

MATERIAL INSPECTION AND RECEIVING REPORT

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"Rapid Identification of Bacterial Virulence Factors"

April 15, 2014

Sponsored by

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Principal Investigator:	Vito G. DelVecchio, Ph.D.
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14. ABSTRACT The main objective of this work is to demonstrate that Rapid Virulence Factor Discovery System (RVFDS) can be used to identify virulence factors of MRSA and <i>Brucella abortus</i> . This will be accomplished by constructing an MRSA and <i>B. abortus</i> random expression library (REL) in <i>E. coli</i> . The MRSA REL contains hundreds of potential virulence factors. Putative virulence factors that interacted with the ECM components fibronectin, laminin, and plasminogen, collagen, and anti-MRSA polyclonal antibodies will be eluted from pull-down columns that contain these bait proteins. Putative MRSA virulence factors will be identified by LC-MS/MS. Additional virulence factors will be identified using a wider range of ECM components as bait proteins. In the Phase I investigation the average size of the REL insert was in the 1.5 to 2.0kb range. In Phase II the average size of the insert will be increased to 3.0kb in order to decrease redundancy. Larger inserts and REL protein sizes will also facilitate LC-MS/MS identification and shorten the time needed for data analysis. Potential proteins that are discovered in Phase II work may serve as targets for future vaccines, antimicrobials, and therapeutic measures.					
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Potential Applications of the Rapid Identification of Bacterial Virulence Factors Project

Summary:

Genomic DNA's of community-acquired MRSA and *Brucella abortus* were physically sheared into fragments that were approximately the size of the average open reading frame of each organism. These pathogens were chosen since MRSA is of such clinical importance and has been subjected to vast investigations on its pathogenesis whereas *B. abortus*, although highly pathogenic, has yet to have many classical virulence factors identified. These pathogens differed in other respects in that MRSA is gram+ and as such does not have a periplasm or outer membrane. *B. abortus* is gram- and has inner and outer membranes enclosing a periplasmic space. Gram- bacteria also differ from gram + in that they form outer membrane vesicles (OMV) that allow the release of complex collections of proteins and lipids into the extracellular environment. Thus these OMV represent a means by which a pathogen interacts with prokaryotic and eukaryotic cells. They are an important component of the host/pathogen relationship.

Each of the genomic libraries (DNA fragments) were then placed in expression vectors and cloned into *E. coli*. The libraries were then expressed, and mass spectroscopy studies confirmed that literally hundreds of proteins were formed from these libraries. This was accomplished in Phase 1 of this project. The importance of this random expression library (REL) strategy is that it circumvents the inherent disadvantages of *in vitro* and *in vivo* studies in that potentially all of the genes of these pathogens were expressed. Thus proteins that would be overlooked or never be expressed in laboratory-grown cultures or identified in host infected tissue investigations would be available for investigation.

The ultimate objective of this project is to identify potential virulence factors and immunogenic proteins thereby providing novel superior antibiotic targets and vaccine candidates to prevent or mitigate the offensive mechanisms these pathogens inflict on their hosts. Thus the virulome, immunome, and therapeutic drug targets would be established by an innovative and novel pull-down/proteomic strategy. We labeled pull-down columns with host extracellular matrix (ECM) components serving as bait to capture any expressed proteins that may be involved in the host/pathogen relationship. These components included plasminogen, fibrinogen, collagen IV, fibronectin, laminin, vitronectin, as well as HeLa whole cell and HeLa cell membrane fractions. Proteins that were identified as binding to these components were included as putative members of the virulome of that pathogen. To define the immunome we utilized anti-MRSA, anti-*B. abortus*, and anti-*B. melitensis* antibodies as bait in pull-down assays. The identity of each potential virulence factor was then determined by LC-MS/MS. Over 25 proteins were included as being potential virulence factors for each of the pathogens.

Of the many potential virulence factors identified in this study, the following protein of MRSA stand out as candidates for further investigations based on exhaustive literature searches: acetyl-CoA carboxylase, acetyltransferase GNAT family protein, acetyl-CoA synthetase, bone fibrinogen/sialoprotein-binding protein, ECM-binding protein homolog, glutamate dehydrogenase, histidine kinase KdpD protein, immunoglobulin-binding protein, K transporting ATPase, membrane-anchored glycerophosphoyl diester phosphodiesterase, MHC class II

analog/extracellular adherence protein, nitroreductase periplasmic-binding protein, peroxiredoxin, phosphoenolpyruvate carboxykinase, serine protease HtrA-like protein, superantigen-like protein, and zinc metalloprotease aureolysin.

The following *B. abortus* proteins that were identified offer extremely promising candidates for vaccine/therapeutic agent targets: 30 ribosomal protein S12, ABC transporter ATPase, copper-translocating P-type ATPase, iron ABC transporter substrate binding protein, spermidine/putrescine ABC transporter periplasmic protein, ABC transporter periplasmic sugar-binding protein, ABC transporter substrate-binding protein, ABC transporter sulfate-binding protein, adenylosuccinate synthetase, antibiotic acetyltransferase, chaperonin ClpA/B protein, cyclic beta 1-2 glucan synthetase, cyclophilin type protein, formyltransferase, HAD superfamily hydrolase, neutral zinc metallopeptidase, OMP 31, and OMP W. It should be noted that many of the potential virulence factors of *B. abortus* are members of the ABC-transporter family. Thus the discovery of a possible conserved component of members of this family could have far-reaching implications on antimicrobial agents.

Task 4 of this project was dedicated to the identification of host target/receptor proteins of putative virulence factors of MRSA. Thus a view of what host components confront the pathogen in its invasive process was obtained. It also provided invaluable information on host/pathogen interactions. Pull-down assays, which used cloned and expressed MRSA acetyl-CoA carboxylase, acetyl-CoA ligase, and K⁺ transporting ATPase B subunit as bait, were employed to pinpoint specific host components that interacted with these potential virulence factors. The data obtained verified that these three proteins may indeed be part of the host/pathogen interaction insofar that they targeted host proteins involved in internalization of invading bacteria and endosome recycling, maturation of phagosomes into phagolysosomes, microtubule formation, response to inflammation caused by pathogens *via* neutrophil recruitment, host membrane components, cytokine and stress responses, upregulation of antimicrobial peptides, activation of complement cascades, as well as combining with the host's ECM components collagen and laminin.

Cloned and expressed *B. abortus* OMP W and ABC transporter ATPase were selected as bait molecules to capture potential HeLa cell components that may participate in the host/pathogen relationship. These host factors captured have been implicated as playing a part in the host/pathogen relationship and as such are ideal candidate for antimicrobial therapies. They captured protein that were involved in correct folding of nascent and stress-induced protein, antigen presentation, immune defense, inflammation response, preventing protein aggregation, internalization of pathogenic bacteria, promotion of bacterial invasion, bacterial adherence and colonization, inflammation, elimination of intracellular bacteria, neutrophil recruitment, and those characteristically found in autophagosomes during bacterial invasion.

The data obtained from this study identified novel targets for therapeutic agents, new antibiotics, vaccines, and next generation diagnostic assays for strain specific pathogens. Many of these targets have been reported to have specific antagonistic molecules. This information will be of great importance for future *in vitro* and *in vivo* investigations. This project validated that the Rapid Identification of Bacterial Virulence Factors can serve as a virulence-factor discovery approach for a wide range of viral, bacterial, and fungal pathogens.

Data obtained from the Rapid Identification of Bacterial Virulence Factors research project:

Genomic DNA's of community-acquired MRSA and *Brucella abortus* were physically sheared into fragments that were approximately the size of the average open reading frame of each organism. These pathogens were chosen since MRSA is of such clinical importance and has been subjected to vast investigations on its pathogenesis whereas *B. abortus*, although highly pathogenic, has yet to have many classical virulence factors identified. These pathogens differed in other respects in that MRSA is gram+ and as such does not have a periplasm or outer membrane. *B. abortus* is gram- and has inner and outer membranes enclosing a periplasmic space. Gram- bacteria also differ from gram + in that they form outer membrane vesicles (OMV) that allow the release of complex collections of proteins and lipids into the extracellular environment. Thus these OMV represent a means by which a pathogen interacts with prokaryotic and eukaryotic cells. They are an important component of the host/pathogen relationship.

Each of the genomic libraries (DNA fragments) were then placed in expression vectors and cloned into *E. coli*. The libraries were then expressed, and mass spectroscopy studies confirmed that literally hundreds of proteins were formed from these libraries. This was accomplished in Phase 1 of this project. The importance of this random expression library (REL) strategy is that it circumvents the inherent disadvantages of *in vitro* and *in vivo* studies in that potentially all of the genes of these pathogens were expressed. Thus proteins that would be overlooked or never be expressed in laboratory-grown cultures or identified in host infected tissue investigations would be available for investigation.

The ultimate objective of this project is to identify potential virulence factors and immunogenic proteins thereby providing novel superior antibiotic targets and vaccine candidates to prevent or mitigate the offensive mechanisms these pathogens inflict on their hosts. Thus the virulome, immunome, and therapeutic drug targets would be established by an innovative and novel pull-down/proteomic strategy. We labeled pull-down columns with host extracellular matrix (ECM) components serving as bait to capture any expressed proteins that may be involved in the host/pathogen relationship. These components included plasminogen, fibrinogen, collagen IV, fibronectin, laminin, vitronectin, as well as HeLa whole cell and HeLa cell membrane fractions. Proteins that were identified as binding to these components were included as putative members of the virulome of that pathogen. To define the immunome we utilized anti-MRSA, anti-*B. abortus*, and anti-*B. melitensis* antibodies as bait in pull-down assays. The identity of each potential virulence factor was then determined by LC-MS/MS. An illustration of this system can be seen in Figure 1.

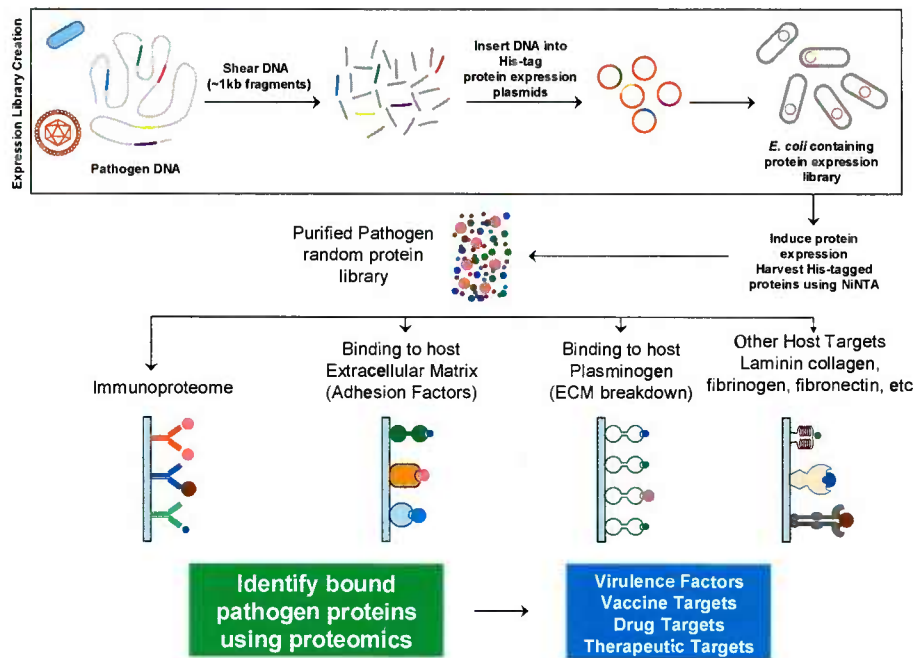


Figure 1. Overview of RVFDS. In Phase 2 the size of the DNA fragment was increased from 1.5 to 3.0kb.

Over 50 proteins were identified as potential virulence factors for each pathogen. Many of these have been reported in the literature as being putative or *bona fide* participants in the pathogenic process in other bacteria. Others have not been recorded as being offensive mechanisms and thus may represent novel vaccine or antimicrobial targets. This study has also found many of the proteins to be immunogenic. On the other side of the equation we have identified additional host components that may play a role in the host/pathogen relationship other than the ECM components bait molecules mentioned above. This was accomplished by reciprocal pull-down assays in which selected cloned potential virulence factors that were identified by more than two ECM components were used as bait in pull-down assays to capture HeLa whole cell and HeLa membrane components that may participate in the host/pathogen interaction. Host components that interacted with selected potential virulence factors were identified. Viewing host participants through the eyes of the pathogen revealed intricacies in the host/pathogen relationship that warrant further studies.

Task 1: Develop a rapid microtiter plate assay that will rapidly determine if an REL contains proteins that interact with host cell and ECM components. Host cell basement membranes are continuous sheets that form an interface between endothelial, epithelial, muscle, neuronal cells, and adjacent structures. Once a pathogen attaches to and penetrates the epithelial or endothelial layers and gains entrance to the basement membrane, dissemination and invasion of the pathogen is likely to occur. Thus a rapid screening system that determines if expressed proteins of an REL interact with specific components of the host ECM, basement membrane, or surface-exposed proteins of epithelial cells will allow us to focus on exactly what ‘bait’ proteins

should be used in a pull-down assays to identify potential virulence factors. The host components that were used as 'bait' in the microtiter plate and pull-down assays were collagen, fibronectin, laminin, fibrinogen, and plasminogen. This would have resulted in a saving of time, labor, and expense for future applications. The rapid screening format was developed in the following manner: Microtiter plates were coated with individual components of the ECM, basement membrane, and surface-exposed proteins. Then His-tagged REL protein libraries were added to each well for possible interaction to occur. Anti-His-HRP-coupled antibodies were then added to reveal the presence of REL proteins. This entire process could have been automated and thus rapidly determine what host component is targeted by proteins found in a particular REL. See Figure 2.

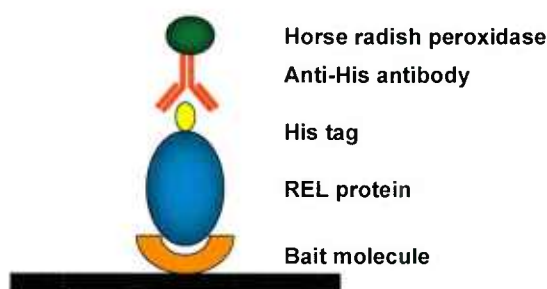


Figure 2. Illustration of the microtiter plate assay that was used to scan the REL fractions.

Initial experimentation employed collagen as the bait molecule to capture components of REL 15, which had significant hits in the LC-MS/MS pull-down assay. Because of this REL 15 was selected as the positive control for all experiments using collagen as the bait. REL 11, which did not produce hits in the LC-MS/MS pull-down assay, was chosen as the negative control. Initial results were promising. However when other RELs were tested no correlation between the microtiter plate and LC-MS/MS pull-down assays was found. Additional investigations were conducted using fibronectin, laminin, and plasminogen as bait molecules and glutamate dehydrogenase as the positive control since it was shown to combine with all of these three ECM components in LC-MS/MS pull-down assays. To this end, *E. coli* cells, which contained the glutamate dehydrogenase gene cloned into an expression plasmid, were induced with IPTG for 3 hours. Cells were then lysed and cell debris removed by centrifugation. Fifty microliters of this lysate that contained the expressed glutamate dehydrogenase was used for the first dilution as the positive control.

The positive control yielded the expected results in the fibronectin assay. As expected there was a downward trend as the dilution of lysate decreased. This downward trend was also seen for all five BaRELs. The microtiter plate results suggest that BA001, 003 and 004 react strongly with fibronectin. Whereas BaRELs 002 and 005 did not react as well. However, when comparing these results to those obtained from the pull-down assay with fibronectin as the bait molecule there was a large discrepancy. BaRELs 001, 002 and 004 did not produce any significant hits upon LC-MS/MS analyses. BaRELs 003 and 005 had only one hit. See Figure 3.

