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**Molecular characterization of the fibronectin-binding
protein BBK32 of *Borrelia burgdorferi* sensu lato**

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A. ABSTRACT

BBK32, a fibronectin (Fn)-binding protein of *Borrelia (B.) burgdorferi* sensu lato (s.l.) which is encoded by the *bbk32* gene located on the 36kb linear plasmid (lp36) of isolate B31, is playing an important role in serological diagnosis of Lyme borreliosis. Firstly, we were interested in the genomic localization of *bbk32* regarding different *B. burgdorferi* s.l. species as well as between strains of the same species. Southern blot analyses based on 23 strains of the species *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii* and *B. spielmanii* revealed that position of *bbk32* is rather variable between the species but also within a given species. *bbk32* could be located on different linear plasmids (lp), mainly on lp23kb, lp24kb, lp25kb, lp31kb and lp36kb. The meaning of this finding remains unclear so far. Secondly, a number of thirteen chimeric polypeptides representing different parts of the N-terminal regions of BBK32 proteins of both *B. burgdorferi* s.s. isolate B31 and *B. garinii* isolate PHei were generated. Fn-binding capabilities of those generated polypeptides were evaluated either by Western-ligand blot-based binding assay or by enzyme-linked immunosorbent assay (ELISA)-based binding assay. Results showed that BBK32 from PHei possesses a higher Fn-binding capability than that from B31. Furthermore, the higher Fn-binding capacity is associated with four amino acids (Lysine₁₃₁, Lysine₁₄₅, Threonine₁₄₇ and Isoleucine₁₅₅) in the 32-amino acid-long segment (from position 131 to 162). Moreover, both gelatin and collagen could partially inhibit the binding of BBK32 to Fn. This suggests that BBK32 might also bind to the collagen-binding domain of Fn (repeat I₆₋₉ and II_{1,2}) and partially to its N-terminal fibrin-binding domain (repeat I₁₋₅). Though the meaning of the different Fn-binding capacities remains unclear so far, such studies may provide us with markers to define the different pathogenic potentials of various *Borrelia* species and strains. Thirdly, eight recombinantly prepared BBK32 homologues (either as partial or as whole) were tested in a line assay to evaluate their contribution for serologic diagnosis of Lyme borreliosis. Though BBK32 homologues could react with sera from Lyme borreliosis patients, compared with other *Borrelia*-antigens established in the Max von Pettenkofer Institute, these BBK32 homologues could not improve the sensitivity and specificity of the class-specific IgG or

IgM antibody tests. Nevertheless, this study underlines the fact that the heterogeneity of Lyme disease *Borrelia* species must be taken into consideration in the microbiological diagnosis of Lyme borreliosis in European patients.

Zusammenfassung

BBK32 ist ein Fibronectin (Fn)-bindendes Protein des Bakteriums *Borrelia* (*B.*) *burgdorferi* sensu lato (s.l.). BBK32 wird durch das *bbk32*-Gen, das im 36 kb linearen Plasmid (*lp36*) des Isolates B31 liegt, kodiert und spielt eine wichtige Rolle in serologischen Verfahren für den Nachweis von Lyme Borreliosis. Als erstes waren wir an der Lokalisierung von *bbk32*-Gen in verschiedenen *B. burgdorferi* s.l. Spezies sowie in verschiedenen Stämmen derselben Spezi interessiert. Southern-Blot-Analysen von 23 Stämmen der Spezies *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii* und *B. spielmanii* zeigten unterschiedliche Lokalisation des *bbk32*-Gens sowohl in den verschiedenen Spezies, als auch in verschiedenen Stämmen derselben Spezi. Das *bbk32*-Gen konnte in verschiedenen linearen Plasmiden (*lp*), hauptsächlich in *lp23kb*, *lp24kb*, *lp25kb*, *lp31kb* und *lp36kb*, lokalisiert werden. Die Relevanz dieser unterschiedlichen Lokalisierung ist nicht klar. Zweitens, es wurden 30 chimäre Polypeptid-Sequenzen aus unterschiedlichen Bereichen der N-terminalen Region des BBK32-Proteins von *B. burgdorferi* s.s. Isolat B31 und *B. garinii* Isolat PHei generiert. Die Fn-Bindungs-fähigkeit dieser hergestellten Polypeptide wurde entweder mittels eines Western-Ligand Blot-basiertes Bindungstestes oder eines Enzymgekoppelter Immunadsorptionstest (ELISA)-basiertes Bindungstestes evaluiert. Die Ergebnisse zeigten, dass BBK32 aus PHei ein höheres Fn-Bindungspotenzial als BBK32-Polypeptide aus B31 aufweist. Außerdem ist die höhere Fn-Bindungs-fähigkeit mit dem Vorhandensein der vier Aminosäuren: Lysin131, Lysin145, Threonin147 und Isoleucin155 im 32-Aminosäuren langen Segment (von Aminosäure-Position 131 bis 162) assoziiert. Darüber hinaus konnten sowohl Kollagen als auch Gelatine die Bindungs-fähigkeit von BBK32 an Fn teilweise inhibieren. Dies deutet darauf hin, dass BBK32 vielleicht auch an

Kollagen-Bindungsdomäne in Fn (Motive I₆₋₉ and II_{1,2}) und teilweise an der N-terminalen Fibrin-Bindungsdomäne (Motive I₁₋₅) binden könnte. Obwohl die Bedeutung der unterschiedlichen Bindungsfähigkeit an Fibronectin noch unklar ist, diese Studie könnte Marker zur Identifizierung diverser *Borrelia*-Spezies und –Stämme mit unterschiedlichem Pathogenitätspotenzial liefern. Drittens, acht rekombinant hergestellte homologe BBK32-Proteine (gesamte oder nur Teil-Sequenzen des Proteins) wurden für ihre mögliche Verwendung für serologische Verfahren zum Nachweis von Lyme Borreliosis in einem Linientest geprüft. Obwohl BBK32-Homologe mit Seren aus Patienten mit Lyme Borreliosis reagierten, konnten diese, gegenüber anderen im Max von Pettenkofer-Institut etablierten *Borrelia*-Antigene, zu keiner Erhöhung der Sensitivität und Spezifität des klassenspezifischen Nachweises von IgG oder IgM im Antikörper-Test führen. Nichtsdestoweniger unterstreicht diese Arbeit die Tatsache, dass die Heterogenität der *Borrelia*-Spezies bei der mikrobiologischen Diagnose von Lyme Borreliosis in Betracht gezogen werden muss.

B. INTRODUCTION

Lyme borreliosis (or Lyme disease) is a multisystem disease involving many organs, mainly the skin, nervous system, joints and heart (Steere, 1989; Pfister *et al.*, 1994; Stanek and Strle, 2003). As the most prevalent and widespread vector-borne infectious disease in the northern hemisphere, Lyme disease is caused by *Borrelia (B.) burgdorferi* sensu lato initially detected in the American tick vector *Ixodes (I.) scapularis* (Burgdorfer *et al.*, 1982). *B. burgdorferi* s.l. is transmitted to humans by the bite of infected hard ticks belonging to *I. ricinus* / *I. persulcatus* species complex (Johnson, 1996).

1. Discovery of Lyme borreliosis

The first record of a condition associated with Lyme borreliosis dates back to 1883 in Breslau, Germany, where a physician named Alfred Buchwald described a degenerative skin disorder now known as acrodermatitis chronica atrophicans (ACA). Then, throughout the early twentieth century, many of the symptoms and signs that constitute Lyme disease were already described independently of each other. Finally, Lyme disease gets its name from a small coastal town in Connecticut called Lyme. In 1975, a woman brought to the attention of Yale researchers an unusual cluster of more than 51 cases of mostly pediatric arthritis. In 1977, Dr. Allen Steere and Yale colleagues identified and named the 51 clusters "Lyme arthritis." In 1979, the name was changed to "Lyme disease," when Steere and colleague Dr. Steven Malawista discovered additional symptoms linked to the disease such as neurological problems and severe fatigue. In 1982, the cause of the disease was discovered by Dr. Willy Burgdorfer. Dr. Burgdorfer published a paper on the infectious agent of Lyme disease and earned the right to have his name placed on the Lyme disease spirochete now known as *Borrelia burgdorferi* (Burgdorfer *et al.*, 1982). Two years later, the isolation of spirochetes from the CSF of a patient with Bannwarth's syndrome further confirmed that spirochetes are the pathogenic agents of Lyme disease (Pfister *et al.*, 1984). In China, Lyme disease cases were first found in Hailin County, Heilongjiang Province in 1986 (Ai *et al.*, 1988). In 1987, *B. burgdorferi* strains were first isolated from *I. persulcatus*

collected from the same area (Zhang *et al.*, 1989). Some important history events in the discovery of Lyme disease are listed in Table B-1.

Table B-1. Timeline of important events in the discovery of Lyme disease

Year	Event
1883	The first record of a condition of Lyme disease was described by Alfred Buchwald. He described it as a degenerative skin disorder now known as acrodermatitis chronica atrophicans (ACA).
1909	Arvid Afzelius presented his research about an expanding, ring like skin lesion, erythema migrans (EM), associated with what would become known as Lyme Disease.
1921	Arvid Afzelius published his work and speculated that the rash came from the bite of an <i>Ixodes ricinus</i> tick and connected the disease with joint problems.
1922	The disease was found to be associated with neurological problems.
1930	The disease was found to also cause psychiatric symptoms.
1934	Patients with benign lymphocytoma also had either EM or ACA. Arthritic symptoms were reported in connection with the disease.
1970	Rudolph Scrimenti reported an EM known with certainty to have been acquired in the United States.
1975	Allen C. Steere, Yale, investigated a group of rashes and swollen joints in Lyme, Connecticut. Scrimenti published about his treatment of the rash with penicillin.
1976	A clustering of cases of the disease was reported in Naval Medical Hospital in Connecticut.
1977	Steere <i>et al.</i> misdiagnosed Lyme disease as juvenile rheumatoid arthritis and named this condition ‘Lyme arthritis’.
1982	The etiological agent of the disease, <i>B. burgdorferi</i> , was discovered by Dr. Willy Burgdorfer.
1984	Pfister HW and his colleagues isolated spirochetes from the CSF of a patient with Bannwarth’s syndrome.
1986	Lyme disease cases were found in Hailin County, Heilongjiang Province in China.

2. Epidemiology

2.1 Frequency of Lyme borreliosis

Although Lyme borreliosis is the most commonly reported tick-borne disease in the northern hemisphere including North America and Eurasia, few data are available about its actual frequency in several regions. Its prevalence is estimated to range between 20 and 100 cases per 100.000 inhabitants in the USA and about 100 to 130 cases per 100.000 in Europe (Huppertz *et al.*, 1999; Hengge *et al.*, 2003). In China, investigations suggested that

around 5.0% of the surveyed residents from 30 provinces were antibody positive for *B. burgdorferi*. Typical cases of Lyme disease were diagnosed in 11 provinces, especially in the northeast forest areas. And more than 130 isolates of *B. burgdorferi* were recovered from patients, ticks or animals in 19 provinces (Cui, 2004; Zhang *et al.*, 1997). However, reported cases of Lyme disease are thought to represent only seventh of the actual number of people with the disease all over the world (Campbell *et al.*, 1998). Lyme borreliosis occurs with similar gender preference with the exception of ACA, which is more frequent in women. Early neuroborreliosis cases showed a bimodal age distribution with a lower frequency in adults whereas ACA occurs primarily in older patients (Wilske, 2005). High-risk groups include people who work or spend time outdoors such as farmers, foresters, lumberjacks, scientific researchers, hikers, trail workers, runners, and vacationers.

2.2 Causative agents

The agents of Lyme borreliosis are borrelia, bacteria of the spirochaete family, which are grouped in the *B. burgdorferi* s.l. species complex and further divided into 14 different genomic species. Only four out of them are assured to cause human disease: *B. burgdorferi* s.s. (the only human pathogenic species present in the USA), *B. afzelii*, *B. garinii* and the only recently described species *B. spielmanii*. *B. spielmanii* so far has only be described in rare cases with erythema migrans. Although the DNA of *B. valaisiana*, *B. lusitaniae*, and *B. bissettii* has already been detected in samples of human origin, or the spirochetes were isolated from the patients with symptoms of Lyme borreliosis, whether these species to be pathogenic for humans is still unclear (Fingerle *et al.* 2008; Richter *et al.* 2006; Stanek *et al.*, 2003; Wilske *et al.*, 2007a, Picken *et al.*, 1996; Zhang *et al.*, 1997; Wan *et al.*, 2002; Zeng *et al.*, 2002; Masuzawa *et al.*, 2001; Chu *et al.*, 2008; Rudenko *et al.*, 2009). *B. burgdorferi* s.l. have a length of 20-30 μ m and a width of only 0.2-0.3 μ m. Hence, most spirochetes cannot be viewed using conventional light microscopy. Dark-field microscopy should be used to view spirochetes (Fig.B-1).



Fig. B-1. Giemsa stained *Borrelia burgdorferi* (with kind permission of Prof. Wilske).

Due to the diversity of the outer membrane protein OspA, the three main pathogenic species (*B. burgdorferi* s.s. *B. afzelii* and *B. garinii*) comprise at least 7 different OspA serotypes in Europe. OspA serotype 1 corresponds to the species *B. burgdorferi* s.s., OspA serotype 2 to *B. afzelii*, and the OspA serotypes 3-7 to the *B. garinii* complex (Wilske *et al.*, 1993) (Table B-2). Distribution of the different OspA serotypes and thus also of the species is very different in isolates from skin biopsy specimens, CSF or ticks. Skin isolates, especially those from ACA, show a predominance of *B. afzelii*, whereas CSF and tick isolates mainly belong to *B. garinii*. However, regarding the latter species, the various specimen types differ in the prevalence of their *B. garinii*-associated OspA serotypes (Table B-2). Though initially Lyme arthritis was exclusively linked to *B. burgdorferi* s.s., OspA-type specific PCR has revealed that Borreliae detected in synovial fluid specimens of patients suffering from Lyme arthritis are heterogeneous (Table B-2) (Eiffert *et al.*, 1998; Vasiliu *et al.*, 1998). The most frequent genomic groups in Europe, *B. afzelii* and *B. garinii* occur across the continent and the islands, whereas the third frequent group *B. burgdorferi* s.s. has only rarely been isolated in eastern Europe (Hubalek *et al.*, 1997). The heterogeneity of OspA has important implications for vaccine development in Europe as well as for diagnostics (e.g. *ospA* PCR). Quite recently, the pathogenic potential of the new species *B. spielmanii* was confirmed by the finding of the corresponding pathogen in patients with EM from the Netherlands, Slovenia, and Germany (Wang *et al.*, 1999a;

Fingerle *et al.*, 2008).

Table B-2. Distribution of *B. burgdorferi* s.l. species and OspA types in European isolates from ticks, cerebrospinal fluid (CSF), skin and synovial fluid specimens (Wilske *et al.*, 2007a).

Species	OspA type	Ticks (%) (n = 90)	CSF (%) (n = 43)	Skin (%) (n = 68) ^a	Synovial fluid (%) (n = 20) ^b
<i>B. burgdorferi</i> s.s.	1	20	19	6	33
<i>B. afzelii</i>	2	9	12	84	29
<i>B. garinii</i> ^c	3–7	71	69	10	38

a. Source of skin specimens known in 46 patients (30 cases of EM, with 1, 26, 1 and 2 cases infected with OspA types 1, 2, 4 and 6, respectively; 16 cases with ACA, with one and 15 cases infected with OspA types 1 and 2, respectively).

b. *B. burgdorferi* s.l. speciation from synovial fluid samples is based on *ospA* PCR results. Culture isolates from this tissue were too few to estimate species distribution.

c. Tick and CSF isolates differ in the percentages of OspA types 4 and 6. OspA type 6 was found in 53% of tick isolates, but in only 23% of CSF isolates. In contrast, OspA type 4 was found in 28% of CSF isolates, but was not isolated from ticks.

2.3 Vectors and reservoirs

B. burgdorferi s.l. complex are transmitted by hard ticks (*Ixodes* spp.): In the U.S. they are transmitted by *I. scapularis* (Eastern regions) and *I. pacificus* (Western regions), in Europe and western Asia by *I. ricinus* (Fig. B-2) and in Asia by *I. persulcatus* (Stanek *et al.*, 2003). *I. persulcatus* played a leading role in the transmission of *B. burgdorferi* to human beings in the northern part China, *I. granuatus* and *Haemaphysalis bispinosa* might serve as principle vector of the agents in the southern region (Wan, 2002; Ai *et al.*, 1990). The larvae and nymphs feed primarily on small rodents whereas adult ticks feed on a variety of larger animals. In Europe small mammals such as mice and voles provide reservoirs for *B. afzelii*, *B. burgdorferi* s.s, and *B. garinii* serotype 4, and birds are reservoirs for *B. garinii* and *B. valaisiana* (Gern *et al.*, 1998; Huegli *et al.*, 2002). Rodents and birds also form the reservoir for *B. burgdorferi* s.s. in North America (Piesman, 2002). Genus *apodemus* and *Clethrionomys* might serve as a major reservoir for *B. burgdorferi* s.l. in China. Some cattle, sheep, dogs and rats from forest areas possessed a high antibody titer for *B. burgdorferi* s.s. (B31) (Wan, 2002), however, whether these animals also serve as reservoirs for *B. burgdorferi* s.l. is unclear so far. Birds might also play an important role as

both hosts of ticks and vectors of *B. burgdorferi* s.l. (Olsen *et al.*, 1995; Kipp *et al.*, 2006; Ishiguro *et al.*, 2000, 2005). In unfed ticks *B. burgdorferi* s.l. lives in the midgut. During the blood meal on humans or other hosts, Borreliae have to migrate to the salivary glands wherefrom they are transmitted via saliva to the host. In most European regions the average infection rates for *I. ricinus* range from 10 - 20 % (Gern *et al.*, 1993). However, there are marked differences in the infection rates of the various developmental stages of the ticks: A study carried out in Southern Germany revealed that of over 3.000 ticks about 20% of the adults, 10% of the nymphs and 1% of the larvae were infected (Fingerle *et al.*, 1994; Wilske *et al.*, 1987). The Borreliae are thus mostly acquired by feeding on infected reservoir hosts during the developmental cycle and only rarely transmitted transovarially. The life cycle of the hard tick *Ixodes* spp and the transmission of Borrelia to human beings are illustrated in Fig. B-3.

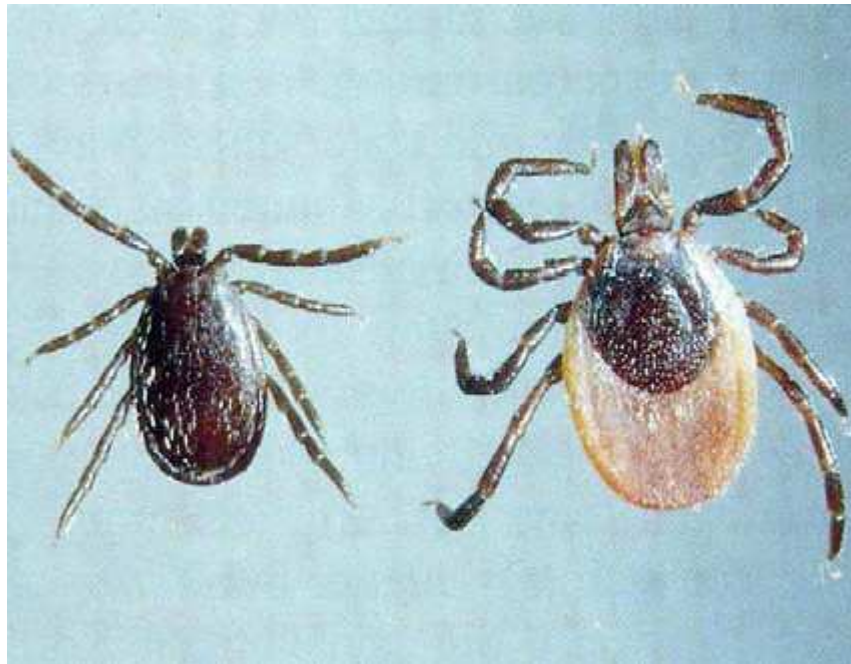


Fig. B-2. Adult *Ixodes ricinus*. Left, male; Right, female. (from Dr. Boesebeck).

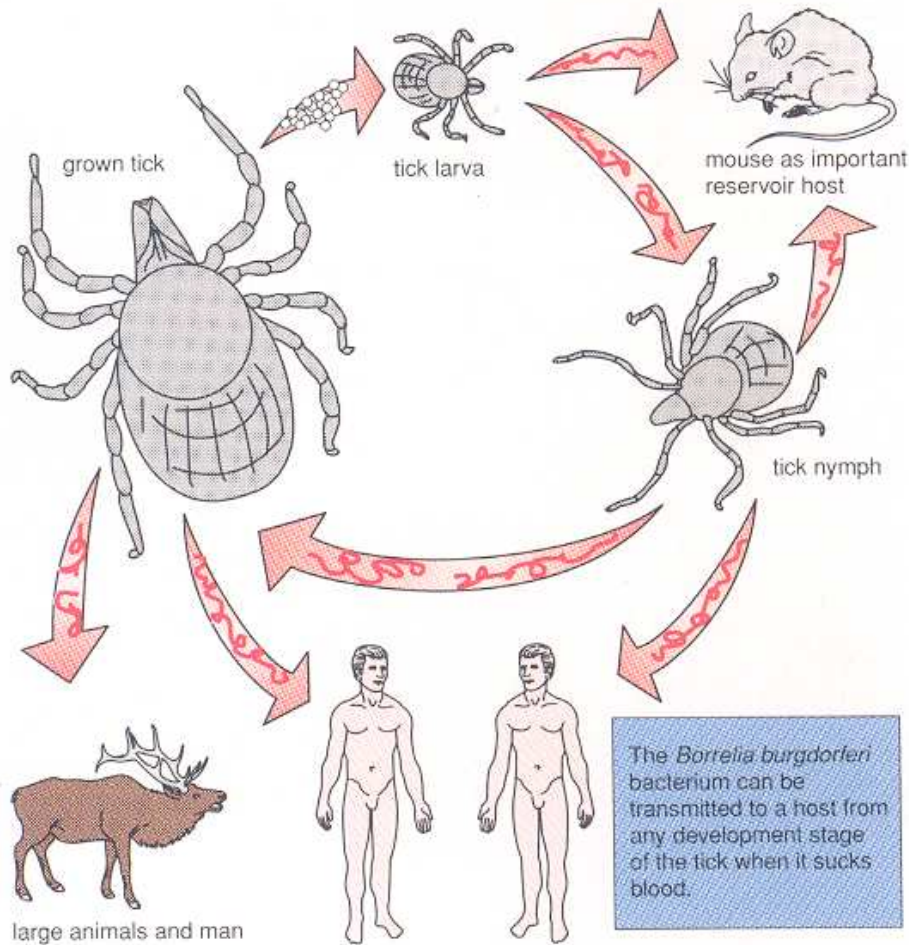


Fig. B-3. Life cycle of the hard tick *Ixodes ricinus* and transmission of the organism that causes Lyme borreliosis. (With kind permission of Dr. Boesebeck, HMR, Germany).

3. Symptoms

Lyme disease comprises three stages: early localized, early disseminated, and late-stage manifestations (Steere *et al.*, 2004). The natural course of untreated *B. burgdorferi* infections varies considerably, and the various clinical manifestations can occur alone or in various combinations (Steere *et al.*, 2004; Oschmann *et al.*, 1999). In most cases, the infection is self-limiting, but in rare cases, *B. burgdorferi* might persist and chronic disease manifestations could develop. The early and late stages are usually separated by an asymptomatic interval.

3.1 Stage I (days through weeks after the tick bite)

The early stage of Lyme disease is often characterized by a distinctive, expanding red rash that usually develops at the site of the tick bite. This rash, known as erythema migrans (EM), is the hallmark and best clinical indicator of Lyme disease (Fig. B-4). The center of the expanding annular lesion often fades to produce a bull's-eye appearance. However, the extension, color intensity and duration of EM vary considerably. In addition, general accompanying symptoms such as low fever, myalgia, headache and, rarely, meningism may occur.



Fig. B-4. Erythema migrans (Source, NRZ Borrelia, http://www.lgl.bayern.de/gesundheits/nrz_borrelien/index.htm)

3.2 Stage II (weeks through months after the tick bite)

In some patients, hematogenous dissemination of the pathogen to other organs and tissues may occur (stage II). Patients sometimes feel quite ill and may present with fatigue, headache, fever, malaise, arthralgia and myalgia, symptoms that may last for weeks. Multiple erythematosa migrantia (Fig. B-5) as sign for dissemination are seen more often in the USA. Neuroborreliosis is the most frequent manifestation of this stage, particularly in its appearance as lymphocytic meningoradiculitis, which usually reveals typical clinical symptoms. Cardinal symptoms are the radicular pain syndrome - characterized by excruciating, burning pains exacerbating mainly during the night - and/or cranial nerve palsy. Pareses of the extremities and the trunk are less frequent. Meningitis or even facial palsy (Fig. B-6) as sole manifestation without any meningitic symptoms is more frequently found in children than in adults. Typical CSF findings characterized by blood/CSF barrier

INTRODUCTION

dysfunction, lymphocytic pleocytosis, elevated CSF protein concentration and oligoclonal IgM are diagnostically indicative. Detection of borrelia-specific intrathecal antibody production assures the diagnosis. Further clinical manifestations of stage II are Lyme carditis, which clinically presents as dysrhythmia, mainly in the form of atrioventricular blocks of changing degrees. Borrelial lymphocytoma, a small reddish to livid nodular swelling of the skin in typical locations such as the earlobe, nipple, or scrotum, is manifested among some patients in Europe as well as in rare cases various forms of ophthalmoborreliosis (Wilske *et al.*, 2007b) (Fig. B-7).



Fig. B-5. Multiple erythema (Source, NRZ Borrelia, http://www.lgl.bayern.de/gesundheit/nrz_borrelien/index.htm)



Fig. B-6. Facial palsy of patient suffered from neuroborreliosis. (Figure remodeled from Nau *et al.*, 2009)



Fig. B-7. Borrelial lymphocytoma. Left, in earlobe; Right, in nipple. (Source, NRZ Borrelia, http://www.lgl.bayern.de/gesundheit/nrz_borrelien/index.htm).

3.3 Stage III (months through years after the initial infection)

Lyme arthritis and acrodermatitis chronica atrophicans (ACA) are the most common manifestations of stage III (Fig. B-8). Lyme arthritis, which typically affects large joints (especially the knee), can take a monoarticular or oligoarticular, intermittent or, less frequently, a chronic course. Here, acute disease manifestations and asymptomatic intervals may alternate. Spontaneous remissions are frequent, transitions into the chronic stage rather seldom. Patients with ACA initially develop an infiltrative stage, followed by the alterations characteristic of the atrophic stage: creased skin with livid discolorations and plastic protrusion of vessels. It is notable that ACA is almost exclusively observed in Europe. Chronic neuroborreliosis is a very rare manifestation of the late stage. Parapareses and tetrapareses are its most common symptoms. Examination of the CSF reveals a marked elevation of protein concentration with a low to moderate increase of CSF cells and oligoclonal IgG-bands, in some cases even IgA- and/or IgM-bands. The detection of intrathecally produced specific antibodies is currently regarded as the best marker for borreliosis and is also the most relevant criterion allowing differentiation from other chronic disease like multiple sclerosis.



Fig. B-8. Lyme arthritis (left) and acrodermatitis chronica atrophicans (ACA) (right) (Source, NRZ Borrelia, http://www.lgl.bayern.de/gesundheit/nrz_borrelien/index.htm)

4. Indications for microbiological diagnosis

If there are no pathognomonic symptoms such as a typical erythema migrans, clinical diagnosis of Lyme borreliosis usually requires confirmation by means of a

laboratory-diagnostic assay. Antibody detection methods mainly are used for this purpose, whereas detection of the causative agent by culture isolation and nucleic acid techniques is confined to special situations.

4.1 Specimens for the microbiological diagnosis

For culture and PCR, skin biopsy samples are the most promising specimens. In general poor results are obtained from body fluids with the exception of PCR from synovial fluid. For antibody determination, serum or CSF can be investigated. CSF examination should always be done together with serum antibody analysis (determination of the CSF/serum antibody index) (Table B-3) (Wilske, 2005). Examination of ticks should be performed only for epidemiological or other scientific studies.

Table B-3. Specimen types used for the diagnosis of Lyme borreliosis (Wilske, 2005).

Clinical manifestation	Specimens for	
	Direct pathogen detection (culture, PCR)	Antibody detection
Stage I (early / localized), (<i>days through weeks after tick bite</i>)		
Erythema migrans	Skin biopsy	Serum
Stage II (early / disseminated) (<i>weeks through months after tick bite</i>)		
Multiple erythemata	Skin biopsy	Serum
Borrelial lymphocytoma	Skin biopsy	Serum
Lyme carditis	Endomyocardial biopsy	Serum
Neuroborreliosis	CSF	Paired serum/CSF ^a
Stage III (late / persistent) (<i>months through years after tick bite</i>)		
Arthritis	Synovial fluid, synovial biopsy	Serum
Acrodermatitis chronica atrophicans	Skin biopsy	Serum
Chronic neuroborreliosis	CSF	Paired serum/CSF ^a

a. from the same day for CSF/serum index determination.

4.2 Direct detection of the pathogen

4.2.1 Culture

B. burgdorferi s.l. can be cultivated in modified Kelly-Pettenkofer (MKP) or Barbour-Stonner-Kelly-II (BSK-II) medium (Preac-Mursic *et al.*, 1991; Wilske and Schriefer, 2003; Barbour, 1984). However, it is very time-consuming and characterized by a low sensitivity, especially in body fluids (Karlsson *et al.*, 1990; Strle, 1999; Arnez *et al.*, 2001; Zore *et al.*, 2002). So, detection of the pathogen by culture usually plays a minor role in the routine diagnosis of Lyme disease and is confined to special indications, e.g. to clarification of clinically and serologically ambiguous findings. Also, application of the method should be restricted to laboratories specialized in this technique.

4.2.2 PCR

For DNA amplification under experimental conditions various target sequences have been used, e.g. from plasmid-borne genes such as *ospA* and *ospB*, or chromosomal genes such as the genes for the flagellar protein or p66, or from gene segments of the 16S rRNA or the 5S/23S rRNA intergenic spacer region (Schmidt, 1997; Agüero-Rosenfeld *et al.*, 2005). *Borrelia* PCR should allow diagnosis of the *Borrelia* species, i.e. the medical report should contain information as to which of the species pathogenic for humans has been found.

4.2.3 Sensitivity of culture and PCR

Table B-4 provides a survey of the sensitivity of direct detection methods in clinical specimens from patients with Lyme borreliosis.

