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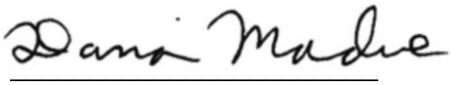
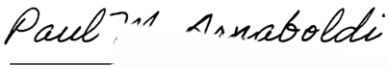
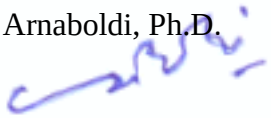


Dr. Dana Mordue, Ph.D. (Sponsor)



Dr. Paul Arnaboldi, Ph.D.



Dr. Chandra Shekhar Bakshi, DVM, Ph.D.

Date of Approval

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Abstract

Babesia microti is a tick born, intraerythrocytic parasite that is endemic to many North American regions. *B. microti* infection causes a disease called babesiosis, a disease whose clinical manifestation causes a wide range of symptoms, from asymptomatic infection to fulminant disease and possible death. Transfusion transmitted babesiosis is becoming an increasing concern as B. microti prevalence continues to grow and donor screening methods are ineffective. Transfusion transmitted babesiosis can be extremely dangerous, with as many as one in five cases resulting in death.

Babesia microti secreted antigen (BmSA1) is an immunogen and reliable biomarker for *B. microti* infection. The aim of this work is to generate a recombinant BmSA1 protein using a trademarked *Pichia pastoris* expression system called "PichiaPink™", for use in the development in an antigen specific ELISA as a protein standard. We hypothesize that expression of recombinant BmSA1 in a eukaryotic yeast system will better preserve the native confirmation and antibody recognition that might have been lost during expression in a prokaryotic expression system. We also use bioinformatics to assess the conservation of homology of the BmSA1 protein between all B. microti strain genomes published in piroplasmaDB.com.

During this work PichiaPink™ yeast strains were successfully cloned with secretion vectors containing the *bmsa1* gene. Probing culture supernatants by Western Blot analysis with hybridoma produced monoclonal antibodies was able to detect the rBmSA1 protein in culture supernatants, but the antibodies also recognize proteins other than rBmSA1 in the culture supernatant as well. Last, bioinformatics analysis of the BmSA1 protein, together with other species of the BMN family of proteins showed great conservation of the BmSA1 protein across

all strains published in the piroplasmaDB.com database, suggesting the BmSA1 protein will be a reliable biomarker as the target of an antigen specific ELISA, as it does not appear to be subject to genetic variation across strain.

Introduction

Background:

Babesia ssp. are intraerythrocytic parasitic protozoa that infect a wide range of mammalian hosts. These protozoa belong to the phylum Apicomplexa, the same as pathogenic Plasmodium, Toxoplasma, and Cryptosporidium (Vannier & Krause, 2012; Krause, 2019). Babesia is responsible for causing babesiosis, a disease first described in cattle by a Hungarian pathologist named Viktor Babes in 1888 (Laha et al., 2015). The first recorded case of human babesiosis was in 1957 (Skrabalo & Deanovic, 1957).

Babesia Epidemiology:

Babesia ssp. responsible for causing human disease are found sporadically throughout the globe, and are endemic to the North American Northeast and Midwest regions (Carpi et al., 2016). Several different species are capable of causing human babesiosis, including *B. microti, B. duncani, B. divergens* and *B. crassa* (Yu et al., 2020). *B. microti* is the most common cause of babesiosis in the United States (White et al., 1998). Since 2011 there has been over 20,000 cases of babesiosis reported in the United States alone (Yang et al., 2021). *B, microti* infection in the US is most commonly reported from late spring to early autumn, when the *Ixodes* tick vector is most active (Krause, 2019). Recent years have seen a rapid increase in case incidence in the United States. Warming climates expanding the geographic regions inhabitable by *Ixodes* ticks and increasing white tail deer populations are likely contributing factors to the increased incidence of babesiosis (Martínez-García et al., 2021; Spielman et al., 1985). Babesiosis is growing in prevalence in regions outside of North America as well. One study conducted in Beijing found that 12% of small mammals screened in the study were infected with *B.*

microti (Wei et al., 2020). While rare, babesiosis is present in parts of Europe as well, where *B. divergens* and *B. venatorum* are the dominant species (Gray et al., 2010). Co-infection of babesiosis with Borrelia burgdorferi, the causative agent of Lyme disease is common and likely underreported because the causative agents of the two diseases are transmitted by the same *Ixodes* tick vector, and these diseases display similar symptoms (Curcio et al., 2016). Tick surveillance studies are now revealing that both Babesia ssp. and *Borrelia burgdorferi* can inhabit in a single tick together and infect humans during the same tick bite transmission event (Milholland et al., 2021).

Babesia Lifecycle and Transmission:

Beginning from the point of vector transmission, *Babesia* enters a vertebrate host as a sporozoite through the saliva of a tick while it is taking a blood meal. The sporozoite travels through the blood stream until it invades host red blood cells (RBCs) (Martínez-García et al., 2021). Once inside the RBC, the sporozoite develops into a trophozoite. Trophozoites undergo asexual binary fission in the RBC forming two (or more, depending on the species) merozoites (Jalovecka et al., 2018; Milholland et al., 2021). Once formed merozoites will eventually lyse the RBC and enter the blood stream, where they go on to infect new RBCs (Chauvin et al., 2009). This merozoite lysis from RBCs is responsible for many of the disease symptoms, including hemolytic anemia, jaundice, and renal deficiencies (Krause, 2019). Some merozoites will differentiate into pre-gametocytes inside the RBC. Pre-gametocytes lyse their resident RBCs and develop into extracellular gametocytes. It is the gametocytes that are capable of surviving ingestion by a new tick (Chauvin et al., 2009).

Once ingested by a new Ixodes tick when it takes a blood meal from an infected host, gametocytes mature into gametes, also known as “ray-bodies”. Ray-bodies undergo sexual reproduction by fission and develop into ookinetes. Ookinetes then penetrate cells of the tick’s gut where they undergo meiotic divisions, developing into kinetes (Milholland et al., 2021). Kinetes disseminate through the tissues of the tick until reaching their salivary glands, where they will differentiate into sporoblasts capable of infecting the next vertebrate that is bitten by the tick, repeating the *Babesia* life cycle (Jalovecka et al., 2018; Mehlhorn & Schein, 1985).

Human Babesiosis Clinical Manifestation:

Many babesiosis infections in immune competent individuals are asymptomatic. In some endemic areas seroprevalence studies have found as many as one in five residents are asymptomatically seropositive, meaning that they have antibodies to *B. microti* indicative of a current of past infection. (Diuk-Wasser et al., 2014). Common clinical presentations of babesiosis typically occur between 1 to 4 weeks after a tick bite and transmission of the parasite. Transfusion-transmitted infections typically have an incubation period of 1 to 9 weeks (Vannier et al., 2015), and severe disease is common with transfusion-transmitted Babesia. Babesiosis presents with a range of clinical symptoms, but most common are fever and fatigue. Other symptoms include: chills, headache, nausea, myalgia, and arthralgia (White et al., 1998). Babesiosis can occasionally cause splenomegaly and hepatomegaly. Severe/fatal disease is rare in immune-competent individuals, but more common in immunocompromised and elderly individuals (Hatcher et al., 2001). Asplenic patients are more likely to develop the severe disease than others (Dumic et al., 2020).

Severe babesiosis may result in ARDS, pulmonary edema, CHF, renal failure, splenic rupture, and possibly death (Hatcher et al., 2001).

Babesiosis Treatment:

Primary treatment regimens for any patient diagnosed with babesiosis is a 7–10-day course of combination atovaquone and azithromycin therapy. For patients unable to take either of these medications due to allergies or other reasons, or for patients for whom this therapy is not effective, a clindamycin and quinone combination is the recommended second line of treatment. Treatment is often extended past 10 days in immunocompromised patients (Krause et al., 2021). Drug treatment is not parasiticidal and in the case of high parasitemia, multiple red blood cell exchanges may be required to decrease parasite burden. While most studies show that the clindamycin and quinone combination therapy is equally as effective as the atovaquone and azithromycin combination therapy, the clindamycin and quinone combination regimen has shown to produce adverse side effects at a much higher rate (Krause et al., 2000) (Vannier et al., 2015). The most commonly reported side effects of the clindamycin and quinone combination therapy are: tinnitus, diarrhea, and temporary hearing loss (Krause et al., 2000).

Transfusion-transmitted infection:

Babesiosis is one of the most frequently acquired transfusion-transmitted diseases in the United States and one of the more common causes of transfusion-transmitted infection fatalities. (Moritz et al., 2016). The first case of transfusion-transmitted babesiosis was in 1994 in Washington State (Herwaldt et al., 1997). As many as 1 in 5 transfusion-transmitted Babesia infections result in death (Bloch et al., 2019). Babesia infections create unique problems to screening in blood banks. Health history screening of

donors is rarely effective due to the high asymptomatic rate of babesiosis, growing prevalence, and the fact that many people have never heard of babesiosis (Leiby, 2011). Studies have shown that *B. microti* can survive in blood samples stored at 4°C for up to 21 days post collection (Eberhard et al., 1995). Almost all confirmed cases of transfusion-transmitted babesiosis are caused by *B. microti* (Leiby, 2011), with a few rare reports of transfusion-transmitted babesiosis being caused by the babesia species B. duncani (Herwaldt et al., 1997).

Diagnosis of babesiosis:

Diagnosis of babesiosis is currently confirmed by a thin-layer blood smear with positive qPCR confirmation (Krause et al., 2021). These methods are adequate for diagnosing patient samples, but are ergonomically unrealistic strategies for screening large volumes of blood donation samples in a cost-effective manner as required by Blood Banks (Simon et al., 2014). There is currently only one approved molecular screening tool for the detection of babesiosis infected blood samples used to prevent TTB called "Procleix Babesia Assay" (from Grifols Diagnostic Solutions). Procleix is a nucleic acid testing (NAT) assay that targets the 18s rRNA transcripts of *B. microti, B. divergens, B. duncani*, and *B. venatorum* species (Tonnetti et al., 2020). The assay runs on the Procleix Panther system, a fully automated platform utilizing Nucleic Acid Testing (NAT) for blood screening. The FDA approval recognized a successful multi-center clinical trial conducted under an Investigational New Drug (IND) study at the American Red Cross, Creative Testing Solutions, and Rhode Island Blood Center (an affiliate of the New York Blood Center, Inc.) in select areas of the U.S., blood samples must be shipped to the company for screening.

The issue with NAT assays like Procleix and qPCR for screening blood donation samples is that these types of transcription-mediated assays are cost-ineffective. Studies have shown that serological assays would be the most economically sensible method for screening blood donations by blood banks (Simon et al., 2014). However, indirect immunologic serology assays like indirect enzyme-linked immunosorbent assay (ELISA) are disadvantageous because high antibody titers are not indicative of active infection, resulting in potentially high false positive-rates (Alvarez et al., 2019). The development of a direct antigen capture ELISA ("sandwich ELISA") would eliminate the complication of relying on antibody titers while still remaining an economically reasonable option. However, in order to develop such an antigen-specific ELISA for use by Blood Banks to prevent TTB, immunogenic Babesia antigens need to be identified, synthesized and antigen-specific monoclonal antibodies need to be developed.

BmSA1:

Babesia microti secreted antigen 1 (BmSA1) is a GPI-linked secreted *B. microti* protein (Cornillot et al., 2016). BmSA1 binds to host RBCs and has been implicated in facilitating RBC invasion by merozoites (Li et al., 2020). This protein belongs to the BMN family of babesia proteins, with the most homology to the BMN1-9 protein (Luo et al., 2011). BmSA1 was identified in the Gray Strain of *B. microti*. The protein was identified as BmGPI12 in other strains of *B. microti*, and the two names (BmSA1 and BmGPI12) are now used synonymously (Cornillot et al., 2016). BmSA1 is a 33-kDa protein with a cleavable N-terminus secretion signal sequence (Luo et al., 2011). This protein is an immunodominant *B. microti* antigen. (Thekkiniath et al., 2018).

BmSA1 as a Biomarker and Immunogen:

Research using BmSA1 as the target of antigen capture assays has proven sensitive and effective. One study by the National Research Center for Protozoan Diseases at the Obihiro University of Agriculture and Veterinary Medicine was able to identify *B. microti* infection in hamsters as early as two days post-infection using a BmSA1 antigen capture ELISA (Luo et al., 2012). Another study out of Yale School of Medicine was able to design a BmSA1 antigen capture ELISA that was more sensitive than real-time qPCR diagnosis (Thekkiniath et al., 2018). Furthermore, in vitro studies have shown that anti-BmSA1 antiserum will significantly inhibit the growth of parasites in RBC tissue cultures, indicating the protein as a potential therapeutic target and vaccine candidate (Li et al., 2020).

Research using Expression of rBmSA1

BmSA1 is quickly becoming an "antigen of choice" for designing diagnostic assays; particularly serological assays. In China, some seroprevalence studies are already being conducted using antigen-capture ELISA assays targeting BmSA1 in blood donor pools (Lin et al., 2019). The growing awareness of BmSA1 as a potential key virulence factor, immunogen for diagnostics, and biomarker for *B. microti* infection has generated a clear need for a reliable method of synthesizing rBmSA1 protein that resembles that of the "wild-type" protein expressed by *B. microti*.

To date, most research using rBmSA1 for immune studies have used *Escherichia coli* bacteria for protein expression (Luo et al., 2011) (Cornillot et al., 2016; Cai et al., 2018; Thekkiniath et al., 2018; Lin et al., 2019; Li et al., 2020; Verma et al., 2020).

However, *B. microti* is a eukaryotic pathogen and *E. coli* is unable to ensure proper post-translational processing of BmSA1. More recent studies have used human cell line models for expression, like human embryonic kidney (HEK) cells (Elton et al., 2019). Human cell lines expression models are not ideal for expressing *B. microti* proteins either, as they might actually hyperglycosylated rBmSA1 proteins as they contain more glycosylase enzymes than Apicomplexan cells (Lombard, 2016). Also, most human cell lines are poor venues for large-scale production of heterologous protein. In a study by Elton et. al., researchers resorted to altering the amino acid sequence inside of an N-linked glycosylation consensus site of the HEK expressed rBmSA1 because Babesia ssp. lack the enzyme that recognizes this specific glycosylation motif (Lombard, 2016). Altering amino acid sequences of a protein could also alter the immunogenicity of an antigen like BmSA1.

Pichia pastoris and PichiaPink™ Expression Systems:

The use of *Pichia pastoris* yeast models for the expression of recombinant proteins has many advantages over other biological expression systems, particularly for heterologous expression of eukaryotic proteins. *Pichia pastoris* ca express large quantities of recombinant heterologous proteins and can be used in the industrial setting (Buckholz & Gleeson, 1991). Pichia pastoris are methylotrophic yeast capable of metabolizing methanol as their sole carbon source (Faber et al., 1995). The ability to metabolize methanol comes from their expression of alcohol oxidase enzymes. Alcohol oxidase metabolizes methanol to formaldehyde and peroxide. The Pichia pastoris genomes contain two alcohol oxidase genes, AOX1 and AOX2. AOX1 is responsible for the majority of alcohol oxidase expression in these cells. The significance of the alcohol oxidase gene in the expression of recombinant protein is that the AOX1 gene is tightly controlled under the regulation of its

own methanol-induced promoter. Using genetic cloning mechanisms, genes of interest can be cloned downstream of this promoter to enable heterologous protein expression through the induction of alcohol oxidase expression. Alcohol oxidase binds to oxygen with poor affinity and *Pichia pastoris* compensates for this by expressing alcohol oxidase at high levels. As such, any genes that are coupled to this expression will be expressed at high levels as well (Invitrogen, 2014).

Pichia pastoris expression models have another key advantage, specifically over other commonly used prokaryotic expression models like *E. coli*. Prokaryotes lack membrane-bound organelles, and as a result, a recombinant protein expressed by these cells will lack any posttranslational modifications that occur in the endoplasmic reticulum, Golgi apparatus, secretory pathway of eukaryotic cells (Buckholz & Gleeson, 1991). *Pichia pastoris* post-translational modifications resemble those of higher eukaryotes, especially glycoprotein modifications. This is important when using recombinant proteins in studies that require bioactive proteins or rely on antibody recognition, like ELISA development. Studies have shown that glycosylated immunogens have their immunogenicity better conserved when expressed in eukaryotic models versus prokaryotic models (Bagno et al., 2020).

The PichiaPink™ Expression System is a trademarked Pichia pastoris expression model that incorporates 4 proprietary yeast strains and unique cloning vectors to facilitate the generation of recombinant proteins. The yeast strains are designed to express varying levels of protease knockouts to prevent the degradation of a recombinantly expressed protein. Strain 1 is a “wild-type” expressing protease strain that has no protease gene knockouts. Strain 2 is a Proteinase A gene *pep4* knockout. Strain 3 contains a proteinase B

gene prb1 knockout. Strain 4 is both a *pep4* and *prb1* knockout. The cloning vectors are constructed with genes for antibiotic resistance, adenine complementation for selection (Figure 1), and cloning sites for insertion of genes of interest, all designed to facilitate the successful cloning of PichiaPink™ strains. The PichiaPink™ Expression System also includes vectors that allow heterologous proteins to be secreted resulting in improvements of purification of the heterologous recombinant proteins (Invitrogen, 2014).

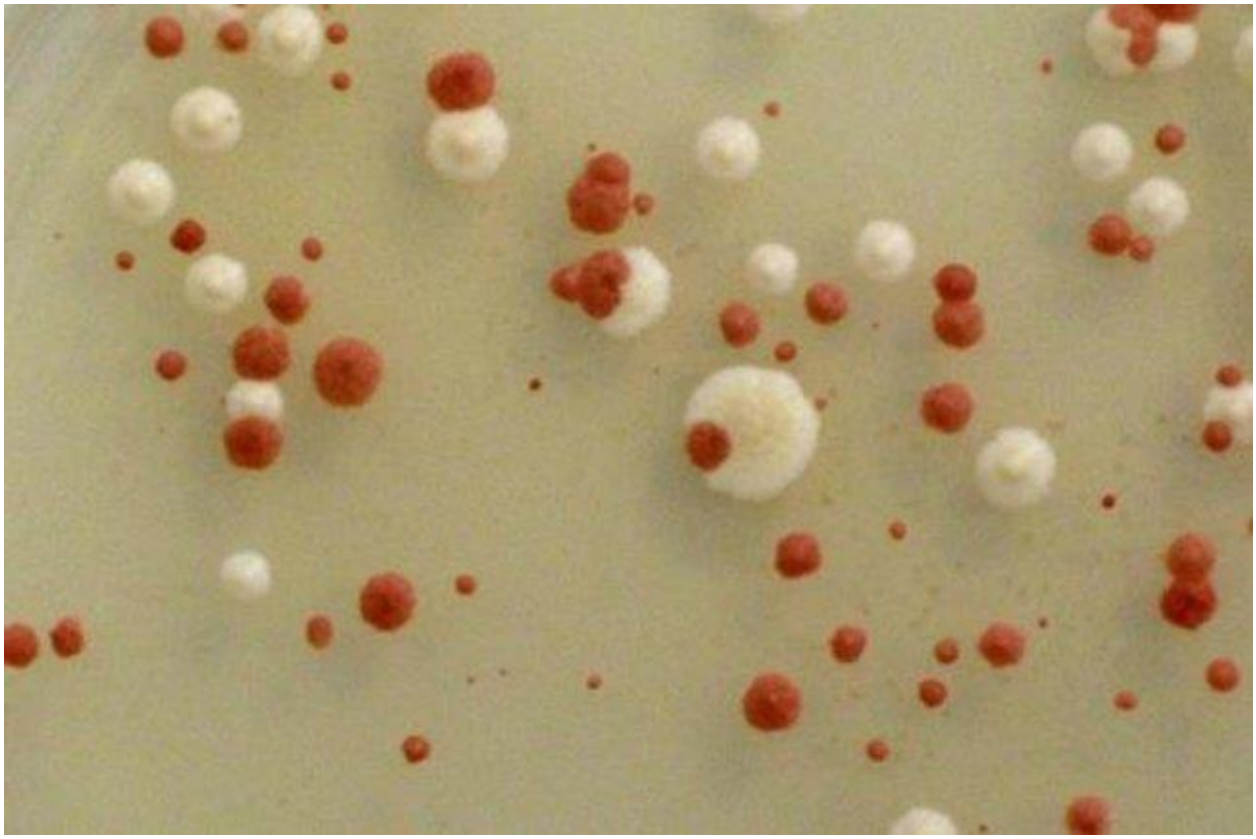


Figure 1: This image is a sample image from ThermoFisher.com that shows the appearance of PichiaPink™ colonies growing on PAD selection agar following electroporation. The red colonies contain low vector insert copy numbers, and the white colonies contain high vector insert copy numbers – (https://www.thermofisher.com/order/catalog/product/A11156#/A11156) (Invitrogen, 2014).

