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Immunological and Molecular Analyses of the Borrelia burgdorferi OspF Protein Family F

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IMMUNOLOGICAL AND MOLECULAR ANALYSES OF THE *BORRELIA BURGDORFERI* OSPF PROTEIN FAMILY

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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TABLE OF CONTENTS

Page

List of Tables

Page

List of Figures

Page

LIST OF ABBREVIATIONS

Abbreviation	Definition
aa	amino acid
B. burgdorferi	*Borrelia burgdorferi*
bp	base pair
BSA	bovine serum albumin
BSK-H	Barbour-Stoenner-Kelly H
°C	degrees Celsius
CDC	Center of Disease Control and Prevention
cp32	32 kilo-base circular plasmid
dATP	deoxyadenosine triphosphate
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
erp	OspEF related protein
EM	erythema migrans
hr	hour(s)
IgG	immunoglobulin G
IPTG	isopropylthio-β-D-galactoside
I. scapularis	*Ixodes scapularis*

kb	kilobase
kDa	kilodalton
LB	Luria Bertani
LIC	ligase independent cloning
M	Molar
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mM	millimolar
mmol	millimole(s)
mw	molecular weight
mRNA	messenger ribonucleic acid
µg	microgram(s)
µL	microliter(s)
nt	nucleotide
Osp	outer surface protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole(s)
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid

SDS	sodium dodecyl sulfate
sec	second(s)
TAE	tris-acetate-EDTA
TIGR	The Institute for Genomic Research
UHB	upstream homology box
V	Volts
VCU	Virginia Commonwealth University
W	Watts

Abstract

IMMUNOLOGICAL AND MOLECULAR ANALYSES OF THE *BORRELIA BURGDORFERI* OSPF PROTEIN FAMILY

By Emily Tran, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2006

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In North America, *Borrelia burgdorferi* is the primary causative agent of Lyme disease which is a growing health concern. The ability of *B. burgdorferi* to maintain chronic infection indicates that they are capable of immune evasion. A distinguishing characteristic of *B. burgdorferi* is the large number of sequences encoding predicted or known lipoproteins, including outer surface protein F (OspF). This study analyzes the specificity of the humoral immune response to *B. burgdorferi* B31MI OspF proteins during murine and human infection. Immunoblot analyses revealed a temporal

expression of OspF proteins during infection and mapped the immunodominant epitopes which lie within the variable domains. To determine if OspF-related proteins are produced by other isolates, immunoblot analyses were performed using sera collected from mice and humans infected with diverse *B. burgdorferi* strains. Differences in the immunoreactivity profile to OspF proteins were seen among the infection sera tested. To identify the molecular basis of these differences, the *ospF* gene was isolated from several strains, sequenced and evolutionary analyses were conducted. These analyses revealed that OspF proteins show little diversity despite the separate geographic locations from which isolates originated. The high degree of OspF protein conservation seen in isolates from two distinct regions emphasizes the potential for OpsF proteins as vaccinogens or in serodiagnostic assays. Altogether, this study demonstrates the potential contribution of OspF proteins to immune evasion through its temporal expression during infection which may play specific roles at different stages of infection. Studies are underway to determine if inactivation of *ospF* genes through allelic exchange mutagenesis impacts on the pathogenicity of the Lyme disease spirochetes.

INTRODUCTION

I. Lyme Disease

A. Etiology

Lyme disease is the most prevalent vector-borne disease in the United States with approximately 25,000 cases reported in 2005 (www.cdc.gov). It was first recognized in 1976 when a group of children from Lyme Connecticut exhibited symptoms of juvenile rheumatoid arthritis which was later determined to be Lyme arthritis, a late manifestation of Lyme disease [1] . Burgdorfer and colleagues identified the causative agent for Lyme disease, a new spirochetal bacterium, named *Borrelia burgdorferi* [2]. *Borrelia* spirochetes are cork-screw shaped bacteria with periplasmic flagella wrapped around the protoplasmic cylinder allowing for mobility and providing its characteristic shape ([3]. These bacterial cells average 0.2 - 0.5 μm by 4 - 18 μm and can be viewed by dark-field microscopy [3].

B. burgdorferi is maintained in a natural cycle involving vertebrate hosts and ticks, in which humans become inadvertently infected thereby making Lyme disease a zoonosis [4]. The vectors are the deer tick, *Ixodes scapularis* in the northeastern and north central US and *Ixodes pacificus* in the western US which acquire and transmit Lyme disease spirochetes by feeding on a variety of small mammalian hosts [5] [6]. During the 20th century, conditions became favorable for exposure of humans to *B. burgdorferi* infection when increasing populations began to move away from cities into rural woodland areas which simultaneously saw an increase in deer population. Since surveillance for Lyme disease was begun in the US by the Center for Disease Control and

Prevention, the number of reported cases has been increasing steadily.

B. Clinical manifestations and pathogenesis

After transmission of the spirochete, human Lyme disease progresses through three stages: localized, disseminated and persistent infection. Each stage is distinguished by different clinical manifestations. The first stage is initiated when an infected tick feeds on a human host, depositing motile *Borrelia* spirochetes through the dermis and causing a characteristic expanding "bull's eye" rash which is seen in 70-80% of cases [7]. Flu-like symptoms can also accompany erythema migrans such as malaise, fatigue, headache, arthralgia and myalgia [8]. Within days to weeks after initial infection, *B. burgdorferi* can disseminate to diverse organs. Spirochetes have been recovered from blood, cerebrospinal fluid and tissues including myocardium, retina, muscle, bone, spleen, liver, meninges and brain [9]. The third stage of Lyme disease is a chronic infection which may involve the cardiological, neurological, musculoskeletal or dermatological systems [10-12]. In the US, 60% of patients that are untreated suffer from Lyme arthritis which is characterized by intermittent attacks of swelling and pain that occur in the large joints, particularly the knee [13].

C. Diagnosis and treatment of Lyme disease

Diagnosis of Lyme disease can be difficult because its symptoms can also be manifestations of other diseases. For instance, fever, malaise and myalgia can be mistaken for viral infections. Therefore, the history of exposure to areas where Lyme disease is endemic must be also considered in the diagnosis. Serological tests are available and include enzyme-linked immunosorbent assays (ELISA) and Western blots

[14]. However, these tests which detect anti-borrelial antibodies are not useful until the later stages of the disease.

Lyme disease can be treated effectively with several antibiotics. For early localized or disseminated infection, treatment with doxycycline for 14 to 21 days is recommended [15]. For treatment of persistent infection, including Lyme arthritis, oral doxycycline or intravenous ceftriaxone are administered for several weeks [16]. Although antibiotic therapy is highly successful, there exists a small number of cases of nonrespondents, emphasizing the need for research into alternative treatments and improved diagnostic assays [12].

II. *Borrelia burgdorferi* genome

The *B. burgdorferi* strain B31MI is the first spirochete whose genome was completely sequenced [17]. Its genome is comprised of a small linear chromosome of approximately one megabase and 21 extrachromosomal elements, the largest number known for any bacterium. The extrachromosomal elements include 9 circular and 12 linear plasmids which add an additional 610,694 bp to the genome [18]. Some of these plasmids are indispensable and could be thought of as mini-chromosomes. Another unique aspect of the *B. burgdorferi* genome is the large number of genes that encode predicted or known lipoproteins including outer surface protein F (OspF). Most of these lipoproteins are predicted to be surface-exposed and consequently are a main research focus because of their potential for direct involvement in the host and tick interaction. These lipoproteins may also play a role in immune evasion; some have been shown to be differentially expressed while others can undergo antigenic variation [19]. In contrast to

the vast number of genes encoding lipoproteins, sequence analyses reveal there are very few proteins for biosynthesis. Hence, *B. burgdorferi* strongly relies on its host for its growth requirements.

III. Outer surface protein F

The survival of *B. burgdorferi* in nature is dependent upon its ability to persist in the enzootic cycle. The outer surface of *Borrelia* spirochetes has been a main research focus because of its diverse protein expression which may play a role for survival in the varied mammal and tick environments. The focus of this study is on the outer surface protein F (OspF) protein family and to elucidate its role in pathogenesis. In *B. burgdorferi* B31MI, the *ospF* gene family contains three paralogs - bbm38, bbo39 and bbr42 (Table 1). *ospF* genes along with *ospE* and *elp* genes have been collectively referred to by some as *erp* (OspEF-related protein) genes [20]. *B. burgdorferi* B31 MI carries 12 highly divergent genes that have been included in the Erp group [21-25]. However, it is now evident that this diverse group of genes actually constitutes three distinct gene families that are transcriptionally responsive to different environmental signals [23,26-29] and differentially expressed [21,27,28,30-34]. One published study suggested that these diverse proteins may still share a common functional role and be key players in factor H-mediated complement evasion [35]. However, the literature shows strong agreement that OspF and Elp proteins do not bind to factor H [35-41]. Therefore, the specific role of OspF in pathogenesis and immune evasion necessitates further investigation.

The *ospF* genes - *bbm38*, *bbo39* and *bbr42* are carried by circular plasmids of 32 kB (cp32); specifically, cp32-6, cp32-7 and cp32-4 respectively (Table 1)[18,24] . Some strains can carry all nine cp32s which are closely related and provide an example of genetic redundancy found in Lyme disease spirochetes [23,26,42,43]. Recent studies have shown evidence to support the hypothesis that cp32s are prophages which could have important implications for the role of OspF in pathogenesis [44-48]. The discovery of bacteriophage-like particles observed by electron microscopy in spirochetal cultures first suggested the presence of bacteriophages [44]. Additional evidence for cp32s as prophages include Southern blot analysis which revealed hybridization of bacteriophage-specific probes to cp32 DNA [44], sequence analysis which showed homology of cp32 genes to bacteriophage proteins [45-48] and treatment of bacterial cultures with a DNA alkylating agent, 1-methyl-3-nitroso-nitroguanidine which demonstrated the existence of a late phage operon [49]. The implication that cp32s are prophages is important because a number of virulence factors have been shown to be encoded by prophages [50]. For example, bacterial toxins have been found to be encoded in the genomes of bacteriophages such as the diphtheria toxin by *Corynebacterium diphtheriae* and the botulinum toxin by *Clostridium botulinum*. Consequently, the recent findings that the *ospF*-carried cp32s may be prophages further supports the role of OspF as a virulence factor in Lyme disease pathogenesis.

BBM38, BBO39 and BBR42 are lipoproteins of 29.4, 26.2 and 25.4 kDa respectively (Table 1). The OspF proteins have been reported to be surface-exposed in *B. burgdorferi* [22] indicating their potential role in host-pathogen interactions during

Table 1. Description of *ospF* gene family carried by *B. burgdorferi* B31MI.

Designation	Plasmid	bp	aa	pI	MW (kDa)
bbm38 (erpK)	M (cp32-6)	768	256	5.34	29.4
bbo39 (erpL)	O (cp32-7)	687	229	6.58	26.1
bbr42 (ospF)	R (cp32-4)	672	224	8.20	25.4

infection. It has been clearly demonstrated that OspF proteins are immunogenic [20,51,52] and elicit a strong antibody response during mammalian infection [20,53-55]. Although *ospF* loci have been shown to be genetically stable during infection [28,34], OspF proteins may use a different mechanism for generating antigenic diversity to evade the immune system. It has been shown that BBM38, BBO39 and BBR42 are antigenically distinct and elicit an antibody response at different times during infection [28]. BBO39 elicits an early antibody response, whereas BBR42 and BBM38 do not elicit an antibody response until late infection [28]. Therefore, antigenic diversity can be generated in populations of spirochetes by changes in temporal patterns of protein expression. Altogether, these studies suggest that OspF plays a role in pathogenesis and is a virulence factor.

IV. Research Objectives:

To investigate if OspF plays a role in pathogenesis and is a potential target for vaccine or diagnostic assay development, the following aims were developed for this study:

1. Identify epitopes of OspF that are exposed during infection and analyze the temporal pattern and specificity of the humoral immune response to OspF paralogs and epitopes.

Full-length and sub-fragments of OspF paralogs will be expressed as recombinant proteins, fractionated under denaturing and non-denaturing conditions and immunoblotted. The immunoblots will be probed with mouse infection sera obtained from different time points during course of infection. These analyses will help to identify

whether linear or conformational epitopes of OspF are presented during infection and determine whether immunodominant epitopes lie within the conserved or variable domains of the protein. Furthermore, these analyses will determine if the humoral immune response to immunodominant epitopes exhibits a temporal pattern.

2. Assess the specificity of the humoral immune response to B31MI OspF proteins in mice and humans with Lyme disease.

Recombinant proteins will be produced for each OspF paralog and used in immuoblot analyses. To analyze the humoral response to OspF proteins in infected mammalian hosts, sera collected from mice infected with heterologous *B. burgdorferi* and from human Lyme disease patients will be used to screen immunoblots. Since multiple strains of *B. burgdorferi* have been isolated from ticks, natural hosts and Lyme disease patients [56-59], it was important to determine whether B31MI OspF proteins are recognized by antibodies from mice and humans infected with heterologous *B. burgdorferi*. These analyses will outline the protective range of B31MI OspF proteins in vaccines and the applicability of these proteins in serodiagnostic assays.

3. Assess OspF sequence heterogeneity through PCR and sequence analyses of *ospF* genes identified from isolates of human Lyme disease patients collected from two distinct geographic regions.

To examine sequence divergence of ospF genes, clinical isolates collected from two distinct geographic locations will be used as template for PCR analysis. PCR amplicons will be selected for sequencing and for comparative analyses of OspF proteins through generation of sequence alignments and phylogram. These analyses will

demonstrate whether Lyme spirochetes from distinct geographic locations have proteins homologous to B31MI OspF proteins or whether sequence variation is localized to a specific region. The assessment of OspF diversity or conservation among *B. burgdorferi* infecting humans will be critical in evaluating the potential use of B31MI OspF proteins in serodiagnostic assays or as vaccinogens.