Table B-4. Sensitivity of direct pathogen detection methods in Lyme borreliosis (Wilske *et al.*, 2007a)

Specimen	Sensitivity
Skin (erythema migrans, acrodermatitis)	50–70% when using culture or PCR
Cerebrospinal fluid (acute neuroborreliosis)	10–30% when using culture or PCR ^a
Synovial fluid ^b (Lyme arthritis)	50–70% when using PCR (culture is only extremely seldom positive)

^a Up to 50% of patients with a disease duration of less than 2 weeks, compared with only 13% of patients with a disease duration of more than 2 weeks (Lebech *et al.*, 2000).

^b Higher sensitivity of direct pathogen detection from synovial biopsy specimens.

4.3 Antibody detection

Analysis of the humoral immune response in patients with Lyme borreliosis with regard to the immunodominant antigens of *B. burgdorferi* reveals specific, stage-dependent characteristics that are also diagnostically relevant. A positive antibody test result can be expected in approx. 20 to 50% of cases with primary-stage Lyme borreliosis. If only a short time has elapsed since onset of the disease, mainly IgM antibodies will be found, whereas in cases of long-term illness IgG antibodies predominate. Approx. 70 –90% of patients going through the secondary stage will show positive antibody test results. In the tertiary stage of Lyme disease, antibodies – in most cases of the IgG type only - can be detected in almost 100% of patients (Wilske *et al.*, 2007a). It is generally accepted that serological examination should follow the principles of a two-step approach (Centers for Disease Control and Prevention 1995; Johnson *et al.*, 1996; Wilske *et al.*, 2000; Wilske and Schriefer, 2003): In the majority of cases it will be sufficient to conduct a serological screening assay and – in the event of a positive or equivocal result – a confirmatory assay is recommended (serological stepwise diagnosis). For screening enzyme linked immunosorbent assay (ELISA) is mostly used, which, when it is reactive, should be confirmed by immunoblot.

4.3.1 ELISA

The ELISA tests used for screening should be at least second-generation tests (Wilske *et al.*, 2000) that have been improved with respect to cross-reactivity with other bacteria (e.g. antigen extract with previous Reiter treponema adsorption) (Wilske and Preac-Mursic, 1993) or use purified intact flagella as antigen (Hansen *et al.*, 1988). Strains used as antigen source should express OspC, the immunodominant antigen of the IgM response, and DbpA, an immunodominant antigen of the IgG response (Wilske *et al.*, 2000). Recently, specific recombinant antigens (i.e. VlsE) or synthetic peptides (i.e. the C6 peptide derived from VlsE) have also been successfully used (Lawrenz *et al.*, 1999; Liang *et al.*, 1999, 2000; Bacon *et al.*, 2003; Goettner *et al.*, 2005).

4.3.2 Immunoblot

As a confirmatory assay, the immunoblot should have high specificity (at least 95%). In whole-cell lysate immunoblots the diagnostic bands are to be specified by the supplier, according to their identification with monoclonal antibodies. With the recombinant immunoblot this is achieved by selection of the respective specific recombinant antigens. For the whole-cell lysate immunoblot the European *B. afzelii* PKo strain, which is well characterized by means of monoclonal antibodies, is recommended as antigen (Wilske *et al.*, 2000). In Europe, recombinant immunoblots with quite high sensitivity and specificity have been developed by the combination of different antigens and homologues of one antigen (Wilske *et al.*, 1999; Panelius *et al.*, 2003; Goettner *et al.*, 2005, Schulte-Spechtel *et al.*, 2006) (Fig. B-9). The American immunoblot interpretation criteria (CDC) are not suitable for application in Europe (Hauser *et al.*, 1997, 1998; Robertson *et al.*, 2000). Interpretation criteria for the immunoblot recommended by the German Society for Hygiene and Microbiology (DGHM) are published in the ‘MiQ 12 Lyme-Borreliose’ (Wilske *et al.*, 2000) which is available in English via internet (<http://www.dghm.org/red/index.html?cname5MIQ>). For the IgG immunoblot at least 2 diagnostic bands are required for a positive result, for the IgM immunoblot the existence of only one diagnostic band is considered sufficient.

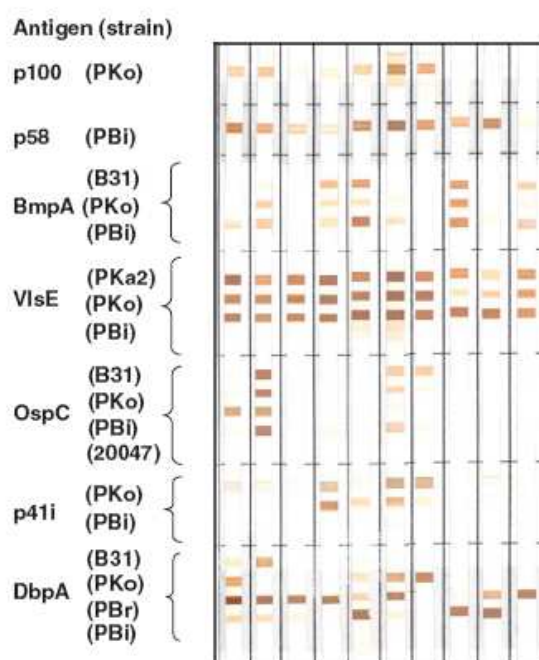


Fig. B-9. Representative IgG line immunoblots of patients with neuroborreliosis. Strains belong to the following species: B31 and PKa2 to *Borrelia burgdorferi* sensu stricto; PKo to *B. afzelii*; PBr to *B. garinii* OspA type 3; PBi to *B. garinii* OspA type 4; 20047 to *B. garinii* unknown OspA type.

4.3.3 Detection of intrathecally produced antibodies

The determination of the CSF/serum index should be performed if neuroborreliosis is considered, as a positive CSF/serum index confirms present or past involvement of the central nervous system. Depending on the time elapsed since the first manifestation of neurological symptoms, this method is successful in 80-90% (8-41 days after onset of the disease), or even in up to 100% (> 41 days after onset) of cases (Hansen *et al.*, 1991). In cases of only short duration, there may be positive CSF findings, while serological results are still negative (Christen *et al.*, 1993). A positive antibody index may be detectable as long as years after successful therapy and is thus not an appropriate means for monitoring treatment success. For diagnosis of late neuroborreliosis detection of a borrelia-specific intrathecal IgG-antibody production is obligate and positive in near by 100%.

5. Treatment of Lyme borreliosis

Most features of Lyme disease respond to antibiotics. The antibiotic, dosage, duration, and route of application depend on the clinical picture and the stage of the disease (Weber and Pfister, 1994; Wormser *et al.*, 2006). For EM and borrelia lymphocytoma, oral treatment with doxycycline, amoxicillin, or cefuroxime axetil is recommended. Patients with acrodermatitis or arthritis receive oral treatment with doxycycline or amoxicillin but in cases of poor therapeutic response, patients should be retreated intravenously with cephalosporins or penicillin G. Parenteral treatment with ceftriaxone, cefotaxime, or high-dose of penicillin G should be performed in the case of severe disseminated infection. Intravenous cephalosporins or penicillin G is also recommended for stage III neuroborreliosis. Antibiotics most often used in treatment of Lyme borreliosis are listed in Table B-5.

Table B-5. Antibiotics most often used for treatment of Lyme borreliosis.

Clinical manifestation	Antibiotics	Dosage per day	Application	Duration (day)
Erythema migrans and borrelial lymphocytoma	Doxycycline*	1 x 200 mg	p.o.	14
	Amoxicillin*	2 x 1000 mg	p.o.	14
	Azithromycin*	2 x 500 mg	p.o.	1

			then 1 x 500 mg	p.o.	2-5
				or	
		Azithromycin*	1 x 500 mg	p.o.	10
		Ceftriaxone*	1 x 1g	i.m.	5
		Minocycline*	2 x 100 mg	p.o.	14
		Cefuroxime*	1 x 500 mg	p.o.	14
		Penicillin V*	3 x 1 g	p.o.	14
Acute neuroborreliosis and carditis		Ceftriaxone*	1 x 2 g	i.v.	14
		Cefotaxime*	3 x 2 g	i.v.	14
		Penicillin G*	4 x 3 g	i.v.	14
Arthritis		Ceftriaxone*	1 x 2 g	i.v.	21
		Cefotaxime*	3 x 2 g	i.v.	21
		Penicillin G*	4 x 3 g	i.v.	21
		Doxycycline*	1 x 200 mg	p.o.	28
Acrodermatitis chronica atrophicans		Doxycycline*	1 x 200 mg	p.o.	21
		Amoxicillin*	2 x 1000 mg	p.o.	21
		Ceftriaxone*	1 x 2 g	i.v.	21
		Cefotaxime*	3 x 2 g	i.v.	21
		Penicillin G*	4 x 3 g	i.v.	21
Chronic neuroborreliosis		Ceftriaxone*	1 x 2 g	i.v.	21
		Cefotaxime*	3 x 2 g	i.v.	21
		Penicillin G*	4 x 3 g	i.v.	21

*alternative regimens; i.m. = intramuscular; i.v. =intravenous; p.o. = oral.

Doxycycline should not be used in children younger than 9 years or in pregnant or breastfeeding women.

6. Prevention

Since the causative agent of the disease is transmitted by ticks, precautions against tick bite should be taken by people in endemic areas. Ticks able to transmit the pathogen are small and difficult to see. Once attached to the skin, they gorge on blood for days. Transmission of *B. burgdorferi* does not usually occur until the infected tick has been in place for several hours. Thus, searching for ticks after potential exposure and removing them can help prevent infection.

In the United States, a vaccine (LYMERix) based on OspA against Lyme borreliosis was licensed for use in humans (Steere *et al.*, 1998). In 2002, the producer withdrew the

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LYMERix vaccine from the market for commercial reasons. However, several other effective preventive measures like repellents remain available to persons living in areas where the disease is endemic. Antibody raised against another surface-associated protein, OspC, has also been shown to protect animals against challenge with homologous *B. burgdorferi* species (Preac-Mursic *et al.*, 1992). Currently, both OspA and OspC vaccines are being developed by industry for use in Europe.

C. AIMS

For many bacterial pathogens, binding to their host extracellular matrix molecules such as Fn or collagen, is believed to be a critical step for their adhesion to and invasion of host tissues. *B. burgdorferi* can bind to a variety of host extracellular matrix molecules (Joh *et al.*, 1999; Menzies, 2003; Cabello *et al.*, 2007; Coburn *et al.*, 2005). A Fn-binding adhesin-BBK32 was originally identified by Probert and Johnson (1998). The Fn-binding feature of the BBK32 and the biochemical mechanism of its binding in case of *B. burgdorferi* s.s. have already been elucidated (Kim *et al.*, 2004; Probert *et al.*, 2001; Raibaud *et al.*, 2005). Overexpression of BBK32 in high-passage *B. burgdorferi* s.s. strains that lacks this protein enhances the organism's binding to Fn, as well as to glycosaminoglycans (Fischer *et al.*, 2006). Furthermore, inactivation of *bbk32* gene in infectious strains of *B. burgdorferi* s.s. reduced spirochetal binding to Fn, as well as its infectivity in mice (Seshu *et al.*, 2006), although the mutations had no apparent defect in tick vectors (Li *et al.*, 2006). These findings suggested that BBK32 might play an important role in the adhesion and invasion activities of *B. burgdorferi* s.s. to its host tissues.

The Fn-binding site in BBK32 was localized to a 32-amino acid-long segment in the protein. This ligand binding segment was shown to share 81–91% amino acid sequence identity with the homologous proteins encoded by *bbk32* genes of *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* (Probert *et al.*, 2001). A former Ph.D student in our laboratory, Dr. Christiane Heimerl, found that the BBK32 of *B. garinii* isolate PHei possesses a stronger Fn-binding capacity than that of *B. burgdorferi* s.s. B31 (C. Heimerl doctoral thesis, 2005). From the observation that gelatin can partially inhibit the binding of Fn to BBK32, the collagen binding domain of Fn was suggested to contain a binding site for BBK32 (Probert *et al.*, 1998).

B. burgdorferi contains a segmented genome that includes a small, 910 kb linear chromosome and as many as 23 circular and linear plasmids, ranging in size from 5 kb to 56 kb (Fraser *et al.*, 1997; Stevenson *et al.*, 1997; 1998; Casjens *et al.*, 2000; Miller *et al.*,

2000). Gene *bbk32* is located on the 36kb linear plasmid (lp36) of *B. burgdorferi* s.s. strain B31 (Fraser *et al.*, 1997). Genes carried by lp36 in B31 are often found on 24-29kbp linear plasmids in other isolates (Palmer *et al.*, 2000).

Besides its Fn-binding character, some studies suggested that BBK32 might also be a good antigen for the serological diagnosis of Lyme borreliosis (Heikkilä *et al.*, 2002; Lahdenne *et al.*, 2003; Panelius *et al.*, 2003; Lahdenne *et al.*, 2006).

The *bbk32* genes are located on linear plasmid and distributed among different *B. burgdorferi* strains. This suggests that these strains have acquired *bbk32* by horizontal transfer and benefit from the presence of *bbk32*. However, the role of *bbk32* for pathogenicities remains to be elucidated. As a first approach in this direction, I investigated (i) the presence and location of *bbk32* genes in a *B. burgdorferi* s.l. strain collection, (ii) the binding properties of the *bbk32*-encoding protein for Fn, and (iii) the serum antibodies response of patients suffering from Lyme borreliosis against recombinant BBK32. A positive antibody response would indicate that BBK32 is expressed during infection and that BBK32 could be used for serological diagnosis.

D. MATERIALS AND METHODS

1. Materials

1.1 Equipments

Centrifuge	Eppendorf centrifuge 5417C, Eppendorf, Hamburg, Germany Sigma centrifuge 4K15, Deisenhofen, Germany Sorvall® RC-5B Refrigerated Superspeed centrifuge, with Rotor: SS34, GSA, GS-3, DuPonts Inst. Kendro
Cycler	Gene Amp PCR System 2700, Applied Biosystems, Darmstadt, Germany
Electroblot apparatus	Semi-Dry Trans Blot Transfer Cell, BioRad, Munich, Germany
Electrode Assembly	Mini-Protean II Power-Pac 200, BioRad, Munich, Germany
Electrophoresis chamber	Horizontal Agarose gel electrophoresis cell, PeQLab, Erlangen, Germany
ELISA reader	SUNRISE, Tecan, Germany
Film Developing Machine	FUJIFILM FPM-100A, Japan
Freezer (−20 °C)	Profi line, Liebherr, Bulle, Switzerland
Freezer (−80 °C)	Forma Scientific, Inc., Marietta, Ohio, USA
French Press	SLM-AMINCO, Rating cell type, 40K, Spectronic Instruments
Gel Dryer	Model 583 Gel Dryer, BioRad, Munich, Germany
Heatblock	TR-L 288, Liebisch
Hybridization oven	Personal Hyb., Stratagene, Amsterdam
Incubator	Hanau Typ B20, Heraeus Instruments, Hanau, Germany
Magnetic stirrer	RCT basic, Ika Labortechnik, Staufen, Germany
Microwave	LG Electronics, Willich, Germany
Microscope	DIALUX 22, with phase contrast and darkfield, Leitz
Orbital shaker	OMV ROM, Fröbel Labortechnik, Lindau, Germany

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PFGE apparatus	CHEF-DR III, Bio-Rad, Munich, Germany
pH Meter	Accumet basic, Fisher Scientific, Schwerte, Germany
Photometer	Spectrophotometer Ultrospec 3000, Pharmacia Biotech
Pipettes	2µl, 10µl, 20µl, 100µl, 200µl and 1000µl Pipetman, Gilson, France 12-Channel 50-300µl Pipette, Labsystems Finnpitette
Scale	MC1 Laboratory LC 220S, Sartorius, Göttingen
Scanner	HP Scanjet 7450C Scanner
SDS-PAGE apparatus	PROTEAN II, Bio-Rad, Munich, Germany
Shaking incubator	Certomat BS-1, B. Braun Biotech International, Melsungen, Germany
Slot blot apparatus	Immunetics; Cambridge, MA
Sterile work bench	Hanau Herasafe HS12, Heraeus
Sterile Filter Units	Millipore
Thermomixer	Comfort, Eppendorf, Hamburg, Germany
Transilluminator	UVT-20M/W, Heralab, Wiesloch
UV-Crosslink	GATC-crosslink, Analysetechnique and Consulting Company, Konstanz, Germany
Vortex	Reax 2000, Heidolph, Schwabach
Water bath	WB/OB7-45, Memmert, Schwabach, Germany

1.2 Enzymes

AmpliTaq® Gold	Applied Biosystems, Darmstadt, Germany
Antarctic Phosphate*	New England Biolabs, Inc.
<i>Bam</i> H I *	Fermentas, Leon-Rot, Germany
Benzonase	Merck, Darmstadt, Germany
<i>Eco</i> R I *	Invitrogen, Karlsruhe, Germany
FastDigest™ <i>Bsa</i> H I *	Fermentas, Leon-Rot, Germany
FastDigest™ <i>Bst</i> F51*	Fermentas, Leon-Rot, Germany

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FastDigest™ <i>Hae</i> III*	Fermentas, Leon-Rot, Germany
Lysozyme	Roche, Karlsruhe, Germany
Proteinase K	Roche, Karlsruhe, Germany
T4 DNA-ligase *	Invitrogen, Karlsruhe, Germany
<i>Xho</i> I *	Fermentas, Leon-Rot, Germany

*Corresponding buffers of these enzymes were also offered by their manufacturers.

1.3 Molecular weight markers

CHEF DNA Size Standards (8~48kb)	Bio-Rad, Munich, Germany
MassRuler™ DNA Ladder (80-1031bp)	Fermentas, Leon-Rot, Germany
Prestained Protein Molecular Weight Marker	Fermentas, Leon-Rot, Germany

1.4 Kits

ELC Direct Nucleic Acid Labelling and Detection Systems kit	Amersham Biosciences, Freiburg, Germany
Glutathione Sepharose 4 Fast Flow	GE Healthcare, Munich, Germany
High Pure PCR Product Purification Kit	Roche, Karlsruhe, Germany
High Pure PCR Template Preparation Kit	Roche, Mannheim, Germany
High Pure Plasmid Isolation Kit	Roche, Karlsruhe, Germany

1.5 Vectors

Cloning vector pGEM®-T Easy Vector was purchased from Promega (USA). Expression vector was prepared from the recombinant plasmid pGEX-4T-Hq22 (Gao *et al.*, 2007), a generous gift from Prof. Dr. Yin Hong from the Lanzhou Veterinary Research Institute, Chinese Academy Agricultural Sciences.

1.6 Antibodies

Polyclonal Swine Anti-Rabbit IgG/ HRP	Dako Denmark
Polyclonal Rabbit Anti- <i>Escherichia coli</i>	Dako Denmark
Polyclonal Rabbit Anti-Human Fibronectin	Dako Denmark

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Polyclonal Rabbit Anti-Human IgG/HRP	Dako Denmark
Polyclonal Rabbit Anti-BBK32 (B31) antibody	Generated by Pineda Antibody Service, Berlin, Germany

1.7 Bacterial strains

1.7.1 *Escherichia coli*

JM109 Competent Cells	Promega, USA
BL21(DE3)pLysS strains	Promega, USA

1.7.2 *Borrelia burgdorferi* s.l. isolates

Borreliae were propagated in Modified Kelly Pettenkofer (MKP) medium as previously described (Preac-Mursic *et al.*, 1986), all the isolates used in this study were listed in Table D-1.

Table D-1. *Borrelia* species

Species	Strain	Biological source	Geographic origin	OspA type
<i>B. burgdorferi</i> s.s.	PKa2 K7/+40	CSF	Germany	1
	PKa2 23/+2	CSF	Germany	1
	PKa2 23/+16	CSF	Germany	1
	B31 27/+7	Tick	United States	1
	B31 27/+8	Tick	United States	1
	B31 K4/+5	Tick	United States	1
	N40 52/+10	Tick	United States	1
<i>B. garinii</i>	PBi 192/300	CSF	Germany	4
	PBi 109/63	CSF	Germany	4
	PBi 196/9	CSF	Germany	4
	PHei	CSF	Germany	5
<i>B. afzelii</i>	PKo	Skin (EM)	Germany	2
	PRui	Skin (ACA)	Germany	2

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	PBo	CSF	Germany	2
<i>B. spielmanii</i>	PMai	Skin	Germany	n.a.*
	PSig	Skin (EM)	Germany	n.a.
	PHaP	Skin (EM)	Germany	n.a.
	ISaue 2	Tick	Germany	n.a.
	DEbelJak	Skin (EM)	Slovenia	n.a.
	DSM	Tick	France	n.a.
	PC-Eq17 DSM	Tick	France	n.a.
	PMew	Skin	Germany	n.a.
	PAnz	Skin	Slovenia	n.a.
	PJes	Skin	Slovenia	n.a.

*n.a., not available.

1.8 Partially purified recombinant BBK32 proteins

Four partially purified recombinant BBK32 proteins from strains B31, PKa2, PHei and TN were available in the laboratory from previous studies (Heimerl, 2005).

1.9 Serum panels

Human serum samples were collected from well-defined clinically Lyme borreliosis patients. 24 with erythema migrans (EM), 40 with early neuroborreliosis (NB), and 8 with acrodermatitis chronica atrophicans (ACA). Controls comprised 20 sera from healthy blood donors, 10 sera from syphilis patients, 10 samples from patients who were positive for rheumatoid factor, and 30 sera from patients with fever of unknown origin.

1.10 Primers

All oligonucleotides used in the PCR reactions were synthesized by biomers.net GmbH. Primers used in this section are shown in Table D-2.

Table D-2. Oligonucleotides

Primer no.	Primer sequence (5'-3') ^a	Restriction enzyme sites
P1	CTGGATCCAGATATGAAATGAAAGAGGA	<i>Bam</i> H I

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P2 ^b	<u>CCACTCGAGTTCATCMTCTTCATCAT</u>	<i>Xho</i> I
P3	<u>CACTCGAGATAATCATCTTCATAATA</u>	<i>Xho</i> I
P4 ^c	<u>TACTCGAGGG</u> <u>C</u> <u>CGTCATCCCTTTATATTCAT</u>	<i>Xho</i> I , <i>Bsa</i> H I
P5	<u>TTGACGCCAAAGAGTTCCTTACAA</u>	<i>Bsa</i> H I
P6 ^b	<u>TAGGATCCMAAGGAAGTTTAAATTCCT</u>	<i>Bam</i> H I
P7	<u>ATCTCGAGCCTTAAATCAGAATCTATAGT</u>	<i>Xho</i> I
P8	<u>TAGGATCCCCAAAGAGTTCCTTACAA</u>	<i>Bam</i> H I
Forward P 1 ^b	<u>CTGGATCCTTATTCAAYAAGAKATGAAAT</u>	<i>Bam</i> H I
Reverse P 1 ^b	<u>CCC TCG AGT CTA GAT AAG ATT SAT ATC</u>	<i>Xho</i> I
Reverse P 2	<u>TACTCGAGTAGTACCAAACACCATTCTT</u>	<i>Xho</i> I
Forward P2 ^b	TTATTCAYAAGAKATGAAAT	
Reverse P3 ^b	TAACACCYTCTAGATAAGATT	

a. Restriction enzyme sites are underlined.

b. M could be A or C; Y could be T or C; K could be T or G; and S could be C or G.

c. The primer contains 2 restriction enzyme sites for different usage. A site-directed silent mutation was carried out in order to create *Bsa*H I site, where the Threonine code change from ACT to ACG. The mutated nucleotide was framed.

1.11 Chemicals and other materials

Acetic acid	Merck, Darmstadt, Germany
Acrylamid	Biozym, Hameln, Germany
Albumin Bovine, No. A-7409	Sigma, Deisenhofen, Germany
6-aminocaproic acid	Sigma, Deisenhofen, Germany
Ammonium persulphate (APS)	BioRad, Munich, Germany
Ampicillin	Biomol, Humberg, Germany
Ampuwa® (pyrogenfreies <i>Aqua dest.</i> H ₂ O _{dest})	Fresenius
Bacto™-Trypton	Becton Dickinson, Heidelberg, Germany
Bisacrylamid	Biozym, Hameln, Germany

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Bitek™-Agar	Difco, Detroit, USA
Boric acid	Merck, Darmstadt, Germany
Brij 58	Sigma, Deisenhofen, Germany
Bromphenol blue	BioRad, Munich, Germany
Calcium Chloride (CaCl ₂)	Merck, Darmstadt, Germany
Citric acid (Trisodium salt), No. C-7254	Sigma, Deisenhofen, Germany
CMRL-1066 (10x) without Glutamine, No. 042-1540	Gibco, Gaitersburg, USA
Coomassie Brilliant Blue R250	Sigma, Deisenhofen, Germany
Decorin (from bovine articular cartilage)	Sigma, Deisenhofen, Germany
Diaminobenzidine	Sigma, Deisenhofen, Germany
Dithiothreitol (DTT)	Biomol, Humberg, Germany
dNTP Mix	Perkin Elmer, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	BioRad, Munich, Germany
ethylenediaminetetraacetate sodium acid (EDTA)	Merck, Darmstadt, Germany
Gelatin, Art. 4070	Merck, Darmstadt, Germany
GenAmp®10×PCR Buffer	Applied Biosystems, Darmstadt, Germany
Glucose, No. G-8270	Sigma, Deisenhofen, Germany
Glycerin	Merck, Darmstadt, Germany
Glycine	Serva, Heidelberg, Germany
Hepes, No. H-3375	Sigma, Deisenhofen, Germany
Human plasma fibronectin	Gibco, Gaitersburg, USA
Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
Hydrogen peroxide (H ₂ O ₂) 30%,	Merck, Darmstadt, Germany
Incert agarose	Biozym, Hilden, Germany
Isopropanol	Merck, Darmstadt, Germany
Isopropyl- β -thiogalaktopyranosid (IPTG)	Sigma, Deisenhofen, Germany

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methanol	Merck, Darmstadt, Germany
N-acetylglucosamine, No. A-8625	Sigma, Deisenhofen, Germany
Neopeptone, No. 0119-01	Difco, Detroit, USA
N-lauroylsarcosine	Sigma, Deisenhofen, Germany
N, N-Dimethylformamide	Sigma, Deisenhofen, Germany
PMSF	Applichem, Darmstadt, Germany
Ponceau-S solution	Serva, Heidelberg, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Darmstadt, Germany
Potassium phosphate dibasic ($\text{K}_2\text{HPO}_4 \times 3 \text{H}_2\text{O}$)	Merck, Darmstadt, Germany
Protein Assay Dye Reagent Concentrate	BioRad, Munich, Germany
Pulsed Field Certified Agarose	Bio-Rad, Munich, Germany
Pyruvic acid (Sodium pyruvat), No. P-2256	Sigma, Deisenhofen, Germany
Rabbit Serum, No. 037-06120	Gibco, Gaitersburg. USA
RNAse A	Roche, Karlsruhe, Germany
Seakem LE agarose	BMA Rockland USA
SIGMA <i>FAST</i> TM OPD	Sigma, Deisenhofen, Germany
Skim milk powder	Glücksklee
Sodium acetate (NaAc)	Merck, Darmstadt Germany
Sodium bicarbonate (NaHCO_3), No.S-8875	Sigma, Deisenhofen, Germany
Sodium carbonate (Na_2CO_3)	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
Sodium deoxycholate	Serva, Heidelberg, Germany
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck, Darmstadt, Germany
Sodium dodecyl sulphate (SDS)	Sigma, Deisenhofen, Germany
Sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
Sodium lauryl sarcosine	Sigma, Deisenhofen, Germany
Sulfuric acid (H_2SO_4) 95-97%	Merck, Darmstadt, Germany
Tetramethylethylendiamin (TEMED)	Serva, Heidelberg, Germany
Thimerosal	AppliChem, Darmstadt, Germany

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Tris (hydroxymethyl) aminomethan (Tris)	Sigma, Deisenhofen, Germany
Triton X 100	Merck, Darmstadt, Germany
Tween® 20	Serva, Heidelberg, Germany
Type I collagen (from calf skin)	Sigma, Deisenhofen, Germany
Urea	Roth, Karlsruhe, Germany
X-Gal	Biomol, Humberg, Germany
Xylencyanol	Sigma, Deisenhofen, Germany

All chemicals used in this work had a degree of purity suitable *pro analysis*. Plastic and related articles were purchased from the following companies: Nunc, Roskilde, DK; Sartorius, Göttingen; Falco/Becton Dickinson, Heidelberg; B. Braun, Melsungen; Eppendorf, Hamburg; Greiner, Nürtingen and Schleicher & Schüll, Dassel. Hybond-N⁺ membranes were purchased from Serva, Heidelberg, NC membranes from PROTRAN, and Whatman-paper (3mm) from Whatman Ltd., Maidstone, England. Hyperfilms were purchased from Amersham Biosciences, England.

1.12 Culture media

Luria-Bertani (LB) broth	10g	Bacto tryptone
	5g	Yeast extract
	5g	NaCl
	Adjust to pH 7.4-7.6 with NaOH, and add H ₂ O _{dest} to 1 liter. Autoclave for 20 min at 121 °C.	
Luria-Bertani agar	10g	Bacto tryptone
	5g	Yeast extract
	5g	NaCl
	15g	Agar
Adjust to pH 7.4-7.6 with NaOH, and add H ₂ O to 1 liter. Autoclave for 20 min at 121 °C.		

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MKP-Medium

MKP-Medium was prepared following Table D-3 (Preac-Mursic *et al.*, 1986).

Table D-3. MKP-Medium

I	CMRL-1066 (10x) without Glutamine	150 ml
	Dist. water	1350 ml
	Neopeptone	4.5 g
	Hepes	9 g
	Citric acid	10.5 g
	Glucose	4.5 g
	Pyruvic acid	1.2 g
	N-acetylglucosamine	0.6 g
	Sodium Bicarbonate	3 g

Adjust to pH 7.6 with 5 N NaOH, sterilize the medium by filtration (0.2µm filter). The durability of the Basic Medium (I) lasts for 3 months at -20°C .

II	7 % Gelatine (autoclave: 115°C /15 Min)	300 ml
	Rabbit Serum - partially hemolyzed (inactivate: 56°C /30 min)	100 ml
	Albumin Bovine (35%)	100 ml

Fill 6.5 ml MKP-Medium into glass tubes (Schott Nr. 26 135 115), or plastic tubes (Falcon No. 2027) and close tightly. The durability of the final medium usually lasts for 3 weeks at 4°C . Incubate several tubes of the end Medium at 33°C for 3 days to check if there is contamination or not.

