as explained under Materials and Methods (Chapter 3.4).

4.4 Selection of protective antigens using a *Borrelia burgdorferi* needle challenge murine model

All 28 successfully expressed vaccine candidates were tested for their capability to protect mice against infection by *Borrelia burgdorferi* s.s. CH3 mice were immunized subcutaneously three times with two weeks interval using 50 µg adjuvanted antigen (Figure 4.11). CFA/IFA was used as adjuvant and each candidate was tested in at least two independent experiments. Immunization with recombinant, lipidated OspA protein derived from the *B. burgdorferi* B31 strain served as positive control. Since OspA is conserved among *B. burgdorferi* s.s. strains (and represent clade 2), the infection with *B. burgdorferi* N40 is considered as a homologous challenge.

Mice were challenged with 10^4 *B. burgdorferi* N40 cells two weeks after the last immunization. In order to monitor the stringency of the challenge model, four negative control groups (only PBS-CFA/IFA immunized mice) were infected with tenfold increasing doses: 10^1 , 10^2 , 10^3 and 10^4 cfu. The real challenge dose was also calculated by counting *Borrelia* and using the most probable number table as described by Materials and Methods. Finally, 4 weeks post challenge mice were sacrificed, blood and organs (bladder, ear) were taken. Organs were incubated for additional 4 weeks and analyzed for the presence of *Borrelia* in the culture medium by microscopy. If any *Borrelia* could be detected, mice were

considered to be infected. In order to avoid contaminations with other bacteria or fungi, BSKII medium was supplemented with Streptomycin and Phosphomycin.

In addition to organ culture analysis, post challenge sera were also analyzed by Western blotting. For that purpose whole cell lysate of the challenge strain *B. burgdorferi* N40 was separated on SDS gel and blotted onto nitrocellulose membrane. Subsequently, membranes were cut in slices and incubated with sera from each mice. Animals were categorized as not infected if no bands or just weak bands were detected compared to control groups. A representative Western blot is shown in Figure 4.12.

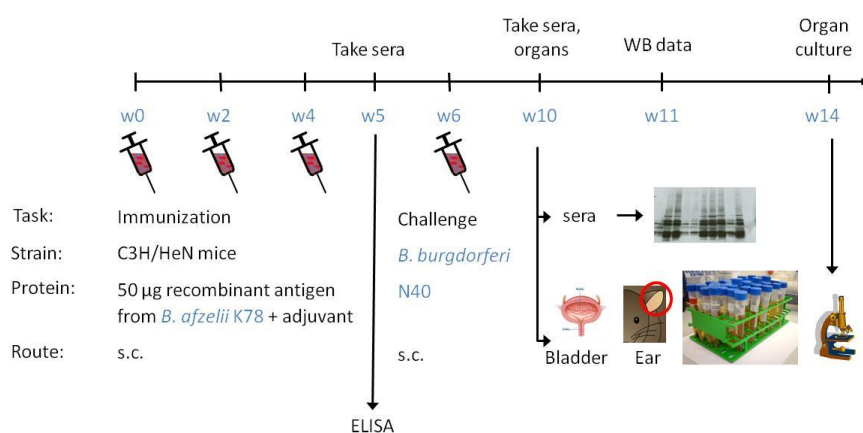


Figure 4.11: Scheme of animal experiment to measure vaccine potential of *Borrelia* antigens. After three immunizations, mice were challenged with *B. burgdorferi* N40 at day 42-48. Four weeks p.i. mice were sacrificed and their organs analyzed for infection culturing bladder and ear for another 4 weeks. Single sera were analyzed by Western blotting to detect infection induced antibodies (Figure 4.12).

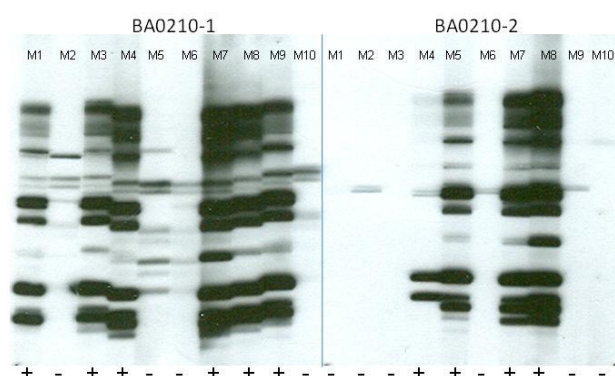


Figure 4.12: Western blot analysis of post-infection sera to detect challenge induced antibodies. *B. burgdorferi* N40 cell lysate was separated by SDS-PAGE and blotted onto nitrocellulose membrane. Membrane was cut in slices and each slice incubated with a single serum. Sera not showing bands or only weak protein bands were visible were considered not infected. +, infected; -, not infected. M1-10, individual mice. BA0210-1 and BA0210-2, two protein constructs representative for antigen BB0210.

The positive control B31 OspA antigen was effective in all experiments with protection levels ranging between 50-100%, with an average of 80%.

The best and most consistent protection was seen with CFA/IFA adjuvanted BA0210-1 (50-70%), BA0210-2 (40-70%) and BAP006-1 (40-60%) (Table 4.11). The flagellar motor switch protein (BA0221), oligopeptide ABC transporter (BA0327) and a hypothetical protein BA0237 also elicited protection in 40% of animals. Three additional recombinant proteins protected 30% of mice. These three proteins are hypothetical protein BA0507, ATP-dependent Clp protease (BA0605), and methyl-accepting chemotaxis protein (BA0675). However, this protection was not consistent in the several repeated challenge experiments, as shown in Table 4.11.

Protein construct	Protection %	
	WB	Culture
BA0056-1	≤10	≤10
BA0135-1	0	0
BA0135-2	0	0
BA0210-1	50-70	50-70
BA0210-2	40-70	40-70
BA0221	≤40	≤40
BA0235-1	0	0
BA0237	≤40	≤30
BA0327	≤40	≤40
BA0329-1	0	0
BA0329-2	0	0
BA0345	0	0
BA0356	0	0
BA0364	0	0
BA0417	0	0
BA0507-2	≤30	≤30
BA0581	≤10	≤10
BA0605	≤30	≤30
BA0653	0	0
BA0675	≤30	≤30
BA0737	0	0
BA0790-2	0	0
BA0790-3	≤10	≤10
BA0792	≤10	≤10
BAP006-1	40-60	40-60
BAP024-2	0	0
BAP038	0	≤10
BAP034	0	0

Table 4.11: Protection potential of vaccine candidates in 2 or 3 independent experiments. %, percentage of mice protected from *Borrelia* infection. WB, Western blot analysis of post challenged sera. Culture, organ culture analysis.

The best protective candidates (BA0210-1, BA0210-2 and BAP006) were further tested for protection in combination with other adjuvants. Beside CFA/IFA, mice were immunized with recombinant proteins adjuvanted also with ALUM and IC31 (Lingnau et al., 2007). In this experiment another positive control was also used: *B. afzelii* ACA1 recombinant OspA protein. Since our recombinant proteins were heterologous to the challenge strain, now we also used heterologous OspA protein to better compare protection results. B31 OspA protected all challenged mice (homologous protection), while ACA1 OspA was effective in only 60% of animals. Our three main candidates (BA0210-1, BA0210-2, BAP006-1) showed equal or higher protection compared to ACA1 OspA. The best protection was achieved when recombinant proteins were adjuvanted with CFA/IFA. Infection was cleared by 40% animals immunized with BA0210-1 (CFA/IFA). The protection level declined when adjuvanted with ALUM (30%) and IC31 (20%). BA0210-2 showed highest protection in this experiment, 70% when combined with CFA/IFA. Using the other two adjuvants (ALUM and IC31) protection level declined also for this candidate, 50% of animals could clear infection in both cases. BAP006-1 did not induce significant protection in this experiment with only 20% animals protected.

BA0210

BA0210 (BB0210), annotated surface located membrane protein 1 (Imp 1), was selected as one of the highly immunogenic proteins by screening with both K78-FhuA and K78-LamB bacterial display libraries. Epitope regions covered by 39 selected hits are shown in Figure 4.13. The gene distribution study showed high prevalence of BA0210 among different *Borrelia* isolates. The gene was detected in 28 of 30 tested strains. Serological analysis of selected epitopes showed less satisfactory results; none of BA0210 peptides was selected as highly immunogenic with human sera. Surface location of the antigen was confirmed by flow cytometry staining of K78 cells with epitope specific immune sera.

BA0210 (3201 bp)



BAP006 (936 bp)

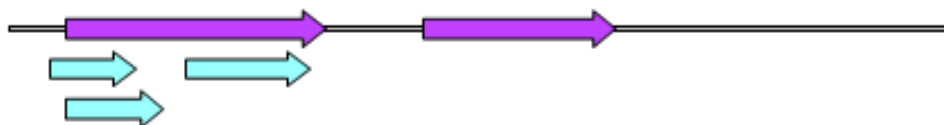


Figure 4.13: Epitope map for two main antigens, BA0210 and BAP006. Violet and blue arrows represent regions recognized by K78-FhuA and K78-LamB clones, respectively.

BAP006

Oligopeptide ABC transporter BAP006 was selected with 10 hits screening the K78-FhuA library. Epitope map is shown in Figure 4.13. The validation analysis was very promising for this candidate since all three validation assays resulted positive. BAP006 was present in 29 of 30 tested *Borrelia* isolates. One of the BAP006 peptides (BAOppA-4.01, Figure 4.7) reacted with 73% of human sera tested in peptide ELISA. FC analysis of *Borrelia* cells stained with epitope specific immune sera confirmed surface location of this antigen.

4.5 Proteomics

In order to define the surface proteome of *Borrelia*, three different proteomic approaches were applied to the three main *Borrelia* species causing human pathology: *B. burgdorferi* B31, *B. afzelii* K78 and *B. garinii* PBi. Using different methods seemed to be advantageous, since all methods have limitations and biases. Some proteins could be missed, because they cannot be solubilised, or are not accessible on the surface while covered by other outer surface proteins (Bunikis et al., 1999), or do not have restriction sites for certain proteases.

To identify surface exposed proteins of the three genospecies three methods were used: fractionation of the outer membrane, partial trypsin digest of outer membrane proteins and biotin-labeling of surface proteins.

4.5.1 A detergent-based fractionation of the outer membrane of *Borrelia*

First, I prepared outer membrane fractions of the three *Borrelia* genospecies, according to Magnarelli et al. (Magnarelli et al., 1989) with slight changes in protocol. For this preparation, a large amount of *Borrelia* culture was necessary, which was a challenge considering the long replication time of borrelias. Preparations were done as described by Materials and Methods and sent to ProtAffin AG for nano-LC-MSMS analysis, which resulted in the identification of 186 proteins in the three membrane preparations: 65 proteins in *B. afzelii* K78 sample, 56 proteins in *B. garinii* PBi preparation and 65 in membrane fraction of *B. burgdorferi* B31. All 186 proteins are listed in Table A5 (Appendices). Analysis showed a significant overlap of 30 proteins identified in the three membrane preparations (Table 4.12). Among these 30 proteins we found well characterized virulence proteins, such as OspA, OspB, OspC, porins (p66 and p13), several lipoproteins and flagellar proteins.

Ba ORF	Bb ORF	Bg ORF	Common name
BAPKO_0636	BB0603	BG0616	Membrane-associated protein p66
BAPKO_2004	BBA03	BGA02	Outer membrane protein
BAPKO_0149	BB0147	BG0147	Flagellar filament 41 kDa core protein
BAPKO_0338	BB0327	BG0330	Oligopeptide ABC transporter
BAPKO_0434	BB0418	BG0421	Hypothetical protein
BAPKO_5018	BBB19	BGB18	Outer surface protein C
BAPKO_0546	BB0519	BG0530	GrpE protein
BAPKO_5016	BBB17	BGB16	IMP dehydrogenase
BAPKO_2016	BBA15	BGA14	Outer surface protein A
BAPKO_2017	BBA16	BGA15	Outer surface protein B
			Membrane protein P13
BAPKO_2059	BBA62	BGA62	Lipoprotein
			M18-family aminopeptidase (<i>yscI</i>)
BAPKO_0735	BB0690	BG0714	Neutrophil activating protein
BAPKO_0346	BB0337	BG0338	Enolase
BAPKO_0057	BB0057	BG0056	Glyceraldehyde-3-phosphate dehydrogenase
BAPKO_0019	BB0020	BG0020	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase
BAPKO_0701	BB0657	BG0680	Ribose 5-phosphate isomerase
BAPKO_0250	BB0241	BG0244	Glycerol kinase
BAPKO_0405	BB0390	BG0391	50S ribosomalprotein L7/L12

Ba ORF	Bb ORF	Bg ORF	Common name
BAPKO_0772	BB0728	BG0750	NADH oxidase, water-forming
BAPKO_0289	BB0279	BG0282	Flagellar protein
BAPKO_0571	BB0543	BG0553	Hypothetical protein
BAPKO_0669	BB0627	BG0648	M18-family aminopeptidase (<i>pepX</i>)
BAPKO_2056	BBA59	BGA59	Lipoprotein
	P58		and/or Surface antigen
BAPKO_0422	BB0405	BG0407	Hypothetical protein
BAPKO_0423	BB0406	BG0408	Hypothetical protein
BAPKO_0593	BB0563	BG0573	Hypothetical protein
BAPKO_0591	BB0562	BG0572	Hypothetical protein

Table 4.12: Overlapping proteins identified in outer membrane fraction from all three genospecies. Proteins identified with SWISS-Prot-ID; BAPKO, *B. afzelii* PKo; BB, *B. burgdorferi* B31; BG, *B. garinii* PBi.

Comparing this data set to proteins identified by the ANTIGENome technology, we found an overlap of 23 proteins identified in outer membrane fraction from *B. afzelii* K78, and 15 proteins in both *B. garinii* PBi and *B. burgdorferi* B31 outer membrane fractions.

4.5.2 Identification of surface exposed proteins by partial trypsin digest

Trypsin is a member of the serine protease S1 family and consists of a single chain polypeptide of 223 amino acids residues. Its possibility to cleave peptides on the C-terminal side of lysine and arginin amino acid residues was used for digestion of borrelial surface proteins. During incubation of *Borrelia* strains (*B. afzelii* K78, *B. burgdorferi* B31 and *B. garinii* PBi) with trypsin, outer surface proteins were digested and aliquots analysed by 2D-LC-MSMS. Proteins that do not have trypsin digestion sites, were resistant to trypsin.

To find the best assay conditions for “shaving off” surface exposed proteins, *Borrelia* cells were incubated with different amounts of trypsin, and aliquots were taken after 1, 2 and 3 hours of incubation. Finally, samples were analysed by 15% Schgger gel and compared to negative control (*Borrelia* cells without trypsin treatment). As shown in Figure 4.14 there were no differences between the three incubation times, however increasing amount of trypsin resulted in greater number of proteins with decreasing size and disappearance of larger ones.

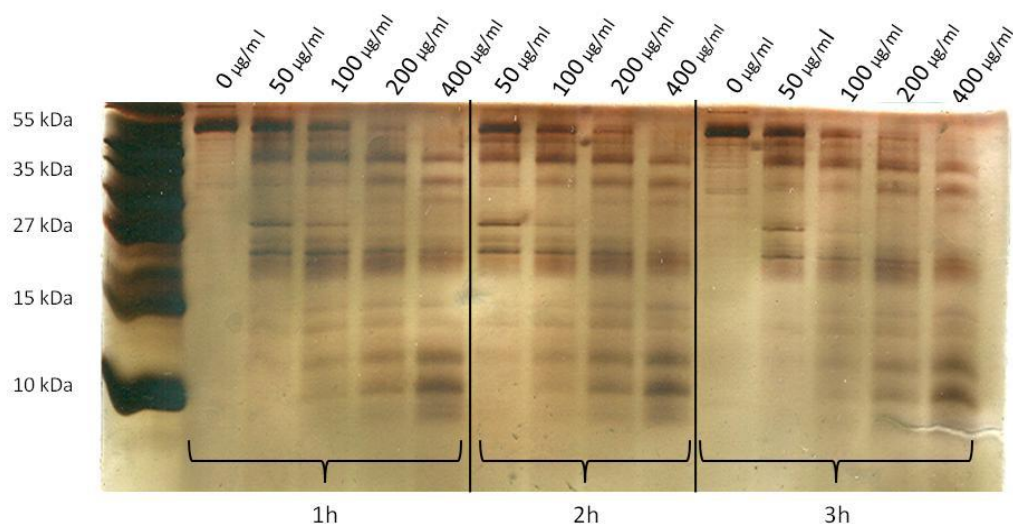


Figure 4.14: Partial trypsin digestion of *B. afzelii* K78 cells. Four different trypsin concentration (50, 100, 200 and 400 µg/ml) were tested in order to find optimal conditions. After different incubation times (1, 2 and 3 hours) aliquots (5 µl) have been taken and analyzed by 15% Schgger gel, and subsequently proteins were visualized by silver staining of the gels.

Aliquots containing shedded outer surface proteins were sent to our collaborator, ProtAffin Biotechnologie AG (Graz, Austria). They performed 2D-LC-MS/MS analysis of all peptides and identified the same by SWISS-Prot-ID. Analysis of digested proteins resulted in identification of altogether 263 proteins: 112 with *B. afzelii*, 133 with *B. garinii* and only 18 with *B. burgdorferi* cells (Table A6, Appendices). The latter sample showed some contaminations that hindered the analysis. In several attempt we tried to identify these non-protein components that caused contaminations, but without any success. However, overlap between proteins identified, resulted with 68 proteins found in both *B. afzelii* K78 and *B. garinii* PBi digest preparations (Table 4.13). 32 proteins from the *B. afzelii* and 35 from the *B. garinii* samples overlapped with proteins selected with ANTIGENome technology.

Protein	<i>B. garinii</i> PBi			<i>B. afzelii</i> K78		
	MW (kDa)	SWISS-Prot ID	#	MW (kDa)	SWISS-Prot ID	#
OspA protein	29,6	Q6LBF1	3	29,6	P0A3N7	3
Outer surface protein B	32,1	Q6ASL0	3	32,4	Q0SLY9	3
Outer surface protein C	22,3	Q45187	3	22,4	Q9Z6C7	3
Antigen, P35	36,2	Q6ASG2	3	36,8	Q0SLU6	3
Antigen, P35, putative	47,1	Q6ASG0	3	47,1	Q0SLU4	3
Antigen, P35, putative	33,2	Q6ASF2	3	34,4	Q0SLT5	3
Antigen, p83/100	79,3	Q660D0	3	75,9	Q0SMB3	3
Flagellar filament 41 kDa	35,8	Q662L6	3	35,8	Q0SP16	3

Protein	<i>B. garinii</i> PBi			<i>B. afzelii</i> K78		
	MW (kDa)	SWISS-Prot ID	#	MW (kDa)	SWISS-Prot ID	#
core protein						
Flagellar filament outer layer protein	38,3	Q660K2	3	38,3	Q0SMI5	3
Outer membrane protein	19,4	Q6ASM3	3	19,4	Q0SM02	3
Lipoprotein LA7	22,1	Q661Q4	3	21,9	Q0SNE9	3
Glyceraldehyde 3-phosphate dehydrogenase	36,2	Q662V7	3	36,2	Q0SPA8	3
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-2)	60,4	Q661T8	3	60,4	Q0SNI3	3
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-3)	62,6	Q661T7	3	62,4	Q0SNI2	1
Decorin binding protein A	19,3	Q6ASK4	3	18,3	Q9ZH93 or Q9ZH89	3
Surface-located membrane protein 1	104,5	Q662F4	3	116,2	Q0SNV2	2
Basic membrane protein A1 [Precursor]	36,9	Q661N6	2	37	Q0SND0	3
Basic membrane protein B	37,2	O31362	3	37,1	O31284	2
Basic membrane protein	38	Q662Q4	1	38	Q0SP56	2
Antigen, S2, putative	28,3	Q662K7	3	28	Q0SP07	1
Lipoprotein	7,9	Q6ASG6	3	8,7	Q0SLV0	3
Lipoprotein	5,8	Q6ASG3	3	6,8	Q0SLU7	1
Antigen, S2	30,4	Q6ASM2	1	28	Q6DRS5	1
Flagellar protein	20	Q661Y6	3	20	Q0SNN2	3
and / or Surface antigen	60,6	Q9K5F7	3	60,5	Q9K5J9	3
Membrane fusion protein	35,1	Q662M1	2	35	Q0SP22	1
Chaperone protein dnaK	69,3	Q661A3	3	69,4	Q0SMZ0	3
Enolase	47,9	Q661T0	3	47,4	Q0SNH5	3
Rod shape-determining protein	38	Q660F8	2	39,8	Q0SME1	3
PTS system, maltose and glucose-specific IIABC component	59,1	Q6ASM5	2	62,4	Q0SP48	3
PTS system, glucose-specific IIA component	20,8	Q660W5	2	20,8	Q0SMV0	3
Glycine betaine, L-proline ABC transporter, binding protein	33,7	Q662L9	3	33,7	Q0SP19	3
60 kDa chaperonin	58,9	Q660M1	3	58,9	Q0SMK4	3
Cell division protein	70,8	Q65ZY5	1	70,7	Q0SM63	3
Ribosomal protein L7/L12	13	Q661M8	1	13,3	Q0SNB7	3
Chemotaxis operon protein	17,6	Q660J9	3	17,6	Q0SMI2	3
DNA-directed RNA polymerase subunit beta	129,7	Q661M9	2	129,9	Q0SNB8	3
DNA-directed RNA polymerase subunit beta'	154,4	Q661N0	2	154,5	Q0SNB9	3
10 kDa chaperonin	9,9	Q660D3	3	9,9	Q0SMB6	3

