CHARACTERIZATION OF ADHERENCE PROPERTIES OF THE VWFA-DOMAIN CONTAINING PROTEINS OF THE CAUSATIVE AGENT OF LYME DISEASE

An Undergraduate Research Scholars Thesis

by

ELAINE CHRISTINE WOOD

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Biology

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Approved by:

Research Advisor: Marie Esteve-Gassent
Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Characterization of Adherence Properties of the VWFA-domain
Containing Proteins of the Causative Agent of Lyme Disease
(May 2012)

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The bacterial spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease (LD) encodes for the von Willebrand Factor A (VWFA) domain proteins BB0172 and BB0173. VWFA domains are commonly known for adhering to extracellular matrix components (ECM), glycoproteins and metaloproteases. *In silico* analysis shows both of these proteins as membrane proteins. Moreover, *in vitro* analysis shows that both proteins have a transmembrane domain that anchors the protein to the cellular membrane with the VWFA domain available outside of the cell. The use of binding assays will help determine whether BB0172 and BB0173 play a role in the adhesion of *Borrelia burgdorferi* to ECM components, such as, Collagen I and IV, Laminin, plasma and cellular Fibronectin, among others, together with the use of commercially available purified integrins ($\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_3\beta_1$). Both $rBB0172_T$ and $rBB0173_T$ showed to be difficult to purified under native conditions, and therefore we developed a new purification protocol in which denatured proteins were purified in a buffer containing 8M Urea, and latter on re-natured by

elimination of the Urea using dialysis and concentration methods. Moreover, both proteins showed binding to the ECM human plasma fibronectin under the presence of the metals Ca^{2+}, Mg^{2+} and Mn^{2+}. No binding was observed to any of the other ECMs (collagen I and IV, laminin, decorin, aggrecan, vitronectin, fibrinogen and elastin) nor the integrins tested.

DEDICATION

I would like to dedicate this Undergraduate Thesis to my family in League City, Texas. Especially to my mother and father, Carolyn and Michael Wood, for without their support and kind words of encouragement I would not be where I am today. Also to my two sisters, Valerie and Brittany, I love all of you.

ACKNOWLEDGMENTS

I would like to give acknowledgment to my supervisor and mentor Dr. Maria Esteve-Gassent. She is a great mentor and has taught me so much about working in a lab setting and what it takes in order to partake in research. In this regard I would also like to acknowledge Dr. May Boggess and Dr. Jay Walton. Both run the UBM program at Texas A & M University. Without them I would not have been given the opportunity to work in Dr. Esteve-Gassent's lab. Also, I wish to thank Joseph Modarelli, Erin McGregor, and Dhwani Kothari. As well as thank the National Science Foundation for the grant to the UBM program, which helps fund my research and the American Heart Association Grant number 11SDG4990006 to Dr. Esteve-Gassent to support the materials required to conduct this study.

NOMENCLATURE

LD	Lyme Disease
Bb	*Borrelia burgdorferi*
VWF	Von Willebrand Factor
EM	Erythema migrans
ECM	Extracellular matrix
Fn	Fibronectin

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CHAPTER I

INTRODUCTION

Lyme disease is the most common tick-borne infectious disease in the US with a total of 22,572 confirmed human cases reported to the CDC in 2010. Lyme disease (LD) is caused by the bacterial spirochetes *Borrelia burgdorferi* senso lato and is transmitted by the tick vector *Ixodes scapularis* in northeastern North American, *Ixodes pacificus* in mid-western North America, *Ixodes ricinus* and *Ixodes persulcatus* in Europe and *Ixodes ovatus* in Asia (2, 3, 17). LD, or also called Lyme borreliosis, is a multisystemic disease characterized by the presence of up to three disease stages when left untreated.

The first stage of LD features the most common symptom, erythema migrans (EM). EM shows 1 to 3 weeks after the initial tick bite and starts as a erythematous papule that extends circularly around the site of infection up to 3 cm a day (3). EM normally occurs at the source of the tick bite mostly on the back, lower extremities or axilla. Overall, only 60-70% of cases have EM with central clearing, but EM without central clearing may occur as well (3). EM tends to occur more often in the USA than Europe (17). Other symptoms that may take place during the first stage of LD are fatigue, fever, headache, pain localized to the tick bite or regional lymphadenopathy (3, 17). The second stage of LD exhibits signs of dissemination with multiple EM sites and complications with

This thesis follows the style of Infection and Immunity.

neurologic and cardiac systems (3, 17). The neurological symptoms can lead to meningitis and cranial neuritis, more common in children. The common cardiac symptom is a first-degree atrioventricular block (3, 17). The process of dissemination may begin as early as the first week of infection to months after the initial tick bite (3, 17). During the second stage of LD also begins the manifestation of arthritis; up to one third of patients may show symptoms of joint pain (3, 6, 17, 18). The third stage of LD comprises neurological involvement, further cardiac problems, and chronic arthritis (6, 18). Neurological involvement starts with lymphocytic meningitis, cranial neuritis and radiculoneuritis in up to 15% of LD patients (3, 17). Neurological symptoms begin during early dissemination, but during the third stage of LD neurological symptoms may lead to more severe neurological complications such as polyneuropathy and cranial neuropathy .