1.13 Softwares and databases

Microsoft Office 2000 (WORD, EXCEL, POWERPOINT)

Adobe Reader 8.0

PDFCreator

HP Precision Scan Pro3.02

Lasergene software package for Windows (DNASTAR, Madison, WI)

DNAMAN Version 5.2.9 software, Lynnon BioSoft, Canada

GenBank <http://www.ncbi.nlm.nih.gov/Genbank/index.html>

NCBI <http://www.ncbi.nlm.nih.gov/>

PubMed <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>

BLAST <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

ExPASy <http://www.expasy.org/>

2. Methods

2.1 Molecular biological methods

2.1.1 Genomic DNA isolation

Genomic DNA from *B. burgdorferi* s.s. strain B31, *B. garinii* strains PBi and PHei, and *B. afzelii* strain PKo was extracted with the High Pure PCR Template Preparation Kit according to the manufacturer's instructions. The final elution volume was 200 μ l.

2.1.2 PCR

PCR was performed in an automatic thermocycler in a 50 μ l reaction mixture containing:

Reaction components:	GenAmp®10 \times PCR buffer	5 μ l
	dNTP Mix (2mM)	8 μ l
	Forward primer (20 pmol/ μ l)	0.5 μ l
	Reverse primer (20 pmol/ μ l)	0.5 μ l
	AmpliTaq Gold™	0.5 μ l
	Template DNA	5 μ l
	H ₂ O	30.5 μ l
Cycling parameters:	Denaturation 95°C	3 min
	Denaturation* 95°C	1 min
	Annealing* T _m	1min
	Elongation* 72°C	1min

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Final extension 72°C

7min

* 30 cycles

T_m: Annealing temperature dependent on the T_m (melting temperature) of primers

2.1.3 Agarose gel electrophoresis

The agarose gels with different concentrations (1% to 2.5%) were prepared by mixing an appropriate proportion of agarose with 1 x TAE buffer, the mixture cooked and after cooling poured into precast agarose gel chambers. The DNA was then mixed with loading buffer, loaded onto spurs on the gel and electrophoretically resolved by voltage application utilizing the 1 x TAE solution as the running buffer. Following the electrophoretic run, gels were stained in ethidium bromide solution (1µg/ml) and the DNA visualized under ultraviolet radiation.

Solutions:

1x TAE buffer	40mM Tris / HCl, pH = 8.2
	20mM Acetic acid
	2mM EDTA, pH 7.6
Loading buffer for agarose gel	0.25 % (w/v) Bromophenol blue
	0.25 % (w/v) Xylencyanol
	30 % (w/v) Glycerin
	20 mM Na ₂ EDTA

2.1.4 Recovery of DNA fragments from agarose gel

Agarose gel electrophoresis separated target DNA fragments were cut from the gel under ultraviolet radiation and recovered using a High Pure PCR Product Purification Kit following the manufacturer's instructions.

2.1.5 Enzymatic modification of DNA

2.1.5.1 Restriction digestion of DNA

Restriction endonuclease digestion of chromosomal or plasmid DNA samples were

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performed as below:

***Bam*H I and *Xho*I double digestion**

DNA	X μ l
10X Buffer <i>Bam</i> HI	1 μ l
<i>Bam</i> HI	0.5 μ l
<i>Xho</i> I	1 μ l
H ₂ O	to 10 μ l*

***Eco*R I digestion**

DNA	X μ l
10X Buffer <i>Eco</i> RI	1 μ l
<i>Eco</i> RI	1 μ l
H ₂ O	to 10 μ l*

Incubate at 37°C for 1 or 2 h.

*The higher concentration of DNA, the more amount of H₂O.

FastDigest® restriction enzyme digestion

DNA	x μ l
10 \times FastDigest® buffer	1 μ l
Restriction enzyme*	1 μ l
H ₂ O	to 10 μ l**

Incubate at 37°C for 5min.

*the enzymes are FastDigest™ *Bsa* H I , FastDigest™ *Bst* F51 or FastDigest™ *Hae*III.

** The higher concentration of DNA, the more amount of H₂O.

2.1.5.2 Dephosphorylation of DNA

To prevent self-religation or dimerization of a restriction endonuclease linearized vector, its 5'-phosphate groups were removed with Antarctic Phosphatase (as shown below) following the manufacturer's instruction. The vector DNA is then free to ligate with an insert DNA of choice.

NE buffer for Antarctic Phosphatase	1 μ l
Antarctic Phosphatase	2 μ l
Linearized vector	7 μ l

Thoroughly but gently mixed and incubated at 37°C for 30 min, followed by heat inactivation at 65°C for 5 min.

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2.1.5.3 Ligation of DNA molecules

Ligation of an insert into arms of a vector or ligation of two DNA fragments to construct a longer linear DNA fragment was carried out with the enzyme T4 DNA ligase as described below:

Ligation of purified PCR products into pGEM®-T easy vector (TA cloning)

2× Rapid Ligation Buffer, T4 DNA Ligase	5µl
pGEM®-T Easy Vector (50ng)	1µl
PCR product	3µl
T4 DNA Ligase (3 Weiss units/µl)	1µl

Mix the reactions by pipetting and incubate at 4 °C overnight.

Ligation of DNA inserts into linearized pGEX vector

5 × T ₄ DNA ligase buffer	2µl
pGEX vector	2µl
Insert	5µl
T ₄ DNA ligase (1 Weiss units/µl)	1µl

Incubate at 16 °C overnight.

Ligation of two DNA fragments with similar sizes

5 × T ₄ DNA ligase buffer	2µl
Fragment 1	3.5µl
Fragment 2	3.5µl
T ₄ DNA ligase (1 Weiss units/µl)	1µl

Incubate at 16 °C overnight.

2.1.6 Preparation of *Escherichia coli* BL21 competent cells

Competent cells were prepared as described by Sambrook (2001). One single colony of *Escherichia (E.) coli* BL21(DE3)pLysS was inoculated into 5 ml LB broth and was incubated overnight at 37 °C and 200 rpm. On next day, the 5 ml overnight culture was inoculated into 250ml LB broth and incubated at 37 °C with vigorous shaking until an

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OD₆₀₀ of 0.6 was achieved (about 3h). The following steps were performed on ice. After being chilled on ice for 10 min, the culture was centrifuged at 4 °C and 4000 rpm (3220 rcf) for 10 min. All supernatants were discarded and the sediments were resuspended with 125 ml 0.1M CaCl₂ and again chilled on ice for 45 min. Chilled cells were centrifuged at 4 °C and 4000 rpm (3220 rcf) for 10 min and supernatants were discarded. The pellets were resuspended with 4 ml ice chilled 0.1M CaCl₂ and 1ml glycerol and 100 µl aliquots of the bacterial suspension was put into 1.5 ml Eppendorf tubes. All the aliquots were stored at –70°C till usage.

2.1.7 Bacterial transformation

2.1.7.1 Transformation into *E. coli* JM109

Ten-microliter of ligation product was gently mixed with 50µl *E. coli* JM109 competent cells and chilled on ice for 20min. After a 1 min heat-shock at exactly 42 °C, the transformation mixture were again chilled on ice for 3 min. LB broth at room temperature was added to the tubes containing cells transformed with ligation reactions to a final volume of 1 ml and incubated at 37 °C for 1.5 h with gentle shaking (~150 rpm). A hundred microliters of transformation culture was spread onto an LB agar plate containing 200 µg/ml of ampicillin (for pGEM-T easy vector, IPTG and x-gal were also required, see below). The rest 900µl culture was centrifuged at 3000 rpm for 3 min and its pellet was cultivated onto another LB agar plate. Plates were incubated at 37 °C overnight.

Preparation of LB/ampicillin/IPTG/X-gal agar plate:

Spread 17µl of 200mM IPTG and 40 µl of X-gal (20 mg/ml in 100 % N, N-Dimethylformamide) onto the surface of an LB agar plate (ø 9 cm) containing 200 µg/ml of ampicillin.

2.1.7.2 Transformation into *E. coli* BL21

One-microliter of isolated plasmid was gently mixed with 100µl *E. coli* BL21(DE3)pLysS competent cells. The following steps were the same as transformation of *E. coli* JM109 cells only with an exception of no IPTG and X-gal adding to the plates.

2.1.8 Recombinant plasmid purification

Three or five *E. coli* white colonies were picked from each plate and inoculated into LB broth (supplied with ampicillin) and cultivated overnight at 37 °C. Plasmids were isolated from the overnight culture by using the High Pure Plasmid Isolation Kit according to the manufacturer's instructions.

2.1.9 Confirmation of inserts

Isolated recombinant plasmids were first digested with *Bam*H I and *Xho* I or *Eco*R I. Plasmids conceiving inserts with expected sizes confirmed by restriction enzyme digestion were sequenced by using the SP6 and T7 primers by Qiagen Company.

2.1.10 Pulsed field gel electrophoresis (PFGE)

2.1.10.1 Preparation of Borrelia DNA in agarose blocks

Borrelia DNA was prepared after embedding the bacterial cells in agarose as described previously (Busch *et al.*, 1995) with some modifications. Cells were washed four times and resuspended in TN buffer (10mM Tris, pH 7.6, 1M NaCl) to approximately 1×10^9 cells/ml. Equal volumes of cells and 2% Incert agarose were mixed at 56°C. Cell-agarose suspension was filled into a mould and incubated at 4°C. The solidified agarose blocks were incubated in a lysis solution (1M NaCl, 20mM Tris-HCl, pH 8.0, 0.1M EDTA Na₂, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% N-lauroylsarcosine, 10µg/ml RNase A, 1 mg/ml chicken lysozyme) for 30 min at 4°C. The agarose blocks were washed with TE buffer (20mM Tris-HCl, pH8.0, 50mM EDTA Na₂) at 4°C for 1 h and digested with digestion buffer (0.5mg/ml Proteinase K, 10mM EDTA, pH8.0, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine) at 50°C for 72 h with gentle shaking. The agarose blocks were washed 3 times (1 h for each wash) with TE buffer at 4°C and stored at 4°C.

2.1.10.2 Gel electrophoresis

PFGE was done with a CHEF (contour-clamped homogeneous electric field), DR (dynamic regulated) III at 14°C in 0.5 X TBE (45mM Tris-borate, 1mM EDTA), with 6 V/cm and

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an electrode angle of 120° with buffer circulation. Half of an agarose block was placed into the sample well of a pre-cast 1% Pulsed Field Certified Agarose gel and sealed with 1% Incert agarose. Separation of plasmids was carried out with a pulse time of 3 s for 30 h. CHEF DNA Size Standards (8~48 kb) was used as a size ladder. The gel was stained with ethidium bromide (EtBr) and photographed under ultraviolet transillumination. For southern blot, placing a ruler near the marker was necessary.

10×TBE:

Tris base	108g
EDTA Na ₂	7.44g
Boric acid	55g

Add water to a final volume of 1 liter and pH 8.3 obtained.

2.1.11 Southern blot

After depurinization (in 0.25M HCl), denaturation (in 0.5M NaOH and 1.5M NaCl) and neutralization (in 0.5M Tris-HCl, pH 7.5, and 1.5M NaCl), DNA was transferred to a Hybond-N⁺ membrane by capillary force and UV cross-linked to the membrane with the GATC-crosslink.

2.1.12 Preparation of labeled probe

Purified PCR product was labelled with the ELC Direct Nucleic Acid Labeling reagent according to the manufacture's instructions. Briefly, PCR product (10 ng/μl) was boiled for 5 min and then chilled on ice for 5min. An equivalent volume of DNA labeling reagent was added to the cooled DNA and mixed gently but thoroughly. The glutaraldehyde solution (the same volume as that of the labeling reagent) was then added to the mixture and spun briefly in a microcentrifuge to collect the contents at the bottom of the tube. After incubation for 10min at 37°C, the mixture was again chilled on ice and mixed with equal volume of glycerol. The prepared probe can be store at -20°C till required.

2.1.13 DNA hybridization and detection

DNA hybridization and detection of the DNA probe were performed using ELC Direct

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Nucleic Acid Labelling and Detection Systems kit following the manufacturer's instructions. Briefly, three *bbk32* PCR fragments were labelled with horseradish peroxidase. Prehybridization (for 1h) and hybridization (overnight) were done in the hybridization buffer (with 0.5M NaCl and 5% [w/v] blocking agent) at 42°C. The three probes were mixed and used at a concentration of 10 ng/ml for each probe. Blots were then washed twice (20 min for each washing) with primary wash buffer (0.5×SSC, 6M Urea, 0.4% SDS) at 42°C and twice with secondary wash buffer (2×SSC) (5 min for each) at room temperature. To visualize positive signals, the blot was incubated for 1 min in a freshly prepared detection mixture of the ECL detection reagents and positive signals were exposed to a sheet of Hyperfilm ECL film at room temperature.

20×SSC:

Na ₃ citrate	0.3M
NaCl	3M
pH 7.0 (adjust with 1M HCl)	

2.2 Biochemical methods

2.2.1 IPTG induction for protein over expression

Three colonies were picked from each plate, inoculated into LB broth (containing 200 µg/ml ampicillin) and cultivated at 37°C overnight. Then 50µl overnight culture was cultivated at 37°C in 5 ml fresh LB broth (with ampicillin adding) with vigorous shaking (~230 rpm) for 3 hours. The culture was then provided with IPTG to a final concentration of 2 mM and incubated at 28°C for additional 5 h.

2.2.2 Sodium-dodecyl-sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were fractionated by sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) using a 12.5% resolving gel and 4% stacking gel (components see below). The electrophoresis system from Bio-Rad was employed in this work and the assembly of glass

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plates and spacers for the production of the gels was according to manufacturer's instructions. Before electrophoresis, protein samples were treated with protein loading buffer, boiled at 95°C for 5 min and loaded into the slots of the gel. The electrophoresis was run at 180 V until the bromophenol blue reached the edge of the gel in a Tris/glycine/SDS running buffer system (pH 8.3). Prestained Protein Molecular Weight Marker was used for the estimation of the molecular mass. Resolved proteins were either viewed directly by staining the gel with Coomassie brilliant blue or transferred to Nitrocellulose (NC) membrane and subjected to Western blot analysis or examined in a Fn-binding assay. Destained gels were dried using a Gel dryer for 2 h and scanned. All the related solutions and buffers were listed below.

25% Acrylamid/ Bis acrylamid (4°C)	40% Acrylamid	60.83ml
	2% Bisacrylamid	33.5ml
	H ₂ O	5.67ml
SDS separation gel (for 2 mini-gels)	H ₂ O	2.35ml
	1.5M Tris, pH8.8	2.5ml
	10% SDS	100µl
	25% Bis-Acrylamid	5.0ml
	10% APS	50µl
	TEMED	5µl
	Total volume	10ml
SDS stacking gel (for 2 mini-gels)	H ₂ O	2.89ml
	0.5M Tris, pH6.8	1.25ml
	10% SDS	50µl
	25% Bis-Acrylamid	0.78ml
	10% APS	25µl
	TEMED	5µl
Total volume	5ml	

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10×SDS-PAGE running buffer (1:10 dilution before usage)	Tris-base	30g
	Glycine	144g
	SDS	10g
	H ₂ O	till 1liter
2×Protein loading buffer (store at — 20°C)	H ₂ O	4.6ml
	0.5 M Tris-HCl pH 6.8	1.0ml
	Glycerin	0.8ml
	10% SDS	1.6ml
	Dithiothreitol (DDT)	8g
	Bromphenol blue	0.05% (w/v)
Gel fix solution	30% (v/v) Ethanol	
	10% (v/v) Acetic acid	
Coomassie Brilliant Blue solution	0.5% (w/v) Coomassie Brilliant Blue (R250)	
	10% (v/v) Acetic acid	
	45% (v/v) Methanol	
SDS-gel Destaining solution	10% (v/v) Acetic acid	
	25% (v/v) Methanol	

2.2.3 Western blot

Electrophoretic transfer of proteins from polyacrylamide gels to a NC membrane was performed using the Semi-Dry Trans Blot Transfer Cell. As shown in Fig. D-1, the blotting sandwich was prepared in the following order: from anodic pad to cathodic, 6 layers of filters soaked in anodic solution I (0.3 mol/L Tris buffer, 20% methanol [v/v], pH 10.5), 3 layers of filters soaked in anodic solution II (25 mM Tris buffer, 20% methanol, pH 10.4),

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anodic solution II moistened NC membrane, cathodic solution equilibrated SDS-PAGE gel and 6 layers of filters soaked in cathodic solution (40 mM 6-aminocaproic acid, 20% Methanol, pH 7.5). Electrophoresis was performed at 60 mA constant current for 45 min per mini-gel. Protein transfer was controlled by staining the membranes with Ponceau S solution. Detection of proteins in a NC membrane was accomplished by using antibodies. Briefly, unbound sites of the membrane were blocked in blocking solution (5% milk in TBST [10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20]) for 1 h at 37° C to suppress non-specific adsorption of antibodies. The membrane was then incubated overnight with first antibody (negative serum, rabbit anti-*E. coli* polyclonal serum or rabbit anti-BBK32_{B31} polyclonal serum) with a dilution of 1:750 at room temperature, followed by a 1:1.000 dilution of swine anti-rabbit IgG-horseradish peroxidase (HRP) conjugate for 2 hours. The dilution solution for both the first and the secondary antibody were TBST containing 1% milk. Between incubations, membranes were washed three times with TBST, 10 min for each washing. Positive signals were detected with 0.5mg/ml diaminobenzidine solution (see below) and the reaction was stopped by 1N sulfuric acid (H₂SO₄).

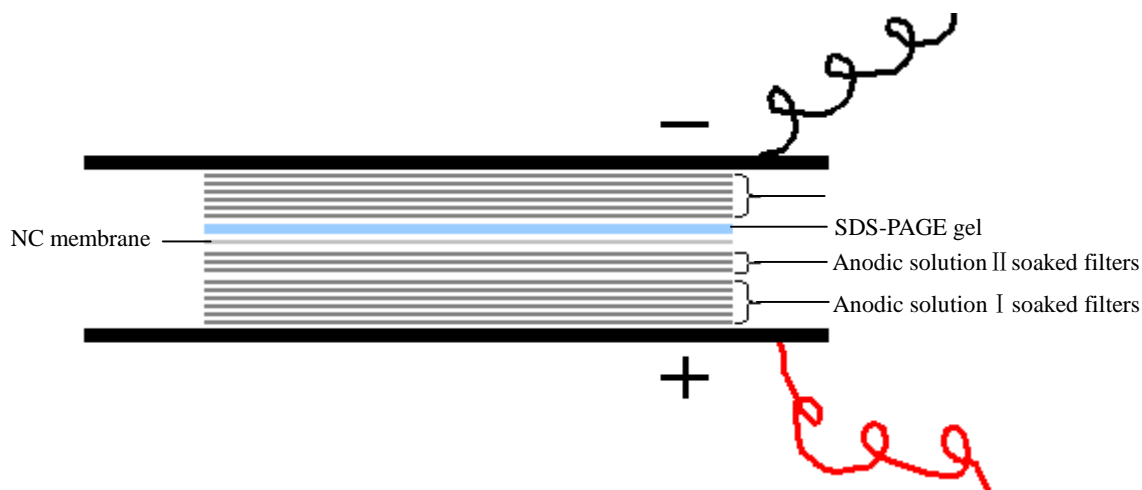


Fig. D-1. Diagram of preparing the gel sandwich

0.5 mg/ml diaminobenzidine solution

Diaminobenzidine	50mg
1M Tris pH 7.4	5ml
H ₂ O	95ml

Just before usage, add 50µl of 30% H₂O₂.

2.2.4 Purification of recombinant proteins

2.2.4.1 Cell extract preparation

About 30 ml overnight culture of *E. coli* BL21(DE3)pLysS expressing expected recombinant protein was inoculated into 500 ml LB broth under ampicillin selection and incubated at 37°C with shaking for 2-3 h till an OD₆₀₀ of 0.7-1.0 was achieved. The cells were then induced with 0.2mM IPTG for another 5 hours at 28°C. Cells were pelleted by centrifugation at 7.000 rpm for 10 min at 4°C, and the pellet were thoroughly resuspended in binding buffer (50 mM Tris-HCl pH 7.5, 0.3M NaCl, 1mM DTT and 0.1% Triton X-100) and brought to a final volume of 28ml. Then 10µl Benzonase and 280µl of 0.2 M PMSF were added to the suspension. The cell extracts were always kept on ice.

2.2.4.2 Lysis of *E. coli* by high pressure (French Press) homogenization

The suspended cells were broken at 1.000 psi by passing through a French Press cell for 3 times. Cell lysate was chilled on ice between runs. Following breakage, lysed samples were centrifuged at 15.000 rpm for 30 min at 4° C (Sorvall Rotor SS34) and the supernatant was ready for protein purification.

2.2.4.3 Batch purification

Supernatant containing recombinant proteins were transferred to 5 ml Glutathione Sepharose 4 Fast Flow beads balanced by Binding buffer beforehand and incubated on ice for 4 hours with gentle agitation. Unbound proteins in the supernatant after centrifugation at 500×g for 5 min were transferred to another freshly prepared beads for a second round of purification. After being washed with Binding buffer (50 mM Tris-HCl pH 7.5, 0.3M NaCl, 1mM DTT and 0.1% Triton X-100) for 3 times, proteins bound to beads were eluted with 10 ml Elution buffer (30mM glutathione, reduced [GSH], pH8.4) on ice for 2 hours. Supernatant containing the purified fusion protein was carefully harvested after centrifugation of the slurry at 500×g for 5 min.

2.2.4.4 Protein purification with polyacrylamide gel

Some proteins, after a first round of purification, were still not pure enough. Thus a second round of purification with polyacrylamide gel was also performed. Briefly, proteins from the first round purification were resolved on a 12.5 % SDS-PAGE gel and transferred to a NC membrane. After being stained with Ponceau S solution, target protein bands were cut from the membrane and eluted with nonvolatile solution (50mM Tris-HCl, pH9.0, 2% SDS [w/v], 1% Triton X-100 [v/v]) at room temperature for 1 hour with rotating. Eluted proteins were concentrated using Vivaspin Concentrators (GE Healthcare) following the manufacturer's instruction.

2.2.5 Protein concentration determination

Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as protein standard. Briefly, six dilutions of a protein standard BSA were prepared and 200 μ l of dye reagent concentrate was added to each tube containing 800 μ l of each standard and mixed well. After an incubation of 5 minutes at room temperature, absorbance at 595 nm of the protein-dye mixture was measured using a spectrophotometer set. A standard curve was thus prepared according to the standard concentrations and their absorbance at 595 nm. Concentrations of purified proteins were then determined by measuring absorbance at 595 nm after appropriate dilution.

20 \times PBS pH7.4

NaCl	78.85g
K ₂ HPO ₄ \times 3 H ₂ O	34.25g
KH ₂ PO ₄	3.9g

Solved to a final volume of 500ml with H₂O, 1:20 diluted before requirement.

2.3 Line assay

For the line assay, each recombinant antigen was individually dissolved in anodic solution II (25 mM Tris buffer, 20% Methanol, pH 10.4), and loaded into a separate line of a slot

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blot apparatus (Miniblotter 45, Immunetics; Cambridge, MA). Proteins were then allowed to bind in horizontal lines to the membrane for 3 hours at room temperature. Protein binding was assessed by staining membranes with Ponceau-S. After being destained with distilled water and blocked in blocking solution (5% milk in TBST), the membranes were cut vertically into 3- to 4-mm-wide strips and incubated overnight at room temperature with human sera diluted 1:200. Bound antibody was detected with horseradish peroxidase-labeled rabbit anti-human IgG secondary antibody. Between incubations, membranes were washed three times with TBST, 5 min for each washing. Positive signals were detected with 0.5mg/ml diaminobenzidine solution and the reaction was stopped by 1N sulfuric acid (H₂SO₄) (all the buffers were the same as in section 2.2.3).

2.4 Fn-binding assays

2.4.1 Western-ligand blot-based binding assay

Proteins were separated by SDS-PAGE gel and transferred to NC membranes. The membranes were incubated first with 5µg/ml human Fn, then with a 1:500 dilution of polyclonal rabbit anti-human Fn, and finally with a 1:1.000 dilution of polyclonal swine anti-rabbit immunoglobulins/ HRP conjugate (in TBST). Prior to the first incubation, membranes were blocked with 5% milk in TBST for 1 hour at 37° C. Between incubations, membranes were washed three times with 10ml TBST. Membranes were developed using 0.5mg/ml diaminobenzidine and stopped by 1N H₂SO₄.

2.4.2 Immunoblot-based inhibition assays

On the one hand, purified proteins (0.5 µg/lane) were separated by SDS-PAGE gel and electroblotted onto NC membrane. On the other hand, strips carrying the purified proteins (100 µg/ml) for line assay were prepared as described in section 2.3. Prior to incubation with Fn, the blots or strips were first incubated with increasing concentrations of either collagen, gelatin, BSA or decorin (in TBST buffer) for 1 h at 37° C. The following steps for binding assays were the same as in section 2.4.1.

2.4.3 ELISA-based binding assay

An enzyme-linked immunosorbent assay (ELISA)-based Fn-binding test was also performed. Briefly, Nunc 96-well microtitre plates were coated with the different purified recombinant proteins (0.01 or 0.05 $\mu\text{g}/\text{well}$) and incubated overnight at 4° C in 0.1 M carbonate buffer, pH 9.6. The plates were washed in washing buffer consisted of PBS containing 0.05% (v/v) Tween 20 (PBST) and then blocked for 1 h with 5% skimmed milk powder in 0.1 M carbonate buffer. After blocking, the plates were incubated first with human Fn (0.1 or 0.2 $\mu\text{g}/\text{ml}$ in PBST), then with a 1:800 dilution of polyclonal rabbit anti-human Fn, and finally with a 1:1.000 dilution of polyclonal swine anti-rabbit immunoglobulins/ HRP conjugate. Each incubation was performed at 37° C for 1 hour. Between incubations, the plates were thoroughly washed for 4 times with washing buffer. After a final wash, 200 μl of o-phenylenediamine dihydrochloride (SIGMA FAST™ OPD) substrate (to dissolve one OPD tablet and one urea hydrogen peroxide/buffer tablet in 20 ml of water to get a final concentration of 0.4 mg/ml OPD, 0.4 mg/ml urea hydrogen peroxide, and 0.05 M phosphate-citrate, pH 5.0) was added to each well and incubated in the dark for 30 min at room temperature. Reactions were then stopped by adding 50 μl of 3 M H_2SO_4 to each well and the absorbance was measured at 492 nm in an ELISA reader.

2.4.4 ELISA-based inhibition assay

To map the functional domain of Fn that interacts with BBK32 proteins, as described above, the microtitre plates were coated with purified proteins with an amount of 0.05 μg protein per well and blocked with skimmed milk powder solution. Before adding to the plates, human Fn (0.1 $\mu\text{g}/\text{ml}$) was incubated for 1h at 37° C with diluent only or increasing concentrations of either BSA, gelatin or collagen. After this incubation step, the Fn was added to the plates. The following steps for binding assays were the same as in section 2.4.3.

2.4.5 Statistical analysis

In ELISA based binding assays, for each concentration of proteins three independent

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experiments (each in duplicate) were performed. For each concentration of Fn, 3 assays in duplicate were conducted. In the ELISA based inhibition tests, for each concentration of gelatin, collagen or BSA, three independent assays (each in duplicate) were carried out. Average of the absorbance value from the duplicate wells in each independent experiment was calculated first. Later, mean and standard deviation (SD) of the three averages were calculated. The two sets of averages (each with 3 numbers) from two different proteins were analyzed using Student's *t*-test. Statistical significance was accepted when the *P*-values were less than 0.05.

2.5 Sequence analysis

BLAST-search analysis was used for the search of homologous sequences. Sequence alignment was carried out with the Lasergene software package for Windows (DNASTAR, Madison, WI) or with the DNAMAN version 5.2.9 software.

2.6 GenBank accession numbers

GenBank accession numbers of *bbk32* genes related to this study are listed in Table D-4.

Table D-4. GenBank accession numbers of *bbk32* genes

Species	Isolate	Accession number
<i>B. burgdorferi</i> s.s.	B31	NP_045605
	156a	YP_002477384
	IA	AF472532
	PKa II	FJ997275
<i>B. garinii</i>	PHei	FJ804148
	PBi	FJ997277
	TN	FJ997278
	50	AF472531
	40	AF472529
	46	AF472530
<i>B. afzelii</i>	PKo	FJ997276

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ACA-1	YP_002455579
570	AF472526
600	AF472528
1082	AF472527
A91	AF472525

E. RESULTS

1. Localization of the *bbk32* gene in the genomes of different *B. burgdorferi* s.l. strains

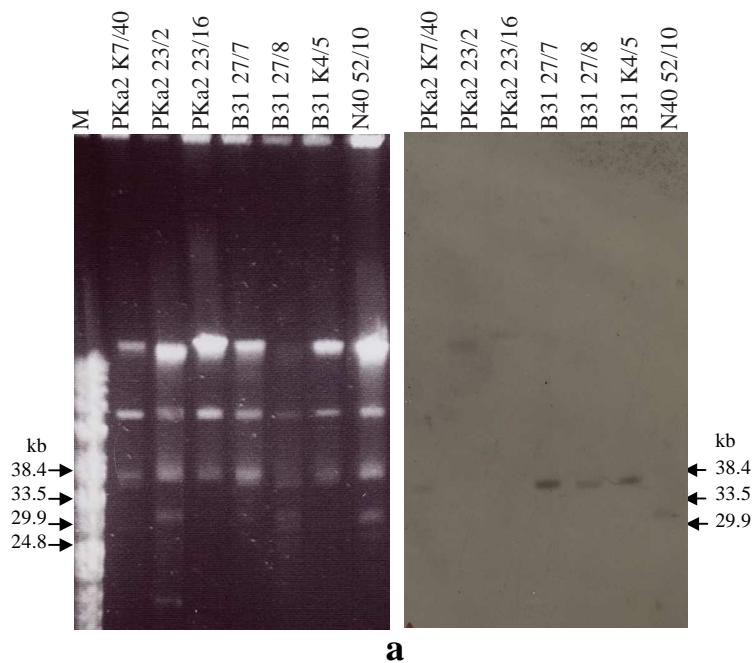
Borrelia burgdorferi s.s. contains a segmented genome that includes a small, 910 kb linear chromosome and as many as 23 circular and linear plasmids, ranging in size from 5 kb to 56 kb (Fraser *et al.*, 1997; Stevenson *et al.*, 1997; 1998; Casjens *et al.*, 2000; Miller *et al.*, 2000). The lp36 plasmid of strain B31 is a linear plasmid of approximately 36kb encoding 54 putative open reading frames (ORFs), seven of which appear to be pseudogenes (Fraser *et al.*, 1997; Casjens *et al.*, 2000). Of the few genes on lp36 that have a putative function (Casjens *et al.*, 2000), *bbk32* has been shown to encode a Fn-binding protein (Probert and Johnson, 1998; Probert *et al.*, 2001) that may be involved in *B. burgdorferi* s.s. mammalian infectivity (Li *et al.*, 2006; Seshu *et al.*, 2006). Former study suggested genes carried by lp36 in B31 are often found on 24-29kbp linear plasmids in other isolates (Palmer *et al.*, 2000). Here we described the plasmid profile of more than twenty isolates of four pathogenic borrelial species and the distribution of *bbk32* genes on these plasmids by PFGE and southern blot.