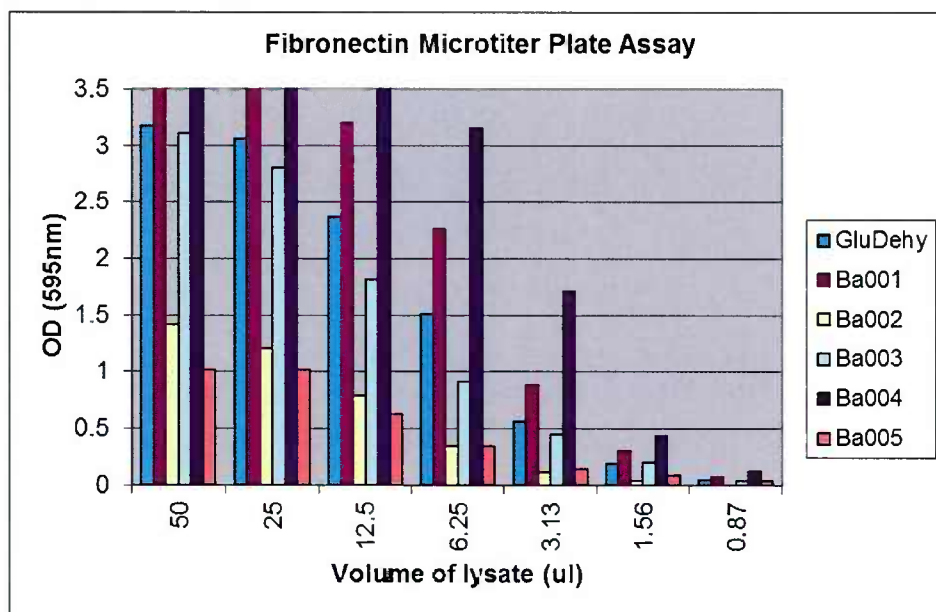


Figure 3. Results of Microtiter plate assay using plates coated with Fibronectin. For dilutions 50, 25 and 12.5 the BaREL 001 and 004 reacted so strongly that it was out of range of detection as indicated by the graph.

VPI was concerned that the discrepancy was due to the fact that each REL contains a different concentration of protein. Therefore, a Bradford assay was performed to determine if the initial starting material was in the same range. The RELs did contain comparable amounts of protein. However, since many proteins may be expressed in a REL at different levels, there is no way to determine the level at which an ECM-interacting protein is present in that particular REL.

In summary the goal of this task was to develop a rapid microtiter plate assay that would initially determine if a REL contains proteins that interact with the specific host ECM, components, thus eliminating unnecessary LC-MS/MS analyses. This did not turn out to be the case since no correlation could be made with the microtiter plate assay data and that of the LC-MS/MS. This may have been due to the RELs having too background protein material. However the most plausible explanation is that the results of one system (LC-MS/MS) do not translate to another experimental level (microtiter plate assay). Thus this task was abandoned. The fact that this proposed task was not successful did not take away from the rapid nature of RVFDS.

Task 2. Expand the pull-down system to include a complete array of bait molecules that will enable the generation of a thorough inventory of potential virulence factors that interact host cell components. Additional RELs of MRSA were generated with various expression times and an increase in the size of the DNA fragments from 1.5 to 3.0kb to decrease redundancy. The proteins found in the RELs were these were captured in pull-down assays with laminin, plasminogen, collagen IV, fibronectin, and vitronectin as bait molecules. In the Appendix portion of this report, Table 1 contains a list of the REL proteins along with the ECM components that they interacted with. Table 2 is a spreadsheet of REL proteins that were captured by the various ECM/bait molecules. The following MRSA proteins were identified in

these pull-down assays:

30S Ribosomal S17: This protein interacted with the anti-MRSA antibodies as a bait molecule. It is one of the primary rRNA binding proteins binding specifically to the 5'-end of 16S ribosomal RNA. This protein has not been cited as being a virulence factor.

50S ribosomal Protein L27: This protein interacted with the anti-MRSA antibodies. The L27 protein stimulates overall peptidyl transferase activity and optimizes cell growth. This protein has not been implicated as a virulence factor.

Acetyl-CoA C-acetyltransferase (3-ketoacyl-CoA acetyltransferase): This protein interacted with the anti MRSA antibodies, HeLa membrane components, HeLa whole cell extract, and also vitronectin. It is a thiolase enzyme that catalyzes a reaction that produces CoA and acetoacetyl-CoA. In *P. gingivalis* the VimA protein has been identified as an acetyl CoA acetyltransferase, and it modulates lipid A and associated protein (Aruni 2012). It is also involved in protein sorting and transport. *VimA*-deletion mutants had decreased invasiveness of HeLa cells when compared to their parental strain, and it has been shown to regulate invasiveness capacity. It also plays a role in autoaggregation and biofilm formation and mediates host cytoskeleton during invasion. In summary it plays a vital role in virulence of this pathogens as well as others.

Acetyl-CoA carboxylase (Carbamoyl-phosphate synthase L chain, N-terminal domain protein (Acetyl-CoA-Carboxyl transferase, beta subunit): This protein interacted with HeLa cell membrane components, HeLa whole cells extract, and fibronectin. It catalyzes the ATP-dependent synthesis of carbamoyl phosphate from glutamine or ammonia and bicarbonate. This enzyme catalyzes the reaction of ATP and bicarbonate to produce carbonyl phosphate and ADP. Carbonyl phosphate reacts with ammonia to yield carbamate. In turn, carbamate reacts with a second ATP to form carbamoyl phosphate and ADP. It catalyzes the initial step in fatty acid synthesis and as such it is essential for the development and maintenance of cellular membranes. Because of the role in maintenance of cell membranes it is thought to be a prime target for antimicrobials and vaccines.

Acetyltransferase GNAT family: This protein interacted with fibronectin and plasminogen. GNAT acetyltransferases is an enormous, superfamily of enzymes universally distributed in nature. They use acetyl-CoA to acetylate their cognate substrates. Two different members of this family were identified in the pull-down assays: gi|320143649, which reacted with plasminogen, and gi|320143065 that interacted with fibronectin. The exact substrates of these proteins have not been characterized. A putative acetyltransferase of *M. abscessus* has been reported to enhance adherence, intracellular survival and antimicrobial resistance (Tsae, 2013).

Addiction module toxin, Txe/YoeB family: This protein was captured by fibronectin. Txe is part of an addiction module that consists of this toxin and a small labile antitoxin that inactivates it. The inactivation prevents Txe from inhibiting initiation of translation. This is accomplished by partial cleavage of mRNA at 3 bases downstream from the

initiation codon regardless of the mRNA sequence (Yoshizumi, 2009). The function of the module has been speculated as being involved in retention of plasmids, many of which harbor antibiotic-resistance genes. Artificial activation of the Txe toxin could lead to an effective antimicrobial agent.

Alanine racemase: This protein was captured by HeLa membrane and HeLa whole cell components in pull-down assays. It belongs to the isomerase family. It plays an essential role in cell wall synthesis as it racemizes L-alanine into D-alanine, a key building block in the biosynthesis of peptidoglycan. In an effort to discover new drugs to treat tuberculosis, Anthony, 2011 chose alanine racemase as the target of a drug discovery effort in *M. tuberculosis*. Good antimicrobial effects have been achieved by inhibition of this enzyme with suicide substrates. Lee (2013) found that certain members of the thiadiazolidinone family, that were inhibitors of alanine racemase, suppressed the growth of this pathogen.

Alkylhydroperoxide reductase subunit C: This protein was captured by HeLa membrane and HeLa whole cell components. It degrades organic peroxides and thus protects bacterial in the host's intercellular environment. As such it contributes to virulence in *Salmonella* and other bacterial pathogens (Hebrard, 2009).

Acetyl-CoA ligase (Acetyl-CoA synthetase): This protein interacted with the HeLa membrane components. It is an enzyme that plays a role in cellular energy homeostasis. This enzyme was found to prevail in *S. epidermitis* strains with characteristically high invasive properties (Yao, 2005). In *P. aeruginosa* redundant species of this enzyme contribute to differential fatty acid degradation and virulence. Mutants deficient for *fad* genes had decreased production of lipases, proteases, rhamnolipid, and phospholipase when compared to their wild-type counterpart. They also were unable to utilize fatty acids and phosphatidylcholine (major components of the host lung surfactant as their sole carbon source (Kang, 2010). Mutants of *C. neoformans* deficient in this enzyme virulence factor expression and virulence in general (Griffiths, 2012).

BglG family transcriptional antiterminator (Putative PTS multi-domain regulator): This protein interacted with collagen IV as the bait molecule. In *E. coli* it inhibits transcription termination within the *bgl* operon in the presence of beta-glucosides. BglG represents a family of transcriptional antiterminators that bind to RNA sequences, which partially overlap rho-independent terminators, and prevent termination by stabilizing an alternative structure of the transcript. No direct participation in pathogenesis has been reported for this protein.

Bone fibrinogen/sialoprotein-binding protein (Bbp, Serine-aspartate repeat protein E, partial): This membrane protein interacted with the ECM component fibronectin. It has been identified as a possible vaccine candidate since it has been shown to be immunogenic against clinical strains of MRSA in the murine abscess model (Prachi, 2013). Antibodies against Bbp have been shown to be indicative of infectious osteomyelites or septic arthritis. Indeed this protein appears to be found in strains of MRSA that cause infectious osteomyelitis suggesting that it may play a role in bone

pathologies in these strains. Bbp has not been found in MRSA strains isolated from patients with endocarditis (Persson, 2009). Bbp has also been reported to bind to other ECM components such as fibrinogen as well as fibronectin by other authors. In fact the binding site for Bbp was mapped to residues 561-575 in the fibrinogen A alpha chain (Vazquez, 2001)

Cysteine synthase protein: This protein interacted with the anti-MRSA antibodies in pull-down assays. It is an enzyme that catalyzes a reaction that produces L-cysteine and acetate. It is thought to serve as a link between metabolism and stress resistance that is a major factor in the ability of *S. aureus* to inhabit so many different environmental niches and thus be a very successful pathogen (Lithgow, 2004). Thus it may offer a prime candidate for the development of novel interventive strategies.

Cytosol aminopeptidase, PepB: This protein reacted with plasminogen. It has not been reported to play a role in microbial virulence.

DNA-binding response regulator (SaeR protein): This cytoplasmic protein interacted with fibronectin. It is part of the SaeR/S component system that is the major control for the expression of many of the virulence factors of MRSA. Upon phosphorylation by SaeS the SaeR protein binds to the P1 promoter that contains the direct repeat sequence GTTAAN₆GTAA (Sun, 2010). The SaeS is a membrane protein that serves as an intermediate sensor to the host environment of MRSA. The SaeR/S system has also been reported to be essential for innate immune evasion by MRSA (Voyich, 2008).

DNA internalization-related competence proteins ComEC/Rec2: This fibronectin-binding putative membrane protein is involved with DNA uptake. It has also been reported as responding to cold stress in *S. aureus*. It may play a role in survival of this pathogen.

ECM-binding protein homologue (EbhB protein): This protein was highly promiscuous in that it reacted with fibronectin, laminin, and plasminogen. EbhB, which has homology to other ECM-binding proteins, has been identified as the largest protein found in MRSA. This 1.1-megadalton cell wall associated protein has also been demonstrated to bind to fibronectin and associated with adhesion to host cells (Clarke, 2002). This multifunctional cell surface protein mediates attachment to host extracellular matrix, biofilm accumulation, and escape from phagocytosis (Christner, 2009). It has also been reported to be involved in tolerance of MRSA to transient hyperosmotic pressure due to repeated dehydration and rehydration occurs on human skin. *EbhB*-deletion mutants cannot survive under such hypervariable water conditions. It has been suggested that EbhB stabilizes the integrity of the MRSA structure by forming bridges between the cell wall and cytoplasmic membrane (Kuroda, 2008).

Glutamate dehydrogenase (RocG): This enzyme has the metabolic function of deaminating glutamate to form α -ketoglutarate. Since the discovery that this protein binds to fibronectin, laminin, and plasminogen, we have been unable to find any reports of this nature or implications of a moonlighting function as a determinant of infectious

disease. It has been reported to be immunogenic (Yang, 2010). GDH is important to glutamate metabolism, which may be a crucial determinant of *M. tuberculosis* survival and growth within infected cells. *M. tuberculosis* GDH is structurally and functionally different from the GDH found in humans, which may make these enzymes potential specific targets for anti-tuberculosis drug development (Viljoen, 2013).

Histidine kinase KdpD (ATPase/histidine kinase/DNA gyrase B/HSP90, Osmosensitive K channel His kinase sensor, PhoB protein): This protein was captured by vitronectin. It is a multifunctional, transmembrane protein of the transferase class that plays a role in signal transduction across the cellular membrane. The kinase domain is responsible for the autophosphorylation of the histidine with ATP, the phosphotransfer from the kinase to an aspartate of the response regulator, and (with bifunctional enzymes) the phosphotransfer from aspartyl phosphate back to ADP or to water. It is significant that four parts of this multi functional protein all were captured by vitronectin.

This protein is part of the two-component system KdpD/KdpE which has a role in potassium transport and recently been identified as a regulator of virulence and intracellular survival of pathogenic bacteria such as *S. aureus*, entero-hemorrhagic *E. coli*, *S. typhimurium*, *Y. pestis*, and *Francisella* species. Such systems are critical for the survival under the stressful conditions a pathogen encounters during invasion of the host, which include phagocytosis, exposure to microbicides, quorum sensing signals, and host hormones (Freeman, 2013). Since there is no corresponding system in humans this protein offers an ideal target for antibiotics, vaccines, and antimicrobials.

Histidine-tRNA ligase: This protein combined with HeLa membrane components. Histidyl-tRNA synthetase (HisRS) is responsible for the synthesis of histidyl-transfer RNA, which is essential for the incorporation of histidine into proteins. This amino acid has uniquely moderate basic properties and is an important group in many catalytic functions of enzymes. This protein has not been reported to be involved in virulence.

Holo acyl carrier protein synthase (Acp): This protein interacted with collagen IV. This enzyme belongs to the family of transferases, specifically those transferring non-standard substituted phosphate groups. This protein has been implicated in the quorum sensing mechanism of certain bacteria and as such may be a target of antimicrobial strategies.

Hypothetical protein SAV1112: This protein reacted with HeLa whole cell components. This protein was similar to NADH-dependent epimerase in *B. megaterium*. It has not been reported to be involved in virulence.

Hypothetical protein SAUSA300_1771: This protein, which was captured by HeLa cell membrane components and did not have similarity to any known proteins in the protein databases.

Hypothetical protein GGSSa03_11236: This putative membrane protein interacted with vitronectin. It did not have any similarity to known proteins in the protein databases.

Hypothetical protein HMPREF9529_02267: This protein interacted with vitronectin. It did not have identity with known protein in protein databases.

Hypothetical protein HMPREF9529_00693: This protein interacted with HeLa whole cell fraction. It did not have identity with any protein in protein databases.

Hypothetical protein (HMPREF9529_02597): This putative membrane protein interacted with plasminogen and consists of 81 amino acid residues. No virulence involvement was found for this protein.

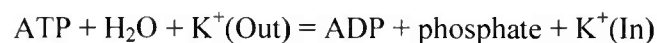
Hypothetical protein (HMPREF9529_00622): This protein was captured by fibronectin and laminin and was composed of 42 amino acid residues. No involvement in virulence was reported for this protein.

Hypothetical protein (HMPREF9529_01618): This protein interacted with fibrinogen and consisted of 148 amino acid residues. Extensive bioinformatic searches could not identify the presence of any putative functional domains in this protein nor involvement in virulence.

Immunoglobulin G-binding protein partial (Protein A): This protein interacted with fibronectin as the bait molecule. It is a 56 kDa surface protein composed of five homologous Ig-binding domains that fold into a three-helix bundle. Each domain is able to bind proteins from many mammalian species, most notably IgGs. It binds the heavy chain within the Fc region of most immunoglobulins and also within the Fab region in the case of the human VH3 family. Through these interactions in serum, where IgG molecules are bound in the wrong orientation (in relation to normal antibody function), the bacteria disrupt opsonization and phagocytosis. Protein A helps inhibit phagocytic engulfment and acts as an immunological disguise. Mutants of *S. aureus* lacking this protein are more efficiently phagocytized *in vitro* and in animal infection models have diminished virulence. Higher levels of Protein A in different strains of *S. aureus* have been associated with nasal carriage of these bacteria.

Isochorismatase synthase: This protein interacted with vitronectin as the bait molecule. No reports of virulence involvement have been reported for this enzyme.

