Identification of an immunogenic Mannheimia haemolytica immunoglobulin-, fibronectin- and fibrinogen-binding protein differentially expressed in vitro

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Identification of an immunogenic *Mannheimia haemolytica* immunoglobulin-, fibronectin- and fibrinogen-binding protein differentially expressed *in vitro*

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Immunobiology

Program of Study Committee:
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Iowa State University
Ames, Iowa
2003
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For the Major Program

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For the Graduate College
DEDICATION

To my children,
Anna Smith Osmundson
and
Caleb Smith Osmundson:

Reach for your stars!
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ABSTRACT

Bovine respiratory disease (BRD), or “shipping fever”, is the cause of large losses to the cattle industry annually and is primarily caused by *Mannheimia haemolytica* serotype (ST) 1. *M. haemolytica*, a normal flora respiratory tract bacterium, rapidly multiplies upon a stressful occurrence and colonizes the upper respiratory tract. This can lead to fibrino-hemorrhagic pneumonia. Bacterial immunoglobulin-binding protein (IgBP) expression is known to play a role in the pathogenesis of a variety of organisms. Far-Western blot was used to demonstrate the presence of an IgBP(s) in whole cell sonicates (WCSs) and a culture supernatant (CS) preparation from *M. haemolytica* ST 1. The IgBP(s) was isolated by affinity chromatography and used in a far-Western blot to show that the IgBP(s) also binds the extra-cellular matrix proteins (ECMPs) fibrinogen and fibronectin. ECMPs are known to play a role in colonization of some bacteria by acting as a bridge enabling binding between bacteria expressing extracellular matrix-binding proteins and epithelial cells. Flow cytometry showed the surface expression of an IgBP(s) on whole cell *M. haemolytica*. Immune sera from convalescent cattle showed an increased antibody response to the 75.0 kDa IgBP when compared to sera from naïve or acutely infected cattle as seen by Western blot. It was demonstrated that all 12 serotypes (1,2, 5-9, 12-14, and 16) bound both bovine Fc IgG and sheep Fc IgG. Significant differences were not found between ST1, most frequently isolated in infections in cattle and ST2, isolated predominantly in sheep manheimiosis. The effect of various *M. haemolytica* growth conditions on IgBP(s) expression was assessed by 2-D gel electrophoresis analysis. Membrane expression of *M. haemolytica* IgBP(s) was increased three-fold when *M. haemolytica* was grown in RPMI-
1640 with 10%FBS and in RPMI-1640 at 40°C and increased two-fold when grown in RPMI-1640 with 50 µM 2,2'-dipyridyl when compared to growth in RPMI-1640 at 37°C. The IgBP(s) constituted up to 19.9% of *M. haemolytica* membrane proteins when *M. haemolytica* was grown in RPMI at 40°C. It is a highly expressed protein when *M. haemolytica* is grown under conditions mimicking factors of the *in vivo* environment. This study shows a possible role in virulence for the *M. haemolytica* IgBP. It is immunogenic, surface expressed and as an IgBP it may enable *M. haemolytica* to evade the host immune response and may play a role in colonization through binding fibronectin or fibrinogen.
CHAPTER 1. GENERAL INTRODUCTION

Bovine respiratory disease (BRD) or "shipping fever" is believed to result in the single largest loss annually to the cattle industry. BRD has a complex etiology but pulmonary fibrinous pneumonia, the ultimate cause of severe clinical disease and death, is primarily caused by *Mannheimia haemolytica* (Houghton and Gourlay, 1984). *M. haemolytica* serotype ST2 is a normal flora bacteria of the upper respiratory tract (URT) in cattle but on occurrence of a stressful incident such as viral infection or shipping from farm to feedlot, isolates of *M. haemolytica* shift from ST2 to ST1 and can grow explosively colonizing the URT (Frank 1979; Frank and Smith, 1983). The bacteria are believed to be inhaled into the lung resulting in macrophage activation and pulmonary serum flooding. An influx of neutrophils along with an *M. haemolytica* secreted leukotoxin exacerbates the response. This leads to lung lesions characteristic of *M. haemolytica*-induced fibrino-hemorrhagic pneumonia. Some animals are able to recover while others succumb to pneumonic mannheimiosis (Whitely et al., 1992).

A number of vaccines are available for *M. haemolytica* induced BRD. Bacterins provide protection of variable efficacy. A number of experimental trials have even shown an exacerbation of BRD in bacterin vaccinates (Friend et al., 1977; Wilkie et al., 1980). While live vaccines generally perform better in field-tests than bacterins (Confer et al., 1985b), live vaccines still have variable efficacy with some trials showing no increased protection due to immunization (Purdy et al., 1986). Live vaccines have inherent problems with production and stability. While not providing full protection, bacterial component vaccines have demonstrated the importance of leukotoxin (Conlon et al., 1991), iron binding proteins
(Potter et al., 1999) and surface antigens (Shewen et al., 1988) in protection. There is a need for a more efficacious vaccine.

*M. haemolytica* expresses a number of known virulence factors. The best characterized is a leukotoxin (Kaehler et al., 1980), a member of the RTX toxin family. Other virulence factors include the capsular polysaccharide (Carter, 1956), LPS (Rimsay et al., 1981), fimbrae (Morck et al., 1987; Potter et al., 1988), iron-regulated proteins, (Donachie and Gilmour, 1988), neuraminidase (Frank and Tabatabai, 1981) and O-sialoglycoprotease (Abdullah et al., 1992). This study demonstrated the presence of an additional *M. haemolytica* virulence factor, an immunoglobulin-binding protein (IgBP).

IgBPs have been identified in a number of other Gram-positive and Gram-negative bacteria. Surface expressed protein A from *Staphylococcus aureus* Cowan 1 is the first IgBP identified (Forsgren and Sjöquist, 1966). Protein A is known inhibits phagocytosis and IgBP surface expression is responsible for this inhibition (Dosset et al., 1969). Protein A in solution fixes complement by forming IgG:protein A complexes (Langone et al., 1978) and to inhibit antibody-dependent cellular cytotoxicity (Rosenblatt et al., 1977). Acting as a B cell superantigen, protein A activates complement (Kozlowski et al., 1996), induces expression of Ig from the \( V_\text{H}3 \) gene family (Kristiansen et al., 1994), increases antigen presentation through soluble protein A:Ig complexes associated with \( V_\text{H}3 \) family surfaced expressed immunoglobulins (Leonetti et al., 1999) and is involved in inflammation as demonstrated by the elicitation of an Arthus reaction through administration of soluble protein A:Ig complexes (Kozlowski et al., 1998). *In vivo* experiments indicate a role for IgBPs in virulence. Mice injected with an *S. aureus* protein A deletion mutant require a marginally higher dose of the mutant for infectivity and take significantly longer to succumb.
to infection and die at a slightly decreased rate than mice infected with *S. aureus* expressing protein A (Patel et al., 1987). These results may have been more dramatic if a second more recently identified *S. aureus* IgBP, Sbi, had also been deleted (Zhang et al., 1998). These experiments demonstrate a role for IgBPs in virulence although their role has not been clearly elucidated.

Most bacteria initiate infection by binding to host cells. Bacterial cell surface components adhere to host cell components or to host extracellular matrix (ECM) proteins that bind host cells and act as a bridge between bacteria and host cells. Extracellular matrix-binding proteins (ECMBPs) from *Staphylococcus aureus* are best characterized and include proteins that bind fibronectin (Fn), collagen (Cn) and a secreted protein that binds fibrinogen (Fg) (Flock, 1999). A number of Gram-negative organisms express ECMBPs including *Eschericia coli* (Westerland et al., 1989), *Haemophilus ducreyi* (Abeck et al., 1992), *H. influenza* (Fink et al., 2002), *Neisseria gonorrhoeae* (van Putten et al., 1998) and *Borellia burgdorferi* (Probert and Johnson, 1998). *In vitro* models demonstrate several additional roles for ECMBPs in virulence. In the presence of Fn and an anti-CD3 monoclonal antibody, *S. aureus* fibronectin-binding protein A (FnbpA) mediates adhesion to T cells and T cell costimulation through the T cell αβ integrin, which binds fibronectin and functions as a costimulatory molecule, leading to T cell activation (Miyamoto et al., 2001). A T helper type 2 inflammatory environment promotes *S. aureus* binding to skin and this binding is mediated by Fn and Fg (Cho et al., 2001a). ECMBPs appear to have a variety of roles in virulence.

The surface components used by *M. haemolytica* to adhere to airway epithelial cells are not known. Two types of fimbriae, a large, rigid fimbriae (Morck et al., 1987) as well a smaller, flexible fimbriae (Potter et al., 1988) have been reported. However, several
investigators have been unable to demonstrate the presence of fimbrae on newly isolated M. haemolytica ST1 using the same procedures (Gonzalez and Maheswaran, 1993) and fimbrial adhesion has not been demonstrated for M. haemolytica. Identification of an M. haemolytica adhesive surface component would open the door to development of a treatment that could decrease colonization and result in reduced development of pneumonic pasteurellosis.

The mechanism for the abrupt change from the predominance of the normal flora M. haemolytica ST2 to explosive growth of stress-associated ST1 is not known. However, change in host environmental factors due to stress is likely to play a role. In an effort to recreate in vivo protein expression while using in vitro systems and also to define culture conditions that affect expression of particular proteins, a number of studies have been done to define protein expression under different culture conditions. Pathogens are known to up-regulate or down-regulate expression of adhesive components under varying conditions of the host environment (Finlay and Falkow, 1989; Francis et al., 1989; and Mekalanos, 1992). Shewen and Wilkie (1982) reported that the addition of 7% fetal bovine serum (FBS) to RPMI-1640 tissue culture media achieves enhanced M. haemolytica leukotoxin production compared to tissue culture media alone. Iron-regulated outer membrane proteins of 71, 77 and 100 kDa are expressed at higher levels in iron-restricted media (Deneer et al., 1989; Morck et al., 1991). No capsular polysaccharide production is detected when M. haemolytica is grown above 40°C (Puente-Polledo et al., 1998). Establishing in vitro growth conditions that closely mimic in vivo conditions may increase the efficacy of vaccines prepared from in vitro M. haemolytica cultures.
Preliminary results in our lab indicated that *M. haemolytica* might express an IgBP(s). When normal bovine serum (NBS) is used as a negative control to label whole cell *M. haemolytica* in an ELISA, similar absorbance values are obtained with the NBS and with an anti-sera specific for an *M. haemolytica* iron-regulated protein (L.B. Tabatabai, unpublished observations). The objectives of the present study were: 1) To identify and isolate an *M. haemolytica* IgBP(s); 2) To determine the presence or absence of IgBP surface expression; 3) To determine whether there is an increased antibody response to the IgP in cattle convalescing from BRD; 4) To determine whether the IgBP may play a role in the dominance of ST1 association with BRD (Wessman and Hilker, 1968 and Frank, 1979) or the dominance of ST2 in sheep mannheimiosis (Fraser, 1982); 5) To establish whether the IgBP binds one or more ECMPs; and 6) To assess whether this protein would be up-regulated when grown under *in vitro* growth conditions that more closely mimic factors of the *in vivo* environment.

**Dissertation Organization**

This dissertation consists of four chapters. Chapter 1 is a general introduction and review of the literature on *Mannheimia haemolytica*, immunoglobulin-binding proteins and extracellular matrix-binding proteins. Chapter 2 and 3 consist of two papers. The first paper, “Identification, isolation and characterization of an immunoglobulin-binding protein expressed by *Mannheimia haemolytica*” is to be submitted for publication in the journal Infection and Immunity. The second paper, “Fibrinogen- and fibronectin-binding of a *Mannheimia haemolytica* immunoglobulin-binding protein differentially expressed *in vitro*,” will also be submitted to Infection and Immunity. Chapter 4 consists of a general discussion
and conclusions. References for the general introduction and the literature review are in alphabetical order and follow the literature review. References for each paper follow the discussion section of each paper while the references for the general discussion and conclusion references follow that section.

**Literature Review**

*Mannheimia haemolytica*

**Taxonomy**

Whether Perroncito in 1878, Pasteur in 1880, or Kitt in 1885, was the first to isolate and describe the bacillus associated with fowl cholera is not clear. Bacilli with similar characteristics were associated with diseases of rabbits, swine and cattle and with fowl cholera. These diseases were characterized as being associated with hemorrhagic septicemia. Italian Count Trevisan first proposed the genus name *Pasteurella* (Holmes et al., 1999) for these organisms in honor of Pasteur's work on fowl cholera. After a series of name changes, Newsom and Cross (1932) proposed that organisms associated with bovine hemorrhagic septicemia and pneumonia be given the name *Pasteurella haemolytica*. Shirlaw (1938) termed the disease bovine pasteurellosis and Smith (1961) described two biotypes of *P. haemolytica* that he termed A and T. These letters stood for arabinose and trehalose fermentation, respectively. Seventeen serotypes (ST) have been defined (Biberstein et. al, 1960; Fodor et al., 1988; Pegram et al., 1979; Younan, 1995), twelve associated with biotype A and four associated with biotype T. The T biotypes were reclassified into a new species *Pasteurella trehalosi* in 1990 and include serotypes 3, 4, 10 and 15 (Bingham et al., 1990).
Through DNA-DNA hybridizations and 16S rRNA sequencing, Angen proposed reclassifying *Pasteurella haemolytica* organisms into the new genus *Mannheimia* (Angen, 1999). This now includes serotypes 1, 2, 5, 6, 7, 8, 9, 12, 13, 14, 16 and 17. *Mannheimia* belongs to the Pasteurellaceae family that consists of the genera *Haemophilus, Actinobacillus* and *Pasteurella*, the HAP organisms.

**Physical characteristics**

*M. haemolytica* are small, non-motile, pleomorphic, gram-negative, coccobacilli. The bacteria have a serotype specific capsular polysaccharide. Log phase cultures tend to be encapsulated while older cultures are generally not encapsulated (Corstvet et al., 1982). After multiple passages *in vitro*, *M. haemolytica* ST1 maintains capsular polysaccharide production (Gentry et al., 1987). There are no flagella on *M. haemolytica* but two types of fimbriae have been reported (Morck et al., 1987; Potter et al., 1988). When grown on ovine or bovine blood agar a narrow zone of β-hemolysis is seen and smooth gray colonies are seen.

**Bovine respiratory disease**

**Etiology and epidemiology.** Because BRD is brought on by multiple factors, the etiology of BRD is not always clear. However, it is generally agreed that *M. haemolytica* ST1 is the primary causative agent of BRD or shipping fever (Frank, 1979 and Confer, 1988). Serotype 1 is the predominant serotype isolated in BRD (Frank, 1979 and Frank et al., 1983) and is almost solely isolated from cattle that have died from BRD (Fox et al., 1971; Allan et al., 1985).
The incidence of BRD is associated with stress. Stress can be due to management practices such as shipping, weaning, crowding, comingling of herds, dehorning and castration. Viral infections are also considered to be predisposing factors in BRD. A study reveals that infectious bovine rhinotracheitis virus (IBR), bovine viral diarrhea virus (BVDV) parainfluenza type three virus (PI-3) and bovine bovine respiratory syncytial virus (BRSV) are associated with respiratory disease (Martin and Bohac, 1986). The greatest incidence of disease occurs in shipping yards and feedlots following shipment of animals; thus BRD is also called shipping fever. Dairy cattle are also known to have the disease. Serotype 2 is most frequently isolated from *M. haemolytica*-infected sheep (Fraser et al., 1982).

**Pathogenesis.** *M. haemolytica* serotype (ST2) is a normal flora bacteria of the upper respiratory tract (URT) in cattle but on occurrence of a stressful incident such as viral infection or shipping from farm to feedlot, isolates of *M. haemolytica* shift from ST2 to ST1 and can grow explosively colonizing the URT (Frank 1979; Frank and Smith, 1983; Jones, 1987; Frank, 1988). The mechanism for the abrupt change from the predominance of the normal flora *M. haemolytica* ST2 to explosive growth of stress-associated ST1 is not known. However, change in host environmental factors due to stress is likely to play a role.