Protein	<i>B. garinii</i> PBi			<i>B. afzelii</i> K78		
	MW (kDa)	SWISS-Prot ID	#	MW (kDa)	SWISS-Prot ID	#
5-methylthioadenosine/S-adenosylhomocysteine nucleosidase, putative	26,7	Q661P4	3	29	Q0SMS0	3
Periplasmic serine protease DO	52	Q662Q8	3	52	Q0SP60	3
Chaperone protein htpG	71,3	Q660W4	3	71,4	Q0SMU9	3
Pyrophosphate--fructose 6-phosphate 1-phosphotransferase	62,5	Q662Z2	3	62,4	Q0SPE4	3
Triosephosphate isomerase	27,9	Q662V9	2	27,8	Q0SPB0	3
L-lactate dehydrogenase	34,9	Q662S5	3	34,9	Q0SP77	3
50S ribosomal protein L11	15,2	Q661M5	3	15,2	Q0SNB4	3
Glucose-6-phosphate isomerase	60	Q660E3	2	60,2	Q0SMC6	3
Elongation factor G 1	77,3	Q660Y4	2	77,3	Q0SMX0	3
30S ribosomal protein S5	17,9	Q661C5	3	17,8	Q0SN12	3
Fructose-bisphosphate aldolase	40	Q661H5	1	40	Q0SN64	3
Lysyl-tRNA synthetase	61	Q937U7	2	61	Q937W6	3
DNA topoisomerase	97,9	Q65ZU6	3	98	Q0SM23	3
5-methylthioadenosine/S-adenosylhomocysteine nucleosidase, putative	29,5	Q660T4	2	26,6	Q0SND8	3
Superoxide dismutase	23,4	Q662L2	3	23,4	Q0SP12	3
DNA gyrase, subunit B	71,4	Q661I5	2	71,5	Q0SN73	2
Phosphocarrier protein HPr	9,4	Q660W7	1	9,4	Q0SMV2	2
Transcription termination factor Rho	57,7	Q662D4	3	57,7	Q0SNT1	2
Pyruvate kinase	53,1	Q661R9	2	53,1	Q0SNG4	2
Glycerol-3-phosphate O-acyltransferase, putative	34,5	Q661U0	2	34,6	Q0SNI5	2
Chemotaxis response regulator	13,7	Q660V4	2	13,7	Q0SMT8	2
ATP-dependent Clp protease ATP-binding subunit clpX	47,7	Q660R1	1	47,6	Q0SMP5	2
Putative N-acetylmannosamine-6-phosphate 2-epimerase	22,7	Q660M6	2	25,7	Q0SMK9	2
50S ribosomal protein L2	30,5	Q661D9	2	30,6	Q0SN26	2
PTS system, glucose-specific IIBC component	55,6	Q660M5	1	55,6	Q0SMK8	1
ABC transporter, ATP-binding protein	34,9	Q660C1	3	35,3	Q0SMA3	1
Putative surface protein	29,6	Q5W285	3	27,9	Q5W297	1
Thioredoxin	13,5	Q000V1/2/6/7/8/9	3	13,4	Q0SPA4	1
2',3'-cyclic-nucleotide 2'-phosphodiesterase	58	Q661B6	1	57,9	Q0SN03	1

Table 4.13: 68 proteins identified in both K78 and PBi digest preparation. #, number of identified peptides.

4.5.3 Biotinylation of surface exposed *Borrelia* proteins

In a third approach, water-soluble, membrane impermeable biotin was used to label membrane proteins of viable spirochetes. Prior to isolation on the streptavidin column, spirochetes were solubilised with ASB-14 (zwitterionic detergent). Since we used SS biotin and, biotin labelled proteins could be eluted by DTT from the streptavidin column. The cleavage enables removing of biotin residue from the surface proteins which allows subsequent proteomic analysis. A representative gel showing the purification procedure with *B. afzelii* K78 lysate is shown in Figure 4.15. Eluates containing surface labeled proteins were sent to our collaborator (ProtAffin) to analyse them by nano-LC-MS/MS. These proteomic analyses of isolated proteins resulted in the identification of 119 proteins from *B. burgdorferi* and 198 proteins from *B. afzelii* strains. All proteins identified with this method are listed in Table A7, Appendices. As expected, no proteins were identified in the negative controls (samples without biotinylation).

Although I used biotin which should be impermeable according to the manufacturer's description (Sulfo-NHS-SS-Biotin, Pierce), bioinformatic analysis showed that many intracellular proteins were also identified. Since these two preparations didn't give promising results based on the presence of too many intercellular proteins, no biotinylation of *B. garinii* was performed (this strain was the most difficult one for *in vitro* growth, especially when larger amounts were needed).

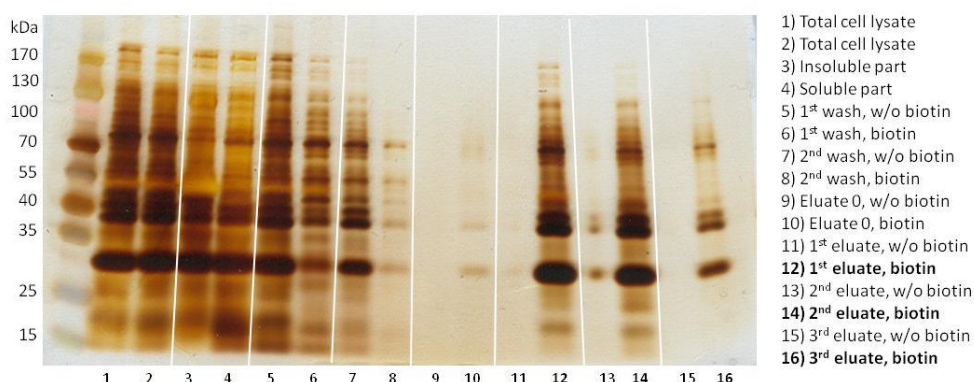


Figure 4.15: Isolation of surface biotinylated *B. afzelii* K78 proteins. Biotinylated (even numbered lanes) and unbiotinylated (uneven numbered lanes) samples were processed parallel. After 30 min incubation at room temperature, cell lysates were prepared, and the soluble fractions were loaded onto Streptavidin Sepharose columns. Proteins were eluted with DTT, separated by SDS PAGE and visualized by Silver staining.

4.5.4 Proteins identified by all three different proteomic approaches

Extensive proteomic analysis of three *Borrelia* genospecies resulted with identification of 297 different *Borrelia* proteins. 151 proteins were identified in *B. burgdorferi* B31 preparations, 240 proteins in *B. afzelii* K78 preparations and 171 proteins in *B. garinii* PBi preparations (Figure 4.16). 104 proteins showed an overlap between all three genospecies (proteins listed in Table A8, Appendices).

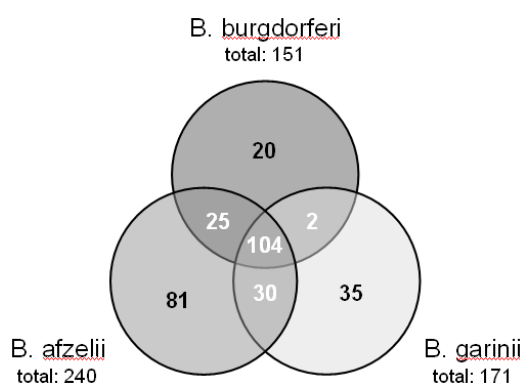


Figure 4.16: Overlap between proteins identified by surface proteome methods in three *Borrelia* genospecies.

54% of these 104 proteins are predicted to be membrane-associated, 20% are hypothetical proteins and 26% were predicted to be intracellular based on gene annotation (www.uniprot.org) as depicted on Figure 4.17. Since the biological information about *Borrelia* proteins in general is rather poor, the cellular localisation of many of these proteins should be regarded as preliminary.

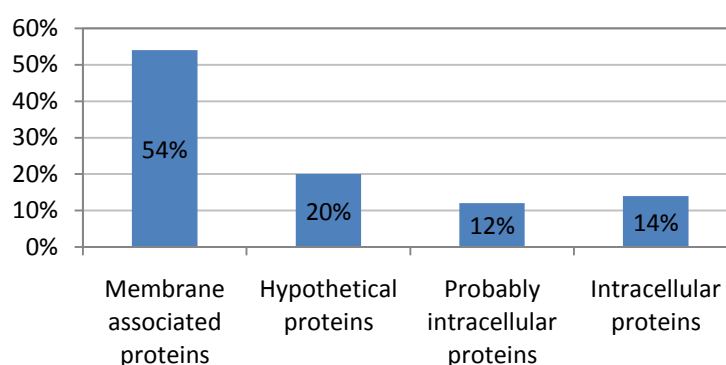


Figure 4.17: Cellular localization of 104 proteins identified in all three genospecies. 56 proteins identified as membrane associated, 21 hypothetical proteins, 12 as probably intracellular proteins and 15 as intracellular proteins.

Compared to the proteins of the ANTIGENome, less hypothetical proteins were identified by proteomic approaches (42 vs 20%). Percentage of proteins predicted to be intracellular was higher than using previous method, probably due to cell lysis during biotinylation.

5 DISCUSSION

Considering diagnostic difficulties, severe health problems caused by chronic infection, progression of LB in endemic regions, and important role of antibodies in immune response, development of prophylactic vaccine would be of great advantage. While *Borrelia* has a very complex biology and alters expression of outer surface proteins according to environment, identification of novel vaccine candidates bear a great challenge.

Even being undoubtedly compulsory, vaccination approaches using *Borrelia* antigens have proven to be cumbersome. Beside OspA used as vaccinogen in LYMERix vaccine (Wallich et al., 1996), also other alternative *Borrelia* outer membrane proteins were used as possible vaccinogen in previous studies. A multivalent OspC-based clinical vaccine has been developed (Earnhart et al., 2007), however, the Phase I showed development of red rash and swelling on the injection site. DbpA showed also strong protection in mice but only by mice infected by syringe inoculated *Borrelia* (Hanson et al., 1998). We at Intercell assumed that our antigen identification technology (Meinke et al., 2005) would be a good tool to identify novel vaccine candidates to aid the development of a broad coverage vaccine against LB caused by the three pathogenic genospecies. This technology relies on the human humoral immune response and screening of bacterial surface display libraries with serum IgGs isolated from the patients' suffering from different LB symptoms and infected with distinct *Borrelia* strains. Theoretically the bacterial surface display libraries allow expression of all potential epitopes and enable detection of *in vivo* expressed antigens under *in vitro* culture conditions (Etz et al., 2001). This is extremely important since *Borrelia* alter its surface expression. In order to monitor differences in human immune response to different disease manifestations, collected sera and later isolated IgGs were analyzed in pools prepared according to patients' disease manifestations. Four serum pools were specific for neuroborreliosis, lyme arthritis, skin disorder (ACA) or erythema migrans. Another two pools were compiled of patients' sera not determined for specific disease symptom and were called Lyme borreliosis I and II. All six serum pools were used for screening of bacterial surface display libraries prepared with genomic DNA of *B. afzelii* strain K78. This genospecies was selected because *B. afzelii*

present the main causative agent of LB in Europe (Hengge et al., 2003), and because the strain K78 was thought to be a virulent human isolate and therefore the best candidate for the identification of novel vaccine antigens.

In order to include linear and potentially structural epitopes two display libraries were generated, LamB and FhuA respectively. Both libraries were screened with all six serum pools resulting in the identification of 151 ORFs selected as antigens after confirmation by Western blot analysis. Interestingly, 45% of selected ORFs are hypothetical proteins with still not determined function. This percentage is even higher (50%) among 10 highly immunogenic proteins, and hypothetical protein BAP001 (BB0844) was the most immunogenic antigen with 313 hits selected in all 12 screens. Applying bioinformatic approach these hypothetical proteins would be excluded from the study and not selected as possible vaccine candidates. This again confirms the advantages of our ANTIGENome technique in discovering novel proteins recognized by immune system. Selected antigens with already known functions could be divided into different groups, outer membrane lipoproteins (e.g. OspC, OspG, OspF); flagellar proteins (e.g. flagellar hook and hook-associated proteins); heat shock proteins (e.g. heat shock proteins 70 and 90); different oligopeptide ABC transporters. Many of the selected antigenic *Borrelia* proteins are involved in pathogenesis, such as decorin binding protein A (Feng et al., 1998), and the Bdr and Erp proteins, important for complement inactivation (Hellwage et al., 2001). Applying our ANTIGENome technology we were able to rediscover most of the proteins identified as antigens in previous studies, especially among the most immunogenic candidates. For example immunogenic protein P35 (BAP024; Fikrig et al., 2000) was selected with 147 hits and antigen p83/100 (BA0737, BB0744) with 54 hits. Previous studies showed diagnostic value of both P35 (Heikkilä et al., 2002; Lahdenne et al., 2006) and p83/100 antigens (Wilske et al., 1990 and 1999; Bruckbauer et al., 1992; Hauser et al., 1998). Especially chromosome coded protein BA0737 is widely used in serological tests as a marker for stage 3 of infection since it is cross-reactive protein among diverse *Borrelia* strains. Interestingly, Outer surface proteins A and B previously identified as protective antigens (Probert et al., 1994), were not selected by screening the bacterial surface display libraries with human IgGs. The most likely reason is the low level of antibodies due to the absence or low concentration of antigen as consequence of down-regulated expression of both OspA and OspB in mammals (Yang et al.,

2004). Soon after first contact with blood during tick bite, *Borrelia* shut down the expression of these two proteins since they are not important for transmission. Therefore the host immune system does not encounter OspA or OspB on the surface of *Borrelia* and no antibodies are produced. Another protective outer surface protein, Outer surface protein C (OspC; Gilmore et al., 1996), was selected with 12 hits screening K78-FhuA library. No hits were selected screening linear K78-LamB library which was expected since conformational organization of the OspC protective epitope is essential for its protective capability (Gilmore and Mbow, 1999).

Beside hypothetical protein BAP001 (313 hits) and immunogenic protein P35 (147 hits), next most immunogenic candidate with 71 hits was flagellar protein FlaB. Being periplasmic and not surface exposed (Charon et al., 1992; Motaleb et al., 2000), borrelial flagella showed high immunogenicity since seven different flagellar proteins were selected.

After defining the *Borrelia* ANTIGENome, several *in vitro* validation assays were applied in order to preselect the most promising vaccine candidates for animal studies. One of the first steps was the gene prevalence determination that allowed pre-selection of antigens that were present in most of the *Borrelia* clinical isolates that were collected from patients and represented diverse OspC types. This validation assay is extremely important for genetically heterogeneous bacterium such as *Borrelia* (Wilske et al., 1992; Wang et al., 1999). The gene prevalence analysis resulted in significant reduction in the number of the possible vaccine candidates. However, this result should be taken with caution due to potential sequence variation of the primer region among the different strains that can lead to false negative data. Another important aspect is that certain borrelial plasmids can be lost during *in vitro* growth affecting plasmid encoded candidates (Schwan et al., 1988; Busch et al., 1997).

The second pre-selection step was to determine human immunogenicity of selected antigens by peptide ELISA. This positive selection criterion identified highly immunogenic epitopes and the corresponding ORFs. The presence of high levels of antibodies indicates *in vivo* expression by the pathogen during infection and immunogenicity of vaccine candidate. This assay also enables further delineation of immunogenic regions. Namely, screening the bacterial surface display libraries we selected clones covering distinct immunogenic regions

(epitopes) within an ORF. The overlapping peptides used in the ELISA analysis, covered smaller regions within these epitopes allowing the delineation of exact linear epitopes. This information is especially useful when the whole protein cannot be expressed or certain domains have to be removed from the recombinant antigens due to toxic effect or homology to human proteins. In addition, it also aids the design of fusion proteins containing immunogenic regions of several antigens.

Another important criterion for a vaccine candidate is its accessibility for antibody binding, e.g. association with the outer membrane. To address this issue, epitope-specific immune sera were generated by immunizing the mice with lysates of *E. coli* clones selected by Borrelia-specific antibodies. The possibility to use selected clones directly, without the generation of recombinant proteins, is a great advantage of the technique applied. Due to the spiral thin shape of Borrelia, application of this flow cytometry based assay was not trivial. However, after several optimization steps reliable results were achieved that confirmed surface localization of 32 candidates.

Combining the results of these three validation assays, the borrelial antigenome was reduced to 25 candidates. 22 of them showed in addition to high gene prevalence (87-100%) also high immunogenicity with human sera and/or were confirmed as surface located. Comparing chromosome vs plasmid encoded antigens, gene distribution results showed much lower prevalence for plasmid encoded candidates, as expected. Especially one of the plasmid encoded antigens, BAP038, showed very low distribution and was present in only 20% of tested strains. The BAP038 however, was determined as surface located with 86% cells shifted compared to the buffer control, and showed also very good reaction with human sera in peptide ELISA. In this case low prevalence of BAP038 among tested Borrelia strains wasn't considered as exclusive, since this can also be a false negative result. As explained before, low gene distribution does not mean automatically that gene is really missing in analyzed strains, but could be the results of plasmid loss in this case due to in vitro culturing and passaging (Purser and Norris, 2000).

Finally, comparing the 25 promising candidates with the complete proteome of *B. burgdorferi* B31 (Figure 5.1), we found that three groups of proteins - cell envelope proteins, proteins involved in transport and binding, and in protein fate (proteases) - constituted 50%

of all identified proteins, which represents an enrichment relative to the complete genome encoded proteome of B31 with 20% of contribution of these three role categories. The biggest group however is the group of proteins with unknown function (hypothetical proteins) which is 36% within the antigenome.

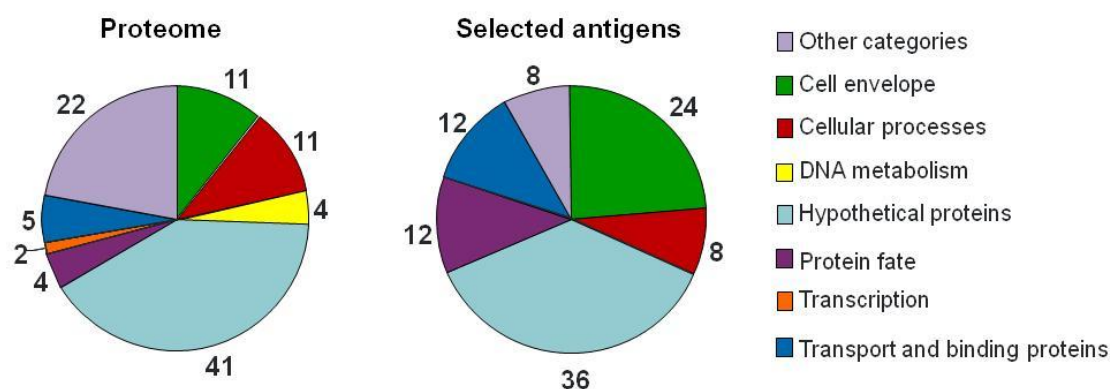


Figure 5.1: Comparison between *Borrelia* genome predicted proteome and selected antigens. The *B. burgdorferi* B31 proteome annotated proteins assigned to role categories.

To select vaccine candidates, antigens were tested for protection in a murine model of borreliosis. The C3H mice, used in this model can develop acute arthritis and carditis (Armstrong et al., 1992). Although, this murine Lyme borreliosis is primary an acute disease and differs from human chronic infections, this model is accepted to be predictive for protection (Feng et al., 2000). Among all antigens tested, two candidates - BAP006 (BBA34) and BA0210 (BB0210) - showed significant and consistent protection in the range of 40-70%. BA0210 is especially promising with the 70% protection level in several independent experiments. Importantly these antigens were able not only to protect, but to cross-protect mice from Lyme borreliosis. As discussed previously, at least three different pathogenic *Borrelia* genospecies can cause Lyme borreliosis in Europe. In our animal studies, we tried to reproduce this complexity by immunizing with recombinant proteins from *B. afzelii* and challenging with a *B. burgdorferi* strain (N40).

BAP006 is plasmid encoded, while the BA0210 gene is on the chromosome and therefore more stable and less heterogeneous expression is expected among different *Borrelia* genospecies for this antigen. Based on the validation results, BA0210 showed excellent gene distribution among the strains and was selected as surface located with flow cytometric analysis. ELISA with synthetic peptides however did not reveal high immunogenicity, in

spite of the frequent selection of this ORF by library screening. It might be explained by the presence of conformational epitopes recognized by the antibodies induced during disease.

BA0210 has a calculated size of 128 kDa. Its function is still not determined, but it was annotated as surface-located membrane protein 1 (Imp-1; Fraser et al., 2007). The surface localization was confirmed in our study. *In silico* analysis identified a Tpr-like domain located in the middle part of the protein, which consists of repeats that are present in varying numbers among different strains. For example, *B. burgdorferi* strain B31 has 7, *B. afzelii* strain K78 6, and *B. garinii* strain PBi only 2 repeats. These repeat motifs or whole Tpr-like domain mediates protein-protein interactions and the assembly of multi-protein complexes. The functional role of these repeats to contribute to protein-protein interactions was shown by Antonara and colleagues (Antonara et al., 2007). In this study, using phage display technology BB0210 was selected as candidate adhesin that could have important role in *Borrelia* dissemination through the mammalian tissue. More recent study (Yang et al., 2009) demonstrated BB0210 as an essential virulence factor important for bacterial pathogenicity. Yang and colleagues generated Imp-1 knock out strain that was able to cause infection, but was rapidly cleared by the immune system being more susceptible to the bactericidal antibodies. Taken all these data together BA0210 is a very promising vaccine candidate. This notion needs to be confirmed in further animal experiments and optimizations have to be done in order to achieve even higher protection. Protection studies in combination with other protective candidates should be also tested.