1.1 Probes for hybridization

To prepare *bbk32* probes, gene fragments of *bbk32* with sizes of around 580 bp were generated by PCR (Fig. E-1) amplification of genomic DNA isolated from *B. burgdorferi* s.s. B31, *B. garinii* PBi, and *B. afzelii* PKo with gene specific primer pairs (Forward P2 and Reverse P3) and confirmed by sequencing. Comparison of the 3 fragments was shown in Fig. E-2. The fragments of *bbk32* from those isolates shared identities from 82.6% to 92.6%. The three *bbk32* PCR fragments were then labeled with horseradish peroxidase and mixed at a concentration of 10 ng/ml for each probe to hybridize *bbk32* genes on the genome of a various of strains of *B. burgdorferi* s.l..

1.2 Localization of *bbk32* among strains

Genomic DNA of 23 isolates of *B. burgdorferi* s.l. were separated by Pulsed Field Gel Electrophoresis (PFGE) and transferred onto Hybond-N+ nylon membrane and *bbk32* genes were probed with the above prepared probes. Results in Fig. E-3 showed that the localizations of *bbk32* were quite various among different species, and even in one species, the localization was different from strains. As previously reported, *bbk32* was localized on the 36 kbp linear plasmid (lp36) of *B. burgdorferi* s.s. B31 (Fraser *et al.*, 1997) and the same pattern was seen in strain PKa2 (K7/40), however the localization of the gene was on a plasmid with a size of around 32kb in strain N40 (Fig. E-3, a). *bbk32* was localized to the 25kbp linear plasmid in both *B. garinii* (PBi) and *B. afzelii* (PKo). The gene was not detected in strains PRui and PBo (Fig. E-3, b and c), In *B. spielmanii*, signals were detected on plasmids with comparable sizes between 23kbp and 28kbp by using the *bbk32* probes. Hybridization signals were not obtained from strains PMai, PSig and DSM (Fig. E-3, d).



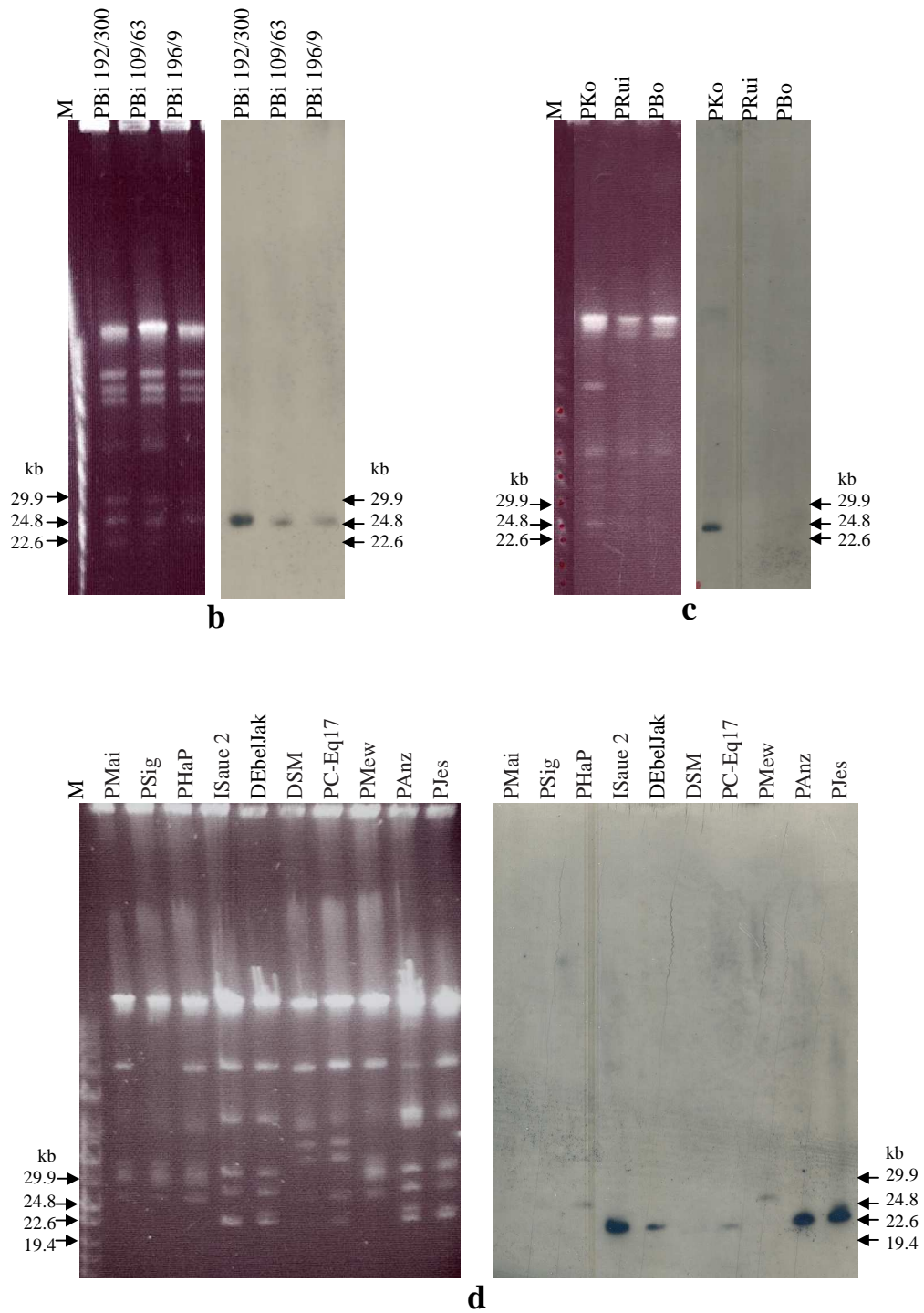


Fig. E-3. Detection of the *bbk32* gene in the genomes of *Borrelia burgdorferi* s.l. strains. DNAs from representative *B. burgdorferi* s.l. isolates (indicated above each lane) were separated by PFGE, the gel was stained with ethidium bromide (left panel in each group), and the DNA was transferred onto membranes for hybridization analysis with labeled *bbk32* probes (right panel in each group). a, *B. burgdorferi* s.s strains; b, *B. garinii* strains; c, *B. afzelii* strains; and d, *B. spielmanii* strains.

2. Comparative assay of Fn-binding capacities of BBK32 proteins from B31 and PHei

For many bacterial pathogens, attachment to their host tissues is believed to be a critical step during colonization and is typically mediated by adhesins, i.e., surface proteins that promote bacterial attachment to host cells (Finlay *et al.*, 1997; Joh *et al.*, 1999; Menzies, 2003). For *B. burgdorferi* s.l., their infectious processes require mobilization from the tick vector following a blood meal into a mammalian host where colonization occurs within the dermis (Duray, 1989; Asbrink *et al.*, 1990; Malane *et al.*, 1991). This location is rich in extracellular matrix (ECM) molecules such as Fn, collagen, and elastin (Cabello *et al.*, 2007; Coburn *et al.*, 2005). Following deposition in the dermis, Borreliae bind to host ligands from which they replicate and disseminate throughout the infected host. Most strikingly, it has become clear that the pathogens encode several adhesion molecules including the Fn-binding protein BBK32, the decorin-binding proteins DbpA and DbpB, the integrin-binding protein P66, and the glycosaminoglycan-binding protein Bgp (Coburn *et al.*, 1999; Guo *et al.*, 1998; Parveen *et al.*, 2000; Probert *et al.*, 1998). All these molecules might run a role in promoting the attachment of Borreliae to cell surfaces or to components of ECM, reinforcing the notion that adhesion is critical to the biology of *B. burgdorferi* and the pathogenesis of Lyme disease (Fig. E-4) (Coburn *et al.*, 2005).

The Fn-binding protein BBK32 was originally identified by Probert and Johnson (1998). The Fn-binding feature of the BBK32 and the biochemical mechanism of the binding have already been elucidated (Kim *et al.*, 2004; Probert *et al.*, 2001; Raibaud *et al.*, 2005). Overexpression of BBK32 protein in high-passage *B. burgdorferi* strain that lacks this protein enhances the organism's binding to Fn, as well as to glycosaminoglycans (Fischer *et al.*, 2006). Furthermore, inactivation of *bbk32* gene in infectious strains of *B. burgdorferi* reduced spirochetal binding to Fn, as well as its infectivity in mice (Seshu *et al.*, 2006), although the mutations had no apparent defect in tick vectors (Li *et al.*, 2006). These findings suggested that BBK32 might play an important role in the adhesion and invasion activities of *B. burgdorferi* to its host tissues.

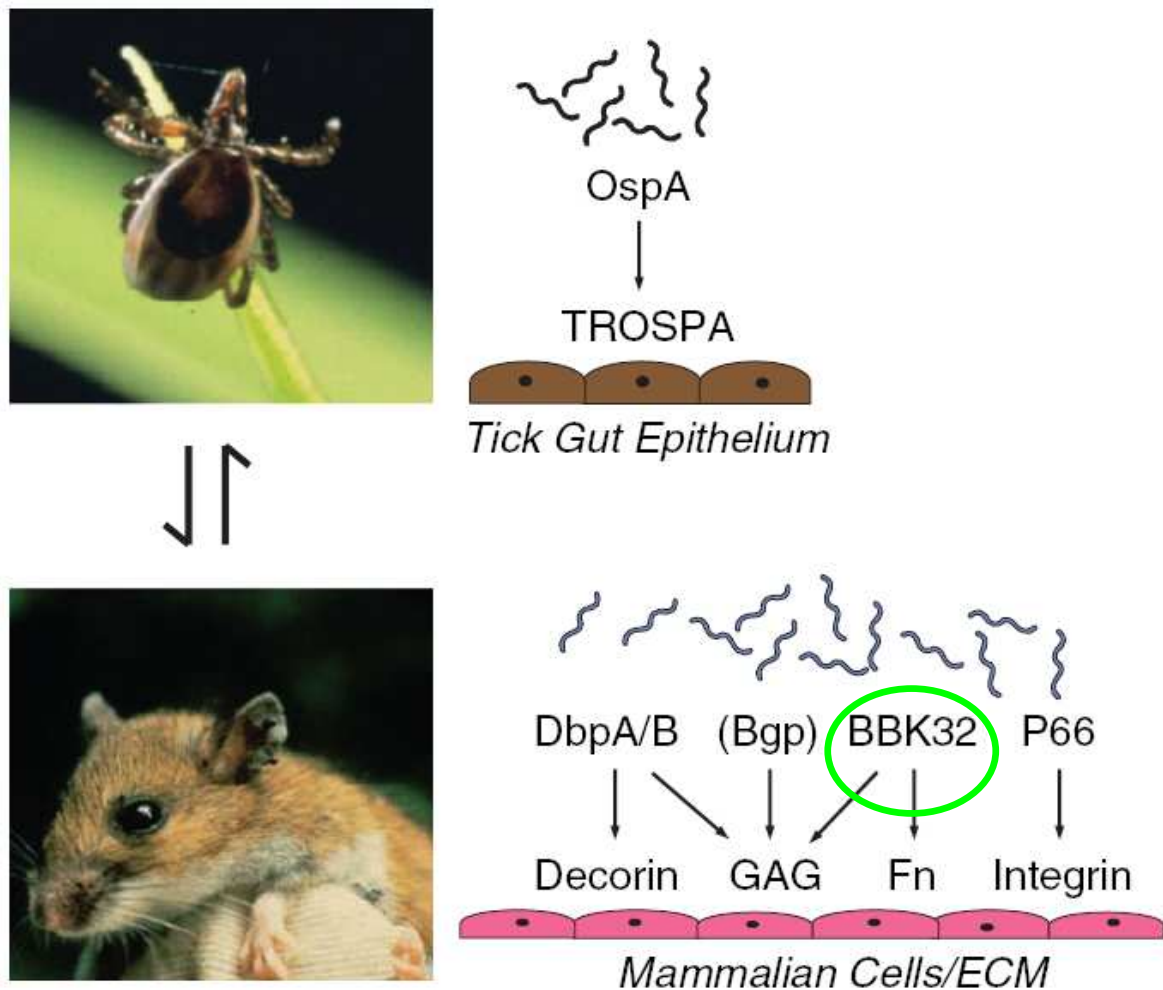


Fig. E-4. Summary of interactions between *B. burgdorferi* and host cells. Top: In the starved *Ixodes* tick, OspA promotes attachment of the spirochete to midgut epithelial cells by recognition of TROSPA on the epithelial cell surface. (Photo provided by the American Lyme Disease Foundation.) Bottom: In the north-eastern USA, the *Peromyscus* mouse is a natural host for *B. burgdorferi*. (Photo provided by S. Telford.) Attachment of the spirochete to mammalian cells can be mediated by DbpA or DbpB, which recognize decorin or GAGs, BBK32, which recognizes fibronectin or GAGs, or P66, which recognizes integrins. In addition, Bgp recognizes GAGs, however, an independent role for Bgp in cell attachment is not well established.

TROSPA: tick receptor for OspA; **GAG:** glycosaminoglycans; **Bgp:** *Borrelia* GAG-binding protein; **Fn:** Fibronectin

(Figure remodeled from Coburn *et al.*, 2005)

BBK32 consists of a C-terminal globular domain and an N-terminal region lacking well-defined secondary structure (Kim *et al.*, 2004). In prior studies, the Fn-binding activity of BBK32 was localized to a 32-amino acid-long segment within the unstructured domain. This ligand binding segment was shown to share 81–91% amino acid sequence

identity with the homologous proteins encoded by *bbk32* genes of *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* (Probert *et al.*, 2001). From the observation that gelatin could partially inhibit the binding of Fn to BBK32 and BBK32 could block the interaction of gelatin with Fn, it was speculated that the region of Fn bound by BBK32 overlapped with the Fn domain that binds gelatin and collagen (Probert *et al.*, 1998).

A former Ph.D student in our laboratory, Dr. Christiane Heimerl, found that the BBK32 of *B. garinii* isolate PHei possesses a stronger Fn-binding capacity than that of *B. burgdorferi* s.s B31 (C. Heimerl doctoral thesis, 2005) (Fig. E-5). In this section the molecular mechanism of the difference will be elucidated.

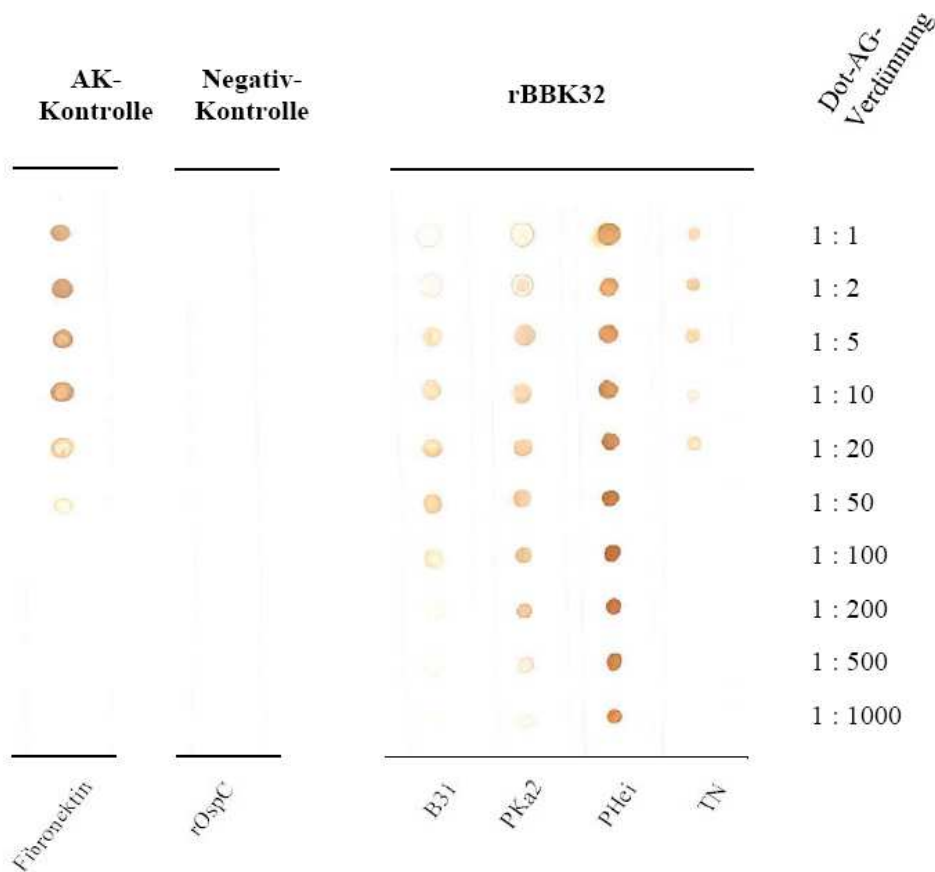


Fig. E-5. BBK32 (PHei) exhibited a stronger Fn-binding capacity than BBK32 (B31) (Result of C. Heimerl doctoral thesis, 2005)

2.1. *bbk32* gene fragment cloning and mutation construction

Former study indicated that the Fn-binding activity of BBK32 was localized to a 32-amino acid-long segment within the N-terminal unstructured region (Probert *et al.*, 2001). In this work, Fn-binding capacities of the N-terminal regions, and especially the 32-amino acid-long segments, of BBK32 proteins of PHei and B31 will be intensively investigated. Sequence alignment showed that 4 amino acids in this 32-amino acid-long segment were different between the two strains (Fig. E-6). Therefore, we suspected that the variation of the 4 amino acids might be responsible for the different Fn-binding capacities.

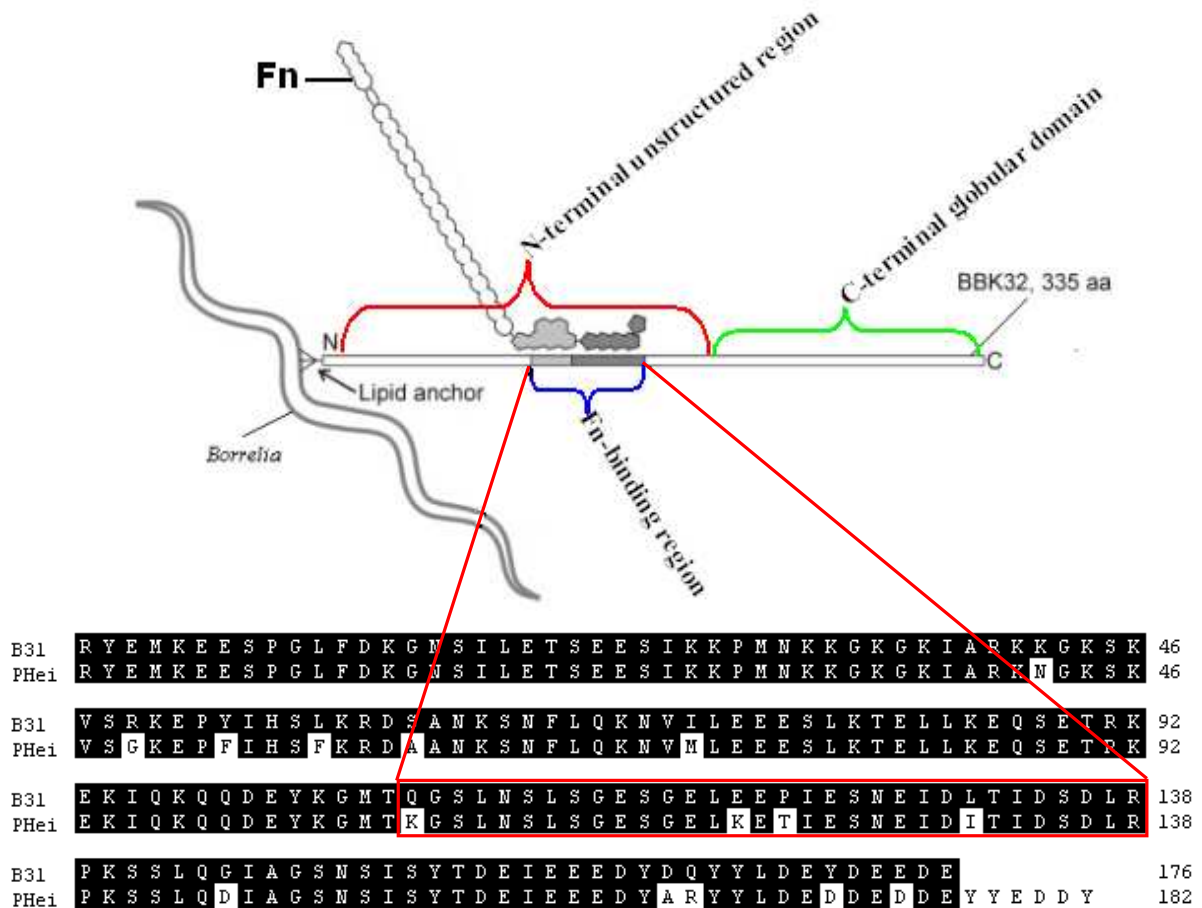


Fig. E-6. Schematic showing the arrangement of Fn binding sites in BBK32 from *B. burgdorferi* and BBK32 sequence comparison between B31 and PHei. The NTD/NTD-binding sites are shown in dark gray, and the GBD/GBD-binding sites are shown in light gray. The 32 amino acids responsible for the Fn binding activities of the proteins were framed. NTD: N-terminal domain; GBD: gelatin-binding domain. (The figure was partially modified from Rainbaud *et al.*, 2005)

In this case, the whole BBK32 N-terminal region of both B31 and PHei was produced as both 3 independent segments (including the 32-amino acid-long segment, segments before and after the 32 amino acids) or as a whole. Deletion mutations without the 32 amino acids and site-specific point mutations were also included. Compared with BBK32 from B31, there is a six-amino acid-insertion (YYEDDY) in BBK32 from PHei, a whole BBK32 N-terminal region of PHei including those additional 6 amino acids will be also prepared.

2.1.1 PCR amplification of *bbk32* fragments

Based on comparison of the BBK32 N-terminal domains of PHei and B31, eight primers were designed. The locations of the eight primers P1 to P8 at the compared *bbk32* sequences of strains B31 and PHei were illustrated in Fig. E-7. By using the primer pairs listed in Table E-1, totally nine *bbk32* gene segments with expected sizes were successfully generated by PCR amplification of genomic DNA isolated from B31 and PHei (Fig. E-8). Amplified PCR products were purified using a High Pure PCR product Kit and cloned into pGEM-T-easy vector and sequenced. Sequencing results showed that all the gene segments were successfully cloned. Detail sequence information were shown in appendix A and B.

Table E-1. Primer pairs and their targeted *bbk32* gene fragments for protein expression

Isolate	Primer pair	Target gene fragment
<i>B. burgdorferi</i> s.s. B31	P1 and P4	<i>bbk32</i> _{B31} (73-390)
	P6 and P7	<i>bbk32</i> _{B31} (391-486)
	P8 and P2	<i>bbk32</i> _{B31} (487-600)
	P1 and P2	<i>bbk32</i> _{B31} (73-600)

<i>B. garinii</i> PHei	P1 and P4	<i>bbk32</i> _{PHei} (73-390)
	P6 and P7	<i>bbk32</i> _{PHei} (391-486)
	P8 and P2	<i>bbk32</i> _{PHei} (487-600)
	P1 and P2	<i>bbk32</i> _{PHei} (73-600)
	P1 and P3	<i>bbk32</i> _{PHei} (73-618)

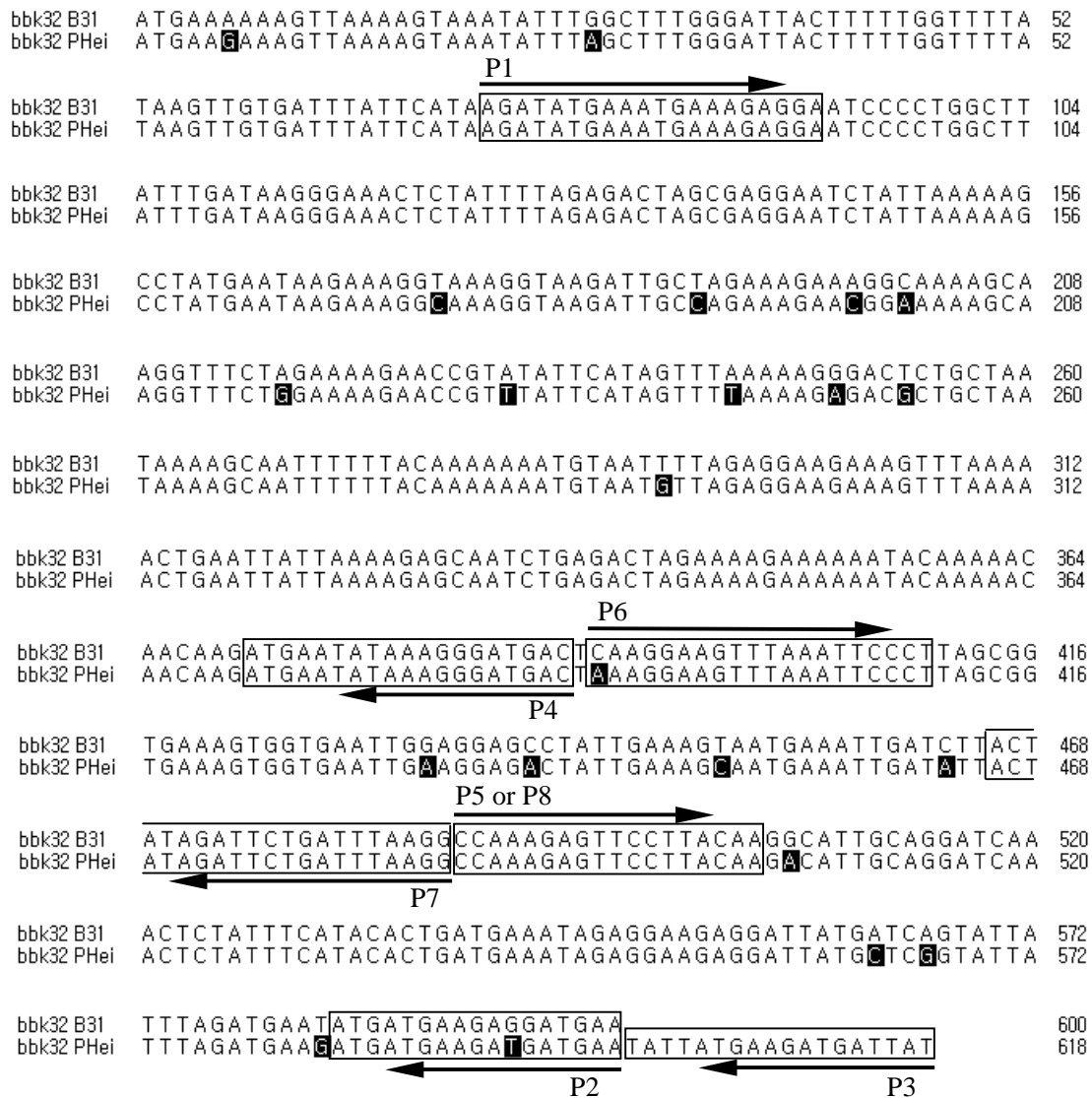


Fig. E-7. Locations of the 8 primers at the compared *bbk32* gene fragments encoding its N-terminal regions of *B. burgdorferi* s.s. strain B31 and *B. garinii* strain PHei. Positions of the corresponding primers were framed and labelled with arrows.

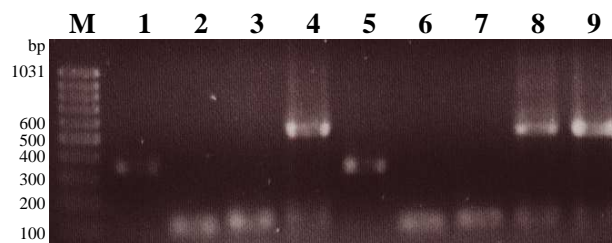


Fig. E-8. Agarose gel electrophoresis analysis of *bbk32* PCR products

PCR products were resolved through a 2.5% agarose gel. Lanes 1 to 9 were *bbk32*_{B31} (73-390), *bbk32*_{B31} (391-486), *bbk32*_{B31} (487-600), *bbk32*_{B31} (73-600), *bbk32*_{PHei} (73-390), *bbk32*_{PHei} (391-486), *bbk32*_{PHei} (487-600), *bbk32*_{PHei} (73-600) and *bbk32*_{PHei} (73-618), respectively. Lane M was the DNA standard ladder.

2.1.2 Construction of the *bbk32* deletion mutations

The strategy of construction of *bbk32* deletion mutations was shown in Fig.E-9. Firstly, four gene segments of *bbk32* from position 73 to 390 and from 487 to 600 were amplified from genomic DNA of strain B31 and PHei, respectively, using the two pairs of primers (P1 and P4; and, P5 and P2) in Table E-2. Amplified PCR products were purified using a High Pure PCR product Kit. After being digested with *BsaH I*, the two segments were ligated together. The ligation product was PCR amplified with primer pairs P1 and P2. Sizes of the amplified PCR products and the ligation product (PCR amplified) were evaluated on an agarose gel as shown in Fig.E-10. The ligation product was cloned into pGEM-T-easy vector and sequenced. Sequencing results showed that nucleotides from position 391 to 486 were successfully deleted from the wild type genes. The two deletion mutations were designated *bbk32*_{PHei} Δ₍₃₉₁₋₄₈₆₎ and *bbk32*_{B31} Δ₍₃₉₁₋₄₈₆₎, respectively. Their sequences were shown in **appendix A and B**.