MATERIALS AND METHODS

I. Gene nomenclature and bacterial cultivation

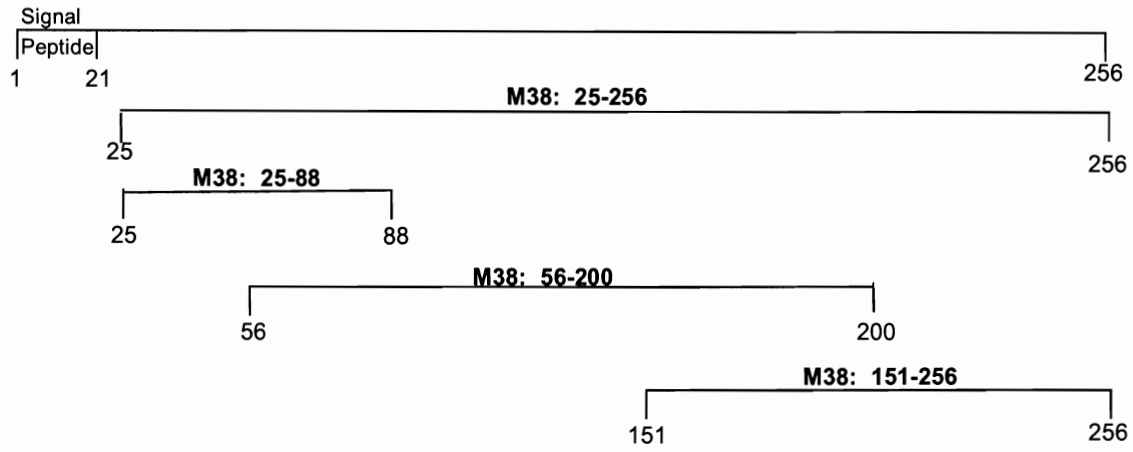
The focus of this research was on the OspF proteins of *B.burgdorferi* B31MI. The complete genome sequence has been determined for this strain and is available on the TIGR website (www.tigr.org)[17]. Although other nomenclature for the OspF proteins may be found throughout the literature, this study uses the TIGR designations given to the OspF protein family which consists of three members: BBM38, BBO39 and BBR42. "BB" designates the bacteria of interest, *B. burgdorferi,* the third letter designates the specific cp32 plasmid which encodes the specific *ospF* gene and the number corresponds to the relative open reading frame on the specific plasmid. *B. burgdorferi* B31MI was cultivated in BSK-H complete media (Sigma) at 33°C.

II. Generation of recombinant OspF proteins

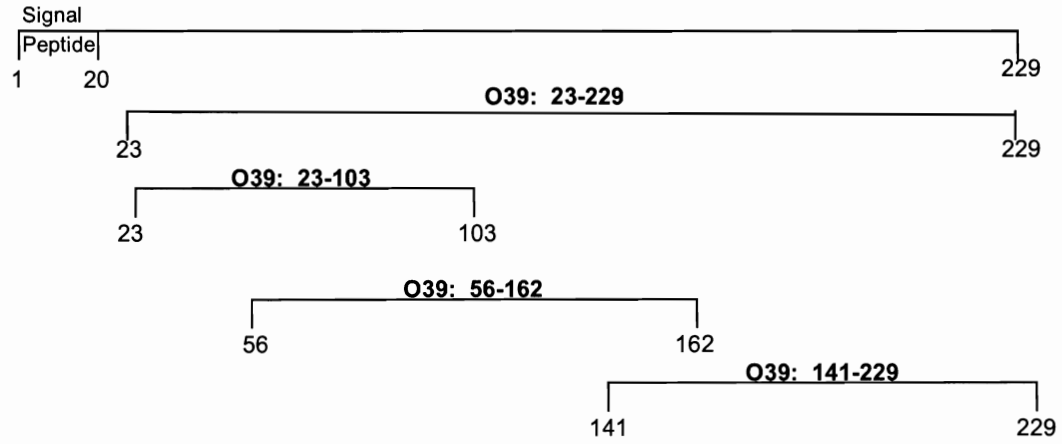
To generate recombinant full-length BBM38, BBO39, BBR42 and OspF sub-fragments, paralog-specific PCR primers were used to amplify each gene from B31 MI genomic DNA. The full-length OspF recombinant proteins exclude the signal peptide and the first three amino acids of the coding region for each OspF paralog. A schematic of the constructs are depicted in Figure 1. Primers were designed with tail sequences complementing the single-stranded overhangs of the pET-32 Ek/LIC vector to allow for ligase-independent cloning (LIC) of the amplicons. Cloning was performed as follows.

Figure 1. Schematic diagrams of the OspF constructs.
The numbers represent the amino acid positions of the unprocessed B31MI OspF proteins. All constructs were generated as S-Tag fusion proteins in *E. coli.*

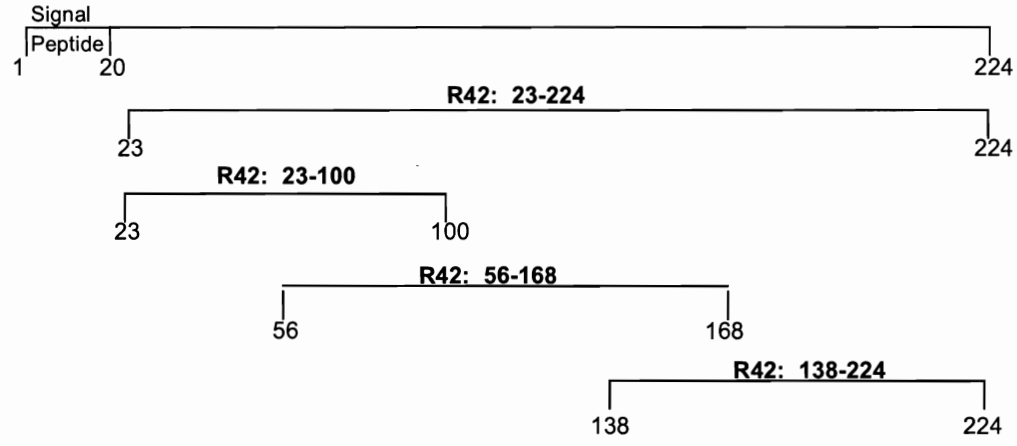
BBM38



BBO39



BBR42



The PCR amplicons were treated with T4 DNA polymerase in the presence of dATP as described by the manufacturer (Novagen) to yield the complementary single-stranded overhangs. To summarize, the 3' to 5'-exonuclease activity of the T4 DNA polymerase digests the amplicon until it reaches the first A-residue in the primers which corresponds to the first base of the start codon and the last base of the stop codon. When these bases are encountered by the DNA polymerase, the 5'-3' polymerase activity counteracts its exonuclease activity resulting in an amplicon with single-stranded overhangs that complement the vector. The treated amplicon and the pET-32 Ek/LIC vector were then annealed and transformed into *E.coli* NovaBlue Singles Competent Cells (Novagen), using a standard transformation protocol. Cells were plated onto LB media containing ampicillin (50μg/mL) and incubated at 37° overnight. To identify colonies harboring the correct recombinant plasmid, colonies were screened using boiled cell lysate as template for PCR. The select recombinants carrying the appropriate plasmid were inoculated into 100 mL of LB containing ampicillin (50 μg/mL) and cultivated at 37°C with shaking (300 rpm) overnight. Plasmids were isolated using the QiaFilter midi plasmid isolation kit (QIAGEN) and *ospF* constructs were verified by automated DNA sequencing (MWG Biotech).

To express the *ospF* constructs as recombinant proteins, purified plasmid was transformed into *E.coli* NovaBlue BL21(DE3) competent cells (Novagen), using a standard transformation protocol. Colonies were screened by PCR analysis as described previously to identify those harboring the correct recombinant plasmid. Selected

colonies were then inoculated into 5 mL of LB containing ampicillin and cultivated overnight at 37°C with shaking (300 rpm). The next morning, 95 mL of LB-ampicillin were added to the cultures and grown to an A_{600} of 0.6. Isopropylthiogalactoside (IPTG) was added to each culture for a final concentration of 1mM to induce protein expression for 3 hrs. The expressed proteins represent a fusion protein with a 62 amino acid S-Tag and His-Tag fused to its N-terminus, thereby adding approximately 17 kDa to the MW of each r-protein. The S-Tag and His-Tag allow for detection and purification of the r-proteins.

The recombinant proteins were purified from IPTG-induced cultures using the His-Bind Column Chromatography kit (Novagen) which selects for His-Tag fusion proteins by metal affinity chromatography. The r-proteins were eluted from columns and dialysed using Slide-a-lyzer dialysis cassettes (Pierce) against PBS buffer overnight and replaced with fresh buffer two additional times for two hours each. Using colorimetric detection, proteins were quantified by the bicinchoninic acid protein assay kit (Pierce) as directed by the manufacturer.

III. Immunoblot analyses

The purified r-proteins were fractionated by SDS-PAGE in 12.5% Criterion Precast gels at a constant voltage of 200 volts for 55 mins. The proteins were transferred from the gels onto PVDF membrane by electroblotting using the Trans-blot system (Bio-Rad) at a voltage of 100 for 1 hr. Immunoblots were blocked in blocking buffer (PBS, 0.2% Tween and 5% nonfat dry milk) for 1 hr and then incubated with a primary

antibody, also for 1 hr. Primary antibodies included 1:1000 dilutions of anti-*B. burgdorferi* B31 MI, -297, -N40,-B331, -LDP84, -LDP73, -LDP89 or 1:400 dilutions of patient infection sera. The anti-S-Tag antibody (HRP-conjugated; Novagen), which detects the N'-terminal S-Tag carried by r-proteins was used at a dilution of 1:5000. Immunoblots were then washed 3 times with wash buffer (PBS, 0.2% Tween), followed by incubation with secondary antibodies for 1 hr. For the analysis of IgG responses, ImmunoPure goat anti-mouse IgG (H+L) peroxidase-conjugated or ImmunoPure goat anti-human IgG peroxidase-conjugated were used at dilutions of 1:40000 and 1:10000, respectively. After incubation was complete, the wash procedure described as above was repeated. The secondary antibodies were detected by chemiluminescence using the Supersignal West Pico Stable Peroxide and the Supersignal West Pico Luminol/Enhancer solutions (Pierce) and the immunoblots were exposed to film.

IV. Non-denaturing polyacrylamide electrophoresis:

To preserve native conformation, r-proteins were fractionated by electrophoresis under non-denaturing conditions on 12.5% Bio-Rad Criterion gels. Hence, both the loading and running buffers lacked SDS, a denaturing detergent. The running buffer consisted of 0.19M glycine and 0.04M Tris-base and the loading buffer consisted of 0.125M Tris-HCl (pH 6.8), 20% glycerol and 1% bromophenol blue. The proteins were then electroblotted onto PVDF membrane as previously described.

V. Polymerase chain reactions (PCR)

Specific *ospF* genes and *ospF* sub-fragments were amplified using isolated

genomic DNA (50ng) or boiled cell lysate templates with primers listed in Table 2. Genomic DNA was isolated as previously described [60]. PCR was performed with *Taq* polymerase (Promega) or with GoTaq (Promega) for 35 cycles in an MJ Research PTC100 thermal cycler with a hot bonnet. Reaction volumes were 30 μL and final primer set concentrations were 1 pmol primer pair per μL. Cycling conditions were as follows: 1 cycle of 5 min, 94°C, 1 min of 50°C and 1.5 min of 72°C. The resulting amplicons were analyzed by agarose gel electrophoresis.

VI. Reverse transcriptase PCR

RNA isolated from *in vitro* cultures of *B. burgdorferi* grown at 33° C was treated with DNase I (37°C, 1h, Invitrogen) followed by inactivation of the DNase with 25mM EDTA (70°C, 10min). cDNA was generated by using Superscript II RT (Invitrogen) with 1 μg of RNA, 1.5 pmol of each specific primer and reagents supplied by the manufacturer, followed by incubation at 42°C for 50 min in the thermocycler. The RT was inactivated by incubation at 70°C for 15 min. Reactions without RT served as the negative controls and also verified that DNA was absent in the RNA preparations. The cDNA was used as the template for amplification in PCR with *Taq* polymerase and the resulting amplicons were analyzed by agarose gel electrophoresis.

VII. Construction of plasmids for inactivation of the *ospF* genes

To inactivate the *ospF* genes *bbm38*, *bbo39* and *bbr42,* the upstream region of the *ospF* coding sequence was amplified with primers designed to introduce *AatII* and *AgeI* restriction sites at the 3' end of the amplicon. Similarly, the downstream region of the

Table 2. Primer sequences and target sites.

PRIMER	SEQUENCE	TARGET SITE
BBM38 F1	GACGACGACAAGATTTACGCAAGTGGTGAAGATGTA	ntd 72-93 of BBM38 on cp32-6
BBM38 F2	GACGACGACAAGATTGGAGATTTTAAAAAACCAGAA	ntd 165-186
BBM38 F3	GACGACGACAAGATAGCTTTAAATGAAAAAATAGAG	ntd 459-480 of BBM38 on cp32-6
BBM38 R1	GAGGAGAAGCCCGGTTTATTTTAATTTTTGATCTTCAGCA	ntd 270-291 of BBM38on cp32-6
BBM38 R2	GAGGAGAAGCCCGGTTTATATTCCAACTTTCCCTTG	ntd 583-600 of BBM38 on cp32-6
BBM38 R3	GAGGAGAAGCCCGGTTTATTCTTTTTTATTAGAATCTTTAG	ntd 746-771 of BBM38 on cp32-6
BBO39 F1	GACGACGACAAGATTTATGCAAGTGGTGAAAATCTA	ntd 66-87 of BBO39 on cp32-7
BBO39 F2	GACGACGACAAGATAGGATTTTTAGAAATTCTAGAGAC	ntd 165-188 of BBO39 on cp32-7
BBO39 F3	GACGACGACAAGATAGCTTTACAAGAGGCC	ntd 420-435 of BBO39 on cp32-7
BBO39 R1	GAGGAGAAGCCCGGTTTACTCAGAATATGTTTCAATAG	ntd 290-309 of BBO39 on cp32-7
BBO39 R2	GAGGAGAAGCCCGGTTTACCCAGTTGAAGTATCTACTTG	ntd 463-483 of BBO39 on cp32-7
BBO39 R3	GAGGAGAAGCCCGGTTTATTCTTTTTTATCTTCTTC	ntd 670-690 of BBO39 on cp32-7
BBR42 F1	GACGACGACAAGATTGATGTAACTAGTAAAGATTTA	ntd 66-87 of BBR42 on cp32-4
BBR42 F2	GACGACGACAAGATAGGATTTTTAGAAATTTTAGAGAC	ntd 165-188 of BBR42 on cp32-4
BBR42 F3	GACGACGACAAGATAGCTTTACAAGAGGCT	ntd 411-426 of