K⁺ transporting ATPase B subunit (*kdpB* gene product): This high affinity importers of potassium protein interacted with the HeLa membrane and HeLa whole cells components. This integral membrane protein catalyzes the reaction:



As such it is one of three importers of potassium in MRSA. In *Salmonella* impairment of potassium transport has been shown to be critical for the pathogenesis in that it interferes with the type three secretion system of the SPL-1 pathogenicity island which consequently impacted upon protein secretion, motility, and virulence characteristic *in vitro* (Lui, 2013).

Membrane domain of membrane-anchored glycerophosphoryl diester

phosphodiesterase: This protein interacted with fibronectin as the bait molecule. It belongs to the family of hydrolases that act on phosphoric diester bonds. The systematic name of this enzyme class is glycerophosphodiester glycerophosphohydrolase, gene *hpd* protein, glycerophosphoryl diester phosphodiesterase, and IgD-binding protein D. This enzyme participates in glycerophospholipid metabolism. In strains of *H. influenzae* this protein is a highly conserved 42 kDa surface lipoprotein. It is involved in the pathogenesis of respiratory tract infections in that it has been shown to impair ciliary function in a human nasopharyngeal tissue culture model and to augment the capacity to cause otitis media in rats. A likely mechanism indicating that it is a virulence factor is its glycerophosphodiesterase activity, which leads to the release of phosphorylcholine from host epithelial cells. PD has been demonstrated to be a promising vaccine candidate against experimental non-typable *H. influenzae* infection (Forsgren, 2008).

MHC class II analog/extracellular adherence protein (77 kDa protein, MAP-W2

domain protein, Eap protein): This protein interacted with the anti-MRSA antibodies and has been shown to resemble mammalian MHC class II proteins in sequence similarities and peptide binding capacity (Jonsson, 1995)s. This 60-70 kDa protein is secreted and then a portion of it adheres to the *S. aureus* surface. It has been reported to be involved in immune avoidance of MRSA (Peacock, 2002), It has also been implicated in biofilm formation in *S. aureus* (Sabiroya, 2013) and has also been documented to be an anti-chemotactic factor in that it blocks the adhesion of leukocytes to the mammalian endothelium. This anti-adhesive property is also anti-inflammatory and thus results in delayed healing at the site of infection (Chavakis, 2002).

Nitroreductase: This protein combined with HeLa whole cell components.

Nitroreductases are a family of evolutionarily related proteins involved in the reduction of nitrogen-containing compounds, including those containing the nitro functional group. In *M. tuberculosis* the *acg*-gene product has been identified as a nitroreductase. This enzyme has been implicated in the survival of this pathogen inside the hypoxic environment of the host macrophage (Purkayasatha, 2002).

Pathogenicity island protein identified as possibly DNA helicase of the superantigen

encoding pathogenicity island SaPI: This protein interacted with the anti-MRSA antibodies. Pathogenicity islands (PAIs) are incorporated in the genome chromosomally or extrachromosomally, of pathogenic organisms, but are usually absent from those nonpathogenic organisms of the same or closely related species. PAIs contribute to microorganism's ability to evolve in the pathogenic relationships. This enzyme most likely has a household function in perpetuating the SaPI but is not directly involved in virulence.

Periplasmic-binding protein, SirA: This transporter protein, which was shown to bind to plasminogen, participates in the capture of iron liberated from transferrin by the siderophore staphyloferrin B. Thus this protein allows *S. aureus* to access the host iron pool and proliferate. Animal studies have shown that this protein, in combination with Hts and Sst transport systems, is needed for full virulence (Beastly, 2011).

Peroxiredoxin, partial (Alkyl hydroperoxide reductase, AhpC protein): This protein interacted with HeLa whole cell components. It is a ubiquitous family of antioxidant enzymes that also control peroxide levels. AhpC activity plays a role in virulence of *M. tuberculosis* in that it is involved in replicative capability and macrophage apoptosis (Rintiswati, 2011) and also Ahp deletion mutants of *E. faecalis* had attenuated virulence in the mouse peritonitis model (La Carbona, 2007).

Phage-related protein: This protein was captured by vitronectin in pull-down assays. After numerous BLASTs the exact identity of this protein could not be determined. Therefore at this point no predictions on its role in virulence can be made.

Phage component proteins: Three proteins that were identified as part of temperate phage components were found. This is not unusual in *S. aureus* for such phage-borne components not only contribute to replication and structural element of the phage but also can sometimes introduce virulence factors (Garcia, 2009).

Two bacteriophage structural proteins and one non-structural protein were identified:

1. **Phage tail tape measure TP901 protein** reacted with plasminogen.
2. **Phage putative tail component protein** interacted with fibronectin.
3. **Virulence-associated protein E, VirE:** gi|320142766. This non structural protein interacted with fibronectin and laminin. VirE is a temperate bacteriophage associated-associated protein and may have a particular role in DNA replication. Although not documented, it may be involved in virulence (Garcia, 2009).

Phosphoenolpyruvate carboxykinase (PEPCK, PckA-gen product): This protein interacted with the anti-MRSA antibodies. (PEPCK) is an enzyme in the lyase family used in the metabolic pathway of gluconeogenesis. It converts oxaloacetate into phosphoenolpyruvate and carbon dioxide. It has been shown to be important for maximum virulence of *A. tumefaciens* (Lui, 2005). PckA-deletion mutants of *M. bovis* have been reported to be attenuated in microphage and mouse models (Lui, 2003). It has also been demonstrated that PEPCK can effectively induce cell-mediated immune response by increasing activity of cytokines and PEPCK may be a promising new subunit vaccine candidate for tuberculosis (2006).

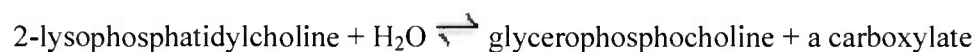
Phosphoenolpyruvate-dependent sugar PTS family porter (PRD domain protein): This integral membrane protein interacted with collagen IV as the bait molecule. It is involved in catalysis of the PEP-dependent, phosphoryl transfer-driven transport of substances across a membrane. The transport happens by catalysis of the reaction: protein

N-phosphohistidine + sugar(out) = protein histidine + sugar phosphate(in). In *S. mutans* the mannose PTS system is a major carbohydrate transport and is involved in carbon catabolite repression and regulates the expression of known virulence genes (Abranches, 2006).

Preprotein translocase, SecA subunit: This protein reacted with plasminogen and is involved in protein export. It interacts with the secY/secE subunits, and it plays a central role in coupling the hydrolysis of ATP for the transfer of pre-secretory periplasmic and outer membrane proteins across the membrane. It is part of the prokaryotic protein translocation apparatus that comprise SecA, SecB, SecD, SecE, SecF, SecG and SecY. It has been shown to be immunogenic in *B. pertussis* and is most likely indirectly involved in virulence.

Putative linear gramicidin synthetase LcrC protein: This protein bonded with laminin. LcrC is part of a tetrameric protein complex (LcrA, B, C, and D) that is classified as a non-ribosomal peptide synthetase. It has also been implicated in surfactin production by microbes. The product of the multimodular synthetase is to form a linear pentadecapeptid gramicidin that consists of 15 hydrophobic amino acids with alternating l- and d-configuration forming a beta-helix-like structure, which interacts with the lipid bilayer of cells.

Putative lysophospholipase: This protein interacted with the anti-MRSA antibodies as a bait molecule. This enzyme is a member of the Alpha/Beta hydrolase fold protein family. The alpha/beta-hydrolase fold family of enzymes is rapidly becoming one of the largest groups of structurally related enzymes with diverse catalytic functions. Members in this family include acetylcholinesterase, dienelactone hydrolase, lipase, thioesterase, serine carboxypeptidase, proline iminopeptidase, proline oligopeptidase, haloalkane dehalogenase, haloperoxidase, epoxide hydrolase, hydroxynitrile lyase and others. Lysophospholipase catalyzes the chemical reaction:



This enzyme has been reported to be involved in the pathogenicity of *C. albicans* (Chen, 2000).

Ribosomal small subunit-dependent GTPase A (RsgA, also known as YjeQ): The RsgA protein has no known function. It is a key factor in late ribosomal biosynthesis and deletions of the gene that encodes this protein impairs maturation of the 30S subunit. RsgA interacted with plasminogen. In deletion studies it has also been found to be required for *S. aureus* virulence in a mouse kidney abscess model (Campbell, 2006). Because it is needed for *in vivo* survival, it may represent an important target for anti-MRSA therapies.

RNA polymerase sigma factor D, RpoD: This protein was found to bind to plasminogen. It is responsible for the majority of mRNA produced in exponentially growing cells. Expression of RpoD has been linked to biofilm formation in *Helicobacter*

pylori (Cellini, 2005). Other members of the Sigma 70 family have been reported to be an essential component of the cell-surface signaling (CSS) system of *P. aeruginosa*.

SasB (truncated FmtB and Mrp protein): It reacted with fibronectin, laminin, and plasminogen. This protein has been indirectly associated with methicillin resistance. After an exhaustive literature search its exact function has not been determined. Further investigations may elucidate the role this protein plays in pathogenicity.

Serine protease HtrA-like protein (Trypsin like serine protease): This protein interacted with fibronectin. This surface protein is involved in virulence of many pathogens mainly because of its role in stress response and bacterial survival. It has been proposed that this protein contributes to *S. aureus* pathogenicity by controlling the production of certain extracellular factors needed for bacterial dispersion. It acts in the agr-dependent regulation pathway carrying out proper folding and maturation of some surface component involved in host tissue invasion (Rigoulay, 2005).

Serine O-acetyltransferase: Anti-MRSA antibodies captured this protein. It is an enzyme that catalyzes a reaction that produces CoA and O-acetyl-L-serine. No virulence role has been reported for this enzyme.

Superantigen-like protein (SSL): This protein interacted with fibronectin as the bait molecule. SSL binds to human IgG and displays striking specificity for the Fc domain of the $\gamma 1$ subclass. This virulence factor shares a common architecture with bacterial superantigens that bind to major histocompatibility complex class II and T-cell receptor to stimulate large numbers of T cells. The majority is produced by the Gram-positive organisms *S. aureus* and *S. pyogenes*, which are the causative agents in toxic shock syndrome, an acute disease caused by the sudden and massive release of T-cell cytokines into the blood stream. The superantigens and SSL molecules demonstrate how a pathogen has employed a simple but adaptable protein to generate potent defense molecules designed to target of the innate and adaptive immune response (Fraser, 2008).

Undecaprenyldipospho-muramoylpentapeptide: This protein interacted with the anti-MRSA antibody as a bait molecule. This protein is an enzyme that catalyzes a reaction that produces UDP and diphosphoundecaprenol. No direct role in virulence has been demonstrated for this protein.

VRR-NUC domain protein: This protein interacted with vitronectin. It is associated with members of the PD-(D/E)XK nuclease superfamily, which include the type III restriction modification enzymes. It is a nuclease with hydrolase activity on ester bonds. No participation in pathogenesis has been reported for this protein.

Zinc metalloprotease aureolysin: In *S. aureus* this protein cleaves complement C3 in order to mediate immune evasion (Laarman, 2011). It has also been shown to effectively inhibit phagocytosis and killing of bacterial by neutrophils. This secreted virulence factor has also been demonstrated to activate plasminogen thereby promoting bacterial dissemination and invasion (Beaufort, 2008). It has also been shown to contribute to

resistance to antimicrobial peptides, inhibition of Ig production by lymphocytes. Recent studies suggested that aureolysin can be expressed within the phagocytic vacuole after phagocytosis of *S. aureus*. Furthermore it has been reported that isogenic aureolysin mutant was more efficiently killed by macrophages upon phagocytosis.

Task 3. Characterize the pathogen immunome. The pull-down system will be extended to include the use of polyclonal antibodies from convalescent patient's sera as bait to identify a pathogen's immune-reactive components. The following proteins reacted with anti-MRSA antibodies: putative lysophospholipase; pathogenicity island protein; 30S ribosomal protein S17; 50S ribosomal protein L27; thermolysin metalloproteinase; serine O-acetyltransferase; alpha/beta hydrolase fold protein; and phosphoenolpyruvate carboxykinase. A description of the involvement of these immunogenic proteins in pathogenesis can be found in the above compilation of ECM-captured proteins.

Task 4. Identify the host target/receptor proteins of putative virulence factors of MRSA. Pull-down assays, which use selected cloned and expressed potential virulence factors as bait, will be employed to determine the specific host components that interact with a pathogen's offensive mechanisms. Acetyl-CoA carboxylase, acetyl-CoA ligase, and K⁺ transporting ATPase B subunit were chosen midway during the Phase 2 portion of the project to be used as bait molecules to capture HeLa whole cell and HeLa cell membrane (host) components. Upon reflection other putative virulence factors may have served as more appropriate bait however at the time the aforementioned proteins were found to have merit as bait molecules.

Acetyl-CoA carboxylase (Carbamoyl-phosphate synthase L chain, N-terminal domain protein (Acetyl-CoA-Carboxyl transferase, beta subunit): This protein interacted with HeLa cell membrane components, HeLa whole cells extract, and fibronectin. It was chosen because of the number of ECM components it interacted with. It catalyzes the ATP-dependent synthesis of carbamoyl phosphate from glutamine or ammonia and bicarbonate. This enzyme catalyzes the reaction of ATP and bicarbonate to produce carbonyl phosphate and ADP. Carbonyl phosphate reacts with ammonia to yield carbamate. In turn, carbamate reacts with a second ATP to form carbamoyl phosphate and ADP. It catalyzes the initial step in fatty acid synthesis and as such it is essential for the development and maintenance of cellular membranes. Because of its role in maintenance of cell membranes it is thought to be a prime target for antimicrobials and vaccines. The maintenance of the cell membrane is critical for it represents a vital line of defense against environmental factors, host immune systems and antibiotic agents. For this reason it offers a prime target for the development of antimicrobial agents. This cloned and expressed bait molecule captured the following HeLa components:

Isoform CRA-a, GRB2-associated-binding protein2, VAMP 4 (Vesicle Associated Membrane Protein 4), VAMP isoform CRB1: This protein was captured from the HeLa membrane fraction. It is an adapter protein which acts downstream of several membrane receptors including cytokine, antigen, hormone, cell matrix and growth factor receptors to regulate multiple host signaling pathways. The vesicle associated membrane proteins (VAMP) or synaptobrevins are calcium-binding proteins specific to eukaryotes. VAMPs, along with synaptosomal associated protein of 25kD (SNAP 25) and syntaxin,

form the core complex of soluble NSF attachment protein receptor (SNARE) proteins that interact with the soluble proteins N-ethylmaleimide-sensitive factor (NSF) and alpha-SNAP. These membrane associated proteins play a key role in the regulation of vesicle membrane fusion with the plasma membrane. The *Clostridium tetani* neurotoxin is a metalloprotease with specificity for VAMP. This protein is key in the zipper model of internalization of *Staphylococcus* and other pathogen. These bacteria express proteins that interact with host cellular receptors initiating signaling cascades that result in close apposition of the cellular membrane around the entering bacterium.

26 S proteasome non-ATPase regulatory subunit 14: This protein was captured from HeLa cell membrane and is a metalloprotease component of the 26S proteasome that specifically cleaves 'Lys-63'-linked polyubiquitin chains. The 26S proteasome is involved in the ATP-dependent degradation of ubiquitinated proteins. This complex was found to be similar to ClpQ/hslV threonine peptidase of *Escherichia coli*. This complex was found to be up-regulated in a microarray analysis of the murine macrophage response to infection with *Francisella tularensis* LVS (Andersson, 2006).

Intraflagellar transport protein 140 homolog (Tetratricopeptide repeats protein 2): This protein was part of the HeLa membrane fraction and is involved in the genesis, resorption and signaling of primary cilia. The primary cilium is a microtubule-based sensory organelle at the surface of most quiescent mammalian cells. It receives signals from its environment, such as the flow of fluid, light or odors, and transduces those signals to the nucleus. Thus it may play a role in the host/pathogen relationship and thus warrants further investigation.