In summary, we were able to show that the ANTIGENome technology is a very powerful tool in identification of novel vaccine candidates. Due to high diversity among the strains, borrelial morphology and extremely long replication cycles, many previously optimized assays performed in our laboratories analyzing other pathogens (Etz et al., 2002; Giefing et al., 2008; Meinke et al., 2009), were not feasible or needed more extensive optimization. However, several protective antigens could be identified. Their protection effect could be induced by combining them in a cocktail. Previous studies showed synergic protection level of mice immunized with both OspA and DbpA (Hanson et al., 2000). Combination vaccine of OspC, BBK32 and DbpA was even more effective protecting mice against *Borrelia* infection (Brown et al., 2005). Immunization with a cocktail of *Borrelia*

proteins could generate long-term immunity. Previous studies showed that reinfection in successfully treated LB patients with more severe disease manifestations are extremely rare, while reinfection in successfully treated patients with developed EM is more common (Narasimhan et al., 2002). These differences in reinfection incidence could be based on an immune response directed against multiple borrelial proteins generated throughout the course of infection.

While the diversity of *Borrelia burgdorferi* s.l. species is very high in Europe and just few studies were done with *B. afzelii* and *B. garinii*, my PhD study comprised another important task – proteomic analysis of all three pathogenic genospecies. The aim of this study was the characterization of borrelial outer membrane and determination of surface located proteins. In order to compare pathogenic *Borrelia* genospecies, different proteomic assays were applied to the three most important species represented by *B. afzelii* K78, *B. burgdorferi* B31 and *B. garinii* PBi strains. Since membrane proteins are difficult to solubilise in general and their accessibility is often limited, especially if only one isolation method is used, combination of three different approaches was aimed to allow us the most comprehensive identification of conserved surface proteins of all three genospecies.

The first method, detergent-based fractionation of outer membrane, seemed to be the best method for identification of high abundant proteins. The total number of identified proteins was much lower compared to other methods used. Second approach, partial trypsin digest, resulted with much higher number of total proteins. However, most likely due to possible cell lysis caused by trypsin, intracellular proteins were also identified. The percentage of identified intracellular proteins was much higher when surface exposed proteins were biotinylated. However, applying this method we were able to identify some known membrane-associated proteins not selected with the other two methods. All three methods showed both advantages and disadvantages in identification of outer membrane proteins. The combination of all three approaches resulted in a most comprehensive approach and so designed proteomic analysis seem to be a good tool for quantitative monitoring of the abundance of proteins in a high-throughput manner.

To our knowledge, this is the first report on the proteomic based identification of a *Borrelia* membrane proteome using three different approaches in a single study. It is also the

first report where *B. burgdorferi* s.s. and *B. garinii*, also *B. afzelii* is included in the study as a major causative agent of LB in Europe. Jacobs and colleagues used three different *B. burgdorferi* strains for extensive global proteomic survey and showed a high degree of overlap of detected proteins (Jacobs et al., 2005). In another study proteins presented in the soluble and membrane-associated fractions of the *B. burgdorferi* strain B31 were examined (Nowalk et al., 2006). Analyzing the membrane composition of the three strains representing each genospecies, we were able to identify 104 proteins that overlap in all three strains and also a number of proteins unique for each genospecies. These species specific proteins could explain differences in disease pathogenesis and manifestations, since each *Borrelia* genospecies disseminate to different tissue or organ. However, in order to receive complete and precise picture of some bacteria also other “omics” analyses are needed. Especially in case of *Borrelia* which has complex biology (environmental induced down- and up-regulation of surface proteins) we should be carefully since culturing conditions are not optimal. Bioinformatic analysis of all proteins identified by these three proteomic approaches showed that only 11 from 25 antigens (44%) selected with ANTIGENome technology could be identified among different proteomic preparations. Some of the protective candidates were not identified at all (such as BAP006 which is one of the main candidates, BA0221, and BA0507), or only in some of the preparations (eg. BA0675). This emphasizes the value of the genomic approach not relying on protein abundance and in vitro expression.

This study show that our ANTIGENome technology is very promising tool to identify novel vaccine candidates in *Borrelia* as in many other pathogens like pneumococcus (Giefing et al., 2008), *Helicobacter pylori* (Meinke et al., 2009) or group A streptococcus (Fritzer et al., 2010), and this new designed proteomic approach helped us to better understand and compare pathogenic *Borrelia* genospecies. All this work with *Borrelia* was extremely challenging, but at the same time exciting, and I hope that my contribution will aid the development of a new Lyme borreliosis vaccine.

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7 APPENDICES

Table A1: Antigen validation – Oligonucleotides used for gene distribution analysis.

Bb ORF	Bg ORF	Primer name	Orientation	Sequence	Fragment size (bp)
BB0058	BG0057	210-4636 210-4672	sense antisense	catttgataataaagtcagggtaaa ctgccttaaaatcaaaaattaaaaa	1000
BB0062	BG0061	210-5117 210-5132	sense antisense	aacttttaatgaattaaaacagga agttttgatgcagtaataatagatg	687
BB0064	BG0063	210-4888 210-4948	sense antisense	ggatgtattaatgttcattctctctt tcaacaatttctctattttctctc	488
BB0074	BG0073	210-4862 210-4922	sense antisense	cgaagacatttgagggaagc cgtccataaccgaagtgggtg	990
BB0077	BG0076	210-4897 210-4957	sense antisense	caaattatttatgcaaaaagcta ttgtagtatatttcttctcatattca	917
BB0081	BG0079	210-4874 210-4934	sense antisense	aaaatagctttaataggttctggaat ttcagaattttcttctccaaa	1030
BB0090	BG0091	210-5103 210-5118	sense antisense	ggtttaataggagtttaattcagcttg aataccattacaaaaagagcaacaga	401
BB0094	BG0095	210-4879 210-4939	sense antisense	actattcgattgatgagcaaa caaacataaatttgacgctctga	1038
BB0109	BG0110	210-4880 210-4940	sense antisense	tgatgaatctcaaaagtgtt taaacaaggccttttcaatgc	920
BB0127	BG0129	210-4984 210-5001	sense antisense	agattcaaatatgaaaatgttctg aaactcttactgaaagccttttg	1050
BB0136	BG0138	210-4637 210-4673	sense antisense	acagcccagacaacacataatc ttcgtctgaccattttgaa	1041
BB0147	BG0147	210-4638 210-4674	sense antisense	acatcagctattaatgcttcaagaaa tctaagcaatgacaaaacataattgg	990
BB0149	BG0148	210-4639 210-4675	sense antisense	ttttgttctggacttgagag ttttccaaaggggttttaaatca	920
BB0151	BG0149	210-4985 210-5002	sense antisense	tcaaaatccgttttaactggaaa tggattgtaagagcttgcttga	1050
BB0181	BG0180	210-4640 210-4676	sense antisense	gtggattcaacattttcagga cttgaactgtatctggagcattg	1201
BB0193	BG0191	210-5104 210-5119	sense antisense	aaaatttagaaattacaaaagacaagc aatttttaagtcttttctgtattgc	452
BB0200	BG0199	210-5105 210-5120	sense antisense	tgaattttcttgcaaatctgctt ttcatgaataaattgttctgattt	886
BB0210	BG0212	210-4641 210-4677	sense antisense	caaattttgcgtattattgctttt tcgttttagatccttaattggcttg	1210
BB0215	BG0218	210-4863 210-4923	sense antisense	caactgtaaaaatcaagacaatgaaa ccctaaaaagccttggttctca	778
BB0221	BG0224	210-4642 210-4678	sense antisense	caggatctaggctttccaag ttttgaaacatttgaaagcatttt	1059
BB0236	BG0229	210-4898 210-4958	sense antisense	caagaagagtttaaatgggctct ttggcatccaaaatagatgaa	1025
BB0238	BG0241	210-4643 210-4679	sense antisense	ttttgaatttattgtctcaagg tcaatttatggaagacaaaacca	729
BB0283	BG0286	210-4644 210-4680	sense antisense	ggcttttatattctgggttttctgg ccattttgatcaatttcataatttc	1000
BB0295	BG0298	210-4986 210-5003	sense antisense	tgagcattgcagttaatatggt ttcaaatcttttgcgttaatttg	1006
BB0315	BG0318	210-4987 210-5004	sense antisense	gaaaaactttacaaaaatcaatgtta agaagggtattgtttattattct	490
BB0323	BG0324	210-4787 210-4800	sense antisense	tttatcgatgcaaaacg tgcagattctaaaaataattcattgg	886

Bb ORF	Bg ORF	Primer name	Orientation	Sequence	Fragment size (bp)
BB0329	BG0330	210-4645 210-4681	sense antisense	gagcagagccaagcagtctt ttttcaataattatcttctgctt	1355
BB0330	BG0331	210-4646 210-4682	sense antisense	gctaaaaattgggaagcctca tggtttgaataaatgcagcaa	941
BB0345	BG0346	210-4988 210-5005	sense antisense	tttgggagttatttgagaaaagt caaaaattcttttcctgctc	1108
BB0347	BG0348	210-5106 210-5121	sense antisense	ccaaatgaaaaatgaagaatca ttttctgcttttgaatta	1087
BB0353	BG0354	210-4647 210-4683	sense antisense	aagagaaatatagcattttgagca caataaaaatacatcgttattccgta	1081
BB0359	BG0359	210-4648 210-4684	sense antisense	cgggcttataaggctggaat cctaatttagacaaatactcacaagc	1001
BB0366	BG0365	210-4864 210-4924	sense antisense	ctgttgctttgctattattgg cccatgctattgttctattcca	1060
BB0382	BG0381	210-4899 210-4959	sense antisense	tccatgttggtataggtgtt aacaccaattcctgctaaacc	600
BB0388	BG0389	210-4900 210-4960	sense antisense	tctttcacagaaaaggactt ttttccagcaatagaagca	1211
BB0389	BG0390	210-4989 210-5006	sense antisense	tcatctgggacaaggaagag ccaagatcatacttcttcaga	1039
BB0418	BG0421	210-4875 210-4935	sense antisense	aattttcaatctgcttattatctc tatcactccccagccttg	724
BB0420	BG0423	210-4788 210-4801	sense antisense	ttaataggaaatgctttaagt aaaatctttatacaattcgctacgc	1450
BB0434	BG0441	210-4889 210-4949	sense antisense	aatagtaggagaaagaaggtaggg aaaaaggaacaattcttctaaa	600
BB0436	BG0443	210-4881 210-4941	sense antisense	aaaaaggcttaagagcttgctt ccattttcaattaaatctctca	1014
BB0446	BG0454	210-4882 210-4942	sense antisense	aatgaattgccatttatgatagaaga aaatccaaatctatctctgattttc	1230
BB0457	BG0466	210-5107 210-5122	sense antisense	atatgggacagtgccttggga ttctgtgtgcttcatctcttaca	1075
BB0467	BG0480	210-4890 210-4950	sense antisense	tgaatttaggatagctgatgtt aaattctccaaattaaagcataac	659
BB0472	BG0484	210-4901 210-4961	sense antisense	tcctaataatgatgtaaaagttgt aatctcgctgcgaccttc	1060
BB0479	BG0490	210-4891 210-4951	sense antisense	ttttctaaagatgggaaagaga tctcttatattttaccagcccttt	504
BB0512	BG0523	210-4649 210-4685	sense antisense	tgttgattcaaatattcaagaaaagc gcaccttgatttcaactcatc	1437
BB0518	BG0529	210-4850 210-4910	sense antisense	ccaaatggttacaaacctga ttttgaagttcttctgttagattt	1597
BB0519	BG0530	210-4789 210-4802	sense antisense	caacaaacaagataaaaaataca ttttgctttgagcaacttt	522
BB0546	BG0556	210-4990 210-5007	sense antisense	tgaagatttgaatgaaaacaatga tgttaacgggtaatatataagttct	856
BB0553	BG0563	210-4902 210-4962	sense antisense	atgtatatcattattggggctaa acataattcctagttcgtgga	1400
BB0560	BG0570	210-4851 210-4911	sense antisense	tcagaaaaagtagaagttacatcaaaa tccttcgtattctggaattagattt	1032
BB0571	BG0582	210-5108 210-5123	sense antisense	gaaataataagcttggaggagga tgaagagagttttgtgctattgg	579
BB0579	BG0591	210-4876 210-4936	sense antisense	tcctttaaagtataattgtcttttg tctctcatcaattattaaatcaaca	1380
BB0588	BG0601	210-4883 210-4943	sense antisense	tggtcttaaaaaagattttaagg aacatgcccattgtctgcac	481
BB0603	BG0616	210-4650 210-4686	sense antisense	aattgcagaacacctttgaa gcttcgctgtaggctatattt	1132
BB0608	BG0621	210-4991 210-5008	sense antisense	tcaaaaattccaagatgttcaaaa tcttcaagatacatctctttatacac	1205
BB0612	BG0628	210-4651	sense	ttgccaactccaacaact	1059

Bb ORF	Bg ORF	Primer name	Orientation	Sequence	Fragment size (bp)
		210-4687	antisense	ttaattgcatttcccactaaaat	
BB0613	BG0629	210-4790	sense	atgttttattgttacaacagccaat	1000
		210-4803	antisense	tcaataaatcaaaaacttcttccaa	
BB0625	BG0644	210-4652	sense	gggtcaaatattattccaagtga	1131
		210-4688	antisense	ttaaacctagaattaccaactttca	
BB0648	BG0671	210-5109	sense	ttgaaattctaagagaacatgagga	1149
		210-5124	antisense	tgctgttatttttaaattagtccttt	
BB0649	BG0672	210-4865	sense	aaaagcttacttagtggcggtga	1050
		210-4925	antisense	ttttctttatcatattcagatgttg	
BB0658	BG0681	210-4653	sense	tcttttactgggtggacagatg	681
		210-4689	antisense	ccttgactagcaacagattcca	
BB0668	BG0691	210-4654	sense	tttgctcaagagactgatgga	958
		210-4690	antisense	ttcttcagggttttcactctcaa	
BB0670	BG0693	210-5110	sense	ttaagggtgttcttttgagcttg	1180
		210-5125	antisense	tgactcttactatccaagaacataaa	
BB0680	BG0703	210-4655	sense	gaagcttaaatacaggtttgttga	1331
		210-4691	antisense	cttctaaactgccttatttcatagc	
BB0687	BG0710	210-4892	sense	gcaattgccatcaacaagag	870
		210-4952	antisense	ttagcatttatcgcttcaaaactt	
BB0710	BG0733	210-4893	sense	ttggatttttaagtgtatggaaa	1191
		210-4953	antisense	aactaaaattttcaacaagctact	
BB0740	BG0761	210-5111	sense	ttcaagcaaattcgatgaca	609
		210-5126	antisense	ttccaaatccttttctatgtg	
BB0744	BG0765	210-4656	sense	aaggactttgttaatatggatctgag	1178
		210-4692	antisense	ttttctttgctagaagctttagaattt	
BB0751	BG0773	210-4903	sense	tgacaaaacaaaaatttctcaa	855
		210-4963	antisense	tgctcttgcattttctcg	
BB0754	BG0776	210-4884	sense	aaacggagccggaaagtc	730
		210-4944	antisense	gtagctaaagagatcttctctgtttt	
BB0760	BG0784	210-5112	sense	gaataccaagaaaaagcaaaaa	286
		210-5127	antisense	cattttcatggcgcttttgt	
BB0765	BG0788	210-4906	sense	aaaaacttaacaaccctaatttgc	860
		210-4966	antisense	ttttattttgattatagggcgata	
BB0772	BG0796	210-4992	sense	tctgaaagcgtaaaattaaaagaa	900
		210-5009	antisense	ttccatcaatttctccaaga	
BB0783	BG0807	210-4657	sense	aattgttttaataatagcaagtgtagt	1126
		210-4693	antisense	atcatctcctaaaataactattacatc	
BB0789	BG0814	210-4885	sense	ttggagcaagtcgtgttagag	994
		210-4945	antisense	gatgcctcttttaacattcttca	
BB0794	BG0820	210-4658	sense	tatgccagttgcaattcagg	1258
		210-4694	antisense	tttaaatctcaagaaatgccta	
BB0796	BG0822	210-4659	sense	tgttgattttgatagaattgtgattg	331
		210-4695	antisense	tgcaacatattgaataaccaga	
BB0832	BG0857	210-4904	sense	cctccaaaacaaaatctaaattac	580
		210-4964	antisense	tttttagtattgggtgattatttg	
BB0844	BGP096	210-4860	sense	tttaggccttatagagaaattcaa	598
		210-4920	antisense	aatctctcagtaattggcatctttt	
BBA03	BGA02	210-4852	sense	ttatgcttaattgcaaaaacaaaag	450
		210-4912	antisense	taagtgtgggttttggaaatctgct	
BBA04	BGA03	210-4877	sense	cggtggataaaaaattaaagccaga	518
		210-4937	antisense	aatgcaaaacttgcgccttt	
BBA21	BGA19	210-5113	sense	gcgttagagaagaagagcaagc	420
		210-5128	antisense	agctgtgaagcttgggattt	
BBA24	BGA21	210-4853	sense	tgttggcgctgttagtagcatgt	483
		210-4913	antisense	tttttattgtctctgttttctttt	
BBA34	BGA28	210-4660	sense	cgggccttcaagctaaa	879
		210-4696	antisense	gcaacactgtatattggaattattgg	
BBA36	BGA30	210-4854	sense	ttttgattggcttctaaggatg	284
		210-4914	antisense	tctctcattgcatttctcagg	
BBA57	BGA58	210-4855	sense	gataaaaacaaagccttaaatgaa	1009
		210-4915	antisense	cttttagttgtctttgtgtttt	

Bb ORF	Bg ORF	Primer name	Orientation	Sequence	Fragment size (bp)
BBA73	BGA73	210-4993 210-5010	sense antisense	ctgttctcatgctctttttattct agtgtatgtgggtcacacagggtt	835
BBB16	BGB15	210-4793 210-4806	sense antisense	tgggtgttagtggtcctttcaa tttttagaatttctgtctttt	831
BBB17	BGB16	210-4994 210-5011	sense antisense	ttttgatgatgtgtctttaattcca catcgtgaggatgggattct	1166
BBB19	BGB18	210-4866 210-4926	sense antisense	tgaaaaagaatacattaagtgcgata caggacttgaagctctttaactgaa	609
BBC01	BGP297	210-5114 210-5129	sense antisense	agaagaaagaaagattaatgaaat ttgtattctatttcttttgggtta	570
BBG10	BGP027	210-4661 210-4697	sense antisense	caagctcaacaacaactgc cttgctctttcttttgcgtga	1100
BBG12	BGP025	210-4995 210-5012	sense antisense	ccctatatcccagactatattagaaa tgaaacacgctcaaccctta	381
BBG21	BGP116	210-4662 210-4698	sense antisense	aaagaaaaacaaaacacacaatca ttgtaacttcgctatctctgcac	953
BBG23	BGP013	210-4796 210-4809	sense antisense	aagtgtatgaagactttgaagaaa ccaatcgtacctgtaaaatga	775
BBG24	BGP012	210-4663 210-4699	sense antisense	tattgtatgatgtctagcgaag gaaagcctgaatgattttctga	1350
BBG30	BGP003	210-5115 210-5130	sense antisense	tgggggaccttaaccgagta acttcaccttggcccttac	255
BBG33	-	210-4867 210-4927	sense antisense	atggaacaactaatagctcaaga aaattgttttctaaatttctaaatc	90
BBH09	BGP199	210-4664 210-4700	sense antisense	ttcgtactgaagaagagcttgaaa tccatggaggatttccaat	1000
BBH13	-	210-4856 210-4916	sense antisense	ctgaagaacaaatatataaagagtcc ttctacggtatcgatcttagcatct	404
BBJ41	BGA66	210-4878 210-4938	sense antisense	tgcaccttttggcaatgtta tgaagtgaataatcccttgatgttt	543
BBK32	BGP100	210-4665 210-4701	sense antisense	gaatcccttgcttatttga ttgttctttgatctttgcaattaaa	867
BBL06	BGP173	210-4666 210-4702	sense antisense	tgcaaaagctgtggcaata tttgccttatgttaacttgcaaaa	929
BBL27	BGP132	210-4857 210-4917	sense antisense	gggaaaattttatggaaacagtg aaaagcaaatattagccctgct	554
BBL28	BGP273	210-4886 210-4946	sense antisense	ttttactaatgctaaacggctgtaa tcactttttatgatcaagtgcaga	281
BBM03	BGP043	210-4895 210-4955	sense antisense	caaaaattaacaagagtttgataa gctctatttccaaaaacactagca	532
BBM15	BGP069	210-4669 210-4705	sense antisense	ttataaacagggtcattgggatt tttagagccgcacccctcat	1000
BBM29	BGP292	210-4905 210-4965	sense antisense	caagaatacaaaaataattcaagca ttttcaacatgcaaaagaattt	887
BBN39	-	210-4869 210-4929	sense antisense	ttttgctgtgataatttcttg tcatcatcacaggcccagta	694
BBO34	BGP314	210-4784 210-4797	sense antisense	tgactaatttagcgtacagaaca ctggatatacttttgttaaagtttt	352
BBO39	BGP260	210-4785 210-4798	sense antisense	aaatgaaaatgttattatttgcgtg tttatcttcttattcccttaagttc	671
BBO40	BGP138	210-5116 210-5131	sense antisense	ttttgctgttttgcactt tttttaactcttctaactcctgattt	1003
BBP28	BGP028	210-4870 210-4930	sense antisense	tgaataatcatcaacatatta tttttaaccagtcaagatgtgtttt	225
BBQ03	BGP310	210-4667 210-4703	sense antisense	tcaggaaacagctgttcaaat tctggagaataataactcgcttataca	354
BBQ13	BGP040	210-4871 210-4931	sense antisense	ttgatgaaaattattatgcaaaagc cctaaaacaaaatcaatgtatgaatga	895
BBQ32	BGP190	210-4668 210-4704	sense antisense	tgcataataacactattggttagg gcattttcacctctttaagtatttg	331
BBQ34	BGP110	210-4858	sense	gactgggtatggaacaactaatagc	446