		BBR42 on cp32-4
BBR42 R1	GAGGAGAAGCCCGGTTTACCCAGAATATGTTTCAATAG	ntd 281-300 of BBR42 on cp32-4
BBR42 R2	GAGGAGAAGCCCGGTTTATCCAGTTGCAGATTCAGCTTG	ntd 454-474 of BBR42 on cp32-4
BBR42 R3	GAGGAGAAGCCCGGTTTATTCTTTTTTACCTTCTACAG	ntd 653-675 of BBR42 on cp32-4
UPM38 KO F	GACGACGACAAGATGGCCTGATTTTTTAAGAAGAATTTT	ntds 25245-225268 on cp32-6
UPM38 KO R	GAGGAGAAGCCCGGTACCGGT(CTA)GACGTCAAGTCTTAAATCTAACGC	ntds 26227-26244 on cp32-6
DWM38 KO F	GACGACGACAAGATGGACGTCAAAAATTTTTTTTACAATC	ntds 27016-27034 on cp32-6
DWM38 KO R	GAGGAGAAGCCCGGTACCGGTAACCCCCAATTTATC	ntds 28001-28015 on cp32-6
UPO39 KO F	GACGACGACAAGATGAGAAGAATTTTAAATTTTTTAG	ntds 25152-25173 on cp32-7
UPO39 KO R	GAGGAGAAGCCCGGTACCGGT(CTA)GACGTCAAGTTACTCCTAAAATCC	ntds 26134-26151 on cp32-7
DWO39 KO F	GACGACGACAAGATGGACGTCCAGTGGTATCTTTTAAGA	ntds 26842-26859 on cp32-7
DWO39 KO R	GAGGAGAAGCCCGGTACCGGTAAATTAGGCTCCTTTTCT	ntds 27825-27842 on cp32-7
UPR42 KO F	GACGACGACAAGATGATGGAGTAACTTATG	ntds 25853-25867 on cp32-4
UPR42 KO R	GAGGAGAAGCCCGGTACCGGT(CTA)GACGTCAAAGATTGTCTCCTA	ntds 26838-26852 on cp32-4
DW R42 KO F	GACGACGACAAGATGGACGTCAGAAAAAGTTTTTATTGTTAATCG	ntds 27528-27551 on cp32-4
DW R42KO R	GAGGAGAAGCCCGGTACCGGTTTATACTTTTCTATTATATGC	ntds 27909-27929 on cp32-4
pKFSS1-AatII 3'F	GGCGAGATCACCAAGGTAGTC	ntds 7-26 of spectinomycin/streptomycin resistance cassette
aad I-5'-R	TCCTTGAAGCTCGGGTATTA	ntds 1179-1199 of spectinomycin/streptomycin resistance cassette

spec/strep F	TGATTTGCTGGTTACGGTGA	ntds 560-579 of spectinomycin/streptomycin resistance cassette
spec/strep R	ATTTGCCGACTACCTTGGTG	ntds 1187-1206 of spectinomycin/streptomycin resistance cassette

ospF coding sequence was amplified with primers that included *AatII* restriction site at the 5' end and *AgeI* restriction site at the 3' end. The upstream regions of *bbm38*, *bbo39* and *bbr42* were approximately 1000 bp. Similarly, the downstream regions of *bbm38* and *bbo39* were also approximately 1000 bp whereas the downstream region of *bbr42* included only 402 bp. Each amplicon was cloned into the pET-32 Ek/LIC vector and transformed in *E. coli* strain Novablue. Transformants were selected for the correct recombinant plasmid and were grown overnight in LB broth containing ampicillin followed by plasmid purification using the Midi Plasmid Purification kit (Qiagen). Each plasmid preparation containing either the upstream region or the downstream region was digested with the restriction enzymes *AatII* and *AgeI*, followed by gel electrophoresis. The upstream-containing plasmid was purified using a PCR purification kit (Qiagen) as only a very small DNA fragment was released by digestion. The downstream fragments were gel extracted from the remaining plasmid sequence and purified. The isolated downstream fragments with its flanking restriction sites allowed for directional insertion and T4 ligation to the cut upstream plasmid. The resulting plasmid was transformed in *E.coli* strain Novablue. The transformants were selected for the combined upstream-downstream insert and a positive clone was used for production and purification of plasmid.

To allow for antibiotic selection of the upstream-downstream containing plasmid, a spectinomycin-streptomycin resistance cassette was selected. This resistance cassette included AatII restriction sites on its ends and was extracted from the pKFSS1/AatII

spectinomycin/streptomycin plasmid by digestion with AatII. The 1.2 kB resistance cassette was then purified using gel extraction. The combined upstream-downstream plasmid was also digested with AatII and purified by a PCR purification kit to allow for T4 ligation of the purified spectinomycin-streptomycin resistance cassette between the upstream and downstream fragments. The resultant plasmid was transformed into *E.coli* Novablue strain. The transformants were screened with primer sets to ensure insertion of the resistance cassette and to assess the orientation of the cassette using primers amplifying outwards from the 5' and the 3' ends of the cassette. The selected transformants which were positive for the upstream-resistance cassette-downstream plasmids were propagated in LB with spectinomycin (50μg/mL), purified using the Midi Kit and linearized by digestion with *MscI*.

VIII. Transformation of *B. burgdorferi* 5A4 and 5A13

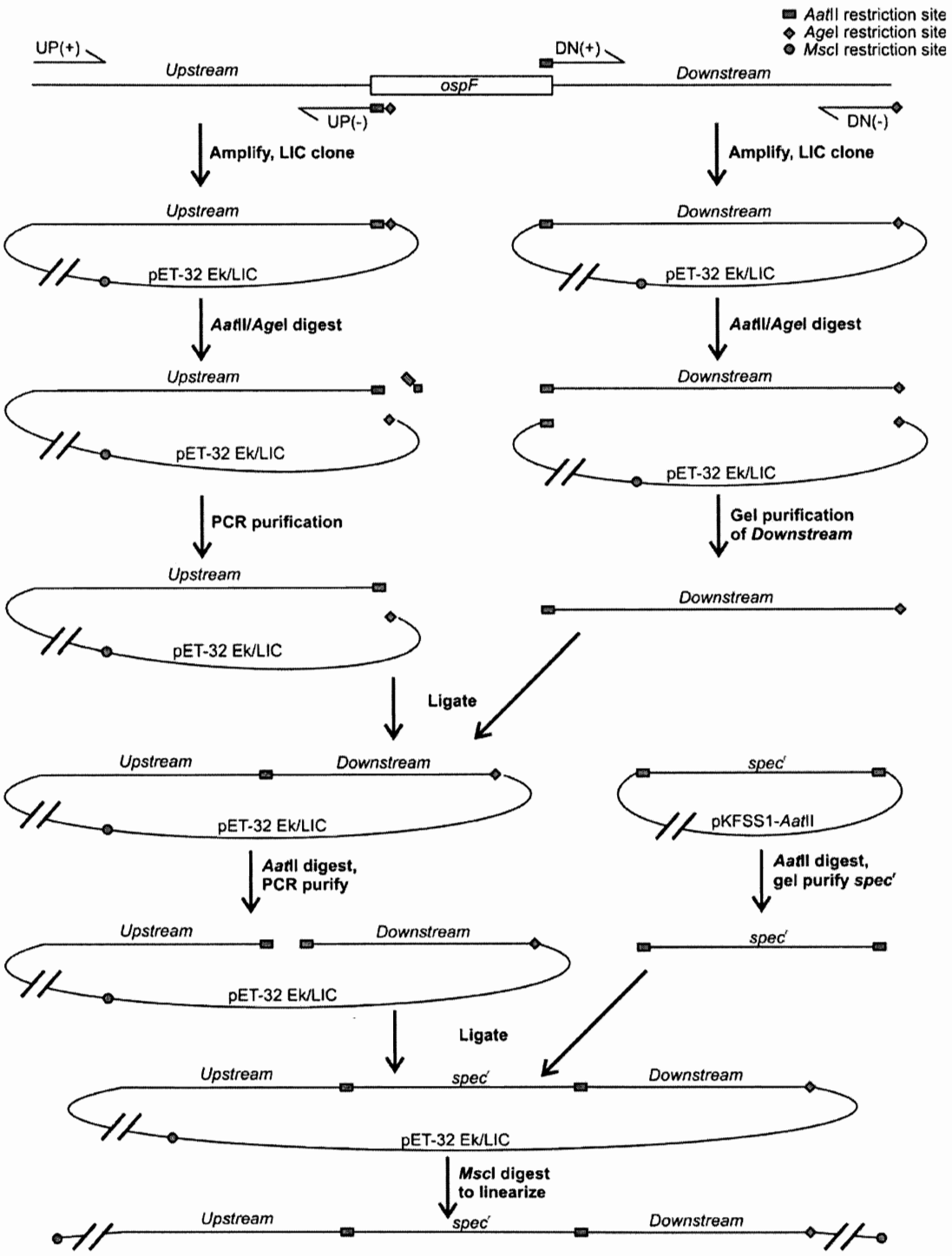
100 mL of log-phase cultures of *B. burgdorferi* 5A4 and 5A13 were pelleted at 4000g for 20 mins at 4°C and washed once with 10mL of cold dPBS (PBS without divalent cations) and pelleted at 3000g for 10 mins at 4°C. Pellets were washed twice with 3 mL of cold EPS (272 mM sucrose, 15% glycerol) with an intermittent spin of 2000g for 10 min at 4°C. The washed pellet was then resuspended in 150 μL of cold EPS and 50μL aliquots of the suspensions were prepared. 10-15μg of the linearized plasmid DNA containing the upstream-spectinomycin/streptomycin resistance cassete-downstream *ospF* regions were mixed with each 50 μL cell suspension on ice and transferred to a 0.2 cm electroporation cuvette (BioRad) at 4°C. One pulse was delivered

from a Gene Pulser with Pulse Controller (BioRad) set at 2.5kV, 25μF and 200Ω, producing a time constant of 4 to 5 ms. 1 mL of BSK media was immediately added to the cuvette and the cells were transferred to 9mL of BSK media. Cultures were incubated at 33°C for 24 hrs followed by the addition of streptomycin antibiotic (50μg/mL). Cultures were incubated at 33° C for three weeks.

IX. Subsurface agarose plating

Subsurface agarose plating was used to obtain clones of the transformed *B. burgdorferi* 5A4 or 5A13. Cultures were prepared in 1 mL of BSK-streptomycin (50μg ml^{-1}) at dilutions of 1×10^{-2}, -10^{-4} and -10^{-6} and then added to 10mL of a 3:2 ratio of 2% GTG agarose (Seakem) and BSK-streptomycin. The diluted culture mixtures were then overlaid onto petri dishes containing 25 mL of a 2:1 mixture of BSK-streptomycin and 2% GTG agarose. After solidification of agarose, plates were incubated at 33° with CO_2. White colonies were apparent within 12-14 days post-inoculation. Colonies were isolated using sterile Pasteur pipettes and inoculated into screw-cap tubes containing 2 mL of BSK-streptomycin and incubated at 33°C for 7 days at which point clones were transferred into 15mL BSK-streptomycin.

Figure 2. Flow chart depicting the construction of plasmid for inactivation of the *ospF* genes.


AatII restriction site
AgeI restriction site
MscI restriction site
UP(+)
DN(+)
Upstream
ospF
Downstream
UP(-)
DN(-)
Amplify, LIC clone
Amplify, LIC clone
Upstream
pET-32 Ek/LIC
Downstream
pET-32 Ek/LIC
AatII/AgeI digest
AatII/AgeI digest
Upstream
pET-32 Ek/LIC
Downstream
pET-32 Ek/LIC
PCR purification
Gel purification of Downstream
Upstream
pET-32 Ek/LIC
Downstream
Ligate
Upstream
Downstream
pET-32 Ek/LIC
spec'
pKFSS1-AatII
AatII digest, PCR purify
AatII digest, gel purify spec'
Upstream
Downstream
pET-32 Ek/LIC
spec'
Ligate
Upstream
spec'
Downstream
pET-32 Ek/LIC
MscI digest to linearize
Upstream
spec'
Downstream

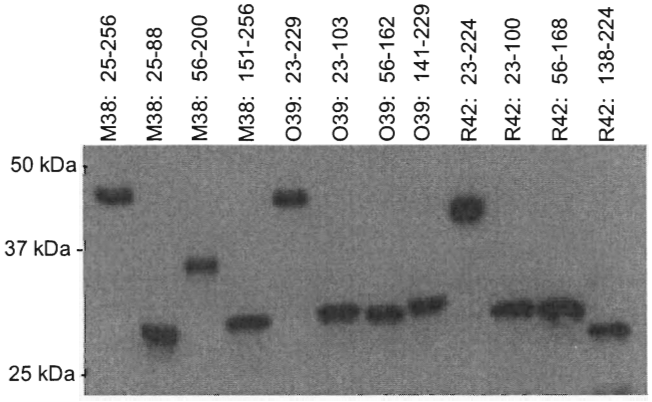
RESULTS

I. Immunoblot analysis of the temporal pattern of the humoral immune response to OspF full-length and subfragment r-proteins during infection of C3H-HeJ mice with *B. burgdorferi* B31MI.

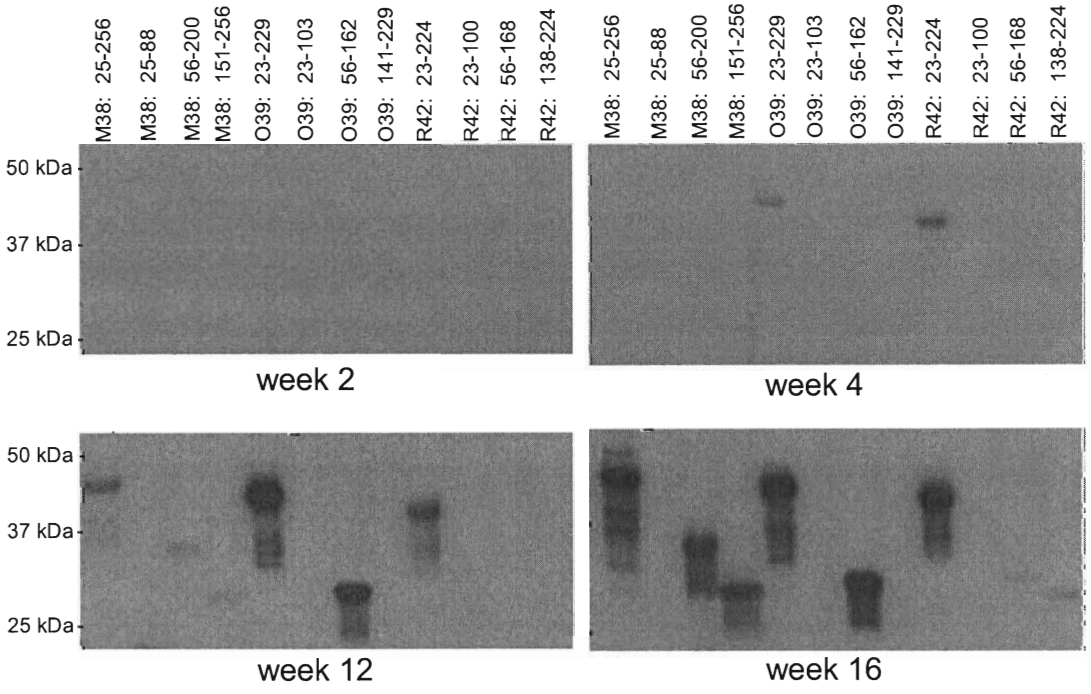
An important step in defining the role of OspF in the host-pathogen interaction is to analyze the specificity and temporal pattern of the humoral immune response to OspF paralogs and to identify the immunodominant epitopes presented during infection. To accomplish this aim, full-length *ospF* genes *bbm38*, *bbo39* and *bbr42* without the signal peptide and constructs spanning the N-terminal, central and C-terminal regions of each gene were PCR-amplified, LIC-cloned and expressed as r-proteins in *E. coli*. To verify r-protein expression, an immunoblot was probed with an HRP-conjugated S-Protein directed against the S-Tag portion of the r-proteins (Figure 3A). The appropriate sizes were detected for the r-proteins thereby confirming successful expression. In addition, equivalent amounts of r-proteins were observed relative to one another which was important for evaluation of the subsequent data. To analyze the temporal pattern of the humoral immune response to OspF protein family members, identical immunoblots were screened with mouse infection sera (Figure 3B). Mice were infected with *B. burgdorferi* B31MI and sera were collected at weeks 2, 4, 12 and 16 post-infection. These analyses revealed a strong IgG response by week 4 to the full-length BBO39 and BBR42 whereas no response was detected to BBM38. At week 12, a response was detected to the full-

Figure 3. Immunoblot analysis of full-length and truncated OspF paralog recombinant proteins expressed in *E. coli.*
The full-length and truncated genes of each *ospF* paralog were PCR-amplified and LIC-cloned. The *E. coli* cultures were induced to express the r-proteins with IPTG and the cell lysates were fractionated by SDS-PAGE and immunoblotted. Identical membranes were screeened with S-protein HRP and infection-derived sera collected from mice. The expression and relative loadings of the r-proteins was demonstrated by immunoblot analysis using S-protein-HRP. The specificity and the temporal pattern of the humoral response during murine infection towards the full-length and truncated OspF r-proteins was demonstrated by screening with sera collected from mice infected with *B. burgdorferi* B31MI at different time points during infection (as indicated on the figure). Molecular mass markers are shown.

