MOLECULAR AND IMMUNOLOGICAL ANALYSES OF 38 kDA AND 45 kDA PROTEIN ANTIGENS OF *Pasteurella haemolytica* S1

By
KARAMJEET PANDHER

Bachelor of Veterinary Science

College of Veterinary Sciences

Punjab Agricultural University

Punjab, India

1993

Submitted to the Faculty of the
Graduate College of
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 1998

MOLECULAR AND IMMUNOLOGICAL ANALYSES

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Thesis Approved:

Thesis Advisor

Dean of the Graduate College

ACKNOWLEDGMENTS

I would like to thank my major advisor, Dr. George L. Murphy for his constant guidance, instruction and constructive criticism, without which this research would not have been possible. I also appreciate his effort and patience in teaching me the techniques involved in molecular biology and technical writing. I would like to express my gratitude to the members of my advisory committee Drs. K.D. Clinkenbeard, A.W. Confer, and J.H. Wyckoff whose suggestions, guidance, and support were extremely helpful. I would especially like to thank Dr. A.W. Confer for providing me with sera used in my research and also for helping with statistical analysis of the data. Thanks are also due to Drs. R.E. Eberle, C.W. Qualls, R.J. Panciera and S. Sangiah for their constant encouragement and support.

I would like to acknowledge my family for their love, moral and financial support during my years as a student. They have always encouraged me to go the extra mile. My years in Stillwater and Oklahoma State University were extremely pleasant ones, because of friends with whom I was fortunate enough to share my time and thoughts.

Finally, I would like to thank the College of Veterinary Medicine for supporting me on a research assistantship through these five years of study.

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INTRODUCTION

Introduction and review of relevant literature

Louis Pasteur is often referred to as the father of medical microbiology. He discovered that fowl cholera was caused by bacteria, thereby providing the pioneering evidence that bacteria could cause disease in animals. In his honor the bacterial causative agent of fowl cholera was named *Pasteurella multocida*. Later similar, Gram-negative, facultatively anaerobic, and fermentative bacteria were included in the family Pasteurellaceae Pohl (Mutters et al., 1989). This family consists of genera *Actinobacillus*, *Haemophilus*, and *Pasteurella*. Organisms in the genus *Pasteurella* can cause haemorrhagic septicemia, bronchopneumonia, meningitis, mastitis, abortion, and localized infection in cattle (Frank, 1989).

In the United States, pneumonic pasteurellosis of cattle is one of the most important diseases associated with Pasteurella (Confer et al., 1990). Annual losses to the beef industry attributed to this disease are about one billion dollars (Dee, 1997). The disease is also called shipping fever and is characterized by acute fibrinous bronchopneumonia and pleuritis (Confer et al., 1988). Pneumonic pasteurellosis is precipitated by stress inducing conditions such as shipping, overcrowding, weaning, viral infections, and inclement weather. Pneumonic pasteurellosis is frequently the immediate cause of death in animals suffering from bovine respiratory disease (BRD) (Frank, 1989). BRD is a bigger entity than pneumonic pasteurellosis and

consists of lower respiratory tract infections of dairy and beef cattle caused by bacteria, viruses, and mycoplasma. Shipping fever is a term used to describe the fibrinous pleuropneumonia caused by *Pasteurella* in beef cattle.

Pasteurella haemolytica serotype 1 (S1) is the organism most commonly associated with shipping fever i.e., pneumonic pasteurellosis of beef cattle (Frank, 1989) (Confer et al., 1988) (Wilkie and Shewen, 1988). The disease has been reproduced experimentally in calves by transthoracic and intratracheal administration of *P. haemolytica* S1 alone (Ames et al., 1985) (Panciera and Corstvet, 1984). *P. haemolytica* have been typed into 17 serotypes based mainly on the antigenic nature of the capsular polysaccharides synthesized by these organisms (Biberstein, 1978) (Fodor et al., 1988) (Younan and Fodor, 1995). Thirteen of the 17 serotypes ferment arabinose and are grouped under biotype A, whereas 4 ferment trehalose and are grouped under biotype T (Biberstein, 1978) (Younan and Fodor, 1995). More recently it has been demonstrated that biotypes A and T are notably different in their 16S rRNA gene sequences and belong to different phylogenetic clusters under the family *Pasteurellaceae* (Dewhirst et al., 1992). It has been proposed that the T strains be given a species name, *P. trehalosi* (Sneath and Stevens, 1990).