Bb ORF	Bg ORF	Primer name	Orientation	Sequence	Fragment size (bp)
		210-4918	antisense	aagttcgcttttaacagtatcaatctt	
BBR27	-	210-4887	sense	tgaccataagggaattttatgg	496
		210-4947	antisense	agcccggtatcattgttat	
BBR35	-	210-4908	sense	agaattaaaaggataataaaggggatt	280
		210-4968	antisense	gatatacttttgcagggtttt	
BBR42	BGP260	210-4670	sense	gaaggatttttagaatttagagaca	470
		210-4706	antisense	tcttctcaatcttttaagcgaat	
BBS36	BGP301	210-4671	sense	ttaagggttaataaaagagatttaagt	502
		210-4707	antisense	tttttaattattctttccatcac	
BBS41	-	210-4861	sense	aattatttgtcagttttgtttga	550
		210-4921	antisense	attttgaggctctgtagctttt	
BBS42	-	210-4894	sense	aagtttaagtcaaatatagaagaaaa	466
		210-4954	antisense	ctaactcttgggcaaatcagc	
BGA26	BGA26	210-4997	sense	tgaaggcaaagaaagatcgag	316
		210-5014	antisense	aaggctgctgctaattttcaa	
BGP022	BGP001	210-4999	sense	atggaaattggagaaaaa	371
		210-5016	antisense	tcctttactaaatcgctgtcca	
BGP036	BGP022	210-4859	sense	tgctgagagcgagtagaca	1000
		210-4919	antisense	gtcaagcacaccctgagcat	
BGP049	BGP036	210-5000	sense	tgaatggaaaaatgagaatgtt	1057
		210-5017	antisense	tcttgacaaccatcactatttgg	
BGP071	BGP049	210-4909	sense	ccagttatcaactattatgatagaaa	272
		210-4969	antisense	agacttgtaattatagctaattctt	
BGP085	BGP085	210-4872	sense	tgaggattttggttggtgtt	469
		210-4932	antisense	tcctttccatctcaaatattgc	
BGP267	BGP267	210-4896	sense	attctattatcaaaccttattaattgc	142
		210-4956	antisense	ccgtcattaatagattttcgtcctt	
BGP321	BGP321	210-4873	sense	gcataatgttagagacaaaggtaaatga	194
		210-4933	antisense	tttcgtcttttacaatactaatttca	

Figure A1: Phylogenetic tree of 62

different OspC types. 34 OspC

types are associated with human disease (H), from which 14 OspC types with invasive human disease (H Inv). In the present study, 9 from 14 OspC types associated with invasive infection were identified and are indicated red. Each type is characteristic for one of three pathogenic *Borrelia* species (Baf, *B. afzelii*; Bbu, *B. burgdorferi*; Bga, *B. garinii*). Two types, 37 and 52, are characteristic for both *B. afzelii* and *B. burgdorferi* genospecies.

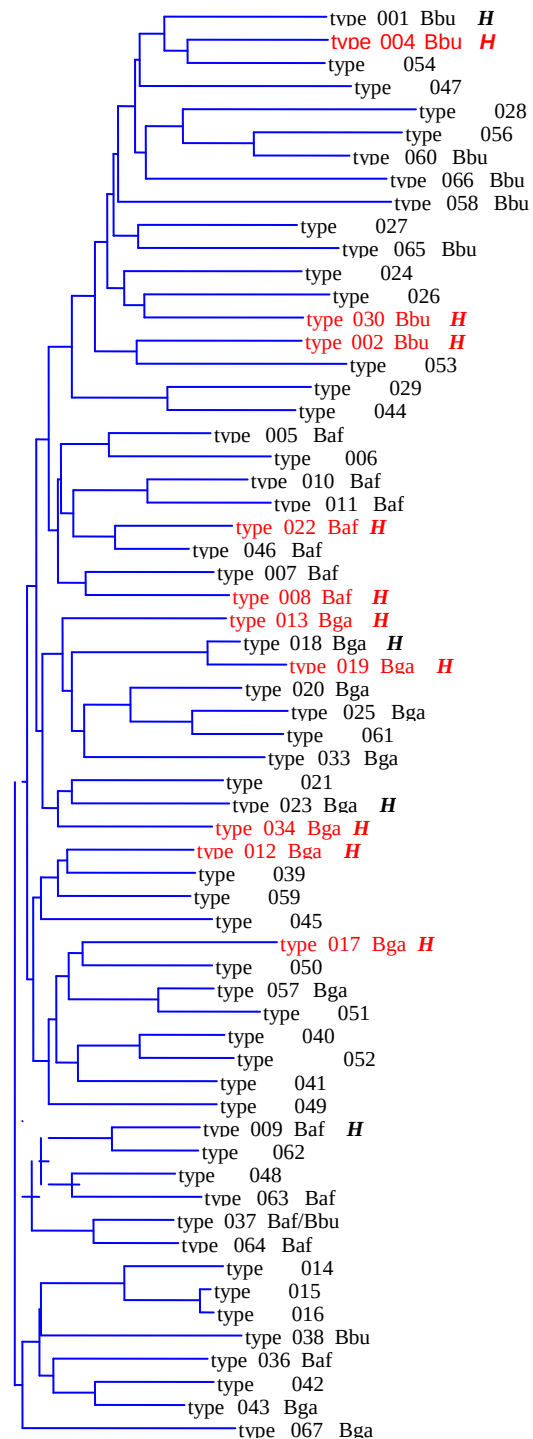


Table A2: Most probable number table.

Positive	MPN ^m	95% CL	Positive	MPN ^m	95% CL	Positive	MPN ^m	95% CL	Positive	MPN ^m	95% CL
10; 1; 0,1			10; 1; 0,1			10; 1; 0,1			10; 1; 0,1		
8 8 7	208	80,9 - 535	8 1 3	5,22	2,03 - 13,4	6 3 2	2,38	0,93 - 6,12	3 4 0	1,01	0,39 - 2,60
8 8 6	139	54,1 - 357	8 1 2	4,27	1,66 - 11,0	6 3 1	2,11	0,82 - 5,42	3 3 1	1,00	0,39 - 2,57
8 8 5	98,2	38,2 - 252	8 1 1	3,50	1,36 - 9,00	6 3 0	1,86	0,72 - 4,78	3 3 0	0,85	0,33 - 2,18
8 8 4	70,2	27,3 - 180	8 1 0	2,87	1,12 - 7,38				3 2 1	0,85	0,33 - 2,18
8 8 3	51,0	19,8 - 131				6 2 2	2,09	0,81 - 5,37	3 2 0	0,70	0,27 - 1,80
8 8 2	38,5	15,0 - 98,9	8 0 2	3,38	1,32 - 8,69	6 2 1	1,84	0,72 - 4,73			
8 8 1	30,1	11,7 - 77,4	8 0 1	2,80	1,09 - 7,20	6 2 0	1,60	0,62 - 4,11	3 1 2	0,84	0,33 - 2,16
8 8 0	24,0	9,34 - 61,7	8 0 0	2,31	0,90 - 5,94				3 1 1	0,70	0,27 - 1,80
						6 1 2	1,82	0,71 - 4,68	3 1 0	0,56	0,22 - 1,44
8 7 8	59,6	23,2 - 153	7 7 1	5,47	2,13 - 14,1	6 1 1	1,58	0,61 - 4,06	3 0 2	0,69	0,27 - 1,77
8 7 7	50,8	19,8 - 131	7 7 0	4,84	1,88 - 12,4	6 1 0	1,35	0,53 - 3,47	3 0 1	0,55	0,21 - 1,41
8 7 6	43,3	16,8 - 111							3 0 0	0,41	0,16 - 1,05
8 7 5	36,9	14,4 - 94,8	7 6 2	5,30	2,06 - 13,6	6 0 2	1,56	0,61 - 4,01			
8 7 4	31,4	12,2 - 80,7	7 6 1	4,71	1,83 - 12,1	6 0 1	1,34	0,52 - 3,44	2 4 0	0,79	0,31 - 2,03
8 7 3	26,7	10,4 - 68,6	7 6 0	4,15	1,61 - 10,7	6 0 0	1,13	0,44 - 2,90	2 3 1	0,79	0,31 - 2,03
8 7 2	22,6	8,79 - 58,1							2 3 0	0,66	0,26 - 1,70
8 7 1	19,1	7,43 - 49,1	7 5 2	4,58	1,78 - 11,8	5 5 1	2,07	0,81 - 5,32	2 2 1	0,65	0,25 - 1,67
8 7 0	15,9	6,19 - 40,9	7 5 1	4,04	1,57 - 10,4	5 5 0	1,85	0,72 - 4,75	2 2 0	0,52	0,20 - 1,34
			7 5 0	3,55	1,38 - 9,12				2 1 1	0,52	0,20 - 1,34
8 6 6	28,4	11,1 - 73,0				5 4 1	1,84	0,72 - 4,73	2 1 0	0,39	0,15 - 1,00
8 6 5	25,0	9,73 - 64,3	7 4 3	4,46	1,74 - 11,5	5 4 0	1,63	0,63 - 4,19			
8 6 4	21,8	8,48 - 56,0	7 4 2	3,95	1,54 - 10,2				2 0 2	0,51	0,20 - 1,31
8 6 3	18,9	7,35 - 48,6	7 4 1	3,47	1,35 - 8,92	5 3 2	1,82	0,71 - 4,68	2 0 1	0,38	0,15 - 0,98
8 6 2	16,3	6,34 - 41,9	7 4 0	3,04	1,18 - 7,81	5 3 1	1,61	0,63 - 4,14	2 0 0	0,26	0,10 - 0,67
8 6 1	13,8	5,37 - 35,5				5 3 0	1,41	0,55 - 3,62			
8 6 0	11,5	4,47 - 29,6	7 3 3	3,86	1,50 - 9,92				1 3 0	0,49	0,19 - 1,26
			7 3 2	3,40	1,32 - 8,74	5 2 2	1,60	0,62 - 4,11	1 2 1	0,49	0,19 - 1,26
8 5 5	18,9	7,35 - 48,6	7 3 1	2,98	1,16 - 7,66	5 2 1	1,40	0,54 - 3,60	1 2 0	0,36	0,14 - 0,93
8 5 4	16,6	6,46 - 42,7	7 3 0	2,59	1,01 - 6,66	5 2 0	1,21	0,47 - 3,11	1 1 1	0,36	0,14 - 0,93
8 5 3	14,4	5,60 - 37,0							1 1 0	0,24	0,09 - 0,62
8 5 2	12,3	4,79 - 31,6	7 2 3	3,33	1,30 - 8,56	5 1 2	1,39	0,54 - 3,57			
8 5 1	10,3	4,01 - 26,5	7 2 2	2,92	1,14 - 7,50	5 1 1	1,20	0,47 - 3,08	1 0 2	0,36	0,14 - 0,93
8 5 0	8,42	3,28 - 21,6	7 2 1	2,55	0,99 - 6,55	5 1 0	1,01	0,39 - 2,60	1 0 1	0,24	0,09 - 0,62
			7 2 0	2,20	0,86 - 5,65				1 0 0	0,12	0,05 - 0,31
8 4 5	14,8	5,76 - 38,0				5 0 2	1,19	0,46 - 3,06			
8 4 4	13,0	5,06 - 33,4	7 1 3	2,87	1,12 - 7,38	5 0 1	1,01	0,39 - 2,60	0 2 0	0,23	0,09 - 0,59
8 4 3	11,1	4,32 - 28,5	7 1 2	2,51	0,98 - 6,45	5 0 0	0,83	0,32 - 2,13			
8 4 2	9,40	3,66 - 24,2	7 1 1	2,17	0,84 - 5,58				0 1 1	0,23	0,09 - 0,59
8 4 1	7,74	3,01 - 19,9	7 1 0	1,86	0,72 - 4,78	4 4 0	1,28	0,50 - 3,29	0 1 0	0,11	0,04 - 0,28
8 4 0	6,22	2,42 - 16,0									
			7 0 2	2,14	0,83 - 5,50	4 3 1	1,27	0,49 - 3,26			
8 3 5	11,8	4,59 - 30,3	7 0 1	1,83	0,71 - 4,70	4 3 0	1,10	0,43 - 2,83			
8 3 4	10,2	3,97 - 26,2	7 0 0	1,55	0,60 - 3,98				0 0 1	0,11	0,04 - 0,28
8 3 3	8,67	3,37 - 22,3				4 2 1	1,09	0,42 - 2,80			
8 3 2	7,18	2,79 - 18,5	6 6 1	3,08	1,20 - 7,92	4 2 0	0,93	0,36 - 2,39			
8 3 1	5,82	2,26 - 15,0	6 6 0	2,77	1,08 - 7,12						
8 3 0	4,67	1,82 - 12,0				4 1 2	1,08	0,42 - 2,78			
			6 5 1	2,73	1,06 - 7,02	4 1 1	0,92	0,36 - 2,36			
8 2 4	8,07	3,14 - 20,7	6 5 0	2,44	0,95 - 6,27	4 1 0	0,76	0,30 - 1,95			
8 2 3	6,72	2,61 - 17,3									
8 2 2	5,50	2,14 - 14,1	6 4 2	2,69	1,05 - 6,91	4 0 2	0,91	0,35 - 2,34			
8 2 1	4,45	1,73 - 11,4	6 4 1	2,41	0,94 - 6,19	4 0 1	0,75	0,29 - 1,93			
8 2 0	3,62	1,41 - 9,30	6 4 0	2,14	0,83 - 5,50	4 0 0	0,60	0,23 - 1,54			

Table A3: Antigen validation – sera used for peptide ELISA.

Intercell No.	Source	Clinical diagnosis
P1755	Uni Graz	Borreliosis
P1766	Uni Graz	Borreliosis
P1772	Uni Graz	Borreliosis
P1806	Uni Graz	Borreliosis
P1811	Uni Graz	Borreliosis
P3055	MUW	Erythema migrans
P3186	MUW	Erythema migrans
P3219	MUW	Erythema migrans
P3077	MUW	Neuroborreliose
P3084	MUW	Neuroborreliose
P3288	MUW	Neuroborreliose
P3150	MUW	Borreliose
P3301	MUW	Borreliose
P3344	MUW	Borreliose
P3394	MUW	Borreliose
P3183	MUW	ACA
P3336	MUW	ACA
P1789	Uni Graz	Borreliosis
P1796	Uni Graz	Borreliosis
P1938	Uni Graz	Borreliosis
P1941	Uni Graz	Borreliosis

Table A4: Summary of peptide ELISA with 22 human sera. Sum indicates how many of the human sera reacted with the respective peptide. From aa and To aa denotes the position of the respective peptide in the protein.

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAOamds.02	BA0619	2	98	125
BAOamds.04	BA0619	7	144	170
BAOamds.05	BA0619	4	165	187
BAOamds.07	BA0619	14	201	223
BAOamds.08	BA0619	15	285	309
BAOamds.09	BA0619	9	305	329
BAOamds.10	BA0619	7	325	348
BAOamds.11	BA0619	19	400	425
BAOamds.12	BA0619	8	513	541
BAOamds.13	BA0619	2	537	564
BAOamds.14	BA0619	2	560	587
BAOamds.15	BA0619	3	583	610
BAOba0077.01	BA0075	20	138	164
BAOba0077.02	BA0075	14	160	187
BAOba0089.01	BA0087	12	160	187
BAOba0128.01	BA0126	15	350	378
BAOba0128.02	BA0126	18	374	402
BAOba0128.03	BA0126	9	398	426
BAOba0128.04	BA0126	17	422	451
BAOba0155.01	BA0152	3	264	288
BAOba0155.02	BA0152	11	284	308
BAOba0155.03	BA0152	10	304	328
BAOba0198.01	BA0192	8	77	91
BAOba0207.01	BA0200	10	111	134
BAOba0242.01	BA0235	6	126	153
BAOba0242.02	BA0235	11	149	176
BAOba0242.03	BA0235	17	172	200
BAOba0303.01	BA0295	12	220	245
BAOba0303.02	BA0295	10	241	266
BAOba0303.03	BA0295	4	262	288
BAOba0323.01	BA0314	1	31	53
BAOba0323.02	BA0314	13	49	71
BAOba0323.03	BA0314	20	67	89
BAOba0356.01	BA0343	13	258	280
BAOba0356.02	BA0343	10	276	298

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAOba0356.03	BA0343	12	294	317
BAOba0356.04	BA0343	11	313	336
BAOba0358.01	BA0345	13	458	472
BAOba0378.01	BA0364	8	82	107
BAOba0378.02	BA0364	14	103	127
BAOba0378.02	BA0364	7	103	127
BAOba0378.03	BA0364	13	212	235
BAOba0378.03	BA0364	2	212	235
BAOba0378.04	BA0364	18	231	253
BAOba0378.04	BA0364	4	231	253
BAOba0394.01	BA0380	12	26	51
BAOba0394.02	BA0380	10	47	73
BAOba0402.01	BA0388	6	1053	1082
BAOba0403.01	BA0389	5	194	217
BAOba0403.02	BA0389	17	213	236
BAOba0403.03	BA0389	15	232	255
BAOba0403.04	BA0389	5	251	274
BAOba0403.05	BA0389	15	270	292
BAOba0444.01	BA0429	10	110	131
BAOba0444.02	BA0429	4	179	201
BAOba0444.03	BA0429	11	197	219
BAOba0444.04	BA0429	2	215	236
BAOba0470.01	BA0429	11	215	236
BAOba0480.01	BA0464	5	12	36
BAOba0480.02	BA0464	3	32	56
BAOba0480.03	BA0464	11	52	77
BAOba0485.01	BA0469	12	281	300
BAOba0485.02	BA0469	16	296	316
BAOba0491.01	BA0475	12	33	57
BAOba0491.02	BA0475	4	53	78
BAOba0557.01	BA0539	9	171	197
BAOba0557.02	BA0539	7	193	219
BAOba0557.03	BA0539	6	215	240
BAOba0565.01	BA0546	13	363	388
BAOba0565.02	BA0546	20	384	409

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAOba0565.03	BA0546	4	405	430
BAOba0573.01	BA0553	5	102	127
BAOba0573.02	BA0553	5	123	148
BAOba0573.03	BA0553	7	144	170
BAOba0584.01	BA0564	13	149	163
BAOba0623.01	BA0601	1	342	366
BAOba0623.02	BA0601	14	362	386
BAOba0670.01	BA0643	17	390	409
BAOba0692.01	BA0665	9	50	64
BAOba0710.01	BA0682	5	223	249
BAOba0710.02	BA0682	13	245	272
BAOba0765.01	BA0733	9	117	131
BAOba0779.01	BA0745	15	116	143
BAOba0779.02	BA0745	3	139	166
BAOba0789.01	BA0754	3	1	21
BAOba0802.01	BA0767	10	245	270
BAOba0802.02	BA0767	11	266	291
BAOba0802.03	BA0767	6	287	312
BAOba0802.04	BA0767	3	308	332
BAOba0819.01	BA0784	2	353	378
BAOba0819.02	BA0784	16	374	399
BAOba0819.03	BA0784	8	395	419
BAOba0832.02	BA0784	7	395	419
BAOba0832.03	BA0784	14	395	419
BAObb0058.01	BA0056	17	128	151
BAObb0058.02	BA0056	6	146	169
BAObb0058.06	BA0056	2	314	336
BAObb0058.07	BA0056	12	339	361
BAObb0058.08	BA0056	15	357	379
BAObb0064.01	BA0062	6	159	188
BAObb0064.02	BA0062	6	184	213
BAObb0074.01	BA0072	9	230	251
BAObb0074.02	BA0072	2	247	267
BAObb0081.01	BA0078	1	97	120
BAObb0081.02	BA0078	2	116	139
BAObb0081.03	BA0078	5	135	158
BAObb0081.04	BA0078	2	154	177
BAObb0094.01	BA0091	6	195	228
BAObb0109.01	BA0106	8	165	192
BAObb0109.02	BA0106	6	188	214