A. S-Protein HRP conjugate



B. B31 MI Infection Sera



length BBM38 and the central fragment of BBO39. By week 16 of infection, immunoblot analyses revealed a strong IgG response to all full-length OspF proteins and to the central fragments of BBM38 and BBO39.

The absence of an early response to BBM38 in mice suggests that this protein may not be expressed during early infection. An alternative explanation is that BBM38 is expressed but is weakly immunogenic. However, earlier studies have demonstrated that OspF related proteins are immunogenic in mice [20,28,51,52]. In addition, immunoblot analyses revealed that OspF epitopes recognized during infection may be located within the central regions of both BBO39 and BBM38 as well as the C-terminal region of BBM38 which were immunoreactive at later stages during infection. To pinpoint the specific amino acids involved in presentation during infection, shorter constructs of these specific regions for BBO39 and BBM38 could be designed. Concerning the immunodominant epitopes of BBR42, it was seen that none of the BBR42 constructs elicited an IgG response at any of the time points tested. Therefore, additional constructs spanning different regions of the protein could be designed in future studies to determine the immunodominant exposed epitopes of BBR42.

II. Binding of OspF-targeting Abs elicited during infection in mice may not require conformational or discontinuous epitopes for optimal binding.

To determine if the Ab response to full-length and fragmented OspF r-proteins targets linear or conformational epitopes, non-denaturing electrophoresis was employed. Epitopes for Ab binding can be classified as linear or conformational. Linear epitopes

involve Abs binding to a stretch of a linear amino acid sequence that is part of the polypeptide chain. Conformational epitopes constitute stretches of primary sequence which are distant from one another but are brought in close proximity by secondary or tertiary folding to form a conformational epitope. Therefore, full-length and fragmented OspF r-proteins were fractionated under non-denaturing conditions to preserve the secondary and tertiary structures of the r-proteins, permitting Ab binding to potential conformational epitopes. Following fractionation, r-proteins were immunoblotted and screened with HRP-conjugated S-protein and mouse infection sera. Unlike SDS-PAGE electrophoresis whereby proteins migrate based on molecular mass, non-denaturing electrophoresis allow for mobility based on the charge and size of the proteins. The immunoblot was screened with S-protein HRP which was necessary for visualizing the relative migration patterns and for demonstrating efficient resolution of the r-proteins. (Figure 4A). Screening with mouse infection sera also revealed the same pattern as seen with the immunoblot analyses under denaturing conditions (Figure 4B). Hence, the r-OspF proteins and their constructs fractionated under non-denaturing conditions provide evidence that the exposed immunodominant epitopes may not be dependent upon conformation.

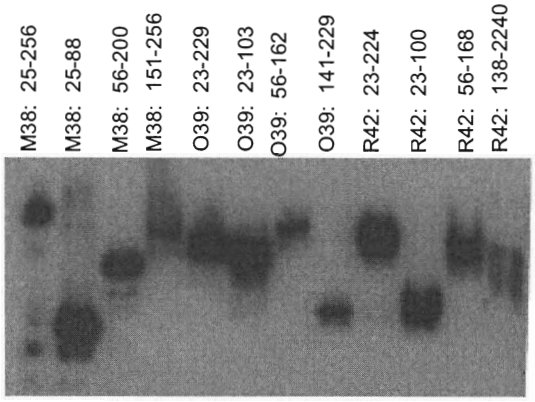
III. Analysis of the antibody response to OspF protein family members in mice infected with heterologous *B.burgdorferi* strains.

To determine if other *B. burgdorferi* isolates express antigenically related proteins, immunoblots of the full-length OspF r-proteins were generated and screened

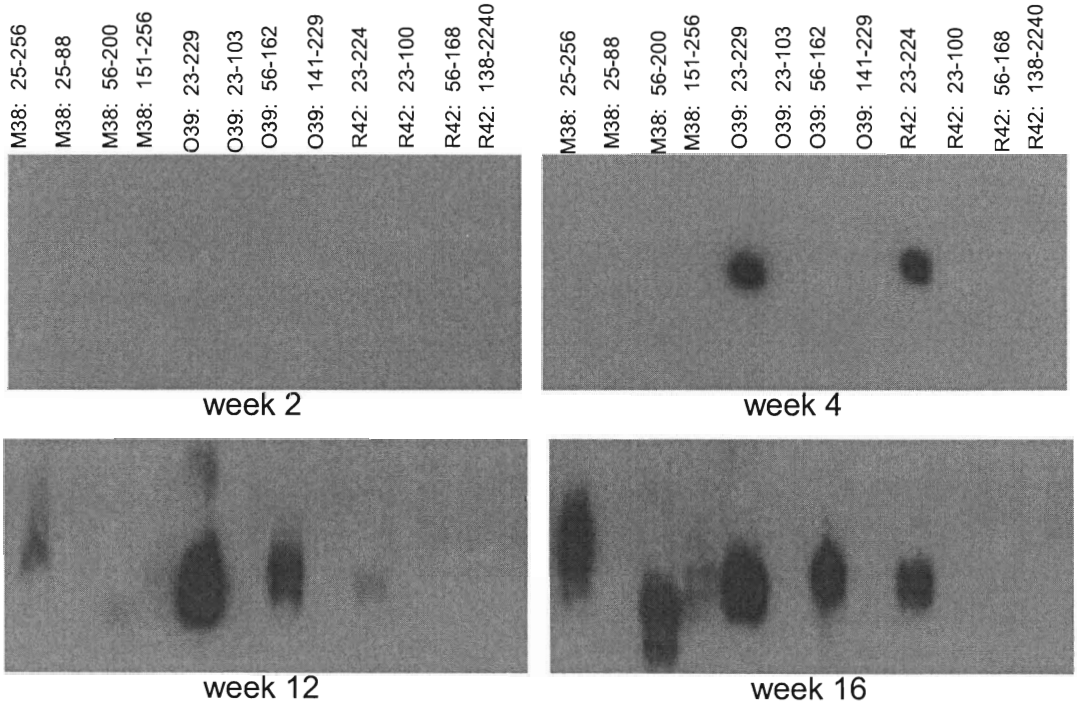
Figure 4. Demonstration of binding of anti-OspF antibodies from murine infection to full-length and subfragments of OspF paralogs fractionated by non-denaturing PAGE.

Cell lysates of *E. coli* induced to express full-length and truncated OspF paralog r-proteins were fractionated by non-denaturing PAGE and immunoblotted. Identical membranes were screened with S-Protein HRP and infection-derived sera collected from mice. The expression, relative loadings and migration of the r-proteins were visualized by immunoblot analysis using S-protein-HRP. The binding of anti-OspF antibodies from murine infection to native OspF full-length and subfragment r-proteins was demonstrated by screening with sera collected from mice infected with *B. burgdorferi* B31MI at different time points during infection (as indicated on the figure).

A. S-Protein HRP conjugate



B. B31 MI Infection Sera

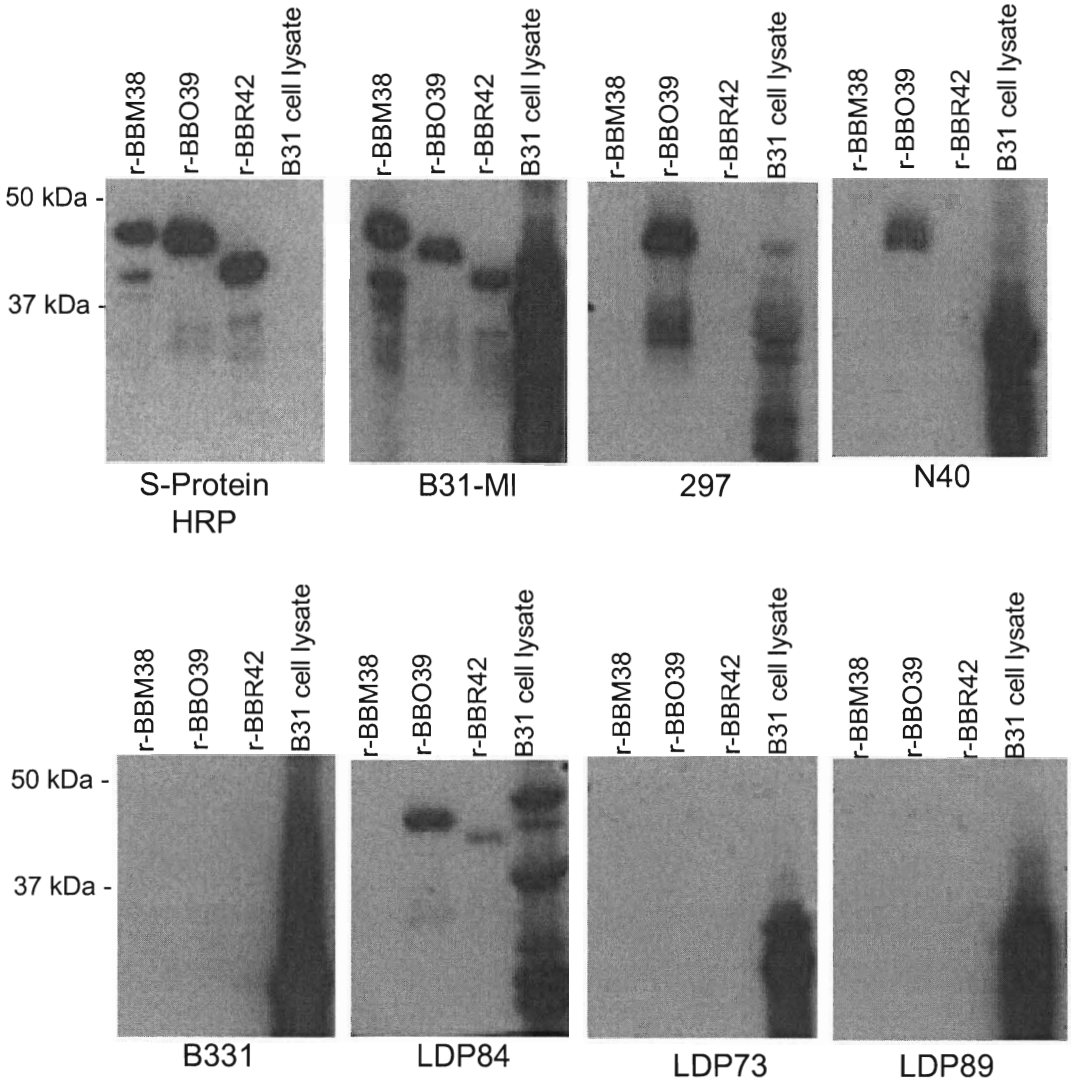


with infection sera from mice. Each mouse was infected with a different *B. burgdorferi* strain which included B31MI, LDP84, LDP73, LDP89, B331,N40 and 297. Sera were collected from mice infected with 297 and N40 at week 10 and from all other infected mice at week 12. The sera from mice infected with B31MI, 297, N40 and LDP84 reacted strongly with BBO39, indicating these strains express BBO39 or an OspF-related protein that has epitopes in common with BBO39 of *B. burgdorferi* B31MI (Figure 5). Similarly, LDP84 infection sera demonstrated reactivity to BBR42 suggesting the presence of an additional OspF-related protein with common epitopes to BBR42 of B31MI. Aside from B31MI infection sera, there was no IgG response detected towards BBM38. With the exception of both B31MI and LDP84, there was no IgG response detected towards BBR42. The absence of antibody response to the OspF proteins in the sera of mice infected with the heterologous *B. burgdorferi* could be due to significant sequence divergence in these proteins or as observed in the B31MI infected mice, expression of these proteins may occur at later time point than was tested.

IV. PCR analyses of *ospF* gene family members in isolates collected from human Lyme disease patients from New York and Maryland

For the application of OspF as a vaccinogen or in a serodiagnostic assay, it was necessary to assess the sequence heterogeneity of *ospF* among isolates collected from human Lyme disease patients. Since Lyme disease is wide-spread throughout North America, it was also important to investigate if *ospF* sequence diversity was localized to a specific area. Therefore, clinical isolates collected from New York and Maryland, two

Figure 5. Immunoblot analysis of murine humoral immune response to OspF paralogs in mice infected with diverse *B. burgdorferi* strains.
Cell lysates of *E. coli* induced to express full-length OspF paralog r-proteins and of *B. burgdorferi* B31MI were fractionated by SDS-PAGE and immunoblotted. Identical membranes were screened with S-Protein HRP and infection sera collected from mice at 10 or 12 weeks after inoculation. The mice were infected with *B. burgdorferi* strains, B31MI, 297, N40, B331, LDP84, LDP73 and LDP89. Molecular mass markers are indicated.