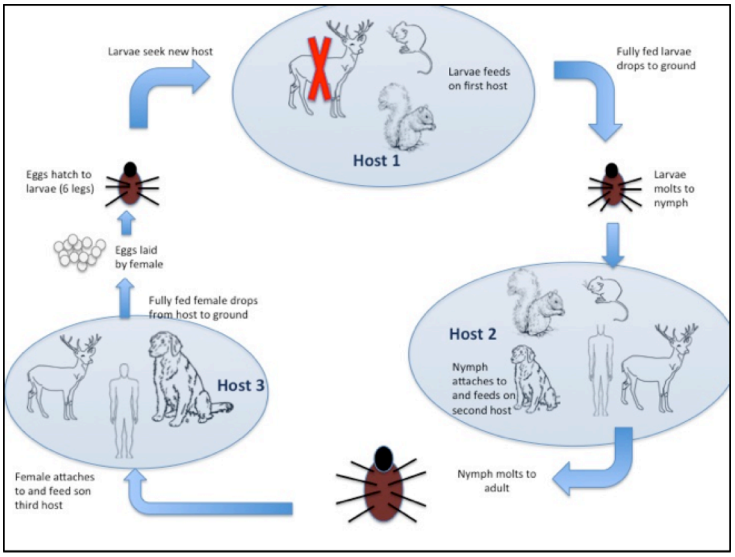

FIG 1. Infectious cycle of *Borrelia burgdorferi.*
B. burgdorferi is transmitted to competent host by the bite of infected Ixodes ticks. Upon hatching larvae are not infected with Bb, and they use to acquire the infectious agent after acquiring their blood meal on an infected mammal. A red cross indicates a less preferred reservoir host. (Adapted from "Lyme borreliosis" Stanek G).

The *Ixodes* tick life cycle plays an important role in *Bb* infection. The *Ixodes* tick life cycle consists of three stages. As observed in figure 1, the first stage comprises eggs hatching and transitioning into larvae in the spring of their first year (3). The second stage begins during summer of year one, when the larvae will mold into nymphs after their first blood meal (3). The nymphs, the most aggressive stage, will then take their blood meal, and transform into adults during the third stage (30). This occurs during the summer of year 2. When the tick feeds on an infected host it then becomes infectious. Transition of *Bb* from the adult tick mother to her eggs is extremely rare (30). Common mammalian reservoirs include the cottontail rabbit, the dusky-footed wood mouse, the cotton rat, cotton mouse, and white-footed mouse for the larvae and nymph stages. Larger mammalian reservoirs such as deer and horses are common hosts for the nymph and adult stages.

Transmission of the *Bb* senso lato takes place while feeding on the infected host. *Bb* remains attached in the tick's midgut epithelium. Once infected the tick will remain so, but rarely transmit the bacteria to its offspring, transmission of *Borrelia* to a host manifests while the tick is feeding (28). The differences in temperature and pH from the tick to host during feeding trigger the bacteria in the tick midgut allowing it to migrate from the tick midgut to the salivary glands and into the mammalian host (28, 29). This process is important to the two *borrelia* proteins BB0172 and BB0173 that will be studied in this thesis, as they have been shown *in vitro* to appear after shifting *in vitro* growth conditions from those mimicking the un-fed tick vector to those mimicking the

mammalian host (Dr. Esteve-Gassent, personal communication, manuscript in preparation).

BB0172 and BB0173 are two von Willebrand Factor A (VWFA) domain carrying proteins in *Borrelia burgdorferi.* Both proteins are membrane proteins by *in silico* analysis using TMMHM 2.0. *In vitro* studies performed in our laboratory showed that the transmembrane domains of each protein anchor these proteins to the membrane with the VWFA domain expanding on the outside of the cell. The topology of BB0172 considering the *in silico* study using TMMHM 2.0 and SignalIP4.0 is shown in Figure 2.

VWFA domain containing proteins performs a central role in the adherence of platelets in areas of vascular damage (39). VWF, a multimeric plasma glycoprotein, play this role by binding to extracellular matrix components (ECM), glycoproteins, and to metalloproteases (15, 23, 32, 39). The absence of VWF proteins, in humans, can result in severe bleeding disorders, or von Willebrand disease due to the lack of coagulation by the removal of the blood clotting factor VIII (27). This disease is the most common inherited bleeding disorder in humans.

VWF proteins have been recognized in other bacterial infections such as *Helicobacter pylori* and *Staphylococcus aureus* (4, 5, 8). The VWFA-domain containing proteins in each of these pathogens help bind to ECM elements as well as adhesion to platelets and endothelial cells. Therefore, this factor can have an important role in the dissemination

of the infection into the mammalian host. The VWFbp protein in *Helicobacter pylori* is known to bind to the Ib glycoprotein on platelets (8). *Staphylococcus aureus* VWFbp domain was able to coagulate the plasma of human's and porcine. Many other VWF binding proteins have been identified and are know to bind to ECM components such as collagen (28, 29, 39). In order to test the role of VWFA domain as an adhesion molecule in the proteins BB0172 and BB0173, a series of binding tests to various ECM components, including Fibronectin from humans and plasma, Collagen I and IV and Laminin as well as binding assays to commercially available integrins ($\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_3\beta_1$) and platelets will be conducted (9, 11-13).