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAObb0181.01	BA0181	10	6	29
BAObb0181.02	BA0181	4	25	47
BAObb0181.03	BA0181	10	43	65
BAObb0181.04	BA0181	1	68	92
BAObb0181.06	BA0181	17	262	288
BAObb0181.08	BA0181	7	306	331
BAObb0181.09	BA0181	2	326	351
BAObb0181.10	BA0181	2	449	471
BAObb0181.11	BA0181	1	467	489
BAObb0181.12	BA0181	3	485	507
BAObb0215.01	BA0215	5	80	103
BAObb0215.02	BA0215	1	99	122
BAObb0215.03	BA0215	5	118	141
BAObb0215.04	BA0215	15	137	159
BAObb0238.01	BA0237	2	15	40
BAObb0238.02	BA0237	16	36	61
BAObb0238.03	BA0237	9	57	82
BAObb0238.04	BA0237	3	78	103
BAObb0238.05	BA0237	5	99	124
BAObb0238.06	BA0237	7	120	146
BAObb0283.01	BA0283	4	35	63
BAObb0283.02	BA0283	2	59	86
BAObb0283.04	BA0283	3	187	213
BAObb0283.05	BA0283	2	209	235
BAObb0283.07	BA0283	3	252	277
BAObb0323.01	BA0321	10	83	108
BAObb0323.02	BA0321	11	104	129
BAObb0323.03	BA0321	8	125	150
BAObb0323.04	BA0321	7	146	172
BAObb0323.05	BA0321	7	272	302
BAObb0323.06	BA0321	9	298	327
BAObb0353.01	BA0351	13	197	222
BAObb0353.02	BA0351	16	218	243
BAObb0353.03	BA0351	15	239	264
BAObb0353.04	BA0351	16	260	284
BAObb0359.01	BA0356	16	378	402
BAObb0359.02	BA0356	17	398	422
BAObb0359.03	BA0356	18	418	442
BAObb0359.04	BA0356	17	438	461
BAObb0418.01	BA0417	15	44	65

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAObb0418.02	BA0417	1	61	82
BAObb0418.03	BA0417	1	78	99
BAObb0420.01	BA0419	5	1230	1254
BAObb0420.02	BA0419	7	150	1275
BAObb0436.01	BA0431	7	372	395
BAObb0436.02	BA0431	4	391	414
BAObb0436.03	BA0431	5	410	434
BAObb0446.01	BA0442	5	439	464
BAObb0512.01	BA0507	17	718	744
BAObb0512.02	BA0507	15	740	766
BAObb0512.03	BA0507	17	762	788
BAObb0512.04	BA0507	17	906	931
BAObb0512.05	BA0507	17	927	951
BAObb0512.06	BA0507	17	947	971
BAObb0518.01	BA0513	1	66	93
BAObb0518.04	BA0513	1	135	161
BAObb0518.05	BA0513	2	494	518
BAObb0519.01	BA0514	7	40	61
BAObb0519.02	BA0514	11	57	79
BAObb0519.03	BA0514	8	75	97
BAObb0579.01	BA0572	2	440	462
BAObb0579.02	BA0572	4	458	480
BAObb0579.03	BA0572	2	476	497
BAObb0588.01	BA0581	5	91	114
BAObb0588.02	BA0581	5	110	133
BAObb0588.03	BA0581	4	129	152
BAObb0588.04	BA0581	3	148	170
BAObb0612.01	BA0605	16	156	183
BAObb0612.02	BA0605	12	179	206
BAObb0612.03	BA0605	16	202	229
BAObb0612.04	BA0605	17	225	252
BAObb0612.05	BA0605	15	248	274
BAObb0613.01	BA0606	11	747	773
BAObb0613.02	BA0606	13	769	795
BAObb0649.01	BA0644	9	6	33
BAObb0649.02	BA0644	9	29	55
BAObb0649.03	BA0644	13	52	79
BAObb0649.04	BA0644	2	74	100
BAObb0658.01	BA0653	15	148	176
BAObb0658.02	BA0653	16	172	200

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAObb0658.03	BA0653	16	196	223
BAObb0680.01	BA0675	14	28	53
BAObb0680.02	BA0675	15	49	73
BAObb0680.03	BA0675	17	165	188
BAObb0680.05	BA0675	16	203	226
BAObb0680.06	BA0675	2	292	320
BAObb0680.07	BA0675	1	316	344
BAObb0680.08	BA0675	2	531	560
BAObb0710.01	BA0703	5	180	208
BAObb0710.02	BA0703	5	200	228
BAObb0754.01	BA0748	4	203	227
BAObb0754.02	BA0748	9	223	247
BAObb0754.03	BA0748	4	243	267
BAObb0754.04	BA0748	6	263	287
BAObb0783.02	BA0778	2	339	363
BAObb0783.03	BA0778	1	359	384
BAObb0794.03	BA0790	7	1372	1398
BAObb0796.01	BA0792	16	14	38
BAObb0796.02	BA0792	3	34	58
BAObb0796.04	BA0792	7	74	98
BAObb0796.05	BA0792	2	94	117
BAObba03.01	BAP002	1	41	67
BAObba03.02	BAP002	3	63	89
BAObba03.05	BAP002	1	129	155
BAObba04.01	BAP003	1	165	185
BAObba04.02	BAP003	5	181	200
BAObba21.01	BAP004	5	26	44
BAObba24.01	BAP005	3	15	44
BAObba24.02	BAP005	2	40	68
BAObba24.03	BAP005	1	64	92
BAObba36.03	BAP007	1	90	114
BAObba36.04	BAP007	1	110	133
BAObba36.05	BAP007	2	140	168
BAObba36.06	BAP007	2	164	192
BAObba36.07	BAP007	2	188	215
BAObba57.01	BAP008	4	91	116
BAObba57.02	BAP008	6	112	137
BAObba57.03	BAP008	6	133	158
BAObba57.04	BAP008	8	154	179
BAObbb16.01	BAP011	8	311	338

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAObbb16.02	BAP011	6	334	361
BAObbb16.03	BAP011	9	357	385
BAObbb16.04	BAP011	7	381	409
BAObbb16.05	BAP011	8	405	433
BAObbb17.01	BAP012	9	331	352
BAObbb17.02	BAP012	9	348	369
BAObbb17.03	BAP012	4	365	386
BAObbb19.01	BAP013	12	116	143
BAObbb19.01	BAP013	6	116	143
BAObbb19.02	BAP013	14	139	166
BAObbb19.02	BAP013	2	139	166
BAObbb19.03	BAP013	20	162	189
BAObbb19.03	BAP013	9	162	189
BAObbb19.04	BAP013	17	185	212
BAObbb19.04	BAP013	9	185	212
BAObbc01.01	BAP014	3	208	230
BAObbg12.01	BAP016	5	32	45
BAObbg12.02	BAP016	4	50	72
BAObbg21.02	BAP017	3	131	153
BAObbg21.03	BAP017	9	149	172
BAObbg21.04	BAP017	5	324	349
BAObbg21.05	BAP017	1	345	370
BAObbg21.06	BAP017	14	366	391
BAObbg23.01	BAP018	8	104	126
BAObbg23.02	BAP018	6	122	143
BAObbg23.03	BAP018	7	148	174
BAObbg23.04	BAP018	2	170	195
BAObbg24.02	BAP019	15	91	115
BAObbg24.03	BAP019	13	111	135
BAObbg24.04	BAP019	5	377	400
BAObbg24.05	BAP019	13	396	419
BAObbg24.06	BAP019	3	532	553
BAObbg24.07	BAP019	3	548	569
BAObbg24.08	BAP019	2	715	744
BAObbg24.09	BAP019	4	740	768
BAObbg33.01	BAP021	7	3	27
BAObbg33.02	BAP021	7	23	47
BAObbg33.03	BAP021	2	43	68
BAObbh09.02	BAP022	2	118	141
BAObbh09.03	BAP022	8	137	160

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAObbh09.05	BAP022	1	521	545
BAObbh13.01	BAP021	4	4	30
BAObbh13.02	BAP021	6	26	52
BAObbh13.03	BAP021	3	48	75
BAObbj41.01	BAP023	5	61	84
BAObbj41.02	BAP023	8	80	103
BAObbj41.03	BAP023	1	99	122
BAObbl06.01	BAP025	5	20	43
BAObbl06.02	BAP025	17	39	62
BAObbl06.03	BAP025	4	95	122
BAObbl06.04	BAP025	16	117	143
BAObbn15.01	BAP028	11	195	219
BAObbn15.02	BAP028	10	215	239
BAObbn15.03	BAP028	8	235	259
BAObbo34.02	BAP030	2	27	54
BAObbo40.01	BAP029	5	197	217
BAObbo40.02	BAP029	3	213	232
BAObbp28.01	BAP032	1	48	73
BAObbp28.02	BAP032	2	69	94
BAObbp28.03	BAP032	1	90	114
BAObbq13.01	BAP025	2	133	159
BAObbq13.02	BAP025	7	155	181
BAObbq32.01	BAP034	1	14	41
BAObbq32.02	BAP034	2	37	63
BAObbq34.02	BAP043	17	29	52
BAObbq34.03	BAP043	9	48	70
BAObbr27.01	BAP026	9	1	29
BAObbr27.02	BAP026	5	24	53
BAObbr27.03	BAP026	3	49	76
BAObbr35.01	BAP036	17	1	21
BAObbr35.02	BAP036	17	17	44
BAObbr42.01	BAP031	2	122	149
BAObbr42.02	BAP031	2	145	172
BAObbs36.01	BAP037/8	1	3	27
BAObbs36.02	BAP037/8	11	23	47
BAObbs36.03	BAP037/8	2	43	66
BAObbs36.04	BAP037/8	1	62	85
BAObbs36.05	BAP037/8	1	136	165
BAObbs36.06	BAP037/8	17	156	185
BAObbs36.07	BAP037/8	0	81	107

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAObbs36.08	BAP037/8	8	103	129
BAObbs41.01	BAP031	8	160	186
BAObbs41.02	BAP031	6	182	208
BAObbs42.01	BAP045	20	27	51
BAObbs42.02	BAP045	10	47	72
BAObgp006.01	BA0060	10	177	191
BAObga26.01	BAP039	4	27	51
BAObga26.02	BAP039	7	47	71
BAObga26.03	BAP039	5	67	91
BAObgp001.01	BAP035	5	7	30
BAObgp001.02	BAP035	8	26	49
BAObgp001.03	BAP035	3	45	69
BAObgp001.04	BAP035	9	65	89
BAObgp003.01	BAP020	9	126	137
BAObgp022.01	BAP040	2	22	43
BAObgp022.02	BAP040	8	39	60
BAObgp022.03	BAP040	10	56	78
BAObgp024.02	BAP040	2	56	78
BAObgp027.01	BAP015	7	693	718
BAObgp027.02	BAP015	6	714	739
BAObgp027.03	BAP015	3	438	460
BAObgp027.04	BAP015	1	456	477
BAObgp027.05	BAP015	10	473	495
BAObgp027.06	BAP015	3	806	828
BAObgp027.07	BAP015	9	824	846
BAObgp027.08	BAP015	11	616	640
BAObgp027.09	BAP015	7	636	659
BAObgp027.10	BAP015	9	655	678
BAObgp027.11	BAP015	8	674	697
BAObgp027.12	BAP015	8	576	598
BAObgp027.13	BAP015	5	594	615
BAObgp027.14	BAP015	7	611	632
BAObgp027.15	BAP015	6	851	877
BAObgp027.16	BAP015	8	873	898
BAObgp027.17	BAP015	9	731	760
BAObgp027.18	BAP015	10	756	784
BAObgp027.19	BAP015	2	3	27
BAObgp027.20	BAP015	5	23	48
BAObgp027.21	BAP015	3	957	982
BAObgp027.22	BAP015	18	978	1002

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAObgp036.01	BAP041	14	157	182
BAObgp036.02	BAP041	5	178	203
BAObgp036.03	BAP041	5	199	224
BAObgp036.04	BAP041	4	220	246
BAObgp085.01	BAP042	13	70	94
BAObgp085.02	BAP042	2	90	113
BAObgp096.01	BAP001	19	20	44
BAObgp096.02	BAP001	7	40	64
BAObgp096.03	BAP001	16	60	84
BAObgp096.04	BAP001	5	80	104
BAObgp096.05	BAP001	4	138	162
BAObgp096.06	BAP001	14	158	182
BAObgp096.07	BAP001	6	178	201
BAObgp321.01	BAP044	13	1	25
BAObgp321.02	BAP044	17	21	45
BAObgp321.03	BAP044	17	41	65
BAObgp43.01	BAP044	3	41	65
BAObgp43.02	BAP044	12	41	65
BAObgp43.03	BAP044	19	41	65
BAOfiaA.01	BA0663	17	12	39
BAOfiaA.02	BA0663	2	35	62
BAOfiaA.03	BA0663	8	58	84
BAOfiaA.05	BA0663	16	175	197
BAOfiaB.01	BA0149	12	18	42
BAOfiaB.02	BA0149	3	38	62
BAOfiaB.03	BA0149	2	58	82
BAOfiaB.04	BA0149	15	78	102
BAOfiaB.05	BA0149	2	98	122
BAOfiaB.06	BA0149	4	118	142
BAOfiaB.07	BA0149	5	138	165
BAOfiaB.08	BA0149	1	256	279
BAOfiaB.09	BA0149	6	275	297
BAOfiaB.10	BA0149	1	293	315
BAOfiaB.11	BA0149	9	163	189
BAOfiaB.12	BA0149	12	185	211
BAOfiaB.13	BA0149	14	207	233
BAOfiaB.14	BA0149	1	229	255
BAOfliD.02	BA0150	3	46	75
BAOfliD.03	BA0150	2	110	131
BAOfliD.04	BA0150	4	127	148

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAOfliD.05	BA0150	7	188	216
BAOfliD.06	BA0150	1	212	240
BAOfliD.07	BA0150	1	344	367
BAOfliD.08	BA0150	5	361	384
BAOfliD.09	BA0150	3	382	405
BAOfliD.10	BA0150	1	401	424
BAOfliG-1.01	BA0221	9	151	180
BAOfliG-1.02	BA0221	7	176	205
BAOfliG-1.03	BA0221	10	245	274
BAOfliG-1.04	BA0221	16	270	299
BAOImp1.01	BA0210	6	937	962
BAOImp1.02	BA0210	4	958	983
BAOImp1.03	BA0210	2	102	130
BAOImp1.04	BA0210	1	126	154
BAOImp1.05	BA0210	2	150	177
BAOImp1.06	BA0210	2	823	849
BAOImp1.07	BA0210	2	845	872
BAOImp1.08	BA0210	3	867	892
BAOImp1.09	BA0210	2	285	314
BAOImp1.10	BA0210	5	310	339
BAOImp1.11	BA0210	3	335	364
BAOomp.01	BAP033	3	97	119
BAOomp.02	BAP033	4	115	137
BAOomp.03	BAP033	1	133	155
BAOomp.04	BAP033	5	151	173
BAOppA-	BA0327	2	20	43
BAOppA-	BA0327	1	39	62
BAOppA-	BA0327	1	58	79
BAOppA-	BA0327	9	69	94
BAOppA-	BA0327	3	90	115
BAOppA-	BA0327	4	167	189
BAOppA-	BA0327	11	185	207
BAOppA-	BA0327	14	203	224
BAOppA-	BA0327	9	296	320
BAOppA-	BA0327	9	316	339
BAOppA-	BA0327	10	335	358
BAOppA-	BA0327	9	354	377
BAOppA-	BA0327	10	404	431
BAOppA-	BA0327	8	427	453
BAOppA-	BA0327	15	449	475

Peptide	<i>B. afzelii</i>	Sum	From aa	To aa
BAOppA-	BA0327	16	471	498
BAOppA-	BA0327	9	494	521
BAOppA-	BA0329	1	40	62
BAOppA-	BA0329	1	76	97
BAOppA-	BA0329	1	251	276
BAOppA-	BA0329	15	272	297
BAOppA-	BA0329	17	291	316
BAOppA-	BA0329	1	333	358
BAOppA-	BAP006	16	335	360
BAOppA-	BAP006	6	356	381
BAOppA-	BAP006	2	422	446
BAOppA-	BAP006	6	442	465
BAOppA-	BAP006	1	461	484
BAOp35.03	BAP024	1	46	69
BAOp35.04	BAP024	11	65	88
BAOp35.05	BAP024	11	84	107
BAOp35.07	BAP024	1	124	149
BAOp35.10	BAP024	4	186	211
BAOp35.11	BAP024	13	207	232
BAOp35.12	BAP024	12	265	294
BAOp35.13	BAP024	1	290	318
BAOp66.02	BA0596	2	56	80
BAOp66.03	BA0596	1	76	100
BAOp66.04	BA0596	16	96	119
BAOp66.05	BA0596	3	343	371
BAOp66.06	BA0596	4	367	394
BAOp66.07	BA0596	3	410	436
BAOp66.08	BA0596	1	432	457
BAOp83.03	BA0737	2	281	307
BAOp83.13	BA0737	1	631	654
BAOpbp-1.01	BA0135	10	41	66
BAOpbp-1.02	BA0135	9	62	86
BAOpbp-1.03	BA0135	2	82	106
BAOpbp-1.04	BA0135	3	102	126
BAOpbp-1.05	BA0135	2	417	444
BAOpbp-1.06	BA0135	2	440	466
BAOpbp-1.07	BA0135	6	528	554
BAOpbp-1.08	BA0135	18	550	575
BAOpbp-1.09	BA0135	3	571	596

Table A5: Proteins identified in outer membrane preparations of three *Borrelia* strains: *B. afzelii* K78 (BA), *B. burgdorferi* B31 (BB), *B. garinii* PBi (BG).

Protein (BA)	MW (kDa)	SWISS-Prot ID	# identifications
Outer surface protein A	29,6	P0A3N7	17
Outer surface protein B	32,4	Q0SLY9	9
Outer surface protein C	22,4	Q9Z6C7	12
Membrane-associated protein p66	68,2	Q0SMQ3	5
Membrane protein P13	19,2	Q9X3U9	4
Outer membrane protein	19,4	Q0SM02	14
Putative uncharacterized protein	24,1	Q0SMW7	4
Putative uncharacterized protein	22,8	Q0SNA1	1
Putative uncharacterized protein	22,2	Q0SNA2	2
Lipoprotein	6,8	Q0SLU7	5
Lipoprotein	8,7	Q0SLV0	1
Flagellar filament 41 kDa core protein	35,8	Q0SP16	4
GrpE protein	22,1	Q0SMY9	1
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein	60,4	Q0SNI3	1
and / or Surface antigen	60,5	Q9K5J9	1
M18-family aminopeptidase (Gene: yscI)	51,5	Q0SNE8	3
M18-family aminopeptidase (Gene: pepX)	47,6	Q0SMM7	2
Neutrophil activating protein	20,8	Q0SMG3	5
Enolase	47,4	Q0SNH5	2
Glyceraldehyde-3-phosphate dehydrogenase	36,2	Q0SPA8	5
Pyrophosphate--fructose 6-phosphate 1-phosphotransferase	62,4	Q0SPE4	2
IMP dehydrogenase	43,8	Q0SL38	3
Ribose 5-phosphate isomerase	25,9	Q0SMJ6	2
Glycerol kinase	55,7	Q0SNS0	1
50S ribosomal protein L7/L12	13	Q5XU58	2
NADH oxidase, water-forming	48,9	Q0SMC8	1
Putative uncharacterized protein	39,8	Q0SN90	1
Flagellar protein	20	Q0SNN2	1
Putative uncharacterized protein	18,9	Q0SMU5	1
Putative uncharacterized protein	19,9	Q0SMU7	1
5-methylthioadenosine/S-adenosylhomocysteine nucleosidase, putative	29	Q0SMS0	2
Serine hydroxymethyltransferase	46,1	Q0SMQ5	2
Putative uncharacterized protein	26,6	Q0SLI8	2
Surface-located membrane protein 1 (Imp1)	116,2	Q0SNV2	4
LGMEQLIAQDLSK (not unique)		?	7
Putative uncharacterized protein	33,8	Q0SLL4	3
Putative uncharacterized protein	21,3	Q0SLT1	2

Putative uncharacterized protein	42	Q0SL48	1
Putative uncharacterized protein	59,6	Q0SMV6	1
Putative uncharacterized protein	28,2	Q0SMW3	1
Putative uncharacterized protein	38,7	Q0SLR0	5
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein	62,4	Q0SNI2	1
Putative uncharacterized protein	20,4	Q0SL93	1
Putative uncharacterized protein	18,7	Q0SLQ8	1
Fibronectin binding protein BBK32	35	Q9L891	3
Putative uncharacterized protein	11,1	Q0SL75	4
Decorin-binding protein B	20,7	Q8RL83	2
Putative uncharacterized protein	44,4	Q0SNI9	4
Putative surface antigen	28,9	Q5W295	3
CRASP-1 outer surface protein	28	Q5W294	2
Xylose operon regulatory protein	34,2	Q0SM20	1
Putative uncharacterized protein	16,5	Q0SLX6	1
Antigen, P35, putative	47,1	Q0SLU4	5
Putative uncharacterized protein	23,8	Q0SPD7	1
Putative uncharacterized protein	11,4	Q0SLY0	3
30S ribosomal protein S13	14	Q0SN07	2
ChpAI protein, putative	17,9	Q0SLZ8	2
Putative uncharacterized protein	12,4	Q0SM95	2
Pyruvate kinase	53,1	Q0SNG4	1
Acetyl-CoA C-acetyltransferase	42,7	Q0SP55	1
DnaK suppressor, putative	14,4	Q0SNZ6	1
Putative uncharacterized protein	29,9	Q0SLN9	1
Superoxide dismutase	23,4	Q0SP12	1
Flagellar filament outer layer protein	38,3	Q0SMI5	1
Putative uncharacterized protein	7,4	Q0SLC8	1
Putative uncharacterized protein	8,8	Q0SMW9	1
Putative uncharacterized protein	6,6	Q0SL56	1
Protein (BB)	MW (kDa)	SWISS-Prot ID	# identifications
Outer surface protein A	29,5	P14013	9
Outer surface protein B	31,8	P17739	11
Outer surface protein C	22,3	Q07337	5
P66 protein	68,2	Q44881	6
Membrane protein P13	19,1	Q7BSC0	3
Putative outer membrane protein BBA03	19,2	Q44849	8
Putative uncharacterized protein BB0543	24,4	O51493	2
Putative uncharacterized protein BB0406	22,8	O51367	1
Putative uncharacterized protein BB0405	22,3	O51366	2
6,6 kDa lipoprotein	7,8	Q57472	5
49kb linear plasmid small 12kDa lipoprotein	9	Q44736	2
Flagellar filament 41 kDa core protein	35,8	P11089	3
Protein grpE	21,9	P28609	3