Monoclonal Antibodies

An antibody library was constructed for the generation of monoclonal antibodies by hybridoma cells against *B. microti*, Gray strain ATCC-30221 cultured in vivo in BALB/c mice. These antibodies were previously validated against rBmSA1 expressed in *E. coli*. The two antibodies used in this study, RD167 and RD261 both recognize *E. coli* expressed rBmSA1 by Western Blot analysis. The *E. coli* expressed rBmSA1 for validation was His-tagged and purified by a nickel column prior to Western Blot analysis. In the unpurified *E. coli* lysate RD167 recognizes a protein bands of higher molecular weight (100kDa and 75kDa). The antibody RD261 was never screened against the unpurified *E. coli* lysate, but it did recognize a protein band of 55kDa in the purified protein sample. The investigators suggest a possible explanation for staining of the higher molecular weight bands in the unpurified lysate by RD167 to be the results of the Alkaline Phosphatase Goat Anti-Mouse IgG secondary antibodies binding to endogenously expressed *E. coli* proteins that contain Fab antibody binding domains. However, this hypothesis was never tested. The investigators suggest a possible explanation for the staining of the 55kDa band by RD261 in the purified protein samples to be the presence of rBmSA1 protein aggregates. This hypothesis has also never been tested. One of the aims of this study is to generate rBmSA1 using a Pichia pastoris expression system to see if these antibodies still recognize molecular weight bands other than those consistent with rBmSA1.

Materials and Methods

Primer Design

All primers were ordered through IDT Technologies. Primer sequences used in the study are listed in Table 1

bmsa1 and *bmn1-21* Gene Amplification:

B. microti Gray Strain ATCC 30221 was used as the source of template DNA for PCR amplification of *B. microti* sequences from parasites. DNA purification from blood tissue samples was performed using Qiagen® DNeasy blood and tissue kit, as per the manufacture's protocol. 400µl of blood from *B. microti* infected mice was frozen upon isolation at -80°C and used for isolation/purification of parasite DNA. The *bmsa1* and *bmn1-21* genes were amplified from the purified template DNA by PCR, using the primers for PCR amplification underlined in (Table 1), using Phusion® High-Fidelity PCR Master Mix. Unique primers were designed to create adaptors for 5' end of the *bmsa1* gene, including synthesis of a phosphorylated 5' end, and a 3'*KpnI* restriction site adapter at the end of the *bmsa1* gene. For *bmn1-21*, a 5' adaptor with a *MlyI* restriction site and 3' adaptor to include a 3'*KpnI* restriction site adapter on the amplified *bmn1-21* gene.

PRIMER DESCRIPTION	PRIMER SEQUENCE
***BMSA1* FORWARD 5' PHOSPHORYLATED PRIMER FOR PCR AMPLIFICATION**	5'-5Phos/GCT-GGT-GGT-AGT-GGT-GGT-AAT-G-3'
***BMSA1* REVERSE PRIMER WITH KPNI RESTRICTION SITE FOR PCR AMPLIFICATION**	5'AAA-GGT-ACC-TTA-GAA-TAG-AAA-CAT-AGC-GAC-CGA-G-3'
***BMN1-21* FORWARD PRIMER WITH MLYI RESTRICTION SITE FOR PCR AMPLIFICATION**	5'-AAA-GAG-TCA-AAA-AGG-AAA-TGG-TGA-TGT-TAA-TCA-ATA-TT-3'
***BMN1-21* REVERSE PRIMER WITH KPNI RESTRICTION SITE FOR PCR AMPLIFICATION**	5'-AAA-GGT-ACC-TTA-ATT-TAG-ATT-TAA-TAT-TAA-TGC-A-3'
VECTOR-SPECIFIC ALPHA-SECRETION SIGNAL SEQUENCE FORWARD PRIMER FOR SEQUENCING CLONES	5'-ACT-ATT-GCC-AGC-ATT-GCT-GCT-AAA-G-3'
VECTOR-SPECIFIC C-GAMMA-C1 TERMINATOR SEQUENCE REVERSE PRIMER FOR SEQUENCING CLONES	5'-TTA-CAT-GAT-ATC-GAC-AAA-GGA-AAA-G-3'
***BMSA1* INTERIOR SEQUENCE FORWARD PRIMER FOR SEQUENCING CLONES 3' JUNCTION**	5'-CCT-CCG-ATA-TCT-CCG-CTA-TTA-AG-3'
***BMSA1* INTERIOR SEQUENCE REVERSE PRIMER FOR SEQUENCING CLONES 5' JUNCTION**	5'-CAT-CCA-CTT-CAG-CAG-CAG-TAT-C-3'

Table 1: A table of all PCR primers used for this research. All primers were synthesized through Integrated DNA Technologies ™

pPinkα-HC Secretion Vector Generation:

The pPinkα-HC secretion vector was obtained from Fisher Scientific as part of the PichiaPink™ Secretion Vector Kit. The vector was used to transform Stellar Chemically Component™ *E. coli* and transformed bacterium was plated overnight on Luria-Bertani (LB) plus ampicillin agar plates (Table 2). Bacterial colonies were selected and evaluated by PCR and restriction enzyme digestion for the presence of the pPinkα-HC secretion vector.

Medium Name	Composition
LB Broth + Carbenicillin	1:1,000 (LB broth:Carbenicillin)
LB Agar + Carbenicillin	1:1,000 (LB agar:Carbenicillin)
YPD agar	2% agar, 2% peptone, 2% yeast extract, 2% dextrose
YPD broth	2% peptone, 2% yeast extract, 2% dextrose
YPDS broth	2% peptone, 2% yeast extract, 2% dextrose, sorbitol 1M
BMGY medium (Buffered Glycerol-complex Medium)	1% yeast extract, 2% peptone, 100mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 0.00004% biotin, 1% glycerol
BMMY medium (Buffered Methanol-complex Medium)	1% yeast extract, 2% peptone, 100mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 0.00004% biotin, 0.5% methanol
PAD agar (Pichia Adenine Dropout)	PichiaPink™ Media Kit

Table 2: A comprehensive table of all media used for this research, with respective media compositions.

Transformed *E. coli* with confirmed expression of the pPinkα-HC secretion vector were prepared in glycerol stock and stored at -80°C prior to use. For experiments, the pPinkα-HC secretion vector containing *E. coli* was propagated in LB broth containing 100µl (1:1,000) of carbenicillin and cultured for 24 hours on a shaker at 37°C. Cultures were then harvested and subject to plasmid template purification using Qiagen™ Spin Midiprep Kit for Plasmid DNA purification, per the manufacture's protocol.

To ensure the identity and integrity of the pPinkα-HC vector plasmids were correct and intact, restriction digest to check for accurate restriction fragment sizes was conducted on a 20µl aliquot of the purified template yield from the Midiprep purified plasmids template. The restriction digest reaction included cleavage with *HindIII*, *NotI*, and *XbaI* restriction enzymes in separate reactions. Restriction enzymes were purchased through New England Bio Labs ™, and 20µl reactions were conducted at 37°C overnight, per the manufacture's protocol. The size of the vector before cleavage with restriction digest enzymes was 8000 base pairs. Restriction digestion with *HindIII* cut the plasmid twice, generating approximately 3000 base pair and 5000 base pair fragments. Restriction digestion *XbaI* also cut the plasmid twice, resulting in an approximately 2000 base pair fragment and a 6000 base pair fragment. Restriction digestion with *NotI* cut the plasmid only once, so the expected fragment size of DNA generated from that reaction was 8000 base pairs. All restriction digestion reactions resulted in the generation of appropriately expected restriction fragment sizes when observed by horizontal gel electrophoresis.

Preparing *bmsa1*, *bmn1-21* and pPinkα-HC for Cloning:

To prepare the *bmsa1* gene for cloning into the pPinkα-HC secretion vector plasmid, the amplified *bmsa1* gene with its adaptor sequence was digested with *KpnI* in a 20µl reaction in order to cleave its 3' *KpnI* site adaptor needed for cloning the gene into the PichiaPink Vector. The restriction enzyme digest was conducted at 37°C for 2 hours, per manufacture's protocol. Since the 5' end of the gene was synthesized with a phosphorylated end, no preparation was necessary to make it compatible with the ligase reaction for cloning. To prepare the *bmn1-21* gene for cloning into the pPinkα-HC secretion vector plasmid, a 20µl restriction digestion reaction was set up using *KpnI* and *MlyI* to cleave the 3'*KpnI* site adapter and 5'*MlyI* site adaptors synthesized onto the gene during amplification. This reaction was also conducted at 37°C for 2 hours, as per the manufacture's protocol. To prepare the pPinkα-HC secretion vector plasmid for cloning, a 20µl restriction digest reaction was set up using KpnI and *StuI* to cleave the 5' *StuI* site and 3' *KpnI* sites of the vector for insertion of GOIs. Restriction enzymes *StuI*, *KpnI* and *MlyI* were purchased from New England Biolabs ®. These restriction reactions were conducted at 37°C for 2 hours, per the manufacture's protocol. These cleavages generated complementary ligation sites for proper placement and orientation of GOIs during the cloning ligation reaction. The cleaved plasmid product was treated with quick CIP from New England Biolabs ® to prevent relegation of gene fragment ends prior to insertion of GOIs. Quick CIP treatment was conducted at room temperature for 10 minutes, as per manufacture's protocol.

Ligation of Plasmid/GOI duplex

Ligation was performed using the Quick Ligation ™ Kit from New England Biolabs ® Inc. with Quick T4 DNA Ligase. 50ng of cleaved vector DNA was ligated to

35ng of GOI DNA in a 5-minute ligation reaction, as per the manufacture's protocol. Ligation product was transformed into Stellar ™ Competent *E. coli* cells as per the manufacture's protocol. Cells were plated on LB agar + Carbenicillin plates (1:1,000 carbenicillin: LB agar) to screen for recombinants. Individual colonies were selected for DNA purification (Figure 2). DNA isolation from transformed bacteria for subsequent PCR amplification with vector-specific primers that spanned the GOIs insertion site was performed using a boil preparation procedure. Colonies were suspended in 20µl of sterile water and heated to 100° for 10 minutes. The cell suspension was then centrifuged at 16,000 x g for 5 minutes. The supernatant containing the DNA was used for PCR. Successful insertion of the GOIs was confirmed by PCR using the "vector-specific primers for screening recombinants" shown in the Table 1, using GoTaq® DNA Polymerase in GoTaq® Master Mix. PCR product was run on horizontal gel electrophoresis for confirmation of a vector size DNA band.

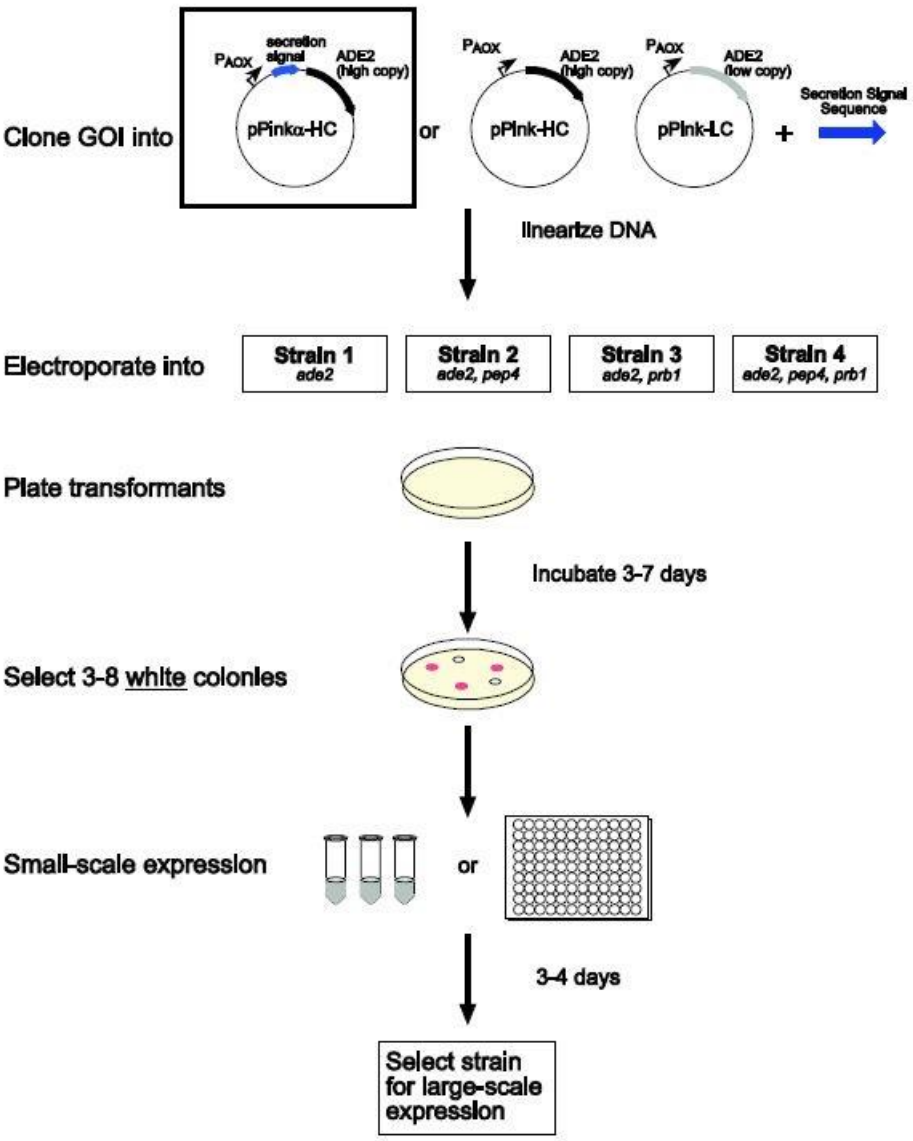


Figure 2: A diagram of the experimental protocol to create heterologous *bmsa1* and *bmn1-21* in the pPinkα-HC secretion vector and for protein expression. On the top left (denoted by the black square) is the pPinkα-HC secretion vector used for cloning.

Preparation of PichiaPink™ Strains for Electroporation:

Four clones of PichiaPink™ yeast were used for transformation with the PichiaPink™ secretion vector containing the GOIs. The four yeast strains differed from one another based on the number of endogenous proteases present as each strain had different combinations of yeast proteases that had been deleted to avoid potential protease cleavage of the heterologous protein if necessary. Competent yeast strains were prepared for transfection according to manufacturer's instructions. The four yeast clones were obtained from frozen glycerol stock and plated for isolation on YPD agar plates (Table 2). Strains were incubated for three days at 28°C in an incubator after which individual colonies were selected for each of the four yeast strains and inoculated in 10mL of YPD media (Table 2) in a 250mL baffled flask. Yeast was grown in suspension on a shaking incubator at 300rpm at 28°C for 24 hours. This starter culture was used to inoculate 100mL of YPD media and grown to an OD_{600} of 0.2. This culture was grown to an OD_{600} of 1.3, then yeast centrifuged at 1,500 x g for five minutes at 4°C. Yeast pellets were resuspended in 250mL of ice-cold, sterile water. Cells were centrifuged a second time at 1,500 x g for five minutes at 4°C then resuspended in 50mL of ice-cold, sterile water. Cells were centrifuged a third time at 1,500 x g for five minutes at 4°C then resuspended in 10 mL of ice-cold 1M sorbitol. Cells were centrifuged a final time at 1,500 x g for five minutes at 4°C then resuspended in 300 µl of ice-cold 1M sorbitol. Cells were kept on ice and used the same day.

Electroporation of PichiaPink ™ :

The following electroporation protocol was used for all electroporation for the transfection of Pichai pastoris yeast. Electrocompetent PichiaPink™ cells (80µl) were mixed with 8µg of linearized *bmsa1* plasmid. Plasmids were linearized with the restriction

enzyme *EcoNI*, cutting the plasmid at position 5326bp to linearize the plasmid and to generate a 1bp overhang. PichiaPink™ cells and Vector/GOI duplex DNA were mixed in ice-cold 2mm cuvettes for electroporation. Cells were pulsed in a BTX Electro Cell Manipulator ® electroporator under the following parameters: 25µF, 200Ω, 150v. Immediately after electroporation, 1mL of ice-cold YPDS media (Table 2) was added directly to the cuvettes by pipetting up and down, and cultures were incubated at 28°C for 2 hours inside the cuvettes. After 2 hours, cells were plated on PAD selection agar at 100µl and 300µl volumes. Cells were incubated on PAD agar for 3 days until colonies began to form, which were allowed to grow for one more day at which point colonies were transferred to fresh PAD selection agar plates (Table 2) for another 3 days. Each colony plate was used as a source to propagate yeast in suspension in order to create glycerol stocks. Transfected yeast clones were frozen for long-term storage in 50% glycerol and stored at -80°C.

PichiaPink™ Heterologous Protein Expression Culture:

From fresh PAD cultures, 25mL of BMGY medium (Table 2) was inoculated with a single transfected yeast colony in 250mL baffled flasks. These cultures were allowed to grow for 24 hours at 28°C in a shaking incubator at 280 rpm. After 24 hours, cells were transferred to separate 50mL conical tubes and centrifuged at 1,500 x g for 5 minutes at room temperature. The BMGY media was discarded and the pellet was resuspended in 2mL of BMMY media (Table 2). Before placing the cultures back on the incubator, caps were replaced with sterile gauze to allow for adequate aeration of cultures. The BMMY cultures were placed back on the shaking incubator for 24 hours at 28°C shaking at 280 rpm. After 24 hours of culture, 100µl was removed for the 0-hour time point collection

sample. Cultures were replenished with 100µl of 40% methanol. For each time point collected after that, 100µl of media were collected, and cultures were replenished with an additional 100µl of 40% methanol.

Supernatant and Pellet Lysate Sample Preparation:

Each 100µl yeast sample collection was transferred into a microcentrifuge tube where 1µl of HALT™ protease inhibitor was added to the sample to a final 1x concentration of protease inhibitor. Samples were then centrifuged at 1,500 x g for 10 minutes. Supernatants were transferred to a new clean microcentrifuge tube and stored at -80°C prior to analysis by either vertical acrylamide electrophoresis for Coomassie staining of proteins or for Western Blot analysis. Cell pellets from each collection were also stored at -80°C prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) separation of proteins for analysis by Coomassie staining or Western Blot analysis. After yeast supernatant and pellets were collected for all time points, the supernatants were thawed and prepared for SDS-PAGE. In preparation for SDS-PAGE, supernatants were mixed in a 1:1 mixture ratio with Laemmli Sample Buffer and 2-Mercaptoethanol (19:1 Laemmli Sample Buffer:2-Mercaptoethanol).