The mechanism used by *M. haemolytica* to colonize the URT is also not clear. The bacteria may adhere to mucosal epithelial cells or proliferate within the mucous layer. Fimbrae have been reported *in vitro* on *M. haemolytica* (Morck et al., 1987, Potter et al., 1988), but fimbrae are not found in *M. haemolytica* isolated from the lung or URT of cattle (Gonzalez et al., 1993). In addition, their role in attachment has not been demonstrated. A number of Gram-negative bacteria express adhesins that are distinct from fimbrae (Minion et
al., 1986; Mooi et al., 1992). Healthy or non-stressed cattle make use of the mucociliary ladder to clear bacteria from the URT. However, in humans, the mucociliary ladder is impaired in Gram-negative bacterial pneumonia (Pavia, 1987). Viral infections may kill ciliated epithelial cells or alter cell ciliary function. Neuraminidase, expressed by *M. haemolytica* ST1 (Frank and Tabatabai, 1981) decreases the viscosity of bovine respiratory mucus (Milligan et al., 1978). Decreased viscosity could inhibit the clearance of *M. haemolytica*. Beyond the aforementioned possibilities, *M. haemolytica* may multiply so rapidly that clearance mechanisms are overwhelmed. It is hypothesized that the large number of *M. haemolytica* in the URT is then inhaled into the lung in aerosolized droplets.

In healthy cattle, macrophage activation initiates an inflammatory response and along with a humoral immune response, the bacteria are cleared. However, in immunologically compromised cattle, the immune response is ineffective at clearing the bacteria and the inflammatory response itself becomes part of the pathology. Activation of alveolar macrophages by bacterial lipopolysaccharide (LPS) and leukotoxin (Lkt) results in secretion of Interleukin-1 (IL-1), tumor necrosis factor alpha (TNFα) and Interleukin-8 (IL-8), proinflammatory cytokines (Yoo et al., 1995; Lafleur et al., 2001). Caswell (2001) demonstrated that IL-8 is an important neutrophil chemoattractant found in bronchoalveolar lavage (BAL) from calves with pneumonic pasteurellosis. After aerosol exposure of calves to *M. haemolytica*, lung lavage fluid contains an influx of neutrophils 30 minutes after exposure and by 60 minutes there is an equal percentage of alveolar macrophages and neutrophils. Four hours post-infection, more than 90% of cells are neutrophils (Walker et al., 1985). In separate experiments macrophages from BAL fluid express IL-1, TNFα and IL-8 within the first four hours after infection with IL-8 being expressed to the greatest extent. By
eight hours post-infection the neutrophils are the predominant source of IL-8 in BAL fluid (Malazdrewich et al., 2001). Neutrophils are stimulated by low concentrations of an \textit{M. haemolytica} secreted Lkt to undergo a respiratory burst that results in the release of superoxide anion, hydrogen peroxide (Czuprynski et al., 1991) and lysosomal proteolytic enzymes (Styrt et al., 1990). The result of the release of oxygen products and proteases is tissue injury and necrosis. An indication of the central role neutrophil activation plays in lesion development is shown in experiments in which neutrophil depletion reduced lesion severity (Breider et al., 1988). The lipid A portion of LPS is known to be able to activate the classical complement pathway (Morrison and Rudback, 1981) while the polysaccharide component activates the alternate complement pathway (Morrison and Ulevitch, 1978). Products of complement activation contribute to the inflammatory response.

Characteristic lesions of pneumonic Mannheimiosis and associated lung damage are believed to occur as a result of host-pathogen interactions and an inflated inflammatory response. Typical lesions of shipping fever involve alveolar edema, hemorrhage, neutrophilic and fibrinous exudates and pulmonary vascular thrombosis. Inflammatory cells and fibrous tissue surround focal areas of coagulation necrosis. Within these lesions are characteristic “streaming macrophages” (Jubb et al., 1985). Experiments by Reeve-Johnson et al. (2001) noted higher clinical scores in calves with temperatures of 39.9 to 41.0°C compared to normal temperatures of 39.2°C. Death is thought to result from hypoxia or toxemia.
Virulence factors

A number of virulence factors have been identified in *M. haemolytica* induced BRD. The role of some virulence factors is well studied while the mechanism of action of others remains to be determined. The role of several virulence factors are deduced from the role of similar virulence factors in other organisms. The most well characterized virulence factors are the leukotoxin and lipopolysaccharide.

**Leukotoxin (Lkt).** In 1978, it was determined that *M. haemolytica* is cytotoxic for bovine leukocytes (Benson et al., 1978). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) determined its molecular weight to be 101-105 kDa (Chang et al., 1987). Shewen and Wilkie (1982) showed that Lkt is specific for ruminant neutrophils, monocytes, macrophages and lymphocytes. It is produced during logarithmic phase but not in the stationery phase of growth (Shewen and Wilkie, 1985). In 1987 the gene was cloned and sequenced (Lo et al., 1987). Leukotoxin is a member of the RTX family of cytolysins that are pore-forming toxins (Whitely et al., 1992). Reports also demonstrate that the Lkt can apoptotically induce cell death (Wang et al., 1998).

The activity of the Lkt is responsible for a great deal of the pathological effects of BRD. One group of researchers showed that subcytolytic concentrations of Lkt stimulate activation of neutrophils which results in a respiratory burst that releases superoxide anion and hydrogen peroxide. This is followed by degranulation and cytolysis (Maheswaran et al., 1992). Release of these oxygen products and proteolytic enzymes would be expected to cause tissue necrosis. High concentrations of Lkt are lytic for ruminant leukocytes. More study is necessary to determine whether low or high concentrations of Lkt are more
detrimental to the host. Other activities of Lkt include stimulating the release of IL-1 and TNFα from macrophages (Yoo et al., 1995) and histamine from mast cells (Adusu et al., 1994). These cytokines are known to contribute to increased capillary permeability, neutrophil influx and the inflammatory response associated with BRD.

**Lipopolysaccharide (LPS).** *M. haemolytica* LPS induces a complex set of responses from a variety of cell types. Endotoxin from *M. haemolytica* LPS is released into the inflammatory exudate and is localized in neutrophils, macrophages, endothelial cells and epithelial cells (Whitely et al., 1990). The interaction of endotoxin and cells leads to cell activation or cell death. Some of the actions mediated by endotoxin include but are not limited to coagulation, inflammation and release of oxygen products. Lipopolysaccharide activated endothelial cells and macrophages produce a wide variety of proinflammatory and procoagulant mediators (Gartner et al., 1988; Cotran, 1987; Russo, 1980; Tipping et al., 1988). Alveolar macrophages produce toxic oxygen radicals and proteases as well as nitric oxide in response to *M. haemolytica* LPS. (Werb, 1983; Dyer et al., 1985; Yoo et al., 1996). Interestingly, Lafleur et al. (2001) reported a synergistic response between LPS and Lkt in inducing cytolysis and inflammatory cytokine (TNFα and IL-8) production in bovine alveolar macrophages. LPS primes neutrophils for release of toxic oxygen products and lysosomal enzymes. Even though LPS effects are multifaceted, antibodies to LPS are not associated with protection (Confer et al., 1988).

**Capsular polysaccharide (CPS).** The *M. haemolytica* CPS has been shown to augment the ability of the bacteria to resist phagocytosis and can hinder complement-mediated lysis (Confer et al., 1990b; Czuprynski et al., 1991). However, recent construction
of an acapsular mutant demonstrates that the mutant is as resistant to complement-mediated killing in colostrum-deprived calf serum as the encapsulated parent strain. Nonetheless, it is more sensitive to killing by immune bovine serum (McKerral and Lo, 2002). Barallo et al. (1999) observed that production of the CPS in ST2 is strictly temperature regulated and that no production is detected above 40°C.

**O-sialoglycoprotease.** In 1992, Abdullah recognized that *M. haemolytica* produces a 35-kDa O-sialoglycoprotease found in the culture supernatant that cleaves O-glycosylated proteins but not N-glycosylated proteins. Antibodies to this enzyme are found in bovine sera (Lee et al., 1994). Tabatabai (1981) observed that fetuin is a substrate for the *M. haemolytica* O-sialoglycoprotease and can be used as an alternative substrate to identify the protease activity. *M. haemolytica* O-sialoglycoprotease enhances bovine platelet adhesion which would indicate a role in thrombosis during mannheimiosis (Nyarko et al., 1998).

**Neuraminidase.** Neuraminidase is implicated as a virulence factor in bacteria. When sialic acid residues are removed from salivary glycoproteins, salivary secretions are less able to protect against pathogenic bacteria (Gottschalk, 1960). This would enhance the ability of microorganisms to survive *in vivo*. Neuraminidase production was first demonstrated in *M. haemolytica* by Scharmann et al. (1970). Frank and Tabatabai (1981) characterized the production of neuraminidase by different *M. haemolytica* serotypes and isolates. They also reported that antiserum to *M. haemolytica* is capable of neutralizing neuraminidase activity. Straus and Purdy (1995) established that neuraminidase is produced *in vivo* in market stressed cattle.
**Fimbriae.** In order for rapidly multiplying *M. haemolytica* to colonize the nasopharynx the bacteria must either adhere to mucosal epithelial cells or proliferate within the mucous layer. Fimbriae are associated with epithelial cell adherence in a number of bacteria and Potter et al. (1988) and Morck et al. (1987) reported *M. haemolytica* associated fimbrae. Two types of fimbrae, a large, rigid fimbrae (Morck et al., 1987) as well a smaller, flexible fimbrae (Potter et al., 1988) have been reported but fimbrae have are not found in *M. haemolytica* isolated from the lung or URT of cattle (Gonzalez et al., 1993). In addition, their role in attachment has not been demonstrated. A number of Gram-negative bacteria express adhesins that are distinct from fimbrae (Minion et al., 1986; Mooi et al., 1992). It is possible that an unknown virulence factor or other known virulence factors such as neuraminidase play a role in adherence so that the involvement of fimbrae may not be essential for colonization.

**Iron-regulated proteins.** The host sequesters iron during infection whereas organisms up-regulate the expression of iron-regulated proteins for iron-uptake when the organism is in an iron-restricted environment such as the lung (Donachie and Gilmour, 1988; Confer et al., 1995). Iron is essential in a variety of cellular functions for both the host and an invading organism. *M. haemolytica* expresses iron-regulated proteins, both outer membrane proteins (Donachie and Gilmour, 1988; Lainson et al., 1991; Confer et al., 1995) termed transferrin binding protein A and B (TbpA and TbpB) and periplasmic proteins (Lainson et al., 1991; Tabatabai and Frank, 1997) termed ferric binding protein A and B (FbpA and FbpB). TbpA and TbpB are receptors for transferring iron (Ogunnariwo and Schryvers, 1990; Gray-Owen, 1996) while FbpA and FbpB function as iron-binding and
iron-transport proteins (Gray-Owen and Schryvers, 1996; Kirby et al., 1998). Davies et al. (1992) showed that expression of outer membrane iron-regulated proteins increases when *M. haemolytica* are grown under iron-restricted conditions and that this increase occurs only in disease-associated strains. Belzer et al. (2000) reported that convalescent calves have high antibody titers to the 35 kDa FbpA. The native FbpA does not cross-react with other *Pasteurella trehalosi* serovars (Tabatabai and Frank 1997).

**Vaccines**

An ideal vaccine would provide long-term protection against disease, be easily administered and have no adverse reactions. A significant degree of protection from pneumonic mannheimiosis is achieved through passive transfer of humoral immunity (Mosier et al., 1995) and calves challenged with *M. haemolytica* after an experimental infection shows no clinical signs of disease (Cho and Jericho, 1986). These results indicate that protection from an *M. haemolytica* challenge is possible. However, the history of vaccine development for *M. haemolytica*-induced BRD has shown that this is not an easy task and full protection for all animals may never be achieved. Although a number of important virulence factors are identified, none of them alone has been sufficient to provide protection. A variety of vaccine formulations have been tested including bacterins, live vaccines and subunit vaccines. Protection is variable. Often there is a significant difference in the efficacy of a particular vaccine between experimental trials and field trials and repetition of results from individual trials has been inconsistent. Experimental trials are hampered by a lack of a suitable laboratory animal model of BRD. Trials are limited by animal size and availability of accommodations keeping many trials to a small number of
animals. More knowledge about the pathogenesis of pneumonic mannheimiosis may be necessary for development of a consistently effective vaccine.

**History.** Early vaccine effectiveness was limited due to confusion over which organisms to incorporate into a vaccine. Vaccines included *M. haemolytica* and *P. multocida* as well as the viruses infectious bovine rhinotracheitis (IBR) and parainfluenza-3 (PI-3), individually and in various combinations. *M. haemolytica* alone was shown to produce fibrinous pneumonia which reduced the focus on bacterial vaccines to *M. haemolytica* (Confer et al., 1989). Because virally induced stress is associated with BRD, viral vaccine formulations remain an area of experimentation. Initial vaccines consisted of bacterins, viral vaccines or bacterin-viral combinations. None of these provided consistent protection. In the 1980s live vaccines at times were successful although at times no effect due to vaccination was found (Purdy, et al., 1986). More recently, efforts have been focused on subunit vaccines incorporating known virulence factors and bacterial cell extracts.

**Bacterins.** Bacterin vaccines in different trials are shown to be somewhat protective (Mosier, 1989), have no effect (Confer et al., 1985b) or at other times result in more severe lesions (Friend et al., 1977; Wilkie et al., 1980). Confer et al. (1985) showed that while no effect is found with a bacterin vaccine, a live vaccine in that same trial results in significant reduction in pulmonary lesions. The focus for vaccine production shifted to live vaccines.

**Live vaccines.** During the 1980s, the protective effect of live and modified-live vaccines was explored. In an experimental transthoracic challenge of calves following vaccination with either a bacterin or a live *M. haemolytica* vaccine, little reduction in mean
lesion scores resulted from a formalin-killed bacterin vaccination (Confer et al., 1985). However, a live *M. haemolytica* vaccine grown on brain-heart infusion agar results in at least a four-fold reduction in lesion scores when compared to control animals. Although nearly 60% of the bacterin-treated calves had antibody titeres that were as high or higher than live-vaccinates, no correlation is found between these antibody titers and resistance to infection. A similar effect was seen in experiments by Panciera et al. (1984). However, Purdy et al. (1986) found no significant effect on performance, morbidity or mortality from a live, freeze-dried vaccine in field trial conditions. A modified-live lyophilized *M. haemolytica* vaccine trial resulted in greater than a two-fold reduction in lesion scores (Blanchard et al., 1987). The reduction in protection afforded by modified-live vaccines compared to live vaccines may be due to the loss of antigens important for protection or the presence of replicating *M. haemolytica* may be necessary. The effectiveness of live vaccines is limited by the concurrent administration of antibiotics and is sensitive to improper storage and administration of the vaccine preparations.

**Subunit vaccines.** More recent vaccination efforts have focused on subunit vaccines. High antibody titers to various antigens of *M. haemolytica* correlate with protection. High antibody responses to both the leukotoxin and the CPS correlate with protection in cattle (Confer et al., 1985a). As more is learned about the pathogenesis of pneumonic mannheimiosis and the role of different virulence factors, it is anticipated that vaccines incorporating or enriched in known virulence factors will provide better protection.

Presponse is a commercial vaccine incorporating an enriched leukotoxin fraction with culture supernatant. The culture supernatant contains ST1 specific antigens and other soluble
capsular material. A number of experiments involving Presponse show an approximately two fold increase in protection following Presponse vaccination (Shewen and Wilkie, 1989; Shewen et. al., 1988; Jim et. al., 1988). In one experimental trial, a vaccine incorporating Presponse enriched with recombinant Lkt is more efficacious than Presponse alone (Conlon et al., 1991).

A vaccine composed of outer membrane proteins of \textit{M. haemolytica} with no leukotoxin resulted in lesion scores similar to scores found with live vaccinates (Morton, et al., 1995). This argues against the importance of Lkt in vaccine formulations. Gilmour et al. (1991) and Potter et al. (1999) demonstrated the importance of iron-regulated proteins in protection. Incorporation of iron-regulated proteins in a sodium salicylate extract vaccine in sheep results in almost complete protection (Gilmour, 1991). Vaccination with iron-regulated proteins TbpA and TbpB alone resulted in a fourfold decrease in clinical scores compared to unvaccinated calves (Potter et al., 1999). There is also experimental evidence for a role for the CPS in protection of goats (Purdy et al., 1993). Vaccines containing \textit{M. haemolytica} chemical extracts show some promise for protection. A recent experiment involving an \textit{M. haemolytica} sodium salicylate extract enriched with LPS, Lkt, iron-regulated outer membrane proteins and CPS resulted in mean clinical lesion scores similar to that found with live vaccines (Sreevatsan et al., 1996). Subunit vaccines avoid the inherent problems a live vaccine presents and appear to hold promise in deriving an effective vaccine.