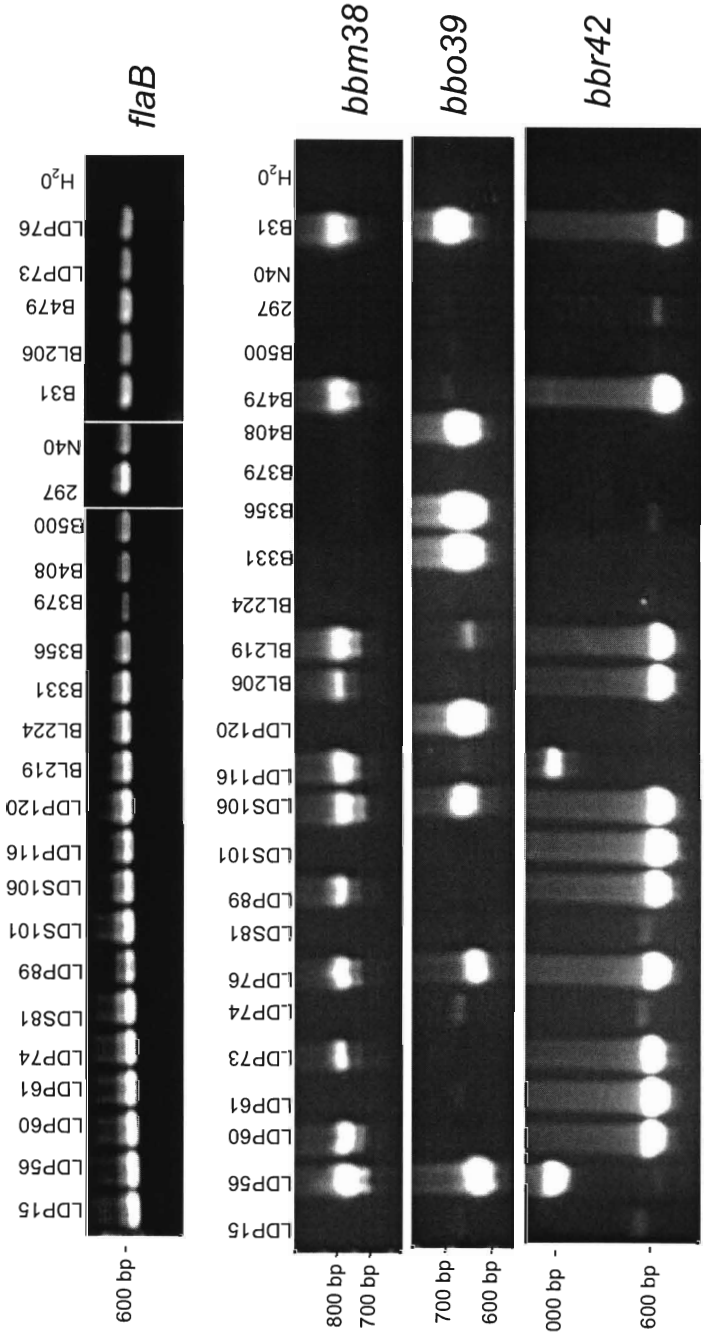

r-BBM38
r-BBO39
r-BBR42
B31 cell lysate
50 kDa -
37 kDa -
S-Protein HRP
B31-MI
297
N40
B331
LDP84
LDP73
LDP89

distinct geographic regions in which Lyme disease is endemic, were used in PCR analysis. Primer sets were designed to amplify full-length *ospF* genes without the signal peptide from clinical isolate templates. These primers were based on the *ospF* sequences from B31MI. To verify the quality of DNA template used in the PCR analysis, a primer set designed to amplify the *flaB* gene was tested for each isolate (Figure 6). A *flaB* gene was detected in all isolates thus confirming that DNA for each isolate could be used for subsequent PCR analysis. Next, the primer sets designed to PCR amplify full-length *ospF* genes were tested on all isolates. PCR amplification generated 13 amplicons out of the 25 isolates tested with the *bbr42* primer set, 11 amplicons for the *bbm38* primer set and 8 amplicons for the *bbo39* primer set. This could be an indication that *bbr42* sequences are more highly conserved than *bbo39* sequences. The majority of amplicons within each primer set tested showed consistency in size with the exception of *bbr42* primer set-derived amplicons from the isolates LDP56 and LDP116. This could indicate the possibility of a divergent *bbr42* sequence with additional insertions to account for the approximate 400 bp increase in size. Overall, PCR-amplification of *ospF* genes was not successful using the B31MI-derived primers on all the isolates tested which indicates the presence of sequence heterogeneity or lack of specific *ospF* gene. Furthermore, there was no significant difference between the number of PCR amplicons from New York or Maryland isolates indicating that *ospF* diversity occurs generally.

V. Sequence analysis of OspF protein family members in clinical isolates.

To assess the conservation of OspF paralogs among clinical isolates from two

Figure 6. PCR analyses of *ospF* paralogous genes in clinical isolates collected from human Lyme disease patients from New York and Maryland.
To verify the quality of cell lysate used as template in the PCR analysis, a primer set designed to amplify the *fla* gene was tested on a panel of isolates collected from human Lyme disease patients in New York and Maryland (indicated above each lane). Once verified, primer sets designed to amplify full-length *ospF* genes without the signal peptide were used on the same isolates. The resulting amplicons were analyzed by electrophoresis in 1% agarose gel or 2% Metaphor agarose gels and visualized by ethidium bromide staining. Molecular size markers are indicated.


flaB
bbm38
bbo39
bbr42
H2O
LDP76
LDP73
B479
BL206
B31
N40
297
B500
B408
B379
B356
B331
BL224
BL219
LDP120
LDP116
LDS106
LDS101
LDP89
LDS81
LDP74
LDP61
LDP60
LDP56
LDP15
600 bp
800 bp
700 bp
1000 bp

distinct geographic locations, New York and Maryland, sequences were determined for select PCR amplicons and translated. *bbm38* sequences were determined, translated and aligned for 7 isolates: BL219, LDP56, LDP60, LDP73, LDP89, BL206, LDP116 and were compared to the reference BBM38 sequence in B31 MI determined by TIGR (Figure 7). It was found that BBM38 sequences were highly conserved with only 1 aa change determined in two isolates - BL206 and LDP116. The substitution took place at different locations in the protein sequence. A change occurred within the N-terminal region in BL206 whereas in LDP116 the change occurred at the C-terminal portion. Overall however, BBM38 amino acid sequences were found to be highly conserved among the select isolates despite the separate geographic locations from which they were initially isolated.

Next, sequences for *bbo39* were determined, translated and aligned for 6 clinical isolates: B331, B356, B408, LDP120, LDP56 and LDS76 (Figure 8). Sequence analyses of BBO39 determined in these isolates revealed more sequence variation with a minimum of 13 amino acid changes in B408 and LDP120 ranging to a maximum of 24 amino acid changes determined in LDP56. A 7 aa-deletion within the N-terminal portion of BBO39 occurred at identical positions in LDP56 and LDS76. Furthermore, all six isolates shared 12 identical amino acid changes within the central region. In contrast to BBM38, the BBO39 sequences of the six isolates demonstrated more sequence variation with a majority of aa changes shared by all isolates.

Figure 7. Alignment of the amino acid sequences of BBM38 in clinical isolates collected from New York and Maryland.
The determined sequences were translated and aligned using the BioEdit program. The isolates analyzed are indicated at the left. In comparison to the reference sequence shown on the first line, identical positions are denoted by dots, gaps introduced by alignment are shown by dashes and amino acid changes are indicated by its specific letter.

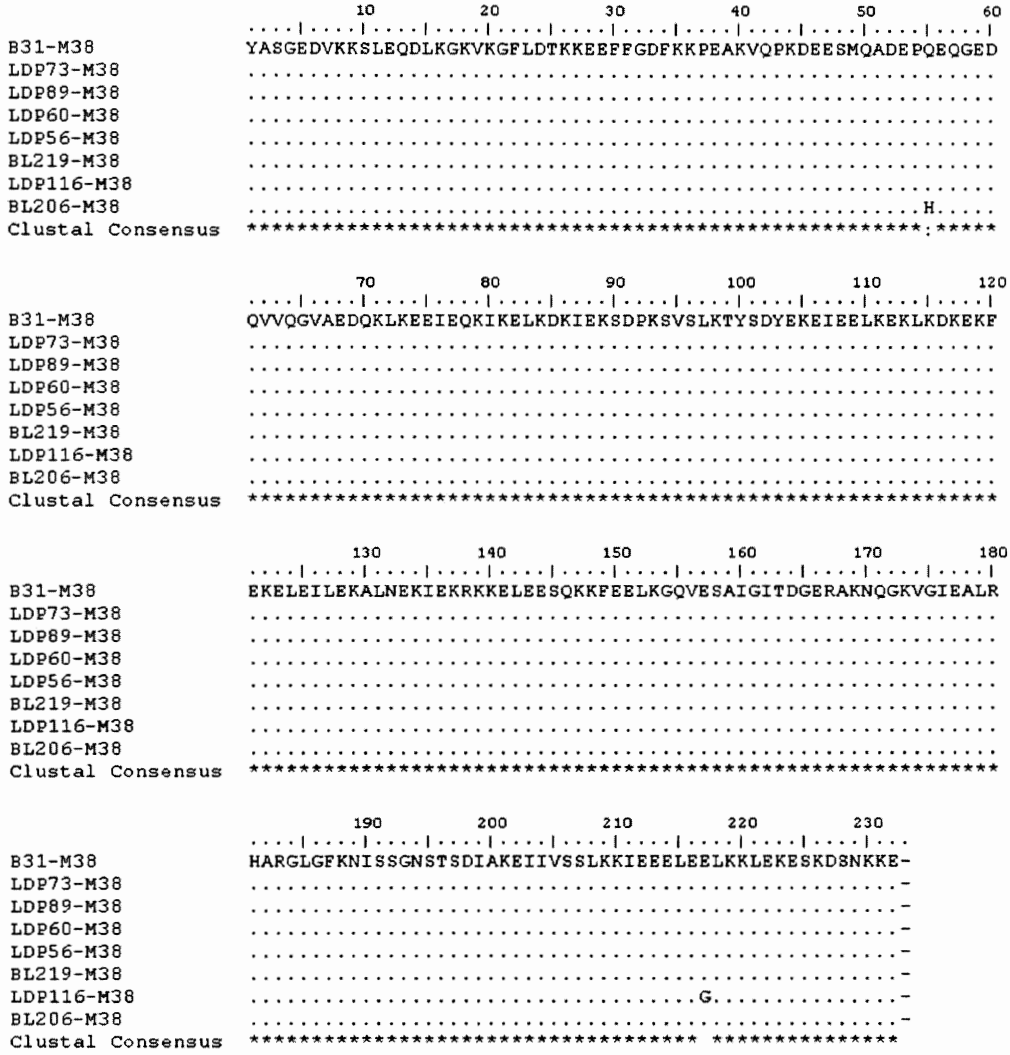

10 20 30 40 50 60
B31-M38 YASGEDVKKSLEQDLKGKVKGFLDTKKEEFFGDFKKPEAKVQPKDEESMQADEPQEQGED
LDP73-M38
LDP89-M38
LDP60-M38
LDP56-M38
BL219-M38
LDP116-M38
BL206-M38 H
Clustal Consensus
70 80 90 100 110 120
B31-M38 QVVQGVAEDQKLKEEIEQKIKELKDKIEKSDPKSVSLKTYSDYEKEIEELKEKLKDKEKF
LDP73-M38
LDP89-M38
LDP60-M38
LDP56-M38
BL219-M38
LDP116-M38
BL206-M38
Clustal Consensus
130 140 150 160 170 180
B31-M38 EKELEILEKALNEKIEKRKKELEESQKKFEELKGQVESAIGITDGERAKNQGKVGIEALR
LDP73-M38
LDP89-M38
LDP60-M38
LDP56-M38
BL219-M38
LDP116-M38
BL206-M38
Clustal Consensus
190 200 210 220 230
B31-M38 HARGLGFKNISSGNSTSDIAKEIIVSSLKKIEEELEELKKLEKESKDSNKKE-
LDP73-M38 -
LDP89-M38 -
LDP60-M38 -
LDP56-M38 -
BL219-M38 -
LDP116-M38 G -
BL206-M38 -
Clustal Consensus

Figure 8. Alignment of the amino acid sequences of BBO39 in clinical isolates collected from New York and Maryland.
The determined sequences were translated and aligned using the BioEdit program. The isolates analyzed are indicated at the left. In comparison to the reference sequence shown on the first line, identical positions are denoted by dots, gaps introduced by alignment are shown by dashes and amino acid changes are indicated by its specific letter.

```
                          10         20         30         40         50         60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
B31-039             YASGENLKNSEQNLESSEQNVKKTEQEIKKQVEGFLEILETKDLSKLDEKDTKEIEKQIQ
B331-039            ..................S.....................................
B356-039            ..................S.....................................
LDP120-039          ............................................................
B408-039            ............................................................
LDP56-039           .......E....-------.ER.....V................................
LDS76-039           .......E....-------.ER.....V................................
Clustal Consensus   *******:****       . :*****:********************************

                          70         80         90         100        110        120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
B31-039             ELKNKIEKLDSKKTSIETYSEYEEKINKIKEKLKGK-GLEDKFKELEESLAKKKGERKKA
B331-039            ..............LK........LKQ......D.KE..K.L....D..K.......S.
B356-039            ..............LK........LKQ......D.KE..K.L....D..K.......S.
LDP120-039          ..............LK........LKQ......D.KE..K.L....D..K.......S.
B408-039            ..............LK........LKQ......D.KE..K.L....D..K.......S.
LDP56-039           ..TE..D..EG...FLK.........KQ......D.KE..K.L....D..K......ES.
LDS76-039           ..TE..D..EG...FLK.........KQ......D.KE..K.L....D..K.......S.
Clustal Consensus   **.:**:**:.*** ::********:::******.*  **.*:****:** ******:.*

                          130        140        150        160        170        180
                    ....|....|....|....|....|....|....|....|....|....|....|....|
B31-039             LQEAKQKFEEYKKQVDTSTGKTQGDRSKNRGGVGVQAWQCANELGLGVSYSNGGSDNSNT
B331-039            ............................................................
B356-039            ............................................................
LDP120-039          ............................................................
B408-039            ............................................................
LDP56-039           ............................................................
LDS76-039           ............................................................
Clustal Consensus   ************************************************************

                          190        200
                    ....|....|....|....|....|....
B31-039             DELANKVIDDSLKKIEEELKGIEEDKKE-
B331-039            ............................-
B356-039            ............................-
LDP120-039          ............................-
B408-039            ............................-
LDP56-039           ...........................N-
LDS76-039           ............................-
Clustal Consensus   ***************************:
```

Finally, BBR42 sequences were determined in 8 clinical isolates and aligned for: LDP73, B479, BL206, LDP60, LDP76, LDP89, LDS106 and BL219 (Figure 9). Upon sequence analyses, it was observed that all determined BBR42 sequences were highly conserved and identical to BBR42 in B31 MI with the exception of two isolates - LDP73 and B479. Amino acid changes occurred in the C-terminal region of both isolates. B479 contained only one amino acid change and LDP73 contained an additional amino acid substitution in the central region of the protein.

In addition, a phylogram was generated using all the BBM38, BBO39 and BBR42 sequences that were sequenced from the New York and Maryland isolates (Figure 10). The phylogram is depicted as a branching tree which allows for a visual representation of the evolutionary analysis in which branch lengths are proportional to the evolutionary change. BBM38, BBO39 and BBR42 sequences derived from human patients form three distinct clades. Both BBM38 and BBR42 show very little branching within each clade confirming sequence conservation. BBO39 showed greater sequence heterogeneity with the formation of 3 clades but amino acid changes were still conserved among the isolates. Overall, sequence alignment and phylogram analyses reveal that BBM38 and BBR42 sequences are highly conserved among the clinical isolates despite the two distinct geographic locations. BBO39 showed more sequence diversity among the clinical isolates but sequence divergence could not be directly correlated with geographic location.

Figure 9. Alignment of the amino acid sequences of BBR42 in clinical isolates collected from New York and Maryland.
The determined sequences were translated and aligned using the BioEdit program. The isolates analyzed are indicated at the left. In comparison to the reference sequence shown on the first line, identical positions are denoted by dots, gaps introduced by alignment are shown by dashes and amino acid changes are indicated by its specific letter.