Cell pellets were thawed and resuspended in a 10:1 pellet volume: Breaking Buffer solution (50 mM sodium phosphate pH 7.4, 1mM PMSF, 1mM EDTA, 5% glycerol) . Then, equal volumes of 0.5µm acid wash beads from VWR North American® were added to the pellet suspension in Breaking Buffer. The bead pellet suspension mixture was subject to vortex at max speed 8 cycles of 30 seconds on, with 30 seconds rest. After vortexing the suspension was centrifuged for 10 minutes at 15,000 x g at 4°C. After centrifugation, the supernatant was carefully transferred to a fresh microcentrifuge tube. As

with the supernatant samples, in preparation for SDS-PAGE, pellet lysates were mixed in a 1:1 mixture ratio with Laemmli Sample Buffer and 2-Mercaptoethanol (19:1 Laemmli Sample Buffer:2-Mercaptoethanol).

SDS-PAGE:

BIO-RAD Mini-PROTEAN ® 10 and 12 lane precast SDS-PAGE gels were used for all SDS-PAGE. Gels were run in 1x Tris/Glycine/SDS loading buffer. Samples were incubated at 100°C for 10 minutes prior to loading into gels. All wells were loaded with 10µl of BIO-RAD Precision Plus® Protein Standard ladder and 10µl of the sample unless otherwise stated in the figure legend. Gels were run at 125v for approximately 45 minutes until the dye front reached the bottom of the gel. These conditions were the same for gels used in Coomassie-stain and gels used in nitrocellulose membrane transfer for Western Blot analysis.

Coomassie Stain

Gels were fixed in fixing solution (50% methanol, 10% glacial acetic acid) for 30 minutes on a shaker set to low. Staining was done with Coomassie dye (0.1% Coomassie Brilliant Blue R-250, 50% methanol, 10% glacial acetic acid) for 1 hour following fixation. After 1-hour gels were destained in destaining solution (40% methanol, 10% glacial acetic acid) until bands began to appear (approximately 20 minutes), and then destaining continued in distilled water until gels were fully destained.

Western Blot Analysis

All protein transfers from SDS-PAGE gels to nitrocellulose membranes were done using a BIO-RAD Trans-Blot® SD Semi-Dry Electrophoretic Transfer cell. Towbin Transfer Buffer (25mM Tris, 192mM glycine (20% methanol), pH8.3 in dH2O) was used

for the semi-dry transfer. The transfer was conducted for 20 minutes at 15 volts. Following the transfer, nitrocellulose membranes were blocked in blocking buffer (TBST with 3% bovine serum albumin) for 45 minutes. After blocking for 45 minutes the nitrocellulose membranes were rinsed for 3 x 5 minutes in TBST (20µm Tris (pH 7.5), 150µm NaCl, 0.1% tween in 1L dH2O). After rinsing in TBST, membranes were incubated in 1:10 dilution of primary antibody in blocking buffer solution, for one hour at room temperature or overnight at 4°C. After incubation with primary antibodies, the nitrocellulose membranes were rinsed in TBST 3 x 5 times, then incubated in a 1:10,000 secondary antibody: blocking buffer solution for 1 hour. Alkaline Phosphatase Goat Anti-Mouse IgG secondary antibodies from Jackson Immunoresearch Laboratories Inc. were used for all western blot analyses. Following the secondary antibody incubation, the nitrocellulose membranes were rinse in TBS (no Tween) 3 x 5 times. Following the TBS rinse, membranes were incubated in Thermo Fisher™ BCIP/NBT alkaline phosphatase substrate for chromogenic detection of antibodies. Once bands were clear, the chromogenic detection reaction was stopped by rinsing the membrane in dH2O.

Genomic DNA Isolation from PichiaPink™:

All genomic DNA isolation from the transfected PichiaPink™ strains was done using Norgen Biotek Corp.® Fungi/Yeast Genomic DNA Isolation Kit, with additional Zymolase treatment for cell wall disruption, per manufacturers protocol.

Genome Sequencing and Alignment:

All Sanger sequencing for genomic DNA was conducted by adding appropriate primers to DNA as per GENEWIZ® instruction and then submitted to GENEWIZ® for Sanger sequencing. Alignments for *bmsa1* and *bmn1-21* genes were done using the NCBI

sequence XP_012648767.1 as a reference. All alignments were done using MUSCLE or Clustal Omega alignment tools through SnapGene Pro® software, unless stated otherwise in the text.

Results

The goal of this study was to use a *Pichia pastoris* heterologous expression vector to create recombinant BmSA1 and BMN1-21 (rBmSA1 and rBMN1-21) protein for use in a pilot antigen-specific antibody capture enzyme-linked immunoassay (ELISA) that is under development. To evaluate genetic diversity in *bmsa1* and *bmn1-21*, both genes were amplified by PCR with gene-specific primers from the *B. microti*, Gray strain obtained from the American Type Culture Collection (ATCC-30221) and propagated in vivo in BALB/c mice. The Gray strain of *B. microti* was isolated from a human patient from Nantucket Island, MA in 1970. The amplified *bmsa1* and *bmn1-21* genes were sequenced and the genomic sequences were compared by Basic Local Alignment Search Tool (BLAST) to the genomic sequence of independent *B. microti* isolates that are referenced on the PiroplasmaDB.org library/search engine, as well as the NCBI library/search engine. Shown in Table 3 and Table 4 are the alignments of *bmsa1* and *bmn1-21* from the *B. microti* Gray strain with the other *B. microti* parasite isolates sequenced to date. The *bmsa1* alignments in Table 3 show an exhaustive list of cataloged genomes for *B. microti* species, indicating all of the known *B. microti bmsa1* genes are highly conserved with 100% sequence homology.

The BMN1 family of proteins are thought to be members of variable surface proteins in *B. microti*. The BLAST alignment of the *bmn1-21* gene did identify variability between the *B. microti* Gray Strain ATCC 30221 *bmn1-21* gene and *bmn1-21* genes from other *B. microti* isolates identified and registered on piroplasmaDB.org (Query covers of 78% for Naushon, ATCC 30222, ATCC PRA-99, and Nan_Hs_2011_N11_50 strains) (Table 4).

bmsa1

Strain Name	Sequence ID	Score	E-value	Query Cover	Identity
***B. microti* strain RI**	LN871598	1781	0.0	100%	100%
***B. microti* strain Naushon**	JGUX01000084.1	1775	0.0	100%	100%
***B. microti* strain ATCC 30222**	JGVA01000085.1	1775	0.0	100%	100%
***B. microti* strain ATCC PRA-99**	JGUZ01000081.1	1775	0.0	100%	100%
***B. microti* strain GI**	JGUY0100088.1	1775	0.0	100%	100%
***B. microti* Greenwich Yale Lab Strain 1**	JGUW01000191.1	1775	0.0	100%	100%
***B. microti* strain Nan_Hs_2011_N11_50**	JGUV01000099.1	1775	0.0	100%	100%

Table 3: The BLAST alignments with the *B. microti* Gray Strain ATCC 30221 *bmsa1* gene sequence. PiroplasmaDB.com BLAST alignment tool was used for alignments.

bmn1-21

Strain Name	**Sequence ID**	**Score**	**E-value**	**Query Cover**	**Identity**
***B. microti* strain RI**	FO082872	1651	0.0	100%	100%
***B. microti* Greenwich Yale Lab Strain 1**	JGUW01000186.1	1609	0.0	100%	100%
***B. microti* strain Naushon**	JGUX01000105.1	201	9e -51	78%	99%
***B. microti* strain ATCC30222**	JGVA01000127.1	201	9e -51	78%	99%
***B. microti* strain ATCC PRA-99**	JGUZ01000069.1	201	9e -51	78%	99%
***B. microti* strain Nan_Hs_2011_N11_50**	JGUV01000066.1	201	9e -51	78%	99%

Table 4: The BLAST alignments with the *B. microti* Gray Strain ATCC 30221 *BMN-1-21* gene sequence. PiroplasmaDB.com BLAST alignment tool was used for alignments.

However, these variations are almost entirely silent as the translated protein products from these genes contain 99% amino acid sequence identity to the Gray Strain ATCC 30221 BMN1-21 protein.

The BmSA1 protein is closely related to the BMN1 family of proteins, but it is divergent from a BMN1 protein subtype called BMN1-9 that shares little homology to the larger BMN1 family of proteins that BMN1-21 belongs to. The *B. microti* Gray Strain ATCC 30221 *bmn1-21* gene and *bmsa1* gene only share 50% sequence identity to each other (Figure 3). Their translated protein products are even more dissimilar. The BMN1-21 translated protein shares only 23% sequence identity to the BmSA1 translated protein, with 42% sequence similarity (Figure 4). Amino acids are considered similar if they are both either: non-polar, polar neutral charge, acidic, or basic amino acids.

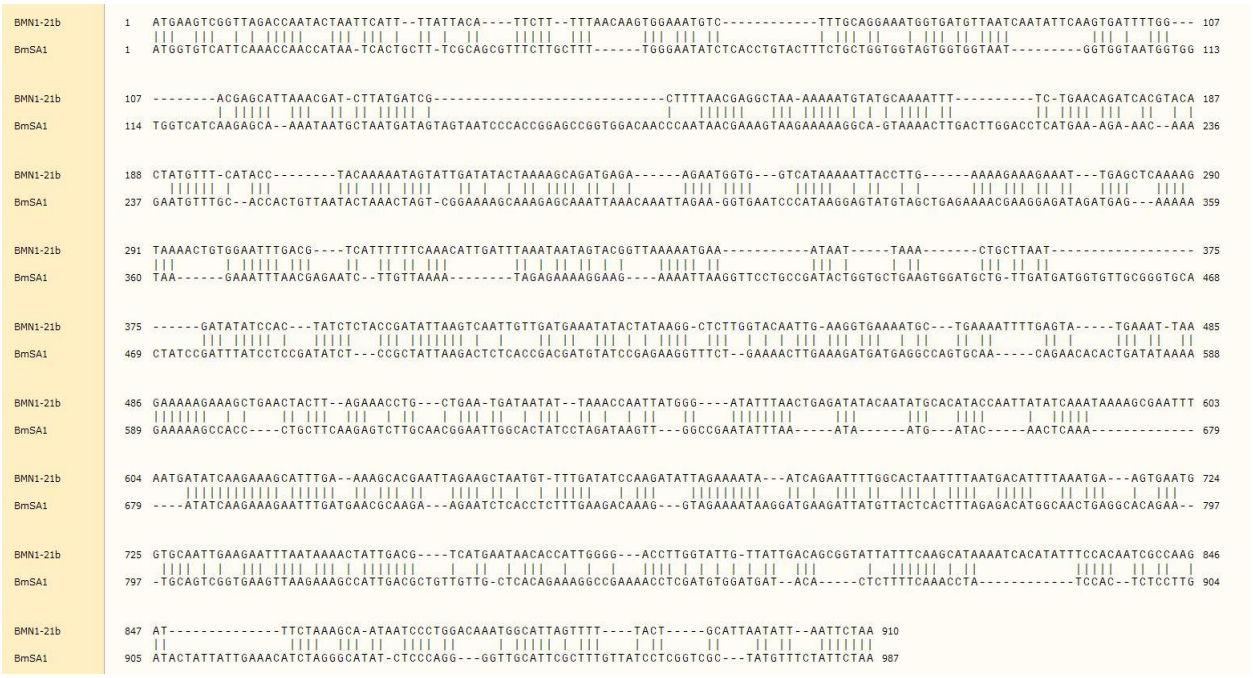


Figure 3: Local alignment of *B. microti* Gray Strain ATCC 30221 *bmn1-21* gene and *bmsa1* gene. The two sequences share 50.09% sequence identity. Local alignment was performed using the Smith-Waterman algorithm.

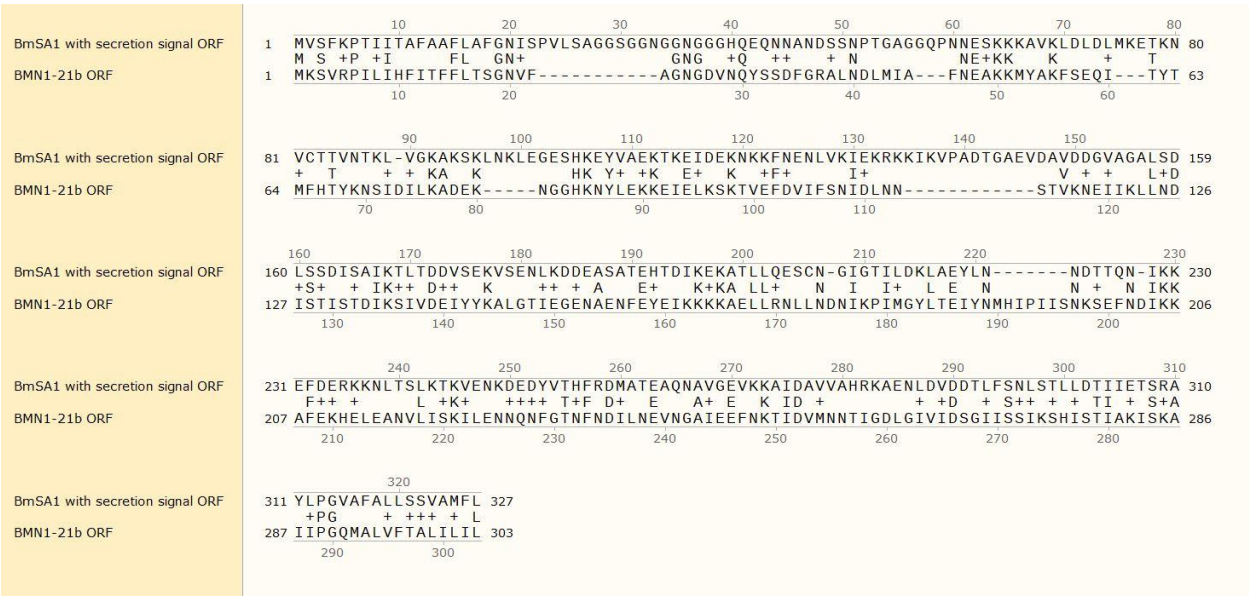


Figure 4: Local alignment of *B. microti* Gray Strain ATCC 20331 BMN1-21 protein and BmSA1 protein. The two sequences share 23.15% sequence identity, and 42.14% similarity. Local alignment was performed using the Smith-Waterman algorithm.

The Gray Strain ATCC 30221 *bmsa1* gene is much more homologous to the *bmn1-9* gene identified in *B. microti* strain MN1. With the exception of the first 27 base pairs at the beginning of the *bmsa1* gene inside of the *bmsa1* secretion signal sequence that is not present in the *bmn1-9* gene, these two genes share 99% sequence identity to each other (Figure 5a). These sequences differ from each other by one base pair in the middle of the gene, where an adenine in the *BMN1-9* gene is replaced with a guanine in the *BmSA1* gene. Their translated protein products share 99% sequence identity and 100% sequence similarity, as the only variation in their protein sequences is a lysine in the BMN1-9 amino acid sequence that is replaced with an arginine in the BmSA1 amino acid sequence (Figure 5b). The BLAST alignment of the *bmsa1* gene identified no variability between the *B. microti* Gray Strain ATCC 30221 *BmSA1* gene and *BmSA1* genes from other *B. microti* isolates identified and registered to piroplasmaDB.org (Table 3). Thus, both BmSA1 and BMN1-21 proteins are well conserved between the *B. microti* parasite isolates that have been sequenced to date.

a)

Score	Expect	Identities	Gaps	Strand
1354 bits(733)	0.0	735/736(99%)	0/736(0%)	Plus/Plus

```
Query  1    CACTGCTTTCGCAGCGTTTCTTGCTTTTGGGAATATCTCACCTGTACTTTCTGCtggtgg  60
Sbjct  27   ............................................................  86

Query  61   tagtggtggtaatggtggtaatggtggtggtCATCAAGAGCAAAATAATGCTAATGATAG  120
Sbjct  87   ............................................................  146

Query  121  TAGTAATCCCACCGGAGCCGGTGGACAACCCAATAACGAAAGTAAGAAAAAGGCAGTAAA  180
Sbjct  147  ............................................................  206

Query  181  ACTTGACTTGGACCTCATGAAAGAAACAAAGAATGTTTGCACCACTGTTAATACTAAACT  240
Sbjct  207  ............................................................  266

Query  241  AGTCGGAAAAGCAAAGAGCAAATTAAACAAATTAGAAGGTGAATCCCATAAGGAGTATGT  300
Sbjct  267  ............................................................  326

Query  301  AGCTGAGAAAACGAAGgagatagatgagaaaaataagaaatttaacgagaatcttgttaa  360
Sbjct  327  ............................................................  386

Query  361  aatagagaaaaagaagaaaattaagGTTCCTGCCGATACTGGTGCTGAAGTGGATGCTGT  420
Sbjct  387  ..........G.................................................  446

Query  421  TGATGATGGTGTTGCGGGTGCACTATCCGATTTATCCTCCGATATCTCCGCTATTAAGAC  480
Sbjct  447  ............................................................  506

Query  481  TCTCACCGACGATGTATCCGAGAAGGTTTCTGAAAACTTGAAAGATGATGAGGCCAGTGC  540
Sbjct  507  ............................................................  566

Query  541  AACAGAACACACTGATATAAAAGAAAAAGCCACCCTGCTTCAAGAGTCTTGCAACGGAAT  600
Sbjct  567  ............................................................  626

Query  601  TGGCACTATCCTAGATAAGTTGGCCGAATATTTAAATAATGATACAACTCAAAATATCAA  660
Sbjct  627  ............................................................  686

Query  661  GAAAGAATTTGATGAACGCAAGAAGAATCTCACCTCTTTGAAGACAAAGGTAGAAAATAA  720
Sbjct  687  ............................................................  746

Query  721  GGATGAAGATTATGTT  736
Sbjct  747  ................  762
```

b)

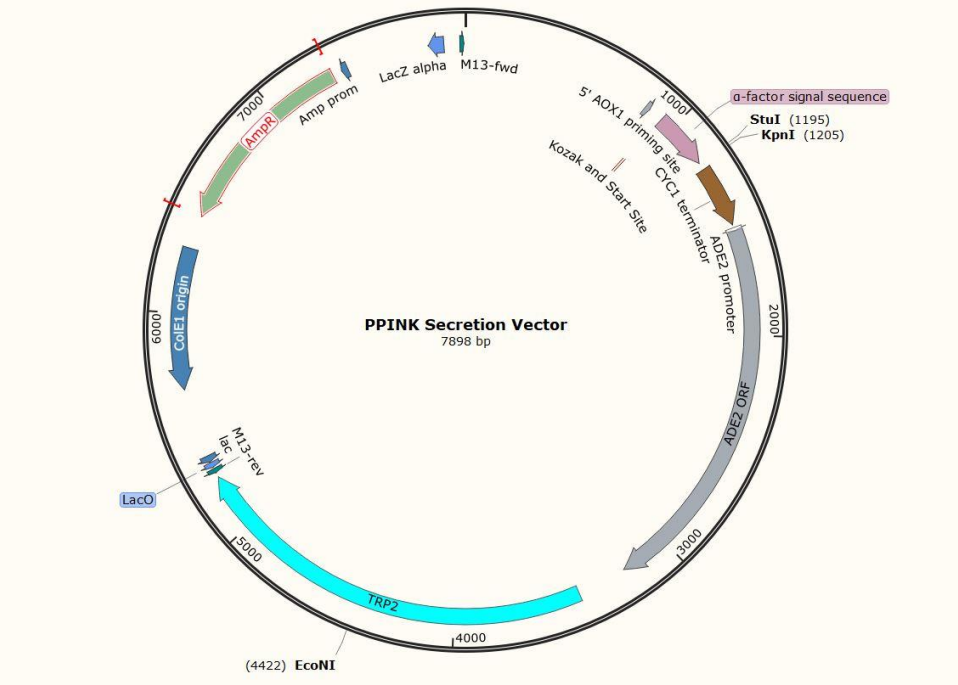
Figure 5: a) Image of the NCBI local alignment tool results aligning the *bmn1-9* gene from *B. microti* MN1 strain (Query) against the *bmsa1* gene from *B. microti* Gray Strain (Subject). The two sequences share 99.86% identity starting at subject position 27, and query position 1. An adenine at query position 374 is replaced with a guanine in the subject. **b)** An alignment of the protein translated from the *bmn1-9* gene and the *bmsa1* gene. There is one amino acid difference between the two proteins, a lysine in BMN1-9 position 124 is replaced with an arginine in BmSA1 position 133. Local alignment was performed using the Smith-Waterman algorithm.