Monocyte chemotactic protein-1 (MCP-1, small inducible cytokine A2, chemokine ligand): This protein was captured from the HeLa membrane fraction. MCP-1 is a small (23 amino acid residues) cytokine that recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection. MCP-1/chemokine (C-C motif) ligand 2 is important for neutrophil-mediated host defense. Reduced bacterial clearance in the lungs was observed in MCP-1^{-/-} mice following *Escherichia coli* infection. Neutrophil influx, along with cytokines/chemokines, leukotriene B4 (LTB4), and vascular cell adhesion molecule 1 levels in the lungs, was reduced in MCP-1^{-/-} mice after infection. *E. coli*-induced activation of NF-κB and mitogen-activated protein kinases in the lung was also reduced in MCP-1^{-/-} mice. Administration of intratracheal recombinant MCP-1 (rMCP-1) to MCP-1^{-/-} mice induced pulmonary neutrophil influx and cytokine/chemokine responses in the presence or absence of *E. coli* infection. *In vitro* migration experiment demonstrates MCP-1-mediated neutrophil chemotaxis. Notably, chemokine receptor 2 is expressed on lung and blood neutrophils, which are increased upon *E. coli* infection. Furthermore, neutrophil depletion impairs *E. coli* clearance and that exogenous rMCP-1 after infection improves bacterial clearance in the lungs. Thus *E. coli*-induced MCP-1 causes neutrophil recruitment directly *via* chemotaxis as well as indirectly *via* modulation of keratinocyte cell-derived chemokine, macrophage inflammatory protein 2, and LTB4 (Balamayooran, 2011). Other studies have unequivocally demonstrated the important role of MCP-1 in monocyte/macrophage-mediated host defense against bacterial infection. During

Streptococcus pneumoniae infection, overexpression of MCP-1 was shown to improve bacterial clearance. Using MCP-1^{-/-} mice it was demonstrated that MCP-1-mediated macrophage recruitment is important to prevent bacterial dissemination following pulmonary *S. pneumoniae* infection. During *Pseudomonas aeruginosa* infection, MCP-1 promoted resolution and repair of the lung by enhancing the uptake of apoptotic neutrophils by alveolar macrophages. In addition, mice deficient in CCR2 showed impairment in macrophage migration and clearance of bacteria from the lungs and extrapulmonary organs after intravenous challenge with *Listeria monocytogenes*. MCP-1 is also shown to be important for the survival of mice during *P. aeruginosa* and *Salmonella enterica* serotype Typhimurium infections, and MCP-1 promotes bacterial killing by macrophages. It should be noted that NF-kappaB was found to interact with OMP W of *B. abortus* in this project.

T cell receptor V-alpha: This 21-amino acid residue peptide was found in the HeLa membrane fraction. Umesaki, 1993 found an increase in alpha-T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice. It most likely is involved in defense against bacterial infection.

Acetyl-CoA ligase (Acetyl-CoA synthetase): This protein interacted with the HeLa membrane components. It is an enzyme that plays a role in cellular energy homeostasis. This enzyme was found to prevail in *S. epidermitis* strains with characteristically high invasive properties (Yao, 2005). In *P. aeruginosa* redundant species of this enzyme contribute to differential fatty acid degradation and virulence. Mutants deficient for *fad* genes had decreased production of lipases, proteases, rhamnolipid, and phospholipase when compared to their wild-type counterpart. They also were unable to utilize fatty acids and phosphatidylcholine a major components of the host lung surfactant as their sole carbon source (Kang, 2010). These defects translated into decreased *in vivo* fitness in the mouse lung infection model. Mutants of *C. neoformans* deficient in this enzyme virulence were found to have decreased virulence (Griffiths, 2012). Because of the reported involvement in virulence it was decided to clone, express, and use this protein as bait in pull-down assays to identify host components that may play a part in the pathogenic process. The following HeLa components were captured by Acetyl-CoA ligase:

Mitochondrial ATP synthase (H⁺ transporting F1 complex beta subunit): This enzyme was captured as part of the HeLa cell membrane and produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. Although this enzyme has not been reported in the host/pathogen relationship, it may be a target of the acetyl-CoA ligase resulting in upset of ATP levels in the host cell. This warrants further investigation.

S-adenosylmethionine synthase isoform type 2: This enzyme catalyzes the formation of S-adenosylmethionine by joining methionine and ATP. Although this enzyme plays a role in pathogenic bacteria quorum sensing no involvement of the host enzyme in pathogenesis was found in the scientific literature.

Beta-cop homolog: This protein was captured from the HeLa cell membrane fraction. The coatomer is a cytosolic protein complex that binds to dilysine motifs and reversibly

associates with Golgi non-clathrin-coated vesicles, which further mediate biosynthetic protein transport from the ER, via the Golgi up to the trans Golgi network. Coatamer complex is required for budding from Golgi membranes, and is essential for the retrograde Golgi-to-ER transport of dilysine-tagged proteins. In mammals, the coatamer can only be recruited by membranes associated to ADP-ribosylation factors (ARFs), which are small GTP-binding proteins; the complex also influences the Golgi structural integrity, as well as the processing, activity, and endocytic recycling of LDL receptors. It plays a functional role in facilitating the transport of kappa-type opioid receptor mRNAs into axons and enhances translation of these proteins. It is also required for limiting lipid storage in lipid droplets. Involved in lipid homeostasis by regulating the presence of perilipin family members PLIN2 and PLIN3 at the lipid droplet surface and promoting the association of adipocyte surface triglyceride lipase (PNPLA2) with the lipid droplet to mediate lipolysis by similarity. It is involved in the Golgi disassembly and reassembly processes during cell cycle. Involved in autophagy by playing a role in early endosome function. It plays a role in organellar compartmentalization of secretory compartments including endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC), Golgi, trans-Golgi network (TGN) and recycling endosomes, and in biosynthetic transport of CAV1. Promotes degradation of Nef cellular targets CD4 and MHC class I antigens by facilitating their trafficking to degradative compartments. This protein is part of an oligomeric complex that consists of at least the alpha, beta, beta', gamma, delta, epsilon and zeta subunits that interacts with anthrax lethal factor (LF); this interaction may facilitate endosomal vesicle membrane translocation of LF and its release from the lumen of endosomal vesicles to external milieu. There is emerging evidence that pathogens directly organize higher-order signaling networks through enzyme scaffolding, and the identity of the effectors and their mechanisms of action are poorly understood. Thus the interaction of this protein with a potential MRSA virulence factor could be of significant importance.

Clustered mitochondrial protein homolog 6: An exhaustive literature search revealed little information on this protein or its involvement in the host/pathogen relationship.

Kinesin-like protein: This protein is involved in the formation of the mitotic spindle and was captured from the HeLa membrane fraction. No reports of involvement of this protein in the host/pathogen relationship have been reported in the literature.

Titin (Connectin) is the largest known protein that functions as a molecular spring, which is responsible for the passive elasticity of muscle. It was captured from HeLa membranes. It is composed of 244 individually folded protein domains connected by unstructured peptide sequences. These domains unfold when the protein is stretched and refold when the tension is removed. It is also a structural protein in chromosomes. Bacterial adhesion to target host cells is enhanced by shear forces (Thomas, 2002). Thus there may be some involvement of this protein in the host/pathogen relationship.

Laminin alpha 1 protein (LAMI1protein) in humans is encoded by the *LAMA1* gene. This protein was captured from HeLa whole cell extracts. The involvement of laminin has been well documented in the scientific literature and addressed in this project

description. Thus this warrants further investigation.

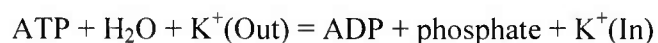
Apoptosis signal-regulating kinase 2 (ASK2, Mitogen-activated protein kinase kinase kinase, MAP3K) was captured from HeLa whole cell extracts and is a highly related serine/threonine kinase to ASK1 and also functions as a MAP3K only in a heteromeric complex with ASK1. It has been reported to play multiple important roles in cytokine and stress responses. MAP kinases also known as extracellular signal-regulated kinases (ERKs). The study of how bacterial pathogens manipulate complex networks like the MAPK and nuclear factor κ B (NF- κ B) kinase pathways could illuminate otherwise inscrutable but important mechanisms of regulation and crosstalk in these pathways and also lead to novel antimicrobial agents. It should be noted that the *Brucella* OMP W interacted with HeLa NF- κ B. Pathogen associated molecular patterns (PAMPs) are detected by eukaryotic pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), and mannose receptors, or the family of cytoplasmic nucleotide-binding oligomerization domain containing protein (NOD)-like receptors. When bound to their PAMP ligand, PRRs induce signaling cascades, most commonly NF- κ B and MAPK pathways, which in turn trigger pro-inflammatory responses such as the up-regulation of cytokines and antimicrobial peptides or activation of the complement cascade. There are numerous examples of such interactions in the literature (See review by Krachler, 2011).

Collagen type VII alpha: This protein was captured from whole HeLa cell extracts. There have been and continue to be numerous citations of the involvement of collagen in the host/pathogen relationship. Collagen is a prominent constituent of the ECM and binds to and aggregates bacteria. Thus this is a significant interaction. This is the first report of its binding to acetyl CoA-ligase. Upon protein BLAST of this protein numerous receptor motifs and binding sites were represented as conserved domains.

Myosin 9: This protein contains an IQ domain and a myosin head-like domain, which is involved in several important functions, including cytokinesis, cell motility and maintenance of cell shape. Defects in this gene have been associated with non-syndromic sensorineural deafness autosomal dominant type 17, Epstein syndrome, and Alport syndrome with macrothrombocytopenia, Sebastian syndrome, Fechtner syndrome and macrothrombocytopenia with progressive sensorineural deafness.

Hanisch, 2011 reported that activation of a RhoA/myosin II-dependent pathway facilitates *Salmonella* invasion and that myosin accumulates in *Salmonella*-induced stress fiber-like structures during entry into nonphagocytic cells. Inhibition of myosin II reduces and its stimulation enhances *Salmonella* invasion, and modulation of myosin activity affects SopB- but not SopE/E2-dependent invasion. It was also shown to be a functional entry receptor for herpes simplex virus-1 (Ari, 2011).

K⁺ transporting ATPase B subunit (*kdpB* gene product): This high affinity importer of potassium protein interacted with the HeLa membrane and HeLa whole cells components. This integral membrane protein catalyzes the reaction:



As such it is one of three importers of potassium in MRSA. In *Salmonella* impairment of potassium transport has been shown to be critical for the pathogenesis in that it interferes with the type three secretion system of the SPL-1 pathogenicity island which consequently impacted upon protein secretion, motility, and virulence characteristic *in vitro* (Lui, 2013). This protein was cloned and expressed in *E. coli* and used as bait to capture the following HeLa components:

ULK1 (Unc51-like protein, Serine/threonine protein kinase): This protein was captured from the HeLa membrane. It plays a key role in the regulation of autophagy. Autophagy is the basic catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the actions of lysosomes. The breakdown of cellular components can ensure cellular survival during starvation by maintaining cellular energy levels. Autophagy ensures the synthesis, degradation and recycling of cellular components. During this process, targeted cytoplasmic constituents are isolated from the rest of the cell within the autophagosome, which are then fused with lysosomes and degraded or recycled. Autophagy has been recognized as an immune mechanism. It plays a role in the destruction of intracellular pathogens, in a process of degradation of dysfunctional intracellular organelles. Intracellular pathogens such as *M. tuberculosis* can survive within the cells by blocking the maturation of their phagosomes into degradative organelles called phagolysosomes. Stimulation of autophagy in infected cells helps overcome the block and aids the cell to eliminate the pathogens. A subset of viruses and bacteria subvert the autophagic pathway to promote their own replication. Thus the finding that ULK1 was captured by the K⁺ transporting ATPase B subunit raises some interesting speculations as to the nature and consequences of this interaction.

CDC42 binding protein kinase alpha (DMPK-like): This protein was captured from the HeLa whole cell extract. The protein encoded by this gene is a member of the Serine/Threonine protein kinase family. This kinase contains multiple functional domains. Its kinase domain is highly similar to that of the myotonic dystrophy protein kinase (DMPK). This kinase also contains a Rac interactive binding (CRIB) domain, and has been shown to bind CDC42. It may function as a CDC42 downstream effector mediating CDC42 induced peripheral actin formation, and promoting cytoskeletal reorganization. CDC42 has been demonstrated to be required for *Salmonella*-induced cytoskeletal and nuclear responses needed for invasion of the host cell (Chen, 1996). Thus the interaction of the K⁺ transporting ATPase B subunit with this protein introduces the possibility that this interaction may play a role in pathogenesis.

Microsomal triglyceride transfer protein large subunit is a protein that in humans is encoded by the *MTTP* gene. MTP encodes the large subunit of the heterodimeric microsomal triglyceride transfer protein. Protein disulfide isomerase (PDI) completes the heterodimeric microsomal triglyceride transfer protein, which has been shown to play a central role in lipoprotein assembly. Mutations in MTP can cause abetalipoproteinemia. No reports in the literature were found for this protein's involvement in pathogenesis.

Kendrin/pericentrin-B: This is a centrosome protein with homology that may be important for centrosome function. Although there have been on reports directly linking this protein with pathogen interactions and virulence, the association of kendrin with K⁺

transporting ATPase may play a role in abnormal centriole functioning and thus contribute to virulence. Further investigation of this interaction is warranted.

Titin (Connectin): This protein was captured from HeLa whole cell extracts. It was also seen in this study to interact with acetyl-CoA ligase. A description of this protein can be seen above in the section on acetyl-CoA ligase.

Task 5. Characterize the host/pathogen interactome and immunome of *Brucella abortus*.

In the Appendix section of this report, Table 3 contains a list of the REL proteins along with the ECM components that they interacted with. Table 4 contains a list of REL proteins that were captured by the various ECM/bait molecules. The following potential virulence factors have been identified for *B. abortus*:

6-phosphogluconate dehydrogenase: This protein interacted with laminin. This enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of donor with NAD⁺ or NADP⁺ as acceptor. No role in pathogenesis was reported for this enzyme.

23S ribosomal RNA methyltransferase: This protein bound to fibronectin as the bait molecule. It methylates the ribose of guanosine 2251 in 23S rRNA. No role in virulence has been reported.

30 S ribosomal protein S12: This protein interacted with fibronectin and laminin. The S12 plays a role in the translational accuracy. It can bind RNA with broad specificity and act as RNA chaperone *in vitro*. In a comprehensive genomic study, This protein has been found to differ significantly in its non synonymous SNP/ synonymous SNP ratio (NS/S ratio) between conserved genes of innocuous and nosocomial infectious strains of *S. epidermitis*. Genes with high NS/S ratios most likely have recently evolved to contribute to the virulence of this pathogen (Wei, 2006). In general SNP's play a significant role in the genesis of pathogenicity along with horizontal transfer and pathogenicity islands. Thus these proteins with high NS/S SNP ratios between commensal and pathogenic strains of the same organism should be considered strong candidate for listing as virulence factors.

ATP-Binding Cassette Transporters (ABC transporter) are found in almost all living organisms and represent one of the largest of all protein super families. Despite their great functional diversity, at their core they all share the same domain architecture and are thought to share a fundamentally similar alternating access transport mechanism and ATPase binding domain which is composed of the sequences known as Walker A and Walker B motifs. Some transporters act as direct pumps to import nutrients or export toxins or other molecules, others flip amphipathic lipids from one membrane leaflet to the other. Its members are mainly, but not exclusively, involved in the transport of a broad range of substrates across biological membranes. Many contribute to multidrug resistance in microbial pathogens.

They are integral membrane proteins that span the membrane of gram positive organisms with the ABC-binding domain located on the cytosolic end. Hydrolysis of ATP supplies energy for the transport of solutes or allocrites through the inner core of the transporter. Many of these transporter involved with uptake of nutrients have been implicated in virulence (Gamory, 2004). In gram- bacteria ABC transporter are characteristically found to span the inner membrane. They import or export solutes from the periplasmic space and cytosol. They require accessory factors for transport of allocrites across the outer membrane to the extracellular milieu. Bacteria with intracellular life styles and have reductive genomes often have many different ABC transporters. This is certainly the case in *B. abortus* as opposed to MRSA.