Pathogenesis

Despite numerous years of research, the pathogenesis of *P. haemolytica* pneumonia is not well characterized. Numerous reviews have put the available knowledge about *P. haemolytica* infection in perspective (Frank,

1989) (Whiteley et al., 1992) (Confer et al., 1995). Healthy calves, even those that have not encountered *P. haemolytica*, have host defense mechanisms that are capable of clearing a *P. haemolytica* infection (Frank, 1989). In contrast, lungs of calves that are immunocompromised due to viral infections, such as bovine herpesvirus-1(BHV-1) or parainfluenza-3 (PI-3) virus, are readily colonized by *P. haemolytica* (Frank et al., 1986) (Frank et al., 1987).

Viral infections play an important role in development of pneumonic pasteurellosis. BHV-1, PI-3 or bovine respiratory syncitial virus (BRSV) damage the mucosa of the respiratory tract and reduce pulmonary clearance (Confer et al., 1988). BHV-1 also modulates leukocyte function (Filion et al., 1983). Although bovine viral diarrhea virus (BVD) does not directly affect pulmonary defense mechanisms, it causes generalized immunosuppression and enhances experimental pneumonic pasteurellosis (Lopez et al., 1982) (Potgieter et al., 1984). Other stress inducing factors such as inclement weather, overcrowding, and shipping also produce similar results, underscoring the role of stress in development of pneumonic pasteurellosis (Whiteley et al., 1992) (Jones, 1987). Stress in general causes immunomodulation which promotes colonization by *P. haemolytica* (Filion et al., 1984). It has been postulated that under stress the upper respiratory tract of cattle favors multiplication of *P. haemolytica* S1 over *P. haemolytica* S2 (Confer et al., 1988). Only *P. haemolytica* S1 seem to have the capacity to

colonize the lung after inhalation, although the mechanism by which they do so is not clear (Gonzalez and Maheswaran, 1993).

It is generally believed that *P. haemolytica* S1 are present in low numbers in the upper respiratory tract (URT) of calves, and under stressful conditions, they proliferate and are aerosolized into the lung (Frank, 1989) (Grey and Thomson, 1971). *P. haemolytica* strains have also been isolated from grass, drinking water, and straw bedding used for sheep, where cold conditions seemed to favor their extended viability (Burriel, 1997). Thus fomites could play an important role in spread of *P. haemolytica* infection among animals in close contact. It is not clear, however, whether the strain that is present in the URT of an individual is also the strain that colonizes the lung or if cattle acquire a new strain during an outbreak of pneumonic pasteurellosis. Limited information is available on the different strains within serotypes of *P. haemolytica*. Ribotyping, restriction endonuclease analysis, minimum inhibitory concentrations (MIC), and plasmid content have been used to compare isolates recovered from feedlot cattle during a pneumonic pasteurellosis outbreak (Murphy et al., 1993). That study identified 6 different strains including 2 different ones from a single animal. Outer membrane protein (OMP), lipopolysaccharide (LPS), and enzyme profiles analyses have also been used for comparing isolates (Davies et al., 1996) (Purdy et al., 1997). Such studies should be helpful in understanding the epidemiology of *P. haemolytica* infection and may provide useful insights into pathogenesis of the disease.

Bacterial factors associated with virulence

Colonization of the URT mucous membrane seems to be the first and indispensable step towards the establishment of *P. haemolytica* infection. It is not clear whether *P. haemolytica* adhere to the URT mucosa or whether they remain in the mucus layer without adhering (Confer et al., 1995). Adherence could potentially take place through pili. Although pili have been demonstrated *in P. haemolytica* by one group of researchers, these results have not been verified by independent groups (Morck et al., 1987) (Potter et al., 1988) (Gonzalez and Maheswaran, 1993) (Confer et al., 1995). Hence, the expression of pili by *P. haemolytica* is debated. Consequently, their role, if any, in adherence of *P. haemolytica* remains undetermined. Outer membrane proteins (OMPs) and capsular polysaccharide preparations of *P. haemolytica* have been shown to adhere to bovine tracheal mucus (Botcher et al., 1993).