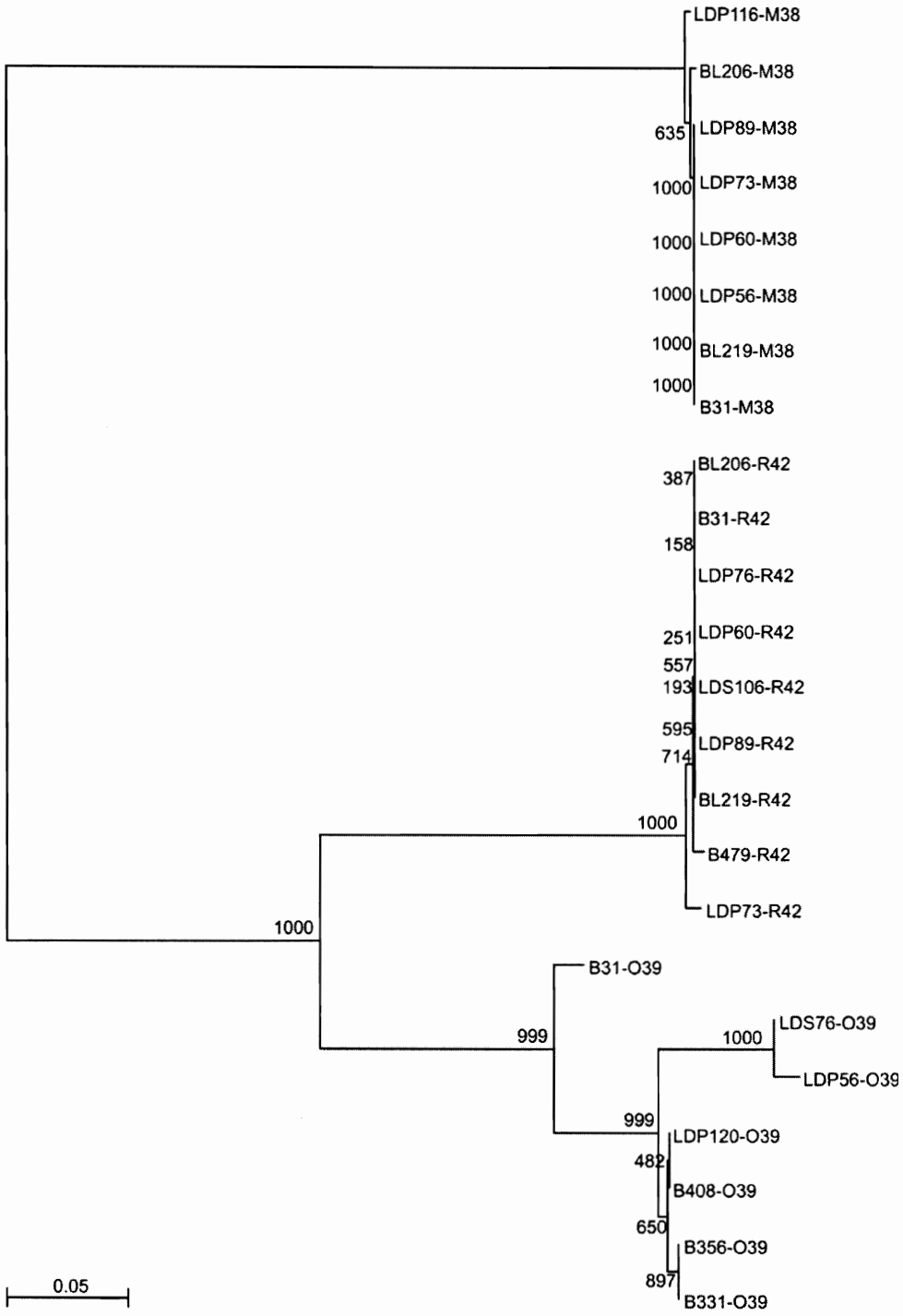
```
                          10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
B31-R42             DVTSKDLEGAVKDLESSEQNVKKTEQEIKKQVEGFLEILETKDLNTLDTKEIEKQIQELK
BL206-R42           ............................................................
B479-R42            ............................................................
LDP60-R42           ............................................................
LDS106-R42          ............................................................
BL219-R42           ............................................................
LDP89-R42           ............................................................
LDP76-R42           ............................................................
LDP73-R42           ............................................................
Clustal Consensus   ************************************************************

                          70        80        90        100       110       120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
B31-R42             NKIEKLDSKKTSIETYSGYEEKINKIKEKLNGKGLEDKLNELSESLKKKKEERKKALQEA
BL206-R42           ............................................................
B479-R42            ............................................................
LDP60-R42           ............................................................
LDS106-R42          ............................................................
BL219-R42           ............................................................
LDP89-R42           ............................................................
LDP76-R42           ............................................................
LDP73-R42           ..............................S.............................
Clustal Consensus   ******************************.*****************************

                          130       140       150       160       170       180
                    ....|....|....|....|....|....|....|....|....|....|....|....|
B31-R42             KKKFEEYKNQAESATGVTHGSQVQRQGGVGLQAWQCANSLGFKNMTSGNNTSDMTNEVIT
BL206-R42           ............................................................
B479-R42            ...........................................................G...
LDP60-R42           ............................................................
LDS106-R42          ............................................................
BL219-R42           ............................................................
LDP89-R42           ............................................................
LDP76-R42           ............................................................
LDP73-R42           ............................................................
Clustal Consensus   ************************************************************ ***

                          190       200
                    ....|....|....|....|...
B31-R42             NSLKKIEEELKNIGETVEGKKE-
BL206-R42           ......................-
B479-R42            ......................-
LDP60-R42           ......................-
LDS106-R42          ......................-
BL219-R42           ......................-
LDP89-R42           ......................-
LDP76-R42           ......................-
LDP73-R42           ..........T...........-
Clustal Consensus   **********.***********
```

Figure 10. Sequence analysis of OspF in clinical isolates from New York and Maryland.
A phylogram was constructed using the translated OspF sequences. The OspF paralog and the clinical isolate in which the gene was initially isolated from is indicated at the end of the branch. The branch length scale is shown (amino acid substitution per 100 residues) and bootstrap values (1000 trials) are indicated at the nodes.

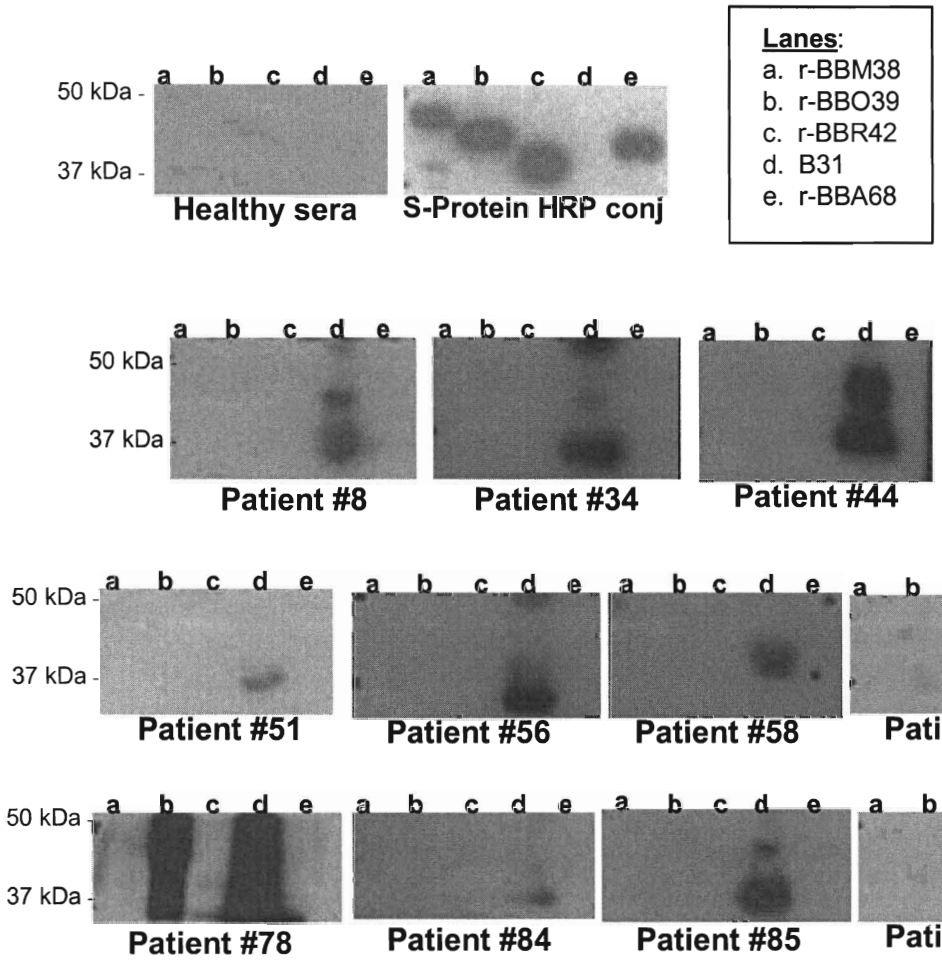

LDP116-M38
BL206-M38
635
LDP89-M38
1000
LDP73-M38
1000
LDP60-M38
1000
LDP56-M38
1000
BL219-M38
1000
B31-M38
BL206-R42
387
B31-R42
158
LDP76-R42
LDP60-R42
251
557
193
LDS106-R42
595
LDP89-R42
714
BL219-R42
1000
B479-R42
LDP73-R42
1000
B31-O39
999
LDS76-O39
1000
LDP56-O39
999
LDP120-O39
482
B408-O39
650
B356-O39
897
B331-O39
0.05

VI. Analyis of humoral immune response to OspF proteins in patients diagnosed at the early stage of Lyme disease

Diverse outer surface proteins expressed by *B.burgdorferi* elicit complex cellular and humoral immune responses in patients with Lyme disease. An example of early immune response in Lyme disease is reactivity with OspC [61-63]. To assess the humoral immune response to OspF proteins in early stage Lyme disease patients, serum samples were collected from patients exhibiting erythema migrans, which usually occurs 3 days to 1 month following the tick bite and from healthy control subjects. Both infection and healthy patient sera were tested for immunoreactivity with r-OspF proteins by immuoblot analysis. Immunoblots of r-OspF proteins, r-BBA68 and B31MI cell lysate were generated. r-BBA68 served as the negative control due to its lack of expression during mammalian infection [40,64,65] and B31MI cell lysate acted as the positive control to confirm Lyme infection.

Immunoblots were screened with S-protein HRP conjugate to verify relative protein loadings and screened with pooled sera from healthy individuals to demonstrate no cross-reactivity with spirochetal proteins (Figure 11). Next, the IgG responses to the r-OspF proteins were tested by screening with 11 samples of sera collected from individual Lyme disease patients with EM, the hallmark indicative of the early stage of Lyme disease (Figure 11). All serum samples showed reactivity with B31MI cell lysate, confirming *B.burgdorferi* infection in these patients. However, no IgG response towards OspF proteins was detected in the patient samples tested except for patient #78 which

Figure 11. Immunoblot analysis of the humoral response to OspF paralogs in early stage Lyme disease patients.
Cell lysates of *E. coli* induced to express OspF paralog and BBA68 r-proteins and of *B. burgdorferi* B31MI were fractionated by SDS PAGE and immunoblotted. The B. burgdorferi protein, BBA68 is not expressed during infection in humans thereby serving as a negative control for immunoblot analysis. Conversely, the *B.burgdorferi* cell lysate served as a positive control to verify Lyme disease infection. Identical membranes were screened with S-Protein HRP and sera collected from healthy individuals and from early stage Lyme disease patients. Molecular mass markers are indicated.

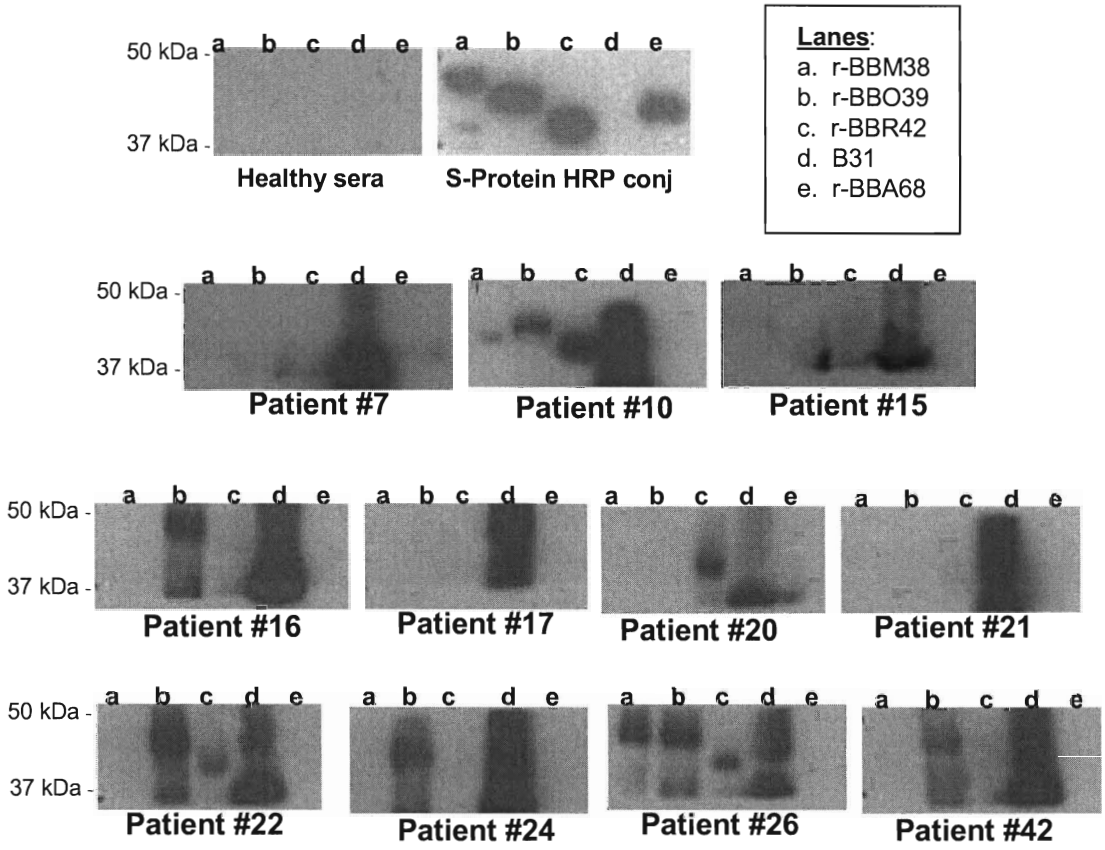

Lanes:
a. r-BBM38
b. r-BBO39
c. r-BBR42
d. B31
e. r-BBA68
50 kDa
37 kDa
a b c d e
Healthy sera
S-Protein HRP conj
Patient #8
Patient #34
Patient #44
Patient #51
Patient #56
Patient #58
Patient #63
Patient #78
Patient #84
Patient #85
Patient #86

only showed immunoreactivity to r-BBO39. These analyses demonstrated that a majority of serum samples collected from patients with early Lyme disease lacked immunoreactivity to OspF proteins with the exception of one patient sample which suggests these proteins are not expressed at the early stage of infection. Since these patients are probably infected with heterologous *B. burgdorferi* strains, their sera may lack antibodies specific to the OspF proteins derived from B31MI, indicating the presence of immunologically distinct OspF proteins.