Many ABC transporter have been found to be immunogenic. This may appear to be paradoxical since the proteins are found in the inner membrane and periplasmic space. However there are many examples of of convalescent antibodies being directed against cytoplasmic proteins. Another possibility is that bacteria with disrupted cell walls may be taken up by antigen presenting cells. Why some of these proteins interacted with ECF components remains a mystery. One possibility is that these protein may be released exposed in bacteria with disrupted cell walls and the interactions with the host membrane may play a role in pathogenesis. Since *B. abortus* is a gram- bacterium it produces outer membran vesicles. These vesicals characteristically harbor outer membrane and periplasmic proteins along with lipids. However under conditions of antibiotic stress inner membrane proteins have been isolated from OMV. Thus this may represent a means for these ABC transporter to gain access into host cells since the OMV have been shown to be engulfed by host cells. In fact OMV have been reported to be involved in pathogenesis of gram- bacteria (Kuehn, 2014). In fact they have been shown to act as long-range virulence factors that protect their luminal cargo from extracellular host defenses and penetrate into host cells and tissues mor readily that the larger bacteris. Since the ABC transporter play a seminal role in the composition of the OMV and their structural similarities they offer attractive targets for post infection immunotherapy, vaccine development, and antimicrobial treatments. The following nine proteins belong to the ABC transporter group:

ATP/GTP-binding protein (YjeE ATPase, 7.5 kDa chromosomal protein):

This protein interacted with anti-*B. melitensis* antibodies. This protein is thought to play a role in cell wall biosynthesis. Although no literature was found relating to any virulence function of this protein because it is involved in cell wall biosynthesis it most likely plays a role in pathogenesis and may be an antimicrobial target and vaccine candidate.

ABC transporter (Elongation factor 3): In fungi this factor exhibits ribosome-dependent ATPase and GTPase activities that are not intrinsic to the fungal ribosome but are nevertheless essential for translation elongation in vivo. The EF-3 polypeptide has been identified in a wide range of fungal species. Little information on bacterial EF3 has been reported in the scientific literature.

ABC transporter ATPase (glycosyl transferase family I): This protein interacted with HeLa whole cells and HeLa membrane proteins. This protein is a transmembrane protein that utilizes the energy supplied by ATP hydrolysis to translocate glycosylconjugates across the cell membrane. It has been shown to be essential for formation of the lipopolysaccharide layer of prokaryotes. As such it has been reported to be involved in adaptation of pathogens to the host microenvironment at the early stages of infection and thus is required for virulence (Jinyun, 2012; Boyce, 2009).

Copper-translocating P-type ATPase (CtpA protein): which is the product of the *ctpA* gene, is a putative inner membrane copper translocating protein. This protein was observed to react with the laminin as the bait protein. PSORT analysis suggests that it is a cytoplasmic protein. Members of this family are ubiquitous in bacteria. This enzyme has two physiological functions: maintenance of cytoplasmic metal levels and assembly of metal proteins. These functions occur at the expense of energy expended during the hydrolysis of ATP. Francis (1997) demonstrated that recovery of a knockout CtpA mutant of *Listeria monocytogenes* from tissue of infected mice was dramatically reduced in comparison to its wild-type counterpart, thus indicating the importance of CtpA in establishing infection in a mouse model. Severely reduced virulence has also been documented in copper-efflux transporter mutants of *Pseudomonas aeruginosa* in mice, and plasmids that increase virulence of *Klebsiella pneumoniae* and *Shigella sonnei* have been shown to harbor putative copper resistant genes (White, 2009). White has also postulated that copper ions produced in macrophages and other host defensive cells acts as a bactericide during invasion by pathogens. Thus CtpA is essential in counteracting the deleterious action of the copper ions by exporting them from the bacterial/pathogen cell.

Iron ABC transporter substrate binding protein (Periplasmic iron compound binding protein): This protein interacted with anti-*B. melitensis* antibodies. This protein transports iron from the outer membrane into the periplasmic space. It contains an ATP-binding cassette, and the hydrolysis of the ATP provides energy for the transport across the membrane. It is thought of as an important virulence factor by means of sequestration of iron.

Spermidine/putrescine ABC transporter periplasmic protein: This integral membrane protein interacted with HeLa membrane component(s). Transport of polyamines such as spermidine and putrescine are required for optimal growth of cells. In *Streptococcus pneumoniae* this enzyme has been reported to be involved in pathogenesis (Ware, 2006). Although the deletion mutant of this gene had the same growth curve as its wild-type counterpart in laboratory grown cultures, growth of the deletion mutant was vastly affected in *in vivo* mouse investigations. Putrescine has been shown to restore virulence gene expression in *Shigella flexneri* (Durand, 2003) and was also reported to be involved in host cell adherence and cytotoxicity of *Trichomonas vaginalis* (Garcia, 2005).

Sugar ABC transporter periplasmic sugar-binding protein (chvE gene product): This protein interacted with anti-*B. melitensis* antibodies. In *Brucella* this protein shares homology with that of plant pathogen *Agrobacterium tumefaciens* wherein it is involved in virulence gene expression. *Agrobacterium* and *Brucella* are phylogenetically closely related. In *B. suis* this protein is essential for sugar utilization but not for survival and replication in macrophages (Alvarez-Martinez, 2001). However this does not necessarily rule out involvement in virulence.

ABC transporter substrate binding protein (twin-arginine translocation protein TatA): This protein interacted with HeLa whole cells components. It is a protein export, or secretion pathway found in plants, bacteria, and archaea. The Tat pathway serves to actively translocate folded proteins having a twin-arginine motif across the cytoplasmic membrane of prokaryotes. To date only phospholipase C proteins are Tat substrates have been experimentally confirmed in several pathogens to be directly involved in virulence (De Buck,). Phospholipase C proteins are important because they are secreted and then hydrolyze phosphatidylcholine, which is a phospholipid that is abundant in lung surfactant. However many studies have suggested that the Tat system may have an indirect role in virulence. It offers a rich source for study in that Tat substrates may extend the list of virulence factors in a complete array of bacterial pathogens. This is especially attractive because *in silico* investigations could easily discover twin arginine containing antagonists for the Tat system and thus yield a new class of antimicrobial compounds.

Sulfate ABC transporter sulfate-binding protein: This protein interacted with the HeLa membrane fraction. ABC transporters are transmembrane proteins that utilize the energy of adenosine triphosphate (ATP) hydrolysis to carry out certain biological processes including translocation of various substrates across membranes and non-transport-related processes such as translation of RNA and DNA repair. In *M. tuberculosis* this enzyme is upregulated in host macrophages and sulfur metabolism has been implicated in virulence, antibiotic resistance, and anti-oxidant defence. Cysteine is the end product of sulfur assimilation (Pinto, 2004). Cysteine availability regulates expression of *B. pertussis* toxin (Bogdan, 2001), and genes in sulfur assimilatory pathway are required for *B. melitensis* virulence (Lestrade, 2000).

Acetyl-CoA synthetase: This protein interacted with anti-*B. melitensis* antibodies as the bait. It is involved in metabolism of carbon sugars. It is in the ligase class of enzymes in that it catalyzes the formation of a new chemical bond between two large molecules. Although it plays a salient role in carbon source utilization it does not appear to contribute to pathogenesis since deletion mutants have no apparent attenuation in virulence (Carman, 2008).

Adenylosuccinate synthetase (*PurA*-gene product): This protein interacted with the HeLa membrane components and plasminogen. It is an enzyme that plays an important role in purine biosynthesis, by catalysing the guanosine triphosphate (GTP)-dependent conversion of inosine monophosphate (IMP) and aspartic acid to guanosine diphosphate (GDP), phosphate and N(6)-(1,2-dicarboxyethyl)-AMP. In *Leishmania donovani* null mutants for this enzyme result in deficiencies in growth and infectivity (Boitz, 2013). Deletion mutants of *L. monocytogenes* were observed to be severely attenuated for systemic infection in intragastrically inoculated A/J mice (Faith, 2012).

Aminoacyl-tRNA synthetase (Phospho 2 dehydro 3 deoxyheptonate aldolase, DAHP synthase): This protein interacted with the HeLa membranes as the bait molecule. It is an enzyme that catalyzes the esterification of a specific amino acid or its precursor to one of all its compatible cognate tRNAs to form an aminoacyl-tRNA. In other words, aminoacyl tRNA synthetase simply attaches the accurate amino acid onto the corresponding tRNA. No role in virulence was found upon review of scientific literature.

Antibiotic acetyltransferase interacted with HeLa whole cells. This is an enzyme belonging to the family of transferases. In *L. monocytogenes* this enzyme acetylates the peptidoglycan layer. This modification confers resistance to various types of antimicrobial compounds that target the bacterial cell wall such as lysozyme, B-lactam antibiotics, and bactericins. It was also shown to be required for growth in macrophages. Deletion mutants of this protein induced early secretion of proinflammatory cytokines and chemokines *in vivo*. Thus this enzyme plays an important role in limiting the innate immune responses and promoting bacterial survival in the infected host (Aubry, 2011).

ATP synthase F0F1 Subunit B: This protein interacted with anti-*B. melitensis* antibodies. This protein complex (Subunits A and B) couples proton translocation to ATP synthesis/hydrolysis and is required for virulence in *S. enterica* (Lee, 2013). Most of complex is exposed to the cytoplasm a short hydrophobic amino terminus being embedded in the membrane. It has also been reported to be the target of novel antibiotics.

Beta-keto-acyl synthase (3-oxoacyl-ACP synthase): This protein was captured with the HeLa membrane fraction. It is an enzyme involved in fatty acid synthesis. It results in the formation of acetoacetyl ACP. It has been reported to regulate virulence factors by N-acyl homoserine lacton and fatty acid synthesis in *Pseudomonas syringae* Taguchi, 2006). N-acyl homoserine lacton is a quorum sensing molecule in this pathogen.

Chaperonin ClpA/B: This protein interacted with HeLa whole cells and HeLa membranes as the bait entities. The proteins are thought to protect cells from stress by controlling the aggregation and denaturation of vital cellular structures. In *Francisella tularensis* ClpB plays a prominent role in resistance to stress and virulence in mice. It is essential for this pathogen to multiply in host tissues and cause disease in mice. ClpB reactivates aggregated protein *in vivo* as part of a stress response induced by the host tissue (Meibom, 2008). In *Yersinia enterocolitica* it plays a critical role in the regulation of virulence factor invasin and motility (Badger, 2000). In a comprehensive genomic study,

ClpB has been found to differ significantly in its non synonymous SNP/ synonymous SNP ratio (NS/S ratio) between conserved genes of innocuous and nosocomial infectious strains of *S. epidermitis*. Genes with high NS/S ratios most likely have recently evolved to contribute to the virulence of this pathogen (Wei, 2006). In general SNPs play a significant role in the genesis of pathogenicity along with horizontal transfer and pathogenicity islands. Thus, proteins with high NS/S SNP ratios between commensal and pathogenic strains of the same organism should be considered strong candidate for listing as virulence factors.

CreB family protein was found to interact with laminin as the bait molecule and has been predicted to be a membrane protein. PSORT analysis suggests that it is located in the cytoplasm. Overproduction leads to camphor resistance and chromosome condensation; deletion increases sensibility to nucleoid decondensation. It is hypothesized to interact with the heat-shock protein CspE, possibly providing a way to anchor the chromosome to the membrane. No virulence function has been assigned to this protein.

Cyclic beta 1-2 glucan synthetase, (also known as glycosyltransferase 36): This protein interacted with fibronectin and laminin. Cyclic beta-1,2-glucans (CbetaG) are periplasmic homopolysaccharides that have been shown to play an important role in several symbiotic and pathogenic relationships. Cyclic beta-1,2-glucan synthase I is the enzyme responsible for the synthesis of CbetaG and is an integral membrane polyfunctional protein that catalyzes the four enzymatic activities (initiation, elongation, phosphorolysis, and cyclization) required for the synthesis of CbetaG. In 2005 Arellano-Reynoso reported that this enzyme is a virulence factor and is required for intracellular survival *Brucella* in host cells. It is also important for circumventing host defenses.

Cyclophilin type (aka peptidyl-prolyl cis-trans isomerase, Chaperone HP017): This protein interacted with HeLa membrane fraction and is part of a family of proteins that bind to cyclosporine, an immunosuppressant that is usually used to suppress rejection after internal organ transplant. This family of proteins also possesses chaperone activity and fold proteins into active configuration by catalyzing slow *cis/trans* isomerization on proline-peptide bonds. In this capacity it is a protein folding catalyst, which regulates cell signaling in gastric epithelial cells and monocytes to modulate the inflammatory response and apoptosis during *Helicobacter pylori* infection (Kundu, M, 2013). In *Legionella pneumophila* it binds to collagen IV and enables this pathogen to transigrate across tissue barriers (Steinert, M, 2013). As reviewed by Kromina, 2008, this enzyme plays multifaceted roles in the pathogenic process of many microorganisms such as: modification of outer membrane porins and channels, components of secretory systems, and secreted proteins themselves. As secreted proteins they also participate in suppression of the host immune response.

Cytoplasmic protein: (Gifsy-2 prophage VmtV protein) interacted with HeLa whole cells. It is thought to be a major tail protein of the inducible prophage Gifsy-2. VmtV has not been implicated in virulence. The prophage does carry virulence genes (Figueroa-Bossi, 1999).

Erythrulose 4-phosphatase dehydrogenase: This protein bound to HeLa whole cell component(s). It is involved in catalysis of an oxidation/reduction reaction. Deletion mutants of the gene that encodes for this protein are avirulent implicating this protein in pathogenesis (Barbier, 2011)

Ffh signal recognition particle protein: This protein interacted with laminin as the bait molecule. It is a signal recognition particle that forms complex with a 4.5S RNA which facilitates translocation of proteins across membranes by binding with the membrane associated FtsY receptor (Rosch, 2008). SRP-deficient mutants were greatly attenuated in the zebra fish necrotic myositis model and the murine subcutaneous ulcer model. Studies in *E. coli* indicated that the SRP pathway is the major means for targeting secretion of integral membrane protein which typically lack cleavable signal sequences (Ulbrandt, 1997). These studies emphasize the important role the SRP pathway plays in pathogenesis.

Fis (Factor for inversion stimulation, *NtrX*-gene product, Nitrogen assimilation regulatory protein) interacted with the HeLa whole cell fraction. This protein plays a pivotal role in turning on the expression of Salmonella pathogenicity island 1 (SPI-1) that is crucial for the invasion and survival of *S. enterolytica* within host cells. It induces the virulence factors harbored on SPI-1 by inducing the expression of *HilA* and *InvF*, two activators of the *SPI-1* genes. Virulence of *fis* mutant is attenuated 100-fold if administered orally but has wild type virulence when administered intraperitoneally. Thus Fis most likely responds to signals found in the intestinal tract environment (Wilson, 2001). It has also been reported to be a key regulator of the plant pathogen *Ewinia chrysanthemi* virulence factors (Lautier, 2007).

Flavin-containing monooxygenase (NADH dehydrogenase): This protein interacted with laminin as the bait molecule. It is a protein family consisting of a group of enzymes that catalyze chemical reactions via the bound cofactor flavin. These reactions involve oxidation of heteroatoms, particularly nucleophilic atoms such as the nitrogen of amines. No direct role in virulence has been reported for this protein.

Formyltransferase, N-terminal (Methionyl tRNA formyltransferase): This protein interacted with anti-*B. abortus* antibodies and HeLa whole cell fraction. This is an enzyme that belongs to the transferase class. It transfers one carbon group. It participates in 3 metabolic pathways (methionine metabolism, one carbon pool by folate and aminoacyl-tRNA biosynthesis). Null mutants of this enzyme of *S. aureus* undergo pleiotropic effect including reduced growth rate, a nonhemolytic phenotype, and drastic reduction of multiple extracellular enzymes such as α -hemolysin and Pantone-Valentine leukocidin that have been associated with pathogenicity (Lewandowski, 2013). This enzyme in its role in formylation of peptides functions as an important virulence factor in *S. aureus* arthritis by mediating neutrophil recruitment, which contributes to joint damage in mice (Gjertson, 2011).

Glutamate 5-kinase (*ProB*-gene product): This protein interacted with anti-*B. melitensis* antibodies. It is part of a multienzyme complex along with glutamylphosphate reductase. It catalyzes the controlling first step of the synthesis of the osmoprotective amino acid proline. It has been reported that it plays no role in the intracellular life cycle and infectious nature of *Listeria monocytogenes* (Sleator, 2001).

Glyoxalase (bleomycin resistance protein, dioxygenase): This protein interacted with anti-*B. melitensis* antibodies. It is suspected that this protein is involved in the breakdown of aromatic hydrocarbons. No involvement in virulence has been reported for *Brucella* and other pathogens.