Since we confirmed that the BmSA1 and BMN1-21 proteins were conserved between the *B. microti* Gray strain and other known isolates of *B. microti*, the *B. microti* Gray strain was used as a template for amplification of both *bmsa1* and *BMN1-21* to clone each gene into the PichiaPink™ heterologous expression system to create recombinant protein. Previous studies in our lab confirmed that both the *bmsa1* and *bmn1-21* genes encode a single exon that codes for the expression of the entire proteins. Figure 2 shows a diagram of the process followed for the expression of the BmSA1 and BMN1-21 as heterologous proteins in the PichiaPink yeast protein expression system:1. Clone the genes of interest (GOIs) in frame into pPinkα-HC expression vectors; 2. Linearize the pPinkα-HC expression vectors encoding the GOIs and introduce it into the PichiaPink™ yeast strains by electroporation, and plate and select transformants on selection media, and; 3. Conduct small scale pilot expression studies of BmSA1 and BMN1-21 proteins to ensure production of the heterologous protein.

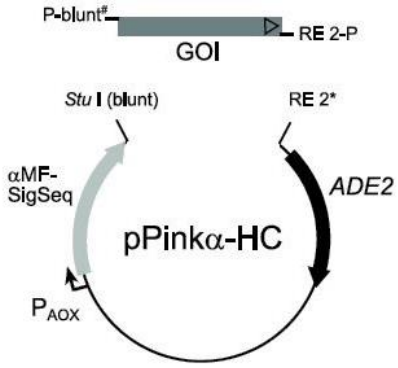
1. Cloning Genes of Interest (GOIs) Into pPinkα-HC Secretion Vector

The pPinkα-HC vector is intricately designed to enable cloning and expression of heterologous protein for secretion by PichiaPink™ yeast strains following induction of protein expression using methanol. A diagram of the pPinkα-HC vector for secreted proteins is shown in figure 6a. The protein-coding DNA for *bmsa11* and *bmn1-21* were inserted downstream of the methanol-induced *AOX1* promoter site in the pPinkα-HC vector. Downstream of the *AOX1* promoter site is the *Saccharomyces cerevisiae* α-mating factor secretion signal harboring the *AOX1* native Kozak start signal with an ATG start site. Immediately downstream of and in-frame with the α-mating factor secretion signal is where the GOIs are inserted for proper transcription and protein expression and secretion.

a)



b)



*RE 2: *Kpn* I, *Nae* I, *Fse* I, or *Swa* I

#blunt: *Mly* I

DNA	5´ end	3´ end
pPinkα-HC	*Stu1*(blunt)	*Kpn1*
Insert containing gene of interest	*BmN1- Mly1* (blunt) *BmSA1*- None	*Kpn1*, phosphorylated

c)

Figure 6. a) Detailed sequence map of the PichiaPink Vector for secreted proteins with all vector genes and regulator regions labeled, before GOI insertion. **b)** Simplified sequence map showing restriction digest schematic for cloning preparation. **c)** Actual sequence with codons, shows features around cloning site with restriction sites. The "multiple cloning site" is the region in between the *StuI* restriction digestion site and the beginning of the CYC1 transcription termination region. It contains *KpnI*, *Nae1*, and *Swa1* restriction sites (Invitrogen, 2014).

The diagram in figure 6b shows the cloning site in the pPinkα-HC vector where the GOIs are directionally cloned into the vector. As shown in figure 6b, the pPinkα-HC vector is specifically designed with a *StuI* restriction site directly following the α-mating factor secretion signal. Restriction digest with *StuI* creates a 5' blunt end cleavage at the exact site where the GOIs need to be inserted. This creates a convenient mechanism for generating an insertion/ligation site for GOI insertion. Downstream of the *StuI* site there is a "multiple cloning site" harboring several restriction enzyme cleavage sites, including a *KpnI* site which we used to generate a 3' overhang end. The purpose of this double digestion with one blunt end and one overhang end is to create non-identical insertion sites on the vector plasmid that ensure proper orientation of our GOIs during the insertion/ligation cloning reaction (Figure 6b, 6c).

Specialized primers were created to amplify the GOIs by PCR in order to create compatible ends on our GOIs for insertion into the vector. This meant that when *bmsa1* and *bmn1-21* genes were amplified by PCR from *B. microti* Gray strain (ATCC 30221), PCR primers were designed to include adapters containing the correct restriction enzyme sites to create GOIs compatible with the *StuI*/*KpnI* sites on the vector. For *bmn1-21*, a *MlyI* restriction site adapter was added to the 5' end, creating a compatible blunt end with the one created on the vector, and a *KpnI* restriction site adapter was added to the 3' end, creating a compatible overhang end with the one created on the vector following restriction enzyme digestion (Figure 7a) (Table 1). Unfortunately, the same technique could not be used when amplifying the *BmSA1* gene because *bmsa1* contains an internal *MlyI* restriction site, and cleavage with this enzyme would have destroyed the *bmsa1* gene. As an alternative, we created a 5' phosphorylated primer with no restriction site for amplifying *bmsa1*. This modification created the same compatible 5' phosphorylated blunt end needed for insertion of the gene in the cloning vector. The 3' PCR primer for the amplification of *bmsa1* was designed with a *KpnI* restriction site adapter to create compatible overhang ends with the vector, the same as with *bmn1-21* (Figure 7b) (Table 1).

a)

b)

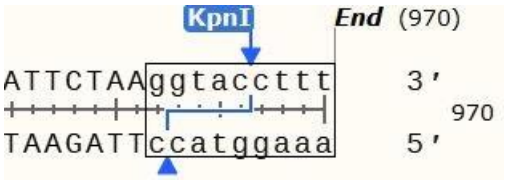


Figure 7. The adaptors were added to the 5’ and 3’ PCR primers to create GOIs compatible for cloning into the vector. **a)** The 5’ and 3’ end adapters of the *bmn1-21* gene after the addition of a 5’ *MlyI* site adapter and a 3’ *KpnI* site adapter. **b)** The 3’ end of the *bmsa1* gene after the addition of a 3’ *KpnI* site adapter.

The *bmsa1* and *bmn1-21* genes were successfully amplified by PCR from *B. microti* Gray strain (ATCC 30221) using the primers described above to amplify the coding region of the gene along with the added 5' and 3' additional adaptors for cloning into the PichiaPink vector. The wild-type *bmsa1* gene contains a 78 base pair secretion signal. The PCR primers amplified the *bmsa1* gene immediately downstream of the secretion signal because the secretion of GOIs secretion signals is directed with the *Saccharomyces cerevisiae* α-mating factor secretion signal in the vector to drive secretion of the cloned protein in yeast strains as previously described. The wild-type *bmn1-21* gene contains a 69 base pair secretion signal which was excluded in the amplification process for the same reason. A proof-reading enzyme (Phusion® High-Fidelity PCR Master Mix) was used for PCR amplification of both *bmsa1* and *bmn1-21* to avoid the introduction of errors during gene amplification that might impact expression of the full-length protein. Shown in Figure 8 are the PCR products for the amplified GOIs with the modified 5' and 3' end adapters to incorporate required restriction enzyme sites. The PCR product was visualized through horizontal agarose gel electrophoresis. *bmsa1* (909 base pairs) is shown in lane 2, and *bmn1-21* (846 base pairs) is shown in lane 3. The 1kb plus Invitrogen TrackIt™ ladder is shown in lane 1 as a comparison in order to estimate the size of the PCR products.

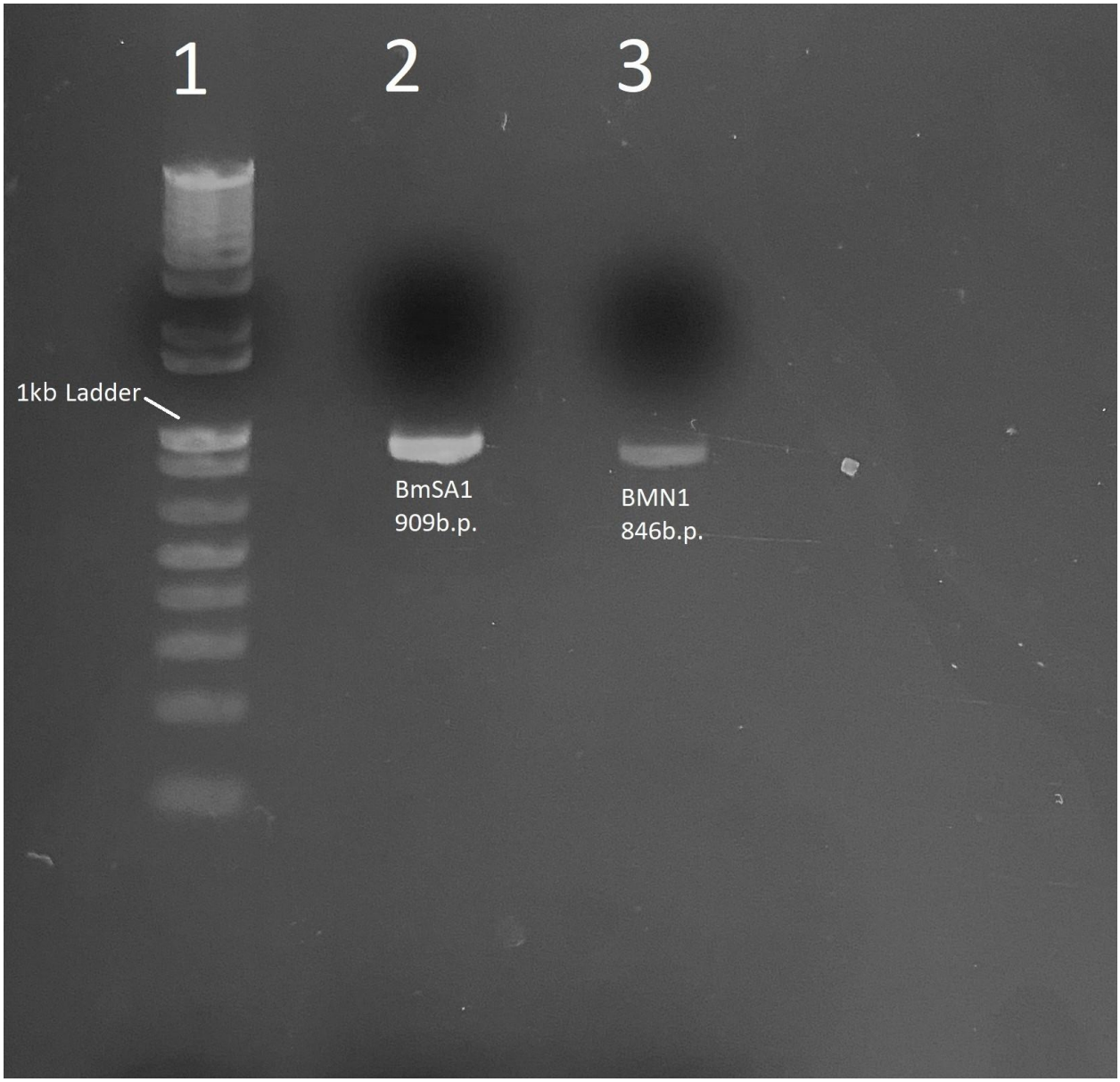
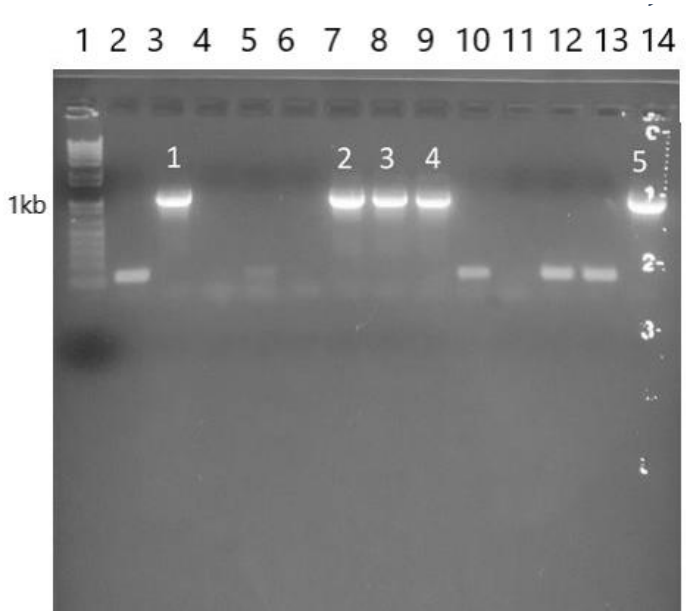


Figure 8: Horizontal gel electrophoresis of the *bmsa1* and *bmn1-21* amplified DNA with the included 5' and 3' adaptors. Lane 1 is a 1kb plus Invitrogen TrackIt™ ladder with the 1kb standard band labeled. Lane 2 shows a band consistent with the 909-base pair (bp) *bmsa1* gene segment expected to be yield from PCR gene amplification. Lane 3 shows a band consistent with the 846-base pair *bmn1-21* gene segment expected to be yield from PCR gene amplification

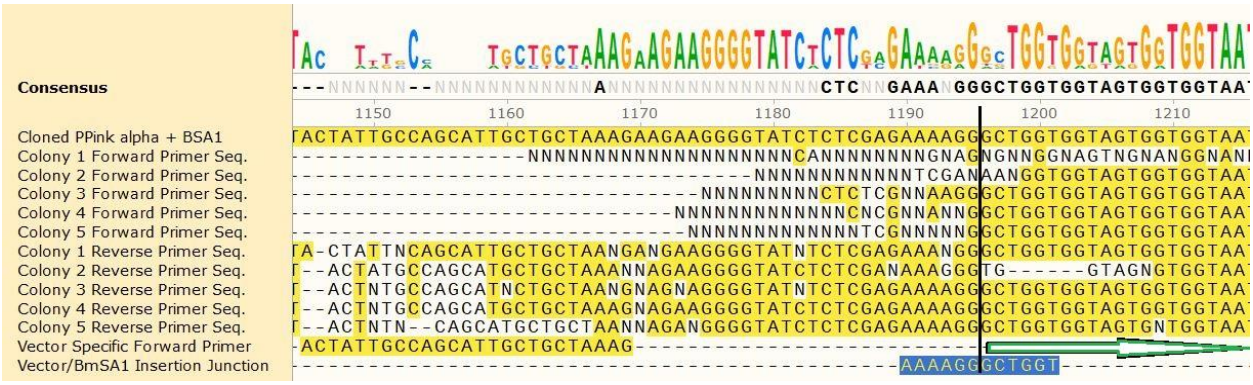
The pPinkα-HC vector and GOIs were digested with the appropriate restriction enzymes for directional cloning of the GOIs into the vector, the digested products purified, and pPinkα-HC vector/GOI duplexes were created by the addition of ligase enzyme. The pPinkα-HC vector/GOI duplexes were used to transform the newly formed DNA duplexes into *E. coli* for propagation and selection. The pPinkα-HC vector includes an ampicillin resistance gene under the control of its own promoter for the selection of the transformants in *E. coli*. This resistance cassette also provides the selection with carbenicillin which we used for selection.

Transformed *E. coli* was grown overnight at 37°C on LB agar plates containing carbenicillin. Thirteen colonies were selected from LB agar plates containing *E. coli* transformed with *BmSA1* construct for validation that the plasmid in each *E. coli* colony contained the correct *BmSA1* insert. DNA was isolated from each bacterial colony and PCR performed with primers 5' and 3' to the GOI insertion site in the vector to amplify the gene insert if present. The sequence of "Vector-specific primers" used for PCR amplification to identify insertion of the GOIs are shown in Table 1. As shown in the horizontal electrophoresis gel in figure 9a, five of the 13 colonies screened by electrophoresis were positive for the insertion of a genomic sequence of roughly 1kb, the expected size for the BmSA1 in the GOI insertion site for the vector (Figure 9a). This means the other 8 colonies that grew on the selection plates did not include the insertion of a gene even though they were resistant to carbenicillin. The plasmid was purified from the bacterial colonies that had an insertion of the correct size in the insertion site of the vector. The plasmid DNA was sequenced using sanger sequencing in separate reactions with either the 5' or 3' primers that spanned the insertion site in the vector in order to sequence the

a)


1 2 3 4 5 6 7 8 9 10 11 12 13 14
1kb
1
2 3 4
5

b)


Consensus
1150
1160
1170
1180
1190
1200
1210
Cloned PPink alpha + BSA1
Colony 1 Forward Primer Seq.
Colony 2 Forward Primer Seq.
Colony 3 Forward Primer Seq.
Colony 4 Forward Primer Seq.
Colony 5 Forward Primer Seq.
Colony 1 Reverse Primer Seq.
Colony 2 Reverse Primer Seq.
Colony 3 Reverse Primer Seq.
Colony 4 Reverse Primer Seq.
Colony 5 Reverse Primer Seq.
Vector Specific Forward Primer
Vector/BmSA1 Insertion Junction

c)

Figure 9: a) Horizontal gel electrophoresis of LB + Carbenicillin selected *E. coli* for validation of vector insertion. DNA was isolated from colonies using a boil preparation procedure. All DNA samples were loaded with 8µl of DNA in 2µl of TrackIt™ Cyan/Yellow loading buffer. Lane 1 is a 1kB plus TrackIt™ ladder with the 1kB standard band labeled (to the left). Colony 1 is shown in lane 3, colony 2 is shown in lane 7, colony 3 is shown in lane 8, colony 4 is shown in lane 9, and colony 5 is shown in lane 14. **b)** Sanger sequencing results of colonies 1-5 aligned with the pPink-α HC vector/*bmsa1* duplex using the *bmsa1* reference sequence on piroplasmaDB.com for *B. microti* strain RI as the query. Sequencing was performed using both the forward and reverse vector-specific primers for sequencing. The forward vector-specific primer is shown for in the second row to the bottom. The bottom row shows the vector/*bmsa1* junction, which is the last 2 codons of the vector followed by the first 2 codons of the *bmsa1* protein-coding gene. The beginning of the *bmsa1* protein-coding gene is denoted by the black line and green arrow. **c)** The chromatograph of the colony 2 forward primer sequence at the vector/*bmsa1* junction. The N at position 20 correlates to the N at position 1998 in Figure 9b. All alignments were performed using Cluastal Omega alignment program through Snap Gene® software.

inserted GOI. The DNA sequence obtained through Sanger sequencing was examined to ensure that the *bmsa1* sequence of the inserted GOI did not contain mutations introduced by PCR amplification and that the sequence was in frame with the pPink-α HC plasmid. Figure 9b shows the alignment of *bmsa1* sequenced from each of the positive plasmids using both forward and reverse primers for sequencing. The Sanger sequencing showed that the sequence was correct for the *bmsa1* gene with the exception that the *bmsa1* junctional sequence between the pPink-α HC vector and *bmsa1* start site in the sequencing chromatograph was unclear (Figure 9c). New primers were designed specifically to amplify the junctional region between the *bmsa1* coding region and the pPink-α HC vector (Table 1). In the meantime, plasmid number 2 (Figure 9a, 9b, 9c) was selected for electroporation into the PichiaPink™ yeast strains 1-4.