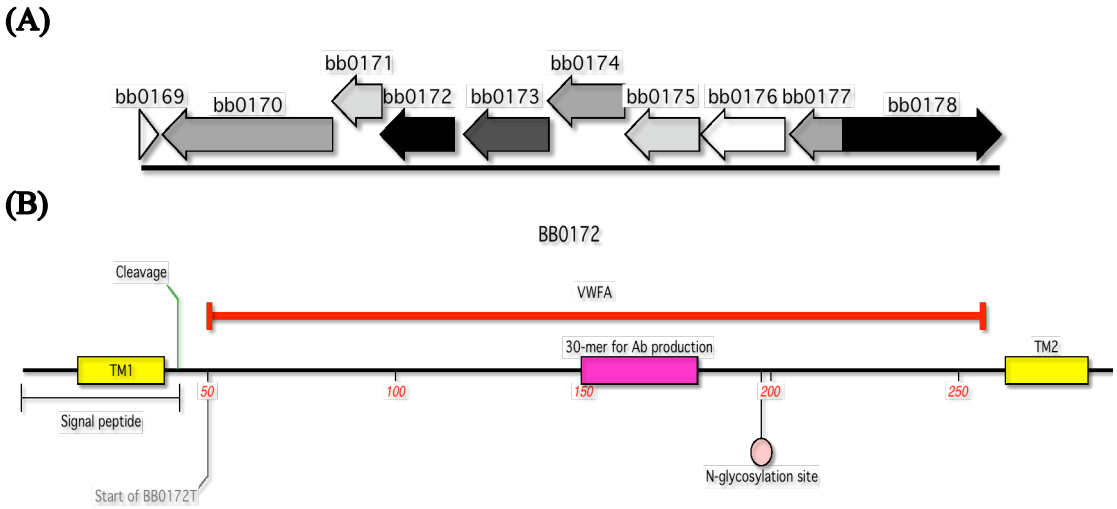

FIG 2. BB0172 Topology**. (A)** Chromosome region where bb0172 and bb0173 are encoded. (B) Structural components of BB0172: the signal peptide comprises amino acids 1-39, with cleavage site in between amino acid 39-40 (QRA-VE), tranmembrane domains extend from amino acid 16-38 and 253-284. A glycosylation site is located in amino acid 198 to 200. The VWFA domain extends from amino acids 45 to 250.

CHAPTER II

METHODS

Recombinant protein expression and purification

Induction and growth conditions

Previously in our laboratory Dr. Esteve-Gassent cloned truncated versions of the Bb proteins BB0172 (*bb0172*) and BB0173 (*bb0173*) without the first 50 or 90 amino acids respectively (so as to remove the signal peptide), P66 (*bb0603*) and BBK32 (*bbk32*) (unpublished information) in the expression vector pET23a (Novagen) and in the expression *E. coli* host Rosetta® and Rosetta-gami® strains (Novagen). Reference Table 1 for the list of plasmid constructs and bacterial strains. To express $rBB0172_T$, $rBB0173_T$, rBBK32 and rP66, overnight cultures were started in 10 mL of LB broth media containing their respective antibiotic and incubated overnight at 37ºC with continuous shaking. The following day the overnight culture was added 1:100 dilution to 1 L of fresh LB broth media with their respective antibiotics and incubated at 37ºC under continuous shaking until the Optical Density (OD) at 600nm reached a value of between 0.5 and 0.8. Proteins were induced by the addition of Isopropil β–D-thiogalactopyranoside (IPTG) to the grown cultures at a final concentration of 1mM and incubated at 37ºC for 2 hours. After the 2 hours incubation, cells were harvested by centrifugation at 4000rpm for 20 minutes and 4ºC. The pellet was then stored at -80ºC for further use.

TABLE 1: Plasmids, bacterial strains and antibiotics used in the expression of the different borrelial proteins.

Protein	Stain	Antibiotic	Concentration (μg/ml)
pET23a(*bb0172*)$_{50\text{-}290}$	Rosetta-gami ®	Ampicillin	100
		Chloramphenicol	20
		Kanamycin	34
		Tetracycline	10
pET23a(*bb0173*)$_{90\text{-}333}$	Rosetta ®	Ampicillin	100
		Chloramphenicol	20
pET23a(*bbk32*)	Rosetta®	Ampicillin	100
		Chloramphenicol	20
pET23a(*p66*)	Rosetta®	Ampicillin	100
		Chloramphenicol	20

Purification of recombinant proteins

The purification of each recombinant protein was achieved under denaturing conditions. The buffers for this process included a lysate buffer, a wash buffer and an elution buffer as per His60 Ni Superflow^{TM} Resin and Gravity Columns Users Manual (Clontech). The lysate buffer consisted of 50 mM NaH_2PO_4, 8 M urea, 300 mM NaCl, and 20 mM imidazole with a pH of 7.4. The wash buffer includes 50 mM NaH_2PO_4, 8 M urea, 300 mM NaCl, and 40 mM imidazole with a pH of 7.4. The elution buffer contains 50 mM NaH_2PO_4, 8 M urea, 300 mM NaCl, and 300 mM imidazole with a pH of 7.4. All buffers were autoclaved and stored at 4ºC. To purify the proteins, harvested cells were thawed on ice and re-suspended in 10 mL of the lysate buffer plus a protease inhibitor. Cells were lyzed by French Press (FP) (Thermo Scientific), running the mixture through the

machine 2-3 times to completely lysate the cells. The lysate was cleared by centrifugation at 4000 rpm for 20 minutes. The supernatant of this centrifugation is then added to 5 mL of previously equilibrated nickel His60 Ni Superflow Resin (Clontech) and incubated at 4ºC overnight with gentle shaking. After binding the recombinant proteins to the beads through the histidine tag, the Ni bead mixture was sedimented at 500 rpms for 5 minutes. Post adsorption supernatant (PASN) was stored at -80ºC until purification of each recombinant protein was confirmed. Ni beads were washed with 10ml of the washing buffer 3 times by sedimenting them at 500rpm for 5 minutes. Beads were re-suspended in 10 mL of the wash buffer and loaded in chromatography columns connected to a peristaltic pump. Each recombinant protein was eluted by adding10 mL of the elution buffer to the gravity column and collecting 0.5 mL to 1 mL fractions. A total of 20 to 25ml of the elution buffer was needed per extraction to completely elute each one of the recombinant proteins purified in this study. Fifty µL of each fraction as well as the washing fractions, the PASN and the FP lysates were mixed with 50 µL of 2x Final Sample Buffer (2x FSB, SDS-PAGE loading buffer), boiled for 5 minutes and stored at -20ºC until use. All the elution fractions, washes and PASN were stored at -80ºC until use.