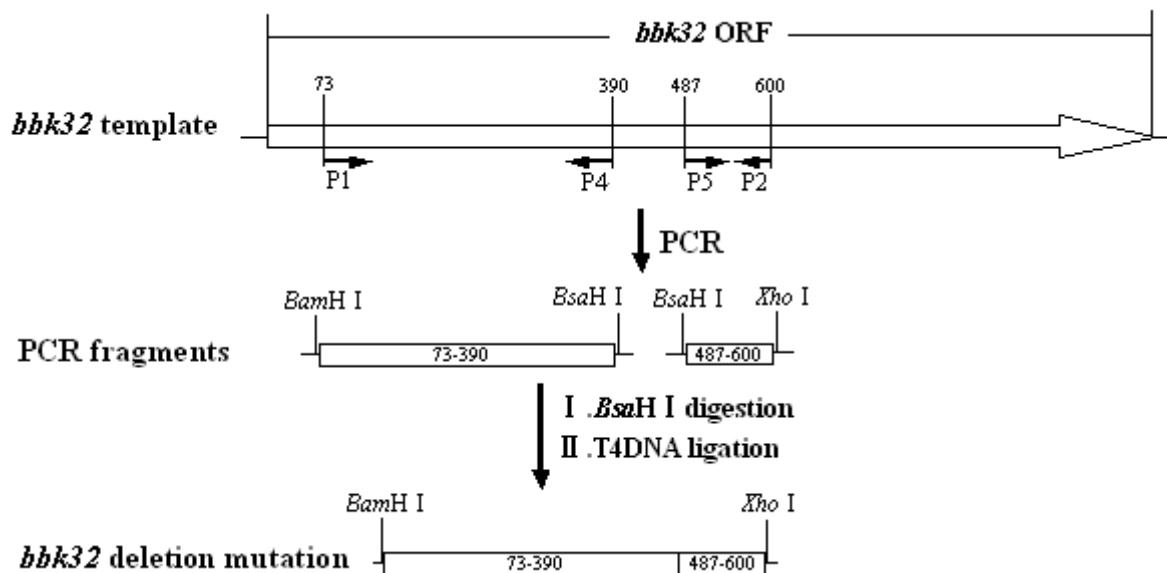


Fig. E-9. A schematic presentation of construction of *bbk32* deletion mutations

DNA fragments before and after nucleotide position 391 to 486 were PCR amplified and ligated to construct deletion mutation *bbk32*_{PHei} Δ₍₃₉₁₋₄₈₆₎ and *bbk32*_{B31} Δ₍₃₉₁₋₄₈₆₎.

Table E-2. Primer pairs and targeted products for deletion mutation construction

Isolate	Primer pair	Target gene fragment
<i>B. garinii</i> PHei	P1 and P4	<i>bbk32</i> _{PHei} (73-390)
	P5 and P2	<i>bbk32</i> _{PHei} (487-600)
<i>B. burgdorferi</i> s.s. B31	P1 and P4	<i>bbk32</i> _{B31} (73-390)
	P5 and P2	<i>bbk32</i> _{B31} (487-600)

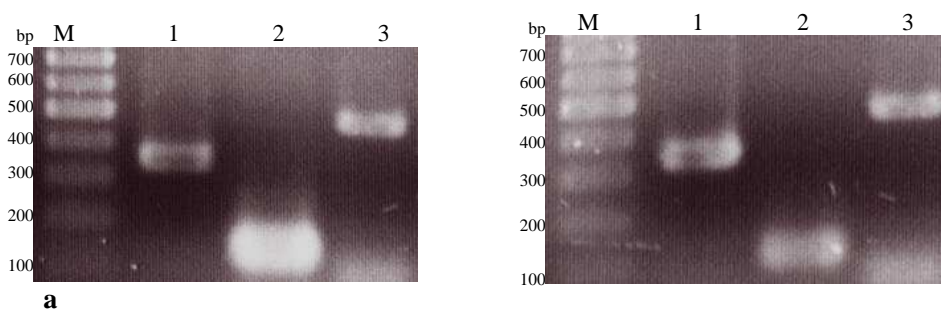


Fig. E-10. Agarose gel electrophoresis analysis of PCR products for construction of *bbk32* deletion mutations and their ligation products. a. gene fragments from PHei and b. gene fragments from B31. M. DNA standard ladder; Lane 1. *bbk32*₍₇₃₋₃₉₀₎, lane 2. *bbk32*₍₄₈₇₋₆₀₀₎ and lane 3. *bbk32* Δ₍₃₉₁₋₄₈₆₎.

2.1.3 Construction of site-specific point mutations

A strategy as shown in Fig.E-11 was used to construct the site-specific point mutations. Purified PCR products *bbk32*_{B31 (73-600)} and *bbk32*_{PHei (73-600)} (Table E-1) were digested with FastDigest™ *Hae*III and thus each of the DNA fragments was cleaved into two fragments with different lengths. The shorter digestion segment from B31 was ligated to the longer part of PHei, while the shorter one from PHei was joined to the longer one of B31. The ligation products were then followed a digestion of FastDigest™ *Bst* F51 and the generated DNA fragments were exchanged in the same way. To guarantee ligation of two fragments with their right direction (ligation happens at the ends with the same restriction site of the two fragments), ligated products were always PCR amplified with a primer pair (P1 and P2) after each ligation and the amplified PCR products were cloned into pGEM-T easy vector and confirmed by sequencing. Sequencing results showed that nucleotides from position 390 to 486 were successfully exchanged between fragments *bbk32*_{B31 (73-600)} and *bbk32*_{PHei (73-600)} and thus they had the substituted nucleotide sequences of (391A, 433A, 439A, 450C and 463A), and (391C, 433G, 439C, 450T and 463C), respectively (see appendix A and B). The produced 2 mutations were named *bbk32*_{B31 (73-389) PHei (390-486) B31 (487-600)} and *bbk32*_{PHei}

(73-389) B31 (390-486) PHei (487-600), respectively. Hereafter, these two mutations in *bbk32* were denoted as *bbk32*_{B31(73-600)}^{mut} and *bbk32*_{PHei (73-600)}^{mut}, respectively. The encoded corresponding polypeptides of the two mutations were designated BBK32_{B31 (25-200)}^{mut} and BBK32_{PHei (25-200)}^{mut}, respectively. The deduced amino acid sequence of the BBK32_{B31 (25-200)}^{mut} had substituted amino acids of lysine₁₃₁, lysine₁₄₅, threonine₁₄₇ and isoleucine₁₅₅, while that of BBK32_{PHei (25-200)}^{mut} had substituted ones of glutamine₁₃₁, glutamic acid₁₄₅, proline₁₄₇ and leucine₁₅₅ (Fig. E-13).

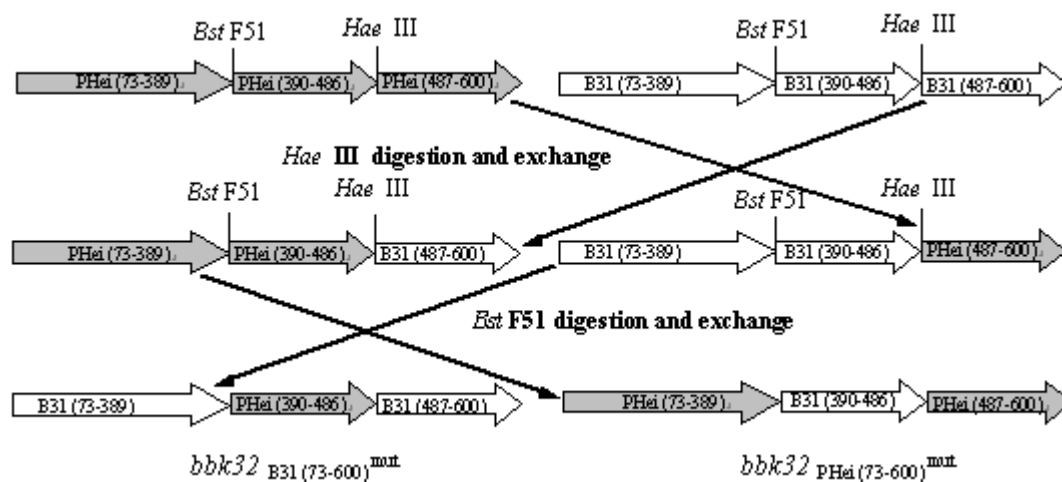


Fig. E-11. A schematic presentation of construction of *bbk32* site-specific point mutations

Nucleotides from position 390 to 486 of wild type fragments *bbk32*_{B31 (73-600)} and *bbk32*_{PHei (73-600)} were exchanged between the two fragments by restriction enzyme treatment and ligation to produce gene segments *bbk32*_{B31 (73-600)}^{mut} and *bbk32*_{PHei (73-600)}^{mut}, respectively.

2.2 Construction of expression plasmids

The 13 *bbk32* gene segments (Table E-3) generated in section 2.1 were cleaved from recombinant cloning plasmids, pGEM-T easy vector conceiving *bbk32* segments, by *BamH* I and *Xho* I double digestion. Obtained *bbk32* fragments were introduced into the *BamH* I / *Xho* I restriction sites of the linearized expression vector pGEX by T₄DNA ligase to construct expression plasmids pGEX-*bbk32* (*bbk32*, any one of the 13 fragments) (Fig.E-12). Inserts of the constructed expression plasmids were confirmed by sequencing. Results showed that all the 13 *bbk32* fragments were successfully inserted into the vectors. The deduced amino acid sequences of 13 *bbk32* fragments were shown in Fig.E-13.

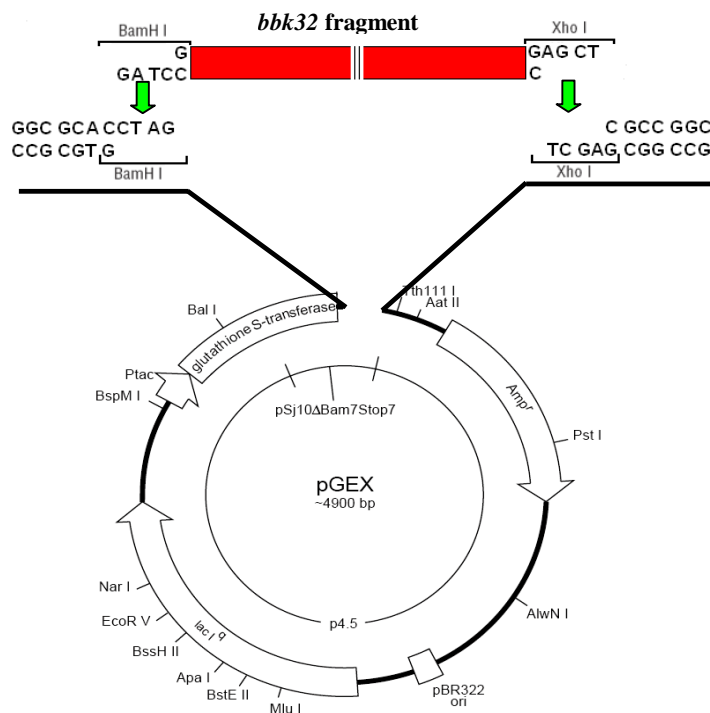


Fig. E-12. A scheme of introduction of *bbk32* gene fragments into the restriction site of the linearized pGEX vector. The red bar indicates any one of the 13 *bbk32* fragments.

Table E-3. *bbk32* gene fragments and their corresponding polypeptides

Gene segments	Polypeptide names	Predicted MW (kDa)*
<i>bbk32</i> B31 (73-600)	BBK32 B31 (25-200)	47
<i>bbk32</i> B31 (73-600) ^{mut}	BBK32 B31 (25-200) ^{mut}	47
<i>bbk32</i> B31 Δ (391-486)	BBK32 B31 Δ (131-162)	43
<i>bbk32</i> B31 (73-390)	BBK32 B31 (25-130)	39
<i>bbk32</i> B31 (391-486)	BBK32 B31 (131-162)	30
<i>bbk32</i> B31 (487-600)	BBK32 B31 (163-200)	31
<i>bbk32</i> PHei (73-618)	BBK32 PHei (25-206)	47
<i>bbk32</i> PHei (73-600)	BBK32 PHei (25-200)	46
<i>bbk32</i> PHei (73-600) ^{mut}	BBK32 PHei (25-200) ^{mut}	46
<i>bbk32</i> PHei Δ (391-486)	BBK32 PHei Δ (131-162)	43
<i>bbk32</i> PHei (73-390)	BBK32 PHei (25-130)	39
<i>bbk32</i> PHei (391-486)	BBK32 PHei (131-162)	20
<i>bbk32</i> PHei (487-600)	BBK32 PHei (163-200)	31

* Molecular masses here referred to those of GST-BBK32 fusion proteins, the vector expressing GST protein's MW 26kDa was added to MWs for every polypeptides.

BBK32 PHei(25-206)	RYEMKEESPGLFDKGNISILETSEESI KKP MNKKGKGKI ARKNGKSK	46
BBK32 PHei(25-200)	RYEMKEESPGLFDKGNISILETSEESI KKP MNKKGKGKI ARKNGKSK	46
BBK32 B31(25-200)	RYEMKEESPGLFDKGNISILETSEESI KKP MNKKGKGKI ARKNGKSK	46
BBK32 PHei(25-200) ^{mut}	RYEMKEESPGLFDKGNISILETSEESI KKP MNKKGKGKI ARKNGKSK	46
BBK32 B31(25-200) ^{mut}	RYEMKEESPGLFDKGNISILETSEESI KKP MNKKGKGKI ARKNGKSK	46
BBK32 PHeiΔ(391-486)	RYEMKEESPGLFDKGNISILETSEESI KKP MNKKGKGKI ARKNGKSK	46
BBK32 B31Δ(391-486)	RYEMKEESPGLFDKGNISILETSEESI KKP MNKKGKGKI ARKNGKSK	46
BBK32 PHei(25-130)	RYEMKEESPGLFDKGNISILETSEESI KKP MNKKGKGKI ARKNGKSK	46
BBK32 B31(25-130)	RYEMKEESPGLFDKGNISILETSEESI KKP MNKKGKGKI ARKNGKSK	46
BBK32 PHei(131-162)	-----	0
BBK32 B31(131-162)	-----	0
BBK32 PHei(163-200)	-----	0
BBK32 B31(163-200)	-----	0
BBK32 PHei(25-206)	VS ^G GKEPFI HSF ^F KRD AANKSNFLQKNVM LEEESLKT ^L TELLKEQSETRK	92
BBK32 PHei(25-200)	VS ^G GKEPFI HSF ^F KRD AANKSNFLQKNVM LEEESLKT ^L TELLKEQSETRK	92
BBK32 B31(25-200)	VSRKEPYI HSLK ^R DSANKSNFLQKNVI LEEESLKT ^L TELLKEQSETRK	92
BBK32 PHei(25-200) ^{mut}	VS ^G GKEPFI HSF ^F KRD AANKSNFLQKNVM LEEESLKT ^L TELLKEQSETRK	92
BBK32 B31(25-200) ^{mut}	VSRKEPYI HSLK ^R DSANKSNFLQKNVI LEEESLKT ^L TELLKEQSETRK	92
BBK32 PHeiΔ(391-486)	VS ^G GKEPFI HSF ^F KRD AANKSNFLQKNVM LEEESLKT ^L TELLKEQSETRK	92
BBK32 B31Δ(391-486)	VSRKEPYI HSLK ^R DSANKSNFLQKNVI LEEESLKT ^L TELLKEQSETRK	92
BBK32 PHei(25-130)	VS ^G GKEPFI HSF ^F KRD AANKSNFLQKNVM LEEESLKT ^L TELLKEQSETRK	92
BBK32 B31(25-130)	VSRKEPYI HSLK ^R DSANKSNFLQKNVI LEEESLKT ^L TELLKEQSETRK	92
BBK32 PHei(131-162)	-----	0
BBK32 B31(131-162)	-----	0
BBK32 PHei(163-200)	-----	0
BBK32 B31(163-200)	-----	0
BBK32 PHei(25-206)	EKI QKQQDEYKGMT KGSLNSLSGESGELKET ESNEIDITIDSDLR	138
BBK32 PHei(25-200)	EKI QKQQDEYKGMT KGSLNSLSGESGELKET ESNEIDITIDSDLR	138
BBK32 B31(25-200)	EKI QKQQDEYKGMT QGSLNSLSGESGEL ^E EPI ESNEIDLTI DSDLR	138
BBK32 PHei(25-200) ^{mut}	EKI QKQQDEYKGMT QGSLNSLSGESGEL ^E EPI ESNEIDLTI DSDLR	138
BBK32 B31(25-200) ^{mut}	EKI QKQQDEYKGMT KGSLNSLSGESGELKET ESNEIDITIDSDLR	138
BBK32 PHeiΔ(391-486)	EKI QKQQDEYKGMT -----	106
BBK32 B31Δ(391-486)	EKI QKQQDEYKGMT -----	106
BBK32 PHei(25-130)	EKI QKQQDEYKGMT -----	106
BBK32 B31(25-130)	EKI QKQQDEYKGMT -----	106
BBK32 PHei(131-162)	----- KGSLNSLSGESGELKET ESNEIDITIDSDLR	32
BBK32 B31(131-162)	----- QGSLNSLSGESGEL ^E EPI ESNEIDLTI DSDLR	32
BBK32 PHei(163-200)	-----	0
BBK32 B31(163-200)	-----	0
BBK32 PHei(25-206)	PKSSLQD AGSNSI SYTDEI EEEDYARYYLDEDD ^D EDDEYYEDDY	182
BBK32 PHei(25-200)	PKSSLQD AGSNSI SYTDEI EEEDYARYYLDEDD ^D EDDE	176
BBK32 B31(25-200)	PKSSLQGI AGSNSI SYTDEI EEEDYDQYYLDEYDEEDE	176
BBK32 PHei(25-200) ^{mut}	PKSSLQD AGSNSI SYTDEI EEEDYARYYLDEDD ^D EDDE	176
BBK32 B31(25-200) ^{mut}	PKSSLQGI AGSNSI SYTDEI EEEDYDQYYLDEYDEEDE	176
BBK32 PHeiΔ(391-486)	PKSSLQD AGSNSI SYTDEI EEEDYARYYLDEDD ^D EDDE	144
BBK32 B31Δ(391-486)	PKSSLQGI AGSNSI SYTDEI EEEDYDQYYLDEYDEEDE	144
BBK32 PHei(25-130)	-----	106
BBK32 B31(25-130)	-----	106
BBK32 PHei(131-162)	-----	32
BBK32 B31(131-162)	-----	32
BBK32 PHei(163-200)	PKSSLQD AGSNSI SYTDEI EEEDYARYYLDEDD ^D EDDE	38
BBK32 B31(163-200)	PKSSLQGI AGSNSI SYTDEI EEEDYDQYYLDEYDEEDE	38

Fig. E-13. Comparison of the deduced amino acid sequences of BBK32 polypeptides.

Protein fragments of the wild type BBK32 from both B31 and PHei and the generated mutations were compared using the ClustalV method in MegAlign component of the Lasergene software package for Windows (DNASTAR, Madison, WI).

2.3 Protein expression in *E. coli* and detection

The constructed expression plasmids, pGEX inserted by *bbk32* fragments, were transformed into BL21(DE3)pLysS competent cells and protein expression was then induced with IPTG. *E. coli* expressing BBK32 recombinant polypeptides were lysed and

analyzed by SDS-PAGE electrophoresis. Results showed that all the recombinant proteins fused with GST were successfully expressed with their expected sizes. A control protein, a 28-kDa GST, was also produced from the expression vector, following induction with IPTG (Fig. E-14, top panel).

To confirm their borrelial origin of the observed recombinant proteins in SDS-PAGE gels, lysate of recombinant *E. coli* expressing different protein fragments of BBK32 N-terminal regions of strain B31 and PHei were transferred to NC membranes for Western blot

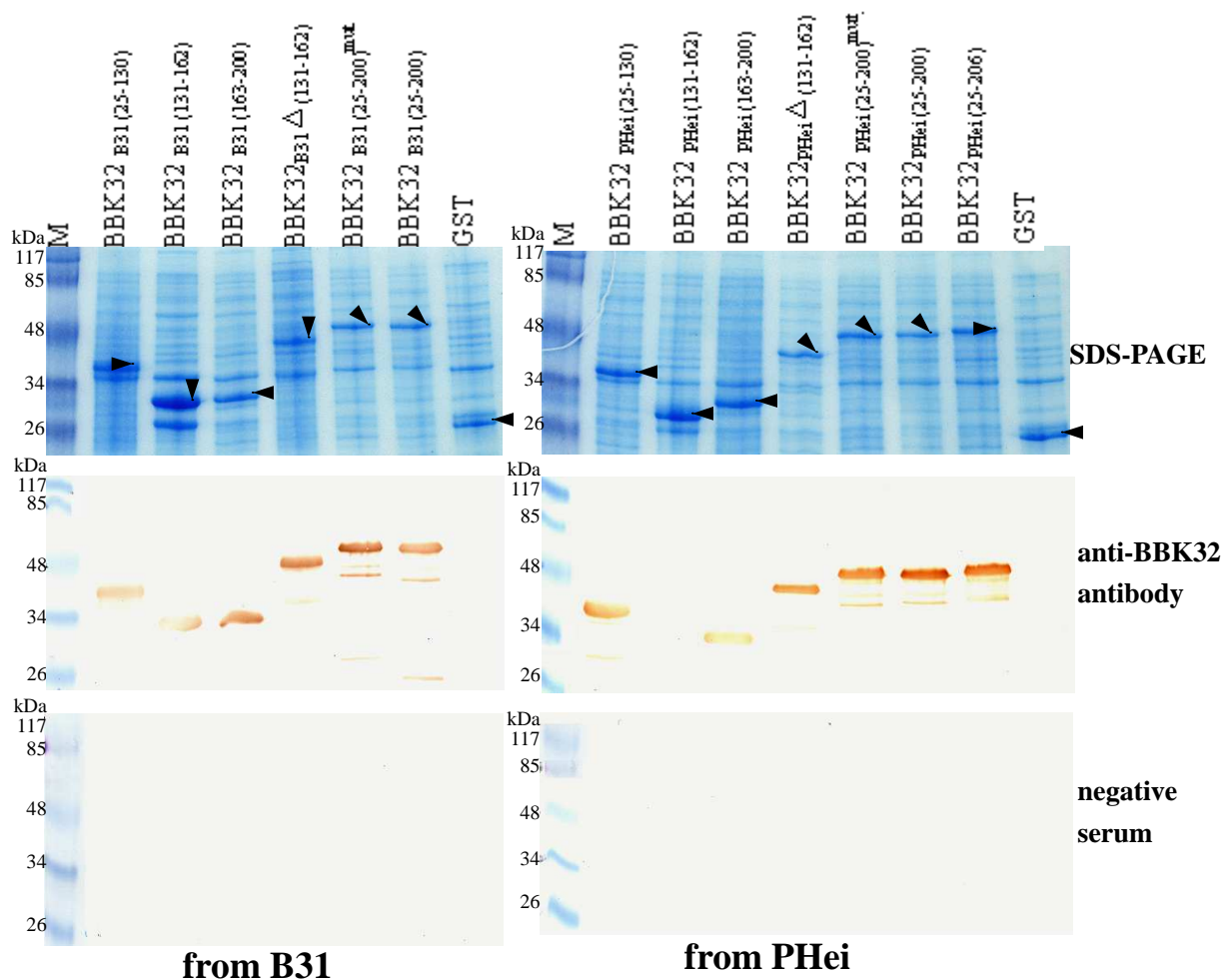


Fig. E-14. SDS-PAGE and Western blot analysis of BBK32-GST fusion proteins

Recombinant proteins were separated by SDS-PAGE and resolved proteins were either viewed directly by staining the gel with Coomassie brilliant blue or transferred to Nitrocellulose (NC) membrane for Western blot analysis. The membranes were immunoblotted with the same secondary antibody, swine anti-rabbit IgG-HRP conjugate. Prior to this, two membranes were incubated with rabbit anti-BBK32_{B31} polyclonal serum (1:750 diluted), while the other two were incubated with normal rabbit serum as negative controls. All the proteins were fused with GST and protein bands in (SDS-PAGE) were marked with arrows (◄). M was the molecular weight marker.

analysis. Results showed that except BBK32_{PHei} (131-162), all the other BBK32 fragments were recognized by the rabbit anti-BBK32_(B31) polyclonal serum, while the control protein GST was not (Fig. E-14, middle panel). Serum collected from a normal rabbit did not recognize any band on the blots (Fig. E-14, bottom panel). Some minor immunoreactive bands of lower MW than the dominant bands probably were degradation products of BBK32.

2.4 Qualitative Fn-binding analysis

So far 13 polypeptides representing different parts of the N- terminal regions and some corresponding mutations as well of BBK32 proteins were produced (Table E-3, Fig. F-14). Fig. E-15 illustrated the basic structures of all those prepared polypeptides.

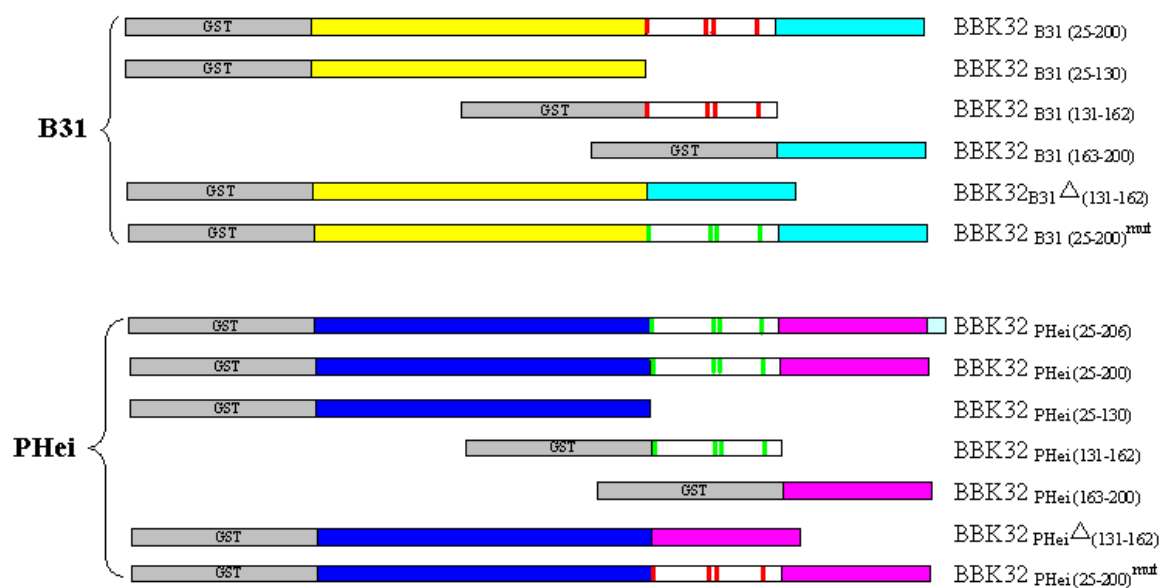


Fig. E-15. Illustration of basic structures of the 13 BBK32 polypeptides.:

Position of amino acid residues

In strain B31: position 25-130; position 131-162; position 163-200.

In strain PHei: position 25-130; position 131-162; position 163-200; position 201-206.

GST

K₁₃₁, K₁₄₅, T₁₄₇ and I₁₅₅.

Q₁₃₁, E₁₄₅, P₁₄₇ and L₁₅₅.

To evaluate whether those polypeptides exhibiting Fn-binding capacities or not, qualitative binding analysis was carried out with non-purified recombinant proteins in a Western-ligand blot-based binding assay. Results in Fig. E-16 showed that the 32 amino acids from position 131 to 162 of BBK32 were almost responsible for the full Fn-binding capacities of the proteins, while amino acids before and after this region had no or quite weak binding capacities. This was further proved by the weak Fn-binding capacities of the two deletion mutations $\text{BBK32}_{\text{B31}} \Delta_{(131-162)}$ and $\text{BBK32}_{\text{PHei}} \Delta_{(131-162)}$, which did not contain the 32 amino acids in their middle parts. Results also showed that both the site-specific point mutations and the wild type N-terminal regions of BBK32 had quite strong Fn-binding capacities. Since all the recombinant proteins were fused with GST, Fn-binding capacity of GST protein was also examined. Results indicated that GST protein had no binding capacity to Fn. This suggested the BBK32 peptide segments were responsible for the whole Fn-binding capacities in the GST-BBK32 fusion proteins. Non-specific reactions might be caused by overloading or degradation of proteins.

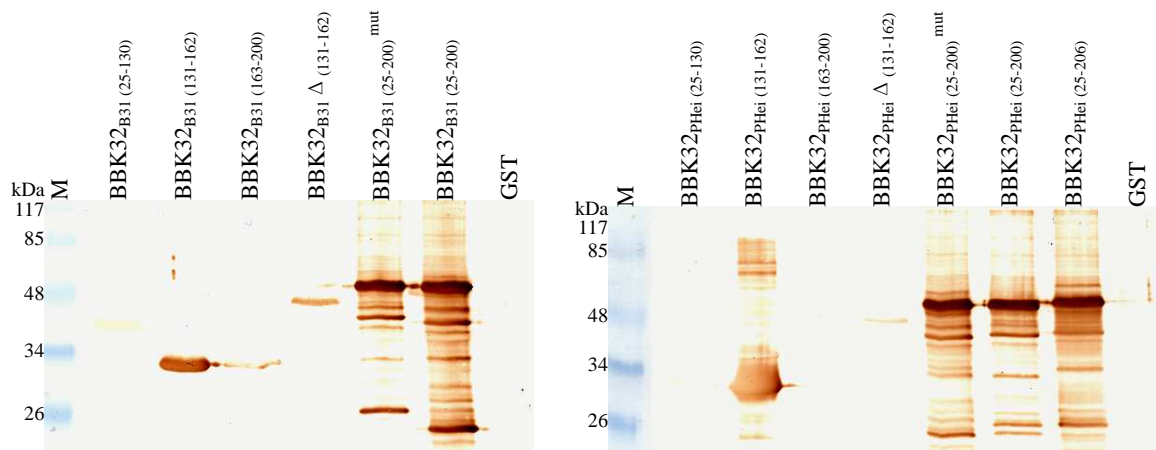


Fig. E-16. Western-ligand blot based fibronectin-binding assay of non-purified BBK32 proteins

Recombinant proteins were separated by SDS-PAGE and transferred to NC membranes. The membranes were incubated first with human fibronectin, then with polyclonal rabbit anti-human fibronectin, and finally with polyclonal swine anti-rabbit IgG-HRP conjugate. All the proteins were fused with GST. M was the molecular weight marker (kDa).

2.5 Purification of the seven polypeptides exhibiting strong Fn-binding capacity

For further quantitative analysis of the Fn-binding capacities of BBK32 polypeptides, the seven GST fusion proteins showing strong Fn-binding capacities were purified with Glutathione Sepharose 4 Fast Flow beads. Protein concentrations were determined by the Bradford method (Bradford, 1976) using the standard curve prepared by application of BSA as a protein standard (Fig. E-17). The final concentration of each purified protein was brought to 100 $\mu\text{g/ml}$ either by dilution with PBS or concentrated using Vivaspin concentrators following the manufacturer's instruction. The purity of the purified proteins was monitored by SDS-PAGE and Western blot. Results indicated 6 out of the 7 BBK32 polypeptides could be recognized by rabbit anti-BBK32_{B31} polyclonal serum, while rabbit anti-*E. coli* serum did not detect any protein bands on the blot. Although BBK32_{PHei (131-162)} could not be recognized by anti-BBK32_{B31} polyclonal serum, the presence of a single band only on the SDS-PAGE gel and non-reactivity with anti-*E. coli* polyclonal serum argued for successful purification of the respective antigen (Fig. E-18). Taken together, 7 proteins were regarded as pure enough for further investigations: BBK32_{PHei (131-162)}, BBK32_{B31 (131-162)}, BBK32_{PHei (25-200)}^{mut}, BBK32_{B31 (25-200)}^{mut}, BBK32_{PHei (25-200)}, BBK32_{B31 (25-200)} and BBK32_{PHei (25-206)}, respectively.