\textbf{Growth condition dependent protein expression.} Pathogenic bacteria may express different components when grown \textit{in vivo} rather than \textit{in vitro} (Davies et al., 1994). In an effort to recreate \textit{in vivo} protein expression while using \textit{in vitro} systems and also to define
culture conditions that affect expression of particular proteins, a number of studies have been done to define protein expression under different culture conditions. Conventional media for \textit{in vitro} growth is usually selected for its ability to support \textit{M. haemolytica} growth. Shewen and Wilkie (1982) reported that the addition of 7% fetal bovine serum (FBS) to RPMI-1640 tissue culture media achieves enhanced \textit{M. haemolytica} leukotoxin production compared to tissue culture media alone. This may be due to the presence of iron in FBS, because Gentry et al. (1986) demonstrated that the addition of iron compounds to culture media results in leukotoxin production levels comparable to FBS induced leukotoxin production levels. Iron-regulated outer membrane proteins of 71, 77 and 100 kDa are expressed at higher levels in iron-restricted media (Deneer et al., 1989; Morck, 1991). Increased expression levels of outer membrane proteins (OMPs) are found in \textit{M. haemolytica} grown in decomplemented fetal calf serum (Davies et al., 1992).

In another study, \textit{M. haemolytica} OMP protein expression was compared between bacteria that were isolated from lungs of experimentally infected calves to bacteria cultured under various \textit{in vitro} growth conditions. OMP expression profiles on SDS-PAGE are similar for \textit{M. haemolytica} isolated from lungs and \textit{M. haemolytica} grown in newborn calf serum (Davies et al., 1994). No CPS production is detected when \textit{M. haemolytica} is grown above 40°C. When \textit{M. haemolytica} is grown at 43°C, enzymes involved in CPS biosynthesis are found at a level at least 25 times lower than \textit{M. haemolytica} grown at 37°C (Barallo, 1999). Establishing \textit{in vitro} growth conditions that closely mimic \textit{in vivo} conditions may increase the efficacy of vaccines prepared from \textit{in vitro} \textit{M. haemolytica} cultures.
Immunoglobulin-binding proteins (IgBPs)

Immunoglobulin-binding proteins are expressed by a number of bacteria and bind immunoglobulins non-specifically, typically on the Fc or Fab portion of the molecule. They are believed to be virulence factors.

**History.** The phenomenon of bacterial surface proteins binding to immunoglobulins in a nonspecific manner was described well before the basis for this reactivity was understood. Jensen (1958) demonstrated reactivity between *Staphylococcus aureus* and 500 normal human serum samples. This reactivity does not correlate with previous known exposure to the bacteria. He termed this a kind of natural immunity or pseudoimmune reactivity and called the bacterial surface antigen involved antigen A. In another demonstration of antigen A reactivity, Cohen et al. (1961) found *S. aureus* binds rabbit sera with no known previous exposure to the bacteria. Antigen A was termed protein A after its protein nature was determined by digestion with trypsin (Grov et al., 1964). Sjöquist combined new found biochemical knowledge of immunoglobulins with work to define the antigens on the surface of *S. aureus* and along with Förgren determined protein A binds to the Fc portion of IgG (Förgren and Sjöquist, 1966). In their research, gel filtration purified protein A and papain digested IgG Fc fragments precipitated in an agar gel Ouchterlony plate. Until recently, bacterial proteins that bind immunoglobulins were termed Fc receptors. More recently, they have been termed immunoglobulin-binding proteins (IgBPs) in recognition of the fact that they have no signaling capability as other cell surface receptors have.
**Protein A.** Further characterization of protein A showed that it is a cell surface protein (Sjöquist et al., 1972) with a molecular weight of 42-kDa and has a distinct extended shape (Bjorck et al., 1972). Protein A is antiphagocytic and chemotactic *in vitro* (Dosset et al., 1969), and IgG complexed with protein A activates complement via the classical pathway (Sjöquist and Stålenheim, 1969). The discovery of extracellular protein A in methicillin-resistant *S. aureus* (Lindmark et al., 1977) led to the development of a method for purification and production of soluble protein A. Work focused towards the widespread utilization of protein A as an immunochemical reagent in detecting and isolating antibodies and little attention was paid to its role in *S. aureus* infections.

**Immunoglobulin-binding protein classification.** An IgBP was discovered on the surface of β-hemolytic streptococci. This protein is called protein G and it is a cell surface expressed and secreted IgBP associated with groups A, C, and G streptococci (Kronvall, 1973). Protein G, like Protein A, is an elongated molecule (Åkerstrom and Björck, 1986). The discovery of protein G led to the concept that proteins on the surface of bacteria that nonspecifically bind immunoglobulins may be a general feature of bacteria-host interactions. Following this finding a systematic study determined that many Gram-positive bacteria express IgBPs that bind Ig from a variety of Ig sources. Myhre and Kronvall (1977, 1980, 1981), and Myhre et al. (1979) described five different types of IgBPs based on functional binding properties of bacteria to Igs from different species and antigenic relatedness. In 1988, Reiss identified a sixth type of IgBP based on these same criteria (Reiss, 1990).

Immunoglobulin binding proteins have been identified on other Gram-positive as well as Gram-negative bacteria. *Coprococcus comes* (Van de Merwe and Stegeman, 1985),
Haemophilus somnus (Widder et al., 1988), Brucella abortus (Bricker et al., 1991), Actinobacillus actinomycetemcomitans (Mintz et al., 1994), Aeromonas salmonicida (Phipps and Kay, 1988) and Escherichia coli (Sandt et al., 1997) are all known to express IgBPs. No published information is available that describes classification of IgBPs from gram-negative organisms.

**Immunoglobulin-binding protein specificity.** IgBPs bind immunoglobulins other than Fc IgG. Protein L from Peptostreptococcus magnus binds κ light chains (Bjorck, 1988) and protein A and protein G bind Fab and F(ab')₂ respectively (Boyle, 2000). Clostridium perfringes expresses an IgBP termed protein P that also binds F(ab')₂ fragments (Boyle, 2000). Neisseria catarrhalis, group A, C and G streptococci, Haemophilus influenzae (Forsgren and Grubb, 1979), Haemophilus haemolyticus and Haemophilus aegypticus (Akkoyunlu, 1991) all bind human IgD. M proteins sir (Stenberg, 1994) and arp (Heden and Lindahl, 1993) and a specific IgA receptor on group B streptococci (Lindahl, 1990) bind human IgA. Protein sir also binds IgM. Additional specificity for IgM has been reported for B. abortus (Nielsen, et al., 1981), and Borrelia burgdorfori (Dorward, et al., 1992).

Immunoglobulins are not the only proteins IgBPs bind. While it has been known since 1987 that protein G binds albumin and IgG (Björck, 1987), more recent work has shown that other IgBPs bind other proteins. Specificity for binding plasminogen (Berge and Sjöbring, 1993), C₄-binding protein (Thern, 1995) fibrinogen and fibronectin (Reichardt, et al., 1995) and factor H (Kotarsky, 1998) has been demonstrated. Protien G has separate binding sites for IgG and albumin (Frick et al., 1994, 1995). The Fc binding portions of protein G bind two plasma proteinase inhibitors, alpha-2 macroglobulin and kininogen.
Binding for all three proteins apparently occurs within the IgG binding domains (Björck et al., 1987; Sjöbring et al., 1989)

**Immunoglobulin-binding proteins and virulence.** Fe-mediated antibody binding to the surface of IgBP expressing bacteria has provided the classical picture for a role for IgBPs in virulence. Antibody bound to the surface of bacteria would provide a means for bacteria to evade the host immune response by inhibiting phagocytosis and complement-mediated killing. Additionally, soluble IgBPs bound to antibodies would decrease the number of specific antibodies available for a humoral immune response. While these effects have been demonstrated *in vitro*, the role of IgBPs in virulence *in vivo* has been more difficult to demonstrate. But this does not preclude a role for IgBPs in virulence. Continuing research shows a greater IgBP role in virulence than had originally been conceived.

**Superantigens.** In 1979, it was established that protein A bound Fab IgG and that this binding took place outside the antigen-binding site (Endressen, 1979). Protein A binds the Fab portion of IgA, IgM and IgE (Inganas, 1981). Protein A binding is restricted to antibodies coded by the VH3 family of genes (Sasso, et al., 1991) and 32-54% of all circulating B cells are capable of this binding. Protein A is also capable of binding membrane bound immunoglobulins. This binding is similar to that found with T cell superantigens.

Protein A, acting as a B cell superantigen, activates complement (Kozlowski et al., 1996), induces expression of Ig from the VH3 gene family (Kristiansen et al., 1994), increases antigen presentation through soluble protein A:Ig complexes associated with VH3 family surfaced expressed immunoglobulins (Leonetti et al., 1999) and soluble protein A:Ig
complexes are involved in inflammation as demonstrated by the elicitation of an Arthus reaction (Kozlowski et al., 1998). Protein A and protein L, acting as superantigens, activate human heart mast cells by binding IgE on the surface of cells (Genovese, 2000).

Although these responses may appear to have a strong role in virulence, recently a perhaps more far reaching role has been found for protein A acting as a superantigen via suppression of $V_h3$ expressing B cells. Following intraperitoneal immunization of both neonatal and adult $V_h3$ “knock in” mice with protein A, researchers demonstrated a selective suppression in $V_h3$ expressing IgM secreting cells in both the spleen and liver and a loss of detectable $V_h3$ expressing B-1 lymphocytes in the peritoneum. This suppression is T cell independent and selectively suppresses a set of $V_h3$ antigen-specific natural antibodies. Mice treated with protein A are tolerant to a whole cell $S. pneumonia$ extract as measured by ELISPOT assay for specific IgM secreting cells. After cessation of protein A treatment, splenic lymphocytes (B-2 B cells) return to normal whereas the suppression of peritoneal lymphocytes (B-1 B cells) is still present 7-21 weeks later (Silverman et al., 2000). This study demonstrates a potentially very important role for IgBP involvement in virulence.

**Mitogens.** Protein A activates lymphocyte proliferation. This activation is limited to B cells with no T cell stimulation found. Optimal stimulation results when protein A is immobilized either on Sepharose or on the surface of $S. aureus$ (Forsgren, et al., 1976). Stimulation occurs with IgG, IgD and IgM bearing B cells (Romagnani, et al., 1980). Mitogenic activity of Protein A persists when tyrosil residues necessary for Fc IgG binding were inactivated and appeared to be due to Fab binding (Romagnani, et al., 1982).
Complement activation and inhibition. Early studies demonstrated that soluble protein A:IgG complexes fixes complement (Sjöquist and Stålenheim, 1969). Langone et al (1978) showed that protein A and IgG complexes in the molecular formula \[(IgG)_2 \text{protein A}_1\] act like IgM molecules in fixing complement and lysing erythrocytes. More recently, protein H was shown to form soluble complement-activating complexes that cleave C3 but to inhibit complement activation by Ig-coated target. Protein H inhibits C1q binding to IgG immobilized on polyacrylamide beads (Berge, 1997).

Antiphagocytes. In 1969, Dosset et al. showed that protein A on the surface of *S. aureus* inhibits phagocytosis and that this effect is dose dependent. This phenomenon has been demonstrated with a number of IgBPs and more recently was shown with protein H from *Streptococcus pyogenes* (Kihlberg et al., 1999).

Antibody-dependent cellular cytotoxicity inhibition. IgBPs have been shown to inhibit antibody-dependent cellular cytotoxicity. Rosenblatt (1977) showed that incubation of antibody-coated target cells with protein A inhibits lysis by lymphocytes.

Virulence role in vivo. The association of disease and IgBP expression has been difficult to show in vivo. This may be because of the complex nature of virulence and because even though an IgBP may have been identified in an organism, it may not be the only IgBP expressed by that organism. This was demonstrated in a protein A deficient mutant *S. aureus* infection. The mutants were constructed with site-directed allelic replacement. The protein A-deficient mutants are marginally less virulent than protein A expressing *S. aureus* as measured by death after intraperitoneal injections of bacteria in mice.
The mice injected with protein A expressing *S. aureus* die significantly more quickly than mice injected with the mutant (Patel et al., 1987). The mechanism for the reduction in virulence is not known and the results may have been more dramatic if a second IgBP *S. aureus* has recently been shown to express, Sbi, (Zhang, 1998) had also been deleted. Further evidence for IgBPs role in virulence was established in *H. somnus* infection in cattle. Pathogenic and carrier isolates were checked for immunoglobulin-Fc binding activity. All isolates express binding activity except for carrier isolates from the prepuce of bulls. None of these binds IgG-Fc (Widders, 1989). Finally, the immunoglobulin-binding domains of protein L form *P. magnus*, a pathogenic vaginal colonizer, were expressed in *Streptococcus godonii*, an oral commensal. The recombinant bacteria show increased murine vaginal colonization and persisted for a longer time in the murine vagina (Ricci et al., 2001). These experiments show a role for IgBPs in bacterial virulence.

**Therapeutic uses of immunoglobulin-binding proteins (IgBPs).** IgBPs fused to antigens increase immunogenicity. This was seen as an increased humoral immune response *in vivo* (Lowenadler et al., 1990) and *in vitro* as increased T cell presentation by antigen presenting cells expressing immunoglobulins on their surface (Leonetti, et al., 1998). Protein A has five Fc IgG binding sites (Hjelm et al., 1975 and Löfdahl et al., 1983) and increased immunogenicity correlates with an increased number of immunoglobulin-binding sites on protein A fused to antigen (Leonetti, et al., 1998). These phenomena may prove important because it increases immunogenicity without an adjuvant.

Protein A also has a number of biomodulatory effects. Protein A acts as a mitogen or induces apoptosis in the same cell depending on concentration. At a particular dose level, it
each with a molecular weight between 235-270 kDa. Each subunit is made up largely of three types of repeating modules, types I, II and III. A variable number of type III repeats contributes to the variability in subunit molecular weight. Fibronectin is a flexible, extended molecule. Fibronectin binds to cell surfaces and dimers polymerize to form fibrils. Cell adhesion is mediated by an arg-gly-glu (RGD) sequence of fibronectin that binds $\beta_1$ integrins. With continued polymerization, Fn fibrils extend into the ECM. Matrix formation is a cooperative process involving interactions between Fn and other ECM components such as collagens, laminin and proteoglycans. Addition of heparin and collagen to cultures has been shown to increase Fn binding to cells (McKeown-Longo and Mosher, 1985). Transforming growth factor-β enhances expression of Fn matrix assembly sites and this is accompanied by an accumulation of Fn fibrils (Allen-Hoffman et al., 1998). Fibronectin also contains binding domains for fibrin, heparin, and collagen.

**Fibrinogen.** Fibrinogen is a 340 kDa glycoprotein synthesized in hepatocytes. It is composed of three pairs of nonidentical chains that are linked by interchain disulfide bonds to form an Fg molecule. Fibrinogen is cleaved by thrombin to form fibrin and thus is involved in clotting. Fibrinogen also binds to a platelet receptor and mediates platelet adhesion and aggregation. Fibrinogen interacts with integrin receptors on endothelial cells (Cheresh, et al., 1989) and leukocytes (Altieri et al., 1990).

**Extracellular matrix-binding proteins**

**Identification.** Kuusela (1978) first reported that fibronectin would bind to *S. aureus*. Following this, two *S. aureus* Fn-binding proteins (FnBPs), FnbpA and FnbpB, were
identified (Signäs et al., 1989 and Jönsson et al., 1991). FnbpA is known to bind Fg as well as Fn. (Wann et al., 2000). ECMBPs of *S. aureus* are best characterized and along with FnbpA and FnbpB include surface expressed type II collagen-binding protein (CnBP), clumping factor A and B (ClfA and ClfB), Fg-binding proteins, and a secreted Fg-binding protein (Efb) (in Flock review, 1999). One *S. aureus* ECMBP, extracellular adherence protein (Eap), binds seven plasma proteins including Fn and Fg (Palma et al., 1999). Although *S. aureus* ECMBPs are the best characterized, a sizeable number of other organisms express ECMBPs. *Streptococcus* spp. express ECMBPs. In addition, a number of Gram-negative organisms including *Escherichia coli* (Westerland et al., 1989), *Haemophilus ducreyi* (Abeck et al., 1992), *H. influenza* (Fink et al., 2002), *Neisseria gonorrhoeae* (van Putten et al., 1998), and *Borellia burgdorferi* (Probert and Johnson, 1998) express ECMBPs. Recently, ECMBP research has increased as their role in virulence is becoming more apparent.