HAD superfamily hydrolase: This protein was captured with anti-*B. abortus* antibodies. This group of proteins is a member of the IIB subfamily of the haloacid dehalogenase (HAD) superfamily of aspartate-nucleophile hydrolases. They are phosphatases and are generally involved in metabolic processes. Some have been implicated in membrane transport, signal transduction, and nucleic acid repair. In eukaryotes it also acts as a regulator of actin dynamics (Gohla, 2005). Several reports point to the multitasking this family of proteins performs in metabolism and pathogenesis. *Porphyromonas gingivalis* has adapted this enzyme to facilitate invasion by modulating the host cell microtubule dynamics (Tribble, 2006). This enzyme is secreted by *P. gingivalis* during infection and has been shown to interact with host glyceraldehyde phospho dehydrogenase (GAPDH) which has been linked to recruitment of microtubules. Microtubules play an important role in internalization of invasive pathogens.

Histidinol aminotransferase (*HisC*-gene product): This protein interacted with plasminogen. It is an enzyme that acts in the chemical reactions and pathways resulting in the formation of histidine, 2-amino-3-(1H-imidazol-4-yl) propanoic acid. No virulence role has been reported for this protein.

Increased membrane permeability protein (*Imp*-gene product, Organic solvent tolerance protein A, *OstA*-gene product): This protein complexed to anti-*B. abortus* antibodies as bait molecule. This outer membrane protein is involved in organic solvent tolerance and also plays a role in cell envelope biogenesis. Although no role in virulence has been reported it may be an ideal vaccine candidate because of its involvement in membrane biogenesis.

Ketol-acid reductoisomerase (*IlvC*-gene product): This protein interacted with anti-*B. abortus* antibodies as the bait molecule. This enzyme belongs to the oxidoreductase class. It participates in valine, leucine and isoleucine biosynthesis and pantothenate biosynthesis. No apparent virulence role has been found for this enzyme in other pathogens.

L-asparaginase type II: This protein interacted with HeLa whole cells. Asparaginase catalyzes the hydrolysis of L-asparagine to produce L-aspartic acid and ammonia. It has not been implicated in virulence according to the literature.

MarR family transcriptional regulator (Transcriptional regulator slyA): This protein interacted with anti-*B. melitensis* antibodies. It regulates the expression of proteins conferring resistance to multiple antibiotics, organic solvents, household disinfectants, oxidative stress, agents and pathogenic factors. Proteins of this family regulate expression of virulence genes. In *S. enterica* it is involved in the modification of the bacterial cell surface that protects it from toxic compounds produced by the host. It has been reported that *Yersinia enterocolitica* utilizes it to regulate expression of a surface protein important for invasion of host tissue (Michaux, 2011).

Mog-gene product: This protein interacted with anti-*B. melitensis* antibodies as the bait molecule. It is required for molybdenum cofactor (Moco) biosynthesis and is believed to function in the addition of molybdenum to the dithiolene of molybdopterin to form Moco. No reported virulence involvement has been reported in the literature.

Neutral zinc metallopeptidase (Zinc-binding region): This protein interacted with anti-*B. abortus* antibodies. This enzyme is believed to catalyze the hydrolysis of peptide bonds by a mechanism in which water acts as a nucleophile, one or two metal ions hold water molecules in place, and charges amino acid chains are ligands for metal ions. Zinc metallopeptidases are widely distributed virulence factors in bacterial and represent attractive targets for the development of antimicrobials. In *M. tuberculosis* it targets several essential host peptides and has the ability to adapt its function to varying substrates (Petrera, 2012). *Burkholderia cenocepacia* also has broad spectrum zinc metalloprotease that is involved with virulence (Kooi, 2006). The Lethal Factor of *B. anthracis* has zinc metalloprotease activity (Klimple, 1994).

OmpA-like transmembrane domain-containing protein (OMP31): This protein interacted with anti-*B. abortus* antibodies. OmpA is the predominant cell surface antigen. It is tightly regulated by a variety of mechanisms. It is a haemin-binding protein and as such is one of the strategies used to obtain iron from host cells. In the brucella its expression appears to be higher when grown under conditions of iron limitation (Delphino, 2006). It has been reported to share 37% identity with OMP25. Its antigenicity has also been reported (Cassataro, 2004). Both of these OMP have been shown to be essential for invasiveness and intracellular survival of virulent *B. ovis* in phagocytes. The absence of these two proteins abolishes invasiveness in HeLa cells and is greatly reduced in J774.A1 cells (Martin-Martin, 2008)

OMP E (Aromatic hydrocarbon degradation membrane protein): This protein interacted with anti-*B. melitensis* antibodies. No scientific literature relating to its involvement in pathogenesis was found.

OmpW family outer membrane protein was found to interact with laminin, HeLa whole cells, HeLa membranes and anti-*B. melitensis* antibodies. OmpW belongs to a family of small outer membrane proteins that are widespread in Gram-negative bacteria. PSORT analysis supports the location in the outer membrane. This eight-stranded β -barrel protein has been found to confer virulence in *E. coli* in that up regulation of this protein conferred increased bacterial survival during phagocytosis and deletion mutants

of OmpW were significantly prone to phagocytosis. In *Vibrio cholerae* it is part of a pathogenicity island (Sharma, 2006). This protein may also be linked to adaptation of the organism during stress (Nandi, 2005). It was also observed to be a protective antigen in mice (Wu, 2013). The fact that this protein adhered to three bait entities and was immunogenic makes it a strong candidate for further investigation. To this end we cloned and expressed the gene for this protein in *E. coli*. OmpW was then used as a bait molecule in pull down assays to identify components of HeLa whole cell and HeLa membrane that interacted with this protein.

PepF/M-gene product (Neutral zinc metallopeptidase): This protein interacted with anti-*B. abortus* antibodies. It belongs to the peptidase M3 family and is a zinc-dependent serine protease that hydrolyzes peptides containing between 7 and 17 amino acids with a rather wide specificity. It is thought to degrade removed signal peptides during protein secretion. This protein has been reported to reside in the cytoplasm and also to be secreted from cells. In general metallopeptidases play important roles in nutrition, degradation of signal peptides, and virulence (Braz, 2002). The secreted form may have a different function than its cytoplasmic counterpart. It has been implicated in adhesion to host cells and in *Aspergillus niger* and *Streptococcus pneumoniae* it is essential for virulence. It is important for survival of *Mycoplasma hyopneumoniae* and thus is a potential target for drugs and vaccine development (Moitinho-Silva, 2013).

Protease: This protein interacted with plasminogen as the bait molecule. It is any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein.

Pyridine nucleotide-disulfide oxidoreductase (Dihydrolipoly dehydrogenase, *LpdA*-gene product, Dihydrolipoamide dehydrogenase): This protein interacted with laminin. It oxidizes dihydrolipoamide to lipoamide, and is part of the pyruvate dehydrogenase enzyme complex. In *P. auregenosa* it is a surface exposed protein that binds to four human proteins, Factor H, Factor H-like protein-1, complement Factor H-related protein, and plasminogen (Hallstrom, 2012). It contributes to survival in human serum and contributes to innate immune escape of this pathogen and most likely contributes to tissue invasion.

Sensory transduction histidine kinase (Blue light activated histidine kinase): This protein interacted with the HeLa membrane components. It is a transmembrane protein that transfers a phosphate group from ATP to a histidine residue within the kinase. Histidine kinases are used by bacteria for environmental sensing and are involved in regulation of gene expression, chemotaxis, phototaxis, and virulence. *B. melitensis* and *B. abortus* contain light-activated histidine kinases that bind a flavin chromophore. Infection of macrophages by *B. abortus* was induced by light but null mutants were not, indicating that the flavin-containing histidine kinase acts as a photoreceptor regulating survival, replication, and virulence in this pathogen (Swartz, 2007).

Transposase IS66: This protein interacted with HeLa whole cells. The function of these proteins is uncertain, but they are probably essential for transposition of DNA

fragments or transposons to another part of the genome. Although this may have had a role in the evolution of antibiotic resistance and virulence, it is doubtful if it plays an immediate virulence function in the host/pathogen relationship.

Ugd-gene product (Uracil DNA glycosylase): This protein interacted with plasminogen as the bait molecule. Catalysis of the reaction: $H_2O + 2 NAD(+) + UDP\text{-}\alpha\text{-D-glucose} = 3 H(+) + 2 NADH + UDP\text{-}\alpha\text{-D-glucuronate}$. Deletion mutants of *L. monocytogenes* were not attenuated in a mouse model of infection (Wilson, 2001)

Uracil phosphoribosyltransferase (Upp-gene product): This protein interacted with laminin. Uracil phosphoribosyltransferase is an enzyme that catalyzes the formation of uridine 5'-monophosphate from uracil and phosphoribosyl- α -L-pyrophosphate (PRPP) in the pyrimidine salvage pathway. This protein has not been reported to be involved in pathogenesis.

The second part of Task 5 was to define the immunome of *B. abortus* by determining what proteins were captured by anti-*B. abortus*- and anti *B. melitensis*-antibodies. The following proteins reacted with anti-*B. abortus* antibodies: *pepF* gene product, neutral zinc metalloproteinase (zinc-binding region), HAD superfamily hydrolase, formyl transferase (N-terminal), OmpA-like transmembrane domain-containing protein, *ilvC*- gene product, *imp*-gene product, and keto-acid reductoisomerase.

The following proteins reacted with anti-*B. melitensis* antibodies: formyltransferase, ATP/GTP-binding protein, OmpW family outer membrane protein, OmpE protein, MarR family transcriptional regulator, ATP synthase F₀F₁ subunit B, acetyl-CoA synthetase, periplasmic binding protein, sugar ABC transporter periplasmic sugar-binding protein, glutamate 5-kinase, *mogA* gene product, glyoxalase/bleomycin resistance protein/dioxygenase, and iron ABC transporter substrate binding protein.

A description of the involvement of these proteins in the host/pathogen relationship can be found in the list of *B. abortus* potential virulence factors above.

OMP W and ABC transporter ATPase were selected as bait molecules to capture potential HeLa cell components that may participate in the host/pathogen relationship. The OmpW family outer membrane protein was selected because of its location on the pathogen and also its potential involvement with virulence. It was found to interact with laminin, HeLa whole cells, HeLa membranes and anti-*B. melitensis* antibodies. OmpW belongs to a family of small outer membrane proteins that are widespread in Gram-negative bacteria. PSORT analysis supports the location in the outer membrane. This eight-stranded β -barrel protein has been found to confer virulence in *E. coli* in that up regulation of this protein conferred increased bacterial survival during phagocytosis and deletion mutants of OmpW were significantly prone to phagocytosis. In *Vibrio cholerae* it is part of a pathogenicity island (Sharma, 2006). This protein may also be linked to adaptation of the organism during stress (Nandi, 2005). It was also observed to be a protective antigen in mice (Wu, 2013). The fact that this protein adhered to three bait entities and was immunogenic makes it a strong candidate for further investigation. To this end we cloned and expressed the gene for this protein in *E. coli*. OmpW was then used as a bait

molecule in pull down assays to identify components of HeLa whole cell and HeLa membrane that interacted with this protein. *B. abortus* OMP W captured the following components of the HeLa cells:

S-adenosylmethionine synthase isoform type-2 was captured from the HeLa cell membrane. It catalyzes the formation of S-adenosylmethionine from methionine and ATP. No reports of involvement in pathogenicity were found in the scientific literature.

Aminomethyltransferase (glycine cleavage system T protein) is an enzyme that catabolizes the creation of methylenetetrahydrofolate. It is part of the system that is triggered by high concentrations of the amino acid glycine. It was captured as a component of HeLa whole cell. This enzyme system is found on the inner mitochondrial membrane. No reports of involvement of this protein in pathogenicity were found in the scientific literature.

Franconi anemia group D2 isoform A: This protein is monoubiquitinated in response to DNA damage, resulting in its localization to nuclear foci with other proteins (BRCA1 and BRCA2) involved in homology-directed DNA repair. This monoubiquitination is required for interaction with the nuclease FANL. Alternative splicing results in two transcript variants encoding different isoforms. This enzyme has not been reported to be involved in the host/pathogen relationship.

Heat shock-related 70 kDa protein (has been found to be a truncated part of HSP 71 by 7 amino acid residues) was captured in three different portions of the protein one with HeLa whole cell fraction and 2 with HeLa membrane components. HSP71 was only captured from the HeLa whole cell extract. This is to be expected since this is the constitutively produced cytosolic form of this protein. Expression of HSP 70 is induced in response to a variety of physiological and environmental insults. In the cytosol these proteins play an essential role as molecular chaperones by assisting the correct folding of nascent and stress-accumulated misfolded proteins, preventing protein aggregation, transport of proteins, and supporting antigen processing and presentation. Following stress, intracellularly located HSPs fulfill protective functions and thus prevent lethal damage. In contrast, membrane-bound or extracellularly located HSPs act as danger signals and elicit immune responses mediated either by the adaptive or innate immune system. Here, HSPs act as carriers for immunogenic peptides, induce cytokine release or provide recognition sites for natural killer (NK) cells. It has been documented that up-regulation of HSP 70 occurs in response to HIV insults to nervous tissue (Lim, 2003). Such up-regulation would have been beneficial to the human nervous tissues. Hirschhausen, 2010 has discovered a novel internalization system for *S. aureus* that involves the pathogens autolysin and a cognate HSP 70 (found in the cytoplasm of host cells) as the host cell receptor. The authors postulated that this might represent a major internalization mechanism playing a role in pathogenesis of this pathogen.

HSP70 is also located in the lumen of endoplasmic reticulum where is known as BiP or Binding Immunoglobulin Protein. In the endoplasmic reticulum it binds newly synthesized proteins as they are translocated into the ER, and maintains them in a state

competent for subsequent folding and oligomerization. BiP is also an essential component of the translocation machinery, as well as playing a role in retrograde transport across the ER membrane of aberrant proteins destined for degradation by the proteasome. BiP is an abundant protein under all growth conditions, but its synthesis is markedly induced under conditions that lead to the accumulation of unfolded polypeptides in the ER. It has also been reported to be an adjuster for sensitivity to various stressors and is characteristically found in autophagosomes during bacteria invasion of macrophages. The fact that there were five pull-down capture events from the HeLa membrane, cytosol, and endoplasmic reticulum points to the strong affinity of OMP W for the HSP70 of the human host cell. It also points to the roles of OMP W and HSP 70 in the host/pathogen relationship. This relationship certainly warrants further investigations.

Hemicentin-1 precursor This precursor was captured from HeLa whole cell extracts. Hemicentins are conserved ECM proteins characterized by a single von Willebrand A (VWA) domain at the amino terminus, a long stretch (>40) of tandem immunoglobulin domains, multiple tandem epidermal growth factors (EGFs), and a single fibulin-like carboxyl-terminal module. It has been shown to be involved in basement membrane removal during the *C. elegans* anchor-cell invasion of vulval epithelium. Sherwood, 2012 has identified the *fos* transcription factor ortholog *fos-1* as a critical regulator of basement-membrane removal by *C. elegans*. They identified ZMP-1, a membrane-type matrix metalloproteinase, CDH-3, a Fat-like protocadherin, and hemicentin, a fibulin family extracellular matrix protein, as transcriptional targets of FOS-1 that promote invasion. These results reveal a key genetic network that controls basement-membrane removal during cell invasion. Hemicentin was also found to be differentially expressed early in *C. elegans* infection (Humphreys, 2013). These reports point to hemicentin playing a salient role in the host parasite relationship.

Multidrug resistance-associated protein (MRP-2) –like protein-2: MRP2 is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. This protein is a member of the MRP subfamily, which is involved in multi-drug resistance. It is expressed in the apical part of the hepatocyte and functions in biliary transport. Substrates include anticancer drugs such as vinblastine; therefore, this protein appears to contribute to drug resistance in mammalian cells. MRP-2 regulates mucosal inflammation by facilitating the synthesis of heparin A (Pazos, 2008). Colonization of *S. typhimurium* on epithelial cells resulted in an up-regulation of MRP-2. MRP-2 is thought to play a role in which epithelial cells respond to infection by driving inflammation and neutrophil recruitment.

Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH): This HeLa membrane protein, along with Pneumococcal cell surface GAPDH, were both found to be ligands of human C1q protein during encounters with this pathogen (Rémi Terrasse, 2012). C1q is a major recognition molecule of the complement system. The membrane-anchored GAPDH on HeLa cells bound C1q through its GRs. C1qGR and GAPDH co-localized at the surface of apoptotic cells, and surface GAPDH exposure increased rapidly at early

steps of apoptosis. The GAPDH recognition by C1q at the early steps of apoptosis could be linked to the uptake of altered self-cells by phagocytes. An interesting aspect was differential effects of human and pneumococcal GAPDHs on complement activation. Bacterial GAPDH activates the complement cascade through the classical pathway. These data indicate that bacterial GAPDH is one of the ligands that lead to complement activation and to the clearance of bacteria. These observations are not consistent with previous reported roles of GAPDH in virulence processes and persistence of the bacteria in the host. This apparent paradox might be scrutinized in light of potential mimicry of apoptotic cells considering that the surface-exposed and/or soluble GAPDH recognition by C1q could be a strategy displayed by the pathogens to evade the immune system in some specific step of infection progression. Sojar, 2005 identified glyceraldehyde-3-phosphate dehydrogenase of epithelial cells as a second molecule that binds to *Porphyromonas gingivalis* fimbriae an essential step in the pathogenesis of periodontal disease. The N-terminal amino sequence was found to be GKVKVGVNGF and showed perfect homology with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Furthermore, purified *P. gingivalis* fimbriae were found to bind to rabbit muscle GAPDH. Antibodies directed against internal peptide 49–68 and 69–90 of fimbrillin were shown to inhibit the binding of *P. gingivalis* and of fimbriae to epithelial cells. Antibodies against these peptides also inhibited the binding of fimbriae to GAPDH. These results confirmed that the amino-terminal domain corresponding to amino residues 49–68 of the fimbrillin protein is the major GAPDH binding domain. These studies point to GAPDH as a major receptor for *P. gingivalis* major fimbriae and, as such, GAPDH likely plays a role in *P. gingivalis* adherence and colonization of the oral cavity, as well as triggering host cell processes involved in the pathogenesis of *P. gingivalis* infections. Several studies have also suggested that GAPDH may be involved in regulating innate immunity. GAPDH is enriched with the NF- κ B family member c-Rel and with other NF- κ B signaling molecules. It has also been reported that GAPDH interacts with molecules in the NF- κ B pathway. GAPDH binding to TRAF2 was enhanced under stress conditions, similar to the interaction between Siah1 and GAPDH. Gao, 2013 postulated that GAPDH may be a co-activator of TRAF2, perhaps acting at an early step in the TNF/NF- κ B signaling pathway, before E1 and E2 enzymes are recruited to TRAF2. They raised the possibility that there may be a network of factors recruited to TRAF2 upon TNFR stimulation that is important to regulating TRAF2 activation, among these, GAPDH.

hCG3005 human protein, immunoresponsive gene 1 (Irg1) Cis aconitate decarboxylase is highly expressed in mammalian macrophages during inflammation. Michelucci, 2013 identify Irg1 as the gene coding for an enzyme producing itaconic acid (also known as methylene- succinic acid) through the decarboxylation of cis-aconitate, a tricarboxylic acid cycle intermediate. Using a gain-and-loss-of-function approach in both mouse and human immune cells, they found Irg1 expression levels correlating with the amounts of itaconic acid, a metabolite previously proposed to have an antimicrobial effect. These authors purified IRG1 protein and identified its cis-aconitate decarboxylating activity in an enzymatic assay. Itaconic acid is an organic compound that inhibits isocitrate lyase, the key enzyme of the glyoxylate shunt, a pathway essential for bacterial growth under specific conditions. They demonstrated that itaconic acid inhibits the growth of bacteria expressing isocitrate lyase, such as *S. enterica* and *M. tuberculosis*. Furthermore, Irg1 gene silencing in macrophages resulted in significantly

decreased intracellular itaconic acid levels as well as significantly reduced antimicrobial activity during bacterial infections. Taken together, our results demonstrate that IRG1 links cellular metabolism with immune defense by the formation of itaconic acid.

Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 2 (NFKBIA): Rauchi, 2006 found that a previously uncharacterized gene, which they named *tlpA* (for TIR-like protein A), was identified in the *S. enterica* serovar Enteritidis genome that is predicted to encode a protein resembling mammalian TIR domains, they showed that overexpression of TlpA in mammalian cells suppresses the ability of mammalian TIR-containing proteins TLR4, IL-1 receptor, and MyD88 to induce the transactivation and DNA-binding activities of NF-kappaB, a downstream target of the TIR signaling pathway. NF-kappaB is one of the most important elements that coordinate stress-induced immune, and inflammatory responses. In addition, TlpA mimics the previously characterized *Salmonella* virulence factor SipB in its ability to induce activation of caspase-1 in a mammalian cell transfection model. Disruption of the chromosomal *tlpA* gene rendered a virulent serovar Enteritidis strain defective in intracellular survival and IL-1 secretion in a cell culture infection model using human THP1 macrophages. Bacteria with disrupted *tlpA* also displayed reduced lethality in mice, further confirming an important role for this factor in pathogenesis. Taken together, our findings demonstrate that the bacterial TIR-like protein TlpA is a novel prokaryotic modulator of NF-kappaB activity and IL-1 secretion that contributes to serovar Enteritidis virulence. Thus the OMP W may play a role in the modulation of mammalian toll/interleukin-1 receptor family proteins and also the intracellular survival of *Brucella*. Camus-Bouclainville (2004) reported that a virulence factor of myxoma virus colocalizes with NF-kappaB in the nucleus and interferes with inflammation. This study focused on the product of the M150R gene that has nine ankyrin repeats (ANKs), with the eighth having a close similarity with the nuclear localization signal-containing ANK of I-kappaB alpha, which regulates NF-kappaB activity by sequestering it in the cytosol. Because the viral protein is targeted to the nucleus, it was named MNF, for myxoma nuclear factor. This localization was lost when the eighth ANK was removed. In tumor necrosis factor alpha-treated cells, MNF and NF-kappaB colocalized as dotted spots in the nucleus. *In vivo* experiments with a knockout virus showed that MNF is a critical virulence factor, with its deletion generating an almost apathogenic virus. Detailed histological examinations revealed an increase in the inflammatory process in the absence of MNF, consistent with the interference of MNF with the NF-kappaB-induced proinflammatory pathway. Because MNF has homologs in other poxviruses, such as vaccinia, cowpox, and variola viruses, this protein is probably part of a key mechanism that contributes to the immunogenic and pathogenic properties of these viruses. Zhou, 2005 demonstrated that *Yersinia* virulence factor YopJ acts as a deubiquitinase to inhibit NF-kappaB activation. YopJ, is essential for the death of infected macrophages and can block host proinflammatory responses by inhibiting both the NF-kappaB and mitogen-activated protein kinase pathways, which might be important for evasion of the host immune response and aid in establishing a systemic infection. Zhang, 2005 reported that inhibition of MAPK and NF-kappa B pathways is necessary for rapid apoptosis in macrophages infected with *Yersinia*. This protein was also shown to be regulated by monocyte chemotactic protein-1 (MCP-1) during *E. coli* pulmonary infections

Balamayooran, 2011). MCP-1 was found to interact with MRSA Acetyl CoA-carboxylase in this project.

ABC transporter ATPase (glycosyl transferase family I): This protein interacted with HeLa whole cells and HeLa membrane proteins. This protein is a transmembrane protein that utilize the energy supplied by ATP hydrolysis translocate of glycosylconjugates across the cell membrane. It has been shown to be essential for formation of the lipopolysaccharide layer of prokaryotes. As such it has been reported to be involved in adaptation of pathogens to the host microenvironment at the early stages of infection and thus is required for virulence (Jinyun, 2012; Boyce, 2009). It was also reported that deletion of the gene for this protein in *B. abortus* resulted in an attenuation of virulence (Zhang, 2013). This protein was also selected as a representative of the ABC Cassette transporters which appear to play an important role in virulence of *B. abortus* as well as other pathogens. The following HeLa cell components interacted with this Brucella membrane protein:

Protein FAM115 isoform 1: This protein was captured from HeLa membrane extracts. The function of this protein is not known at the present time. There have been no reports in the literature on the involvement of it in the host/pathogen relationship.

Hereditary multiple exostoses isolog protein product: This protein was captured from the HeLa membrane extract. It is a single-pass membrane protein which functions as a glycosyltransferase and catalyzes the transfer of N-acetylglucosamine to glycosaminoglycan chains, which is important in heparin and heparin sulfate synthesis. There have been no reports in the literature on the involvement of it in the host/pathogen relationship.

Enolase: This protein was captured from HeLa whole cell extracts. Although there are numerous reports of bacterial enolase being important in pathogenesis, no reports of the involvement of human enolase were found in the scientific literature.

Hla-Dr1TP1: This protein was captured from HeLa whole cell extracts. It was also found to be complexed to Staphylococcal enterotoxin C3 variant 3b2. It belongs to the HLA class II eta chain paralogues. It is a heterodimer consisting of DRA and DRB both of which are anchored in the membrane. They are expressed in antigen presenting cells. It plays a central role in the immune system by presenting peptides derived from extracellular proteins.

Nuclear distribution gene C homolog (NudC domain containing protein 3): This protein was captured from the HeLa whole cell fraction and it functions to maintain the stability of dynein intermediate chain. Depletion of this gene product results in aggregation and degradation of dynein intermediate chain, mislocalization of the dynein complex from kinetochores, spindle microtubules, and spindle poles, and loss of gamma-tubulin from spindle poles. The protein localizes to the Golgi apparatus during interphase. It has been identified and found to be highly conserved in different species from fungi to mammals. The high degree of conservation, in special in the nudC domain, suggests that they are genes with essential functions. Most of the identified genes in the family have

been implicated in cell division through the regulation of cytoplasmic dynein. As for mammalian genes, human NUDC has been implicated in the migration and proliferation of tumor cells and has therefore been considered a possible therapeutic target. There is evidence suggesting that mammalian NudC is also implicated in the regulation of the inflammatory response and in thrombopoiesis. The presence of these other functions not related to the interaction with molecular motors agrees with that these genes and their products are larger in size than their microbial orthologous, indicating that they have evolved to convey additional features. Thus the interaction of this host protein with *Brucella* ABC transporter ATPase may be of significance and therefore should be subjected to further investigations.

Tripartite motif protein TRIM9 isoform alpha (E3 ubiquitin-protein ligase TRIM9):

This component was captured from HeLa whole cell extracts. It ubiquitinates itself in cooperation with an E2 enzyme and serves as a targeting signal for proteosomal degradation. **Proteasomes** are protein complexes inside all eukaryotes and archaea, and in some bacteria. In eukaryotes, they are located in the nucleus and the cytoplasm.[1] The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis. Proteasomes which are part of a major mechanism by which cells regulate the concentration of particular proteins and degrade misfolded proteins. The degradation process yields peptides of about seven to eight amino acids long, which can then be further degraded into shorter amino acid sequences and used in synthesizing new proteins. Proteins are tagged for degradation with a small protein called ubiquitin. The tagging reaction is catalyzed by enzymes called ubiquitin ligases. Once a protein is tagged with a single ubiquitin molecule, this is a signal to other ligases to attach additional ubiquitin molecules. The result is a polyubiquitin chain that is bound by the proteasome, allowing it to degrade the tagged protein. Proteosomal degradation has also been implicated in elimination of intracellular bacterial and viral proteins during infection.

Dhingra, 2007, found that infection with wild-type rabies virus resulted in down-regulation of soluble NSF attachment receptor proteins (SNAREs) such as alpha-synaptosome-associated protein (SNAP), tripartite motif-containing 9 (TRIM9), syntaxin, and pallidin, all of which are involved in docking and fusion of synaptic vesicles to and with presynaptic membrane. As a consequence, accumulation of synaptic vesicles was observed in the presynapses of mice infected with rabies virus. These data demonstrate that infection with wild type RV results in alteration of host protein expression, particularly those involved in ion homeostasis and docking and fusion of synaptic vesicles to presynaptic membrane, which may lead to neuronal dysfunction.

Immunoglobulin heavy chain variable region: The involvement of antibodies in mitigation of the effects of microbial infections has been well documented.

Hypotheical protein hCG1808509: All attempts determine the function of this protein were futile. Thus we could not search for reports of involvement of this protein in the host/pathogen relationship.

Isoform CRA_a (Granulin, PC cell- derived growth factor): This protein is a high molecular weight secreted mitogen. It is abundantly expressed in rapidly cycling epithelial cells, in the immune system and in neurons, such as cerebellar Purkinje cells. Progranulin contributes to tumorigenesis in diverse cancers, including breast cancer, clear cell renal carcinoma, invasive ovarian carcinoma and glioblastoma. It regulates the rate of epithelial cell division in responsive epithelial cells, and confers an invasive phenotype on these cells. It is involved in the wound response. It has been reported that granulin-deficient mice were unable to rapidly clear an infection of *Listeria monocytogenes* and displayed elevated bacterial burdens in the spleen, liver, and brain (Yin et al., 2010). Seo, 2008 has shown that macrophages had elevated levels of granulin upon infection with *B. anthracis* spores.

Research project conclusions:

A thorough inventory of molecules that play a role in the pathogenic process was established which included some well-known virulence factor along with many novel or potential participants that have not been cited as playing a role in pathogenesis. These novel proteins should be subjected to further investigations to determine if they are salient virulence factors. Of the many potential virulence factors identified in this study, the following protein of MRSA stand out as candidates for further investigations based on exhaustive literature searches: acetyl-CoA carboxylase, acetyltransferase GNAT family protein, acetyl-CoA synthetase, bone fibrinogen/sialoprotein-binding protein, ECM-binding protein homolog, glutamate dehydrogenase, histidine kinase KdpD protein, immunoglobulin-binding protein, K transporting ATPase, membrane-anchored glycerophosphoyl diester phosphodiesterase, MHC class II analog/extracellular adherence protein, nitroreductase periplasmic-binding protein, peroxiredoxin, phosphoenolpyruvate carboxykinase, serine protease HtrA-like protein, superantigen-like protein, and zinc metalloprotease aureolysin. The following *B. abortus* proteins that were identified offer extremely promising candidates for vaccine/therapeutic agent targets: 30 ribosomal protein S12, ABC transporter ATPase, copper-translocating P-type ATPase, iron ABC transporter substrate binding protein, spermidine/putrescine ABC transporter periplasmic protein, ABC transporter periplasmic sugar-binding protein, ABC transporter substrate-binding protein, ABC transporter sulfate-binding protein, adenylosuccinate synthetase, antibiotic acetyltransferase, chaperonin ClpA/B protein, cyclic beta 1-2 glucan synthetase, cyclophilin type protein, formyltransferase, HAD superfamily hydrolase, neutral zinc metalloprotease, OMP 31, and OMP W. It should be noted that many of the potential virulence factors of *B. abortus* are members of the ABC-transporter family. Thus the discovery of a possible conserved component of members of this family could have far-reaching implications on antimicrobial agents.

In addition to ECM components, host proteins that are targeted by several potential virulence factors, were also identified. Their participation in the pathogenic process provided a view from the eyes of the pathogen and will be indispensable in defining the pathogenic process. Task 4 was dedicated to the identification of host target/receptor proteins of putative virulence factors of MRSA. Pull-down assays, which used cloned and expressed MRSA acetyl-CoA carboxylase, acetyl-CoA ligase, and K⁺ transporting ATPase B subunit as bait, were employed to pinpoint specific host components that interacted with these potential virulence factors. These three

virulence factors were chosen midway during Phase 2 to be used as bait molecules to capture HeLa whole cell and HeLa cell membrane (host) components. Upon reflection other putative virulence factors may have served as more appropriate bait however the aforementioned proteins were found to have merit as bait molecules. Nonetheless the data obtained verified that these three proteins may indeed be part of the host/pathogen interaction insofar that they targeted host proteins involved in internalization of invading bacteria and endosome recycling, maturation of phagosomes into phagolysosomes, microtubule formation, response to inflammation caused by pathogens *via* neutrophil recruitment, host membrane components, cytokine and stress responses, upregulation of antimicrobial peptides, activation of complement cascades, as well as combining with the host's ECM components collagen and laminin.

Cloned and expressed *B. abortus* OMP W and ABC transporter ATPase were selected as bait molecules to capture potential HeLa cell components that may participate in the host/pathogen relationship. These host factors captured have been implicated as playing a part in the host/pathogen relationship and as such are ideal candidate for antimicrobial therapies. They captured protein that were involved in correct folding of nascent and stress-induced protein, antigen presentation, immune defense, inflammation response, preventing protein aggregation, internalization of pathogenic bacteria, promotion of bacterial invasion, bacterial adherence and colonization, inflammation, elimination of intracellular bacteria, neutrophil recruitment, and those characteristically found in autophagosomes during bacterial invasion.