Twenty-two colonies were selected from LB agar plates containing *E. coli* transformed with the *bmn1-21* gene for validation of the correct gene insert. DNA was isolated from each bacterial colony and PCR performed with primers 5' and 3' to the GOI insertion site in the vector to amplify the GOI if present. "Vector-specific primers" from Table 1 were used for identifying the *bmn1-21* insert as described for the *bmsa1* colonies. Eight of the 22 colonies screened by electrophoresis were positive for the presence of a genomic sequence of approximately 1kb inserted into the insertion site in the vector (Figure 10). The plasmid DNA from each of the eight bacterial colonies that showed the presence of an insert of the correct size were sequenced using sanger sequencing in separate reactions with either the 5' or 3' primers that spanned the insertion site in the vector in order to sequence the inserted GOI. Sanger sequencing was not effective for the *bmn1-21* plasmid eight due to lack of priming during sequencing. *bmn1-21* plasmid 4 had multiple mutations

throughout with no insertions or deletions. The other six *bmn1-21* plasmids all contained the same sequence which differed by a few silent mutations from the *B. microti* BMN strain *bmn1-21* sequence published on PiroplasmaDB (Figure 11). Because the sequence homology was consistent between each of the *bmn1-21* plasmids the sequence was believed to be accurate for the *bmn1-21* sequence from the Gray Strain of *B. microti* that was used. The six *E. coli* clones with a plasmid containing the correct in-frame sequence of *bmn1-21* were frozen down in glycerol stock for future studies. At this point, the decision was made to not immediately transform Pichia pastoris strains with *bmn1-21* as the first priority was the creation of recombinant *bmsa1* for use with a pilot BmSA1 antigen specific ELISA under development.

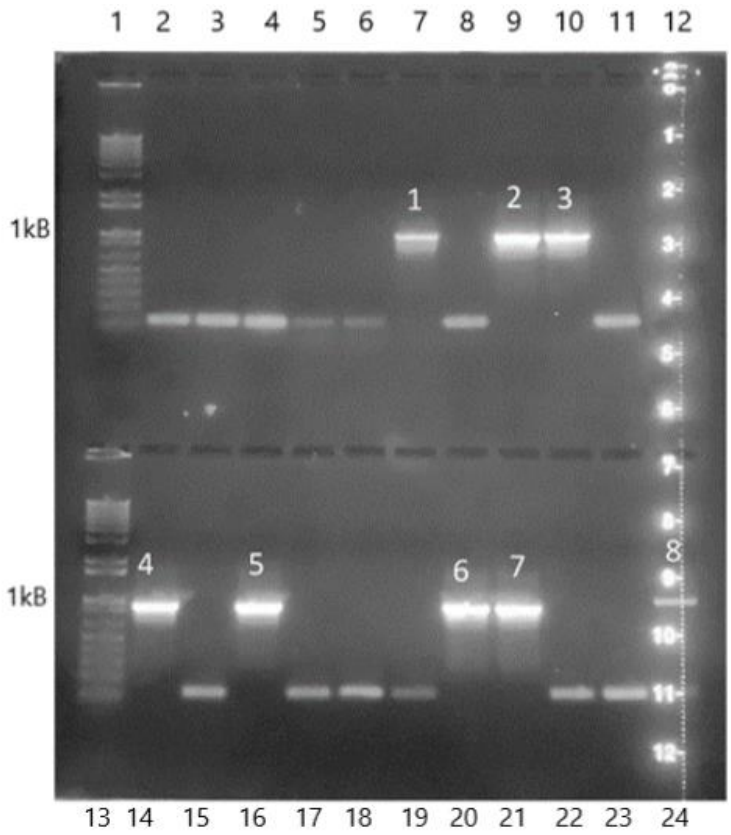


Figure 10: Horizontal gel electrophoresis of LB + Carbenicillin selected *E. coli* for validation of Vector/*bmn1-21* DNA insertion. DNA was isolated form colonies using a boil preparation procedure. All DNA samples were loaded with 8µl of DNA mixed with 2µl of TrackIt™ Cyan/Yellow loading buffer.
Lane 1 and 13: a 1kb plus TrackIt™ ladder with the 1kb standard band labeled (to the left).
Lane 7: Insert from bacterial colony termed "Colony 1"
Lane 9: Insert from bacterial colony termed "Colony 2"
Lane 10: Insert from bacterial colony termed "Colony 3"
Lane 14: Insert from bacterial colony termed "Colony 4"
Lane 16: Insert from bacterial colony termed "Colony 5"
Lane 20: Insert from bacterial colony termed "Colony 6"
Lane 21: Insert from bacterial colony termed "Colony 7"
Lane 24: Insert from bacterial colony termed "Colony 8"
*all other lanes contained colonies screened that were negative for presence of Vector/*bmn1-21* insert by PCR product visualized through horizontal gel electrophoresis.

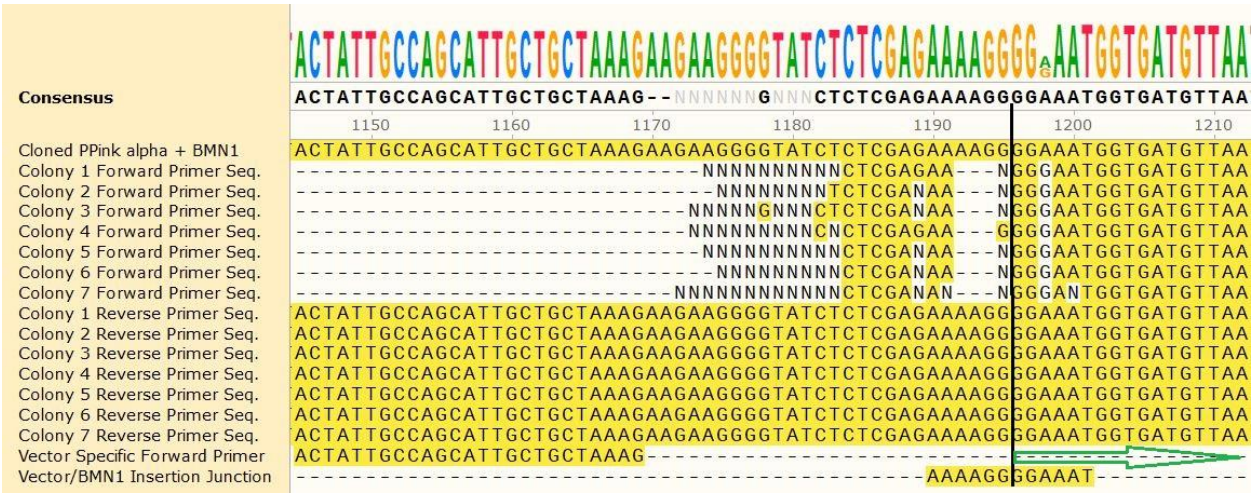


Figure 11: Sanger sequencing results of colonies 1-8 aligned with the pPink-α HC vector/*bmn1-21* duplex using the *bmn1-21* reference sequence on piroplasmaDB.com for *B. microti* strain RI as the query. Sequencing was performed using both the forward and reverse vector-specific primers for sequencing. The forward vector-specific primer is shown for in the second row to the bottom. The bottom row shows the vector/*bmn1-21* junction, which is the last 2 codons of the vector followed by the first 2 codons of the *bmn1-21* protein-coding gene. The beginning of the BmSA1 protein-coding gene is denoted by the black line and green arrow.

2) Electroporation of PichiaPink™ strains with linearized plasmid DNA

The BmSA1 expressing pPinkα-HC vector was linearized by restriction enzyme digestion within the *TRP2* gene of the vector. *TRP2* encodes a metabolic enzyme in the PichiaPink™, and integration of linearized vector can only occur at the *TRP2* locus of PichiaPink™ strains. The restriction enzyme EcoN1 was used to linearize the *bmsa1* expressing pPinkα-HC vector within the *TRP2* gene (Figure 6a). Following restriction enzyme digestion to linearize *bmsa1* expressing pPinkα-HC vector, the linearized plasmid was compared to uncut plasmid through horizontal gel electrophoresis verifying linearization of the plasmid. Electrophoresis validated that the restriction enzyme digestion resulted in linearization of the plasmid as the linearized product ran slower than the supercoiled non-linearized plasmid (data not shown).

Electroporation was conducted in 2mm electroporation cuvettes under the following electroporation conditions: 25μF, 200Ω, 150v, with readouts of between 1452V-1475V, all of a duration of 5 milliseconds or less. After electroporation, PichiaPink™ cells were gently resuspended in YPDS, allowed to recover for 2 hours, then replated on PAD selection plates for selection.

3) Isolation of Transfected PichiaPink™ Strains

Purified linearized *bmsa1* expression pPinkα-HC vector duplex was introduced into four different PichiPink™ strains by electroporation. Transfected PichiaPink™ yeast colonies were selected using adenine deficient selection plates. Prior to transfection PichiaPink™ yeast are *ADE2* deficient. *ADE2* encodes phosphoribosylaminoimidazole carboxylase, a necessary enzyme in the biosynthesis of purine nucleotides (jones and fink, 1992). Additionally, *ADE2* mutants are adenine auxotrophs and cannot grow on adenine

deficient media. Shown on Figure 6 is the *ADE2* gene under the control of its own 13 base pair promoter inside pPinkα-HC vector. As a result, only successfully transfected yeast containing the linearized pPinkα-HC vector/*bmsa1* duplex can grow on adenine deficient selection plates. In addition to being adenine auxotrophs, *ADE2* mutants will accumulate purine precursors in vacuoles when the gene is under expressed, causing colonies to display a red color. This creates a second quantitative level of expression to evaluate transfected colonies. Only colonies that have taken up the vector genome will be able to grow on adenine deficient plates, but colonies that have only taken up a low copy number of the vector will under-express the ADE2 gene, displaying a red color colony instead of a white color colony. This is why it was important to select for only white colonies during the plate selection. If colonies contain higher copy levels of the *bmsa1* gene, then theoretically they should express higher levels of protein under methanol induction.

All four yeast strains (that differ in the number of endogenous protease genes they contain) were transfected with the *bmsa1* containing vector by electroporation and plated on PAD selection plates. However, only strains 1 and 2 formed any colonies on the PAD selection plates. The strain 1 culture contained 3 white colonies, while the strain 2 culture contained 2 white colonies. These colonies were expanded on fresh PAD selection plates where they were used to generate glycerol stocks and inoculate starter cultures for pilot expression studies of rBmSA1 protein as well as for the creation of glycerol stocks of the transfected yeast colonies for long-term storage.

4) Pilot Expression Studies of Heterologous BmSA1 Protein Expression

As mentioned previously, expression of the cloned *bmsa1* gene was designed to fall under the control of a methanol induced *AOX1* promoter in the yeast PichiaPink™ vector. Another consideration for expression of heterologous proteins in PichiaPink™ yeast strains are the use of four different strains that differ in the number of protease genes that have been deleted in each yeast. While *Pichia pastoris* yeast generally secrete very low levels of native proteins, they have been reported to secrete proteases into expression media, particularly during fermentation. While this study was designed to express proteins under aerobic conditions, protease secretion and possible degradation of BmSA1 was still taken into consideration. PichiaPink™ strain 1 is the "protease wild-type" that has no genetic alteration to any endogenous protease genes. PichiaPink™ strain 2 is a *pep4* knockout. The *pep4* gene encodes proteinase A. This proteinase is a vacuolar, self-activating aspartyl protease, that is also involved in activating other proteases. Out of an abundance of caution, all samples were harvested in the presence of HALT™ protease inhibitors to prevent any degradation of secreted rBmSA1 protein during the pilot expression studies.

Yeast Culture to Induce Protein Expression

To examine heterologous expression of the BmSA1 protein, transfected PichiaPink™ strains 1 and 2 were first cultured in a BMGY to generate a starter culture of PichiaPink™ yeast cells in the absence of methanol. Cells were grown for 24 hours in a shaking incubator (280rpm) at 28°C. After 24 hours, cells were harvested and centrifuged at 1,500 x g for 5 minutes. The BMGY media was decanted and the cell pellet was resuspended in 2mL of BMMY. The methanol in the BMMY medium induces expression of the heterologous cloned recombinant protein by PichiaPink™ strains. The BMMY

cultures were grown in a shaking incubator (280rpm) at 28°C for 24-hours before the first time point collection. 100µl of culture was collected for each time point, and cultures were replenished with 40% methanol after each time point collection. Depending on the study, 24-hour, 30-hour, 48-hours, 72-hour, and 96-hour time points were collected.

SDS-PAGE Coomassie Staining:

An SDS-PAGE Coomassie stain is used to determine relative concentrations of protein in a sample separated by size. Samples from yeast cultures were examined by SDS-PAGE gel run under reducing conditions after heating the samples at 100°C for ten minutes in loading dye that contained 2-mercaptoethanol (2ME).

Protein expression by the PichiaPink yeast was first examined by SDS-PAGE followed by Coomassie staining to detect proteins on the gel. Supernatant from the PichiaPink™ cultures was first examined to evaluate whether a protein of the correct size was induced by methanol and secreted by the yeast into the supernatant. The yeast pellet was also examined to determine if the BmSA1 protein was secreted or retained within the yeast cell. Samples were collected at 24-hour, 48-hour, 72-hours, and 96-hour increments for supernatant samples. For the cell pellet lysate, samples were collected at 24-hour, 48-hour, and 72-hour increments. Gels were stained with Coomassie stain to evaluate the relative production of secreted proteins, as well as the production and secretion of a protein that is the same size of BmSA1 protein. Gels were loaded with 20µl of protein sample. Coomassie stain showed several protein bands in the yeast supernatant, one protein of approximately 40kDa in size was the approximately correct size for BmSA1, which is 35 kDa in size (Figure 12 and Figure 13). This protein band appeared in both the supernatant and cell pellet lysate (Figure 12, Figure 13, and Figure 14). In the supernatant, this band

was present at low amounts at the 24-hour time point compared to 48-hour time point collections (Figure 12). The protein bands were even more robust after 72 hours, compared to the 48-hour collections. (Figure 13). In the cell pellet lysate samples, protein concentration was relatively equal between 24- and 48-hour samples as shown by the equal band robustness between the two time points in Figure 13. The expression of a protein band approximately the size of BmSA1 in the supernatant with relatively low expression of other secreted proteins was encouraging because it suggests the BmSA1 protein was being expressed by the PichiaPink™ yeast and was in the secreted fraction which would be helpful for purification of the protein.

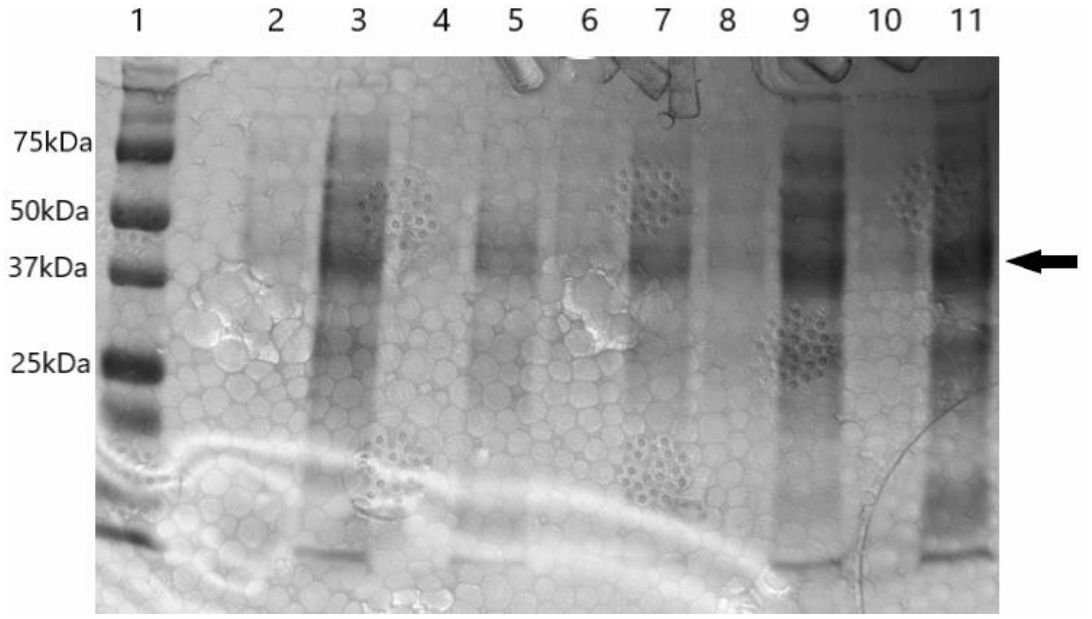


Figure 12: SDS-PAGE gel electrophoresis Coomassie stain of supernatant from methanol induced PichiaPink™ cultures. All lanes were loaded with 20µl of sample.
Lane 1: Precision Plus Protein™ Standard.
Lane 2: Strain 1 Colony 1 culture supernatant, 24 hours post-induction.
Lane 3: Strain 1 Colony 1 culture supernatant, 48 hours post-induction.
Lane 4: Strain 1 Colony 2 culture supernatant, 24 hours post-induction.
Lane 5: Strain 1 Colony 2 culture supernatant, 48 hours post-induction.
Lane 6: Strain 1 Colony 3 culture supernatant, 24 hours post-induction.
Lane 7: Strain 1 Colony 3 culture supernatant, 48 hours post-induction.
Lane 8: Strain 2 Colony 1 culture supernatant, 24 hours post-induction.
Lane 9: Strain 2 Colony 1 culture supernatant, 48 hours post-induction.
Lane 10: Strain 2 Colony 2 culture supernatant, 24 hours post-induction.
Lane 11: Strain 2 Colony 2 culture supernatant, 48 hours post-induction.