A number of IgBPs are known to also bind ECMPs. For example, Protein H, present in some strains of *S. pyogenes* and first known to bind Fc IgG, also binds fibronectin. A fibronectin binding protein also from *S. pyogenes*, SfbI, binds Fc IgG (Frick et al, 1995; Medina et al, 1999). A Group C streptococci expresses protein FAI which binds fibrinogen, albumin and IgG (Talay et al 1996). In addition, FgBP, a fibrinogen-binding protein of *Streptococcus equi*, has recently been shown to bind equine Fc IgG (Meehan et al, 2001).

**Virulence association.** Unlike IgBPs which were originally valued as reagents to isolate immunoglobulins, studies on extracellular matrix-binding proteins were focused on their role in virulence soon after these protein were identified. Although the mechanisms are
generally unclear, a number of roles for ECMBPs in virulence have been determined. Fibronectin-binding proteins in particular have been associated with virulence and once again, most work has been done with \textit{S. aureus}.

Exogenous Fn is required for internalization of \textit{S. aureus} by HEp-2 cells. HEp-2 cells are unable to produce Fn. In this same study, monoclonal antibody to $\beta_1$ integrins significantly reduced invasion which suggests a bridge is formed by Fn between an FnBP and $\beta_1$ integrins (Dziewanowska et al., 2000). Recent work by Dziewanowska (2002) demonstrated that invasion into human corneal epithelial cells is reduced by 99% with an FnBP-deficient isogenic strain of \textit{S. aureus}. Clearly, FnBPs play a role in \textit{S. aureus} internalization.

In relation to respiratory diseases, two sets of experiments illustrate the importance of \textit{S. aureus} FnBPs in bacterial adherence to human endothelial and airway epithelial cells. Mongodin et al. (2002) established that an FnBP-deficient strain of \textit{S. aureus} have a five-fold decrease in the level of adherence to human airway epithelial cells suggesting that FnBPs are involved in adherence. In 1999, Peacock et al., using a number of strains deficient in individual ECMBPss or protein A showed that only the mutant deficient in FnbpA and B production lowered adherence to endothelial cells. They conclude that their results indicate that Fnbp binding to endothelial cell Fn is the dominant pathway for \textit{S. aureus} adherence to human endothelial cells \textit{in vitro}. Bacterial cell adherence to host cells is a required initial step in most bacterial infections and ECMBPs, particularly FnBPs, may play a large role in this process or may be required for infection.

\textit{In vivo} and \textit{in vitro} models demonstrate several additional roles for ECMBPs in virulence. Both FnBP and ClfA, a fibrinogen-binding protein, enhance \textit{S. aureus} binding to
atopic skin as was found with *S. aureus* strains that did not produce one or the other of these proteins (Cho et al., 2001a). On human T cells, integrins α₄β₁ and α₅β₁ bind fibronectin and function as a costimulatory molecule for T cell activation. Miyamoto et al. (2001) established that *S. aureus* FnbpA through the T cell α₅β₁ integrin mediates adhesion to T cells and T cell activation *in vitro* in the presence of Fn and an anti-CD3 monoclonal antibody. In relation to this, *S. aureus* preferentially binds to skin sites of T helper-2-mediated sites of inflammation when compared to T helper-1-mediated sites of inflammation in a murine model. Fibrinogen and Fn mediate this binding (Cho et al., 2001b). An isogenic mutant unable to produce the Fn- and Fg-binding protein of *Streptococcus suis* ST2 decreases colonization of the joints and CNS of piglets while colonization of the tonsils is unaffected (de Greeff et al., 2002). The role of ECMBPs is not limited to adherence.

**Vaccine potential.** In 1999, Brennan et al. demonstrated that antibodies raised to one of three fibronectin-binding sites on *S. aureus* Fnbp are able to block adherence of *S. aureus* to Fn *in vitro*. Work previous to this showed that antibodies to Fnbp opsonize *S. aureus* as seen in an *in vitro* phagocytosis assay. Also, mice clear opsonized bacteria from the peritoneal cavity and liver more rapidly than controls (Rozalska and Wadstrom, 1993). Mamo et al. (1995) described a 1000 fold reduction in the number of opsonized *S. aureus* recovered from mammary glands of mice injected with opsonized bacteria as well as a significant reduction in pathology. Mamo et al. (1994) showed that mice vaccinated with an Fn-binding domain from *S. aureus* Fnbp have a decreased number of bacteria recovered from mammary glands as well as a significantly reduced incidence of severe mastitis. In an organism other than *S. aureus*, immunization with *Streptococcus pyogenes* fibronectin-
binding protein, FB54, results in mice that survive significantly longer than control mice. In the same study, an increased IgG titer to FB54 is increased in actively infected human patients compared to healthy volunteers (Kawabata et al., 2001). Hap is a broad-spectrum \textit{H. influenza} expressed ECMBP that binds fibronectin, laminin and collagen IV. Cutter, et al. (2002) demonstrated that mice immunized intranasally with purified Hap significantly reduced the density of nasopharyngeal colonization compared to controls challenged with a heterologous strain of \textit{H. influenza}. The overall results from the above-mentioned studies suggest that a vaccine-induced immune response to ECMBPs may reduce the incidence of disease.

References


CHAPTER 2. IDENTIFICATION, ISOLATION, AND CHARACTERIZATION OF AN IMMUNOGLOBULIN-BINDING PROTEIN EXPRESSED BY MANNHEIMIA HAEMOLYTICA

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A paper to be submitted to Infection and Immunity

Abstract

*Mannheimia haemolytica* is the primary causative agent of bovine respiratory disease (BRD) or “shipping fever”. Bacterial immunoglobulin-binding protein (IgBP) expression is known to play a role in the pathogenesis of a variety of organisms. This study demonstrated the presence of an IgBP in whole cell sonicates (WCSs) and a culture supernatant (CS) preparation from *M. haemolytica* serotype (ST) 1. Far-Western blot demonstrated binding of bovine Fc IgG to *M. haemolytica* expressed IgBP. An *M. haemolytica* IgBP(s) protein was isolated by affinity chromatography using bovine gamma globulin linked to Sepharose and flow cytometry showed the surface expression of an IgBP on whole cell *M. haemolytica*. Immune sera from convalescent cattle showed an increased antibody response to the 75.0 kDa IgBP when compared to sera from naïve or acutely infected cattle as seen by Western blot. These results demonstrated the presence of an IgBP on the surface of *M. haemolytica* and that an immune response to the 75.0 kDa IgBP may play a role in protection from *M. haemolytica* infection. It was demonstrated that all 12 serotypes (1, 2, 5-9, 12-14, and 16) bound both bovine Fc IgG and sheep Fc IgG without appreciable differences between ST1, most frequently isolated in infections in cattle and ST2, isolated predominantly in sheep
mannheimiosis. Serotype-specific Fc IgG binding did not appear to play a role in species-specific infection by IgBPs from different serotypes of *M. haemolytica*.

**Introduction**

Bovine respiratory disease (BRD) or “shipping fever” is believed to result in the single largest loss annually to the cattle industry. BRD has a complex etiology but pulmonary fibrinous pneumonia, the ultimate cause of severe clinical disease and death, is primarily caused by *Mannheimia haemolytica* (20). Calves are chiefly affected and are predisposed to *M. haemolytica* infection following stressful incidents such as shipping, weaning or viral infection. *M. haemolytica*, a normal flora bacterium of the nasopharynx, increases in number and colonizes the upper respiratory tract following a stressful incident. The increased number of *M. haemolytica* are believed to be inhaled into the lung resulting in macrophage activation and pulmonary serum flooding. An influx of neutrophils along with an *M. haemolytica* secreted leukotoxin exacerbates the response. This leads to lung lesions characteristic of *M. haemolytica*-induced fibrinohemorrhagic pneumonia. Some animals are able to recover while others succumb to pneumonic mannheimiosis (45).

A number of vaccines are available for *M. haemolytica* induced BRD. Bacterins provide protection of variable efficacy. A number of experimental trials even show an exacerbation of BRD in bacterin vaccinates (17, 47). While live vaccines generally perform better in field-tests than bacterins (7), live vaccines still have variable efficacy with some trials showing no increase in protection due to immunization (35). Also, live vaccines have inherent problems with production and stability. While not providing full protection, bacterial component vaccines demonstrate the importance of leukotoxin (8), iron binding
proteins (34) and surface antigens (39) in protection. There is a need for a more efficacious vaccine.

*M. haemolytica* expresses a number of known virulence factors. The best characterized is a leukotoxin (21), a member of the RTX toxin family. While the leukotoxin is a major virulence factor, immunization with the leukotoxin alone is not sufficient to provide protection (43). Other virulence factors include the capsular polysaccharide (5), LPS (36), fimbriae (31), iron-regulated proteins, (9), neuraminidase (13) and O-sialoglycoprotease (1). This study demonstrated the presence of an additional *M. haemolytica* virulence factor, an immunoglobulin-binding protein (IgBP).

IgBPs have been identified in a number of other gram-positive and gram-negative bacteria. Protein A from *Staphylococcus aureus* Cowan 1 was the first IgBP identified (12). Protein A inhibits phagocytosis and protein A surface expression is responsible for this inhibition (10). Protein A in solution fixes complement by forming IgG:protein A complexes (27) and inhibits antibody-dependent cellular cytotoxicity (38). Streptococcal protein H, an Fc IgG binding protein, forms soluble complement-activating complexes with IgG but decrease complement activation by inhibiting C1q binding to IgG of IgG-coated cells (2). A *Moraxella catarrhalis* IgD-binding protein, MID, activates human B cells and in the presence of Th2 cytokines induces immunoglobulin (Ig) secretion (18). Acting as a B cell superantigen, protein A activates complement (23), induces expression of Ig from the V_{H}3 gene family (25) and increases antigen presentation through soluble protein A:Ig complexes associated with V_{H}3 family surface expressed immunoglobulins (28). It is involved in inflammation as demonstrated by the elicitation of an Arthus reaction through administration of soluble protein A:Ig complexes (24). Following intraperitoneal immunization of both
neonatal and adult V_{H3} "knock in" mice with protein A, researchers demonstrated a selective suppression in V_{H3} expressing IgM secreting cells in both the spleen and liver and a loss of detectable V_{H3} expressing B-1 lymphocytes in the peritoneum (40). *In vivo* and *in vitro* evidence shows that protein A acts as a B cell superantigen.

*In vivo* experiments also indicate a role for IgBPs in virulence. Mice injected with an *S. aureus* protein A deletion mutant require a marginally higher dose of the mutant for infectivity and infected mice take significantly longer to succumb to infection and die at a slightly decreased rate than mice infected with *S. aureus* expressing protein A (32). These results may have been more dramatic if a recently discovered second *S. aureus* IgBP, Sbi, had also been deleted (49). When immunoglobulin-binding domains of peptostreptococcal protein L are expressed on *Streptococcus gordonii*, vaginal colonization is enhanced and colonization persists for a longer time (36). These experiments demonstrate a role for IgBPs in virulence although their role has not been clearly elucidated.

Early work with Gram-positive bacteria focused on utilizing IgBPs as immunochemical reagents. For example, protein G binds Igs from a wider range of species while protein A binds Igs from fewer species and with lower affinity (5). These features have been used to isolate Igs from different species. No work has been published concerning the possible relationship between species-specific IgBP binding and species-specific infection or concerning a possible relationship between serotype-specific IgBP binding and serotype-specific infection.

Preliminary results in our lab indicated that *M. haemolytica* might express an IgBP. When normal bovine serum (NBS) is used as a negative control to label whole cell *M. haemolytica* in an ELISA, similar absorbance values are obtained with the NBS and with an
anti-sera specific for an *M. haemolytica* iron-regulated protein (Tabatabai, unpublished results). The objectives of the present study were: 1) To identify and isolate an *M. haemolytica* IgBP, 2) To determine the presence or absence of IgBP surface expression, 3) To determine whether there is an increased antibody response to the IgBP in cattle convalescing from BRD, 4) To determine whether the IgBP may play a role in the dominance of ST1 association with BRD or the dominance of ST2 in sheep mannheimiosis.

**Materials and Methods**

**Bacterial isolates**

*M. haemolytica* ST 1 (isolate L101) was isolated from an infected bovine lung (14). Serotypes 1,2, 5-9, 12-14, and 16 (3, 33, 11) were gifts from G. Frank (USDA/ARS National Animal Disease Center, Ames, Iowa). Serotype 17 (48) was unavailable for this study. *Haemophilus somnus* strain 8025 was a gift from Ron Griffith (Iowa State University).

**Whole cell sonicate and supernatant preparations**

Cells stored at -70°C were cultured overnight on blood agar plates, colonies transferred and cultured with shaking in 3 ml RPMI (Sigma; St. Louis, MO) with 20mM HEPES (pH 7.2) for 6 hours at 37°C. Ten ml of these cultures were used to culture 1 L RPMI for 20 hours with shaking at 37°C. The culture was centrifuged (6,000 × g for 20 minutes) and the CS was retained and stored at -70°C. The cell pellet was washed with 0.1M phosphate buffered saline (PBS), pH 7.2, and centrifuged (6,000 × g for 20 minutes) three times. The cells were lysed in water. The lysate was sonicated for four minutes (4 cycles of 1” on, 1” off, power at 7, duty cycle at 8) using a Branson Sonifier 250. The lysate was
ultracentrifuged (50,000 × g for 2 hours at 4°C). The supernatant was retained as the whole cell sonicate (WCS) preparation, dialyzed in 5 mM NH₄HCO₃ (pH 7.2), aliquoted and stored at -70°C. Prior to IgBP affinity isolation, *M. haemolytica* WCS was dialyzed in 50 mM Tris (pH 8.0). This procedure was used for all *M. haemolytica* serotypes and *H. somnus* 8025.

**Gel electrophoresis and immunoblotting**

SDS-PAGE was conducted on denatured *M. haemolytica* WCSs, CS and isolated IgBP in 12.5% acrylamide gels with a 4% acrylamide stacking gel (26). Protein concentrations for all preparation were determined by Folin-Lowry (29). Proteins were transferred onto 0.45μm Protran nitrocellulose (Schleicher & Schuell, Keene, NJ) using a Bio-Rad Trans-Blot Cell apparatus. Nitrocellulose blots were blocked with 0.25% fish gelatin (Norland Products, Inc., New Brunswick, NJ) in 0.1M PBS, pH 7.2 for 15 minutes. In far-Western blots, which detect non-antibody mediated protein:protein interactions, transferred IgBP was bound to bovine Fc IgG by incubating nitrocellulose blots overnight in bovine Fc IgG (44 μg/10 ml 0.1M PBS, pH 7.2, Jackson Laboratories, West Grove, PA) or sheep Fc IgG (180 μg/10 ml 0.1M PBS, pH 7.2, Jackson Laboratories, West Grove, PA). In Western blots, which detect antigen:antibody interactions, transferred WCS was incubated with cattle sera (1:100) in 0.25% fish gelatin, 0.1M PBS, pH 7.2. After washing (0.1M PBS, pH 7.2 with 0.05% Tween 80) the blots were developed with horseradish-peroxidase (HRP)-conjugated rabbit anti-bovine Fc IgG, sheep Fc IgG or bovine IgG.

**Bovine antisera**

Sera from acutely infected animals were obtained from 10 feed yard calves three days after experimental infection with *M. haemolytica*. Sera from convalescent animals were
collected two weeks post-infection. The $\log_2$ indirect hemagglutination titers (14) ranged from 4 to 7 for the infected calves and from 7 to 10 for the convalescent calves.

**Affinity isolation**

Cyanogen bromide (CNBr)-activated Sepharose 4B (Sigma, St. Louis, MO) was coupled to bovine gamma globulin (Jackson Immunoresearch Labs, West Grove, PA) according to manufacturer’s procedure. *M. haemolytica* WCS in 50 mM Tris (pH 8.0) was adsorbed with bovine gamma globulin-Sepharose 4B by rotating the sample for 48 hours at 4°C. IgBP was eluted from the Sepharose in a column with 0.9% NaCl-1M propionic acid. IgBP was concentrated by Speedvac vacuum concentration (Savant, Holbrook, NY).