Protein clean up and concentration

For each recombinant protein, elution fractions, PASN and washes were separated in a 12% SDS-PAGE gel, to determine the elution fractions containing the majority of the protein. . Once the appropriate fractions were determined they were placed in a dialysis

Cassettes (Slide-A-Lyzer, Thermo Scientific) and stirred for 2 hours at room temperature in a dialysis buffer (50 mM NaH_2PO_4 and 300 mM NaCl). After 2hrs, the old dialysis buffer was replaced with fresh buffer and incubated stirring for 1 more hour. Once dialysis was finished the clean protein fraction was recuperated and spun using AmicoUltra filtration systems to concentrate the protein before storing them at -80ºC. The protein concentration in each one of the clean protein fraction's was determined using the BCA Protein Assay Kit (Thermo Scientific) following manufacturer's recommendations. Briefly, serial dilutions of a known protein standard provided in the kit (BSA, 2mg/ml) were done ranging from 0 to 250µg/ml). Each recombinant protein was diluted 1:5 and 1:10. Twenty-five µl of each one of the samples or the standards were added in triplicates to 96-well plates (Corning). After adding the working reagent (provided by the kit) samples were incubated at 37ºC for 30 minutes. Protein concentration was measured using the BGI OMEGA plate reader and adjusted in the downstream binding assays performed.

Binding Assays

Plates coated in extra cellular matrix components

Pre-coated 96 well plates (BioCoat, BD) with Lamin, Collagen I, Collagen IV, and Fibronectin were used in this part of the study. The plates were blocked with blocking buffer (Phosphate Buffer Saline (PBS: 137mM sodium chloride, 2.7mM potassium chloride, 4.3mM sodium phosphate 1.4mM potassium phosphate, pH 7.4), 0.5% tween 20, and 3% Bovine Serum Albumin (BSA)) for 2 hours at room temperature. The

binding of the purified proteins was done by adding 500 ng/well and 100 ng/well of each of the recombinant proteins ($BB172_T$, $BB0173_T$ and BBK32) in PBS-T (PBS containing 0.5% Tween 20) for 1 hour at room temperature. The plates were then washed with PBS-T and incubated with a 1:1000 dilution of mouse anti-BB0172 or anti-BB0173 or 1:2000 dilution of the anti-BBK32 for 1 hour at room temperature. The plates will then be washed and a secondary anti-mouse-HRP conjugated antibodies at a 1:3000 dilution will be added and incubated for 1 hour at room temperature. After a final wash the plates were developed using OPD tablets (Thermo Scientific) and the OD at 450 mm read in a BGI Omega plate reader. Wells with no recombinant protein were used as blanks.

Plates coated with $BB0172_T$, $BB0173_T$ and BBK32

Plates were first coated with 500ng/well of each of the respective recombinant protein. After coating overnight at 4ºC plates were washed and blocked using the PBS-T with 3% BSA buffer for 2 hours at room temperature. Blocked plates were washed and incubated for 1 hour at room temperature with 500 ng/well of each of the ECM components specified in table 2 (1, 10). After washing, plates were incubated for 1hr at room temperature with the respective anti-ECM component for 1 hour at room temperature. The plates will then be washed and a secondary HRP conjugated antibody was applied for 1 hour at room temperature before being developed with the OPD tablets (Thermo scientific) as described above. Wells without ECM components were used as blanks. In addition we also ran this protocol using HBS buffer (25 mM HEPES, 150 mM NaCl, 1

mM $MnCl_2$, 1 mM $MgCl_2$, 0.25 mM $CaCl_2$) containing Ca^{2+}, Mg^{2+} and Mn^{2+} instead of the PBS. The same percent of BSA or Tween was used for blocking, washing and antibody and ECM component incubation (10).

Integrin coated plates

Plates were first coated with 500ng/well of each of the respective integrins specified in Table 3. After coating overnight at 4ºC plates were washed and blocked using the HBS-T with 3% BSA buffer for 2 hours at room temperature. Blocked plates were washed and incubated for 1 hour at room temperature with 500 ng/well of each of the recombinant proteins ($BB0172_T$, $BB0173_T$ and P66). After washing, plates were incubated for 1hr at room temperature with 1:1000 dilution of the mouse anti-BB0172 or anti-BB0173 or with 1:2000 dilution of the mouse anti-P66 specific antibody. The plates will then be washed and a secondary anti-mouse-HRP conjugated antibodies at a 1:3000 dilution will be added and incubated for 1 hour at room temperature. After a final wash the plates were developed using OPD tablets (Thermo Scientific) and the OD at 450 mm read in a BGI Omega plate reader. Wells without integrins were used as blanks. All samples were studied in triplicates.

TABLE 2: List of ECM components used in Binding Assays

ECM component	Purified protein	Specific antibody
Fibronectin (from human plasma)	F0895-1mg	F3648-0.2ml (in Rabbit)
Fibronectin (from human fibroblast culture)	F0556-100µl	
Fibrinogen (from human plasma)	F3879-250mg	F8512-2ml (in Goat)
Decorin (from Bovine articular cartilage)	D8428-0.5mg	SAB2501273-100µg (in Goat)
Aggrecan (from Bovine anticular cartilage)	A-1960-1mg	SAB4500662-100µg (in Rabbit)
Vitronectin (human recombinant)	SRP3186-250µg	SAB2102690-50µg (in Rabbit)
Elastin (from human aorta)	E6777-2mg	HPA018111-100µL
Anti-Goat Peroxidase labeled		A5420-1ml