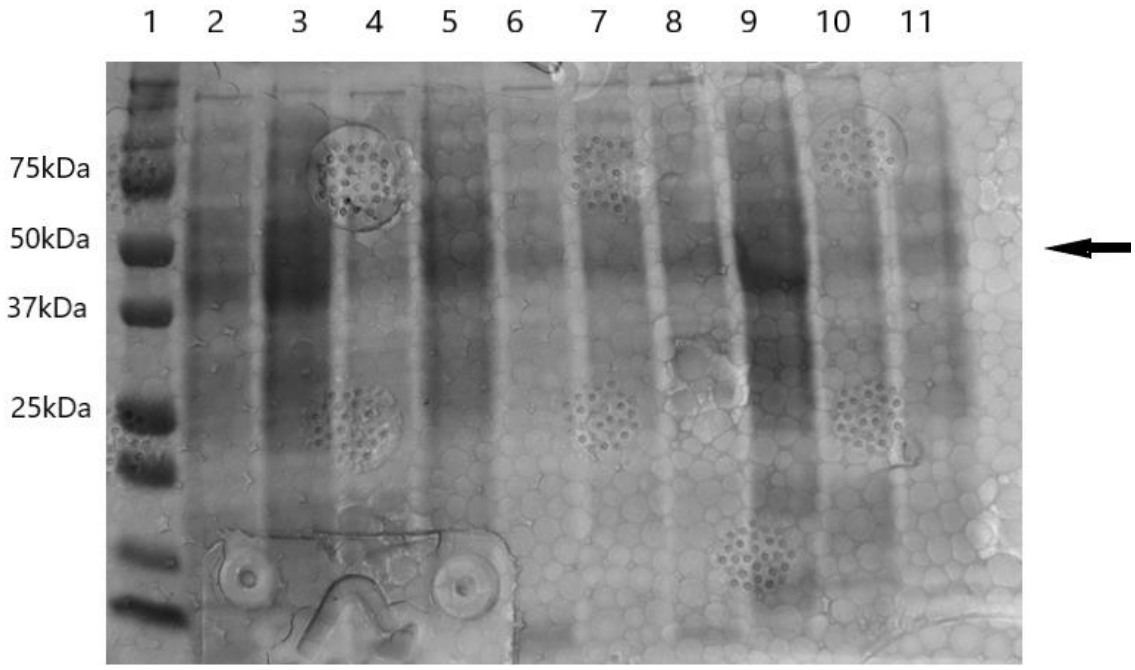


Figure 13: SDS-PAGE gel electrophoresis Coomassie stain of supernatant from methanol induced PichiaPink™ cultures. All lanes were loaded with 20µl of sample.
Lane 1: Precision Plus Protein™ Standard.
Lane 2: Strain 1 Colony 1 culture supernatant, 48 hours post-induction.
Lane 3: Strain 1 Colony 1 culture supernatant, 72 hours post-induction.
Lane 4: Strain 1 Colony 2 culture supernatant, 48 hours post-induction.
Lane 5: Strain 1 Colony 2 culture supernatant, 72 hours post-induction.
Lane 6: Strain 1 Colony 3 culture supernatant, 48 hours post-induction.
Lane 7: Strain 1 Colony 3 culture supernatant, 72 hours post-induction.
Lane 8: Strain 2 Colony 1 culture supernatant, 48 hours post-induction.
Lane 9: Strain 2 Colony 1 culture supernatant, 72 hours post-induction.
Lane 10: Strain 2 Colony 2 culture supernatant, 48 hours post-induction.
Lane 11: Strain 2 Colony 2 culture supernatant, 72 hours post-induction.

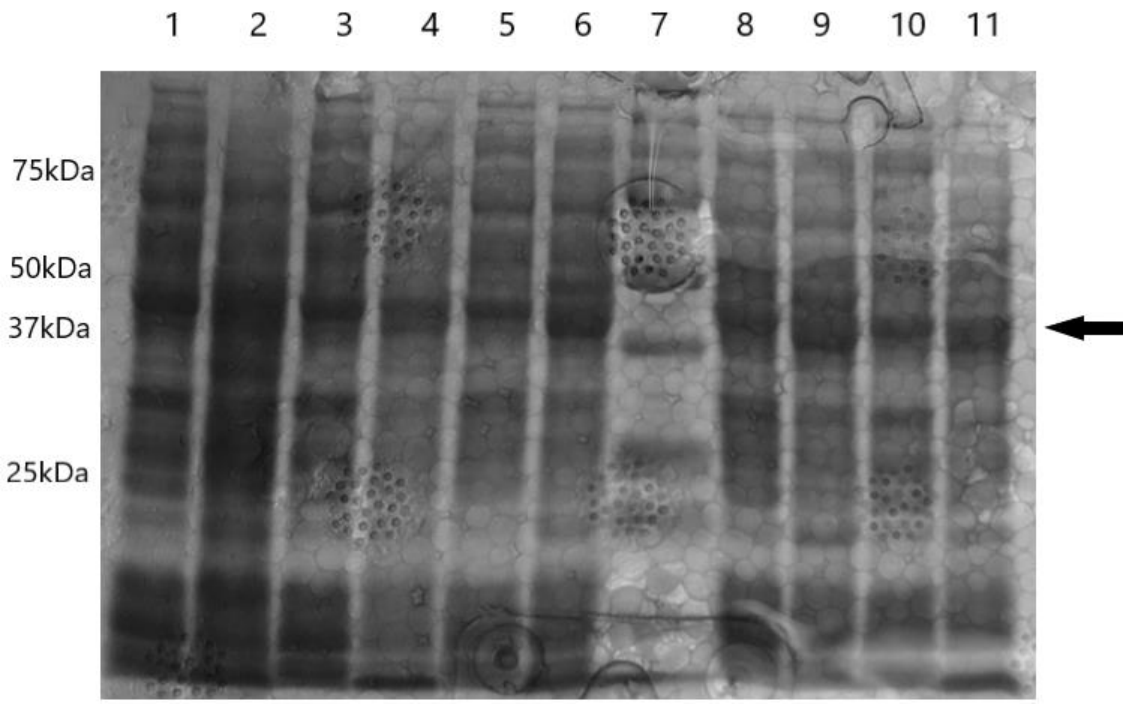


Figure 14: SDS-PAGE gel electrophoresis Coomassie stain of cell pellet lysate from methanol induced PichiaPink™ cultures. All lanes were loaded with 20µl of sample.
Lane 1: Strain 1 Colony 1 cell lysate, 24 hours post-induction.
Lane 2: Strain 1 Colony 1 cell lysate, 48 hours post-induction.
Lane 3: Strain 1 Colony 2 cell lysate, 24 hours post-induction.
Lane 4: Strain 1 Colony 2 cell lysate, 48 hours post-induction.
Lane 5: Strain 1 Colony 3 cell lysate, 24 hours post-induction.
Lane 6: Strain 1 Colony 3 cell lysate, 48 hours post-induction.
Lane 7: Precision Plus Protein™ Standard.
Lane 8: Strain 2 Colony 1 cell lysate, 24 hours post-induction.
Lane 9: Strain 2 Colony 1 cell lysate, 48 hours post-induction.
Lane 10: Strain 2 Colony 2 cell lysate, 24 hours post-induction.
Lane 11: Strain 2 Colony 2 cell lysate, 48 hours post-induction.

Western Blot Analysis:

To confirm that the secreted 40kDa protein was rBmSA1, western blot with BmSA1 specific antibodies were used to evaluate the protein in PichiaPink™ supernatants. Supernatants were probed by Western Blot analysis with the hybridoma antibodies RD167 and RD261 because of their previous validation against *E. coli* expressed rBmSA1, as previously described. Both antibodies recognized the protein of approximately 40kDa consistent with the size of BmSA1, and consistent with the SDS-PAGE Coomassie stain results (Figure 15, and Figure 16). It was immediately apparent that the antibodies were recognizing proteins of other sizes as well. The RD167 antibody also stained a protein of approximately 70kDa, and another of approximately 30kDa (Figure 15). However, this was consistent with the previous validation of RD167 against *E. coli* expressed rBmSA1, which also showed that RD167 recognized larger molecular weight protein bands as well. The RD261 antibody recognized a protein of approximately 70kDa as well (Figure 16). While the recognition of protein bands with sizes not consistent with BmSA1 was established for RD167 in previous validation studies, the recognition of protein bands with sizes not consistent with BmSA1 by RD261 was not consistent with previous validation studies. Even more unusual was that these bands appeared more robust than the 40kDa rBmSA1 band itself (Figure 15 and Figure 16). Because of this, we decided to repeat the western blot assays in the presence of a *B. microti* lysate as a positive control, to more accurately compare the size of the protein bands that the antibodies recognized in the *B. microti* parasite lysate compared to the size of the protein bands secreted by the transfected PichiaPink™ yeast. In theory, it was possible that the 70kDa protein was a BmSA1 dimer

and the 30kDa product a degradation product form BmSA1 that still contained the BmSA1 epitope recognized by the antibodies to BmSA1.

RD167

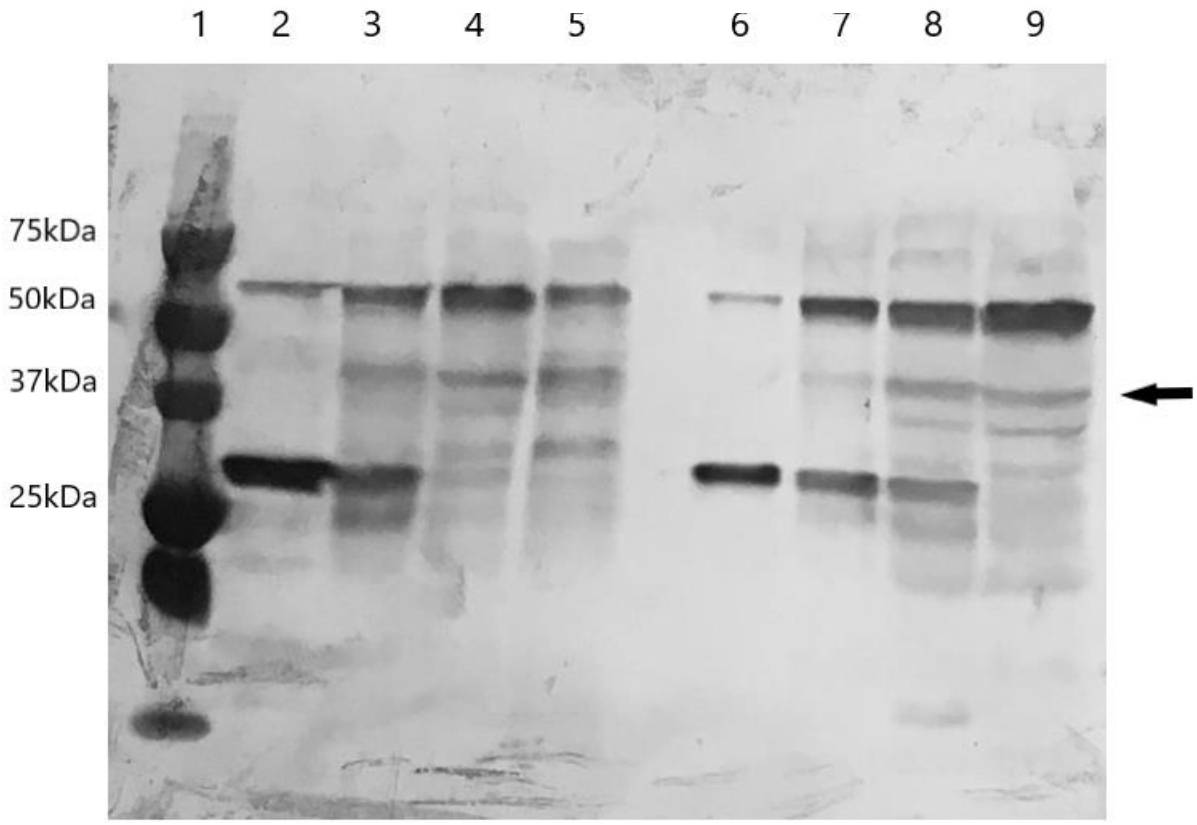


Figure 15: Western Blot analysis of supernatant samples of methanol induced PichiaPink ™. This membrane was probed with mAb RD167 (1:10). To detect mAbs, blots were incubated with 2° antibody, goat anti-mouse IgG (1:10,000). The Arrow is pointing to the BmSA1 protein detected in the B. microti cell lysate.
Lane 1: Precision Plus Protein™ Standard.
Lane 2: Strain 1 Colony 1 culture supernatant, 24 hours post-induction (20µl).
Lane 3: Strain 1 Colony 1 culture supernatant, 48 hours post-induction (10µl).
Lane 4: Strain 1 Colony 1 culture supernatant, 72 hours post-induction (10µl).
Lane 5: Strain 1 Colony 1 culture supernatant, 96 hours post-induction (10µl).
Lane 6: Strain 2 Colony 1 culture supernatant, 24 hours post-induction (10µl).
Lane 7: Strain 2 Colony 1 culture supernatant, 48 hours post-induction (10µl).
Lane 8: Strain 2 Colony 1 culture supernatant, 72 hours post-induction (10µl).
Lane 9: Strain 2 Colony 1 culture supernatant, 96 hours post-induction (10µl).

RD261

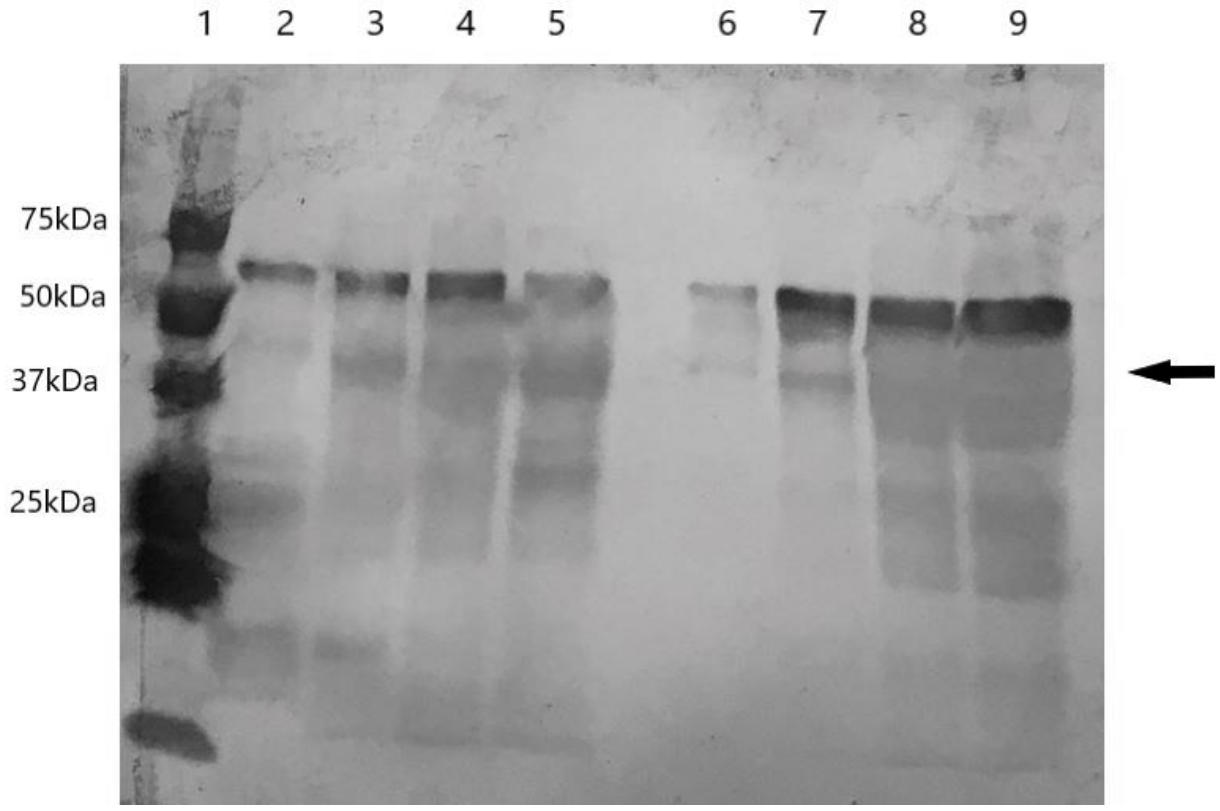


Figure 16: Western Blot analysis of supernatant samples of methanol induced PichiaPink ™. This membrane was probed with mAb RD621 (1:10). To detect mAbs, blots were incubated with 2° antibody, goat anti-mouse IgG (1:10,000). The Arrow is pointing to the BmSA1 protein detected in the B. microti cell lysate. All lanes were loaded with 10µl of sample.
Lane 1: Precision Plus Protein™ Standard.
Lane 2: Strain 1 Colony 1 culture supernatant, 24 hours post-induction.
Lane 3: Strain 1 Colony 1 culture supernatant, 48 hours post-induction.
Lane 4: Strain 1 Colony 1 culture supernatant, 72 hours post-induction.
Lane 5: Strain 1 Colony 1 culture supernatant, 96 hours post-induction.
Lane 6: Strain 2 Colony 1 culture supernatant, 24 hours post-induction.
Lane 7: Strain 2 Colony 1 culture supernatant, 48 hours post-induction.
Lane 8: Strain 2 Colony 1 culture supernatant, 72 hours post-induction.
Lane 9: Strain 2 Colony 1 culture supernatant, 96 hours post-induction.

Results from the repeat western blot analyses with *B. microti* lysate control showed that both of these antibodies did recognize protein bands of various sizes, including the 70kDa and 30kDa bands recognized in the PichiaPink™ supernatant (Figure 17 and Figure 18). Another observation from these repeat western blot analyses was the size of the *B. microti* lysate BmSA1 protein band; the size of the *B. microti* lysate BmSA1 was slightly smaller than the PichiaPink™ rBmSA1 (Figure 18). The 70kDa and 30kDa protein bands could be endogenous PichiaPink™ secreted proteins, since we had not cloned any *B. microti* genes into the yeast other than the 35kDa rBmSA1. Since it was apparent that the antibodies were recognizing some endogenous PichiaPink™ proteins, we wanted to test these antibodies against non-transfected vector negative PichiaPink™ strains' supernatants collected under the same induction conditions.

RD167

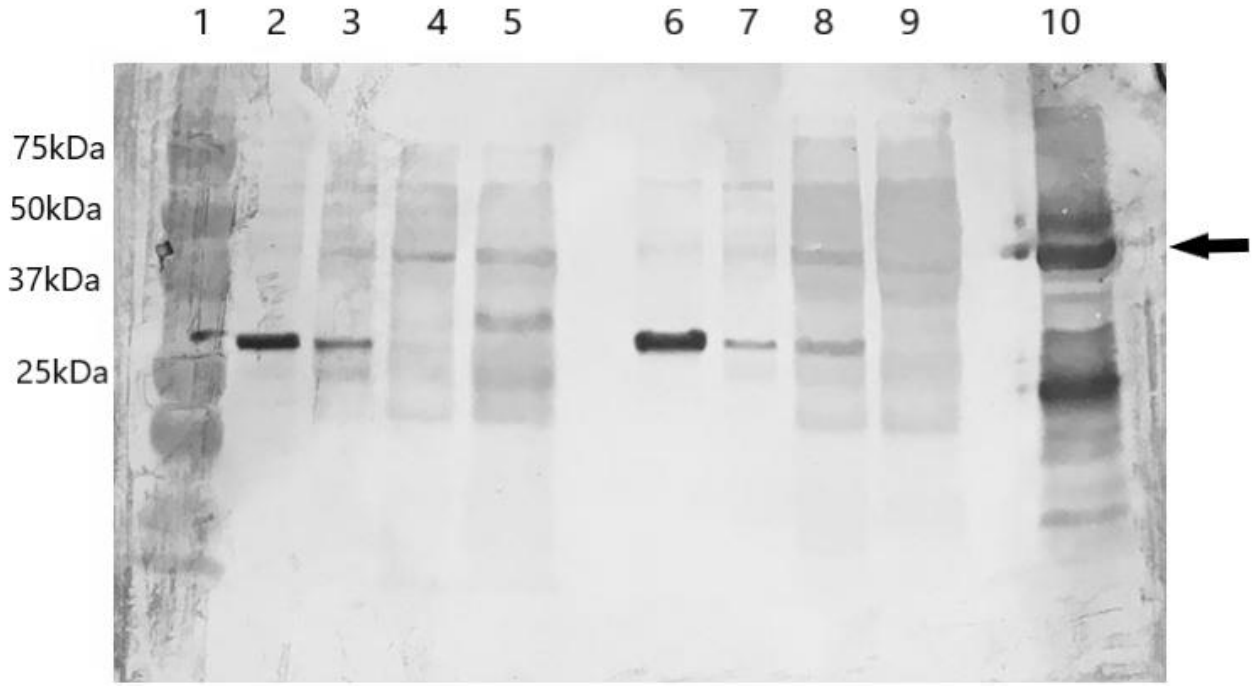


Figure 17: Western Blot analysis of supernatant samples of methanol induced PichiaPink ™. This membrane was probed with mAb RD167 (1:10). To detect mAbs, blots were incubated with 2° antibody, goat anti-mouse IgG (1:10,000). The Arrow is pointing to the BmSA1 protein detected in the B. microti cell lysate.
Lane 1: Precision Plus Protein™ Standard.
Lane 2: Strain 1 Colony 1 culture supernatant, 24 hours post-induction (20µl)
Lane 3: Strain 1 Colony 1 culture supernatant, 48 hours post-induction (10µl)
Lane 4: Strain 1 Colony 1 culture supernatant, 72 hours post-induction (10µl)
Lane 5: Strain 1 Colony 1 culture supernatant, 96 hours post-induction (10µl)
Lane 6: Strain 2 Colony 1 culture supernatant, 24 hours post-induction (20µl)
Lane 7: Strain 2 Colony 1 culture supernatant, 48 hours post-induction (10µl)
Lane 8: Strain 2 Colony 1 culture supernatant, 72 hours post-induction (10µl)
Lane 9: Strain 2 Colony 1 culture supernatant, 96 hours post-induction (10µl)
Lane 10: *B. microti* Gray Strain ATCC 30221 cell lysate.