**Flow cytometry**

*M. haemolytica* whole cells were blocked with 0.25% fish gelatin in 0.1 M PBS (pH 7.2) for 15 minutes at room temperature before labeling. Cells (0.5x $10^6$) were labeled with either 0.1 M PBS (pH 7.2) or 1.9 mg bovine Fc IgG (Jackson Immunoresearch Laboratories, West Grove, PA) for two hours at room temperature. Fluorescein isothiocyanate (FITC) labeled goat F(ab')2 anti-bovine IgG, Fc fragment specific (Jackson Immunoresearch Laboratories) was conjugated to all cells for two hours at room temperature. Cells were washed across a 0.22 μm filter five times (0.1M PBS, pH 7.2 with 0.05% Tween-80) before and after adding conjugate. The final wash after incubation of cells in conjugate was done with 0.01 M PBS, pH 7.2. Fluorescence was detected on a Beckman Coulter EPICS XL-MCL (Miami, FL). Absorbance was at 488 nm and emission was read at 550 DL/525 BP.
Results

Identification of *M. haemolytica*-expressed IgBP(s)

Both WCS and CS preparations from *M. haemolytica* ST1 expressed at least one IgBP as seen on an immunoblot (Fig. 1). A dominant band at 75.0 kDa, a band at 40.7 kDa and several less distinct bands ranging from 74.9 kDa to 62.0 kDa were present in both WCS and CS preparations. A band at 107.5 kDa was present only in the supernatant. Although the *M. haemolytica* supernatant was at a higher concentration than the WCS preparation in this immunoblot, the 107.5 kDa band appeared to be unique to the supernatant as this band did not appear when concentrated isolated IgBP(s) was run on SDS-PAGE at higher concentrations (Fig. 2). The *H. somnus* WCS preparation was a positive control known to express IgBPs (46). No binding was evident between the conjugate and the WCS or supernatant preparations (results not shown).

Isolation of *M. haemolytica*-expressed IgBP

*M. haemolytica* IgBP(s) was isolated by affinity chromatography (Fig. 2). An *M. haemolytica* WCS preparation was adsorbed with CnBr activated-Sepharose 4-B to which bovine gamma globulin had been bound. IgBP(s) was eluted in a column with 1 M propionic acid in 0.9% NaCl. At the IgBP concentration collected in the fractions, bands are seen at 77.2, 63.2 and 40.2 kDa. After IgBP fractions were concentrated by vacuum centrifugation, additional bands were evident at 168.7, 132.5, 56.5, and 20.0 kDa. All bands were able to bind bovine Fc IgG as seen on far-Western blot (results not shown). Efforts to determine the N-terminal sequence of the 77.2 and 20.0 kDa bands were unsuccessful. The N-terminus might be blocked or there may have been insufficient protein to be sequenced.
Surface expression of *M. haemolytica* IgBP

Whole cell *M. haemolytica* (0.5x10^6 cells) were labeled with bovine Fc IgG or PBS. Bovine Fc IgG bound to whole cells was detected with FITC-conjugated goat F(ab')2 anti-bovine IgG, Fc fragment specific. Propidium iodide incorporated *M. haemolytica* cells were used to set the gate identifying *M. haemolytica* whole cells. Flow cytometry measurements showed that the number of FITC positive cells was increased by 64.9% on *M. haemolytica* whole cells labeled with bovine Fc IgG compared to PBS labeled cells (Fig. 3).

Humoral immunogenicity of an *M. haemolytica* IgBP

Sera from naïve cattle, acutely infected cattle and convalescent cattle were used to probe an *M. haemolytica* WCS preparation in a Western blot. Binding was detected with an HRP-conjugated rabbit anti-bovine Fc IgG. The humoral immune response to the dominant 75.0 kDa IgBP was increased in convalescent cattle when compared to naïve or acutely infected cattle (Fig. 4). The location of the IgBP in the WCS preparation was identified in lane c. This lane shows the WCS preparation probed with bovine Fc IgG. *H. somnus* in lane b was a positive control.

Involvement of IgBP from different serotypes with species-specific infection

*M. haemolytica* WCS preparations from all serotypes (1, 2, 5-9, 12-14, and 16) were probed with either bovine Fc IgG or sheep Fc IgG in a far-Western blot (Fig. 5 and 6). Detection was with HRP-conjugated rabbit anti-bovine or anti-sheep Fc IgG. The same WCS preparation was used for both blots and all lanes have the same concentration of *M. haemolytica* WCS. At the conjugate dilution used, rabbit immunoglobulin (Ig) binding to *M. haemolytica* IgBP(s) was not apparent (Results not shown).
All serotypes bound both bovine Fc IgG and sheep Fc IgG (Fig. 5 and 6). The banding patterns found by probing with either bovine Fc IgG or sheep Fc IgG were quite dissimilar. More bands were evident with sheep Fc IgG binding than with bovine Fc IgG binding. The dominant bands detected from bovine Fc IgG binding were 74.1 63.5 and 33.2 kDa whereas the dominant sheep Fc IgG IgBP(s) bands were 58.4 and 50.1 kDa.

Serotypes 1 and 2 both bound bovine Fc IgG with dominant bands at 74.1 and 63.5 kDa (Fig. 5). There was no significant difference between the serotypes in the intensity of the staining of either band. The 74.1 kDa band was more intense in both serotypes. Serotype 2 had an additional band at 33.2 kDa that was not evident in ST 1. The remaining serotypes had various similar IgBP binding patterns and no serotype has a band that is unique to that serotype alone.

Serotype 1 bound sheep Fc IgG less intensely than ST2 binds sheep Fc IgG (Fig. 6). Although both serotypes had similar binding patterns, ST2 IgBP(s) bound sheep Fc IgG more intensely at 79.4, 58.4, 50.1, 30.1 and 24.7 kDa than did ST1. Serotypes 5, 8, 13 and 14 had intense bands 58.4 kDa although all serotypes expressed an IgBP that binds at 58.4 kDa. Generally, all serotypes had a similar sheep Fc binding pattern.

**Discussion**

This study showed that an IgBP(s) present in WCS and CS preparations binds bovine Fc IgG as shown on far-Western blot (Fig. 1). Both preparations have a dominant band at 75.0 kDa and a less intensely staining band at 40.2 kDa. This suggests that the same protein or proteins is expressed in the WCS and supernatant. Both bands are of similar intensity in the WCS and CS preparations although the CS has almost twice the amount of total protein
in lane b as compared to the WCS in lane a. This could indicate that less IgBP(s) is secreted than is cell bound. The supernatant preparation has an additional band at 107.5 kDa. This band appears unique to the supernatant because isolated, concentrated IgBP(s) from a WCS preparation in figure two shows a number of additional bands on SDS-PAGE but no band comparable to the 107.5 kDa band found in the supernatant.

Numerous molecular weight (MW) determinations by SDS-PAGE have shown the dominant *M. haemolytica* IgBP to have MW ranging from 58.0 kDa to 77.2 kDa. Molecular weight variability is indicative of elongated molecules. Protein A displayed a variable MW (40.2 kDa to 56.0 kDa) and is an elongated molecule (4). This suggests that the dominant *M. haemolytica* IgBP may be an elongated molecule.

There are a number of possible explanations for the appearance of more than one IgBP band on far-Western blot. IgBPs from other organisms have repeat binding sites for Fc Ig. Protein A has five (30) while protein G has three repeat Fc Ig binding regions (19). It is possible that the different *M. haemolytica* IgBP(s) bands evident on far-Western blot are due to the IgBP(s) expressing a different number of Fc IgG binding sites which would result in number of different molecular weight bands. Multiple bands could also be due to a unique protein in each Western blot band or the bands might be degradation products of one or more proteins. There is evidence for the latter possibility as seen by SDS-PAGE of the isolated, concentrated IgBP (Fig. 2).

*Mannheimia haemolytica* IgBP(s) was isolated by affinity chromatography by binding bovine gamma globulin to CNBr-activated Sepharose (Fig. 2). When affinity-isolated IgBP(s) is concentrated (Fig. 2, lane c), five additional bands are evident. The 63.2 and 22.4 kDa bands appear to be breakdown products of larger proteins because these bands
are much more intense when the affinity-isolated IgBP(s) was concentrated while the 77.2 KDa band is of equal intensity in both preparations. Further work with the isolated protein is planned in an effort to compare the *M. haemolytica* IgBP(s) to other better-characterized IgBPs from gram-positive bacteria.

Several *M. haemolytica* IgBP(s) characteristics determined in this study indicate a role for the IgBP in virulence. First, flow cytometry shows the IgBP(s) to be surface expressed on *M. haemolytica* (Fig. 3). Surface expression of protein H, an Fc IgG-binding protein from *Streptococcus pyogenes*, reduces phagocytosis of the bacteria (22) and inhibits complement activation by blocking C3 deposition on the bacterial surface (2). Increased protein A surface expression on *S. aureus* leads to increased inhibition of phagocytosis (10). These virulence mechanisms may be active in *M. haemolytica*. Secondly, this study identifies a secreted form of the *M. haemolytica* IgBP. Soluble protein A reduces antibody-dependent cellular (38) and fixes complement in solution thus consuming complement components (42). Soluble protein H interacts with IgG to form complement-activating complexes (2). Thus, the CS IgBP(s) may play a role in inhibiting phagocytosis and limiting complement activation as well as consuming soluble complement components. Lastly, convalescent cattle had an increased antibody response to the 70.5 kDa *M. haemolytica* IgBP when compared to naïve or acutely infected cattle (Fig. 4). The increased antibody response in convalescent cattle suggests that an immune response to the IgBP(s) may be important in protection. These results indicate a role in virulence for the *M. haemolytica* IgBP(s).

*M. haemolytica* ST1 is generally associated with BRD in cattle (15, 44) and ST2 is most frequently associated with pneumonic mannheimiosis in sheep (16). In an effort to determine whether the *M. haemolytica* IgBP(s) is a factor in serotype-specificity of species-
specific infection, far-Western blots were run to determine whether differences are evident between binding of bovine Fc IgG or sheep Fc IgG to ST1 and ST2 and whether bovine Fc IgG and sheep Fc IgG binds to all 12 serotype WCS preparations regardless of whether they are known to be involved in infection. Differences among infectious serotypes or between infectious and noninfectious serotypes might indicate a role for serotype-specific infection in cattle and sheep. Serotypes 1 and 2 both bind bovine Fc IgG and have protein bands at 74.1 and 63.5 kDa that stain with similar intensity in the two serotypes (Fig. 5). The 74.1 kDa band appears equally dominant in these two serotypes. A 33.2 kDa band is evident in ST2 that is not found in ST1. It is possible that this difference is responsible for serotype-specificity of infection in cattle and sheep. However, the remaining serotypes express the 33.2 kDa protein band and these are not typically isolated from infections in sheep. This suggests that *M. haemolytica* expressed IgBPs do not play a role in serotype-specificity in infection of cattle. All 12 serotypes express an IgBP that binds bovine Fc IgG and all serotypes have similar banding patterns. No serotype has a protein band that is unique to that serotype. This is further evidence that *M. haemolytica* IgBP(s) does not play a role in the dominance of ST1 found in infections in cattle. Differences among the serotypes in binding bovine Fc IgG may be due to; 1) serotypes expressing dissimilar amounts of IgBP(s), 2) different serotypes expressing IgBP(s) with diverse numbers of Fc IgG binding sites, 3) *M. haemolytica* expressing more than one bovine Fc IgG IgBP in different serotypes.

Serotype 2 binds sheep Fc IgG more intensely than does ST1 (Fig. 6). However, as in bovine Fc IgG binding, other serotypes not known to be infective have protein-banding patterns as intense as found with ST 2. This would suggest that the difference found between ST1 and ST2 is not responsible for serotype-specific infection in sheep. All serotypes bind
sheep Fc IgG in a similar protein-banding pattern. Four serotypes (5, 8, 13 and 14) have a particularly dominant band at 62.1 kDa. What role, if any, this may play in bacterial virulence or lack of virulence in these serotypes is not known.

Bovine Fc IgG bound to *M. haemolytica* IgBP(s) in a different protein-band pattern than sheep Fc IgG in all STs. *M. haemolytica* may express only one IgBP that has separate binding sites for sheep Fc IgG and bovine Fc IgG or *M. haemolytica* may express different proteins for binding bovine Fc IgG and sheep Fc IgG. Whether or not this plays any role in virulence is not known and is again doubtful because these same differences occur among infective serotypes and non-infective STs.

The fact that all serotypes express an IgBP(s) but not all serotypes are infective could indicate that the IgBP(s) are not involved in virulence. However, several lines of evidence demonstrate that this may not be true: 1) Protein A from *S. aureus* (41) and protein H from *Streptococcus pyogenes* are surface expressed. The *M. haemolytica* IgBP(s) was also shown to be surface expressed. Protein A and protein H surface expression inhibits phagocytosis (10, 22). 2) Convalescent cattle show an increased antibody response to *M. haemolytica* ST 1 IgBP. 3) *M. haemolytica* IgBP(s) may be a virulence factor in infection of cattle and sheep but may not be a factor sufficient by itself to cause infection.

In summary, this study identifies an *M. haemolytica* surface expressed IgBP(s) as well as a secreted IgBP(s). The IgBP(s) does not appear to play a role in serotype-specificity of species-specific infection as all serotypes bind both bovine Fc IgG and sheep Fc IgG. However, sheep Fc IgG binds *M. haemolytica* IgBP(s) with a different protein band pattern on far-Western blot than does bovine Fc IgG. This difference may indicate a different virulence role for the IgBP(s) in cattle and sheep but more likely it may be that more than one
IgBP may be expressed by *M. haemolytica* or there are different binding sites for sheep Fc IgG and bovine Fc IgG. The *M. haemolytica* IgBP(s) from the WCS preparation was isolated by affinity chromatography. Western blot results in this study showed an increased antibody response to *M. haemolytica* IgBP in cattle recovering from mannheimiosis indicating a possible role for the IgBP in protection from *M. haemolytica* induced BRD. The *in vivo* and *in vitro* virulence association of protein A and other previously identified IgBPs, the similarity in surface expression of protein A, protein H and *M. haemolytica* IgBP(s), the presence of a secreted *M. haemolytica* IgBP(s) and the increased immune response to an *M. haemolytica* IgBP in convalescent cattle suggest a role for *M. haemolytica* IgBP in virulence. The *M. haemolytica* IgBP may be an important factor in future subunit vaccines.