In addition, a neuraminidase produced by *P. haemolytica* can cleave bovine mucin and could be a factor in promoting adherence of the organism to mucous membranes (Botcher et al., 1993) (Confer et al., 1995). The removal of sialic acid residue from salivary glycoproteins reduces their capability to function as a protective covering on the mucous membranes (Gottschalk, 1960). Thus by cleaving the sialic acid residue from salivary glycoproteins, neuraminidase could serve as an important virulence factor for *P. haemolytica*, aiding in colonizing nasal mucous membranes (Straus et al., 1993). However, this study could not determine if strains of *P. haemolytica*

which lack the ability to produce neuraminidase were less virulent when compared to neuraminidase producing strains. Recently, antibodies against a sialoglycoprotease of *P. haemolytica* have been reported in sera of calves that were administered a commercial vaccine (Chiang et al., 1994). It was demonstrated that on challenge with live *P. haemolytica*, calves producing these anti-sialoglycoprotease antibodies had fewer pulmonary lesions, when lungs were examined post mortem (Chiang et al., 1994). However, a direct role for these specific antibodies, in protection against *P. haemolytica* infection was not established.

Recently, a gene encoding serotype specific antigen (Ssa) was discovered in *P. haemolytica* S1, and its homologues in serotypes 2, 5, 6, 7, 9, and 12 were also demonstrated (Lo et al., 1991) (Gonzalez-Rayos et al., 1986)(Gonzalez et al., 1991). The gene encoding Ssa1, when transformed into *E. coli*, was able to confer a serotype 1 phenotype to the recipient due to expression of Ssa1 protein (Gonzalez et al., 1991). This protein has been suggested to be another potential adherence factor for *P. haemolytica* S1 (Gonzalez et al., 1991). However, evidence to support this suggestion is lacking.

A capsular polysaccharide (CPS) produced by *P. haemolytica* was identified by Corstvet et al., 1982. CPS is serotype specific and its structure was studied using chemical analysis and NMR spectroscopy (Adlam et al., 1984). CPS causes diminished phagocytosis and killing by bovine neutrophils and macrophages (Czuprynski et al., 1991a) (Czuprynski et al., 1987). Also encapsulated *P. haemolytica* were more resistant to serum agglutination and

complement-mediated killing as compared to decapsulated organisms (Chae et al., 1990).

Leukotoxin (Lkt) is an important virulence factor of *P. haemolytica* that seems to help not only in overpowering the host immune response, but also in orchestrating the inflammatory response associated with the disease. Lkt is a secreted, pore-forming cytolysin, which is specific for bovine leukocytes and platelets (Clinkenbeard et al., 1989) (Clinkenbeard and Upton, 1991) (Shewen and Wilkie, 1982). Lkt also induces apoptosis in bovine leukocytes (Stevens and Czuprynski, 1996), down-regulates major histocompatibility complex class II (MHC-II), and induces histamine release by pulmonary mast cells (Hughes et al., 1994) (Adusu et al., 1994). Lkt is secreted in an aggregated form and this form is not as potent at lysing leukocytes as the disaggregated form. Bovine serum albumin was shown to be a potent stimulator of Lkt activity (Waurzyniak et al., 1994). It has been proposed that exudation of BSA-rich serum into lung alveoli during pneumonic pasteurellosis could cause the disaggregation of Lkt, thus enhancing its lytic activity on bovine leukocytes (Confer et al., 1995). On the other hand, when present in low concentration Lkt is a potent stimulator of bovine neutrophils *in vitro* (Czuprynski et al., 1991b). Activation of leukocytes could cause release of oxygen-free radicals in the surrounding tissue thereby increasing tissue necrosis (Confer et al., 1995). Thus it is not clear whether Lkt is more harmful in low doses by increasing neutrophil-mediated injury in the lung parenchyma or in high dose by causing neutrophil lysis. It is certain, however, that Lkt plays a major role in

the inflammatory process that leads to fibrinous pleuropneumonia as a result of *P. haemolytica* infection.

Lipopolysaccharide (LPS) of *P. haemolytica* is similar to that of other Gram-negative bacteria and contains biologically active lipid A (Rimsay et al., 1981) (Confer et al., 1990). LPS induces adherence of neutrophils to bovine pulmonary artery endothelial cells and causes activation of arterial endothelial cells (release of arachidonic acid), processes that are important in the inflammatory pathways, like leukocyte extravasation, associated with pneumonic pasteurellosis (Paulsen et al., 1989). LPS also induces the release of proinflammatory cytokines in bovine alveolar macrophages (Yoo et al., 1995). An LPS-associated protein (LAP) has also been demonstrated to induce pulmonary inflammation when deposited in the lung of calves (Brogden et al., 1995a). Thus LPS of *P. haemolytica* is an important virulence factor involved in the development of an acute inflammatory response.