The data obtained from this study identified novel targets for therapeutic agents, new antibiotics, vaccines, and next generation diagnostic assays for strain specific pathogens. Many of these targets have been reported to have specific antagonistic molecules. This information will be of great importance for future *in vitro* and *in vivo* investigations. This project validated that the Rapid Identification of Bacterial Virulence Factors can serve as a virulence-factor discovery approach for a wide range of viral, bacterial, and fungal pathogens.

Recommendations for further investigation:

Comparison of putative virulence factors with secreted and surface exposed proteins in the analysis. In previous investigations on MRSA our laboratory performed proteomic studies on its secretome and surface exposed proteins. The secretome was analyzed by isolation of proteins found in the growth medium followed by mass spectrometry. Surface exposed proteins were identified by LC.MS/MS after “shaving” whole MRSA cell with trypsin, followed by centrifugation, and filtration to remove the whole cells. This comparison is important since these sub proteomes are often enriched for virulence factors. This will also add to the inventory of potential virulence factors. It should be pointed out that these investigations were performed in laboratory-grown cultures.

Determine the *in silico* 3D structure of selected proteins to aid in the elucidation of their role in pathogenesis. An exhaustive analyses of the selective potential virulence factors involving advanced literature searches, BLAST analysis to determine the conserved nature of a selected protein in various strains of a pathogen and also to ascertain that the virulence factor does not have homologous components in the human proteome should be undertaken. Several other software programs could be utilized to convert the amino acid sequence into a 3D-

computer model to elucidate important properties of the protein such as what part of the protein is conserved or variable, and what amino acid residues are located on the surface or in the core? The secondary structure can also be determined and this will provide intrinsic information on structure and function. This will also aid in determining a proteins placement in a lipid bilayer by identifying trans-membrane helices and their topologies. Molecular docking software programs could be used to elucidate how the virulence factor and host component interact. Such investigations will be extremely important in pinpointing the function of potentially salient hypothetical proteins found in the study.

Epitope and host's critical binding site (CBS) can be determined for the selected virulence factors using the LC.MS/MS extraction method. This technology uses cloned putative virulence factors to locate the area on a host protein that interacts with that virulence factor. Almost all of the host CBS are available commercially. Identification of these CBS's or regions where virulence factors attach to specific host components can be accomplished by first obtaining the spectrum of the trypsin-digested cloned host protein. The digested peptide fragment can then be mixed with its corresponding virulence factor. Any fragment that interacts with the virulence factor will be extracted from the spectrum and its amino acid sequence determined. The resulting host CBS peptides may act as competitive inhibitors to the pathogenic process. they can then be analyzed by molecular docking software which will predict how a the host CBS interacts with the suspected virulence factor.

Micro titer plate assay to determine if a CBS peptide interferes with virulence factor/host receptor interactions and thus may have antimicrobial properties. Molecular docking analysis will yield important data on the ideal structure of the CBS peptide and how it interacts with its virulence factor counterpart. Candidate CBS peptides can then be tested in an *in vitro* microtiter plate assay. Essentially, cloned host receptor proteins will be attached to microtiter plates and the ability of the corresponding host CBS peptide to inhibit binding to the potential virulence factor will be assessed. Synthetic CBS's and reported known inhibitors of the specific interaction as found on drug-target websites will also be tested.

Testing of efficacy of CBS peptide to arrest or mitigate infection in an animal model. Mouse animal models are available for both *B. abortus* and MRSA. Thus the efficacy of the CBS peptide therapy can be tested.

Development of deletion mutants for selected virulence factors and testing for attenuation, safety, and efficacy in mouse animal model. Clean deletions of selected virulence factors can be carried out and the safety, efficacy, and attenuation investigated and compared to the wild-type counterpart.

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APPENDIX 1

(Tables 1 – 4)

MRSA Protein Identified	laminin	plasminogen	ant MRSA	HeLA Membrane	HeLA whole Cell	collagen IV	fibronectin	vitronectin
30S ribosomal protein s17			✓					
acetyl-CoA carboxylase, carboxyl transferase, beta subunit				✓	✓		✓	
acetyl-CoA C-acetyltransferase			✓	✓	✓			✓
acetyltransferase, GNAT family		✓					✓	
addiction molecule toxin, Txe/YoeB family							✓	
alanine racemase, N-terminal domain protein								
alkyl hydroperoxide reductase subunit C				✓	✓			
alpha/beta hydrolase fold protein			✓					
AMP binding enzyme/Acetyl-CoA ligase				✓				
AMP-binding protein, putative gramicidin synthetase, LgrC	✓							
ATPase, histidine kinase-, DNA gyrase B-, and HSP90-like domain protein								✓
ATPase/histidine kinase/DNA gyrase B/HSP90 domain protein								✓
BglG family transcriptional antiterminator						✓		
carbamoyl-phosphate synthase L chain, N-terminal domain protein							✓	
cysteine synthase A		✓	✓					
cytosol aminopeptidase family, catalytic domain protein		✓						
DNA-binding response regulator							✓	
DNA internalization-related competence protein ComEC/Rec2							✓	
ECM-binding protein, EbnB protein	✓	✓					✓	
glutamate dehydrogenase (NAD(P) ⁺)	✓	✓					✓	
histidine kinase internal region								
histidine-IRNA ligase				✓				
holo-(acyl-carrier-protein) synthase						✓		
hypothetical protein HMPREF_9529_00593					✓			
hypothetical protein GGSa03_11236					✓			
hypothetical protein CGSSa03_11256							✓	
hypothetical protein HMPREF9529_00622	✓						✓	
hypothetical protein HMPREF9529_01618							✓	
hypothetical protein HMPREF9529_02267								✓
hypothetical protein HMPREF9529_02597		✓						
hypothetical protein SAR2120					✓			
hypothetical protein SAUSA300_1771				✓				
hypothetical protein SAV1112					✓			
immunoglobulin G-binding protein A, partial							✓	
isochorismatase family protein								✓
K ⁺ transporting ATPase				✓	✓			
MAP domain protein			✓					
membrane domain of membrane-anchored glycerophosphoryl diester phosphodiesterase							✓	
O-acetyltransferase OatA family protein				✓	✓			
osmosensitive K ⁺ channel His kinase sensor domain protein								✓
pathogenicity island protein			✓					
periplasmic binding protein, SirA protein		✓			✓			
peroxiredoxin, partial							✓	
phage putative tail component protein								✓
phage-related protein								
phage tail tape measure protein, TP 901 family, core region		✓						
phosphoenolpyruvate carboxykinase			✓					
phosphoenolpyruvate-dependent sugar PTS family porter								
preprotein translocase, SecA subunit		✓				✓		
putative serine protease HtrA-like protein							✓	
RNA polymerase sigma factor RpoD		✓						
ribosomal protein L27			✓					
ribosome small subunit-dependent GTPase A, RsgA/YieO protein		✓						
SasB protein	✓	✓					✓	
sensor histidine kinase KdpD								✓
serine O-acetyltransferase			✓					
serine-aspartate repeat protein E, partial							✓	
superantigen-like protein							✓	
thermolysin metalloproteinase			✓					
undecaprenylphospho-muramoyl pentapeptide			✓					
VRR-NUC domain protein								✓
virulence-associated protein E	✓						✓	

Table 1. List of the REL proteins along with the ECM components that they interacted with.

Table 2

anti MRSA antibody	
REL 001	Thermolysin metallopeptidase
	undecaprenyldiphospho-muramoylpentapeptide
	ribosomal protein L27
REL 004	serine O-acetyltransferase
	MAP domain protein
	cysteine synthase A
REL 006	acetyl-CoA C-acetyltransferase
	alpha/beta hydrolase fold protein
	phosphoenolpyruvate carboxykinase
REL 013	pathogenicity island protein
REL 015	30S ribosomal protein s17

HeLA Membrane	
REL 001	acetyl-CoA C-acetyltransferase
	AMP binding enzyme
	K ⁺ transporting ATPase
	hypothetical protein
REL 002	O-acetyltransferase OatA family protein
	hypothetical protein SAUSA300_1771
	acetyl-CoA carboxylase, carboxyl transferase, beta subunit
	histidine--tRNA ligase
	alkyl hydroperoxide reductase subunit C
REL 004	AMP binding enzyme

HeLA Whole Cell	
REL 001	acetyl-CoA carboxylase, carboxyl transferase, beta subunit
	alanine racemase, N-terminal domain protein
	alkyl hydroperoxide reductase subunit C
REL 002	hypothetical protein SAR2120
	hypothetical protein SAV1112
REL 004	hypothetical protein
REL 006	K ⁺ -transporting ATPase, B subunit
	hypothetical protein
	acetyl-CoA C-acetyltransferase
	O-acetyltransferase OatA family protein
	Peroxiredoxin, partial

Collagen IV	
REL 001	phosphoenolpyruvate-dependent sugar PTS family porter
	BglG family transcriptional antiterminator
REL 004	holo-[acyl-carrier-protein] synthase

Fibronectin	
REL 004	Carbamoyl-phosphate synthase L chain, N-terminal domain protein
	acetyl-CoA carboxylase, biotin carboxylase
	DNA-binding response regulator
	serine-aspartate repeat protein E, partial
	superantigen-like protein
	immunoglobulin G-binding protein A, partial
REL 006	putative serine protease HtrA-like protein
	Membrane domain of membrane-anchored glycerophosphoryl diester phosphodiesterase

Table 2 continued

Vitronectin	
REL 001	VRR-NUC domain protein
	Phage-related protein
	Histidine kinase internal region
REL002	ATPase, histidine kinase-, DNA gyrase B-, and HSP90-like domain protein
	hypothetical protein HMPREF9529_02267
	acetyl-CoA C-acetyltransferase
REL 004	isochorismatase family protein
	hypothetical protein CGSSa03_11256
REL 006	sensor histidine kinase KdpD
	osmosensitive K ⁺ channel His kinase sensor domain protein
	ATPase/histidine kinase/DNA gyrase B/HSP90 domain protein

Table 2. Spreadsheet of REL proteins that were captured by the various ECM/bait molecules.
Color coded proteins were identified in multiple RELs using various ECM/bait molecules

Ba Protein Identified		Fibronectin	Laminin	Plasminogen	Bait protein used			
					HeLA whole Cells	HeLA Membrane	abortion	mellitensis
23S ribosomal RNA methyltransferase		✓						
30S ribosomal protein S12		✓	✓					
hypothetical protein found in the genus <i>Brucella</i>			✓					
Cyclic beta 1-2 glucan synthetase, (also known as glycosyltransferase 36)			✓					
<i>mfi</i> gene product			✓					
Copper-translocating P-type ATPase			✓					
CrcB family protein			✓					
OmpW family outer membrane protein			✓		✓	✓		✓
<i>upp</i> gene product (uracil phosphoribosyltransferase)			✓					
pyridine nucleotide-disulfide oxidoreductase			✓					
flavin-containing monooxygenase (FMO)			✓					
6-phosphogluconate dehydrogenase				✓		✓		
hsc gene product (histidinol aminotransferase)				✓				
protease				✓				
<i>purA</i> (adenylosuccinate synthetase)				✓				
<i>ugd</i> gene product				✓				
hypothetical protein				✓				
hypothetical protein					✓			
cytoplasmic protein					✓			
hypothetical protein BR1101					✓			
twin-arginine translocation protein TaaA					✓			
ABC transporter ATPase						✓		
ABC transporter					✓			
transposase IS66					✓			
L-asparaginase type II					✓			
D-erythritolose 4-phosphate dehydrogenase					✓			
<i>ntrX</i> gene product					✓			
formyltransferase					✓		✓	
antibiotic acetyltransferase					✓			
chaperonin ClpA/B					✓	✓		
cyclophilin type peptidyl-prolyl cis-trans isomerase						✓		
spermidine/putrescine ABC transporter periplasmic protein						✓		
<i>fabB</i> gene product						✓		
Beta-ketoacyl synthase						✓		
sulfate ABC transporter sulfate-binding protein						✓		
aminoacyl-tRNA synthetase						✓		
hypothetical protein							✓	
HAD superfamily hydrolase							✓	
Formyl transferase, N-terminal							✓	
OmpA-like transmembrane domain-containing protein							✓	
<i>ilvC</i> gene product							✓	
ketol-acid reductoisomerase							✓	
ATP synthase F0 subunit B								✓
acetyl-CoA synthetase								✓
periplasmic binding protein								✓
sugar ABC transporter periplasmic sugar-binding protein								✓
glutamate 5-kinase								✓
<i>mogA</i> gene product								✓
glyoxalase/bleomycin resistance protein/dioxygenase								✓
outer membrane protein							✓	✓
<i>purA</i> gene product								✓
adenylosuccinate synthetase								✓
Sensory transduction histidine kinase								✓
<i>pepF</i> gene product							✓	
Neutral zinc metalloproteinase, zinc-binding region								✓
ATP/GTP-binding protein								✓
MarR family transcriptional regulator								✓

Table 3. List of the REL proteins along with the ECM components that they interacted with.

Table 4

Fibronectin	
BaREL 003	23S ribosomal RNA methyltransferase
BaREL 005	30S ribosomal protein S12
Laminin	
BaREL 001	hypothetical protein found in the genus <i>Brucella</i>
	Cyclic beta 1-2 glucan synthetase, (also known as glycosyltransferase 36)
	<i>ffh</i> gene product
	Copper-translocating P-type ATPase
	CrcB family protein
BaREL 002	OmpW family outer membrane protein
BaREL 003	<i>upp</i> gene product (uracil phosphoribosyltransferase)
BaREL 004	pyridine nucleotide-disulfide oxidoreductase
	flavin-containing monooxygenase (FMO)
BaREL 005	30S ribosomal protein S12
Plaminogen	
BaREL 001	6-phosphogluconate dehydrogenase
	<i>hisC</i> gene product
	protease
	<i>purA</i>
BaREL 002	<i>ugd</i> gene product
BaREL 003	hypothetical protein
HeLA Whole Cells	
BaREL 001	hypothetical protein
	cytoplasmic protein
	hypothetical protein BR1101
BaREL 002	OmpW family outer membrane protein
BaREL 003	twin-arginine translocation protein TatA
	ABC transporter ATPase
	ABC transporter
	transposase IS66
BaREL 004	L-asparaginase type II
	hypothetical protein
	D-erythrulose 4-phosphate dehydrogenase
	<i>ntrX</i> gene product
Ba REL 005	formyltransferase
	antibiotic acetyltransferase
	twin-arginine translocation pathway signal
	chaperonin ClpA/B
HeLA Membrane	
BaREL 001	<i>purA</i> gene product
	adenylosuccinate synthetase
BaREL 002	chaperonin ClpA/B
BaREL 003	Sensory transduction histidine kinase
	6-phosphogluconate dehydrogenase
BaREL 004	cyclophilin type peptidyl-prolyl cis-trans isomerase
	spermidine/putrescine ABC transporter periplasmic protein
	<i>fabB</i> gene product
	Beta-ketoacyl synthase
	sulfate ABC transporter sulfate-binding protein
BaREL 005	OmpW family outer membrane protein
	aminoacyl-tRNA synthetase
	ABC transporter ATPase

Table 4 continued

Anti-<i>Brucella abortus</i>	
BaREL 001	pepF gene product
	Neutral zinc metallopeptidase, zinc-binding region
BaREL 002	No Significant hits
BaREL 003	No Significant hits
BaREL 004	hypothetical protein
	HAD superfamily hydrolase
	Formyl transferase, N-terminal
	OmpA-like transmembrane domain-containing protein
BaREL 005	ilvC gene product
	ketol-acid reductoisomerase

Anti-<i>Brucella melitensis</i>	
BaREL 001	formyltransferase
	ATP/GTP-binding protein
BaREL 002	OmpW family outer membrane protein
BaREL 003	MarR family transcriptional regulator
BaREL 004	ATP synthase F0 subunit B
	acetyl-CoA synthetas
	periplasmic binding protein
BaREL 005	sugar ABC transporter periplasmic sugar-binding protein
	glutamate 5-kinase
	mogA gene product
	glyoxalase/bleomycin resistance protein/dioxygenase
	outer membrane protein

Table 4. List of REL proteins that were captured by the various ECM/bait molecules.
Color coded proteins were identified in multiple RELs using various ECM/bait molecules