Oligopeptide permease periplasmic binding protein (OppA-2)	60,7	O54584	6
Probable M18 family aminopeptidase 1	51,5	Q45055	2
Probable M18 family aminopeptidase 2	47,4	O51572	3
Neutrophil activating protein	22,4	O51633	6
Enolase	47,3	O51312	2
Glyceraldehyde-3-phosphate dehydrogenase	36,3	P46795	12
Pyrophosphate-fructose 6-phosphate 1-phosphotransferase	62,4	P70826	2
Inosine-5,monophosphate dehydrogenase	43,8	P49058	4
Ribose-5-phosphate isomerase A	25,9	O51601	4
Glycerol kinase	55,6	O51257	2
50S ribosomal protein L7/L12	12,9	O51351	2
NADH oxidase, water-forming	49,1	O51670	2
Putative uncharacterized protein BB0418	38,8	O51379	1
Flagellar fliL protein	20,1	Q57442	1
Putative uncharacterized protein BB0563	19,8	O51511	1
Putative uncharacterized protein BB0562	19,8	O51510	1
Pfs protein (Pfs-2)	29,1	O51534	1
and / or Glycosaminoglycan-binding protein Bgp	29,1	Q9F952	1
Serine hydroxymethyltransferase	45,9	O51547	3
Putative uncharacterized protein	27,2	O50817	3
LMP1 (Surface-located membrane protein 1)	128,1	Q7BI20	4
LGMEQLIAQDLSK (not unique)		?	7
Putative uncharacterized protein	34,6	O50726	1
Putative uncharacterized protein	34,1	O50805	1
Outer surface protein, putative	41,9	O50983	1
Putative uncharacterized protein BB0553	59,3	O51503	3
Putative uncharacterized protein BB0546	32,4	O51496	3
ErpB2	43,6	O08264	1
and / or ErpB	25,3	Q44782	1
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (OppA-3)	62,4	O51308	4
BdrA protein	24,1	Q45025	1
Immunogenic protein P35	40,8	O50835	1
Uncharacterized lipoprotein BBF20	11,1	O51025	1
Decorin-binding protein B	20,4	O50918	1
Decorin-binding protein A	21,2	O50917	4
Pfs protein (Pfs-1)	26,6	O50162	2
Outer surface 22 kDa lipoprotein	21,9	Q05903	2
Conserved hypothetical integral	22,3	O51527	1

membrane protein			
Immunogenic protein P37, putative	35,3	O50846	5
Immunogenic protein P37	37,3	O50850	2
Putative uncharacterized protein	35,3	O50692	2
Putative uncharacterized protein	30,5	O50958	3
Phosphate ABC transporter, periplasmic phosphate-binding protein	31,3	O51233	2
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (OppA-1)	60,6	O51307	2
Uncharacterized protein BB_0539	25,7	O51489	1
Outer membrane porin protein Oms28	28	P70854	1
Probable cysteine desulfurase	48,1	O51111	2
Putative uncharacterized protein BB0142	50,8	O51167	2
Rare lipoprotein A	31,2	O51677	2
Outer surface protein D	28,4	Q05051	6
Bbk47-like	37,1	Q6VEY0	1
Rev protein	17,9	Q9S0B8	2
Putative uncharacterized protein BB0110	53,4	O51137	1
Putative uncharacterized protein	39,7	O50789	1
Putative uncharacterized protein	24,3	O50823	1
Protein (BG)	MW (kDa)	SWISS-Prot ID	# identifications
OspA protein	29,6	Q6LBF1	18
Outer surface protein B	32,1	Q6ASL0	17
Outer surface protein C	22,3	Q45187	20
Membrane-associated protein p66	68,5	Q660R9	13
Membrane protein P13	19,3	Q9X3V0	5
Outer membrane protein	19,4	Q6ASM3	8
Putative uncharacterized protein	24,2	Q660Y1	4
Putative uncharacterized protein	22,8	Q661L3	4
Putative uncharacterized protein	22,3	Q661L4	3
Lipoprotein	5,8	Q6ASG3	5
Lipoprotein	7,9	Q6ASG6	5
Flagellar filament 41 kDa core protein	35,8	Q662L6	8
GrpE protein	22	Q661A2	3
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein	60,4	Q661T8	2
<i>and / or</i> Surface antigen	60,6	Q9K5F7	2
M18-family aminopeptidase (Gene: yscl)	51,3	Q661Q3	5
M18-family aminopeptidase (Gene: pepX)	47,7	Q660P4	2
Neutrophil activating protein	20,9	Q660I0	6
Enolase	47,9	Q661T0	9
Glyceraldehyde 3-phosphate dehydrogenase	36,2	Q662V7	17
Pyrophosphate--fructose 6-phosphate	62,5	Q662Z2	6

1-phosphotransferase			
IMP dehydrogenase	44	Q6ASN5	3
Ribose 5-phosphate isomerase	25,8	Q660L3	2
Glycerol kinase	55,7	Q662C3	2
Ribosomal protein L7/L12	12,9	Q661M8	1
NADH oxidase, water-forming	48,9	Q660E5	1
Putative uncharacterized protein	39,5	Q661K0	3
Flagellar protein	20	Q661Y6	1
Putative uncharacterized protein	18,8	Q660W1	1
Putative uncharacterized protein	19,6	Q660W2	1
Decorin binding protein A	19,3	Q6ASK4	2
5-methylthioadenosine S-adenosylhomocysteine nucleosidase	26,7	Q661P4	1
Lipoprotein LA7	22,1	Q661Q4	1
Conserved hypothetical integral membrane protein	22,4	Q660U3	1
Putative uncharacterized protein	44,1	Q661U4	7
Putative uncharacterized protein	27,4	Q6ASF7	2
Putative uncharacterized protein	29,3	Q6ASF4	2
Xylose operon regulatory protein	34,3	Q65ZU3	2
Putative uncharacterized protein	14,1	Q6ASJ9	2
Antigen, P35, putative	47,1	Q6ASG0	1
Putative uncharacterized protein	24	Q662Y5	1
Putative uncharacterized protein	28,2	Q661F1	2
Putative uncharacterized protein	30,5	Q6ASF8	4
Putative uncharacterized protein	27,4	Q660P3	2
ATP-dependent Clp protease proteolytic subunit	22,2	Q660B8	1
Chaperone protein dnaK	69,3	Q661A3	1
60 kDa chaperonin	58,9	Q660M1	1
Antigen, p83/100	79,3	Q660D0	1
Thymidylate kinase	23,8	Q65ZY0	1
Putative uncharacterized protein	29	Q6ASF9	1
Putative uncharacterized protein	21,4	Q661J4	1
Putative uncharacterized protein	21,1	Q65ZV1	1
Putative uncharacterized protein	14,2	Q661U3	1
V-type ATPase, subunit K, putative	14,9	Q662S2	1
4-methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis protein	19,9	Q660Q1	1
RRNA methylase	28,1	Q661A5	1
Antigen, S2, putative	28,3	Q662K7	1

Table A6: Proteins identified in trypsin digest preparations.

Protein BA	MW (kDa)	SWISS-Prot ID	# identifications
Outer surface protein A	29,6	P0A3N7	3
Outer surface protein B	32,4	Q0SLY9	3
Outer surface protein C	22,4	Q9Z6C7	3
Antigen, P35	36,8	Q0SLU6	3
Antigen, P35, putative	47,1	Q0SLU4	3
<i>and/or</i> Putative p35 surface antigen	47,1	Q5W299	3
<i>and/or</i> Outer surface protein [Precursor]	47,1	Q8KKG8	3
P83/100	75,8	Q06AZ1/2/3/4/5	3
<i>and/or</i> Antigen, p83/100	75,9	Q0SMB3	3
Putative p35 surface antigen	34,4	Q5W292	3
<i>and/or</i> Antigen, P35, putative	34,4	Q0SLT5	3
Basic membrane protein A [Precursor]	37	Q0SND0	3
<i>and/or</i> Basic membrane protein A [Precursor] [Fragment]	35,4	P0C223	3
Chaperone protein dnaK	69,4	Q0SMZ0	3
Flagellar filament 41 kDa core protein	35,8	Q0SP16	3
Flagellar filament outer layer protein	38,3	Q0SMI5	3
<i>and/or</i> Flagellin A	38,3	Q937Y5	3
Flagellar protein	20	Q0SNN2	3
Fibronectin binding protein BBK32	35	Q9L891	3
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-1)	60,1	Q0SNI4	3
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-2)	60,4	Q0SNI3	3
<i>and/or</i> Surface antigen	60,5	Q9K5J9	3
Oligopeptide ABC transporter, permease protein	39,5	Q0SNI0	3
Putative surface antigen	28,9	Q5W295	3
Decorin binding protein A	18,3	Q9ZH93 or Q9ZH89	3
Enolase	47,4	Q0SNH5	3
Outer membrane protein	19,4	Q0SM02	3
Lipoprotein LA7	21,9	Q0SNE9	3
Putative surface antigen [Precursor]	30,8	Q5W296	3
<i>and/or</i> Putative uncharacterized protein	29,7	Q0SLU1	3
Lipoprotein	8,7	Q0SLV0	3
Translation elongation factor TU	43,3	Q0SN31	3

Glyceraldehyde 3-phosphate dehydrogenase	36,2	Q0SPA8	3
Rod shape-determining protein	39,8	Q0SME1	3
PTS system, maltose and glucose-specific IIABC component	62,4	Q0SP48	3
PTS system, glucose-specific IIA component	20,8	Q0SMV0	3
Glycine betaine, L-proline ABC transporter, binding protein	33,7	Q0SP19	3
60 kDa chaperonin	58,9	Q0SMK4	3
Cell division protein	70,7	Q0SM63	3
Ribosomal protein L7/L12	13,3	Q0SNB7	3
Chemotaxis operon protein	17,6	Q0SMI2	3
DNA-directed RNA polymerase subunit beta	129,9	Q0SNB8	3
DNA-directed RNA polymerase subunit beta'	154,5	Q0SNB9	3
Phosphoglycerate kinase	42,5	Q0SPA9	3
10 kDa chaperonin	9,9	Q0SMB6	3
5-methylthioadenosine/S-adenosylhomocysteine nucleosidase, putative	29	Q0SMS0	3
Periplasmic serine protease DO	52	Q0SP60	3
Chaperone protein htpG	71,4	Q0SMU9	3
Probable M18-family aminopeptidase 1	51,5	Q0SNE8	3
Pyrophosphate--fructose 6-phosphate 1-phosphotransferase, beta subunit	62,4	Q0SPE4	3
Triosephosphate isomerase	27,8	Q0SPB0	3
L-lactate dehydrogenase	34,9	Q0SP77	3
50S ribosomal protein L11	15,2	Q0SNB4	3
50S ribosomal protein L4	23,7	Q0SN28	3
Glucose-6-phosphate isomerase	60,2	Q0SMC6	3
Elongation factor G 1	77,3	Q0SMX0	3
30S ribosomal protein S5	17,8	Q0SN12	3
50S ribosomal protein L13	17,3	Q0SNH3	3
Putative uncharacterized protein	14,7	Q0SN80	3
Fructose-bisphosphate aldolase	40	Q0SN64	3
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	28,5	Q0SMJ5	3
Putative uncharacterized protein	44,4	Q0SNI9	3
Lysyl-tRNA synthetase	61	Q937W6	3
DNA topoisomerase	98	Q0SM23	3
5-methylthioadenosine/S-adenosylhomocysteine nucleosidase, putative	26,6	Q0SND8	3
ChpAI protein, putative	17,9	Q0SLZ8	3
Putative uncharacterized protein	33,8	Q0SLL4	3

Cell division protein ftsZ	42,5	Q0SNL2	3
Superoxide dismutase	23,4	Q0SP12	3
Long-chain-fatty-acid CoA ligase	72,8	Q0SMR5	3
Putative uncharacterized protein	21,3	Q0SLT1	3
Putative uncharacterized protein	29,9	Q0SLN9	3
Putative uncharacterized protein	24,1	Q0SMW7	3
Putative uncharacterized protein	11,4	Q0SLY0	3
Basic membrane protein	38	Q0SP56	2
CRASP-1 outer surface protein	28	Q5W294	2
Surface-located membrane protein 1	116,2	Q0SNV2	2
DNA gyrase, subunit B	71,5	Q0SN73	2
Phosphocarrier protein HPr	9,4	Q0SMV2	2
Transcription termination factor Rho	57,7	Q0SNT1	2
Pyruvate kinase	53,1	Q0SNG4	2
Glutamyl-tRNA synthetase	56,8	Q0SNE1	2
Putative uncharacterized protein	29,7	Q0SNS3	2
Putative uncharacterized protein	43,5	Q0SPE0	2
30S ribosomal protein S4	23,7	Q0SMP1	2
Trigger factor	52,8	Q0SMP7	2
Putative uncharacterized protein	40,3	Q0SLJ7	2
Flagellar basal-body rod protein	65,2	Q0SNM0	2
N-acetylglucosamine-6-phosphate deacetylase	44,4	Q0SP14	2
UDP-N-acetylmuramate dehydrogenase	34,4	Q0SMQ9	2
Glycerol-3-phosphate O-acyltransferase, putative	34,6	Q0SNI5	2
Basic membrane protein B [Precursor]	37,1	O31284	2
Chemotaxis response regulator	137	Q0SMT8	2
ATP-dependent Clp protease, subunit X	47,6	Q0SMP5	2
Putative N-acetylmannosamine-6-phosphate 2-epimerase	25,7	Q0SMK9	2
50S ribosomal protein L2	30,6	Q0SN26	2
Putative uncharacterized protein	11,1	Q0SL75	2
ATP-dependent protease LA	90,9	Q0SNQ8	2
Flagellin	35,7	Q44650	2
Carboxyl-terminal protease	53,5	Q0SNF4	1
Conserved hypothetical integral membrane protein	50,8	Q0SN33	1
RNA polymerase sigma-70 factor	73,6	Q0SME4	1
PTS system, glucose-specific IIBC component	55,6	Q0SMK8	1
DNA-directed RNA polymerase subunit alpha	38,6	Q0SN05	1
Putative uncharacterized protein	31,8	Q0SLD9	1
Oligopeptide ABC transporter,	62,4	Q0SNI2	1

periplasmic oligopeptide-binding protein			
ABC transporter, ATP-binding protein	35,3	Q0SMA3	1
Ribosomal protein S1	63,3	Q0SP36	1
Outer membrane protein	94,6	Q0SM56	1
PTS system, fructose-specific IIABC component	67	Q0SN99	1
Putative surface protein	27,9	Q5W297	1
<i>and/or</i> Putative uncharacterized protein	27,9	Q0SLU2	1
Translation initiation factor IF-2	86,4	Q0SM50	1
Thioredoxin	13,4	Q0SPA4	1
Putative uncharacterized protein	58,6	Q0SPC4	1
2',3'-cyclic-nucleotide 2'-phosphodiesterase	57,9	Q0SN03	1
Ferric uptake regulation protein	20	Q0SMK6	1
Membrane fusion protein	35	Q0SP22	1
Lipoprotein	6,8	Q0SLU7	1
Putative uncharacterized protein	29,2	Q0SLK6	1
S2 lipoprotein	28	Q6DRS5	1
<i>and/or</i> Antigen, S2, putative	28	Q0SP07	1
Protein BB	MW (kDa)	SWISS-Prot ID	# identifications
Outer surface protein A	29,5	P14013	3
Outer surface protein C	22,3	Q07337	3
Flagellar filament 41 kDa core protein	35,8	P11089	3
6,6 kDa lipoprotein	7,8	Q57472	3
Oligopeptide permease periplasmic binding protein (OppA-2)	60,7	O54584	3
60 kDa chaperonin	58,9	P27575	3
Putative uncharacterized protein BB0238	30,4	O51254	3
Outer surface protein B	31,8	P17739	2
Enolase	47,3	O51312	2
Flagellar filament outer layer protein [Precursor]	38,4	P70856	2
Glyceraldehyde-3-phosphate dehydrogenase	36,3	P46795	1
Membrane protein P13	19,1	Q7BSC0	1
Outer surface 22 kDa lipoprotein	21,9	Q05903	1
Putative uncharacterized protein	27,2	O50817	1
Pfs protein (Pfs-2)	29,1	O51534	1
<i>and / or</i> Glycosaminoglycan-binding protein Bgp	29,1	Q9F952	1
P66 protein (Membrane-associated protein p66)	68,2	Q44881	1
<i>and / or</i> P66 protein	68,2	Q44644	1
Protein BG	MW (kDa)	SWISS-Prot ID	# identifications

OspA protein	29,6	Q6LBF1	3
Outer surface protein B	32,1	Q6ASL0	3
Outer surface protein C	22,3	Q45187	3
Antigen, P35, putative	47,1	Q6ASG0	3
Antigen, P35, putative	33,2	Q6ASF2	3
Antigen, P35	36,2	Q6ASG2	3
Outer membrane porin	27,8	Q6ASF1	3
Antigen, p83/100	79,3	Q660D0	3
Flagellar filament 41 kDa core protein	35,8	Q662L6	3
Flagellar filament outer layer protein	38,3	Q660K2	3
Outer membrane protein	19,4	Q6ASM3	3
Antigen, S2, putative	28,3	Q662K7	3
Membrane protein P13	19,3	Q9X3V0	3
Glyceraldehyde 3-phosphate dehydrogenase	36,2	Q662V7	3
Lipoprotein LA7	22,1	Q661Q4	3
Surface-located membrane protein 1	104,5	Q662F4	3
Putative uncharacterized protein	31,2	Q5XYR1	3
Putative uncharacterized protein	30,5	Q6ASF8	3
Putative uncharacterized protein	27,4	Q6ASF7	3
Putative uncharacterized protein	24,5	Q5XYT9	3
and / or Surface-exposed lipoprotein [Fragment]	22	P70895	3
Basic membrane protein B	37,2	O31362	3
Periplasmic serine protease DO	52	Q662Q8	3
L-lactate dehydrogenase	34,9	Q662S5	3
Elongation factor Tu	43,4	Q661E5	3
Putative uncharacterized protein	40,9	Q5XZ32	3
Putative uncharacterized protein	29,3	Q6ASF4	3
Putative uncharacterized protein	32,5	Q5XYN0	3
Pyrophosphate--fructose 6-phosphate 1-phosphotransferase	62,5	Q662Z2	3
Decorin binding protein A	19,3	Q6ASK4	3
Phosphoglycerate kinase	42,6	Q662V8	3
Putative uncharacterized protein	18	Q5XYK6	3
Putative uncharacterized protein	44,1	Q661U4	3
Putative uncharacterized protein	43,3	Q6ASG7	3
Putative uncharacterized protein	44	Q5XYE8	3
Putative uncharacterized protein	29	Q6ASF9	3
50S ribosomal protein L11	15,2	Q661M5	3
60 kDa chaperonin	58,9	Q660M1	3
Chaperone protein dnaK	69,3	Q661A3	3
10 kDa chaperonin	9,9	Q660D3	3
Putative uncharacterized protein	57,4	Q660C3	3
Superoxide dismutase	23,4	Q662L2	3
Oligopeptide ABC transporter, periplasmic oligopeptide-binding	62,6	Q661T7	3

protein (oppA-3)			
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-2)	60,4	Q661T8	3
and / or Surface antigen	60,6	Q9K5F7	3
5-methylthioadenosine/S-adenosylhomocysteine nucleosidase, putative	26,7	Q661P4	3
Lipoprotein	7,9	Q6ASG6	3
Lipoprotein, putative	39,6	Q662Y4	3
Lipoprotein	5,8	Q6ASG3	3
Lipoprotein, putative	62,3	Q65ZT2	3
Flagellar protein	20	Q661Y6	3
Hydrolase, alpha/beta fold family	37,5	Q660M4	3
ABC transporter, ATP-binding protein	34,9	Q660C1	3
Putative surface protein	29,6	Q5W285	3
Surface lipoprotein P27	32,1	Q6ASG5	3
Putative uncharacterized protein	14,2	Q661U3	3
Putative uncharacterized protein	43,3	Q661P5	3
Putative uncharacterized protein	30,9	Q5XYN3	3
Putative uncharacterized protein	11,1	Q5XZ25	3
Putative uncharacterized protein	17,9	Q5XZ47	3
Putative uncharacterized protein	17,9	Q660I1	3
Putative uncharacterized protein	60	Q660X1	3
Putative uncharacterized protein	29,8	Q6ASF3	3
Putative uncharacterized protein	14,1	Q6ASJ9	3
Putative uncharacterized protein	24,2	Q660Y1	3
Aminoacyl-histidine dipeptidase	53,3	Q660R4	3
Glycerol kinase	55,7	Q662C3	3
Chemotaxis operon protein	17,6	Q660J9	3
Thioredoxin	13,5	Q000V1/2/6/7/8/9	3
Chaperone protein htpG	71,3	Q660W4	3
ATP-dependent Clp protease, subunit C	84,8	Q65ZU0	3
Polyribonucleotide nucleotidyltransferase	80,2	Q65ZW8	3
Glycine betaine, L-proline ABC transporter, binding protein	33,7	Q662L9	3
Transcription termination factor Rho	57,7	Q662D4	3
Enolase	47,9	Q661T0	3
DNA topoisomerase	97,9	Q65ZU6	3
30S ribosomal protein S5	17,9	Q661C5	3
5-methylthioadenosine/S-adenosylhomocysteine nucleosidase, putative	29,5	Q660T4	2
Translation initiation factor IF-3	26,7	Q662H4	2
Putative uncharacterized protein	39,5	Q660C4	2
Putative uncharacterized protein	28,1	Q5XZ45	2