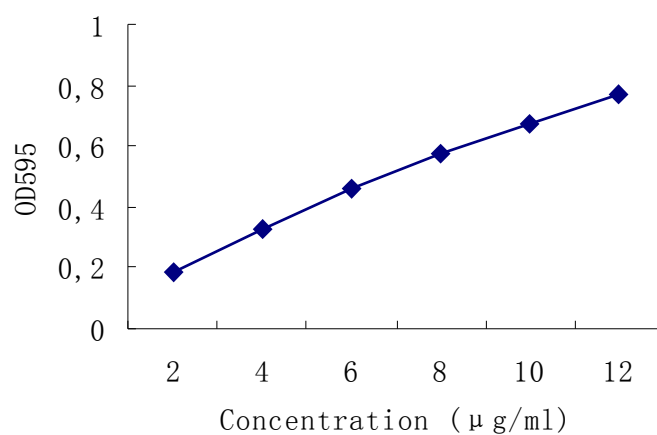


Fig. E-17. Standard curve for the Bio-Rad Protein Microassay (1-12 $\mu\text{g/ml}$) prepared from bovine serum albumin.

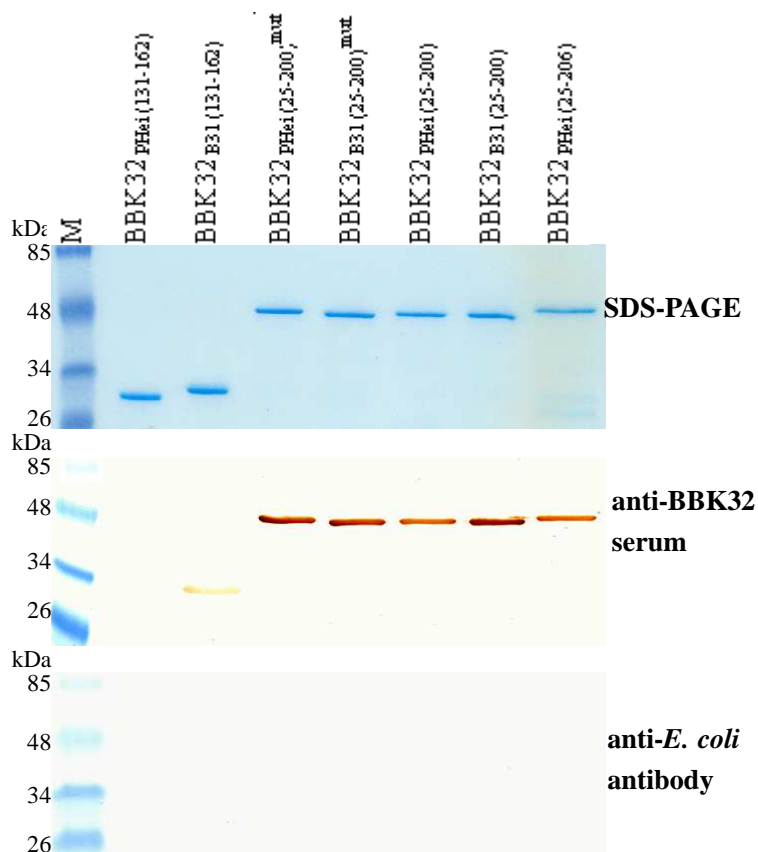


Fig. E-18. SDS-PAGE and western blot analysis of the 7 purified BBK32-GST fusion proteins

Purified proteins were separated by SDS-PAGE and then stained with Coomassie brilliant blue or transferred to NC membranes. The membranes were incubated with the same secondary antibody, swine anti-rabbit IgG-HRP conjugate. Prior to this, membrane in the middle was incubated with rabbit anti-BBK32_{B31} polyclonal antiserum, while membrane at bottom was incubated with rabbit anti-*E. coli* polyclonal antiserum. All the proteins were fused with GST. M was the molecular weight marker.

2.6 Quantitative Fn-binding assay

To quantitatively compare the Fn-binding capacities of BBK32 from strains B31 and PHei, ELISA-based binding tests were performed using the seven purified BBK32 polypeptides. Microtitre plates were coated with 0.01 or 0.05 $\mu\text{g}/\text{well}$ of each of the purified recombinant proteins overnight. Immobilized BBK32 proteins on the plates were incubated with 0.1 $\mu\text{g}/\text{ml}$ human Fn to evaluate their Fn-binding capacities. Results (Fig. E-19, panel A) showed that BBK32_{PHei(25-200)} had a stronger Fn-binding capacity than BBK32_{B31(25-200)} ($P < 0.05$ in Student's *t*-test). Further, in the case of the two 32-amino acid long-segments,

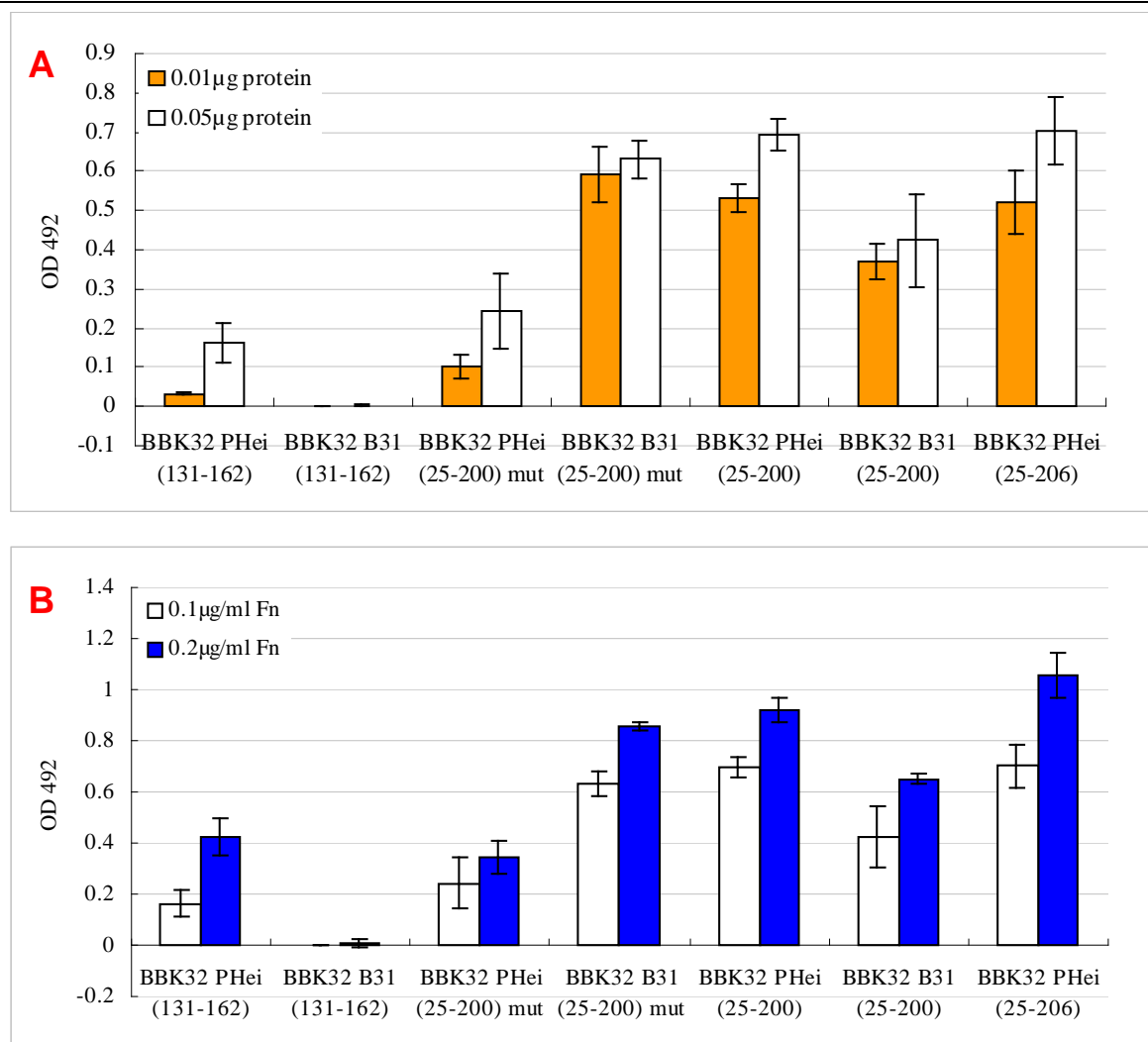


Fig. E-19. Binding of human fibronectin to immobilized BBK32 polypeptides.

Ninety-six-well plates were coated with BBK32 polypeptides and subsequently incubated with human fibronectin to determine the capacity of these proteins adhere to fibronectin. The value shown indicated the mean of three binding assays per peptide tested and the errors bars represent the standard deviation observed. A. Microtitre plates were coated with 0.01 or 0.05 µg/well of BBK32 polypeptides and subsequently incubated with 0.1 µg/ml human fibronectin. B. Microtitre plates were coated with 0.05 µg/well of BBK32 polypeptides and subsequently incubated with 0.1 or 0.2 µg/ml human fibronectin.

BBK32_{PHei (131-162)} and BBK32_{B31 (131-162)}, the former showed stronger capability to attach to Fn than the latter ($P < 0.05$ in Student's *t*-test). Sequence alignment showed that 4 amino acids were different between the two 32-amino acid long-segments. Obviously, the variation of the 4 residues could be responsible for the difference in the Fn-binding capacities. We speculated that the higher Fn-binding capacity of the whole BBK32 N-terminal region of strain PHei might also be caused by the variation of the 4 amino acids. To prove our hypothesis, two mutations BBK32_{PHei (25-200)}^{mut} (Q₁₃₁, E₁₄₅, P₁₄₇ and L₁₅₅) and

BBK32_{B31 (25-200)}^{mut} (K₁₃₁, K₁₄₅, T₁₄₇ and I₁₅₅) were then constructed by exchanging all the 4 amino acids between the whole N-terminal regions of BBK32s from PHei and B31. After substitution of the 4 amino acids, Fn-binding capacity of the constructed mutant BBK32_{PHei (25-200)}^{mut} decreased greatly compared with its wild type BBK32_{PHei (25-200)} ($P < 0.05$) and even was the same as that of BBK32_{B31(25-200)} ($P > 0.05$). On the contrary, Fn-binding capacity of BBK32_{B31 (25-200)}^{mut} was increased to the same intensity as that of BBK32_{PHei (25-200)} ($P > 0.05$), which was much stronger than that of its wild type BBK32_{B31 (25-200)}. In addition, compared with BBK32 from B31, there is a 6 amino acid insertion (YYEDDY, from position 201 to 206) in BBK32 from isolate PHei, but these 6 amino acids did not influence the Fn-binding capacity because BBK32_{PHei (25-200)} and BBK32_{PHei (25-206)} showed similar Fn-binding capacity ($P > 0.05$).

In another trail, microtitre plates coated with 0.05 µg/well each of the 7 purified proteins were incubated with 0.1 or 0.2 µg/ml human Fn. Likewise, similar results as above were again obtained (Fig. E-19, panel B).

2.7 Inhibition of the BBK32-Fn interaction with ligands

Fn contains distinct domains that interact with heparin, collagen (gelatin), integrins and other plasma and tissue components (Proctor, 1987; Pankov and Yamada, 2002; Owens and Baralle, 1986; Mardon and Grant, 1994; Matsuka *et al.*, 1994; Coburn *et al.*, 2005). One study indicated that the region of Fn bound by BBK32 overlapped with the Fn domain that binds gelatin and collagen (Probert *et al.*, 1998). A former study also demonstrated the 32-residue peptide (from position 131 to 162 in the N terminus of BBK32), which had been mapped to be the Fn-binding region of the protein, shared sequence homology to the upstream Fn-binding region of an Fn-binding protein (FnBP) (SfbI) from *Streptococcus pyogenes* (Probert *et al.* 2001). This region of SfbI had been shown to bind to the gelatin-binding domain (GBD) of Fn (Talay *et al.*, 2000). Moreover, it has been shown that, like FnBPs from other Gram-positive pathogens *Staphylococcus aureus* and *S. pyogenes*, the unstructured N-terminus of BBK32 undergoes a significant conformational change upon binding to the F1 module in the N-terminal domain (NTD) of Fn with an increase in β -sheet content in the complex (Kim *et al.*, 2005; Raibaud *et al.*, 2005). The observation

suggests that BBK32 could interact with the NTD of Fn in a similar manner to that of the FnBPs of *S. aureus* and *S. pyogenes*. To ascertain the region of Fn that interacts with BBK32s, we attempted to block specific binding sites using some known ligands of Fn.

2.7.1 Western blot-based inhibition test

Samples of purified BBK32 proteins (0.6 μ g/lane) were resolved by SDS-PAGE and transferred to NC membranes for Fn-binding assay. As shown in the upper panel in Fig. E-20, all the purified BBK32 polypeptides exhibited Fn-binding capacities. Again we found BBK32_{PHei} (131-162) had a stronger Fn-binding capacity than BBK32_{B31} (131-162). In the lower panel, prior to incubation with human Fn, blot carrying BBK32 proteins was incubated

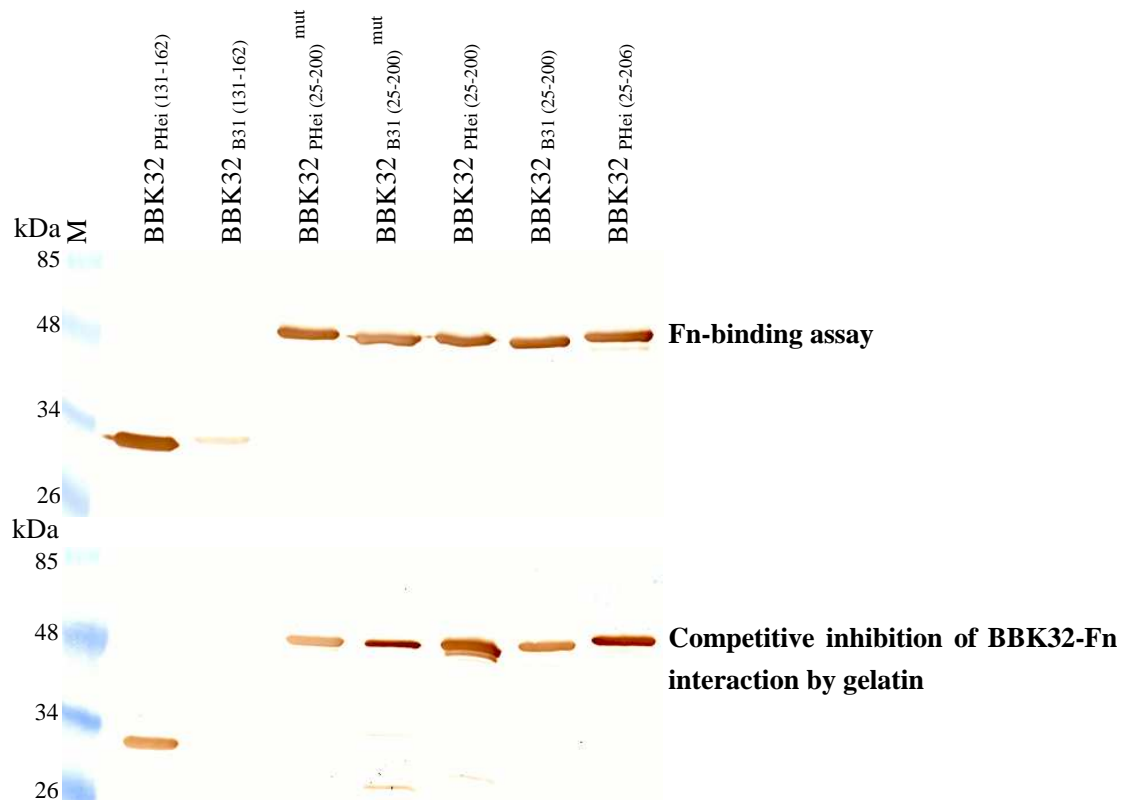


Fig. E-20. Western blot based Fn-binding assay of purified BBK32-GST fusion proteins

Recombinant proteins were separated by SDS-PAGE and transferred to NC membranes. Successive incubations of the blots were carried out in human fibronectin, polyclonal rabbit anti-human fibronectin, and polyclonal swine anti-rabbit IgG-HRP conjugate. Prior to reaction with human fibronectin, membrane in the bottom panel was incubation with 5mg/ml gelatin. All the proteins were fused with GST. M was the molecular weight marker.

with 5mg/ml gelatin. Result showed that the Fn-binding capacity of BBK32_{B31 (131-162)} could be inhibited completely by 5 mg/ml gelatin, and gelatin could also weaken the Fn-binding capacities of BBK32_{B31(25-200)} and BBK32_{PHei (25-200)^{mut}} as well. The minor bands migrated faster than the predicted BBK32 fragments did, suggesting that degradation of corresponding BBK32 polypeptides might take place (Fig.E-20, lower panel).

2.7.2 Line assay-based inhibition test

Line assay-based binding tests were developed to evaluate inhibition of BBK32 attachment to Fn. Prior to incubation with human Fn, the bound proteins on the strips were incubated with increasing concentrations of known Fn ligands collagen and gelatin, or with decorin and BSA as controls. Results showed that with the increasing concentrations of collagen and gelatin, continuous decreases in the Fn-binding capacity of BBK32_{B31(131-162)} was observed (Fig. E-21). This suggested both collagen and gelatin could inhibit the Fn-binding capacity of BBK32_{B31(131-162)}. While neither BSA nor decorin could affect the attachment of BBK32_{B31 (131-162)} to Fn.

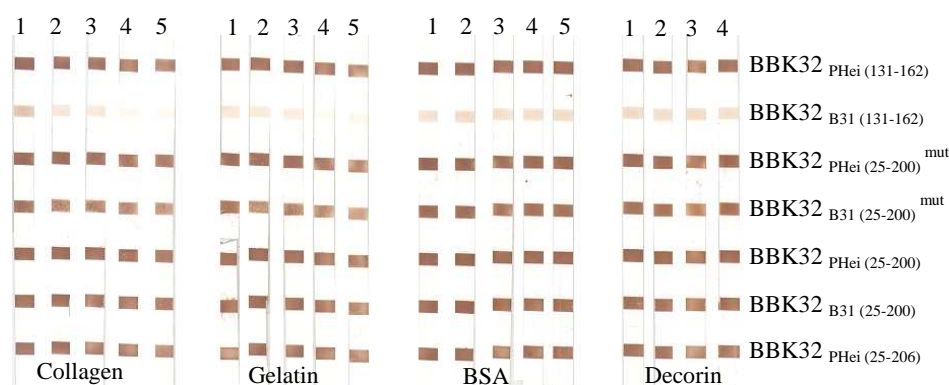


Fig. E-21. Effect of ligand addition on the interaction of BBK32 polypeptides with fibronectin.

Strips with same amount of BBK32 recombinant proteins at different positions were prepared as described in Materials and methods. Successive incubations of the strips were carried out in human Fn, polyclonal rabbit anti-human Fn, and polyclonal swine anti-rabbit IgG-HRP conjugate. Prior to incubation with human Fn, proteins on the blots were incubated with increasing concentrations of collagen, gelatin, BSA or Decorin. The concentrations of the 4 ligands from strip 1 to 5 in each group were 1, 5, 10, 50 and 100 µg/ml.

2.7.3 ELISA-based inhibition test

To evaluate the inhibition impact caused by gelatin and collagen on the interaction between BBK32 and Fn in a quantitative manner, an ELISA-based inhibition assay was carried out. Human Fn was first incubated with diluent only or either gelatin, collagen or BSA before incubation with the immobilized BBK32 polypeptides on the microtitre plates. As shown in Fig. E-22 (panels A and B) both gelatin and collagen could inhibit the attachment of Fn to most of the BBK32 polypeptides in a dose dependent manner ($P < 0.05$) (except BBK32_{B31[131-162]} and BBK32_{B31 [25-200]^{mut}). Since Fn-binding capacity of BBK32_{B31 (131-162)} was not visible at this low concentration (0.05 μg/well), inhibition impact caused by gelatin or collagen on it could not be observed. In the case of BBK32_{B31(25-200)^{mut}}, the addition of 50 μg/ml of gelatin could cause a minor decrease in its Fn-binding capacity ($P < 0.05$), while collagen could not inhibit the Fn-binding capacity of BBK32_{B31(25-200)^{mut}}. Moreover, addition of gelatin and collagen caused a greater inhibition impact on BBK32_{B31(25-200)} than on BBK32_{PHei (25-200)} and BBK32_{PHei (25-206)} (Table E-4). The mutation of the 4 amino acids in BBK32_{PHei (25-200)} greatly increased the inhibition effect of both gelatin and collagen on BBK32_{PHei (25-200)^{mut}} binding to Fn. On the contrary, after the mutation of the 4 amino acids in BBK32_{B31(25-200)}, the inhibition impact caused by gelatin and collagen on BBK32_{B31(25-200)^{mut}} was reduced dramatically or even no inhibition was observed. These results indicated that the sites of BBK32 interaction overlapped with the gelatin or collagen binding domains of Fn. However, BBK32 from isolate B31 might possess a higher percentage of overlap with the gelatin or collagen binding domain of Fn than protein from isolate PHei had. In contrast, preincubation of Fn with BSA had no effect on BBK32 polypeptides/ Fn binding activities (Fig. E-22, panel C).}

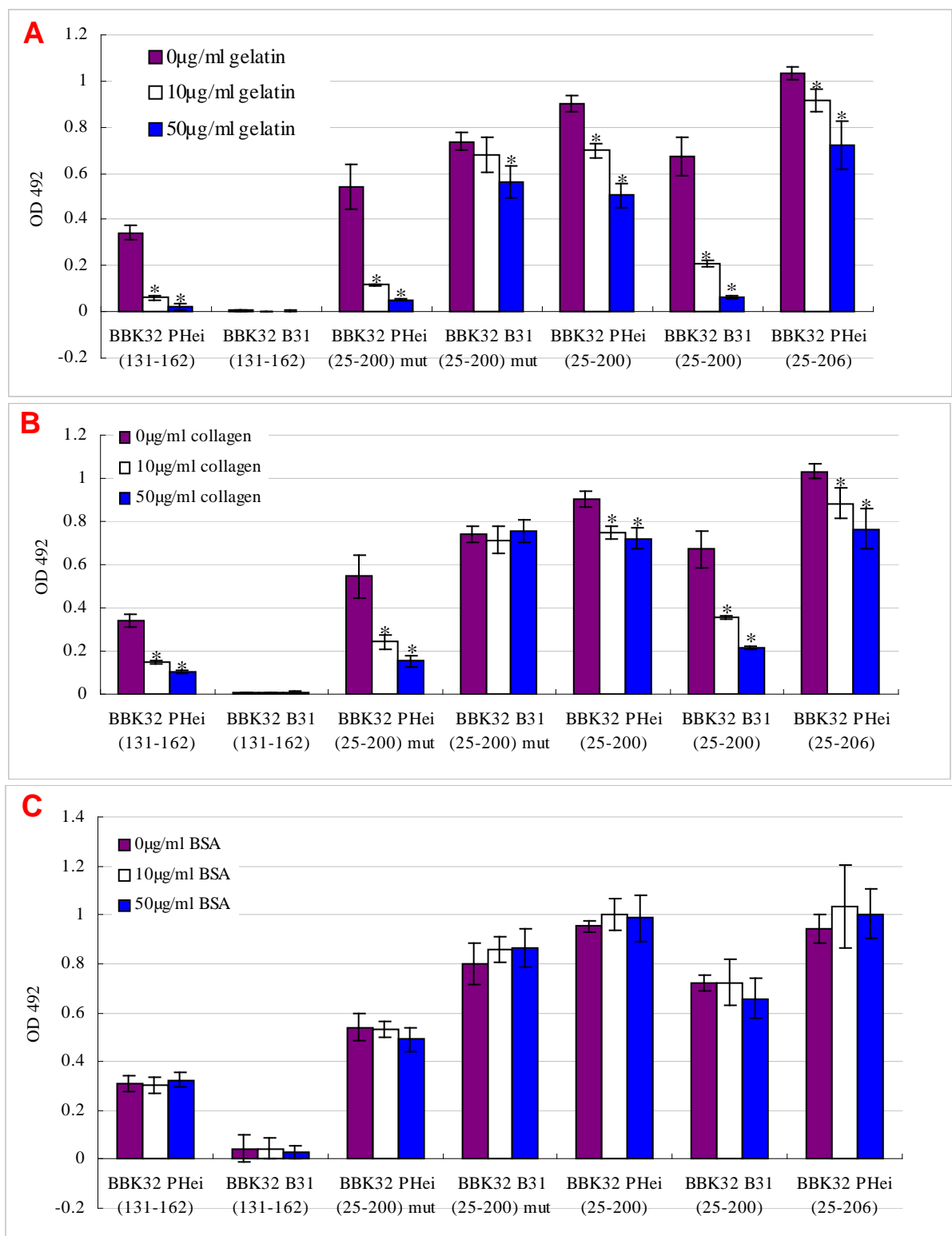


Fig. E-22. Effect of preincubation of ligand and fibronectin on BBK32-fibronectin interaction.

Ninety-six-well plates were coated with 0.05µg BBK32 polypeptides per well. Before being used to probe BBK32 proteins in the plates, human Fn was incubated with either diluent or different concentrations of ligands. Ligand in panel A, B and C were collagen, gelatin and BSA, respectively. The value shown indicated the mean of three binding assays per peptide tested and the errors bars represent the standard deviation observed. Asterisks indicated that the ligand could significantly inhibit the interaction of BBK32 peptide and Fn ($P < 0.05$).

Table E-4. Effect of preincubation of ligand and Fn on the interaction of BBK32 polypeptides and Fn.

Protein	Percent fibronectin binding activity (%) ^a					
	10µg/ml BSA	50µg/ml BSA	10µg/ml gelatin	50µg/ml gelatin	10µg/ml collagen	50µg/ml collagen
BBK32 _{PHei (131-162)}	99	105	18	7	43	30
BBK32 _{B31 (131-162)} ^b	—	—	—	—	—	—
BBK32 _{PHei (25-200)} ^{mut}	99	91	21	10	44	28
BBK32 _{B31 (25-200)} ^{mut}	108	108	92	76	97	102
BBK32 _{PHei (25-200)}	105	103	77	56	83	80
BBK32 _{B31 (25-200)}	101	91	31	10	53	32
BBK32 _{PHei (25-206)}	110	107	89	70	86	74

a. (average absorbance of wells receiving ligand/average absorbance of wells receiving diluent)×100.

b. Since Fn-binding capacity of BBK32_{B31 (131-162)} was not visible at this low concentration (0.05µg/well), inhibition impact caused by ligands could not be observed.

3. Application of recombinant BBK32 in serodiagnosis of Lyme borreliosis

Due to limitations in direct detection of *B. burgdorferi* s.l. in clinical specimens, antibody detection methods have been the main laboratory modality used to support a clinical diagnosis of Lyme borreliosis. Serological diagnosis should follow the principle of a two-step procedure: a sensitive enzyme-linked immunosorbent analysis as the first step, followed by immunoblot (IgM and IgG) if reactive (Wilske *et al.*, 2007a). An in-house recombinant immunoblot previously developed by Wilske and her colleagues have been improved significantly by the addition of several other antigens such as VlsE and DbpA (Wilske *et al.*, 1999; Schulte-Spechtel *et al.*, 2003, 2006; Goettner *et al.*, 2005). As the improvement of immunoblot for serological test is desirable, we checked the suitability of BBK32 proteins as antigens.

Several studies suggested that BBK32 might be a good antigen in serological diagnosis of Lyme borreliosis (Heikkilä *et al.*, 2002; Lahdenne *et al.*, 2003; Panelius *et al.*, 2003; Lahdenne *et al.*, 2006). One investigation also indicated that BBK32 fragments might

improve the early IgG serology of Lyme borreliosis compared to the BBK32 whole protein (Lahdenne *et al.*, 2006). Moreover, In Europe and Asia, the development of a uniform approach for the serological evaluation of Lyme borreliosis is always complicated by the prevalence of organisms from the three or more genospecies of *B. burgdorferi* s.l. and by the antigenic diversity due to variations in the sequences and expression of immunogenic proteins in these different *Borrelia* species (Wang *et al.*, 1999b; Baranton *et al.*, 1992; Robertson *et al.*, 2000; Roessler *et al.*, 1997; Goettner *et al.*, 2005; Schulte-Spechtel *et al.*, 2003; Schulte-Spechtel *et al.*, 2006).

We investigated here the implication of recombinant BBK32s in line assay for serological diagnosis of Lyme borreliosis. In order to cover all the frequent pathogenic *Borrelia* species that cause human Lyme borreliosis, we prepared 8 BBK32 homologues from different strains of the three pathogenic borrelial species, *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii*. The respective variant rBBK32s were tested for use in Lyme borreliosis serology by using serum samples from patients suffering from the disease at early- and late-stage.

3.1 Cloning of *bbk32* gene fragments

In order to produce recombinant BBK32 proteins as both whole and partial as antigens for serological diagnosis of Lyme borreliosis, four *bbk32* gene segments were amplified from genomic DNA isolated from strain B31, PBi and PKo by using the primer pairs in Table E-5. Agarose electrophoresis analysis showed that 3 of the amplified PCR products were with a size of around 580 bp, and the other one was about 1000bp length (Fig. E-23). The amplified PCR products were ligated into pGEM-T-easy vector and sequenced. Sequencing results showed that the four *bbk32* gene segments were successfully cloned. Since the shorter *bbk32* segment (around 580 bp) was only encoding the N-terminal part of the whole BBK32, the 3 short *bbk32* segments from strain B31, PBi and PKo were designated *bbk32*_{B31 partial}, *bbk32*_{PBi partial} and *bbk32*_{PKo partial}, respectively. And their encoding proteins were named as BBK32_{B31 partial}, BBK32_{PBi partial} and BBK32_{PKo partial}, correspondingly. The 1000bp-length *bbk32* gene segment amplified from strain PKo was given a name of *bbk32*_{PKo whole} and BBK32_{PKo whole} for its encoding protein. The cloned nucleotide sequence

and its deduced amino acid sequence *bbk32*_{PKo whole} were shown in Fig. E-24.