TABLE 3: List of integrins used in Binding Assays

Integrin	Catalog #	Function
$\alpha_1\beta_1$	CC1012	It is a receptor for laminin and collagen. It recognizes the proline-hydroxylated sequence G-F-P-G- E-R in collagen
$\alpha_3\beta_1$	CC1092	It is a receptor for fibronectin, laminin, collagen, epiligrin, thrombospondin and CSPG4. Alpha- 3/beta-1 may mediate with LGALS3 the stimulation by CSPG4 of endothelial cells migration
$\alpha_V\beta_3$	CC1018	α_V is a receptor for vitronectin, cytotactin, fibronectin, fibrinogen, laminin, matrix metalloproteinase-2, osteopontin, osteomodulin, prothrombin, thrombospondin and von Willebrand factor. They recognize the sequence R-G-D in a wide array of ligands.
$\alpha_V\beta_5$	CC1024	α_V is a receptor for vitronectin, cytotactin, fibronectin, fibrinogen, laminin, matrix metalloproteinase-2, osteopontin, osteomodulin, prothrombin, thrombospondin and von Willebrand factor. They recognize the sequence R-G-D in a wide array of ligands
$\alpha_5\beta_1$	CC1052	It is a receptor for fibronectin and fibrinogen. It recognizes the sequence R-G-D in its ligands
GPIIbIIIa	528240	Native, platelet membrane glycoprotein IIbIIIaA from human platelets. A platelet integrin that binds to fibrinogen via the recognition sequence Arg-Gly-Asp-Ser. Acts as a receptor for such adhesive ligands as fibrinogen, fibronectin and von Willebrand factor during platelet stimulation. Glycoproteins IIb and IIIa constitute the fibrinogen receptor and are required for platelet aggregation. Glycoprotein IIb consists of two disulfide-linked subunits, GPIIbα and GPIIbβ, whereas GPIIIα has only one polypeptide chain

CHAPTER III

RESULTS

Recombinant protein purification and expression

SDS-PAGE gel

After growing the bacterial clone expressing each of the recombinant proteins as described above, we induced their expression for 2hrs at 37ºC. Once the induction was terminated, cells were disrupted by French Press in the cold to prevent any protein degradation. Recombinant proteins were purified utilizing their binding to Ni chromatography through the 6*His tag engineered in their C terminus end. Each one of the eluted fractions together with the induction lysate and the post-absorbion supernatant (resulting from the binding of the recombinant proteins to the Ni beads) were separated in a 12% separating SDS-PAGE gel (Figure 3). These gels were used to determine which fractions to use in the downstream assay. As observed in figure 3A and B, $BB0172_T$ and $BB0173_T$ expressed in very low amounts giving positive elution fraction 4-9 for $BB0172_T$ and fractions 5-10 for $BB0173_T$. This result was expected since both proteins are membrane proteins and they tend to be toxic when expressed in *E. coli*. Thus, by eliminating the signal peptide and transmembrane domain in the N-terminus of these proteins during the cloning process (data not shown), we made them soluble and easy to purify in enough concentrations to carry out the downstream binding assays, as well as to generate specific antibodies for other applications. On the other hand, BBK32 was

induced in higher concentrations (figure 3C). For this recombinant protein elution fractions E2 though E12 and above had enough protein to do the whole proposed study. Selected fractions were then pooled for each recombinant protein and dialyzed to eliminate the Urea and imidazole used in the purification process. Samples were concentrated and stored at -80ºC for further use.