RD261

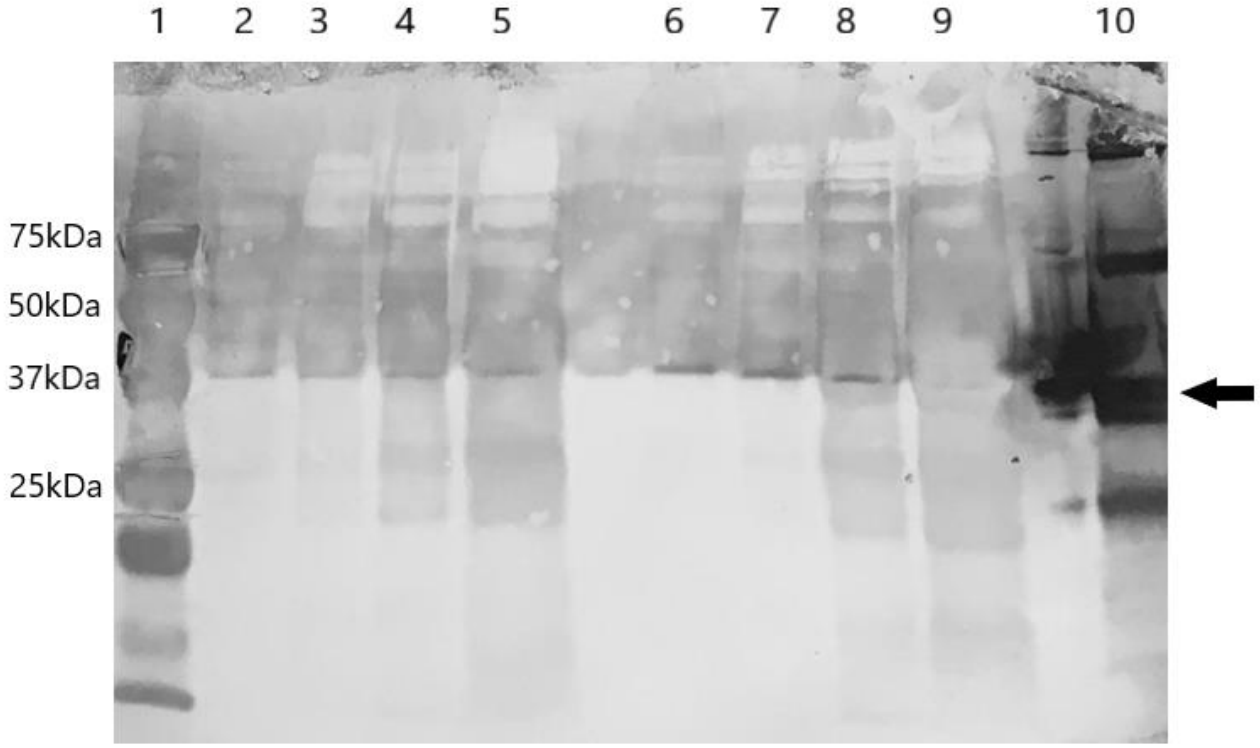


Figure 18: Western Blot analysis of supernatant samples of methanol induced PichiaPink ™. This membrane was probed with mAb RD621 (1:10). To detect mAbs, blots were incubated with 2° antibody, goat anti-mouse IgG (1:10,000). The Arrow is pointing to the BmSA1 protein detected in the B. microti cell lysate.
Lane 1: Precision Plus Protein™ Standard.
Lane 2: Strain 1 Colony 1 culture supernatant, 24 hours post-induction
Lane 3: Strain 1 Colony 1 culture supernatant, 48 hours post-induction
Lane 4: Strain 1 Colony 1 culture supernatant, 72 hours post-induction
Lane 5: Strain 1 Colony 1 culture supernatant, 96 hours post-induction
Lane 6: Strain 2 Colony 1 culture supernatant, 24 hours post-induction
Lane 7: Strain 2 Colony 1 culture supernatant, 48 hours post-induction
Lane 8: Strain 2 Colony 1 culture supernatant, 72 hours post-induction
Lane 9: Strain 2 Colony 1 culture supernatant, 96 hours post-induction
Lane 10: *B. microti* Gray Strain ATCC 30221 cell lysate.

There was concern that the identified protein in the PichiaPink™ supernatant was not strongly induced by methanol and that the monoclonal antibodies did not provide strong signals for the protein in western blots. In order to evaluate whether the 40kDa protein in the PichiaPink™ supernatant was a yeast protein instead of BmSA1, Western Blot analysis was performed using PichiaPink yeast that had not been transfected. Non-transfected vector negative PichiaPink™ strains' supernatants were collected under the same induction conditions as transfected PichiaPink™ yeast.

Western Blot analysis with antibody RD261 comparing transfected PichiaPink™ yeast to control non-transfected yeast showed that this antibody recognized a protein of the same size as the PichiaPink™ rBmSA1 in the non-transfected vector negative PichiaPink™ strains' supernatants (Figure 19). Based on the fact that BmSA1 protein did not seem to be expressed in yeast, DNA was isolated from transfected yeast and primers were used to amplify the vector *bmsa1* insert by PCR to re-evaluate the DNA sequence and to confirm that the *bmsa1* gene was cloned in frame with the vector. The PCR product was sequenced using Sanger sequencing with primers 5' and 3' to the vector that spanned the insertion site. New primers were designed to the *bmsa1* insert in order to sequence the junction site (Table 1, "*bmsa1* interior primers"). Sequencing with these primers revealed that there was a 4 base pair deletion mutation starting at the +2 position of the *bmsa1* gene coding region (Figure 20). This mutation altered the entire amino acid sequence of the translated protein and resulted in a protein sequence that was out of frame. This setback revealed that the 40kDa protein band observed by SDS-PAGE Coomassie stain and Western Blot analysis was not rBmSA1, which explained the discrepancy in size between

the 35kDa *B. microti* lysate BmSA1 and the 40kDa protein recognized by the RD167 and RD261 antibodies.

RD167

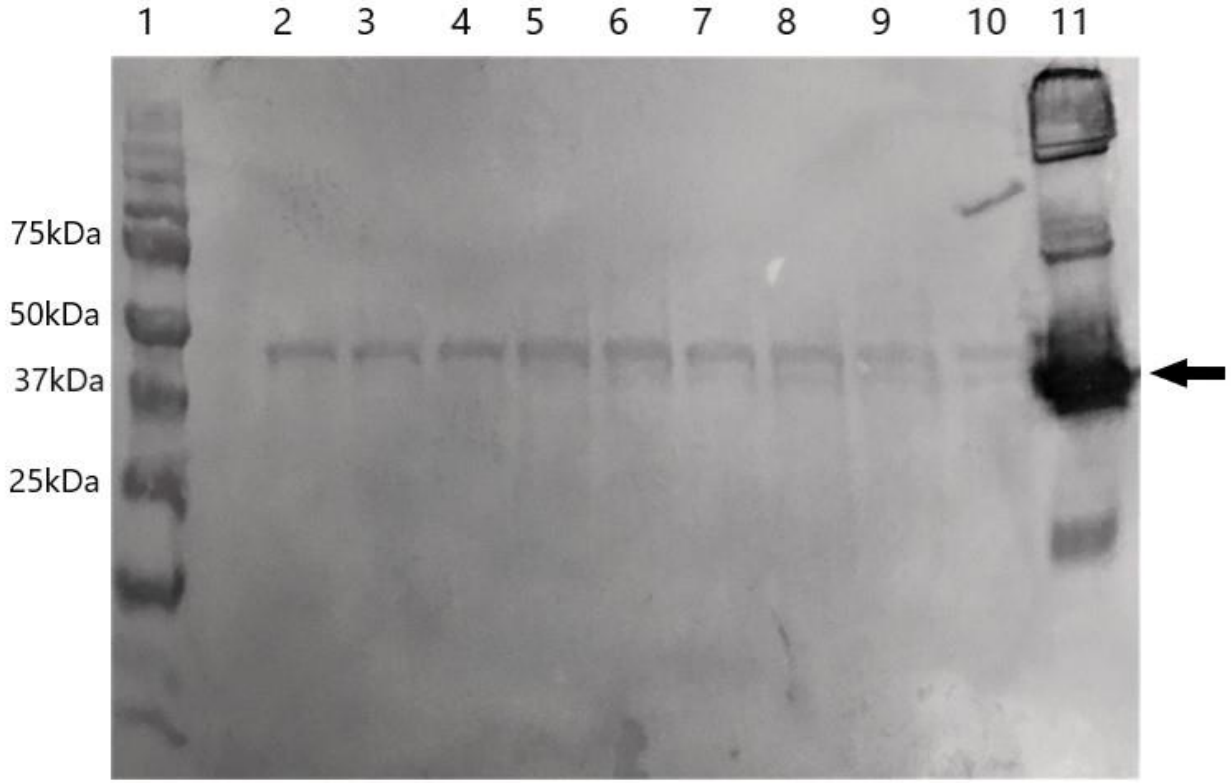


Figure 19: Western Blot analysis of supernatant samples of methanol induced PichiaPink ™. This membrane was probed with mAb RD261 (1:10). To detect mAbs, blots were incubated with 2° antibody, goat anti-mouse IgG (1:10,000). The Arrow is pointing to the BmSA1 protein detected in the B. microti cell lysate. All lanes were loaded with 10µl of sample.
Lane 1: Precision Plus Protein™ Standard.
Lane 2: Strain 1 Colony 1 culture supernatant, 24 hours post-induction.
Lane 3: Strain 1 Colony 1 culture supernatant, 48 hours post-induction.
Lane 4: Strain 1 Colony 1 culture supernatant, 72 hours post-induction.
Lane 5: Strain 2 Colony 1 culture supernatant, 24 hours post-induction.
Lane 6: Strain 2 Colony 1 culture supernatant, 48 hours post-induction.
Lane 7: Strain 2 Colony 1 culture supernatant, 72 hours post-induction.
Lane 8: Vector negative PichiaPink™ (negative control) supernatant, 24 hours post-induction
Lane 9: Vector negative PichiaPink™ (negative control) supernatant, 48 hours post-induction
Lane 10: Vector negative PichiaPink™ (negative control) supernatant, 72 hours post-induction
Lane 11: *B. microti* Gray Strain ATCC 30221 cell lysate.

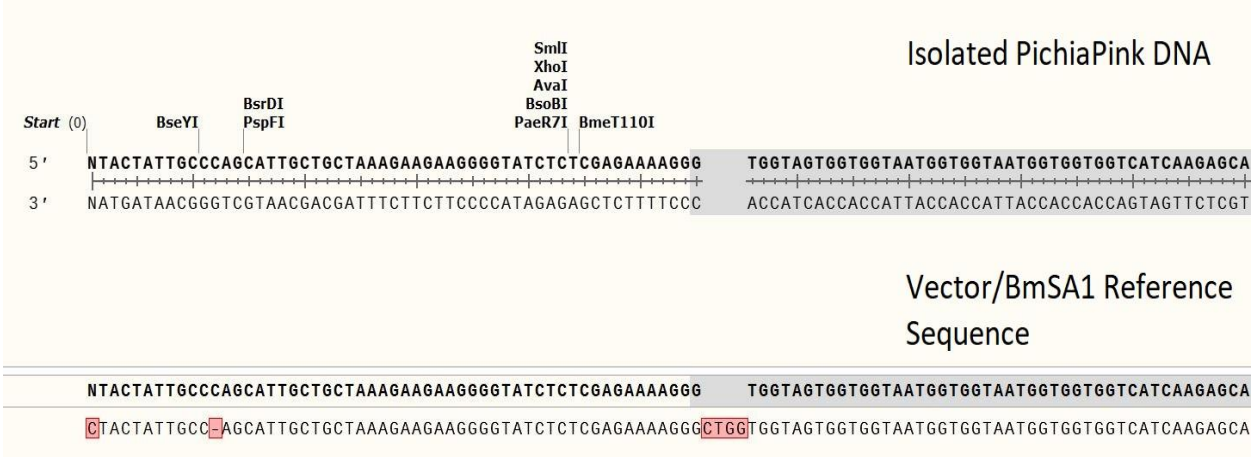


Figure 20: Saner sequencing from genomic DNA isolated from the transfected PichiaPink™ and amplified using sequencing primers from Table 1. On the top is the reverse compliment of the gene sequence returned from Sanger sequencing on the transfected PichiaPink™ purified vector using the interior reverse primers for sequencing the 5' vector/*bmsa1* junction. On the bottom is the 5' strand alignments of the purified vector and the *bmsa1* reference strand. Highlighted in red is the 4 base pair deletion presents in the purified vector sequence.

Electroporation of PichiaPink™ with Plasmid Insert 3 *bmsa1*:

After discovering the transfected PichiaPink™ strains created for this study contained a deletion mutation that completely altered the identity of the protein being expressed, it was necessary to generate new PichiaPink™ clones with an in frame *bmsa1* gene before conducting any further studies. Sanger sequencing conducted earlier in the study showed colony 3 (Figure 10a, 10b) could be a good candidate for insertion into the vector for heterologous protein expression. However, the sequencing results were imperfect, so plasmids were purified from *E. coli* with the colony 3 plasmid *bmsa1* insert and sent for Sanger sequencing using the vector-specific primers, as well as the interior primers designed to capture insertion junctions (Table 1). Figure 21 shows the sequencing results for this plasmid using all 4 of the aforementioned primers, validating that this Vector/*bmsa1* duplex was in frame and contained 100% sequence identity to the Gray Strain ATCC 30221 *B. microti bmsa1* gene.

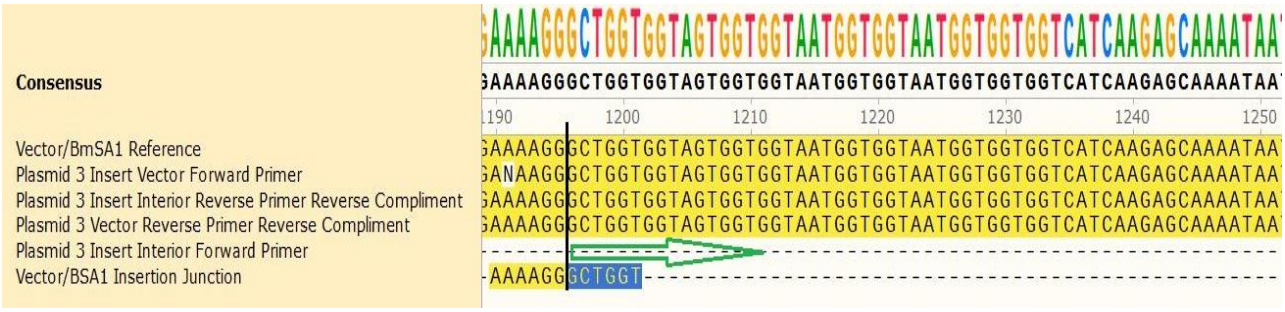


Figure 21: Sequence alignment of the Vector/*bmsa1* compared to the Vector/*bmsa1* reference sequence. a) Purified Vector/*bmsa1* duplex DNA from transfected *E. coli* colony 3 (Figure 9a,9b) sanger sequencing results aligned with reference Vector/*bmsa1* sequence. All 4 primers for sequencing clones (Table 1) were used for PCR amplification of the Vector/BmSA1 duplex. The beginning of the BmSA1 protein-coding gene is represented by the black line and green arrow.

After validating the sequence of the purified colony 3 DNA Vector/*bmsal* duplex, linearization of the plasmid with the restriction enzyme EcoN1 was performed exactly as described previously, with successful linearization being verified by horizontal gel electrophoresis (Figure 22). Electroporation of PichiaPink™ strains 1 and 2 was performed using the linearized plasmid. Selection on PAD selection plates resulted in 2 white colonies from transfected PichiaPink™ strain1 and 3 white colonies from transfected PichiaPink™ strain 2. These colonies were expanded on fresh PAD selection plates where they were used to generate glycerol stocks and inoculate starter cutlers for pilot BmSA1 expression studies. Also, DNA was purified from these expanded colonies for sequence validation using the vector-specific and *bmsa1* interior primers (Table 1) to validate the

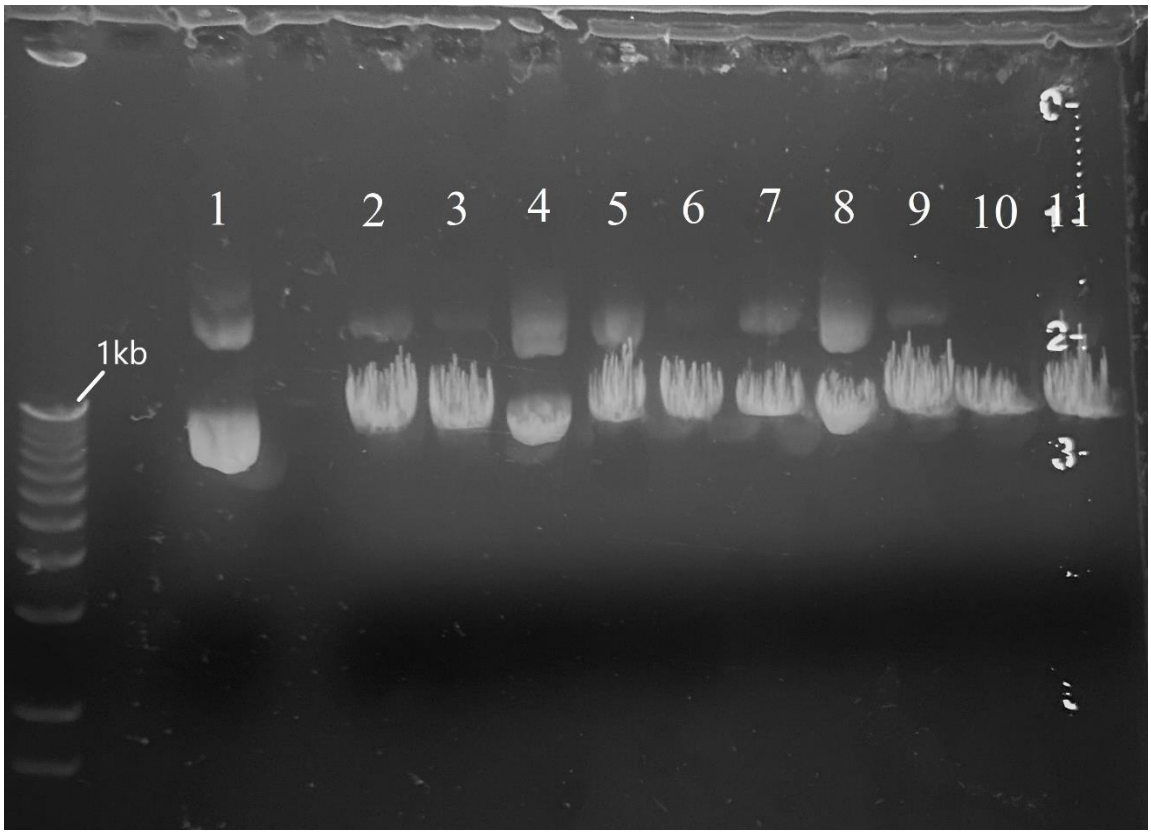


Figure 22: Horizontal gel electrophoresis of the linearized Vector/*bmsa1* plasmid duplex from *E. coli* colony 3. Plasmid was linearized with the restriction enzyme *EcoNI*.
Lane 1: The 1 kb plus standard.
Lane 2: The uncut plasmid control
Lanes 3-12: 8 µl aliquots of 1:10 dilutions of restriction digested DNA in sterile water mixed with 2 µl of TrackIt™ Cyan/Yellow loading buffer, each from separate 20 µl restriction digest reactions.