P. haemolytica express novel periplasmic protein of 35 kDa and outer membrane proteins of 71, 73, and 100 kDa when grown in iron-restricted culture conditions or *in vivo* (Murray et al., 1992) (Deneer and Potter, 1989) (Morck et al., 1991) (Ogunnariwo and Schryvers, 1990). These proteins might be considered as virulence factors, because they are expressed *in vivo* and may be important in the acquisition of iron in an environment where no free iron is available (Yu et al., 1992) (Morck et al., 1991).

Immunological importance of *P. haemolytica* molecules

Leukotoxin

As described above Lkt is a pore-forming cytolysin, belonging to the family of RTX toxins, and it specifically lyses ruminant leukocytes and platelets (Confer et al., 1995). If Lkt is an important virulence factor, it is logical to assume that antibodies against this molecule would aid in development of resistance to pneumonic pasteurellosis by neutralizing the harmful effects of leukotoxin on its target cells. Indeed serum-neutralizing antibodies against Lkt directly correlated with resistance to experimental *P. haemolytica* challenge (Gentry et al., 1985). Addition of recombinant leukotoxin containing supernatant to a commercial vaccine, enhanced the protective capacity of the vaccine; however, vaccination of cattle with recombinant Lkt alone failed to protect cattle against experimental challenge (Conlon et al., 1991) (Purdy et al., 1993). These results corroborate earlier findings that antibody responses against both Lkt and other surface antigens are required for development of immunity to *P. haemolytica* infection (Shewen and Wilkie, 1988). The use of Lkt in commercial vaccines is discussed in a later section on *P. haemolytica* vaccine studies.

Capsular polysaccharide

The capsular polysaccharide (CPS) from *P. haemolytica* S1 has been purified and characterized, and its immunological properties have been studied (Adlam et al., 1984). Antiserum raised against the partially purified form of *P. haemolytica* S1 capsule was found to be specific against the

homologous serotype; and in an immunodiffusion test, it did not precipitate antigens from other serotypes (Adlam et al., 1984). Indeed, antiserum raised against the capsular antigen is used to differentiate *P. haemolytica* into 16 serotypes (Frank, 1989). Immunization of calves with *P. haemolytica* capsular antigen or with live or killed *P. haemolytica* induces an immune response to capsular polysaccharide; however, this immune response does not correlate with development of resistance to pneumonic pasteurellosis (Confer et al., 1989). Vaccination of calves with CPS failed to protect cattle against experimental challenge (Conlon and Shewen, 1991). In a recent study, however, it was demonstrated that the protective ability of CPS as an immunogen could be significantly increased by using muramyl dipeptide analogs as adjuvants (Brogden et al., 1995b). In another study, preparturient vaccination of dams was used as a technique to enhance passive immunity of dairy calves to CPS of *P. haemolytica* S1 (Hodgins and Shewen, 1996). However, this study did not analyze the correlation between these passively acquired antibodies and resistance to pneumonic pasteurellosis. Confer et al. (Confer et al., 1989) studied the role of carbohydrate protein subunit (CPS), which contains capsular carbohydrate along with low concentrations of LPS and outer membrane proteins, as an immunogen. They reported that serum antibodies that are important in resistance to pneumonic pasteurellosis are mainly directed against the periodate-resistant (protein) epitopes of CPS; however, some carbohydrate epitopes may also be important. Hence it appears that although CPS may be an important virulence factor of *P.*

haemolytica (discussed previously), its role as an immunogen in development of resistance to *P. haemolytica* infection may be rather limited and needs additional study.

Lipopolysaccharide

Although LPS might be an important virulence factor of *P. haemolytica*, it does not seem to be an important target of the host immune response. High serum antibodies to LPS O-antigens did not correlate with resistance to experimental bovine pneumonic pasteurellosis (Confer et al., 1986). Vaccination with LPS failed to protect calves against experimental transthoracic challenge with *P. haemolytica* (Purdy et al., 1993). An LPS subunit vaccine was also ineffective in development of resistance to pneumonic pasteurellosis in a experimental goat model (Purdy et al., 1993).