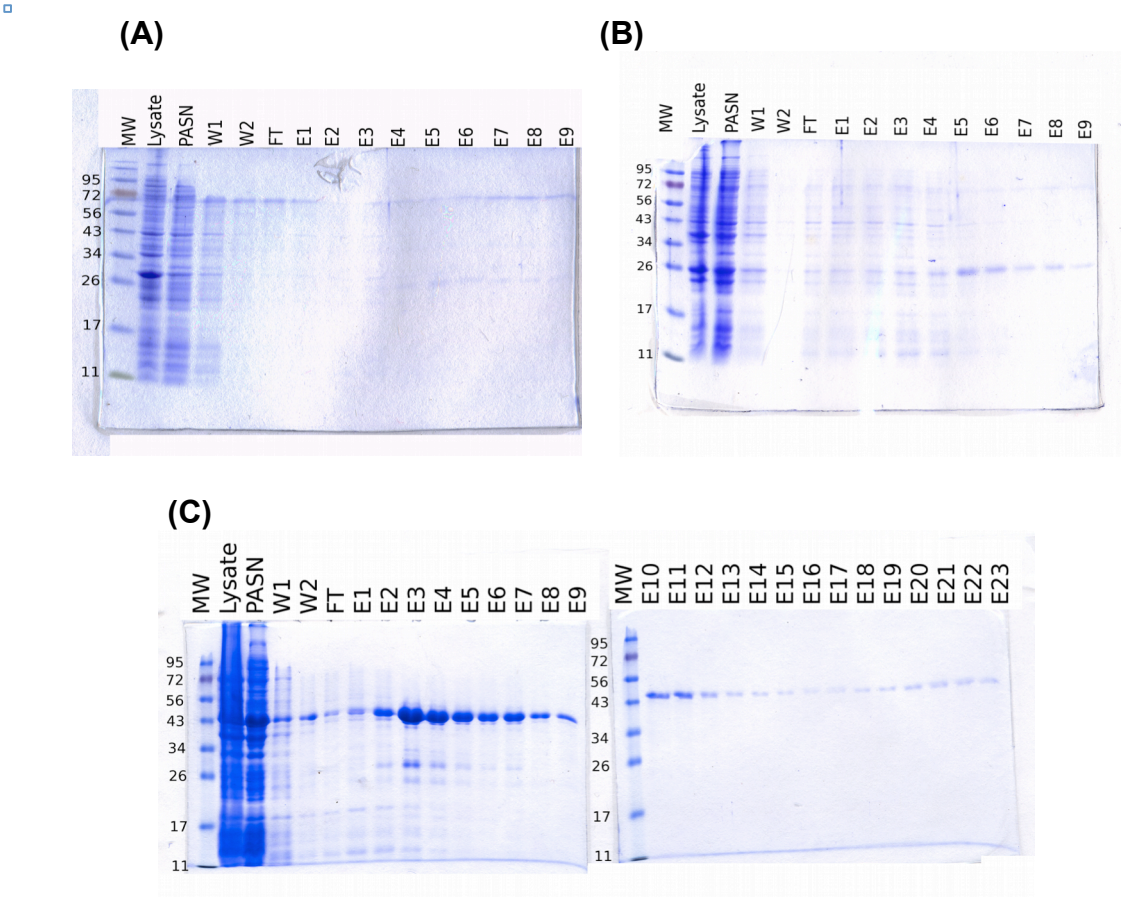

FIG 3**.** Recombinant BB0172$_T$ **(A)** rBB0173$_T$ **(B)** and rBBK32 **(C)** purification. Fractions were separated by 12% SDS-PAGE gels and stained with Coomassie blue. MW is the molecular weight in kDa (Ez-Run, Thermo Fisher). The whole cell lysate, the post adsorption supernatant (PASN) and the wash 1 and 2 (W1 and W2) were loaded to determine whether the protein was expressed correctly and whether or not it was lost in the different purification steps. All the collected elution fractions were loaded to determine those containing the majority of the purified protein (E1 though E 23).

BCA assay

After dialysis and filtration the purified proteins were quantified using the BCA Assay (Thermo Scientific). This kit uses a set of 6 standards ranging from 0 µg/mL to 250 µg/mL. Replicates of 1:5 and 1:10 dilutions of each recombinant protein were compared to these standards to determine their concentration. $rBB0172_T$ and $rBB0173_T$ concentrations ranged from 25 µg/mL to 125 µg/mL, and rBBK32 concentrations averaged 625 µg/mL. A standard curve was used to establish the fitness of each concentration (figure 4). Fitness, r^2, was measured to be above 0.99.

FIG 4. Representative Standard curve of BCA Assay Kit.

Binding Assays

Extra cellular matrix components binding assays

Two different approaches were used in this study. In the first approach we used pre-coated plates with different human ECM (Table 2, BD BioCoat^{TM}). In those plates, both $BB0172_T$ and $BB0173_T$ showed very little to no binding to each of the ECM components tested, compared to the control BBK32 (figure 5). BBK32 showed high affinity for Fibronectin, Collagen I and IV and much lower binding to Laminin.

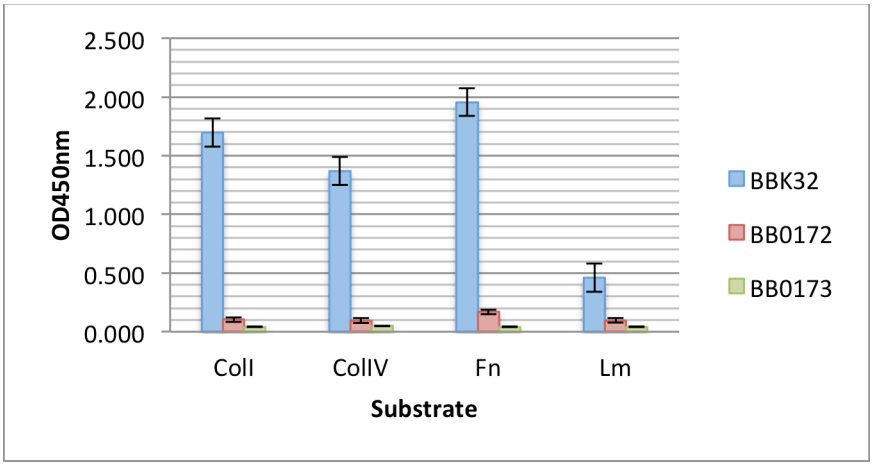

FIG 5. ECM Binding Assay with pre-coated plates. ECM Binding Assay to pre-coated plates with ECM components Collagen I, Collagen IV, Fibronectin, and Laminin (BD BioCoat)

Since BB0172 showed a little binding with Fibronectin, we decided to repeat the binding assay using human plasma and tissue fibronectin. Moreover, it has been previously

reported that proteins such as BBK32 bind different to either one of them (14, 19, 22, 24, 33-36). To test whether BB0172 and BB0173 had differential binding such as the one observed with BBK32, we coated 96-well plates with 500ng/well of each one of our recombinant proteins. After incubating with 500ng/well of the human plasma or tissue fibronecting plates were developed and OD_{450nm} was read using a plate reader. As observed in Figure 6, the control rBBK32 showed high binding to the human plasma Fibronectin while it bound with lower intensity to tissue fibronectin, $BB0173_T$ showed no binding to either Fibronectin substrate. $BB0172_T$ shows very little binding to Human Fibronectin from tissue, but no binding to Human Fibronectin from plasma.