References


FIG. 1. Far-Western blot identification of an IgBP expressed by *M. haemolytica*. Lanes: a, 7.0 µg *M. haemolytica* WCS; b, 12.0µg *M. haemolytica* supernatant; c, 36.0 µg *H. somnus* WCS. All lanes were probed with 0.58 mg bovine Fc IgG and developed with HRP-conjugated rabbit anti-bovine IgG, Fc fragment specific (1:1000). Location of molecular size markers is indicated on the left. All molecular weights are in kDa.
FIG. 2. SDS-PAGE of IgBP isolation from *M. haemolytica* WCS. Lane: a, MW markers; b, 0.35 μg IgBP isolated from *M. haemolytica* WCS; c, 3.50 μg isolated, speed-vacuum concentrated IgBP from *M. haemolytica*. IgBP was isolated on a bovine gamma globulin-CNBr Sepharose column. All molecular weights are in kDa.
FIG. 3. Flow cytometry analysis of whole cell *M. haemolytica* IgBP surface expression. (A) Whole cell *M. haemolytica* (0.5x10^6) labeled with 0.1M PBS, pH 7.2. (B) Whole cell *M. haemolytica* (0.5x10^6) labeled with 1.9 mg bovine Fc IgG in PBS. FITC-conjugated rabbit F(ab')_2 anti-bovine IgG, Fc fragment specific revealed the presence of bound bovine Fc IgG.
FIG. 4. Antibody responses to *M. haemolytica* WCS IgBP in pooled sera from naïve, acutely infected and convalescent cattle. Lanes: a, MW markers; b, *H. somnus* WCS; c, 7.2 µg isolated *M. haemolytica* IgBP; d, e, f, 7.0 µg *M. haemolytica* WCS. Lanes a, b and c were probed with 0.58 mg bovine Fc IgG. Lane d was probed with naïve cattle sera. Lane e was probed with sera from cattle with acute *M. haemolytica* infection. Lane f was probed with sera from cattle convalescing from *M. haemolytica* induced BRD. Cattle sera were diluted 1:100. All binding was detected with HRP-conjugated rabbit anti-bovine IgG, Fc fragment specific.
FIG. 5. Bovine Fc IgG binds to all serotypes of *M. haemolytica* WCS preparations. In a far-Western blot, SDS-PAGE separated proteins from 10.0μg *M. haemolytica* WCS preparations from all serotypes were transferred onto nitrocellulose and probed with bovine Fc IgG (0.5 mg). Blots were developed with HRP-conjugated rabbit F(ab')2 anti-bovine Fc IgG. Molecular weight markers are in the first lane. The serotype of each *M. haemolytica* WCS preparation is identified above succeeding lanes. The molecular weight of all bands resulting from bovine Fc IgG binding is indicated on the right.
FIG. 6. Sheep Fc IgG binds to all serotypes of *M. haemolytica* WCS preparations. In a far-Western blot, SDS-PAGE separated proteins from *M. haemolytica* WCS preparations from all serotypes (10.0 μg each) were transferred onto nitrocellulose and probed with sheep Fc IgG (0.5 mg). Blots were developed with HRP-conjugated rabbit F(ab')2 anti-sheep Fc Ig. Molecular weight markers are in the first lane. The serotype of each *M. haemolytica* WCS preparation is identified above each lane. The molecular weight of all bands resulting from bovine Fc IgG binding is indicated on the right.
CHAPTER 3. FIBRINOGEN- AND FIBRONECTIN-BINDING AND IN VITRO GROWTH CONDITION-DEPENDENT EXPRESSION OF A MANNEHIMIA HAEMOLYTICA IMMUNOGLOBULIN-BINDING PROTEIN

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A paper to be submitted to Infection and Immunity

Abstract

Bovine respiratory disease (BRD) is the cause of large losses to the cattle industry annually and is primarily caused by Mannheimia haemolytica serotype (ST) 1. M. haemolytica, a normal flora respiratory tract bacteria, rapidly multiplies upon a stressful occurrence and colonizes the upper respiratory tract. Extra-cellular matrix proteins (ECMPs) are known to play a role in colonization by some bacteria by acting as a bridge enabling binding between bacteria expressing extracellular matrix-binding proteins (ECMBPs) and epithelial cells. A previously identified immunoglobulin-binding protein (IgBP) expressed by M. haemolytica was shown here to have bound the ECMPs fibrinogen (Fg) and fibronectin (Fn) Far-Western blot results demonstrated that the isolated IgBP bound bovine Fc IgG and either Fg or Fn simultaneously. The effect of various M. haemolytica growth conditions on IgBP expression was assessed by 2-D gel electrophoresis analysis. Membrane expression of M. haemolytica IgBP was increased in growth conditions more closely replicating in vivo conditions. IgBP expression was increased a minimum of twofold when M. haemolytica was grown in RPMI-1640 media with 2,2'-dipyridyl, and threefold when M. haemolytica was grown in RPMI-1640 supplemented with 10% fetal bovine sera (FBS) and
RPMI-1640 at 40°C when compared to IgBP expression when *M. haemolytica* was grown in RPMI-1640 at 37°C. Up to 19.9% of *M. haemolytica* membrane proteins were IgBPs when *M. haemolytica* was grown in RPMI at 40°C. This study shows a possible increased role in virulence for the previously identified *M. haemolytica* IgBP. It may play a role in colonization through binding Fg or Fn and is a highly expressed protein when *M. haemolytica* is grown under conditions mimicking aspects of *in vivo* conditions.

**Introduction**

Bovine respiratory disease (BRD) has a complex etiology but pneumonic pasteurellosis, more commonly called shipping fever, is believed to be caused by *Mannheimia haemolytica*. *M. haemolytica* serotype (ST2) is a normal flora bacteria of the upper respiratory tract (URT) in cattle but on occurrence of a stressful incident such as viral infection or shipping from farm to feedlot, isolates of *M. haemolytica* shift from ST2 to ST1 and can grow explosively colonizing the URT (Frank 1979; Frank and Smith, 1983). The bacteria are believed to be inhaled into the lungs. This is followed by an influx of neutrophils into the lungs, serum flooding, and an inflammatory response that leads to fibrinohemorrhagic pneumonia. Morbidity and mortality due to shipping fever are among the leading causes of economic loss in the cattle industry.

Adherence to epithelial cells is a necessary component for *M. haemolytica* colonization of the URT in cattle. The surface components used by *M. haemolytica* to adhere to airway epithelial cells are not known. Two types of fimbrae, a large, rigid fimbrae (Morck et al., 1987) as well as smaller, flexible fimbrae (Potter, et al., 1988) have been reported but fimbrae have not yet been found in *M. haemolytica* isolated from the lung or URT of cattle.
(Gonzalez and Maheswaran, 1993). In addition, their role in attachment has not been demonstrated. A number of Gram-negative bacteria express adhesins that are distinct from fimbriae (Minion et al., 1986 and Moo et al., 1992). Identification of an *M. haemolytica* adhesive surface component would open the door to development of a treatment that could decrease colonization and result in reduced development of pneumonic pasteurellosis.

Bacteria bind to host cells when bacterial cell surface components adhere to host cell components or to host extracellular matrix proteins (ECMPs) that bind host cells and act as a bridge between bacteria and host cells. The latter group of adhesive bacterial cell surface components is termed extracellular matrix-binding proteins (ECMBPs), adhesins, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), or receptins. ECMBPs from *Staphylococcus aureus* are best characterized and include proteins that bind fibronectin (Fn), collagen and a secreted protein that binds fibrinogen (Fg) (Flock, 1999). One *S. aureus* ECMBP, extracellular adherence protein (Eap), binds seven plasma proteins including Fg and Fn (Palma et al., 1999). A number of Gram-negative organisms express ECMBPs including *Eschericia coli* (Westerland et al., 1989), *Haemophilus ducreyi* (Abeck et al., 1992), *H. influenza* (Fink et al., 2002), *Neisseria gonorrhoeae* (van Putten et al., 1998) and *Borellia burgdorferi* (Probert and Johnson, 1998). In addition, a number of immunoglobulin-binding proteins are known to bind ECMPs. For example, Protein H, present in some strains of *S. pyogenes* and first known to bind Fc IgG, also binds fibronectin. A fibronectin binding protein also from *S. pyogenes*, Sfbl, has been shown to bind Fc IgG (Frick et al., 1995; Medina et al., 1999). A Group C streptococci has been shown to express protein FAl which binds fibrinogen, albumin and IgG (Talay et al., 1996). In addition,
FgBP, a fibrinogen-binding protein of *Streptococcus equi*, has recently been shown to bind equine Fc IgG (Meehan et al., 2001).

ECMBPs are believed to contribute to virulence in a number of bacteria. Mongodin et al. (2002) established that a fibronectin-binding protein-deficient strain of *S. aureus* has a fivefold-decrease in the level of adherence to human airway epithelial cells suggesting that Fnbps are involved in adherence. An isogenic mutant unable to produce the Fn- and Fg-binding protein of *Streptococcus suis* ST2 shows decreased colonization of piglets in the joints and central nervous system, organs specifically involved in *S. suis* infection (de Greeff, et al., 2002). *In vitro* models demonstrate several additional roles for ECMBPs in virulence. In the presence of Fn and an anti-CD3 monoclonal antibody, *S. aureus* FnbpA mediates adhesion to T cells and T cell costimulation through the T cell αβ1 integrin, which binds Fn and functions as a costimulatory molecule, leading to T cell activation (Miyamoto et al., 2001). A T helper type 2 inflammatory environment promotes *S. aureus* binding to skin and this binding is mediated by Fn and Fg (Cho et al., 2001). ECMBPs appear to have a variety of roles in virulence.

The mechanism for the abrupt change from the predominance of the normal flora *M. haemolytica* ST2 to explosive growth of stress-associated ST1 is not known. However, change in host environmental factors due to stress is likely to play a role. In an effort to recreate *in vivo* protein expression while using *in vitro* systems and also to define culture conditions that affect expression of particular proteins, a number of studies have been done to define protein expression under different culture conditions. Shewen and Wilkie (1982) reported that the addition of 7% fetal bovine serum (FBS) to RPMI-1640 tissue culture media achieves enhanced *M. haemolytica* production of leukotoxin, a secreted RTX cytolysin,
compared to tissue culture media alone. Iron-regulated outer membrane proteins of 71, 77 and 100 kDa are expressed at higher levels in iron-restricted media (Deneer and Potter, 1989; Morck et al., 1991). *M. haemolytica* OMP protein expression was compared between bacteria that were isolated from lungs of experimentally infected calves to bacteria cultured under various *in vitro* growth conditions. OMP expression profiles on SDS-PAGE are similar for *M. haemolytica* isolated from lungs and *M. haemolytica* grown in newborn calf serum (Davies et al., 1994). No capsular polysaccharide production is detected when *M. haemolytica* is grown above 40°C (Puente-Polledo et al., 1998). Experiments by Reeve-Johnson et al. (2001) noted higher clinical scores in calves with temperatures of 39.9–41.0°C compared to normal temperatures of 39.2°C. In addition, pathogens are known to upregulate or downregulate expression of adhesive components under varying conditions of the host environment (Finlay and Falkow, 1989; Francis et al., 1989; Mekalanos, 1992). Establishing *in vitro* growth conditions that closely mimic *in vivo* conditions may increase the efficacy of vaccines prepared from *in vitro* *M. haemolytica* cultures.

The hypotheses for this study were that a previously identified *M. haemolytica* ST1 immunoglobulin-binding protein (IgBP) binds one or more ECMPs and that this protein would be upregulated when grown under *in vitro* growth conditions that more closely mimic *in vivo* conditions.
Material and Methods

Bacterial isolate and growth conditions

*M. haemolytica* ST1 (isolate L101) was isolated from an infected bovine lung (Frank and Smith 1983).

Fetal bovine sera

FBS was obtained from National Animal Disease Center, Ames, Iowa. It was heated to 56°C for 25 min before use.

IgBP isolation

Cyanogen bromide (CNBr)-activated Sepharose 4B (Sigma, St. Louis, MO) was coupled to bovine gamma globulin (Jackson Immunoresearch Labs, West Grove, PA) according to manufacturer’s procedure. *M. haemolytica* WCS in 50 mM Tris (pH 8.0) was adsorbed with bovine gamma globulin-Sepharose 4B by rotating the sample for 48 hours at 4°C. IgBP was eluted from the Sepharose in a column with 0.9% NaCl-1M propionic acid. IgBP was concentrated by Speedvac vacuum concentration (Savant, Holbrook, NY).

Gel electrophoresis and immunoblotting

SDS-PAGE was conducted on denatured *M. haemolytica* WCS, isolated IgBP, Fn (from bovine plasma, Sigma, St. Louis MO) and Fg (from bovine plasma, Sigma, St. Louis, MO) in 12.5% acrylamide gels with a 4% acrylamide stacking gel (Laemmli, 1970) or 4-12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA). Protein concentrations for all preparations were determined by Folin-Lowry (Lowry et al., 1951). Proteins were
transferred onto 0.45µm Protran nitrocellulose (Schleicher & Schuell, Keene, NJ) using a Bio-Rad Trans-Blot Cell apparatus. Nitrocellulose blots were blocked with 0.25% fish gelatin (Norland Products, Inc., New Brunswick, NJ) in 0.1M PBS, pH 7.2 for 15 minutes. Transferred IgBP was detected by incubating nitrocellulose blots overnight in bovine Fc IgG (44 µg/10 ml, Jackson Laboratories, West Grove, PA). After washing (0.1M PBS, pH 7.2 with 0.05% Tween 80) the blots were developed using horseradish-peroxidase (HRP)-conjugated rabbit antibodies to fibronectin (1:1000) or bovine Fc IgG (1:1000).

**WCS and membrane protein preparation**

Cells stored at -70°C were cultured overnight on blood agar plates (Becton Dickinson, Sparks, MD), colonies transferred and cultured in 3 ml RPMI-1640 (Sigma; St. Louis, MO) with 20mM HEPES (pH 7.2) for 6 hours at 37°C with shaking and 10 ml was used to culture 1 L RPMI-1640 at 37°C, RPMI-1640 with 50 µM 2,2'-dipyridyl at 37°C, RPMI-1640 with 10% FBS at 37°C, or RPMI-1640 at 40°C for 20 hours with shaking. Optical density readings were taken at 600 nm on a Beckman DU 640 spectrophotometer (Beckman, Fullerton, CA). Streaking on blood agar plates checked purity. The culture was centrifuged (6,000 × g for 20 min). The cell pellet was washed with 0.01M phosphate buffered saline (PBS), pH 7.2, and centrifuged (6,000 × g RPMI-1640 for 20 minutes) three times. After washing the pellet two more times, the pellet was resuspended in 0.01 M PBS (pH 7.2) and sonicated on ice for four minutes (8 cycles of 30 sec on, 30 sec off, power at 7, duty cycle at 5) using a Branson Sonifier 250. The sonicated cells were centrifuged (6000 × g for 20 min) and the supernatant was ultracentrifuged (50,000 × g for 2 hours at 4°C). The supernatant was retained as the WCS and the pellet, the membrane preparation, was
resuspended in 50 mM Tris HCl, pH 7.5 containing 50 μg/ml phenylmethylsulfonylfluoride (PMSF) (Sigma). The solution was set on ice for 1 h and protein concentrations were determined using Amersham Bioscience’s PlusOne 2-D Quant kit (San Francisco, CA).

2-D gel electrophoresis

Membrane samples (50 μg protein/50 μl) were added to 75 μl rehydration buffer (0.5% v/v carrier ampholytes, pH 3-10 (Amersham Bioscience, San Francisco, CA), 2M thiourea, 2mM tributylphosphine (TBP), 1% w/v tetradecanoylamido-propyl-dimethyl ammonia-propane-sulfonate (ASB-14), 2% w/v CHAPS, 8M urea, 0.5% IPG buffer, pH3-10 (Amersham Bioscience), 10 ml E-pure water and 0.2% Pefabloc (Boehringer Ingelheim (Ingelheim, Germany), and 0.28 mg dithiothreitol (Sigma) were added. The samples were briefly centrifuged to remove particulate matter and 125 μl of each sample was loaded onto Immobiline IPG DryStrip gels (7 cm, pH 3-10,) (Amersham Biosciences). Strips were rehydrated for 10h at 30°C and proteins were focused on an IPGphor electrophoresis unit (Amersham Bioscience) for approximately 33kVh as follows:

<table>
<thead>
<tr>
<th>Volts</th>
<th>500</th>
<th>1000</th>
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<td>1:00</td>
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</table>

IPG strips were equilibrated in SDS equilibration buffer [50mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) 87% glycerol, 2% (w/v) SDS, trace bromophenol blue in E-pure water] with 100 mg dithiothreitol/10 ml buffer for 15 min. Then IPG strips were equilibrated in SDS equilibration buffer with 250 mg iodoacetamide/10 ml solution for 15 min. Strips were sealed in place on top of an SDS-PAGE gel using 0.5% agarose in SDS electrophoresis.
buffer containing bromophenol blue, SDS-PAGE was carried out on 4-12% Bis-Tris gel (Invitrogen) polyacrylamide gels using MOPS Running Buffer (Invitrogen). NuPAGE antioxidant (0.25%) (Invitrogen) was added to the cathode buffer. Second-dimension electrophoresis was carried out using 15mA/gel constant current for 15 min then at 200V (constant voltage) for 45 min in a Mighty Small II electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, CA). Gels were stained overnight with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid. Gels were destained in 25% ethanol and 10% acetic acid and imaged on an Amersham Biosciences Image Scanner.

**2-D gel analysis**

Far-Western blot and 2-D gel analysis were done using PDQuest 6.1 gel analysis software (Bio-Rad Laboratories, Hercules, CA). A match set was created with all four gels from the four different growth conditions and the far-Western immunoblot identifying immunoglobulin-binding protein. Spots identified on the immunoblot were matched to spots on the four gels. Spot analysis was performed for each gel. Normalization was done for each spot by dividing the raw pixel density quantity of each spot in a member gel by the total pixel density quantity of all pixels in the gel image. Pixel density was determined from a Gaussian image of each gel.