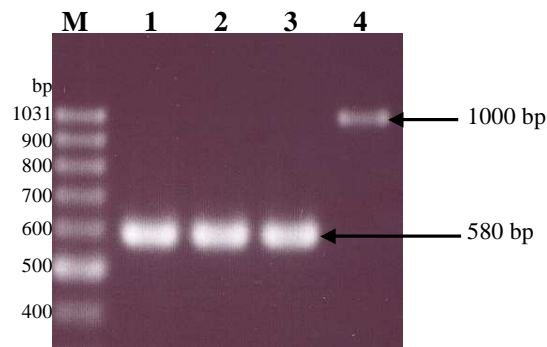


Fig. E-23. Amplified PCR products of *bbk32* gene partial and whole fragments

PCR products were electrophoresed through a 2.5% agarose gel. Lanes 1 to 4 were *bbk32*_{B31 partial}, *bbk32*_{PBi partial}, *bbk32*_{PKo partial} and *bbk32*_{PKo whole}, respectively. M was the DNA standard ladder.

```

tta ttc ata aga gat gaa ata aaa gag aaa tct ctt ggc ttg tgt gat gag gaa agt tct 60
L F I R D E I K E K S L G L C D E E S S
att tta gag act ggt gac aaa tct gtt aaa aag tct ctt aat aag aaa ggc aaa gat aag 120
I L E T G D K S V K K S L N K K G K D K
gtt gct aga aag aaa gtt gaa ggt aat gct gtt aaa aaa gac cgc ttt aat cat cat gta 180
V A R K K V E G N A V K K D P F N H H V
aag agg gag tct gtt aat aat agt aat cta tca caa aaa aat gtg ata tcg gaa gaa gaa 240
K R E S V N N S N L S Q K N V I S E E E
att ttg aaa act aaa tta tta aga gaa cga cct gag act aga aaa gaa gaa ata caa aaa 300
I L K T K L L R E R P E T R K E E I Q K
cag caa gat gag cat aaa agg atg ctt caa gga agt tta agt ttt ctt agt ggt gaa agt 360
Q Q D E H K R M L Q G S L S F L S G E S
ggt gaa ttg aag gat act att gaa agc aat gaa att gat ttt act ata gat tct gat tta 420
G E L K D T I E S N E I D F T I D S D L
aga ctg aag agt gat tta caa gct att tca ggc tca aat tct att tca tat act gat gaa 480
R L K S D L Q A I S G S N S I S Y T D E
ata gaa gaa gaa gat tat gat cag tat tct tta gaa gaa gat tat tat tat gat gag gaa 540
I E E E D Y D Q Y S L E E D Y Y Y D E E
aca aga tta agt aat aga tat gaa tct tat cta gag ggt gtt aaa tat aat gta agt tca 600
T R L S N R Y E S Y L E G V K Y N V S S
gca att aaa aca att gtt aag ata tat gat aat tat acc tta ctt tca aca aag caa acc 660
A I K T I V K I Y D N Y T L L S T K Q T
caa atg tat tct aca cgt ctt gac aac ctt gct aaa gcc aaa gct aga gaa gaa gct aaa 720
Q M Y S T R L D N L A K A K A R E E A K
aag ttt aca aaa gaa gaa ctt gaa aaa gat ctt aag acc tta ttg aac tat att caa gtg 780
K F T K E E L E K D L K T L L N Y I Q V
agt gca agg act gcg aca aat ttt gta tat gca aga gaa ata tat tca aaa aga aaa tta 840
S A R T A T N F V Y A R E I Y S K R K L
gat gcc att gaa aca gaa ata aaa aat tta gtt tta aag atc aaa gga caa tct gat tta 900
D A I E T E I K N L V L K I K G Q S D L
tac gag gca tat aaa gca ata gta agg tca atc tta tta atg aaa gat tct ctt aaa ata 960
Y E A Y K A I V R S I L L M K D S L K I
atc gaa ata gtc att gat aag aat ggt gtt tgg tac ta 998
I E I V I D K N G V W Y

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Fig. E-24. Nucleotide sequence of the cloned *bbk32*_{PKo whole} and its deduced amino acid sequence

Table E-5. Primer pairs and their targeted *bbk32* fragments for protein expression

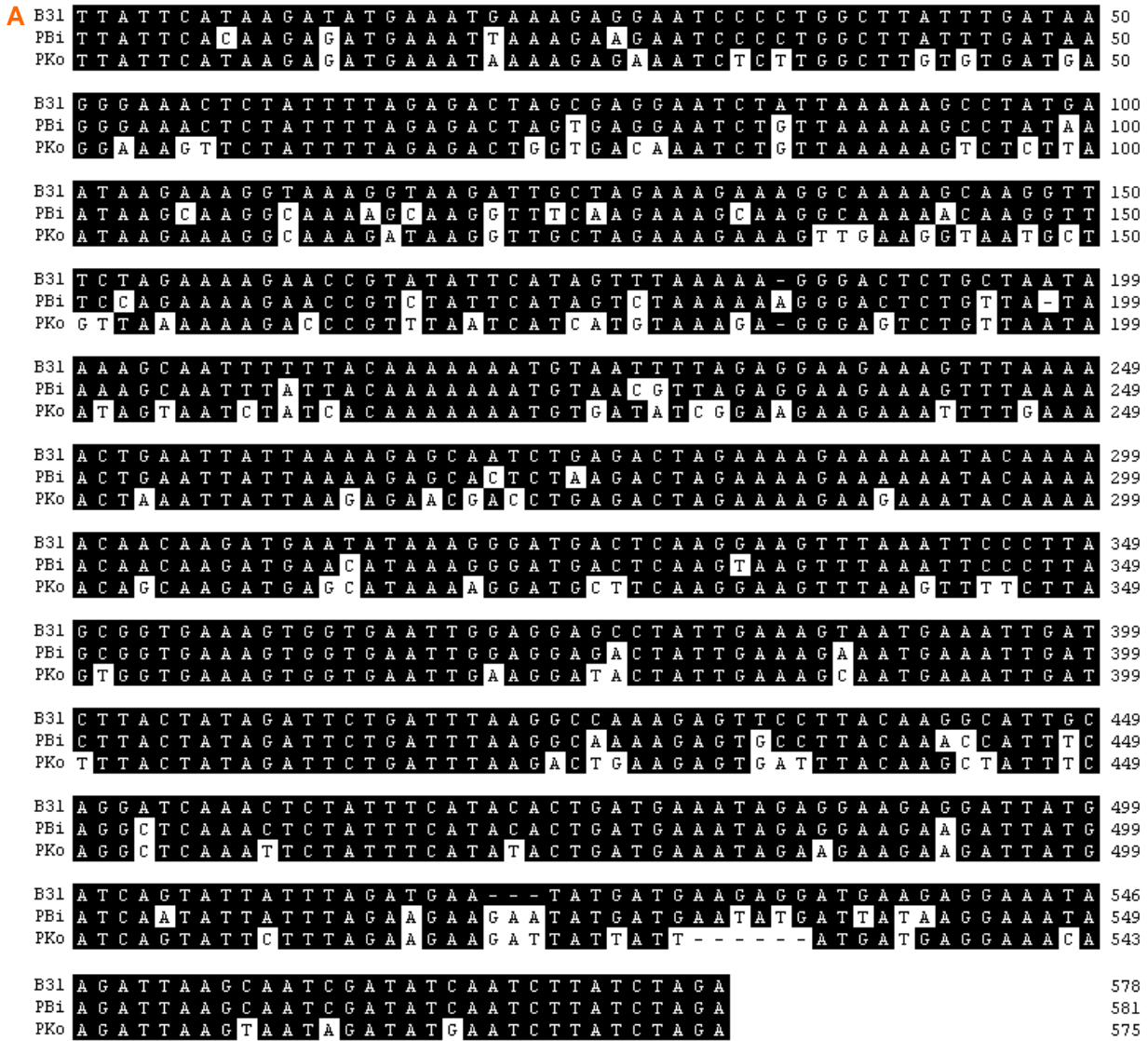
Isolate	Primer pair	Target gene fragment
<i>B. burgdorferi</i> s.s. B31	Forward P1 and Reverse P1	<i>bbk32</i> _{B31 partial}
<i>B. garinii</i> PBi	Forward P1 and Reverse P1	<i>bbk32</i> _{PBi partial}
<i>B. afzelii</i> PKo	Forward P1 and Reverse P1	<i>bbk32</i> _{PKo partial}
	Forward P1 and Reverse P2	<i>bbk32</i> _{PKo whole}

3.2 Sequence comparative analysis

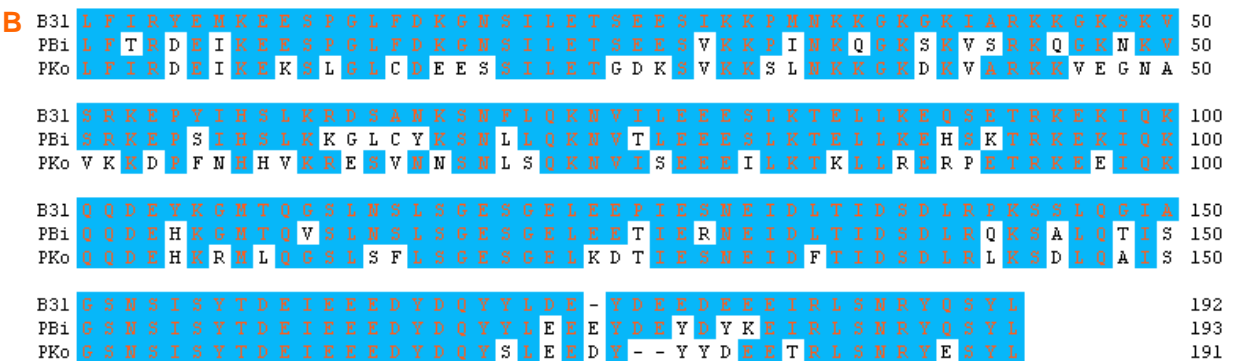
The 3 shorter gene fragments *bbk32*_{B31 partial}, *bbk32*_{PBi partial} and *bbk32*_{PKo partial} and their deduced amino acid sequences were aligned using the Clustal V method in MegAlign component of DNASTAR. At the nucleotide level, identities shared by the three shorter *bbk32* segments ranged from 78% to 89%, and at their amino acid level the identities were between 65% and 81%. Comparison of the three partial sequences was shown in Fig. E-25. Obviously, BBK32 has a much higher amino acid sequence heterogeneity compared to the DNA sequence heterogeneity.

To further confirm this, open reading frames (ORFs) of 16 *bbk32* sequences from the GenBank database were aligned and a sequence homology tree (DNA tree) was created with the DNAMAN software. Identity shared by *bbk32* DNA sequences was relatively high ranging from 84 to 100%. In the tree, the selected *bbk32* DNA sequences were divided into two big groups (group I and group II). Group I comprised all the *B. afzelii* strains and *bbk32* sequences in this group were highly conserved (showing 100% identity). While all the strains from *B. garinii* and *B. burgdorferi* s.s. were clustered into group II. As a whole, *bbk32* sequences in group II were also quite homologous, with the exception of PBi, which showing more variation (Fig. E-26). Another tree (AA tree) was constructed by alignment of the BBK32 amino acid sequences derived by the 16 ORFs with the same software. Like their corresponding DNA sequences, again the BBK32 amino acid sequences were clustered into two big groups. However, identities shared by the amino acid sequences were lower compared to their DNA sequences (Fig. E-26). Comparison of the amino acid sequence homology tree with the DNA sequence identity revealed a decrease of minimal BBK32 sequence identities from 84 to 71% from the DNA to the amino acid level.

RESULTS



Decoration 'Decoration #1': Shade (with solid black) residues that match *bbk32* B31 partial.seq exactly.



Decoration 'Decoration #1': Shade (with solid bright turquoise) residues that match *BBK32* B31 partial.pro exactly.

Fig. E-25. Sequence comparison of *bbk32* partial gene fragments of strains B31, PBi and PKo and their deduced amino acid sequences.

The cloned *bbk32* partial gene fragments *bbk32*_{B31 partial}, *bbk32*_{PBi partial} and *bbk32*_{PKo partial} (in panel A) and their encoding amino acid sequences *BBK32*_{B31 partial}, *BBK32*_{PBi partial} and *BBK32*_{PKo partial} (in panel B) were aligned using the Clustal V method in MegAlign component of DNASTAR.

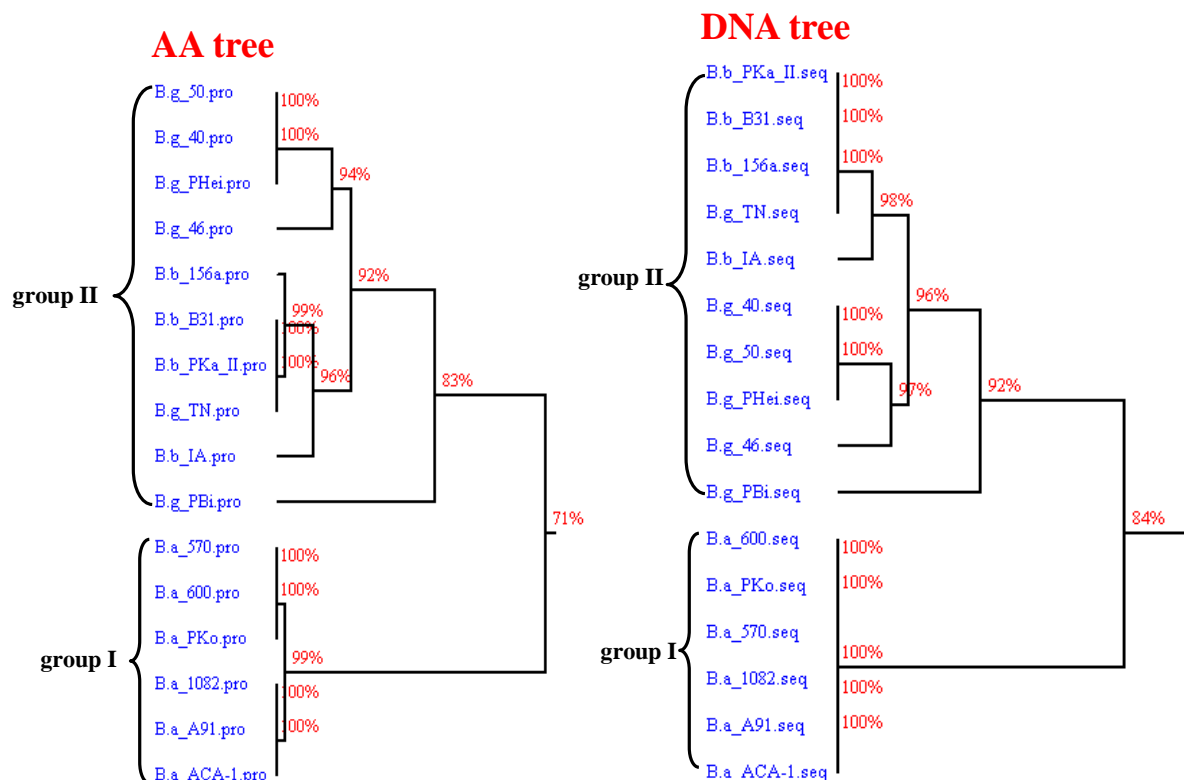


Fig. E-26. Sequence homology trees of BBK32 sequences of *Borrelia burgdorferi* s.l. strains.

Both DNA sequences and their deduced amino acid (AA) sequences of BBK32s from a variety of borrelial strains were aligned with the DNAMAN software and two homology trees (DNA tree and AA tree) were created. In the figures: B.b, *Borrelia burgdorferi* s.s.; B. g, *Borrelia garinii*; and B. a, *Borrelia afzelii*. GenBank accession numbers of involved *bbk32* genes see **Table D-4**.

3.3 Construction of expression plasmids and protein expression

Recombinant cloning plasmids, pGEM-T easy vector inserted by *bbk32*_{B31} partial, *bbk32*_{PBi} partial, *bbk32*_{PKo} partial or *bbk32*_{PKo} whole, were cleaved by *Bam*H I and *Xho* I double digestion. Released *bbk32* gene fragments were subcloned into the *Bam*H I /*Xho* I restriction sites of the linearized expression vector pGEX by T₄DNA ligase. The constructed expression plasmids, pGEX inserted by *bbk32* fragments, were transformed into BL21(DE3)pLysS competent cells and protein expression was then induced with IPTG. Expression products were examined by SDS-PAGE electrophoresis. Results showed that the 4 GST fusion proteins were successfully expressed with their expected sizes. GST protein was also produced as a control (Fig. E-27).

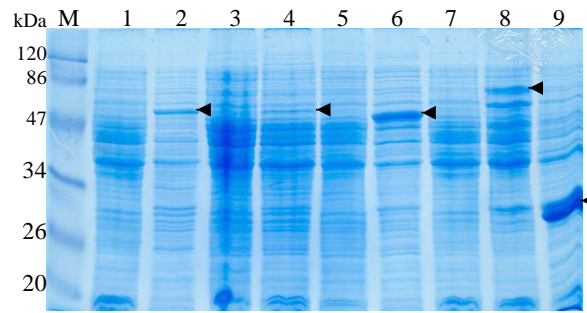
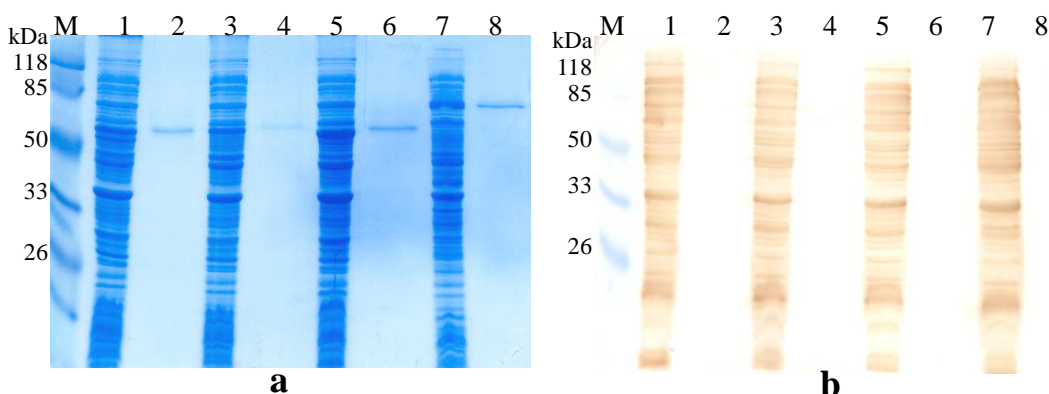


Fig. E-27. SDS-PAGE analysis of BBK32-GST fusion proteins

Recombinant proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. Lanes 1, 3, 5 and 7 were overnight cultures without induction with IPTG; lanes 2, 4, 6 and 8 were IPTG-induced proteins BBK32_{B31} partial, BBK32_{PBi} partial, BBK32_{PKo} partial and BBK32_{PKo} whole, respectively; and lane 9 was the GST control. All the proteins were fused with GST and the target protein bands were marked with arrows (◀). M was the molecular weight marker.

3.4 Purification of recombinant proteins

At first, the four recombinant proteins BBK32_{B31} partial, BBK32_{PBi} partial, BBK32_{PKo} partial and BBK32_{PKo} whole were purified with Glutathione Sepharose 4 Fast Flow beads. However, after purification, they were still contaminated by *E. coli* proteins. The 4 proteins together with some other partially purified BBK32 proteins (whole BBK32 from strains B31, PKa2, PHei and TN) produced before (Heimerl, 2005) were again purified with a Polyacrylamide gel. After the second round of purification, a single band for each purified protein was observed on the SDS-PAGE gel (Fig. E-28, a and c). In Western blot analysis, before purification, all the protein samples had cross-reactions with rabbit anti-*E. coli* polyclonal antibody, while all those non-specific bands disappeared after this purification (Fig. E-28, b and d). All the 8 purified BBK32 homologues could be recognized by rabbit anti-BBK32_{B31} polyclonal antibody in Western blot analysis (Fig. E-29)



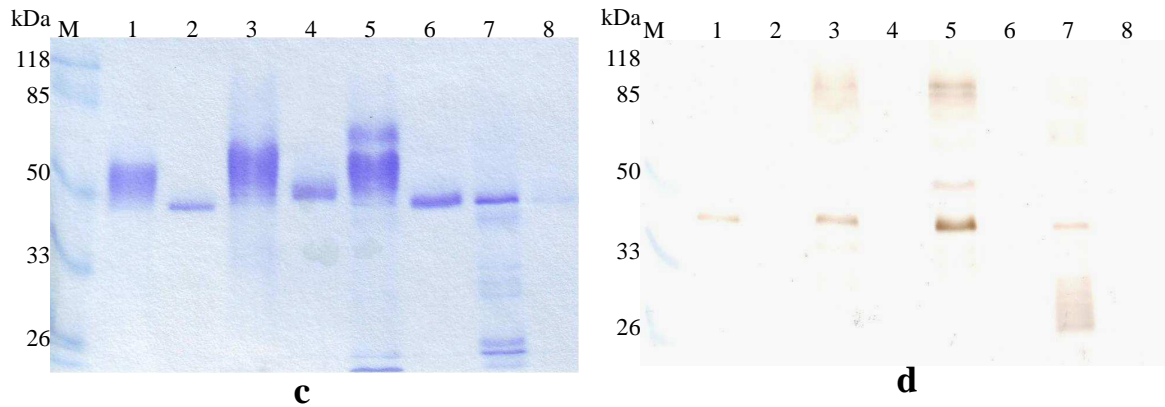


Fig. E-28. Detection of BBK32 proteins after being purified with Polyacrylamide gel

BBK32 proteins before and after purification were separated by SDS-PAGE and then stained with Coomassie brilliant blue (panel a and c) or transferred to a membrane (panels b and d). The two membranes were first incubated with rabbit anti-*E. coli* polyclonal antiserum, and then immunoblotted with the secondary antibody, swine anti-rabbit IgG-HRP conjugate. In panels a and b, lane 1, 3, 5 and 7 were *E. coli* lysate expressing BBK32_{B31 partial}, BBK32_{PBi partial}, BBK32_{PKo partial} and BBK32_{PKo whole} respectively; while lane 2, 4, 6 and 8 were purified corresponding proteins. In panel c and d, lane 1, 3, 5 and 7 were partially purified recombinant BBK32 whole proteins originated from strains B31, PHei, PKa2 and TN; Lane 2, 4, 6 and 8 were purified BBK32 whole proteins from corresponding strains. M was the molecular weight marker.

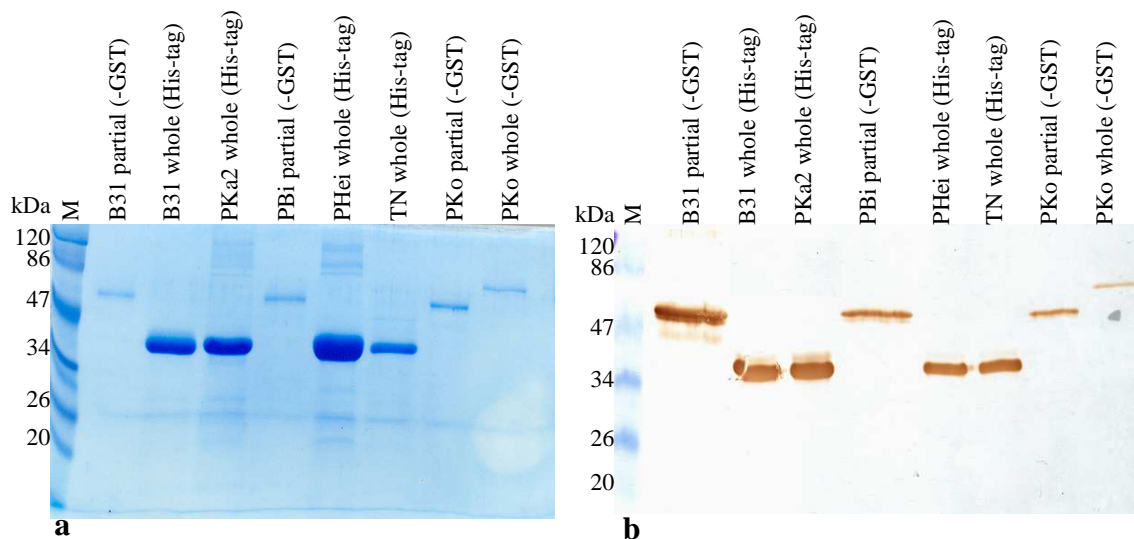


Fig. E-29. SDS-PAGE and western blot analysis of the 8 purified BBK32 proteins

Purified BBK32 proteins were separated by SDS-PAGE and then stained with Coomassie brilliant blue (panel a) or transferred to a membrane (panels b). The membrane was immunoblotted with rabbit anti-BBK32_{B31} polyclonal antiserum, and the bound first antibody was detected with swine anti-rabbit IgG-HRP conjugate. Lanes were labelled with corresponding strain designation from which BBK32 proteins were produced. -GST, the protein was fused with GST; His-tag: the protein was fused with a 6-His tag. M was the molecular weight marker.

3.5 Application of BBK32 proteins in line assay

Eight BBK32 homologues (both partial and whole as shown in Table E-6) from different borrelial stains were tested with a panel of 72 serum samples of Lyme borreliosis patients. Fig. E-30 exemplarily shows the reaction of sera from 8 patients with ACA with the 8 BBK32s.

Table E-6. BBK32 proteins from *B. burgdorferi* s.l. used in line assay

Species	Proteins
<i>B. burgdorferi</i> s. s.	BBK32 _{B31} partial, GST fused
	BBK32 _{B31} whole, His-tag fused
	BBK32 _{PKa2} whole, His-tag fused
<i>B. garinii</i>	BBK32 _{PBi} partial, GST fused
	BBK32 _{PHei} whole, His-tag fused
	BBK32 _{TN} whole, His-tag fused
<i>B. afzelii</i>	BBK32 _{PKo} partial, GST fused
	BBK32 _{PKo} whole, GST fused

Results in Table E-7 showed 13 out of 24 (54.1%) sera from patients with EM, 6 out of 40 (15%) sera from patients with NB, and 8 out of 8 (100%) sera from patients with ACA reacted positive with rBBK32 proteins from strains B31, PKa2, PHei, PBi and TN. No sera from patients with EM, only 1 out of 40 sera from patients with NB and 5 out of 8 sera from patients with ACA reacted positive with rBBK32 from strain PKo. No negative sera could react with the rBBK32 proteins. The results indicated rBBK32 from *B. burgdorferi* s.s. and *B. garinii* could be valuable antigens for diagnosis of Lyme borreliosis, especially in ACA. Notably, partial and whole rBBK32 proteins from *B. burgdorferi* s.s. shared the same sensitivity in the diagnosis of Lyme borreliosis, while whole BBK32 proteins from *B. garinii* exhibited a higher sensitivity than their corresponding partial ones.

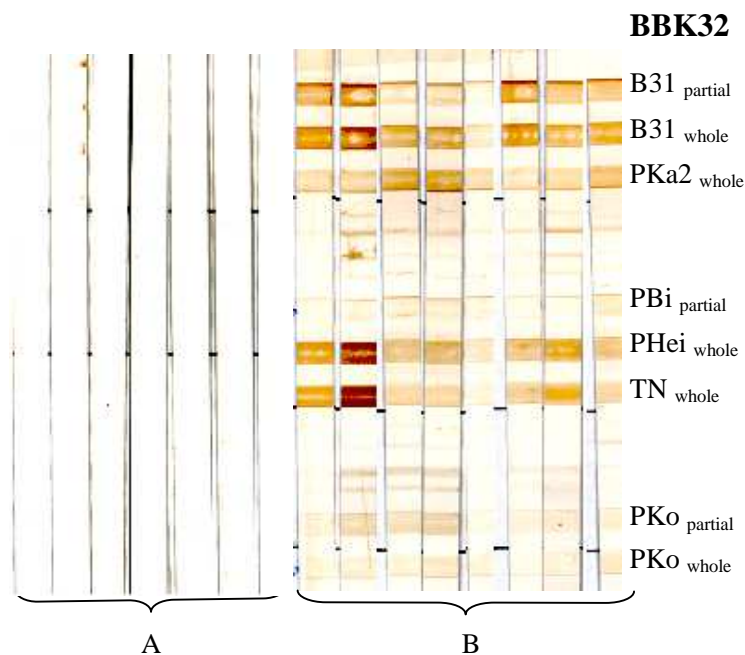


Fig. E-30. Reactivity of recombinant BBK32s from *B. burgdorferi* s.s. (B31 and PKa2), *B. garinii* (PBi, PHei and TN), and *B. afzelii* (PKo) using the line immunoblot. (A) Exemplary reactivity of negative sera (n=7, from healthy blood donors). (B) Exemplary reactivity of patient sera (n=8, from patients with ACA).

Table E-7. Reactivity of human Lyme borreliosis sera with rBBK32s

Group ^a	No. of sera	No. (%) of positive sera (IgG)	
		rBBK32	
		<i>B. burgdorferi</i> s.s. and <i>B. garinii</i>	<i>B. afzelii</i>
EM	24	13*(54.1)	0 (0.0)
NB	40	6 (15)	1 (2.5)
ACA	8	8 (100)	5 (62.5)
All cases	72	27 (37.5)	6 (8.3)
Controls	20	0 (0.0)	0 (0.0)

^a EM, erythema migrans (stage I)

NB, early neuroborreliosis (stage II)

ACA, acrodermatitis chronica atrophicans (stage III)

* partially weak reactions

F. DISCUSSION

1. The various locations of *bbk32* on the borrelial genome

B. burgdorferi conceives a segmented genome that includes a small, 900 kb linear chromosome and as many as 23 circular and linear plasmids, ranging in size from 5 kb to 56 kb (Fraser *et al.*, 1997; Stevenson *et al.*, 1997; 1998; Casjens *et al.*, 2000; Miller *et al.*, 2000). The gene *bbk32* was sequenced from the 36kbp linear plasmid (lp36) of B31 (Fraser *et al.*, 1997). Here we also localized *bbk32* on lp36 of strains *B. burgdorferi* s.s. B31 and PKa2. However, the gene was localized no a 31kpb plasmid in strain N40 in this species. Moreover, our study showed that the location of *bbk32* was quite various among different species and even differ between strains within one species. In strains of *B. garinii*, *B. afzelii* and *B. spielmanii*, *bbk32* is carried by plasmids with sizes ranging from 23 kb to 28 kb (Fig. E-3). Our results here are consistent with that of Palmer's in the aspect that genes carried by 36kbp linear plasmid in B31 are often found on 24-29kbp linear plasmids in other isolates (Palmer *et al.*, 2000).

The *B. burgdorferi* genome is unstable during *in vitro* passage and many of the plasmids can be lost during this process (Busch *et al.*, 1997; Schwan *et al.*, 1988; Norris *et al.*, 1995; Xu *et al.*, 1996; Xu *et al.*, 2005; Purser and Norris, 2000; Labandeira-Rey and Skare, 2001; McDowell *et al.*, 2001; Labandeira-Rey *et al.*, 2003; Grimm *et al.*, 2004; 2005; Lawrenz *et al.*, 2004; Strother *et al.*, 2005; Glöckner *et al.*, 2006). Here gene *bbk32* was not detected in some strains of the strain collection suggests loss of corresponding plasmids harboring the gene *bbk32* during cultivation.

Other reasons for lack of hybridization signal might be that *bbk32* genes are ploymorph and cannot be detected with the DNA-probes derived from strains B31, PKo and PBi.

2. Comparative analysis of the Fn-binding capacities of BBK32 proteins

For many bacterial pathogens, binding to host tissues is an essential step during

colonization and is typically mediated by adhesins, i.e., surface proteins that promote bacterial attachment to host cells (Boyle *et al.*, 2003; Finlay *et al.*, 1997; Joh *et al.*, 1999; Menzies, 2003). To gain entry into the host through mucosal surfaces, bacterial pathogens express fimbrial and/or afimbrial adhesins that bind to various abundant sugar moieties of glycolipids and glycoproteins on epithelial cells. Mucosal colonization by pathogens often results in inflammation and tissue damage that exposes the underlying extracellular matrix. Binding to integrin and components of the extracellular matrix, such as Fn and collagen, by pathogens not only promotes bacterial colonization but also facilitates bacterial invasion into deeper tissues, which may lead to systemic dissemination (Cabello *et al.*, 2007; Coburn *et al.*, 2005).

Fn is a large, dimeric glycoprotein that is produced by a broad range of cell types. It exists as a soluble molecule in body fluids such as blood plasma and as a splice-variant as insoluble cellular Fn of cell membranes and the extracellular matrix. Its two nearly identical subunits of 220-250 kDa are linked covalently near their carboxyl-termini by a pair of disulfide bonds. Each monomer is comprised of three different types of homologous repeating domains, termed Type I, II and III (Hynes, 1990). Fn contains 12 type I repeats, two type II repeats and 15-17 type III repeats. Through these functional domains, Fn can interact with a variety of macromolecules such as fibrin, heparin, collagen, and integrins as well. Many of these functional domains are also targeted by adhesins expressed by pathogenic microorganisms (Fig. F-1) (Proctor, 1987; Pankov and Yamada, 2002). Streptococcal fibronectin binding protein I (SfbI) and its allelic variant (F1) mediate adherence to and invasion of *S. pyogenes* into human epithelial cells by binding to the whole N-terminal fragment of Fn (includes the N-terminal fibrin binding domain of Fn, repeat I₁₋₅, and the collagen-binding domain of Fn, repeat I₆₋₉ and II_{1, 2}) (Joh *et al.*, 1998; Talay *et al.*, 2000; Ozeri *et al.*, 1996). The collagen-binding domain (repeat I₆₋₉ and II_{1, 2}) binds far more effectively to denatured collagen (gelatin) than to native collagen. Thus Fn interactions with collagens in general may be due to its binding to unfolded regions of the collagen triple helix (Pankov and Yamada, 2002).

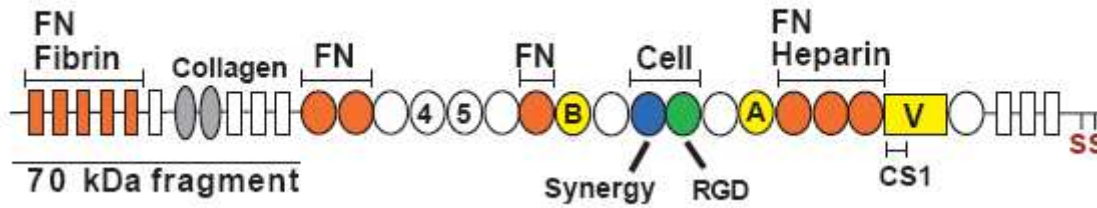


Fig. F-1. Domain structure of fibronectin (Fn).

Fn consists of type I (rectangles), type II (ovals) and type III (circles) repeats. Sets of repeats constitute binding domains for fibrin, FN, collagen, cells and heparin, as indicated. The assembly domain and FN-binding sites are highlighted in orange. SS indicates the C-terminal cysteines that form the dimer. (Figure remodeled from Wierzbicka-Patynowski and Schwarzbauer, 2003).

Up to now, several adhesins expressed by *Borrelia* species that can bind Fn have been identified (Grab *et al.*, 1998; Kopp *et al.*, 1995; Probert *et al.*, 1998; Schorey *et al.*, 1996). BBK32, a 47-kDa lipoprotein, is a primary Fn-binding adhesin identified in *B. burgdorferi* (Probert *et al.*, 1998). Overexpression of BBK32 protein in high-passage *B. burgdorferi* strain that lacks this protein enhances the organism's binding to Fn, as well as to glycosaminoglycans (Fischer *et al.*, 2006). Furthermore, inactivation of *bbk32* gene in infectious strains of *B. burgdorferi* reduced spirochetal binding to Fn, as well as its infectivity in mice (Seshu *et al.*, 2006), although the mutations had no apparent defect in tick vectors (Li *et al.*, 2006). These findings suggested that BBK32 might run an important role in the adhesion and invasion activities of *B. burgdorferi* to its host tissues

BBK32 consists of a C-terminal globular domain and an N-terminal region lacking well-defined secondary structure (Kim *et al.*, 2004). In prior studies, the Fn-binding activity of BBK32 was localized to a 32-amino acid-long segment within the unstructured domain. This ligand-binding segment was relatively conserved among strains (Probert *et al.*, 2001). Previously, this research group found that BBK32 protein from strain PHei possessed a stronger Fn-binding capacity than that from B31 (C. Heimerl doctoral thesis, 2005). In this study the molecular mechanism of this difference was clarified. Since it has already been described that the N-terminal region, and especially the 32-amino acid-long segment in this region is mainly responsible for the Fn-binding activity of BBK32 (Probert *et al.*, 2001), Fn-binding capacities of the N-terminal region, and especially the 32-amino

acid-long fragment from both strain PHei and B31 were intensively investigated in this thesis. By sequence comparison, four amino acids in this 32-amino acid-long segment were found different between the two strains (Fig. E-6). We speculated that the 4 different amino acids should be responsible for the different Fn-binding capacities. In this case, a variety of protein fragments including some mutated proteins (see Fig. E-15) were generated and their Fn-binding capacities were evaluated. In Western blot (Fig. E-14), 12 out of the 13 produced BBK32 polypeptides could be recognized by rabbit anti-BBK32_{B31} polyclonal antibody, suggesting that the protein bands with expected sizes observed with Coomassie staining were in fact BBK32 protein fragments. The non-reactivity of BBK32_{PHei (131-162)} with the rabbit immune serum might be due to the fact, that the antigen used to generate the polyclonal antibody in rabbit was from strain B31, while 4 out of the 32 amino acids of BBK32_{PHei (131-162)} are different to the corresponding region of BBK32 from B31.