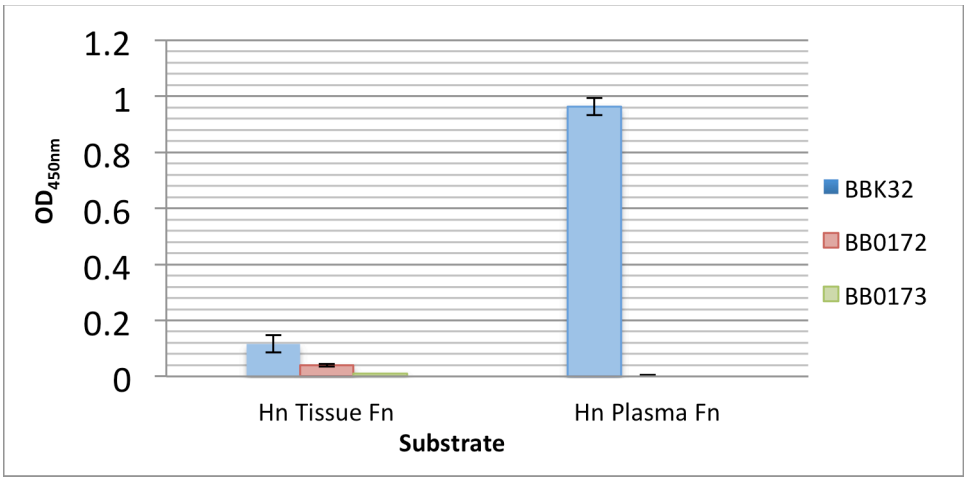

FIG 6. ECM Binding Assay coated with recombinant proteins**.** ECM Binding Assay to plates coated with recombinant protein showing their binding to Human Fibronectin from Plasma and tissue.

The last ECM binding assay was also done using coated plates with each recombinant protein. At this point we used the ECMs listed in Table 2. Since we observed very little binding to the ECM components showed above we decided to change some of the conditions to incorporate metals in our reaction buffer since both BB0172 and BB0173 show *in silico* a metal binding domain (MIDAS). To this end the HBS buffer containing Mg^{2+}, Ca^{2+}, Mn^{2+} was used. As shown in figure 7, $rBB0172_T$ and $rBB0173_T$ display a much higher adhesion to the ECM components tested in the presence of metals than without them (figure 5). The ECM Fibronogen exhibited the highest affinity to each recombinant protein, while Aggrecan, Vitronectin, Decorin, Elastin and Fibrinogen showed the least affinity. Finally, an integrin binding assay, plates coated with each integrin, with incubation steps using the HBS buffer containing Ca^{2+}, Mg^{2+}, Mn^{2+} no binding occurred to either recombinant protein BB0172 and BB0173.

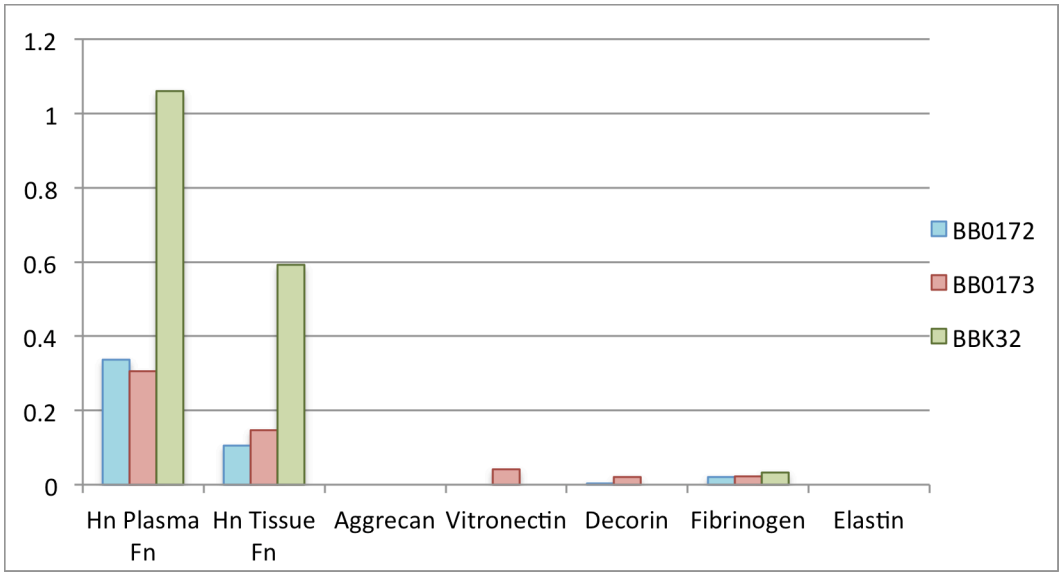

FIG. 7. ECM Binding Assay using plates coated with recombinant proteins. Binding Assay was done with HBS buffer (containing the metals Ca^{2+}, Mg^{2+}, Mn^{2+}). The origin of each one of the ECMs I specified in Table 2.

CHAPTER IV

DISCUSSION

The ability of *Borrelia burgdorferi* to colonize the mammalian host is dependent on its ability to rapidly alter gene expression in response to highly disparate environmental signals following transmission from infected ticks. Some of the open reading frames (ORFs), up-regulated upon infection, are members of a large family of proteins termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), which facilitate adherence of *B. burgdorferi* to extra-cellular matrix components of the host. By comparative genome analysis, a family of von Willebrand factor A (VWFA) domain-containing proteins have been identified in *B. burgdorferi* (31, 37, 39). These chromosomally encoded proteins (BB0172, BB0173, BB0175 and BB0325) could be involved in the interactions of *B. burgdorferi* with the mammalian host (37). The VWFA domains present in extracellular matrix (ECM) proteins and on eukaryotic cells are involved in cell adhesion and protein-protein interactions (4, 5, 8, 21). Therefore, these borrelial proteins with VWFA domains might be involved in the adhesion of *B. burgdorferi* to eukaryotic cells, ECM components or to activated platelets and thus play a role in the virulence mechanisms of *B. burgdorferi.* Our first approach to understand the role of the borrelial VWFA containing proteins was to evaluate the adhesion of those exposed to the bacterial surface to extracellular matrix components.