Vector/*bmsa1* duplex was in frame and homologues to the Gray Strain ATCC 30221 *bmsa1* gene. Sequencing confirmed that the insert in the PichiaPink™ strains was correct, with the exception of 1 base pair (Figure 23a, 23b). This 1 base pair SNP resulted in an arginine in the Gray Strain ATCC 30221 BmSA1 amino acid sequence being exchanged for a threonine (Figure 24).

a)

b)

Figure 23: a) Sanger sequencing from purified Vector/*bmsa1* duplex DNA from electroporated transfected PichiaPink™ strains. The forward vector-specific primer is shown for in the second row to the bottom. a) All 5 colonies were sequenced with both vector-specific forward primers for sequencing clones and interior reverse primers for sequencing clones 5' junction. The forward vector-specific primer is shown in the second row to the bottom. The bottom row shows the vector/*bmn1-21* junction, which is the last 2 codons of the vector followed by the first 2 codons of the BMN1-21 protein-coding gene. The beginning of the BmSA1 protein-coding gene is denoted by the black line and green arrow) **b)** Shows the SNP difference between the Vector/*bmsa1* reference genome and the *B. microti* Gray Strain ATCC 30221 *bmsa1* gene.

Figure 24: An alignment of the protein translated from the *bmsa1* gene purified from the PichiaPink™ clone, and the *bmsa1* gene as it appears in the *B. microti* Gray Strain ATCC 30221. There is 1 amino acid difference between the two proteins, an arginine in the PichiaPink purified *bmsa1* (position 107) is replaced with a threonine in the Gray Strain ATCC30221 *BmSA1*. The middle line shows the amino acids that are identical in both sequences. Local alignment was performed using the Smith-Waterman algorithm

Western Blot Analysis of PichiaPink™ BmSA1 Insert:

After validating through sequencing that the vector/*bmsa1* duplex was integrated into the PichiaPink™ genome in frame and with sequence homology to the *B. microti* Gray Strain ATCC 30221, BmSA1 protein pilot expression studies were initiated. Figure 25 shows the Western Blot analysis of methanol induced PichiaPink™ supernatants at 24-hour and 28-hour collection points. One clear protein band of approximately 45kDa was observed in both strains at the 24-hour time points. At 28 hours, this protein band had almost completely dissipated. At this point there was no way of validating that the protein recognized by the antibody RD-261 in this Western Blot analysis was rBmSA1. This protein could have been the one expressed endogenously by non-transfected PichiaPink™ that was observed in the earlier analyses. Unlike the earlier analysis however, we are certain that the PichiaPink™ strains used in this later study did contain a pPinkα-HC vector/*bmsa1* duplex that was in frame and homologous to the *B. microti* Gray Strain ATCC 30221 *bmsa1*.

RD261

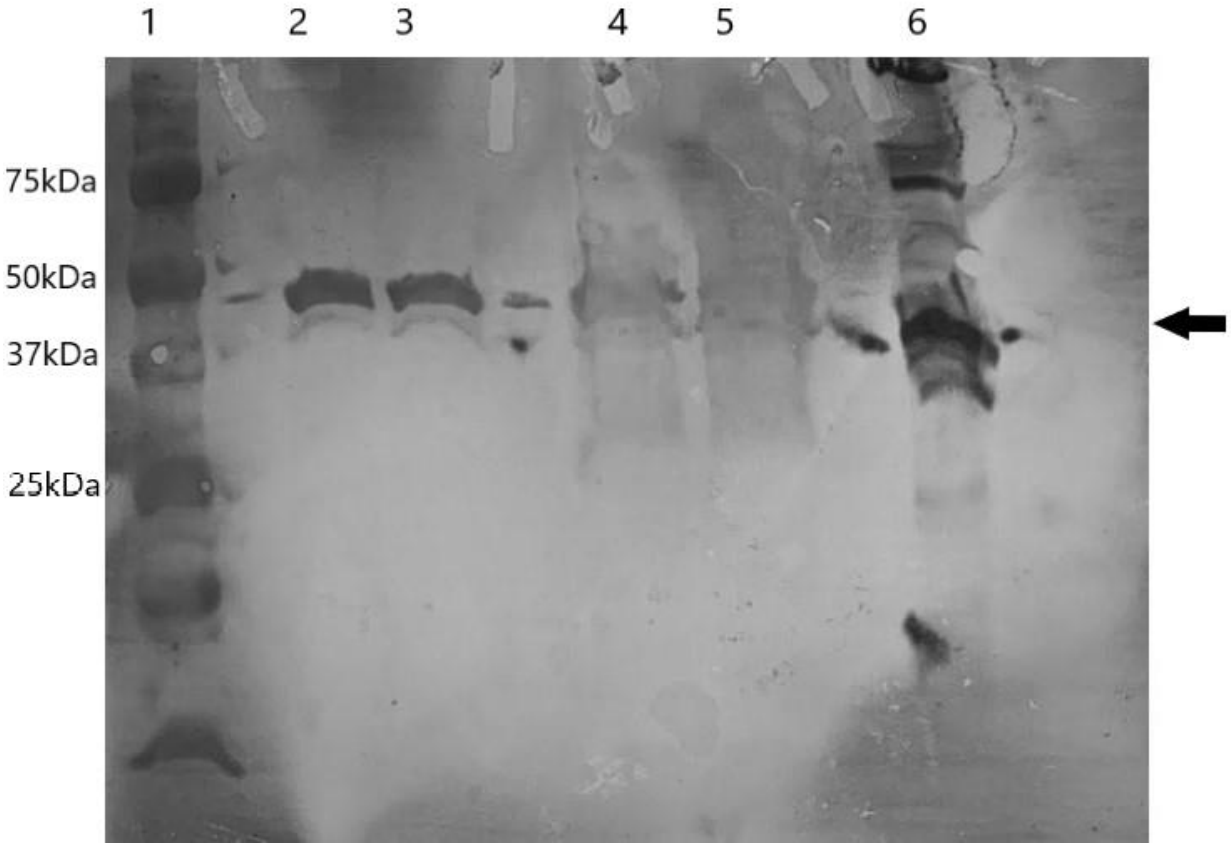


Figure 25: Western Blot analysis of supernatant samples of methanol induced PichiaPink ™. This membrane was probed with mAb RD621 (1:10). To detect mAbs, blots were incubated with 2° antibody, goat anti-mouse IgG (1:10,000). All lanes were loaded with 10µl of sample.
Lane 1: Precision Plus Protein™ Standard.
Lane 2: Strain 1 Colony 1 culture supernatant, 24 hours post-induction.
Lane 3: Strain 2 Colony 1 culture supernatant, 24 hours post-induction.
Lane 4: Strain 1 Colony 1 culture supernatant, 28 hours post-induction.
Lane 5: Strain 2 Colony 1 culture supernatant, 28 hours post-induction.
Lane 6: *B. microti* Gray Strain ATCC 30221 cell lysate.

Discussion

The main goal of this study was to generate rBmSA1 that accurately represents that of the wild-type protein secreted by *B. microti*. The objective behind this was to validate a library of hybridoma produced antibodies generated via fusion with *B. microti* infected BALB/c mice B-cells and suspected to target BmSA1 proteins according to liquid chromatography-mass spectrometry (LC-MS/MS) of proteins immunoprecipitated by the antibodies. Previous studies testing rBmSA1 produced in *E. coli* against this hybridoma library generated mixed results. While many BmSA1 antibodies validated by LC-MS/MS did recognize the *E. coli* rBmSA1, many did not. There were also antibodies in the hybridoma library that recognized proteins other than BmSA1 in the *E. coli* cell lysate. There was reason to believe that a possible explanation for these discrepancies was the lack of eukaryotic post-translational modifications altering the specificity of these antibodies for the rBmSA1 protein produced in *E. coli*. The goal was to use the heterologously expressed rBmSA1 in the PichiaPink™ *Pichia pastoris* expression as a BmSA1 protein standard for generating an antigen-capture ELISA using the aforementioned antibody library. It would also be used to identify additional monoclonal antibodies from the mAb library that specifically targeted BmSA1.

In theory, the expression of rBmSA1 in the PichiaPink™ *Pichia pastoris* expression system should address many of the complications that the earlier antibody screening studies using *E. coli* expressed rBmSA1 faced. One of those issues was the need to histidine tag the rBmSA1 and purify it through a nickel column from *E. coli* cell lysate. The addition of histidine tags to a protein has the potential to alter the protein's structure, function, and/or immunogenicity if the tags cannot be removed (Booth et al., 2018)., which

was the case with the *E. coli* rBmSA1 in previous studies. The rBmSA1 protein in *E. coli* included both 5' and 3' histidine tags and only one of the two tags had the potential to be removed. The Pichia pastoris expression model allows for proteins to be secreted into the culture supernatant, eliminating the need for the inclusion of a histidine tag to purify proteins from cell lysates. Pichia pastoris also secrete very low volumes of proteins endogenously (Sreekrishna et al., 1997) making it easier to purify heterologous proteins like rBmSA1 because fewer endogenous proteins have to be removed. Our research supports this notion, as very little protein was observed through SDS-PAGE Coomassie staining in the methanol-induced PichiaPink™ culture supernatants (Figure 12 and Figure 13). The ability of Pichia pastoris to secrete proteins into culture without the presence of many other proteins generates the potential for protein purification by means other than protein tagging, like ammonium sulfate precipitation, ion-exchange chromatography or size exclusion chromatography (Shi et al., 2021; Steglich et al., 2020)

Another issue with the *E. coli* expression model is that *E. coli* lack post-translational modification mechanisms present in eukaryotic organisms like *B. microti* and *Pichia pastoris*. We do not know if the lack of post-translational modifications of the *E. coli* expressed rBmSA1 was altering the antibody specificity of the rBmSA1, but we do know that many of the antibodies that had been confirmed as BmSA1 specific by LC-MS/MS of immunoprecipitated proteins did not recognize the *E. coli* expressed rBmSA1. Research has shown that glycosylated immunogens have their immunogenicity better conserved when expressed in eukaryotic models compared to prokaryotic models (Bagno et al., 2020). Since BmSA1 is a glycophosphatidylinositol (GPI) linked protein, it is reasonable to predict that the structure of *E. coli* expressed rBmSA1 could have altered

antibody specificity since *E. coli* do not have the pathway for synthesizing GPI anchors (Kinoshita, 2016*).* Heterologous expression and secretion of recombinant proteins by *Pichia pastoris* results in the synthesis of eukaryotic proteins that closely resemble their native conformation, structure, immunogenicity, and recognition by antibodies (Balamurugan, et. al).

It is likely that additional studies to optimize the expression of rBmSA1 will identify optimum induction strategies with methanol for secretion of the heterologous protein. In this thesis only one study was performed to evaluate the expression of rBmSA1, and it examined secreted culture media from yeast by Western Blot analysis with mAbs to BmSA1 for detection. The results were promising as the BmSA1 protein was present in the transformed yeast culture supernatant. The process for evaluating optimal expression conditions for rBmSA1 will follow the process that was outlined in the thesis for the out-of-frame rBmSA1. Optimization will include performing time-course analysis of rBmSA1 in the yeast culture supernatants and cell lysate before and after methanol induction followed by an analysis of protein expression by Coomassie staining of total proteins in the gel and by Western Blot analysis with mAbs confirmed to recognize BmSA1.

In this study, the yeast with pPinkα-HC secretion vector containing the incorrect *bmsa1* gene exhibited poor expression induction after methanol induction. It is also possible that the yeast with the pPinkα-HC secretion vector containing the correct *bmsa1* gene will also exhibit poor expression induction after methanol induction (Figure 12 and Figure 13). There are several mechanisms that could impair methanol induction of heterologous proteins in PichiaPink™ yeast. First, the *AOX1* promoter is highly repressed by glucose (Ahmad et al., 2014). The protocol used in these studies to grow a starter

culture of PichiaPink™ yeast prior to expression involved growing the cultures in BMGY medium containing glucose. While the cultures were centrifuged, and the BMGY medium was decanted before the cultures were resuspended in BMMY methanol containing media for induction, there was no wash step in between to ensure all glucose was absent from the cell pellets before resuspension in BMMY methanol media. Allowing cultures to grow for longer durations in the BMMY methanol media might have allowed the yeast to grow to higher cell densities and given them more time to completely metabolize all of the residual glucose in the medium to alleviate the *AOX1* promoter repression.

There is also evidence to support the possibility that proteolysis could be occurring inside of the yeast cultures. In figure 25, it is clear that rBmSA1 protein was present at 24 hours and had dissipated by 28 hours. In this study we only successfully transfected PichiaPink™ strains 1 and 2 with the pPinkα-HC secretion vector/*bmsa1* gene duplex. Strain 1 has no protease knockouts and strain 2 has only one protease knockout which is the gene *pep4* that encodes proteinase A. Both of these strains still have the *prb1* gene that encodes proteinase B. Studies have shown the best way to prevent proteolytic cleavage of *Pichia pastoris* heterologously expressed proteins is to clone expression vectors into *pep4 prb1* double knockout strains; some have successfully hampered the proteolytic activity of Pichia pastoris expressed proteases by altering culture conditions in ways like adjusting pH and temperature of culture conditions, lowering salt concentrations, or adding casamino acids and increasing peptone concentration of culture mediums (Ahmad et al., 2014; Zhao et al., 2008).

It is also possible that the PichiaPink™ rBmSA1 is not stable in methanol. The protocol for sample collection used in these studies involved replenishing induction

cultures with 100µl of 40% methanol after removing 100µl of sample for protein analysis. The addition of 100µl of 40% methanol brings the methanol concentration of the BMMY induction media from 0.5% to 2.475%. Methanol metabolism has been associated with the degradation of secreted recombinant proteins in *Pichia pastoris* expression models by inducing the expression and secretion of vacuolar proteases (Sinha et al., 2005). The increased methanol concentration between the 24-hour collection and the 28-hour collection could have triggered the secretion of proteases into the yeast culture and degraded the rBmSA1 protein. The higher methanol concentration could also be microbicidal to the yeast, and cell lysis has also been shown to contribute to protease secretion and degradation of secreted *Pichia pastoris* recombinant proteins (Sinha et al., 2005).

The two antibodies RD167 and RD261 had recognized proteins of larger and smaller molecular weights than BmSA1 in both the previous studies using *E. coli* expressed rBmSA1 and in this study (Figure 15 and Figure 16). In the E. coli expressed rBmSA1 studies the investigators speculated that these bands could have been protein aggregates (in the case of the 70kDa protein) and protein degradation products (in the case of the 30kDa protein). Similar-sized proteins were evident in the protein culture samples from the yeast with pPinkα-HC secretion vector containing incorrect *bmsa1* genes, suggesting that at least in this case, there are endogenous yeast proteins that are secreted that are approximately 30kDa and 70kDa. It is possible that they represent conserved proteins in both Pichia pastoris as well as in *E. coli*.

Considering these new findings, an alternative hypothesis for the identity of the protein bands recognized by these antibodies could be ubiquitin. Ubiquitin is a 9kDa

protein that is covalently attached to target proteins as a tagging step in ATP-dependent protein degradation (Ludemann et al., 1993). Ubiquitin is also one of the few protein species secreted into the matrix by *Pichia pastoris* during growth in expression media (Sreekrishna et al., 1997) If BmSA1 was ubiquinated during hybridoma screening, it is possible that the antibodies were bound to BmSA1 protein not by one of the protein's own epitopes but rather by the ubiquitin that was covalently bound to it. Not only could free ubiquitin polymers or other ubiquitinated proteins be responsible for the 30kda and 70kDa protein bands, but a single monoubiquitylation on the rBmSA1 would explain the size discrepancy of roughly 10kDa difference between the known size of native *B. microti* translated BmSA1 (33kDa) and the observed size of rBmSA1 by Western Blot in our study (Figure 25). This size discrepancy was also observed in the previous work using *E. coli* expressed rBmSA1, and while prokaryotes do not have the genes for ubiquitin, they do encode ubiquitin-like modification proteins that could be cross-reactive to eukaryotic ubiquitin (Xu et al., 2019). However, one flaw to this hypothesis is that the hybridoma cells were produced through fusion with B-cells from *B. microti* infected mice. Like all mammals, mice express ubiquitin endogenously, and due to self-tolerance, it is unlikely that they would have produced anti-ubiquitin antibodies. Immunoscreening samples with anti-ubiquitin antibodies would easily support or reject this hypothesis.

In order for an antigen-capture ELISA to be accurate, the antigens targeted by the antibodies in the assay need to be well conserved across circulating strains. The BLAST alignments revealed that the *bmsa1* gene is perfectly conserved across all identified *B. microti* strains sequenced and uploaded to piroplasmaDB.org (Table 3). This justifies the continued efforts in synthesizing a reliable, near-native conformation recombinant BmSA1

for use as a standard in the development of an antigen-capture ELISA. BmSA1 shares near-perfect gene and amino acid identity with the *B. microti* BMN1-9 protein, and the *bmn1-9* gene has not been annotated in many of the published *B. microti* strain genomes (data not shown). As current *B. microti* genomic characterization studies have already begun to re-assign the BMN family of proteins based on biological function and immunorecognition (Verma et al., 2020), it is possible that BmSA1 and BMN1-9 could become classified as variants of the same protein. The BMN1-21 protein is also well conserved, but shares only 23% amino acid sequence identity and 42% sequence similarity to BmSA1. Because these proteins are both well conserved but not very similar to each other, BMN1-21 would make a good candidate for the future development of antigen capture ELISA that might compliment a BmSA1 antigen capture ELISA without cross-reacting with the BmSA1 antigen.

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