**Results**

*M. haemolytica growth in different in vitro growth conditions*

*In vitro* growth conditions are usually chosen to support growth. Typical laboratory culture conditions for *M. haemolytica* are growth in a chemically defined media, RPMI-1640,
at 37°C. To mimic aspects of in vivo growth conditions, *M. haemolytica* was grown in three different culture conditions: RPMI-1640 with 50 μM 2,2'-dipyridyl, RPMI-1640 with 10% fetal bovine serum and RPMI-1640 cultured at 40°C. The iron-chelator 2,2'-dipyridyl was added to RPMI-1640, a medium without added iron, to chelate any exogenous iron. Most pathogenic organisms upregulate a number of proteins under iron-restricted conditions (Gray-Owen and Schryvers, 1996). Fetal bovine sera (10%) was added to RPMI-1640 given that expression of leukotoxin, a cytolytic toxin secreted by *M. haemolytica*, is increased in vitro when 7% FBS is added to the media (Shewen and Wilkie, 1982). In addition, pneumonic mannheimiosis is known to lead to serum flooding in the lungs. Finally, *M. haemolytica* was grown in RPMI-1640 at 40°C because infected cattle have temperatures of 40°C or above. Normal cattle temperature range is 38.3-38.9°C (discussions with G. Frank, NADC, Ames, IA). Growth in RPMI-1640 at 40°C resulted in a culture density almost half that of *M. haemolytica* grown in the same media at 37°C (Table 1). When grown at 40°C, pellets of *M. haemolytica* cells appeared more viscous than when grown at 37°C. When 10% FBS was added to RPMI-1640, cell culture density was nearly twice that of the density of cells when grown in RPMI at 37°C. Culture density of *M. haemolytica* grown in RPMI-1640 with the iron-chelator 2,2'-dipyridyl was slightly less than growth in RPMI-1640 at 37°C.

*M. haemolytica* IgBP also binds Fg and Fn

Earlier work indicated that the *M. haemolytica* IgBP(s) might bind fibronectin. A mass-peptide fingerprint of a tryptic digest of one band of the IgBP(s) from an SDS-PAGE gel did not establish identity of the IgBP(s) with other proteins in the database (MS-Fit, University of California, San Francisco, CA). However, a degree of homology was found to
other Fn-binding proteins. Far-Western blots validated that isolated *M. haemolytica* IgBP(s) does bind Fn (Fig. 1, lane 2). One band was evident at 29.8 kDa. To determine whether the IgBP(s) would concurrently bind Fn and bovine Fc IgG, Fn was electrophoresed by SDS-PAGE and electroblotted to nitrocellulose. The blot was incubated with the IgBP(s) in 0.1 M PBS and after washing was incubated with bovine Fc IgG in 0.1 M PBS. After washing and development with conjugate [rabbit F(ab')2 anti-bovine Fc IgG-HRP], the blots revealed that the IgBP bound bovine Fc IgG and Fn at the same time (Fig. 1, lane 4). Bands were evident at 214.9 and 36.8 kDa (Fig. 1A). No binding was seen on far-Western blot between Fn and bovine Fc IgG or Fn and the conjugate (results not shown). The same procedure showed that isolated IgBP(s) binds bovine Fc IgG and Fg simultaneously (Fig. 2, lane 2). This lane had nine bands ranging from 35.1–159.0 kDa. The control (Fig. 2, lane 5) showed slight evidence of conjugate-Fg binding. This lane revealed three faintly evident bands on the blot (71.0, 84.5, and 92.2 kDa).

**Comparative analysis of total IgBP pixel density**

IgBP expression was determined by 2-D gel analysis from *M. haemolytica* grown under culture conditions that more closely mimic *in vivo* conditions. The identity and location of the IgBP(s) are shown on the far-Western blot (Fig. 3A). The MW markers are indicated (Fig. 3A). The cross hairs indicate the location of the IgBP(s) on the blot and gels (Fig. 3, B through F). The pixel density of IgBP spots and total gel pixel density was determined with PDQuest 2-D gel analysis software. Dividing spot pixel density by total gel pixel density normalized the pixel density value of each spot. Normalization was necessary to account for differences in gel loading and/or staining efficiency.
Expression of membrane IgBP(s) was increased a minimum of twofold under all test conditions while the addition of 10% FBS or growth at 40°C increased IgBP(s) expression at least threefold (Table 2). When membrane IgBP expression was analyzed as a percentage of total membrane expression, all three test conditions show an approximately twofold increase in the percentage of the IgBP(s) compared to the control. Growth conditions of RPMI-1640 at 40°C yielded the highest percentage membrane IgBP(s) at 19.9%.

**Comparative analysis of individual spot pixel density**

The quantitative relatedness of IgBP spots from each of the three gels generated from *M. haemolytica* grown in RPMI-1640 with 50 μM 2,2'-dipyridyl, RPMI-1640 with 10% FBS and RPMI-1640 at 40°C to the IgBP spots from *M. haemolytica* grown in RPMI-1640 at 37°C was determined with PDQuest 2-D gel analysis software. The pixel density of each IgBP spot in the gel from *M. haemolytica* grown in RPMI-1640 at 37°C (x-axis) was plotted on a log scale against the corresponding IgBP spot density in each of the three remaining gels containing proteins expressed under different conditions. If the spot quantities from the gels being compared are equal, the indicating dot will appear on the regression line with a slope of one. Deviations indicate an increase or decrease in expression for a given spot between the two gels. The correlation coefficient indicating the relatedness of all the spots between two gels as well as the equation for a linear regression defining the indicating dots from the plot is shown (Fig. 4). The IgBP(s) spots from the gels generated by *M. haemolytica* grown in RPMI-1640 at 37°C and RPMI-1640 with 10% FBS showed the largest amount of deviation (correlation coefficient of 0.20). Neither of the remaining analysis sets had a correlation coefficient greater than 0.50.
Discussion

Bacterial cell adherence to host cells is a required initial step in most bacterial infections and ECMBPs may play an important role in this process. ECMBPs may be required for infection. The S. aureus FnbpA is known to bind Fg as well as Fn (Wann et al., 1999), and FnbpA has been shown to be involved in adherence to epithelial cells (Mongodin et al., 2002). The mechanism used by M. haemolytica to adhere to the URT in colonization is not known. Whereas M. haemolytica ST2 is most frequently found as a commensal of the nasopharynx, ST1 is most frequently isolated in infection. This change is initiated by stress and possibly involves a change in the host environment. Results here indicated that the M. haemolytica IgBP binds Fg and Fn. Fibrinogen and Fn are involved in bacterial cell adhesion as extracellular matrix proteins and experiments indicate a role for ECMBPs in bacterial adhesion to host cells. Up to 19.9% of M. haemolytica membrane proteins were IgBPs in conditions modeling *in vivo* conditions which could indicate an important IgBP role in virulence. The M. haemolytica IgBP(s) might play a role in adherence and colonization. Additional roles for ECMBPs in virulence are being found and as a result the M. haemolytica IgBP(s) may have other roles in virulence.

Far-Western blot results demonstrated that the M. haemolytica IgBP can bind bovine Fc IgG and Fg as well as bovine Fc IgG and Fn simultaneously. The significance of this is not known. There has been no previously demonstrated virulence role that IgBPs and ECMBPs have in common. It is possible that the M. haemolytica IgBP(s) binds one protein with a higher affinity than the other proteins or that steric hindrance could result in preferential binding of one or more proteins, particularly when IgBP(s) expression is upregulated.
Bacterial growth in RPMI-1640 with 10% FBS resulted in the highest number of bacteria while the least growth occurred when bacteria were grown in RPMI-1640 at 40°C. However, both of these conditions resulted in the greatest and nearly equal relative IgBP expression. It is possible that one environmental factor trigger induces upregulation under both conditions or the upregulation may be due to independent factors. Regardless, bacterial growth or lack of growth can occur independently of IgBP expression and IgBP expression can be upregulated under conditions that are not optimal for growth.

The membrane proteins were separated over a pH 3–10 range. The predominance of the IgBP(s) spots were identified at 24 kDa and at higher pi values. These spots were 60% of the IgBP(s) from *M. haemolytica* grown in RPMI-1640 at 37°C and 76% of the IgBP(s) from *M. haemolytica* grown in RPMI-1640 with 10% FBS. It is not known if the multiple spots of *M. haemolytica* IgBP(s) are breakdown products of one protein or consist of a number of different proteins. Previous research suggested that the *M. haemolytica* IgBP(s) may proteolyze with products found at approximately 20 kDa (Osmundson, 2003, manuscript in preparation). A number of other IgBPs have multiple binding sites for immunoglobulins (Moks et al., 1986; Sjöbring et al., 1989). It is possible that breakdown products bind bovine Fc IgG as long as the binding sites are intact.

When *M. haemolytica* was grown in control media, 8.2% of membrane proteins were IgBP(s). Up to 19.9% of membrane proteins consisted of the IgBP(s) when grown in media mimicking factors of *in vivo* growth. The smallest increase was seen when *M. haemolytica* were grown in RPMI-1640 with 50 μM 2,2-dipyridyl where the percentage of IgBP(s) is 1.9 times that found in minimal media alone. These results suggest that the *M. haemolytica*
IgBP(s) may have a significant role in virulence under various growth conditions and any role it may play in virulence might be increased during *M. haemolytica* infection.

Growth in both RPMI-1640 with 10% FBS and RPMI-1640 at 40°C resulted in a threefold increase in total IgBP pixel density and growth in RPMI-1640 with 2,2′-dipyridyl resulted in a twofold increase compared to IgBP expression in controlled conditions. This again argues for a role in the IgBP(s) in virulence. Even though there was a threefold increase in IgBP pixel density in the first two growth conditions, the scatter plot analysis indicated a differential distribution at the level of expression. The correlation coefficient of pixel density of individual spots from IgBP(s) grown from cells in RPMI-1640 with 10% FBS compared to IgBP(s) from cells grown in RPMI at 37°C was 0.2. This compares to a correlation coefficient of 0.4 in the same comparison in conditions involving RPMI-1640 at 40°C and RPMI-1640 at 37°C. The average pixel density deviation of upregulated protein(s) was 5494.4 for IgBPs from RPMI-1640 with 10% FBS grown *M. haemolytica*. This same value for IgBPs from RPMI-1640 at 40°C was 2986.9. Therefore, both growth conditions resulted in similar absolute total pixel density values, but there was a larger average deviation in individual spot density from *M. haemolytica* grown in RPMI-1640 with 10% FBS. There is an apparent difference in the upregulation of IgBP(s) expressed in the two different growth conditions.

The *M. haemolytica* IgBP(s) has previously been shown to bind bovine Fc IgG and to be immunogenic (Osmundson, 2003, manuscript in preparation). These results suggest that the IgBP(s) has a further role in virulence because it also binds Fg and Fn. Other bacterial ECMBPs are involved in cell adhesion to airway epithelial cells. Similarly, upregulation of IgBP(s) expression in conditions mimicking *in vivo* conditions further suggests an important
role in virulence. The *M. haemolytica* IgBP(s) might be an important component of an *M. haemolytica* subunit vaccine.

References


FIG. 1. Far-Western blot identifying IgBP(s) binding to Fn. (A) Lanes: 1, MW markers; 2, Far-Western blot of SDS-PAGE of 7.0 µg isolated IgBP(s) blotted to nitrocellulose and bound with 300.0 µg Fn followed by labeling with rabbit anti-Fn-HRP (1:1000); 3, MW markers; 4, Far-Western blot of SDS-PAGE of 10.0 µg Fn blotted to nitrocellulose and bound to 44.0 µg isolated IgBP(s) followed by binding to 44.0 µg bovine Fc IgG and rabbit F(ab')₂ anti-bovine Fc IgG-HRP; 5, SDS-PAGE of 10.0 µg Fn
FIG. 2. Far-Western blot identifying IgBP(s) binding to Fg. Lanes: 1, MW markers; 2, Far-Western blot of SDS-PAGE of 10.0 µg Fg blotted to nitrocellulose and bound to 7.0 µg isolated IgBP(s) followed by binding to 44.0 µg bovine Fc IgG and rabbit F(ab')₂ anti-bovine Fc IgG-HRP; 3, SDS-PAGE of 10.0 µg Fg; 4, MW markers; 5, Far-Western blot of SDS-PAGE of 10.0 µg Fg blotted to nitrocellulose followed by binding to 44 µg bovine Fc IgG and rabbit F(ab')₂ anti-bovine Fc IgG-HRP; 6 SDS-PAGE of 10.0 µg Fg.
FIG. 3. 2-D SDS-PAGE gels and far-Western blot of *M. haemolytica* membrane proteins (50 μg) grown in four *in vitro* growth conditions. (A,B) Far-Western blots of 2-D SDS-PAGE gel of membrane proteins (50 μg) labeled with 44 μg bovine Fc IgG and labeled with rabbit F(ab′)2 anti-bovine Fc IgG-HRP (1:1000). (A) MW of spots and MW markers. 2-D SDS-PAGE of membrane proteins (50 μg) grown in (C) RPMI-1640 at 37°C (D) RPMI-1640 with 50 μM 2,2'-dipyridyl (E) RPMI-1640 with 10% FBS (F) RPMI-1640 at 40°C. Crosshairs mark IgBP(s) location.
FIG. 4. Comparative analysis of individual spot pixel density. Individual spot pixel density values for IgBP(s) from *M. haemolytica* grown in RPMI-1640 (x-axis, panel A-C) is plotted versus individual spot pixel density for IgBP(s) from *M. haemolytica* grown under different conditions (y-axis): panel A, RPMI-1640 with 50μM 2,2’-dipyridyl; panel B, RPMI-1640 with 10% FBS; panel C, RPMI-1640 at 40°C.
TABLE 1. Bacterial growth *in vitro*

<table>
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<th>Growth Condition</th>
<th>RPMI-1640 37°C</th>
<th>RPMI-1640 50μM 2,2'-dipyridyl</th>
<th>RPMI-1640 with 10% FBS</th>
<th>RPMI-1640 40°C</th>
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<tr>
<td>A&lt;sub&gt;600&lt;/sub&gt;</td>
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<td>0.54</td>
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</table>

TABLE 2. Comparison of total IgBP expression from four *in vitro* growth conditions

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>IgBP pixel density</th>
<th>Fold-increase of IgBP pixel density</th>
<th>% IgBP pixel density of total pixel density</th>
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<td>64,265</td>
<td>2.11</td>
<td>15.8</td>
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<td>RPMI-1640 with 10% FBS</td>
<td>94,880</td>
<td>3.12</td>
<td>16.1</td>
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<tr>
<td>RPMI-1640 at 40°C</td>
<td>96,710</td>
<td>3.18</td>
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CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS

This study identified an IgBP expressed by *M. haemolytica*. Results based on virulence studies of IgBPs of other organisms suggest that the *M. haemolytica* IgBP(s) may have a significant role in virulence. IgBPs are present in a number of Gram-positive and Gram-negative bacteria.

Experiments *in vitro* and *in vivo* point to an IgBP role in virulence both in terms of evasion of the host immune response and modulation of the immune response. The *M. haemolytica* IgBP was shown to be expressed in the WCS and also to be secreted. Both preparations had a dominant band on SDS-PAGE at 75.0 kDa and a less intensely staining band at 40.2 kDa. The supernatant preparation has an additional band at 107.5 kDa. This band appears unique to the supernatant because isolated, concentrated IgBP in Chapter 2. Fig. 2 shows a number of additional bands on SDS-PAGE but no band comparable to the 107.5 kDa band found in the supernatant. Additionally, 2-D gel results from *M. haemolytica* membrane preparations have no spots greater than 95 kDa. This additional supernatant band could be due to post-translational modification or perhaps an additional secreted IgBP that is not found in the WCS or membrane preparations.

There are a number of possible explanations for the appearance of more than one IgBP band and multiple spots on SDS-PAGE and 2-D far-Western blots. IgBPs from other organisms have repeat binding sites for Ig. Protein A has five (Mocks et al., 1986) while protein G has three repeat Ig binding regions (Guss et al., 1986). It is possible that the different *M. haemolytica* IgBP bands evident on far-Western blot are due to a single protein differing only in the number of Fc IgG binding sites at a particular molecular weight band.
Multiple bands could also be due to a unique protein in each Western blot band or the bands might be degradation products of one or more proteins. There is evidence for the latter possibility as seen by SDS-PAGE of the isolated, concentrated IgBP (Chapter 2, Fig. 2).