As stated previously, both BB0172 and BB0173 are membrane proteins by *in silico* analysis. Also, *in vitro* studies performed in our laboratory showed that each protein has a transmembrane domain with the VWFA domain located on the outside of the cell (Maria Esteve-Gassent, manuscript in preparation). Consequently, the expression of these two proteins as recombinant proteins has proven challenging due to the transmembrane domain and signal peptides, located within the first 50 to 90 amino acids, inducing toxicity to *E. coli*. To overcome this toxicity effect, the first 50 amino acids on BB0172 and the first 90 amino acids on BB0173 were removed during the cloning process. The result of this elimination was the truncated version of BB0172 and BB0173 and allowed expression of both recombinant proteins in *E. coli* during the first 2-3 hours of induction and at enough concentrations that allowed further experiments possible.

The goal of our study was to purify each of the recombinant proteins in its native form for their use in the Binding Assays. Therefore, the first purification was done under native conditions with buffers containing 250 mM of imidazole. Both proteins were found to be insoluble and were located in the lysate pellet. The next purification attempt was done under denaturing conditions with buffers containing 8 M Urea. Both proteins were soluble, but still unable to be eluted do to a lack of binding to the Ni beads. A mixture of native and denaturing conditions was used for the third trial of purification. The buffers for this purification included both 8 M Urea and 300 mM imidazole. The 300 mM imidazole addition to the denaturing buffer increased the stringency to compete both imidazole and our His*tagged proteins to the Ni beads. This change enabled the

purification of each recombinant protein at concentrations that were appropriate for further studies.

Since both rBB0172$_T$ and rBB0173$_T$ were purified with 8 M Urea the re-naturing of each protein was required. To this end, each of the protein was dialyzed, either for 48 hours at 4ºC or by three hours at room temperature, changing the buffer each hour, with an additional over night dialysis at 4ºC. After dialysis each purified protein was concentrated, measured using the BCA Assay kit, and then stored at -80ºC. A confirmation of dialysis is shown during thawing of each recombinant protein by the lack of crystals that normally form when Urea is at colder temperatures.

Consequently, in this study we were able to rescue the lytic phenotype that these type of membrane proteins use to have in surrogate hosts, and were able to express and purify both borrelial membrane proteins at enough concentration to re-nature and utilize in further binding assays. The first binding assay was done using pre-coated plates of Fibronectin, Laminin, Collagen I and IV (BioCoat^{TM}, BD). These ECMs were chosen because of their relevance during infection processes and because of their commercial availability. In addition, the borrelial protein BBK32 was selected as a control for ECM binding assays. BBK32 is a known *B. burgdorferi* Fibronectin binding protein that has been extensively studied in recent years (14, 19, 22, 24, 33-36). Since, little to no binding occurred for either rBB0172$_T$ or rBB0173$_T$ three changes in the approach to the binding assays were made. First, the plates were coated with each recombinant protein

instead of using the commercially available pre-coated plates. Second, more ECM components commercially available were included in the study, as seen in table 2. BB0172 and BB0173 have a region within their WVFA domain with high similarity to typical integrin metal binding domain (MIDAS) (21). Therefore, the new experiments were done in the presence of metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}) (10) to determine whether or not this condition facilitated the binding to any of the ECMs by either recombinant proteins (15, 23, 32, 39). Under these conditions, both recombinant proteins were able to bind to Human Plasma Fibronectin while no binding was observed towards the other ECMs tested, which suggests certain specificity.

The ability of Borrelial $rBB0172_T$ and $rBB0173_T$ to bind to Fibronectin could explain why *B. burgdorferi* BBK32 knock out mutant strains are still able to bind to Fibronectin as observed in different studies (20, 24, 36). It has been shown that *B. burgdorferi* tends to encode for multiple proteins with similar adherence fuction such as BBK32 and RevA (7, 9, 14, 16, 36) binding to Fibronectin and DbpA/B to decorin (11, 25, 26, 38). Consequently, BB0172 and/or BB0173 could be support system to BBK32 binding to Fibronectin. In addition, we have observed that BB0172 is expressed when bacteria cultures are shifted from RT/pH7.6 (un-fed tick conditions) to 37ºC /pH6.8 (fed tick conditions) and stops its expression when adapted to 37ºC. Consequently, BB0172 could be binding to Fibronectin during the initial transmission out of the tick and into the host, before BBK32 is expressed in enough levels so as to take over the fibronectin binding.

Another plausible explanation for BB0172 and BB0173 binding to Fibronectin could be that they are used to bind in different tissues other than the tissues BBK32 target.

Taken together, during this study we were able to purify two borrelial outer membrane proteins BB0172 and BB0173. Both proteins are encoded in the chromosome and contain a VWFA-domain in the central region of the protein, with metal biding capabilities. The presence of metals showed to be a limiting factor that regulated the biding of these VWFA-domain containing proteins to ECM components. Currently we are investigating which of the metals utilized in this study will be the one turning on and off the binding of BB0172 and BB0173 to human plasma fibronectin, and whether or not they will also be involved in the binding of the borrelial VWFA-domain containing proteins to integrins. Moreover, these two proteins could be part of the adhesin collection encoded in *B. burgdorferi* to bind to different tissues in different stages of the transmission from the tick to the mammalian host. Having different adhesins expressing at different times during transmission, could be a critical step in the biology of *B. burgdorferi*, since it has to rearrange its gene expression to survive during the transition in between two complete different environments, the arthropod tick *versus* the mammalian host. In summary, our study started to elucidate the potential role of VWFA-domain containing proteins in *B. burgdorferi* and their relevance during the infection process.

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