Putative uncharacterized protein	27,6	Q662V2	2
Putative uncharacterized protein	18,6	Q5XYF2	2
Putative uncharacterized protein	38,5	Q660D9	2
Rod shape-determining protein	38	Q660F8	2
Putative uncharacterized protein	14,7	Q661J1	2
Putative uncharacterized protein	37,4	Q5XZ44	2
Membrane fusion protein	35,1	Q662M1	2
Glycerol-3-phosphate O-acyltransferase, putative	34,5	Q661U0	2
Glucose-6-phosphate isomerase	60	Q660E3	2
Putative uncharacterized protein	22,3	Q661L4	2
Putative uncharacterized protein	22,9	Q5XZ39	2
Putative uncharacterized protein	35,1	Q662V1	2
Putative uncharacterized protein	43	Q661U2	2
Putative uncharacterized protein	29,6	Q662C6	2
Putative uncharacterized protein	39,5	Q661K0	2
Putative uncharacterized protein	58,2	Q661G1	2
Putative uncharacterized protein	43,7	Q662Y8	2
Basic membrane protein D	37,2	Q661N3	2
Chemotaxis response regulator	13,7	Q660V4	2
DNA gyrase, subunit B	71,4	Q661I5	2
Lysyl-tRNA synthetase	61	Q937U7	2
Membrane-associated protein p66	68,5	Q660R9	2
Putative N-acetylmannosamine-6-phosphate 2-epimerase	22,7	Q660M6	2
PTS system, glucose-specific IIA component	20,8	Q660W5	2
Methionyl-tRNA synthetase	84,2	Q660T5	2
Elongation factor G 1	77,3	Q660Y4	2
50S ribosomal protein L2	30,5	Q661D9	2
Triosephosphate isomerase	27,9	Q662V9	2
DNA-directed RNA polymerase subunit beta'	154,4	Q661N0	2
DNA-directed RNA polymerase subunit beta	129,7	Q661M9	2
Elongation factor Ts	31,3	Q662P0	2
Putative uncharacterized protein	49,4	Q6ASN1	2
Putative uncharacterized protein	23,5	Q660D5	2
PTS system, maltose and glucose-specific IIABC component	59,1	Q6ASM5	2
Basic membrane protein A1 [Precursor]	36,9	Q661N6	2
Methyl-accepting chemotaxis protein (gene:mcp-5)	70,9	Q660I9	2
Rare lipoprotein A	29,7	Q660D8	2
Acriflavine resistance protein	119	Q662M2	2
Pyruvate kinase	53,1	Q661R9	2
Cell division protein	70,8	Q65ZY5	1
Putative uncharacterized protein	31,7	Q5XYH0	1
Ribosomal protein L7/L12	13	Q661M8	1

Fructose-bisphosphate aldolase	40	Q661H5	1
Basic membrane protein	38	Q662Q4	1
Antigen, S2	30,4	Q6ASM2	1
Putative uncharacterized protein	48,6	Q661B1	1
Methyl-accepting chemotaxis protein (gene:mcp-4)	84,7	Q660J0	1
30S ribosomal protein S6	16,5	Q662P7	1
3-hydroxy-3-methylglutaryl-CoA synthase	45,7	Q660I7	1
Phosphocarrier protein HPr	9,4	Q660W7	1
2',3'-cyclic-nucleotide 2'-phosphodiesterase	58	Q661B6	1
PTS system, glucose-specific IIBC component	55,6	Q660M5	1
ATP-dependent Clp protease ATP-binding subunit clpX	47,7	Q660R1	1
S-adenosylmethionine synthetase	43,2	Q661P3	1

Table A7: List of the surface biotinylated proteins.

Protein BA	MW (kDa)	SWISS-Prot ID
DNA-directed RNA polymerase subunit beta'	154,5	Q0SNB9
DNA-directed RNA polymerase subunit beta	129,9	Q0SNB8
Transcription elongation factor	105,4	Q0SP31
Zinc protease, putative	108,8	Q0SMX4
Outer membrane protein	94,6	Q0SM56
Chaperone protein dnaK	69,4	Q0SMZ0
Methyl-accepting chemotaxis protein (gene:mcp-3)	84,3	Q0SMR0
Methyl-accepting chemotaxis protein (gene:mcp-2)	80,3	Q0SMR1
Chaperone protein htpG	71,4	Q0SMU9
DNA gyrase, subunit B	71,5	Q0SN73
Cell division protein	70,7	Q0SM63
Long-chain-fatty-acid CoA ligase	72,8	Q0SMR5
V-type ATP synthase alpha chain	64	Q0SP70
Membrane-associated protein p66	68,2	Q0SMQ3
Flagellar basal-body rod protein	65,2	Q0SNM0
60 kDa chaperonin	58,9	Q0SMK4
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-1)	60,1	Q0SNI4
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-2)	60,4	Q0SNI3
and / or Surface antigen	60,5	Q9K5J9
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-3)	62,4	Q0SNI2
Oligopeptide ABC transporter, periplasmic (oppA-4)	61,1	Q0SLX3
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-5)	60,9	Q0SL40
Glucose-6-phosphate isomerase	60,2	Q0SMC6
2',3'-cyclic-nucleotide 2'-phosphodiesterase	57,9	Q0SN03
Transcription termination factor Rho	57,7	Q0SNT1
Pyruvate kinase	53,1	Q0SNG4
Probable M18-family aminopeptidase 1	51,5	Q0SNE8
Heat shock protein 70	54,7	Q0SNP7
Carboxyl-terminal protease	53,5	Q0SNF4
V-type ATP synthase beta chain	48,1	Q0SP71
Periplasmic serine protease DO	52	Q0SP60
ATP-dependent Clp protease, subunit X	47,6	Q0SMP5
Putative uncharacterized protein	57,4	Q0SMA5

Putative uncharacterized protein	57,3	Q0SPD2
Enolase	47,4	Q0SNH5
M18-family aminopeptidase	47,6	Q0SMM7
Pyrophosphate--fructose 6-phosphate 1-phosphotransferase	49,8	Q0SMC9
Elongation factor Tu	43,4	Q0SN31
3-hydroxy-3-methylglutaryl-CoA synthase	45,9	Q0SMH0
Antigen, P35, putative	47,1	Q0SLU4
and / or Putative p35 surface antigen	47,1	Q5W299
Xylose operon regulatory protein	46	Q0SMG1
Flagellar filament 41 kDa core protein	35,8	Q0SP16
Serine hydroxymethyltransferase	46,1	Q0SMQ5
Cell division protein ftsZ	42,5	Q0SNL2
Putative uncharacterized protein	46,5	Q0SNG7
Fructose-bisphosphate aldolase	40	Q0SN64
Fibronectin binding protein BBK32	35	Q9L891
Putative uncharacterized protein	49,4	Q0SL29
S-adenosylmethionine synthetase	43,2	Q0SND7
Phosphoribosyl pyrophosphate synthetase	46	Q0SMW5
Phosphoglycerate kinase	42,5	Q0SPA9
Methyl-accepting chemotaxis protein (gene:mcp-1)	44,5	Q0SMT0
Putative uncharacterized protein	38,7	Q0SLR0
Basic membrane protein A [Precursor]	37	Q0SND0
and / or Basic membrane protein A [Precursor] [Fragment]	35,4	P0C223
Flagellin	35,7	Q44650
Putative uncharacterized protein	39,8	Q0SMA6
DNA-directed RNA polymerase subunit alpha	38,6	Q0SN05
Rod shape-determining protein	39,8	Q0SME1
Outer surface protein A	29,6	P0A3N7
Outer surface protein B	32,4	Q0SLY9
Outer surface protein C	22,2	Q49578
Basic membrane protein B [Precursor]	37,1	O31284
Neutrophil activating protein	20,8	Q0SMG3
Glyceraldehyde 3-phosphate dehydrogenase	36,2	Q0SPA8
Heat shock protein	40,6	Q0SMZ1
Flagellar filament outer layer protein	38,3	Q0SMI5
Basic membrane protein	38	Q0SP56
Oligopeptide ABC transporter, ATP-binding protein	37,1	Q0SNH7
Xylose operon regulatory protein	34,2	Q0SM20
Basic membrane protein D	37,2	Q0SNC2
Ribosomal protein S2	29,3	Q0SP41
Oligopeptide ABC transporter, ATP-binding protein	32,9	Q0SNH8

PTS system, cellobiose-specific IIC component	48,4	Q0SL51
Lambda CII stability-governing protein	37,2	Q0SNV8
Lipoprotein, putative	39,1	Q0SPD6
Membrane fusion protein	35	Q0SP22
Antigen, P35	36,8	Q0SLU6
Putative uncharacterized protein	21,3	Q0SLT1
ABC transporter, ATP-binding protein	35,3	Q0SMA3
Phosphate ABC transporter, periplasmic phosphate-binding protein	31,1	Q0SNU7
Putative uncharacterized protein	29,2	Q0SLK6
5-methylthioadenosine/S-adenosylhomocysteine nucleosidase, putative	29	Q0SMS0
Lipoprotein, putative	31,9	Q0SM19
50S ribosomal protein L3	22,2	Q0SN29
CRASP-1 outer surface protein	28	Q5W294
Flagellar motor rotation protein A	28,3	Q0SNN0
Putative surface protein	27,9	Q5W297
and / or Putative uncharacterized protein	27,9	Q0SLU2
S2 lipoprotein	28	Q6DRS5
and / or Antigen, S2, putative	28	Q0SP07
Putative surface antigen	28,9	Q5W295
Triosephosphate isomerase	27,8	Q0SPB0
Putative N-acetylmannosamine-6-phosphate 2-epimerase	25,7	Q0SMK9
Outer membrane protein	19,4	Q0SM02
Putative uncharacterized protein	28,8	Q0SLT9
Putative uncharacterized protein	29,7	Q0SNS3
ABC transporter, ATP-binding protein	25,8	Q0SP85
50S ribosomal protein L25	20,5	Q0SM66
Putative uncharacterized protein	24,1	Q0SMW7
Flagellar protein	20	Q0SNN2
Lipoprotein LA7	21,9	Q0SNE9
30S ribosomal protein S6	16,4	Q0SP49
30S ribosomal protein S9	15,4	Q0SNH4
30S ribosomal protein S7	18,2	Q0SNC1
Membrane protein P13	19,2	Q9X3U9
Decorin binding protein A	18,3	Q9ZH93 or Q9ZH89
Probable chemoreceptor glutamine deamidase cheD	18,1	Q0SMQ0
30S ribosomal protein S10	11,8	Q0SN30
50S ribosomal protein L7/L12	13	Q5XU58
Putative septation protein spoVG	11,2	Q0SM67
30S ribosomal protein S11	13,9	Q0SN06
Putative uncharacterized protein	44,4	Q0SNI9
Lipoprotein	6,8	Q0SLU7
Putative uncharacterized protein	9,2	Q0SMF8
30S ribosomal protein S14 type Z	7,2	Q0SN16

DNA polymerase III, subunit alpha	132,7	Q0SMS9
Isoleucyl-tRNA synthetase	122,5	Q0SM18
Surface-located membrane protein 1	116,2	Q0SNV2
Methyl-accepting chemotaxis protein	84,7	Q0SMH3
ATP-dependent helicase	95	Q0SM24
Translation initiation factor IF-2	86,4	Q0SM50
DNA topoisomerase	98	Q0SM23
Protein translocase subunit secA	102,1	Q0SP11
ATP-dependent protease LA	90,9	Q0SNQ8
Chemotaxis histidine kinase	98,4	Q0SMI4
Leucyl-tRNA synthetase	98,2	Q0SNR0
Elongation factor G 1	77,3	Q0SMX0
Polyribonucleotide nucleotidyltransferase	80,4	Q0SM46
RNA polymerase sigma-70 factor	73,6	Q0SME4
Methyl-accepting chemotaxis protein (gene:mcp-5)	70,7	Q0SMH2
DNA recombinase	78,7	Q0SMS7
Putative uncharacterized protein	85,1	Q0SNQ2
Putative uncharacterized protein	70,6	Q0SNP4
DNA mismatch repair protein mutL	71,4	Q0SNV1
Long-chain-fatty-acid CoA ligase	70,9	Q0SP26
Ribosomal protein S1	63,3	Q0SP36
Peptidase, putative	67,7	Q0SP97
Phenylalanyl-tRNA synthetase beta chain	65,1	Q0SMZ4
PTS system, fructose-specific IIABC component	67	Q0SN99
N-utilization substance protein A	54,8	Q0SM51
Putative uncharacterized protein	70,4	Q0SMY3
Pyrophosphate--fructose 6-phosphate 1-phosphotransferase, beta subunit	62,4	Q0SPE4
PTS system, fructose-specific IIABC component	67,3	Q0SMM5
Protein-export membrane protein	65,1	Q0SMK1
Trigger factor	52,8	Q0SMP7
Putative uncharacterized protein	55,6	Q0SND2
Glycerol kinase	55,7	Q0SNS0
Glycyl-tRNA synthetase	52,3	Q0SNE2
Heat shock protein	51	Q0SNL6
IMP dehydrogenase	43,8	Q0SL38
PTS system, maltose and glucose-specific IIABC component	56,6	Q0SL27
Glycine betaine, L-proline ABC transporter, binding protein	33,7	Q0SP19
Acetyl-CoA C-acetyltransferase	42,7	Q0SP55
Nucleotide sugar epimerase	40,2	Q0SN65
Putative uncharacterized protein	44,3	Q0SNW9
Putative uncharacterized protein	43,6	Q0SP29
Spermidine/putrescine ABC transporter,	40,5	Q0SML4

binding periplasmic protein		
N-acetylglucosamine-6-phosphate deacetylase	44,4	Q0SP14
PTS system, glucose-specific IIBC component	55,6	Q0SMK8
Putative uncharacterized protein	37,3	Q0SNC7
and / or Putative uncharacterized protein	37,3	Q0SNC4
Glutamate transporter	49,5	Q0SMC7
Basic membrane protein C	39,6	Q0SNC3
Preprotein translocase secY subunit	48,7	Q0SN09
Oligopeptide ABC transporter, permease protein	39,5	Q0SNI0
Putative uncharacterized protein	37,4	Q0SMK7
DNA gyrase, subunit A	91,2	Q0SN74
Putative uncharacterized protein	36,2	Q0SMN7
50S ribosomal protein L2	30,6	Q0SN26
L-lactate dehydrogenase	34,9	Q0SP77
Putative surface antigen [Precursor]	30,8	Q5W296
and / or Putative uncharacterized protein	29,7	Q0SLU1
Flagellar assembly protein	35,3	Q0SNM2
Putative p35 surface antigen [Precursor]	34,4	Q5W292
and / or Antigen, P35, putative	34,4	Q0SLT5
Elongation factor Ts	31,2	Q0SP42
Outer membrane porin	30	Q0SLT3
Putative uncharacterized protein	29,9	Q0SLN9
Prolipoprotein diacylglycerol transferase	37,2	Q0SNF2
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	28,5	Q0SMJ5
5-methylthioadenosine/S-adenosylhomocysteine nucleosidase, putative	26,6	Q0SND8
Oligopeptide ABC transporter, permease protein	34,2	Q0SNI1
Lipoprotein, putative	28,6	Q0SN47
30S ribosomal protein S4	23,7	Q0SMP1
Putative uncharacterized protein	26,5	Q0SMI9
GrpE protein	22,1	Q0SMY9
Putative uncharacterized protein	23,7	Q0SMT1
Putative uncharacterized protein	23,9	Q0SLD2
Putative uncharacterized protein	28,1	Q0SNT5
Putative uncharacterized protein	27,5	Q0SMM6
50S ribosomal protein L1	25,7	Q0SNB5
Putative uncharacterized protein	28,6	Q0SL85
Glycine betaine, L-proline ABC transporter, permease protein	33,7	Q0SP18
50S ribosomal protein L4	23,7	Q0SN28
PTS system, glucose-specific IIA component	20,8	Q0SMV0
Putative uncharacterized protein	23,9	Q0SMW2
Putative uncharacterized protein	22,2	Q0SNA2

Superoxide dismutase	23,4	Q0SP12
ATP-dependent Clp protease proteolytic subunit	22,2	Q0SM98
Putative uncharacterized protein	24,7	Q0SMX3
Ferric uptake regulation protein	20	Q0SMK6
ATP-dependent protease hslV	19,6	Q0SNL5
50S ribosomal protein L13	17,3	Q0SNH3
50S ribosomal protein L10	18,1	Q0SNB6
Putative uncharacterized protein	19,4	Q0SL41
30S ribosomal protein S13	14	Q0SN07
Putative uncharacterized protein	17,8	Q0SMG4
50S ribosomal protein L11	15,2	Q0SNB4
50S ribosomal protein L22	13,7	Q0SN24
Putative uncharacterized protein	18,9	Q0SMU5
50S ribosomal protein L18	13,8	Q0SN13
Lipoprotein	8,7	Q0SLV0
50S ribosomal protein L24	11,4	Q0SN18
Putative uncharacterized protein	14,7	Q0SN80
50S ribosomal protein L23	11,1	Q0SN27
Putative uncharacterized protein	11,1	Q0SL75
30S ribosomal protein S21	8,6	Q0SNQ5
Protein BB	MW (kDa)	SWISS-Prot ID
DNA-directed RNA polymerase subunit beta'	154,5	O51349
DNA-directed RNA polymerase subunit beta	129,6	Q59191
Putative uncharacterized protein BB0326	107,5	O51305
Zinc protease, putative	108,5	O51486
Valyl-tRNA synthetase	101,9	O51680
Protein translocase subunit secA	102,1	O07497
Acriflavine resistance protein	115,4	O51165
Transcription elongation factor greA	105,3	O51157
Methyl-accepting chemotaxis protein (gene:mcp-4)	84,6	O51623
ATP-dependent protease La homolog	92,3	O51558
RNA polymerase sigma factor rpoD	73,6	P52323
Outer membrane protein	94,6	O51735
Putative uncharacterized protein	54,3	O50870
<i>and / or</i> Virulent strain associated lipoprotein	55,5	Q9ZIU2
Chemotaxis histidine kinase	79,4	O51515
<i>and / or</i> Histidine kinase	79,8	Q44875
Methyl-accepting chemotaxis protein (gene:mcp-3)	84,6	O51543
Chaperone protein dnaK	69,3	P28608
Methyl-accepting chemotaxis protein (gene:mcp-2)	80,5	O51542
Methyl-accepting chemotaxis protein (gene:mcp-5)	71,5	O51624
Chaperone protein htpG	71,2	P42555

Long-chain-fatty-acid CoA ligase	72,9	O51539
P66 protein	68,2	Q44881
DNA gyrase subunit B	72,1	P33769
tRNA uridine 5-carboxymethylaminomethyl modification enzyme gidA	70,1	P53362
Outer surface protein A	29,5	P14013
Outer surface protein B	31,8	P17739
Outer surface protein C	22,2	Q07337
Outer surface protein D	28,4	Q05051
Long-chain-fatty-acid CoA ligase	70,8	O51162
Cell division protein	70,8	O51729
V-type ATP synthase alpha chain	64	O51121
60 kDa chaperonin	59	P27575
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-1)	60,6	O51307
Oligopeptide permease periplasmic binding protein (oppA-2)	60,7	O54584
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-3)	62,4	O51308
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppAIV)	60,8	O31315
Peptidase, putative	67,6	O51094
PTS system, fructose-specific IIABC component	67,1	O51369
Transcription termination factor rho	57,6	P33561
Trigger factor	52,9	O51555
Oligoendopeptidase F homolog	69,7	O51264
Ribosomal protein S1	63,5	O51153
Protein-export membrane protein secD	65	O51596
Probable M18 family aminopeptidase 1	51,5	Q45055
Pyruvate kinase	53	O51323
Glycerol kinase	55,6	O51257
Glycerol-3-phosphate dehydrogenase, anaerobic	60,3	O51259
Pyrophosphate-fructose 6-phosphate 1-phosphotransferase	62,4	P70826
GMP synthase [glutamine-hydrolyzing]	60,2	P49056
Carboxyl-terminal protease	53,5	O51334
Heat shock protein 70	54,9	O51279
Glutamyl-tRNA synthetase	56,8	O51345
PTS system, maltose and glucose-specific IIABC component	58,9	O51000
Enolase	47,3	O51312
Periplasmic serine protease DO	53,1	O51131
Probable M18 family aminopeptidase 2	47,4	O51572
Uncharacterized protein BB_0752	57,4	O51693

Pyrophosphate--fructose 6-phosphate 1-phosphotransferase	49,9	O51669
Putative uncharacterized protein BB0142	50,8	O51167
Flagellum-specific ATP synthase	48,4	P52607
Flagellar filament 41 kDa core protein	35,8	P11089
Elongation factor Tu	43,4	P50062
Putative uncharacterized protein	39,7	O50789
Nucleotide sugar epimerase	40,2	O51400
Fructose-bisphosphate aldolase	40	O51401
3-hydroxy-3-methylglutaryl-CoA synthase	46	O51626
Neutrophil activating protein	22,4	O51633
Inosine-5'-monophosphate dehydrogenase	43,8	P49058
Phosphoglycerate kinase	42,3	Q59181
Cell division control protein 27, putative	44,1	O51213
Putative uncharacterized protein BB0345	46,5	O51320
Rod shape-determining protein	39,8	O51657
Basic membrane protein A [Precursor]	37	Q45010
and / or Immunodominant antigen P39	37	Q9S6C1
Basic membrane protein B	37,5	Q45011
and / or Membrane lipoprotein BmpB	37,1	Q8KWS0
Basic membrane protein D [Precursor]	37,2	Q44743
Spermidine/putrescine ABC transporter, spermidine/putrescine-binding periplasmic protein	40,7	O51584
Glyceraldehyde-3-phosphate dehydrogenase	36,3	P46795
PTS system, glucose-specific IIBC component	55,8	O51590
D-alanine--D-alanine ligase	40,2	O51218
Putative uncharacterized protein	35,3	O50692
Putative uncharacterized protein BB0751	41,9	O51692
Flagellar filament outer layer protein [Precursor]	38,4	P70856
Uncharacterized protein BB_0028 [Precursor]	39,7	O51059
Oligopeptide permease homolog F	37,1	O31310
Basic membrane protein	38	O51135
Putative uncharacterized protein	34,6	O50726
Putative uncharacterized protein	34,1	O50805
Antigen, P35, putative	32,9	O50796
and / or Putative uncharacterized protein	33	O50891
ABC transporter, ATP-binding protein	35,2	O51695
L-lactate dehydrogenase	34,8	O51114
Glycine betaine, L-proline ABC transporter, glycine/betaine/L-proline-binding protein	33,3	O51169
BbCRASP-1 protein	29,2	Q5W2C1

and / or Complement regulator acquiring protein 1	28,4	Q66ZC6
and / or Putative uncharacterized protein	29,2	O50957
Outer membrane porin protein Oms28	28	P70854
Phosphate ABC transporter, periplasmic phosphate-binding protein	31,3	O51233
Putative uncharacterized protein	30,5	O50958
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	28,4	O51602
Putative uncharacterized protein BB0238	30,4	O51254
Putative uncharacterized protein	27,2	O50817
Triosephosphate isomerase	27,8	Q59182
Antigen, P35, putative	28,2	O50725
Chemotaxis protein motA	28,4	Q44902
S2 lipoprotein	28	Q6DRT2
and / or Antigen, S2, putative	28	O51180
Putative uncharacterized protein BB0664	26,5	O51608
Putative uncharacterized protein	25,6	O50883
Outer surface 22 kDa lipoprotein	21,9	Q05903
Decorin-binding protein A	21,2	O50917
Flagellar fliL protein	20,1	Q57442
Putative outer membrane protein BBA03	19,2	Q44849
ErpN	20,1	Q9S060
and / or ErpA	19,6	Q44781
Membrane protein P13	19,1	Q7BSC0
49kb linear plasmid small 12kDa lipoprotein	9	Q44736
Putative septation protein spoVG	11,2	O51726
DAEICISCGVR (not unique)		?
UPF0109 protein BB_0696	9,3	O51639
6,6 kDa lipoprotein	7,8	Q57472
10 kDa chaperonin	9,9	O51683

Table A8: 104 overlapping proteins identified in three different proteomic approaches. M*:

Method; 1, membrane preparation; 2, shedding approach; 3, Biotinylation approach.