Outer membrane proteins (OMPs) including IROMPs

Antibodies against OMPs of Gram-negative bacteria can be important in development of resistance to infection, by promoting phagocytosis and by impairing the function of these OMPs. Similarly, an immune response against *P. haemolytica* OMPs could be important in the development of resistance to pneumonic pasteurellosis (Squire et al., 1984). Indeed vaccination of cattle with OMP-enriched fractions of *P. haemolytica* S1 significantly reduced lung damage upon experimental exposure with homologous serotype (Morton et al., 1995). Earlier research demonstrated that antibodies against the protein and not the carbohydrate component of the carbohydrate-protein subunit (CPS) correlated with development of resistance

to experimental *P. haemolytica* infection (Gentry et al., 1982) (Confer et al., 1989). Further immunological analysis of CPS indicated that antibody responses against antigens of 86, 66, 51, 49, 34, 31, and 16 kDa correlated with resistance to pneumonic pasteurellosis (Mosier et al., 1989a). It was also demonstrated that antibodies against surface antigens of *P. haemolytica*, including OMPs, are important in protective efficacy of a recombinant Lkt-containing commercial vaccine, Presponse® (Conlon et al., 1991). OMPs can also serve as potentially important cross-protective antigens. Antisera from cattle vaccinated with *P. haemolytica* S1 showed significant antibody responses against various OMPs of eight untypeable strains of *P. haemolytica* (Simons et al., 1992). These data suggest that an immune response against *P. haemolytica* OMPs might be an important component of a protective immune response.

OMPs are promising immunogens of *P. haemolytica*, and the nature of immune response against these antigens has been further scrutinized. Because antibodies directed against the surface antigens are considered to be important in aiding antibody-dependent host defense mechanisms, it is important to determine which OMPs exhibit surface-exposed epitopes. ^{125}I-labeling was employed to determine that 94, 84, 53.5, 49, 43, 41, 29.5 and 16 kDa OMPs have surface-exposed domains (Morton et al., 1996). This technique relies on reaction of ^{125}I-iodine with surface-exposed tyrosine residues, consequently, a surface-exposed OMP that lacks an exposed tyrosine residue would go undetected (Richardson and Parker, 1985). Knights et al., 1990,

identified surface-exposed OMPs of *P. haemolytica* S2 by studying their susceptibility to extracellular proteases and their ability to absorb out antibodies from hyperimmune rabbit serum. However, the results of this study cannot be directly extrapolated to *P. haemolytica* S1 OMPs, because the OMP profiles of these two serotypes have been shown to quite different (McCluskey et al., 1994). McCluskey et al., 1994, also compared the OMP profiles of 18 isolates within serotype 1 and found four different patterns of OMP-profiles. Hence, a study using more comprehensive techniques than ^{125}I-iodine labeling is needed to establish the repertoire of surface-exposed OMPs of *P. haemolytica* S1. This need has been addressed in the research presented in chapter 1 of this dissertation.

Study of surface-exposed OMPs could benefit from the production of surface-reactive monoclonal antibodies (MAbs) against these proteins. MAbs were raised against OMPs of 29 kDa and 66 kDa, (Austin et al., 1992); however, these antibodies did not recognize surface-exposed epitopes. Doig et al. produced MAbs against OMPs of *Helicobacter pylori* and screened them on the basis of their reactivity to whole cells in ELISA, immunoflourescence, and immunoelectron microscopy (Doig and Trust, 1994). This technique resulted in isolation of 17 MAbs against surface-exposed epitopes and could be applied to isolate surface-reactive MAbs against *P. haemolytica.*

Studies have also been directed towards understanding the roles of individual *P. haemolytica* OMPs in the pathogenesis of *P. haemolytica* infections and in development of resistance to pneumonic pasteurellosis.