At first, Fn-binding capacities of all the produced polypeptides were evaluated by using a Western blot-based Fn-binding assays. Just as reported by Probert and his colleagues (2001), we also found that the 32-amino acid-long segment (from position 131-162) was mainly responsible for the Fn-binding capacity of BBK32 proteins. Strikingly, amino acid residues proximal and distal from the 32-amino acid-long segment had quite weak or even no Fn-binding capacity. And this was further confirmed by the weak Fn-binding capacities of the two deletion mutations, which did not contain the 32 amino acids in their middle parts. The whole N-terminal regions of BBK32 from both strains and two other proteins containing the four substituted amino acids in the 32-amino acid-long region also exhibited strong Fn-binding capacities (Fig. E-16).

To compare the Fn-binding capacities of those proteins quantitatively, seven purified BBK32 polypeptides were evaluated in an ELISA-based Fn-binding assay. Again, the 32-amino acid-long segment from PHei showed a stronger Fn-binding capacity than the corresponding region from B31 ($P < 0.05$). Furthermore, compared with its wild type protein BBK32_{PHei (25-200)}, Fn-binding capacity of BBK32_{PHei (25-200)}^{mut} decreased dramatically ($P < 0.05$) to such an extent that there was no difference in the Fn-binding capacity between

BBK32_{PHei (25-200)}^{mut} and the wild type protein BBK32_{B31 (25-200)} ($P>0.05$). While on the contrary, Fn-binding capacity of BBK32_{B31 (25-200)}^{mut} which containing the 4 substituted amino acids increased to as strong as that of the wild type BBK32_{PHei (25-200)} ($P<0.05$) (Fig. E-19). All these data demonstrated that the replacement of the 4 amino acids motif in the 32-amino acid-long segment of PHei greatly increased its attachment to Fn and this even led to the high affinity of the whole BBK32 protein to Fn. In addition, it must be emphasized that although the 32- amino acid-long segment in BBK32 plays a critical role in binding to Fn, it could not be responsible for the whole Fn-binding capacity of BBK32. On the one hand, although quite weak, protein segments before and after this 32-amino acid-long segment also can bind to Fn (Fig. E-16). On the other hand, whole N-terminal region of BBK32 exhibited a stronger Fn-binding capacity than the 32-amino acid-long segment in its middle part ($P<0.05$).

Like Fn-binding proteins (FnBPs) from other Gram-positive pathogens such as *S. aureus* and *S. pyogenes*, binding of BBK32 to the F1 module in the N-terminal domain (NTD) of host Fn converts the unstructured amino-terminus of BBK32 into a stable β -sheet structure (Kim *et al.*, 2005; Raibaud *et al.*, 2005; Joh *et al.*, 1999; Menzies, 2003). The observation suggests that BBK32 could interact with the NTD of Fn in a similar manner to that of the FnBPs of *S. aureus* and *S. pyogenes*. Previous studies also demonstrated that the ligand-binding region of BBK32 (the 32 amino acids from position 131 to 162) shared sequence homology to the upstream Fn-binding region of an FnBP (SfbI) from *S. pyogenes* (Probert *et al.* 2001). This region of SfbI had been shown to specifically bind to the collagen-binding domain of Fn (Talay *et al.*, 2000). From the observation that gelatin could partially inhibit the binding of Fn to BBK32 and BBK32 could block the interaction of gelatin and Fn, it was speculated that the collagen-binding domain of Fn might contain a binding site for BBK32 (Probert *et al.*, 1998). To ascertain the region of Fn that interacts with BBK32 proteins, we attempted to block specific binding sites using some known ligands of Fn such as collagen and gelatin.

In a Western blot-based binding assay, preincubation of gelatin with BBK32 proteins could

reduce the binding of Fn to the whole N-terminal region of BBK32 (B31) and especially the 32-amino acid-long segment of the strain (Fig.E-20). Further, in a line assay, with the increase of the concentration of gelatin or collagen, continuous decreases in the binding of Fn to the 32-amino acid-long segments (from both B31 and PHei) were observed (Fig. E-21). This suggests the entire N-terminal region of BBK32 from B31 also can bind to collagen/gelatin, and the collagen/gelatin binding region of the BBK32 should be in the 32-amino acid-long segment. Once this region was occupied by collagen/gelatin, its capability of binding to Fn was highly inhibited. Thus we suggest that BBK32 from *B. burgdorferi* s.s. B31 should also be a collagen/gelatin binding protein.

In an ELISA based inhibition test, preincubation of Fn with both gelatin and collagen could inhibit the attachment of Fn to wild BBK32 polypeptides of the two strains. However, gelatin and collagen caused a greater inhibition impact on BBK32 polypeptides from B31 (Fig. E-22, Table E-4). This different inhibition effect was further confirmed in the two mutations, BBK32_{B31(25-200)}^{mut} and BBK32_{B31(25-200)}^{mut}. The mutation of the 4 amino acids in BBK32_{PHei (25-200)} greatly increased the inhibition effects of both gelatin and collagen on BBK32_{PHei (25-200)}^{mut} binding to Fn. On the contrary, after the mutation of the 4 amino acids in BBK32_{B31(25-200)}, the inhibition impact caused by gelatin and collagen on BBK32_{B31(25-200)}^{mut} was reduced dramatically or even no inhibition was observed (Fig. E-22, Table E-4). These results indicated that BBK32 proteins mainly bind to the collagen-binding domain of Fn (repeat I_{6,9} and II_{1,2}). However, preincubation of Fn with gelatin or collagen could not thoroughly block the interaction between Fn and BBK32, this indicates that BBK32 might also partially bind to the N-terminal fibrin binding domain of Fn (repeat I₁₋₅) in a similar manner to that of the FnBPs of *S. aureus* and *S. pyogenes* (Joh *et al.*, 1998; Talay *et al.*, 2000; Ozeri *et al.*, 1996). Sequence homology shared by the 32-amino acid-long segment of BBK32 and the upstream Fn-binding region of an FnBP (SfbI) from *S. pyogenes* also supports our hypothesis (Probert *et al.* 2001).

Moreover, on the one hand, the higher affinity of the 32-amino acid-long segment from BBK32_{PHei} to Fn might be difficult to be inhibited by collagen or gelatin. On the other hand,

BBK32 from isolate B31 might possess a higher percentage of overlap with the gelatin or collagen binding domain of Fn than protein from isolate PHei. In addition, since other regions rather than the 32-amino acid-long segment of BBK32 could also bind to other domains of Fn (presumably the repeat I₁₋₅) and/or because of the high concentration of proteins on the blot in the immunoblot-based inhibition assays, the inhibition impact of gelatin and collagen on other polypeptides were not visible (Fig. E-20; E-21). With an amount of 0.05µg BBK32_{B31} (131-162) per well in the microtiter plate, the polypeptide exhibited a quite weak Fn-binding capacity, so the inhibition impact caused by gelatin or collagen on it could not be observed (Fig. E-19, E-22, Table E-4).

Our results also suggested compared to collagen, gelatin exhibited a higher inhibition effect on the interaction of BBK32 and Fn, this is consistent with the fact that the collagen-binding domain (repeat I₆₋₉ and II_{1, 2}) binds far more effectively to denatured collagen (gelatin) than to native collagen (Pankov and Yamada, 2002).

From a strategy point of view, by using restriction enzyme digestion and T₄DNA ligation, the construction of the four mutations was largely simplified compared with traditional PCR directed mutation. For construction of the two deletion mutations, *bbk32*_{PHei} Δ₍₃₉₁₋₄₈₆₎ and *bbk32*_{B31} Δ₍₃₉₁₋₄₈₆₎, a site-directed silent mutation was carried out in order to create *Bsa*H I site (GACGCC) at the 3'-end of *bbk32*₍₇₃₋₃₉₀₎, where the threonine codon (nucleotide 388-390) was changed from ACT to ACG. And the same restriction enzyme site was also created at the 5'-end of the gene fragment *bbk32*₍₄₈₇₋₆₀₀₎. After digestion with *Bsa*H I, the two gene fragments were connected head to tail. Thus nucleotides from position 391 to 486 were successfully deleted from the gene (Fig. E-9). As for construction of the 2 site-specific point mutations, *bbk32*_{PHei} (73-600)^{mut} and *bbk32*_{B31} (73-600)^{mut}, 4 amino acids of BBK32 from each isolate were mutated by codon replacement. We found two unique restriction enzyme sites (*Bst*F51 and *Hae*III) proximal and distal the region conceiving the 4 amino acids to be replaced. Thus the regions between the two restriction enzyme sites were exchanged between *bbk32* gene fragments from PHei and B31 after a series of digestion and ligation treatments (Fig. E-11).

In summary, the interaction of *B. burgdorferi* s.l. BBK32 and human Fn is mainly mediated by the N-terminal region, and especially the 32-amino acid-long segment of BBK32 by binding to the collagen-binding domain of Fn (repeat I₆₋₉ and II_{1, 2}) and partially to its N-terminal fibrin binding domain (repeat I₁₋₅). Because of the variation of the 4 amino acids (K₁₃₁, K₁₄₅, T₁₄₇ and I₁₅₅) in BBK32 of isolate PHei, its binding capacity to Fn increases greatly compared with BBK32 proteins from isolate B31, and this high affinity to Fn is only partially inhibited by gelatin or collagen in comparison to that of its homologue from B31. Surprisingly, we found BBK32 from *B. burgdorferi* s.s. B31 should also be a collagen/gelatin binding protein.

3. Application of recombinant BBK32 proteins in serological diagnosis of Lyme borreliosis

Some studies indicated that BBK32 might be a good antigen for detection of specific antibodies of patients suffering from Lyme borreliosis (Heikkilä *et al.*, 2002; Lahdenne *et al.*, 2003; Panelius *et al.*, 2003; Lahdenne *et al.*, 2006). One investigation also indicated that BBK32 fragments might improve the early IgG serology of Lyme borreliosis compared to the BBK32 whole protein (Lahdenne *et al.*, 2006). An in-house recombinant immunoblot assay for serodiagnosis of Lyme borreliosis was previously established in our laboratory (Max von Pettenkofer institute) (Wilske *et al.*, 1999; Schulte-Spechtel *et al.*, 2003, 2006; Goettner *et al.*, 2005). Therefore, we checked the possibility of improving the immunoblot established by our laboratory by addition of some BBK32 proteins as antigens.

In Europe and Asia, the development of a uniform approach for the serological evaluation of Lyme borreliosis is complicated by the prevalence of organisms from the three or more genospecies of *B. burgdorferi* s.l. and by the antigenic diversity due to variations in the sequences and expression of immunogenic proteins in these different borrelial species (Wang *et al.*, 1999b; Baranton *et al.*, 1992; Robertson *et al.*, 2000; Roessler *et al.*, 1997; Goettner *et al.*, 2005; Schulte-Spechtel *et al.*, 2003, 2006). Both former studies (Heikkilä *et al.*, 2002) and our results indicated BBK32 proteins were quite heterogeneous (Fig. E-25).

We also realised that BBK32 proteins had a much higher heterogeneity at amino acid sequence level than at DNA sequence level (Fig. E-26). This may be due to the immune selection as *Borrelia* binding to Fn might evade humoral immunity of its host. In order to cover all pathogenic *Borrelia* species that cause human Lyme borreliosis, we prepared 8 BBK32 homologues from different strains of the three pathogenic species, *B. burgdorferi* s.l., *B. afzelii*, and *B. garinii*.

The respective variant BBK32 recombinant proteins were tested for use in Lyme borreliosis serology by using serum samples from patients with early- and late-stage Lyme borreliosis. Results indicated rBBK32 from *B. burgdorferi* s.s. and *B. garinii* could be valuable antigens for diagnosis of Lyme borreliosis, especially in ACA. Our results also suggested for improvement of sensitivity, that entire BBK32 proteins should be applied rather than truncated ones.

Former studies suggested that epitope specific antibodies might be induced by different species of *B. burgdorferi* s.l. (Heikkilä *et al.*, 2002; Lahdenne *et al.*, 2003). As shown in the AA homology tree (Fig. E-26), BBK32 sequences in *B. afzelii* were quite different from those in *B. garinii* and *B. burgdorferi* s.s.. We speculated that most of the pathogenic agents of those borreliosis patients of our sera sources in this study were likely infected with *B. garinii* or *B. burgdorferi* s.s.. And this might explain the low percentage reactivity of antibodies with BBK32 proteins from strain PKo of *B. afzelii*. To prove this, pathogenic *Borrelia* should be isolated from patients and *ospA* PCR should be performed in order to differentiate related *B. burgdorferi* s.l. isolates as described elsewhere (Michel *et al.*, 2003). This further implies that in regions where Lyme borreliosis is caused by different species of *B. burgdorferi* s.l., variant BBK32 antigens are probably needed to cover all the EM cases.

After the first round of purification of recombinant BBK32 proteins with either Ni-NTA beads or Glutathione Sepharose 4 Fast Flow beads, resulted to some contaminations from *E. coli* proteins. To get rid of this, a second round of purification with Polyacrylamide gel was performed. As demonstrated in this study there were no non-specific reaction bands visible after the second round of purification (Fig. E-28). Although with its short-coming of quite low efficiency, the method is still reliable in some experimental test where only small

amounts of proteins are needed. In the future, some other methods will be tried for large-scale purification of proteins.

However, though BBK32 homologues could react with Lyme borreliosis patient sera, compared with other antigens on the in-house immunoblot established in the Max von Pettenkofer Institute (Wilske *et al.*, 1999; Schulte-Spechtel *et al.*, 2003, 2006; Goettner *et al.*, 2005), these homologues could not improve the method in detection of either IgG or IgM antibodies. Nevertheless, the study underlines the fact that the heterogeneity of *Borrelia* strains and species must be taken into consideration in the serological diagnosis of Lyme borreliosis in European patients.

G. CONCLUSION

The location of *bbk32* gene varies among different *Borrelia* species and might even differ between strains. The gene was not detected by PCR and Southern-blot in some strains, which suggest the loss of corresponding plasmids harboring the *bbk32* gene in those strains during *in vitro* cultivation. Other reasons might be that *bbk32* genes in some strains have distinct sequences and are not recognized by the three probes prepared from strains B31, PKo and PBi.

BBK32 from *B. garinii* strain PHei exhibits a much stronger Fn-binding capability when compared to its homologue from *B. burgdorferi* s.s. strain B31. The 32 amino acids (from position 131 to 162) in the N-terminal region are mainly responsible for the Fn-binding capability of the protein. The substitution of the 4 amino acids (K 131 Q, K 145 E, T 147 P and I 155 L) in the Fn-binding region of BBK32(PHei) is abrogated the higher affinity of BBK32_{PHei} to Fn. The interaction of BBK32 and human Fn is mainly mediated by the N-terminal region, and especially the 32-amino acid-long segment of BBK32 by binding to the collagen-binding domain of Fn (repeat I₆₋₉ and II_{1, 2}) and partially to its N-terminal fibrin binding domain (repeat I₁₋₅).

Though BBK32 homologues react with serum antibodies from some Lyme borreliosis patients, these homologues could not improve the serological test in detection of either IgG or IgM antibodies. Nevertheless, the study underlines the fact that the heterogeneity of Lyme disease *Borrelia* species must be taken into consideration in the microbiological diagnosis of Lyme borreliosis in European patients.

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I. LIST OF ABBREVIATIONS

ACA	acrodermatitis chronica atrophicans
APS	Ammonium persulphate
bp	Base pairs
BSA	Bovine Serum Albumine
BSK-II medium	Barbour-Stonner-Kelly-II medium
CDC	Center for disease control
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylendiamintetraacetic acid
EM	erythema migrans
EtBr	ethidium bromide
Fig.	Figure
Fn	fibronectin
FnBP	Fn-binding protein
g	gram
GBD	gelatin-binding domain
h	hour
HRP	Horseradish peroxidase
IPTG	Isopropyl- β -thiogalaktopyranosid
kb	kilobase
kDa	kilodalton
l	liter
LB broth	Luria Bertani broth
M	molar
MCS	multiple cloning site
Min	minute

LIST OF ABBREVIATIONS

MKP-medium	Modified Kelly-Pettenkofer medium
ml	milliliter
mM	milimolar
MW	Molecular weight
µl	microliter
NB	neuroborreliosis
NC	Nitrocellulose
NCBI	National Center for Biotechnology Information
NTD	N-terminal domain
OD	Optical density
o/n	overnight
ORF	open reading frame
Osp	outer surface protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
pH	power of hydrogen
PMSF	phenylmethanesulphonyl fluoride
rpm	revolutions per minute
RT	Room temperature
Sec	second
SfbI	Streptococcal fibronectin binding protein I
SD	standard deviation
SDS	Sodium dodecyl sulphate
TEMED	<i>N,N,N',N'</i> -Tetramethylethylenediamine
Tris	Tris-hydroxymethyl-aminomethane
Tween 20	Polyoxyethylensorbitanmonolaureate
v/v	volume/volume

LIST OF ABBREVIATIONS

w/v	weight/volume
X-Gal	5-Bromo-4-Chloro-3-Indolyl- β -D-Galaktosid

J. APPENDIX

Nucleotide sequences of the cloned gene fragments and constructed mutations of *bbk32*. A. gene fragments of isolate B31, and B. gene fragments of isolate PHei.

A

bbk32 B31 (73-600)	AGATATGAAATGAAAGAGGAATCCCTGGCTTATTTGATAAAGGGAAACTCTATTT	55
bbk32 B31 (73-600) ^{mut}	AGATATGAAATGAAAGAGGAATCCCTGGCTTATTTGATAAAGGGAAACTCTATTT	55
bbk32 B31Δ(391-486)	AGATATGAAATGAAAGAGGAATCCCTGGCTTATTTGATAAAGGGAAACTCTATTT	55
bbk32 B31 (73-390)	AGATATGAAATGAAAGAGGAATCCCTGGCTTATTTGATAAAGGGAAACTCTATTT	55
bbk32 B31 (391-486)	-----	0
bbk32 B31 (487-600)	-----	0
bbk32 B31 (73-600)	TAGAGACTAGCGAGGAAATCTATTAAAAAGCCTATGAATAAGAAAGGTTAAAGGTTAA	110
bbk32 B31 (73-600) ^{mut}	TAGAGACTAGCGAGGAAATCTATTAAAAAGCCTATGAATAAGAAAGGTTAAAGGTTAA	110
bbk32 B31Δ(391-486)	TAGAGACTAGCGAGGAAATCTATTAAAAAGCCTATGAATAAGAAAGGTTAAAGGTTAA	110
bbk32 B31 (73-390)	TAGAGACTAGCGAGGAAATCTATTAAAAAGCCTATGAATAAGAAAGGTTAAAGGTTAA	110
bbk32 B31 (391-486)	-----	0
bbk32 B31 (487-600)	-----	0
bbk32 B31 (73-600)	GATTGCTAGAAAGAAAGGCAAAAAGCAAGGTTTCTAGAAAAGAACCGTATATTCAT	165
bbk32 B31 (73-600) ^{mut}	GATTGCTAGAAAGAAAGGCAAAAAGCAAGGTTTCTAGAAAAGAACCGTATATTCAT	165
bbk32 B31Δ(391-486)	GATTGCTAGAAAGAAAGGCAAAAAGCAAGGTTTCTAGAAAAGAACCGTATATTCAT	165
bbk32 B31 (73-390)	GATTGCTAGAAAGAAAGGCAAAAAGCAAGGTTTCTAGAAAAGAACCGTATATTCAT	165
bbk32 B31 (391-486)	-----	0
bbk32 B31 (487-600)	-----	0
bbk32 B31 (73-600)	AGTTTAAAAAAGGACTCTGCTAATAAAAAGCAATTTTACAAAAAATGTAATTT	220
bbk32 B31 (73-600) ^{mut}	AGTTTAAAAAAGGACTCTGCTAATAAAAAGCAATTTTACAAAAAATGTAATTT	220
bbk32 B31 (391-486)	AGTTTAAAAAAGGACTCTGCTAATAAAAAGCAATTTTACAAAAAATGTAATTT	220
bbk32 B31 (73-390)	AGTTTAAAAAAGGACTCTGCTAATAAAAAGCAATTTTACAAAAAATGTAATTT	220
bbk32 B31 (391-486)	-----	0
bbk32 B31 (487-600)	-----	0
bbk32 B31 (73-600)	TAGAGCAAGAAAGTTTAAAAACTGAATTATTAAGAAGCAATCTGAGACTAGAAA	275
bbk32 B31 (73-600) ^{mut}	TAGAGCAAGAAAGTTTAAAAACTGAATTATTAAGAAGCAATCTGAGACTAGAAA	275
bbk32 B31Δ(391-486)	TAGAGCAAGAAAGTTTAAAAACTGAATTATTAAGAAGCAATCTGAGACTAGAAA	275
bbk32 B31 (73-390)	TAGAGCAAGAAAGTTTAAAAACTGAATTATTAAGAAGCAATCTGAGACTAGAAA	275
bbk32 B31 (391-486)	-----	0
bbk32 B31 (487-600)	-----	0
bbk32 B31 (73-600)	AGAAAAAATACAAAAACAACAAGATGAATATAAAGGGATGACTCAAGGAAGTTTA	330
bbk32 B31 (73-600) ^{mut}	AGAAAAAATACAAAAACAACAAGATGAATATAAAGGGATGACTCAAGGAAGTTTA	330
bbk32 B31Δ(391-486)	AGAAAAAATACAAAAACAACAAGATGAATATAAAGGGATGACTCAAGGAAGTTTA	318
bbk32 B31 (73-390)	AGAAAAAATACAAAAACAACAAGATGAATATAAAGGGATGACTCAAGGAAGTTTA	318
bbk32 B31 (391-486)	-----	12
bbk32 B31 (487-600)	-----	0
bbk32 B31 (73-600)	AATTCCCTTAGCGGTGAAAGTGTTGAATTTGGAGGAGCCTATTGAAAGTAATGAAA	385
bbk32 B31 (73-600) ^{mut}	AATTCCCTTAGCGGTGAAAGTGTTGAATTTGGAGGAGCCTATTGAAAGTAATGAAA	385
bbk32 B31Δ(391-486)	-----	318
bbk32 B31 (73-390)	-----	318
bbk32 B31 (391-486)	AATTCCCTTAGCGGTGAAAGTGTTGAATTTGGAGGAGCCTATTGAAAGTAATGAAA	67
bbk32 B31 (487-600)	-----	0
bbk32 B31 (73-600)	TTGATCTTACTATAGATTCTGATTTAAGGCCAAAGAGTTTCCTTACAAGGCATTGC	440
bbk32 B31 (73-600) ^{mut}	TTGATCTTACTATAGATTCTGATTTAAGGCCAAAGAGTTTCCTTACAAGGCATTGC	440
bbk32 B31Δ(391-486)	TTGATCTTACTATAGATTCTGATTTAAGGCCAAAGAGTTTCCTTACAAGGCATTGC	344
bbk32 B31 (73-390)	TTGATCTTACTATAGATTCTGATTTAAGGCCAAAGAGTTTCCTTACAAGGCATTGC	318
bbk32 B31 (391-486)	-----	96
bbk32 B31 (487-600)	-----	26
bbk32 B31 (73-600)	AGGATCAAACCTCTATTTTCATACACTGATGAAATAGAGGAAAGAGGATTATGATCAG	495
bbk32 B31 (73-600) ^{mut}	AGGATCAAACCTCTATTTTCATACACTGATGAAATAGAGGAAAGAGGATTATGATCAG	495
bbk32 B31Δ(391-486)	AGGATCAAACCTCTATTTTCATACACTGATGAAATAGAGGAAAGAGGATTATGATCAG	399
bbk32 B31 (73-390)	AGGATCAAACCTCTATTTTCATACACTGATGAAATAGAGGAAAGAGGATTATGATCAG	318
bbk32 B31 (391-486)	-----	96
bbk32 B31 (487-600)	-----	81
bbk32 B31 (73-600)	TATTATTTAGATGAATATGATGAAGAGGATGAA	528
bbk32 B31 (73-600) ^{mut}	TATTATTTAGATGAATATGATGAAGAGGATGAA	528
bbk32 B31Δ(391-486)	TATTATTTAGATGAATATGATGAAGAGGATGAA	432
bbk32 B31 (73-390)	TATTATTTAGATGAATATGATGAAGAGGATGAA	318
bbk32 B31 (391-486)	-----	96
bbk32 B31 (487-600)	-----	114

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M. SCIENTIFIC PUBLICATIONS

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