VII. Analysis of humoral immune response to OspF proteins in Lyme arthritis patients.

Since previous immunoblot analyses suggested a temporal pattern in OspF expression in the murine Lyme model, with delayed immunoreactivity towards r-BBM38, the next step was to assess the humoral immune response to OspF proteins in late stage Lyme disease patients. The sera collected from these patients all had Lyme arthritis, typically characterized by pain and swelling in one or more large joints, commonly the knees. If an infected patient is not diagnosed with Lyme disease and left untreated, Lyme arthritis can arise within months to years after infection. Once again, identical immunoblots of r-OspF proteins were screened with S-protein HRP conjugate to confirm equal protein loadings and screened with sera pooled from healthy individuals to confirm lack of cross-reactivity with r-proteins in healthy serum (Figure 12). Next, immunoblots were screened with 11 samples of sera collected from individual patients diagnosed with

Figure 12. Immunoblot analysis of the humoral response to OspF paralogs in late stage Lyme disease patients.
Cell lysates of *E. coli* induced to express OspF paralog and BBA68 r-proteins and of *B. burgdorferi* B31MI were fractionated by SDS PAGE and immunoblotted. The *B. burgdorferi* protein, BBA68 is not expressed during infection in humans thereby serving as a negative control for immunoblot analysis. Conversely, the *B.burgdorferi* cell lysate served as a positive control to verify Lyme disease infection. Identical membranes were screened with S-Protein HRP and sera collected from healthy individuals and from late stage Lyme disease patients. Molecular mass markers are indicated.


50 kDa
37 kDa
a b c d e
Healthy sera
S-Protein HRP conj
Lanes:
a. r-BBM38
b. r-BBO39
c. r-BBR42
d. B31
e. r-BBA68
Patient #7
Patient #10
Patient #15
Patient #16
Patient #17
Patient #20
Patient #21
Patient #22
Patient #24
Patient #26
Patient #42

therefore do not correspond with the previous set of samples from patients with early Lyme disease. In contrast to the analyses obtained from early Lyme disease infection sera, immunoreactivity to r-OspF proteins occurred more frequently when screened with sera from Lyme arthritis patients. All 11 samples showed immunoreactivity with the B31MI cell lysate positive control, confirming *B. burgdorferi* infection in these patients. Of the 11 samples tested, 7 showed immunoreactivity to at least one r-OspF protein. More specifically, 6 sera samples were immunoreactive to r-BBO39, thereby making it the most frequently detected r-OspF protein. Although the samples in these studies were collected from different Lyme disease patients, there was a significant increase in immunoreactivity to r-OspF proteins when testing with Lyme arthitis sera in comparison to early arthritis sera. These results correlate with the immunoblot analyses in the murine Lyme model which demonstrated early expression of BBO39 and BBR42 and much later detection of BBM38.

VIII. Transcriptional analyses of *ospF* genes during *in vitro* cultivation of B31MI at 33 degrees.

Studies have shown differential expression of some *B.burgdorferi* genes between the *in vivo* and *in vitro* environments [52,53,66] . Previous immunoblot analyses established that OspF proteins are expressed *in vivo* based upon the specific antibody response that is seen in *B. burgdorferi*-infected mice and humans towards OspF proteins [20,28,53-55]. Therefore, it was important to assess transcription of *ospF* genes during *in vitro* cultivation for utilization of *B. burgdorferi* cultures in future OspF-related

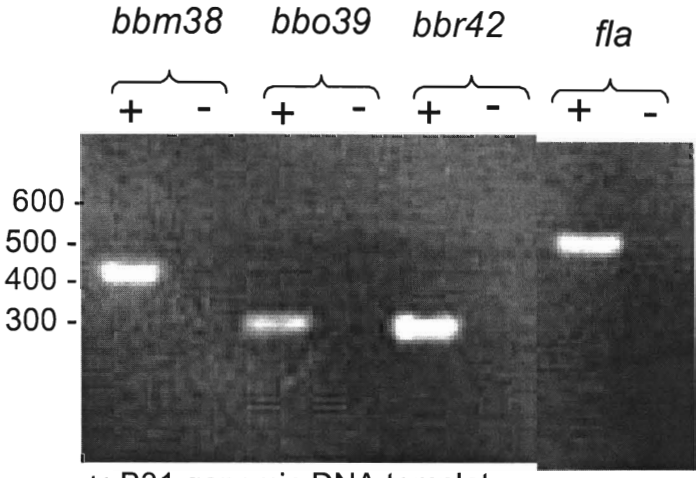
analyses. Total RNA was isolated from cultures grown at 33°C and specific primers were designed for each *ospF* gene - *bbm38*, *bbo39* and *bbr42* for employing RT-PCR analysis. Prior to performing the RT-PCR analyses, it was necessary to establish allelic specificities for the primers which was accomplished by PCR analysis using isolated *B. burgdorferi* B31MI genomic DNA as a template (Figure 13A). All primer sets yielded a single amplicon of the expected size. Next, RT-PCR was employed to assess the expression of each individual *ospF* gene in *B. burgdorferi* cultivated at 33°C. Reactions lacking reverse transcriptase did not yield products, indicating that the RNA preparations were free of contaminating genomic DNA (Figure 13B). RT-PCR products were obtained in all of the reactions and were of the expected size indicating the *ospF* genes *bbm38*, *bbo39* and *bbr42* are transcribed during *in vitro* cultivation of *B. burgdorferi* B31MI at 33°C.

IX. Inactivation of OspF by allelic exchange mutagenesis

To investigate the role of OspF in *B. burgdorferi* pathogenesis, it was necessary to inactivate *ospF* genes by allelic exchange. Sequences directly flanking *ospF* upstream and downstream were PCR amplified with primers designed with restriction sites and LIC-tails, sequences complementing the single-stranded overhangs of the pET-32Ek/LIC vector to allow for ligase-independent cloning (LIC). Following a series of PCR amplification, LIC cloning and restriction digests, plasmids were constructed to contain the upstream and downstream regions of each *ospF* gene with a spectinomycin-streptomycin resistance cassette in replacement of the *ospF* gene (Figure 2). PCR

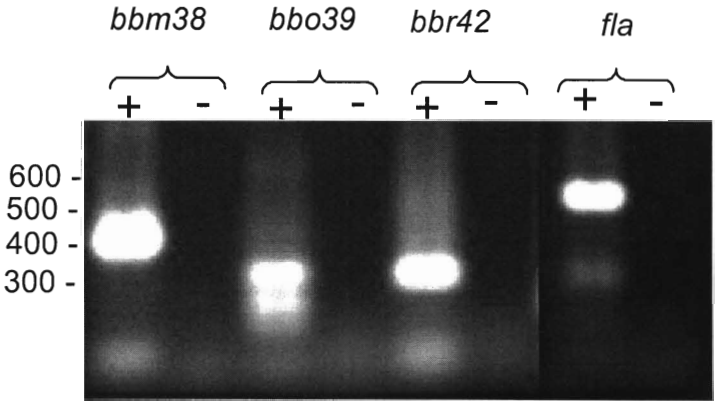
Figure 13. PCR and RT-PCR analyses of *ospF* genes in *in vitro* cultivation of *B. burgdorferi* B31MI.
To demonstrate the specificity of the primer sets designed to amplify the central regions of the *ospF* genes, PCR analyses were performed. To determine if *ospF* genes are transcribed during *in vitro* cultivation, RT-PCR was performed using RNA extracted from *B. burgdorferi* B31MI grown at 33°C. The resulting amplicons were analysed on 1% agarose gels. Controls for these analyses included *flaB* and reactions lacking template in the PCR analyses or reactions in which RT was omitted in the RT-PCR analyses.

A. PCR



+: B31 genomic DNA template
-: H_20 template

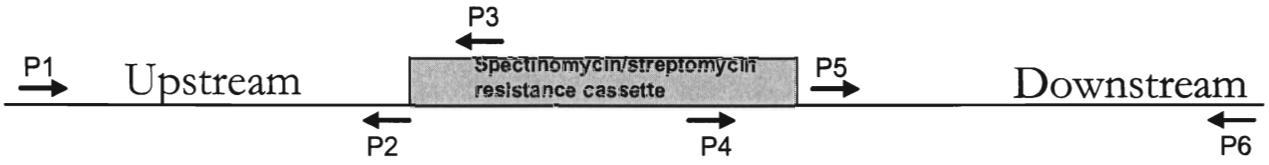
B. RT-PCR

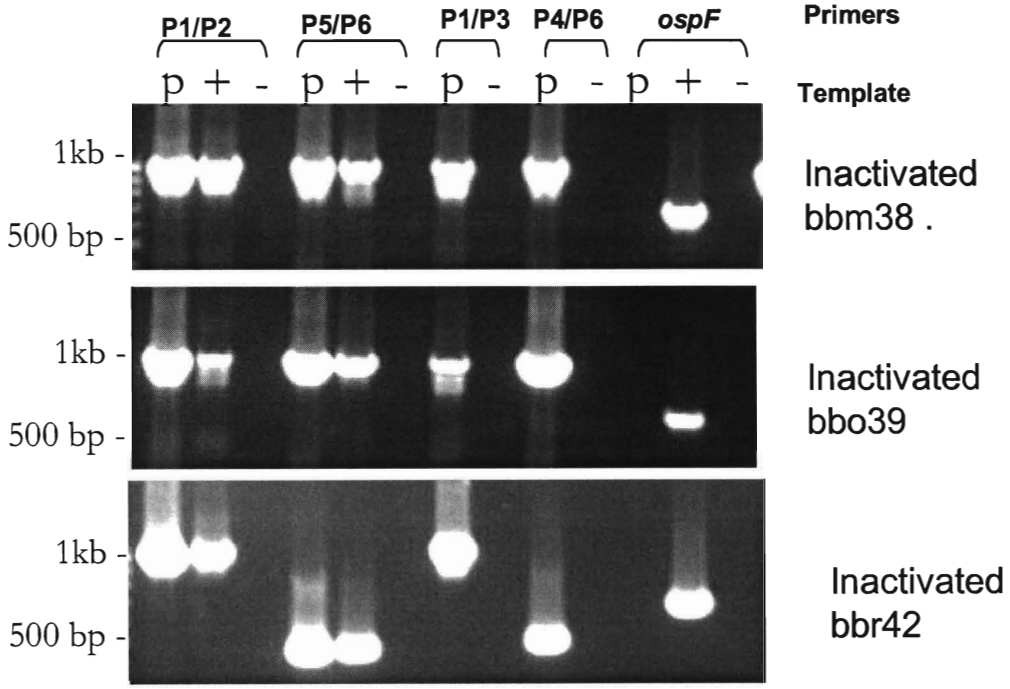


+: RT enzyme present
- : RT enzyme absent

which included the presence of the upstream and downstream regions, the proper insertion of the resistance cassette and the deletion of the *ospF* gene (Figure 14). All PCR amplicons were of the expected size, confirming successful plasmid construction. The gene knockout plasmids were then linearized by restriction digest in preparation for electroporation into *B. burgdorferi* clones 5A4 and 5A13. Thus far, clonal populations for *Δbbm38* in *B. burgdorferi* 5A4 and *Δbbr42* in *B. burgdorferi* 5A13 have been obtained by sub-surface plating. All clones tested by PCR analysis demonstrated successful inactivation of *bbm38* or *bbr42* by homologous recombination and replacement with the antiobiotic resistance cassette. The clones 5A4 and 5A13 differ by infectivity phenotypes with 5A4 exhibiting high infectivity in contrast to the low infectivity phenotype of 5A13. The disparity in infectivity phenotypes is due to the lack of one plasmid in 5A13, lp25, which has been shown to be required for infectivity in mice [67, 68]. Since loss of plasmids can occur during electroporation and ultimately affect infectious potential, it was important to determine the plasmid composition of the clonal populations by PCR analysis. Primer sets specific for each plasmid were designed and tested on select clonal populations. Results from PCR analyses reveal a loss of plasmid lp25 in 5A4 clones and a loss of lp28-1 in both 5A4 and 5A13 clones (Table 3). Both lp25 and lp28-1 have been shown to be important plasmids for infectivity in mice [67-69]. Therefore, PCR analyses on additional clonal populations is necessary to identify deletion mutant clones with intact plasmid composition or clones

Figure 14. PCR analyses for verification of plasmid constructs targeted at inactivating *ospF* genes.
To verify the proper construction of plasmid for inactivation of *ospF* genes, primer sets (shown by the arrows) were designed to target the upstream and downstream regions and to confirm insertion of the spectinomycin/streptomycin resistance cassette in the correct orientation. In addition, primer sets designed to amplify *ospF* genes were used to confirm absence of the specific gene. The templates used in the PCR analyses included plasmid DNA, B31MI DNA as a positive control and water as the negative control.





DNA templates:
p: plasmid construct
+: B31
- : H20

TABLE 3. Summary of plasmid profiles for clones with Δbbm38 or Δbbr42 in *B.burgdorferi* 5A4 and 5A13

Plasmid	B31	M38 KO c1 (5A4)	M38 KO c2 (5A4)	M38 KO c4(5A4)	R42 KO c3(5A13)	R42 KO c4(5A13)	R42 KO c13 (5A13)	R42 KO c15 (5A13)	R42 KO c19 (5A13)
lp54	+	+	+	+	+	+	+	+	+
cp26	+	+	+	+	+	+	+	+	+
cp9	+	+	+	+	-	-	-	-	-
lp-17	+	+	+	+	+	+	+	+	+
lp25	+	-	-	-	-	-	-	-	-
lp28-1	+	-	-	-	-	-	-	-	-
lp28-2	+	-	-	-	+	+	-	-	+
lp28-3	+	+	+	+	-	+	-	-	-
lp38	+	+	+	+	+	-	+	+	+
lp-36	+	-	-	-	+	+	-	-	+
cp32-8	+	+	+	+	+	+	+	+	+
cp32-6	+	+	+	+	+	+	+	+	+
cp32-9	+	+	+	+	+	+	-	-	+
cp32-7	+	+	+	+	-	-	+	+	-
cp32-1	+	+	+	+	+	+	+	+	+
lp56	+	+	+	+	+	+	+	+	+
cp32-4	+	+	+	+	+	+	+	+	+
cp32-3	+	+	-	+	+	+	+	-	-
lp21	+	-	-	-	-	-	-	-	-

that have maintained the necessary plasmids for infectivity. Ultimately, *in vivo* experiments will be performed to determine whether loss of OspF expression in the mutants have adverse effects on Lyme disease pathogenicity. Both wild-type and mutant spirochetes can be inoculated into experimental Lyme disease mouse models to examine and compare pathogenesis. Hence, the ability to inactivate OspF by allelic exchange mutagenesis is an important tool in helping to define the role of OspF in *B. burgdorferi* pathogenesis.