Using recombinant DNA technology a segment of *P. haemolytica* DNA was cloned that encoded proteins of 28, 30, and 32 kDa (Craven et al., 1991). Rabbit antibodies raised against *P. haemolytica* 30 kDa protein caused agglutination of whole *P. haemolytica,* suggesting that this protein exhibits surface-exposed epitopes (Craven et al., 1991). Analysis of the DNA sequence revealed that the three proteins of 28-30 kDa are transcribed off a single promoter (Murphy and Whitworth, 1993). The recombinant forms of these three proteins were utilized to prove that they are lipid modified and that they are immunologically distinct (Dabo et al., 1994). Bovine antibody response against one of these 28-30 kDa lipoproteins correlated with resistance to pneumonic pasteurellosis (Dabo et al., 1994). Recently two OMPs of 32 and 35 kDa (PomA and PomB, respectively) have been characterized (Mahasreshti et al., 1996). PomA was determined to be the *P. haemolytica* homologue of an *E. coli* protein OmpA and was demonstrated to exhibit surface-exposed domains (Mahasreshti et al., 1996). Also, cattle vaccinated with live *P. haemolytica* showed a significant increase in antibodies to PomA and PomB (Mahasreshti et al., 1996). Further investigation into the role of PomA in immunity to *P. haemolytica* infection is currently under progress in Dr. Murphy's laboratory. A secreted 60 kDa protein of *P. haemolytica* has also been recently cloned; however, this study did not characterize the nature of this antigen (Lo and Mellors, 1996). Antibody responses against the 60 kDa protein correlated with resistance to pneumonic pasteurellosis (Weldon et al., 1994). Similarly a 100 kDa, serotype-specific OMP of *P. haemolytica* has been cloned and sequenced

(Lo et al., 1991). Others have determined that the DNA encoding this protein is also present in other serotypes; however, its expression, which is controlled at the transcriptional level, is only allowed in serotype 1 (Gonzalez et al., 1995) (Gonzalez et al., 1991). The role of this protein in immunity and/or pathogenesis has been speculated (discussed previously); although significant data has not been collected to support or prove it (Gonzalez et al., 1995) (Lo et al., 1991).

Although the above mentioned studies provide circumstantial evidence supporting the role of antibodies against these specific proteins in development of a protective immune response, a more detailed investigation into the role of antibodies in host defense mechanisms is lacking. Such investigations are critical if these proteins are to be considered in the development of an efficient subunit vaccine, especially in light of the fact that antibodies against certain OMPs of other Gram-negative pathogens have been shown to block host immune mechanisms (Joiner et al., 1985).

Several other researchers have studied OMPs for their roles in mechanisms other than immunity. A 35 kDa *P. multocida* protein has been shown to facilitate the binding of the organism to respiratory mucus and to epithelial cell wall preparation (Lübke et al., 1994). A similar 44 kDa *P. haemolytica* antigen was also identified in this study. N-terminal regions of these proteins were found to be similar to *E. coli* OmpC and OmpF porins (Lübke et al., 1994). However, no trimers (characteristic of porins) of the 44 kDa *P. haemolytica* protein were observed. Mpa1, a *P. haemolytica* protein

that is located in the cytoplasmic membrane and is homologous to bacterial PurK proteins has been cloned and sequenced (Chang et al., 1993). PurK is involved in *de novo* purine biosynthesis, but the function of Mpa1 has not been studied. A gene encoding a periplasmic binding protein of *P. haemolytica*, LapT was cloned and overexpressed in *E. coli* (Caskey et al., 1996). LapT was purified from these recombinant *E. coli* and binding assays were employed to determine that LapT is an arginine-binding protein (Caskey et al., 1996). This study also reported that arginine is not important for growth of *P. haemolytica*, hence a LapT mutant may have little potential as an attenuated live *P. haemolytica* vaccine candidate. However, previous studies by the same laboratory suggest that the arginine transport locus has a negative effect on leukotoxin expression (Highlander et al., 1993). Further studies will be required to understand the role of the above mentioned proteins in the physiology and virulence of *P. haemolytica*.

Mutational analysis of *P. haemolytica* antigens

As described above, numerous *P. haemolytica* antigens have been associated with pathogenesis of the pneumonic pasteurellosis and in development of immunity to *P. haemolytica* infection. Null mutations of genes responsible for the production of these molecules would be extremely helpful in delineating their role in *P. haemolytica* infection and immunity. A chemical mutagenesis approach, using nitrosoguanidine, was employed to abolish the production of Lkt in *P. haemolytica* (Chidambaram et al., 1995)

(Petras et al., 1995). This approach yielded two mutants that lacked Lkt production. One of these mutant strains was shown to have reduced virulence in goat and cattle challenge experiments (Petras et al., 1995). This mutant could serve as an important tool in understanding the role of Lkt in pathogenesis of *P. haemolytica* infection. However, chemical mutagenesis is a random process so there is always a possibility that more than one gene has been affected thereby making it difficult to interpret the effects of the mutation (Chidambaram et al., 1995).