Bacterial cell adherence to host cells is a required initial step in most bacterial infections and ECMBPs may play a large role in this process or may be required for infection. The *S. aureus* FnbpA is known to bind Fg as well as Fn (Wann et al., 2000) and FnbpA is involved in adherence to epithelial cells (Mongodin et al., 2002). The mechanism used by *M. haemolytica* to adhere to the URT initiating colonization is not known. *M. haemolytica* ST2 is most frequently found as a commensal of the nasopharynx while ST1 is most frequently isolated in infection. This change is initiated by stress and possibly involves a change in the host environment. Results here indicate that the *M. haemolytica* IgBP binds Fg and Fn. Fibrinogen and Fn are involved in bacterial cell adhesion as extracellular matrix proteins and experiments indicate a role for ECMBPs in bacterial adhesion to host cells. Up to 19.9% of *M. haemolytica* membrane proteins were IgBPs in conditions modeling *in vivo* conditions which could indicate an important IgBP role in virulence. The *M. haemolytica* IgBP(s) might play a role in adherence and colonization. Additional roles for ECMBPs in virulence are being found and as a result the *M. haemolytica* IgBP(s) may have other roles in virulence.

Far-Western blot results demonstrated that the *M. haemolytica* IgBP bound bovine Fc IgG and Fg as well as bovine Fc IgG and Fn simultaneously. The significance of this is not known. There is no previously demonstrated virulence role that IgBPs and ECMBPs have in common. It is possible that the *M. haemolytica* IgBP(s) binds one protein with a higher
affinity than the other proteins or that steric hindrance could result in preferential binding of one or more proteins, particularly when IgBP(s) expression is upregulated.

Several physical characteristics of the IgBP(s) determined in these experiments are consistent with the proposal that the IgBP(s) plays a role in virulence. First, flow cytometry showed the IgBP to be surface expressed on *M. haemolytica* (Chapter 2, Fig. 3). Surface expression of protein H, an Fc IgG-binding protein from *Streptococcus pyogenes*, reduces phagocytosis of the bacteria (Kihlberg, et al., 1999) and inhibites complement activation by blocking C3 deposition on the bacterial surface (Berge, et al., 1997). Increased protein A surface expression on *S. aureus* leads to increased inhibition of phagocytosis (Dosset, et al., 1969). These virulence mechanisms may be active in *M. haemolytica*. Second, this study identified a secreted form of the *M. haemolytica* IgBP. Soluble protein A reduces antibody-dependent cellular (Rosenblatt, et al., 1977) and fixes complement in solution thus consuming complement components (Sjöquist and Stålenheim, 1969). Soluble protein H interacts with IgG to form complement-activating complexes (Berge, et al., 1997). Thus, the CS IgBP may play a role in inhibiting phagocytosis and limiting complement activation as well as consuming soluble complement components. Additionally, convalescent cattle were shown to have an increased antibody response to the 70.5 kDa *M. haemolytica* IgBP when compared to naïve or acutely infected cattle (Chapter 2, Fig. 4). The increased antibody response in acutely infected cattle suggests that inhibiting the effectiveness of the IgBP may play a role in recovery. Lastly, growth in both RPMI-1640 with 10% FBS and RPMI-1640 at 40°C resulted in a three-fold increase in total IgBP pixel density on 2-D gel analysis and growth in RPMI-1640 with 2,2'-dipyridyl resulted in a two-fold increase compared to IgBP expression in controlled conditions. When *M. haemolytica* was grown in control media,
8.2% of membrane proteins were IgBP(s). Up to 19.9% of membrane proteins consisted of the IgBP(s) when grown in media simulating factors of in vivo growth. The smallest increase was seen when *M. haemolytica* are grown in RPMI-1640 with 50 μM 2,2-dipyridyl where the percentage of IgBP(s) was 1.9 times that found in minimal media alone (Chapter 3, Table 2). These results suggest that the *M. haemolytica* IgBP(s) may have a significant role in virulence under various growth conditions and any role it may play in virulence may be increased during *M. haemolytica* infection.

The mechanism for the abrupt change from the predominance of the normal flora *M. haemolytica* ST2 to explosive growth of stress-associated ST1 is not known. However, change in host environmental factors due to stress is likely to play a role. There is no significant difference in the bovine Fc IgG SDS-PAGE banding patterns between ST1 and ST2 WCSs prepared with the same growth conditions. However, IgBP(s) membrane expression was up-regulated in growth conditions that mimic factors of in vivo growth during infection. If the change from the commensal ST2 to infective ST1 is due to a phase variation in *M. haemolytica* and is due to environmental factors, increased IgBP(s) expression may be part of that change.

*M. haemolytica* ST1 is generally associated with BRD in cattle (Whitely, et al., 1992 and Frank, 1979) and ST2 is most frequently associated with pneumonic mannheimiosis in sheep (Fraser, et al., 1982). In an effort to determine whether the *M. haemolytica* IgBP is a factor in serotype-specificity of species-specific infection, far-Western blots were run to determine whether differences were evident between binding of bovine Fc IgG or sheep Fc IgG to ST1 and ST2 and whether bovine Fc IgG and sheep Fc IgG binds to all 12 serotype WCS preparations regardless of whether they are known to be involved in infection.
Differences among infectious serotypes or between infectious and noninfectious serotypes might indicate a role for serotype-specific infection in cattle and sheep. Serotypes 1 and 2 both bound bovine Fc IgG and they all had protein bands at 74.1 and 63.5 kDa that stain with similar intensity in the two serotypes (Chapter 2, Fig. 5). The 74.1 kDa band appeared equally dominant in these two serotypes. A 33.2 kDa band was evident in ST2 that is not found in ST1. It is possible that this difference is responsible for serotype-specificity of infection in cattle and sheep. However, the remaining serotypes expressed the 33.2 kDa protein band and these are not implicated in infection in cattle. This suggests that *M. haemolytica* expressed IgBPs do not play a role in serotype-specificity in infection of cattle. All 12 serotypes expressed an IgBP that bound bovine Fc IgG and all serotypes had similar banding patterns. No serotype had a protein band that is unique to that serotype. This is further evidence that *M. haemolytica* IgBP does not play a role in the dominance of ST1 found in infections in cattle. Differences among the serotypes in binding bovine Fc IgG may be due to: 1) Serotypes expressing dissimilar amounts of IgBP; 2) different serotypes expressing IgBPs with diverse numbers of Fc IgG binding sites; or 3) *M. haemolytica* expressing more than one bovine Fc IgG IgBP in different serotypes.

Serotype 2 bound sheep Fc IgG more intensely than does ST1 (Chapter 2, Fig. 6). However, as in bovine Fc IgG binding, other serotypes not known to be infective had protein-banding patterns as intense as found with ST 2. This would suggest that the difference found between ST1 and ST 2 is not responsible for serotype-specific infection in sheep. All serotypes bound sheep Fc IgG in a similar protein-banding pattern. Four serotypes (5, 8, 13 and 14) have a particularly dominant band at 62.1 kDa. What role if any this may play in bacterial virulence or lack of virulence in these serotypes is not known.
Interestingly, bovine Fc IgG bound to *M. haemolytica* IgBP in a different protein-band pattern than sheep Fc IgG in all STs. *M. haemolytica* may express only one IgBP that has separate binding sites for sheep Fc IgG and bovine Fc IgG or *M. haemolytica* may express different proteins for binding bovine Fc IgG and sheep Fc IgG. Whether or not this plays any role in virulence is not known and is again doubtful because these same differences occur among infective serotypes and noninfective STs.

The fact that all serotypes expressed an IgBP(s) but not all serotypes are infective could indicate that the IgBP(s) are not involved in virulence. However, several lines of evidence demonstrate that this may not be true: 1) Protein A from *S. aureus* (Sjöquist et al., 1972), protein H from *Streptococcus pyogenes* are surface expressed. These experiments showed *M. haemolytica* IgBP(s) to be surface expressed. Protein A surface expression on *S. aureus* is involved in inhibiting phagocytosis (Dosset et al., 1969) and Protein H surface expression inhibits phagocytosis (Kihlberg et al., 1999). 2) Convalescent cattle showed an increased antibody response to *M. haemolytica* ST1 IgBP. 3) *M. haemolytica* IgBP may be a virulence factor in infection of cattle and sheep but may not be a factor sufficient by itself to cause infection.

Bacterial growth in RPMI-1640 with 10% FBS resulted in the highest number of bacteria while the least growth occurred when bacteria were grown in RPMI-1640 at 40°C. However, both of these conditions resulted in the greatest and nearly equal relative IgBP expression. It is possible that one environmental factor trigger induces upregulation under both conditions or the upregulation may be due to independent factors. Regardless, bacterial growth or lack of growth can occur independently of IgBP expression and IgBP expression can be upregulated under conditions that are not optimal for growth.
Even though there was a three-fold increase in IgBP pixel density in the first two growth conditions the scatter plot analysis indicated a differential distribution in the level of expression. The correlation coefficient of pixel density of individual spots from IgBP(s) grown in RPMI-1640 with 10% FBS compared to IgBP(s) grown in RPMI at 37°C was 0.2 (Chapter 3, Fig. 3) This compared to a correlation coefficient of 0.4 in the same comparison in condition involving RPMI-1640 at 40°C and RPMI-1640 at 37°C. The average pixel density deviation of upregulated protein(s) was 5494.4 for IgBPs from RPMI-1640-FBS grown *M. haemolytica*. This same value for IgBPs from RPMI-1640 at 40°C was 2986.9. Therefore, both growth conditions result in similar absolute total pixel density values, but there is a larger average deviation from the spot density of individual IgBP(s) spots grown in control by IgBP(s) grown in RPMI-1640 with 10% FBS. There is an apparent difference in the upregulation of IgBP(s) expressed in the two different growth conditions.

Even though *M. haemolytica* ST2 is found as a commensal in cattle while ST1 is generally isolated in *M. haemolytica* induced BRD, there was no apparent difference in the bovine Fc IgG SDS-PAGE banding patterns between ST1 and ST2 WCSs prepared with the same growth conditions. However, IgBP(s)membrane expression was upregulated in growth conditions that mimic factors of in vivo growth during infection. If the change from the commensal ST2 to infective ST1 is due to a phase variation in *M. haemolytica* and is due to environmental factors, expression may be part of that change.

The bovine bacterial pathogenesis in Table 1 is consistent with information demonstrated in bacterial infections and *M. haemolytica* induced BRD.

Table 1. Bovine bacterial pathogenesis
The proposed IgBP role in virulence model in Table 2 is consistent with what has been shown about bacterial infections, BRD pathogenesis and with the results of this study. In this scenario, whether attachment or evasion dominates when Fn, Fg and bovine Fc IgG are available might depend on several factors. First, the protein bound to IgBPs would be a determining factor in the outcome and Fn or Fg binding would facilitate attachment while bovine Fc IgG binding would provide protection from the host immune response. Secondly, the degree of upregulation may determine which protein is bound as one protein may have a steric advantage or disadvantage in binding at a given expression level. Finally, the affinity of the IgBP(s) for a particular protein would affect which protein is bound. Perhaps in the LRT when serum IgG, Fn, and Fg would be abundant during infection, the IgBP(s) would have a greater affinity for Fc IgG than the ECMPs and evasion of the host immune response would be the dominant role of the IgBPs. Because pathogenesis is a process and because it is not likely that only one moiety of protein will be bound to IgBPs at a given time, the proportion of any of these proteins bound at a particular time might affect the virulence role of the IgBP(s).
Table 2. Proposed IgBP role in virulence

<table>
<thead>
<tr>
<th>IgBP(s) Expression</th>
<th>Nasopharynx (Commensal)</th>
<th>URT (Colonization)</th>
<th>LRT (Infection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expression</td>
<td>Homeostatic</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>ECMP Binding</td>
<td>Fn/Fg binding</td>
<td>Fn/Fg binding</td>
<td>Fn/Fg binding</td>
</tr>
<tr>
<td></td>
<td>No Fc IgG binding</td>
<td>No Fc IgG binding</td>
<td>Bovine Fc IgG binding</td>
</tr>
<tr>
<td>Function</td>
<td>Attachment</td>
<td>Attachment</td>
<td>Attachment Evasion</td>
</tr>
</tbody>
</table>

Future research could take a number of interesting directions. Biochemical and biophysical characterization of the IgBP(s) would establish similarities or dissimilarities between the *M. haemolytica* IgBP(s) and other known IgBP(s). This information could help to determine whether roles in virulence established for other IgBPs might also be true for the *M. haemolytica* IgBP(s). Biologically, it can be determined whether or not Fn or Fg can act as a bridge between the *M. haemolytica* IgBP(s) and airway epithelial cells thus facilitating adhesion. Genetic analysis would clarify whether the IgBP(s) is one protein or more than one protein. This work would open the door to creation of a deletion mutant lacking expression of the IgBP(s). Infectivity trials with the deletion mutant would help determine the importance of the IgBP(s) in virulence.

In conclusion, this study identified an *M. haemolytica* surface expressed IgBP as well as a secreted IgBP. The IgBP did not appear to play a role in serotype-specificity of species-specific infection as all serotypes bound both bovine Fc IgG and sheep Fc IgG. However, sheep Fc IgG binds *M. haemolytica* IgBP with a different protein band pattern on far-Western blot than did bovine Fc IgG. This difference may indicate a different virulence role for the IgBP in cattle and sheep but more likely it may be that more than one IgBP may be
expressed by *M. haemolytica* or there are different binding sites for sheep Fc IgG and bovine Fc IgG. The *M. haemolytica* IgBP from the WCS preparation was isolated by affinity chromatography. Western blot results in this study showed an increased antibody response to *M. haemolytica* IgBP in cattle recovering from mannheimiosis indicating a role for the IgBP in protection from *M. haemolytica* induced BRD. The *in vivo* and *in vitro* virulence association of protein A and other previously identified IgBPs, the similarity in surface expression of protein A, protein H and *M. haemolytica* IgBP, the presence of a secreted *M. haemolytica* IgBP and the increased immune response to *M. haemolytica* IgBP in convalescent cattle suggest a role for *M. haemolytica* IgBP in virulence. The *M. haemolytica* IgBP(s) bound Fg and Fn. These results suggest that the IgBP(s) has a further role in virulence. Other bacterial ECMBPs are involved in cell adhesion to airway epithelial cells. Similarly, upregulation of IgBP(s) expression in conditions mimicking *in vivo* conditions further suggests an important role in virulence. The *M. haemolytica* IgBP(s) might be an important component of an *M. haemolytica* subunit vaccine.
REFERENCES


ACKNOWLEDGMENTS

I am ever grateful to my children. They have been with me in the good times and the bad while I’ve gone through graduate school. They shared their pride about my accomplishments and encouraged me when the going was rough. They have sacrificed at times they may not have wanted to sacrifice. And they’ve shared in the joy as I’ve accomplished my goals. May I be as good at supporting them toward their goals in life as they’ve been with mine.

I am grateful to my major professor, Dr. Louisa B. Tabatabai. She had faith in me before I had faith in myself. At times when I wasn’t sure whether I wanted to take the next step, she showed me where to put my foot. Her guidance in my research has often opened a door for exploration when the next stage looked dark to me. She has encouraged me to look for and try new, creative solutions. She taught me by her example to find different ways to generate more information so that more questions can be answered. She has freely shared her initiative and curiosity with me, and helped me to learn to use those same qualities. Thank you, Louisa.

I would like to thank my committee members; my co-major professor Dr. James Roth who has been helpful with discussions, Dr. Joan Cunick who has shared her expertise about flow cytometry, Dr. Merlin Kaeberle who has added his wisdom, and Dr. James Dickson who has made extra efforts to learn about my research after joining my committee after much of the research was done. I wasn’t able to thank Dr. Donald Graves who retired before the completion of my research. He helped me keep perspective about my work.
Dr. Carol Belzer was a graduate student in our lab when I started. I’m grateful that she helped me learn many practical aspects about lab work and shared her roadmap through graduate school. Thank you, Carol.

I also want to thank my parents, Isabelle Smith and Gordon Smith. I would not be the person I am today without the start they gave me in life.