DISCUSSION

A distinct characteristic of *B.burgdorferi*, the causative agent of Lyme disease is the large number of genes that encode for lipoproteins. Since the surface-exposed outer membrane proteins have direct interactions with the mammalian and tick host environments, these diverse proteins are likely to be important for establishing disease and maintaining chronic infection. Studies have shown that OspF proteins are surface-exposed, expressed during infection and elicit an antibody response. For these reasons, the role of OspF in Lyme disease pathogenesis was the focus of this study.

Previous studies have shown that OspF proteins are antigenic and may be temporally expressed as seen with an early response to BBO39 and a delayed response to BBM38. In light of this, immunoblot analyses were performed to identify epitopes of OspF that are exposed during infection and to analyze the temporal pattern and specificity of the immune response to OspF constructs. Full-length and truncated r-proteins spanning the N-terminal, central and C-terminal regions of each OspF paralog were expressed and fractionated under denaturing conditions. Identical immunoblots were generated and screened with sera collected from mice infected with B31 at weeks 2, 4, 12 and 16 post-infections. These analyses showed a response by week 4 to the full-length BBO39 and BBR42 whereas no response was detected to BBM38 until week 12 of infection. The temporal pattern of response to BBO39 and BBM38 is consistent with the study by McDowell et al [28]. Differing from the previous study is the early response to

BBR42 detected at week 4 seen in these analyses which may be due to relative protein loadings or due to a different immune response generated in a different mouse. Despite the difference in time points in which BBR42 elicited a specific antibody response, the overall trend confirming temporal expression of OspF proteins is the same in both studies. Furthermore, immunoblot analyses revealed the immunodominant epitopes of BBM38 which included central and C-terminal regions (amino acids (aa) 56-256) and BBO39 which included the central region (aa 56-162). The epitopes for BBR42 were unable to be identified due to the lack of IgG response towards the BBR42 subfragment r-proteins. This indicates that the full-length protein of BBR42 is required to elicit an immune response. An alternative explanation for lack of response to BBR42 subfragments is that BBR42 epitopes may be conformational. Consequently denaturing conditions would disrupt protein conformation and prevent Ab binding. To address this issue, OspF r-proteins were also fractionated under non-denaturing conditions to preserve and to identify conformational epitopes. Identical immunoblots were generated and screened with the mouse infection sera. The immunoreactivity pattern was identical to that seen in the immunoblot analyses under denaturing conditions. This demonstrates that the immunodominant OspF epitopes are not dependent upon conformation for Ab binding and also suggests that full-length BBR42 is required to elicit an Ab response.

Overall, immunoblot analyses confirm the temporal expression of OspF proteins during murine infection. As all OspF-related proteins have been demonstrated to be immunogenic in mice [20,51], it is likely that the delayed response to BBM38 results from the temporal expression of these proteins during infection. It has also been shown that antibodies that develop late in infection are specific and the late immunoreactivity seen is not a result of cross-reactive antibodies [28].

Multiple strains of *B. burgdorferi* have been isolated from ticks, natural hosts and Lyme disease patients [56-59]. We were interested in determining the conservation of the anti-OspF humoral immune response in mice infected with various *B. Burgdorferi* strains. Immunoblot analyses were performed to assess the humoral immune response to OspF paralogs in mice infected with heterologous *B. burgdorferi* strains. Individual mice were infected with the common laboratory strains B31MI, 297 and N40 and with clinical isolates LDP84, LDP73, LDP89 and B331. Identical immunoblots of BBM38, BBO39 and BBR42 r-proteins were generated and screened with infection sera of mice infected with the heterologous *B. burgdorferi*. Only the B31MI infection sera showed an immune response to all OspF paralogs, which is not surprising since these proteins were derived from the B31MI isolate. BBO39 was the most frequently detected OspF protein by the heterologous infection sera B31MI, 297, N40 and LDP84. This would indicate these strains express OspF-related proteins that have epitopes in common with BBO39 of *B. burgdorferi* B31MI. Immunoblot analyses also revealed a weak response to BBR42 detected in LDP84 infection sera and no response to BBM38 in any of the infection sera

(with the exception of B31MI). The absence of immunoreactivity to B31MI OspF proteins in the infection sera is likely due to sequence divergence, lack of the gene or due to repression of protein expression among the various strains. Considering the temporal expression of OspF proteins during B31MI infection, it is also possible that the OspF-related proteins in diverse isolates are expressed at a later time point than what was tested. These analyses demonstrate the variability of anti-OspF immune responses in mice infected with different *B. burgdorferi* strains.

To further investigate sequence divergence of OspF proteins, PCR analyses were conducted on a panel of clinical isolates from Maryland and New York. Primer sets were designed using *ospF* sequences from B31MI and were tested on all isolates. PCR amplification was not successful for all isolates tested, indicating lack of the specific *ospF* gene or the presence of genes that are divergent from the B31MI *ospF* sequences. The majority of amplicons showed consistency in size within each primer set tested indicating the possibility of sequence conservation. However, the *bbr42* primer set-derived amplicons from the isolates LDP56 and LDP116 were an exception to the amplicon size uniformity indicating possible sequence divergence. These amplicons showed an approximate 400 bp increase in size compared to the other *bbr42* primer set-derived amplicons which may be due to sequence insertions or gene duplication.

To assess the conservation of OspF paralogs among clinical isolates a number of PCR amplicons were selected for sequencing. Alignment of OspF amino acid sequences revealed that BBM38 and BBR42 are highly conserved despite the two distinct

geographic locations from which these isolates originated. On the other hand, BBO39 sequences showed greater variation between the clinical isolates but all six BBO39-derived sequences shared a number of identical amino acid changes. Therefore none of the OspF sequence variation could be correlated with geographic location. This is in contrast to what has been shown with another well-studied *B.burgdorferi* outer surface protein, OspC, which exhibits significant genetic diversity even within a single geographic location [70]. Further sequence analyses on isolates obtained from additional geographic regions is necessary but these present findings support OspF proteins as good candidates for vaccine or diagnostic assays because of their broad application.

Sequence analyses were also used as a tool for interpretation of the previous immunoblot analyses using heterologous infection sera. *OspF* orthologs have already been identified and sequenced in strains N40 and 297. The nomenclature for *ospF* in these two strains include *ospF* and *bbk2.10* and an additional *ospF* gene in 297 named *bbk2.11*. The OspF proteins in strains 297 and N40 reveal sequence variation from the OspF proteins belonging to B31MI, with identity values ranging from 31.9% to 79.1% (Table 4). Identity values demonstrate the extent to which two amino acid sequences are invariant.

There were no anti-BBM38 antibodies detected in the heterologous infection sera tested except in B31MI mouse infection sera, the strain from which these proteins originated (Figure 3). BBM38 proteins identified and sequenced from LDP73 and LDP89 strains showed 100% identity to BBM38 from B31MI. Therefore, sequence

Table 4. Pairwise sequence comparison of OspF amino acid sequences

	BBM38_B31	BBO39_B31	BBR42_B31	BBK2.10_297	OSPF_297	BBK2.11_297	BBK2.10_N40	OSPF_N40
BBM38_B31		40.1	40.5	43	38.7	48.1	43.4	39.1
BBO39_B31	65.5		70	36	68.9	79.1	36.4	68.1
BBR42_B31	62.1	82.1		33.2	69.2	70.6	31.9	67.6
BBK2.10_297	57.3	61.4	55.4		35.2	33.3	98.9	35
OSPF_297	63.8	86.5	82.6	58.9		63.4	35.7	86.7
BBK2.11_297	70.7	89.1	80.1	57.8	81.5		34.3	66.2
BBK2.10_N40	58.2	61.8	54.5	98.9	59.9	58.8		33.8
OSPF_N40	64.2	84.8	81	58.1	92.9	81.5	58.6	

Percent amino acid identity values are presented in the upper half of the table shaded region and percent amino acid similarity values are given in the lower half of the table.

differences could not account for the lack of immunoreactivity to BBM38 r-proteins in the immunoblots probed with LDP73 and LDP89 infection sera. Earlier immunoblot analyses revealed delayed immune response to BBM38 proteins in B31MI infection sera. This observation may explain the lack of immunoreactivity to r-BBM38 in LDP73 and LDP89 infection sera. Subsequently, a response to the homologous r-BBM38 could occur at a later timepoint than 12 weeks post-infection with LDP73 and LDP89 strains. In addition, there was no immune response to r-BBM38 detected in 297, N40, B331 or LDP84 infection sera. PCR analyses showed lack of *bbm38* genes in strains 297, N40 and B331 and sequence analyses of OspF sequences in 297 and N40 revealed 48.1% to be the highest identity value to BBM38. Therefore, the lack of immunoreactivity to BBM38 in B331, 297 and N40 infection sera is likely due to sequence divergence of BBM38 in these respective strains.

In contrast to BBM38, detection of anti-BBO39 antibodies was seen in B31MI, 297, N40 and LDP84 infection sera. LDP84 was not included in the PCR and sequence analyses but it is evident that a BBO39-related protein is present in LDP84. Although PCR amplification of *bbo39* from 297 and N40 was unsuccessful, published sequences of BBK2.11 in 297 and OspF from N40 show 77.7% and 67.9% identity values respectively to BBO39. Furthermore, the immunodominant epitope of BBO39 was localized to amino acids 56-162 which included a region of homology to BBK2.11 in 297 (aa 56-130) and stretches of homology to OspF from 297. Sequence analysis of BBO39 identified in the B331 strain shared 93.3% identity BBO39 in B31MI. Despite the high identity value,

B331 infection sera showed no immune response to r-BBO39 in the immunoblot analyses. The absence of immunoreactivity is likely contributed by the amino acid changes that occur in BBO39 from the strain B331 which lie within the immunodominant epitope range 56-162 aa. Immunodetection of BBO39 beyond the tested 12 weeks post-infection is not a likely explanation, since previous immunoblot analyses show that BBO39 can be detected as early as 4 weeks post-infection.

An immune response to r-BBR42 was detected in B31MI and LDP84 infection sera. Since LDP84 was not included in the PCR or sequence analyses, it can be speculated that a BBR42-like protein is present in this strain. However, sequences for BBR42 were determined in the strains LDP89 and LDP73, both which lacked an immune response to r-BBR42 in the immunoblot analyses. Interestingly, sequence analyses reveal complete homology of BBR42 in LDP89 to BBR42 in B31MI whereas there are 2 amino acid changes in the BBR42 sequence from the LDP89 strain. Both BBM38 and BBR42 proteins were identified and sequenced from LDP89 and LDP73 strains which showed complete or high sequence conservation to the OspF proteins from the B31MI strain. However, despite the sequence homology, immunodetection of these proteins is not observed at this 12 week post infection time point. Both these observations support the finding of McDowell et al [28], which demonstrated delayed expression of BBM38 and BBR42. Therefore, during infection with LDP89 and LDP73 strains, expression of both BBM38 and BBR42 probably occur at a later time point than 12 weeks post-infection. Furthermore, sequence analyses of all OspF proteins in 297 and N40 have

identity values to BBR42 that range from 31.9 - 70.6% with a majority of conserved amino acids within the N-terminal portion of BBR42. Hence, the absence of anti-BBR42 antibodies observed in 297 and N40 infection sera is explained by sequence variation.

In addition to examining the humoral immune response to OspF proteins in mice, sera collected from Lyme disease patients were used for immunoblot analyses. Infection sera were collected from patients at distinct stages of disease. A majority of patients with early Lyme disease lacked immunoreactivity to OspF proteins. In contrast, sera collected from patients with late stage Lyme disease showed an increase in immunoreactivity to OspF proteins, of which BBO39 was the most frequently detected OspF protein. These results using human infection sera correlate with the temporal pattern of OspF expression seen in the murine Lyme disease model. An earlier study conducted by Nguyen et al [54] involving patient sera, also demonstrated an increase in immune response to OspF-related proteins amplified from *B. burgdorferi* N40. At the time of Nguyen's study, the genome sequence had not been completed, and therefore the specific OspF proteins being expressed were unknown. These analyses demonstrate that infected humans produce antibodies directed against B31MI OspF proteins emphasizing the use of OspF proteins in serodiagnostic assays and vaccines.

Finally, RT-PCR analyses revealed that *ospF* genes are transcribed during *in vitro* cultivation of B31MI. An earlier study conducted by Akins et al [51] revealed differences in transcription of ospF paralogs in 297 where transcript of *bbk2.10* was detected during *in vivo* but not *in vitro* cultivation. Therefore, the temporal expression of

OspF proteins may be regulated at a transcriptional level. Further studies investigating transcription levels of *ospF* genes during in vivo infection may explain how OspF proteins are temporally expressed during infection.

CONCLUSIONS

B. burgdorferi is the causative agent of Lyme disease, the leading arthropod-borne disease in the United States. This important health concern necessitates advanced research into the molecular mechanisms of Lyme disease pathogenesis for development of reliable diagnostic assays and effective vaccines. Research has been focused on the outer surface proteins of *B. burgdorferi* because of their direct interactions with the varied mammalian and arthropod environments and as targets for vaccine and diagnostic assays.

This study focused on the immunological and molecular characterization of the OspF proteins in *B. burgdorferi* B31MI for elucidation of their role in pathogenesis and investigation of their potential for vaccine and diagnostic assays. The immunodominant epitopes for OspF proteins were identified to lie within the variable domains accounting for the specificity of the immune response elicited to OspF paralogs during murine infection. The temporal expression of these antigenically distinct OspF proteins provides a mechanism for immune evasion and allows OspF proteins to play stage-specific roles during Lyme pathogenesis. We demonstrate the presence of antibodies and the variability of immunoreactivity profiles elicited towards B31MI OspF proteins in mice and humans infected with heterologous *B. burgdorferi* isolates. To identify the molecular basis for differences in OspF immunoreactivity profiles among the different infection

sera, PCR and sequence analyses were performed. OspF proteins identified in clinical isolates showed little diversity despite the separate geographic regions from which these isolates originated. The high degree of conservation observed among the identified OspF proteins is ideal for vaccine and serodiagnostic assay development with the desired goal for it to be administered over a wide geographic distribution. Lastly, *in vivo* studies are underway to determine the role of OspF in Lyme disease pathogenesis using allelic exchange mutagenesis for inactivation of *ospF* genes.

In summary, this thesis presents greater insight into the role of OspF proteins in immune evasion using immunological and molecular analyses. The data presented here for the first time show the immunodominant epitopes of OspF proteins and the conservation of OspF proteins in isolates from two highly endemic areas of Lyme disease. These studies expand our knowledge on the multiple immune evasion mechanisms exploited by *B. burgdorferi* for establishing chronic infection and will facilitate future research on improving treatment, diagnosis and prevention of Lyme disease.

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