Protein	<i>B. burgdorferi</i> B31			<i>B. afzelii</i> K78			<i>B. garinii</i> PBi		
	MW (kDa)	SWISS-Prot ID	M*	MW (kDa)	SWISS-Prot ID	M*	MW (kDa)	SWISS-Prot ID	M*
10 kDa chaperonin	9,9	O51683	3	9,9	Q0SMB6	2	9,9	Q660D3	2
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	28	O51602	3	29	Q0SMJ5	2,3	28	Q660L2	2
3-hydroxy-3-methylglutaryl-CoA synthase	46	O51626	3	46	Q0SMH0	3	46	Q660I7	2
49kb linear plasmid small 12kDa lipoprotein	9	Q44736	1,3	8,7	Q0SLV0	1,2,3	7,9	Q6ASG6	1,2
50S ribosomal protein L7/L12	13	O51351	1	13	Q5XU58	1,3	13	Q661M8	1,2
6,6 kDa lipoprotein	7,8	Q57472	1,2,3	6,8	Q0SLU7	1,2,3	5,8	Q6ASG3	1,2
60 kDa chaperonin	59	P27575	2,3	59	Q0SMK4	2,3	59	Q660M1	1,2
ABC transporter, ATP-binding protein	35	O51695	3	35	Q0SMA3	2,3	35	Q660C1	2
Acriflavine resistance protein	115	O51165	3	119	Q0SP23	3	119	Q662M2	2
Antigen, P35, putative	35	O50962	3	34	Q5W292 or Q0SLT5	2,3	33	Q6ASF2	2
Antigen, P35, putative	28	O50725	3	29	Q0SLK6	2,3	32	Q5XYH0	2
Antigen, P35, putative or Putative uncharacterized protein	32,9 or 33	O50796 or O50891	3	32	Q0SLD9	2	30	Q6ASF3	2
Antigen, p83/100	80	Q45013	3	75,8 or 75,9	Q06AZ1/2/3/4/5 or Q0SMB3	2	79	Q660D0	1,2
Basic membrane protein	38	O51135	3	38	Q0SP56	2,3	38	Q662Q4	2
Basic membrane protein A [Precursor] or Immunodominant antigen P39	37	Q45010 or Q9S6C1	3	37 or 35,4	Q0SND0 or P0C223	2,3	37	Q661N6	2
Basic membrane protein B or Membrane lipoprotein BmpB	37,5 or 37,1	Q45011 or Q8KWS0	3	37	O31284	2,3	37	O31362	2
Basic membrane protein D [Precursor]	37	Q44743	3	37	Q0SNC2	3	37	Q661N3	2
BbCRASP-1 protein	29	Q5W2C1	3	28	Q5W294	1,2,3	29	Q6ASF4	1,2
Cell division protein	71	O51729	3	71	Q0SM63	2,3	71	Q65ZY5	2
Chaperone protein dnaK	69	P28608	3	69	Q0SMZ0	2,3	69	Q661A3	1,2
Chaperone protein htpG	71	P42555	3	71	Q0SMU9	2,3	71	Q660W4	2
Chemotaxis response regulator (CheY-2)	14	O51517	3	137	Q0SMT8	2	14	Q660V4	2
Complement regulator acquiring protein 1	28	Q66ZC6	3	29	Q0SLT9	3	31	Q6ASF8	1,2
Conserved hypothetical integral membrane protein	22	O51527	1	22	Q0SMS8	3	22	Q660U3	1
Decorin-binding protein A	21	O50917	1,3	18	Q9ZH93 or Q9ZH89	2,3	19	Q6ASK4	1,2
Decorin-binding protein B	20	O50918	1	21	Q8RL83	1	20	Q9ZH94	2
DNA gyrase subunit B	72	P33769	3	72	Q0SN73	2,3	71	Q661I5	2
DNA-directed RNA polymerase subunit beta	130	Q59191	3	130	Q0SNB8	2,3	130	Q661M9	2
DNA-directed RNA polymerase subunit beta'	155	O51349	3	155	Q0SNB9	2,3	154	Q661N0	2
Elongation factor Tu	43	P50062	3	43	Q0SN31	2,3	43	Q661E5	2
Enolase	47	O51312	1,2,3	47	Q0SNH5	1,2,3	48	Q661T0	1,2

Protein	<i>B. burgdorferi</i> B31			<i>B. afzelii</i> K78			<i>B. garinii</i> PBi		
	MW (kDa)	SWISS-Prot ID	M*	MW (kDa)	SWISS-Prot ID	M*	MW (kDa)	SWISS-Prot ID	M*
ErpB2 or ErpB	43,6 or 25,3	O08264 or Q44782	1	39	Q0SLR0	1,3	44	Q5XYE8	2
Flagellar filament 41 kDa core protein	36	P11089	1,2,3	36	Q0SP16	1,2,3	36	Q662L6	1,2
Flagellar filament outer layer protein [Precursor]	38	P70856	2,3	38	Q0SMI5 or Q937Y5	1,2,3	38	Q660K2	2
Flagellar fliL protein	20	Q57442	1,3	20	Q0SNN2	1,2,3	20	Q661Y6	1,2
Fructose-bisphosphate aldolase	40	O51401	3	40	Q0SN64	2,3	40	Q661H5	2
Glyceraldehyde-3-phosphate dehydrogenase	36	P46795	1,2,3	36	Q0SPA8	1,2,3	36	Q662V7	1,2
Glycerol kinase	56	O51257	1,3	56	Q0SNS0	1,3	56	Q662C3	1,2
Glycine betaine, L-proline ABC transporter, glycine/betaine/L-proline-binding protein	33	O51169	3	34	Q0SP19	2,3	34	Q662L9	2
Glycosaminoglycan-binding protein Bgp and / or Pfs protein (Pfs-2)	29	Q9F952 or O51534	1,2	29	Q0SMS0	1,2,3	30	Q660T4	2
Immunogenic protein P35	41	O50835	1	35	Q9L891	1,2,3	41	Q5XZ32	2
Inosine-5-monophosphate dehydrogenase	44	P49058	1,3	44	Q0SL38	1,3	44	Q6ASN5	1
L-lactate dehydrogenase	35	O51114	3	35	Q0SP77	2,3	35	Q662S5	2
LMP1 (Surface-located membrane protein 1)	128	Q7BI20	1	116	Q0SNV2	1,2,3	105	Q662F4	2
Membrane fusion protein (MtrC)	35	O51166	3	35	Q0SP22	2,3	35	Q662M1	2
Membrane protein P13	19	Q7BSC0	1,2,3	19	Q9X3U9	1,3	19	Q9X3V0	1,2
Methyl-accepting chemotaxis protein (mcp-2)	81	O51542	3	80	Q0SMR1	3	80	Q660S6	2
Methyl-accepting chemotaxis protein (mcp-3)	85	O51543	3	84	Q0SMR0	3	85	Q660S5	2
Methyl-accepting chemotaxis protein (mcp-4)	85	O51623	3	85	Q0SMH3	3	85	Q660J0	2
Methyl-accepting chemotaxis protein (mcp-5)	72	O51624	3	71	Q0SMH2	3	71	Q660I9	2
NADH oxidase, water-forming	49	O51670	1	49	Q0SMC8	1	49	Q660E5	1
Neutrophil activating protein	22	O51633	1,3	21	Q0SMG3	1,3	21	Q660I0	1
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppA-1)	61	O51307	1,3	60	Q0SNI4	2,3	60	Q661T9	2
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (OppA-3)	62	O51308	1,3	62	Q0SNI2	1,2,3	63	Q661T7	2
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (oppAIV)	61	O31315	3	61	Q0SL40	3	61	Q6ASN6	2
Oligopeptide permease homolog F	37	O31310	3	37	Q0SNH7	3	37	Q661T2	2
Oligopeptide permease periplasmic binding protein (OppA-2)	61	O54584	1,2,3	60,4 or 60,5	Q0SNI3 or Q9K5J9	1,2,3	60,4 or 60,6	Q661T8 or Q9K5F7	1,2
Outer membrane porin protein Oms28	28	P70854	1,3	30	Q0SLT3	3	28	Q6ASF1	2
Outer surface 22 kDa lipoprotein	22	Q05903	1,2,3	22	Q0SNE9	2,3	22	Q661Q4	1,2
Outer surface protein A	30	P14013	1,2,3	30	P0A3N7	1,2,3	30	Q6LBF1	1,2
Outer surface protein B	32	P17739	1,2,3	32	Q0SLY9	1,2,3	32	Q6ASL0	1,2
Outer surface protein C	22	Q07337	1,2,3	22	Q9Z6C7	1,2,3	22	Q45187	1,2
Outer surface protein, putative	42	O50983	1	42	Q0SL48	1	42	Q6ASP4	2

	<i>B. burgdorferi</i> B31			<i>B. afzelii</i> K78			<i>B. garinii</i> PBi		
Protein	MW (kDa)	SWISS-Prot ID	M*	MW (kDa)	SWISS-Prot ID	M*	MW (kDa)	SWISS-Prot ID	M*
P35 antigen protein	34	P70848	3	37	Q0SLU6	2,3	36	Q6ASG2	2
P66 protein	68	Q44881	1,2,3	68	Q0SMQ3	1,3	69	Q660R9	1,2
Periplasmic serine protease DO	53	O51131	3	52	Q0SP60	2,3	52	Q662Q8	2
Pfs protein (Pfs-1)	27	O50162	1	27	Q0SND8	2,3	27	Q661P4	1,2
Phosphate ABC transporter, periplasmic phosphate-binding protein	31	O51233	1,3	31	Q0SNU7	3	31	Q662E9	2
Phosphoglycerate kinase	42	Q59181	3	43	Q0SPA9	2,3	43	Q662V8	2
Probable M18 family aminopeptidase 1	52	Q45055	1,3	52	Q0SNE8	1,2,3	51	Q661Q3	1
Probable M18 family aminopeptidase 2	47	O51572	1,3	48	Q0SMM7	1,3	48	Q660P4	1
Protein grpE	22	P28609	1	22	Q0SMY9	1,3	22	Q661A2	1
Protein-export membrane protein secD	65	O51596	3	65	Q0SMK1	3	62	Q660L8	2
PTS system, fructose-specific IIBC component	67	O51369	3	67	Q0SN99	2,3	67	Q661L1	2
PTS system, glucose-specific IIBC component	56	O51590	3	56	Q0SMK8	2,3	56	Q660M5	2
PTS system, maltose and glucose-specific IIBC component	59	O51000	3	57	Q0SL27	3	59	Q6ASM5	2
Putative outer membrane protein BBA03	19	Q44849	1,3	19	Q0SM02	1,2,3	19	Q6ASM3	1,2
Putative uncharacterized protein	35	O50726	1,3	34	Q0SLL4	1,2	28	Q5XZ45	2
Putative uncharacterized protein	34	O50805	1,3	21	Q0SLT1	1,2,3	28	Q5XZ45	2
Putative uncharacterized protein	31	O50958	1,3	30,8 or 29,7	Q5W296 or Q0SLU1	2,3	29	Q6ASF9	1,2
Putative uncharacterized protein	29	O50957	3	29	Q5W295	1,2,3	27	Q6ASF7	1,2
Putative uncharacterized protein BB0238	30	O51254	2,3	30	Q0SNS3	2,3	30	Q662C6	2
Putative uncharacterized protein BB0405	22	O51366	1	22	Q0SNA2	1,3	22	Q661L4	1,2
Putative uncharacterized protein BB0406	23	O51367	1	23	Q0SNA1	1	23	Q661L3	1
Putative uncharacterized protein BB0418	39	O51379	1	40	Q0SN90	1	40	Q661K0	1,2
Putative uncharacterized protein BB0543	24	O51493	1	24	Q0SMW7	1,2,3	24	Q660Y1	1,2
Putative uncharacterized protein BB0553	59	O51503	1	60	Q0SMV6	1	60	Q660X1	2
Putative uncharacterized protein BB0562	20	O51510	1	20	Q0SMU7	1	20	Q660W2	1
Putative uncharacterized protein BB0563	20	O51511	1	19	Q0SMU5	1,3	19	Q660W1	1
Putative uncharacterized protein BB0751	42	O51692	3	40	Q0SMA6	3	40	Q660C4	2
Putative uncharacterized protein or Virulent strain associated lipoprotein	54,3 or 55,5	O50870 or Q9ZIU2	or 3	30	Q0SLN9	1,2,3	31	Q5XYR1	2
Pyrophosphate-fructose 6-phosphate 1-phosphotransferase	62	P70826	1,3	62	Q0SPE4	1,2,3	63	Q662Z2	1,2
Pyruvate kinase	53	O51323	3	53	Q0SNG4	1,2,3	53	Q661R9	2
Rare lipoprotein A	31	O51677	1	31	Q0SMC1	3	30	Q660D8	2
Ribose-5-phosphate isomerase A	26	O51601	1	26	Q0SMJ6	1	26	Q660L3	1
Rod shape-determining protein	40	O51657	3	40	Q0SME1	2,3	38	Q660F8	2

Protein	<i>B. burgdorferi</i> B31			<i>B. afzelii</i> K78			<i>B. garinii</i> PBi		
	MW (kDa)	SWISS-Prot ID	M*	MW (kDa)	SWISS-Prot ID	M*	MW (kDa)	SWISS-Prot ID	M*
S2 lipoprotein or Antigen, S2, putative	27,5 or 27,3	Q6DRT2 or O51180	3	28	Q6DRS5 or Q0SP07	2,3	28	Q662K7	1,2
Spermidine/putrescine ABC transporter, spermidine/putrescine-binding periplasmic protein	41	O51584	3	41	Q0SML4	3	41	Q660N1	2
Transcription termination factor rho	58	P33561	3	58	Q0SNT1	2,3	58	Q662D4	2
Trigger factor	53	O51555	3	53	Q0SMP7	2,3	53	Q660R3	2
Triosephosphate isomerase	28	Q59182	3	28	Q0SPB0	2,3	28	Q662V9	2
Uncharacterized lipoprotein BBF20	11	O51025	1	11	Q0SL75	1,2,3	11	Q5XZ25	2
Uncharacterized protein BB_0028 [Precursor]	40	O51059	3	39	Q0SPD6	3	40	Q662Y4	2
Uncharacterized protein BB_0752	57	O51693	3	57	Q0SMA5	3	57	Q660C3	2

8 ABBREVIATIONS

ARF	Alternative Reading Frame
ARIS	Antigen Research Information System
BSA	Bovine Serum Albumine
BSK	Barbour-Stoenner-Kelly
CDC	Center for Disease Control and Prevention
CFA	Complete Freund's Adjuvant
CFU	Colony Forming Unit
CRF	Complementary Reading Frame
CSF	Cerebro Spinal Fluid
DNA	Desoxyribo Nucleic Acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FITC	Fluoresceinisothiocyanat
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
His	Histidine
His-tag	Histidine-tag
IFA	Incomplete Freund's Adjuvant
IPTG	Isopropyl β -D-Thiogalacto-Pyranoside
LB medium	Luria Bertani
MACS	Magnetic Cell Sorting
M _w	Molecular weight
NAD	Nicotinamide adenine dinucleotide
OD	Optical Density

ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with 0.1% Tween-20
rRNA	ribosomal Ribonucleicacid
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	TRIZMA®base

CURRICULUM VITAE

Personal information

Name	Albina Poljak
Place of birth	10040 Zagreb, Croatia
Date of birth	13th December 1980
Nationality	Croatian

Education and training

2009-2010	Maternity leave
2006-2009	PhD Thesis "Identification and characterization of Borrelia antigens as potential vaccine candidates" at Intercell AG, Campus Vienna Biocenter 3, 1030 Vienna, Austria with Dr. Urban Lundberg and Dr. Eszter Nagy and supervisor Prof. Dr. Alexander von Gabain
2007-2008	Part of the Organization Committee of VBC Student Symposium I and II Campus Vienna Biocenter, Vienna, Austria
Aug-Sep 2006	Generation of the knock out strains at the Umeå University, Department of Molecular Biology, in Prof. Dr. Sven Bergström group, Umeå, Sweden
2004-2005	Diploma thesis "Defining the antigenome of Borrelia by bacterial surface library" at Intercell AG, Campus Vienna Biocenter 3, 1030 Vienna, Austria with Dr. Urban Lundberg and supervisor Prof. Dr. Alexander von Gabain

2002-2005	Studies of Genetics and Microbiology at the University of Vienna
2001-2002	Study of Biology at the University of Vienna
1999-2001	Studies of Biology and Chemistry at the University of Zagreb, Croatia
1995-1999	VII. Gimnazija, Zagreb Croatia
1991-1995	Secondary school, Zagreb, Croatia
1987-1991	Elementary school, Zagreb, Croatia

THESIS-RELATED PUBLICATIONS

Publications

Albina Poljak, Bernd Gesslbauer, Claudia Handwerker, Urban Lundberg, Andreas Meinke, Eszter Nagy and Andreas J. Kungl. **Comparative membrane proteome analysis of three *Borrelia* species.** Manuscript in preparation.

Poster and oral presentations at scientific conferences

A. Poljak, U. Lundberg, B. Noiges, S. Prustomersky, C. Triska, U. Stierschneider, D. Gelbmann, M. Hanner, E. Nagy, A. von Gabain and A. Meinke. **Identification of common antigens for the three European pathogenic *Borrelia burgdorferi* sensu lato genospecies.** Poster presentation at *ASM general Meeting 2006, Orlando, Florida*.

A. Poljak, G. Swatosch, J. Pikalo, C. Satke and U. Lundberg. **Identification of novel vaccine candidates against Lyme borreliosis.** Poster presentation at *VBC Student Symposium I, 2007, Vienna, Austria*.

A. Poljak, G. Swatosch and U. Lundberg. **Identification of novel vaccine candidates against Lyme borreliosis.** Poster presentation at *Semmering Conference 2007, Baden, Austria*.

A. Poljak, U. Lundberg, G. Swatosch and E. Nagy. **Identification of novel vaccine candidates against Lyme disease.** Poster presentation at *Gordon Research Conference, Biology of Spirochetes*, 2008, Ventura, California.

A. Poljak. **Identification of candidate antigens in Borrelia.** Oral presentation at *11th International Conference on Lyme Borreliosis and Other Tick-Borne Diseases*, 2008, Irvine, California.

A. Poljak, B. Gesslbauer, A. Kungl, U. Lundberg, A. von Gabain and E. Nagy. **Identification of candidate antigens in Borrelia.** Oral presentation at *VBC Student Symposium II*, 2007, Vienna, Austria.