Specific gene targeting is a more desirable approach to mutagenesis and is possible through homologous recombination. Mutants developed through this approach are genetically defined and hence more useful for studying the functions of particular genes. Homologous recombination has recently been used to develop a mutant *P. haemolytica* S1 that is deficient in production of the three contiguous lipoproteins (Lpp) around 30 kDa (mentioned above) and also to develop a mutant lacking in production of Lkt (Murphy and Whitworth, 1994) (Murphy et al., 1995). The *lpp30* mutant had reduced growth rate and increased susceptibility to bovine serum antibodies in complement-mediated killing (Murphy et al., submitted). This mutation causes the production of IROMPs under iron-sufficient conditions. The *Lkt* mutant failed to show β-hemolytic activity on sheep and rabbit blood agar plates confirming the role of Lkt as a hemolysin (Murphy et al., 1995). This *Lkt* mutant has also served as a useful control in studying the role of Ca^{++} influx in neutrophil activation and lysis mediated through Lkt (personal

communication with Dr. K.D. Clinkenbeard and Dr. C.R. Clark). This mutant is also likely to be deficient in production of other proteins encoded by the operon *Lkt CABD*. As a result this mutant could also serve as a useful tool to determine if *P. haemolytica* employs the Lkt secretory mechanism to transport proteins other than Lkt. Very recently, Tatum et al., 1998, have reported the construction of another Lkt mutant by allelic replacement. This mutant has a marked reduction in virulence, as determined by decreased lung lesion scores in an experimental calf model of pneumonic pasteurellosis. Histologically, lung of Lkt mutant-infected calves showed reduced necrosis and lack of leukocyte degeneration. This study has fortified the view that Lkt plays an important role in the pathogenesis of *P. haemolytica* bronchopneumonia

Attenuated mutant strains can potentially be used as live vaccines. With this objective in mind, two auxotrophic mutants have been produced. Both these mutants were produced by allelic exchange of *aroA* gene (Homchampa et al., 1994) (Tatum et al., 1994). The product of this gene is involved in biosynthesis of aromatic amino acids, and bacteria with defective aromatic amino acid synthesis pathways have been shown to be attenuated and avirulent (reviewed in Tatum et al., 1994). The *aroA* mutant developed by Homchampa et al., 1994, was demonstrated to be highly attenuated in a mouse septicemia model of *P. haemolytica* infection. The efficacy of these mutants as live vaccine candidates in cattle remains to be determined. Similarly, *galE* mutants of *Niesseria gonnorrhoeae* and *Salmonella*

typhimurium have been shown to have decreased virulence (Hone et al., 1987) (Robertson et al., 1993). GalE is involved in production of LPS and to some extent in CPS. The *galE* locus of *P. haemolytica* has been cloned and characterized (Potter and Lo, 1996). A *GalE*$^-$ strain of *P. haemolytica* could be potentially useful as an attenuated live vaccine candidate. However, no such mutant strains have so far been reported.

Vaccines

Vaccination of cattle with live *P. haemolytica* has been shown to confer the most consistent resistance to experimental challenge with *P. haemolytica* (Purdy et al., 1993) (Confer et al., 1985) (Blanchard-Channell et al., 1987). However, field trials using live vaccines have given inconsistent results (Purdy et al., 1986) (Smith et al., 1985). Also, intradermal vaccination with live *P. haemolytica* is associated with development of localized swelling, while vaccination through an intramuscular route may lead to lameness and fever (Confer et al., 1988). Zeman et al., 1993, reported systemic *P. haemolytica* infection as result of vaccination with an avirulent *P. haemolytica* strain. On the other hand, vaccination of cattle with *P. haemolytica* bacterins has either failed to protect cattle against the disease or caused an enhancement of the disease (Confer et al., 1985) (Friend et al., 1977) (Martin, 1983) (Wilkie and Markham, 1979). However, a recent study demonstrated that vaccination with a tissue culture derived formalin fixed *P. haemolytica* vaccine caused

inhibition of nasal and tonsillar colonization by homologous serotype (Frank et al., 1994).

Other attempts at developing vaccines have focused on the use of bacterial components. A vaccine containing recombinant Lkt along with *P. haemolytica* culture supernatant, was studied for its ability to induce a protective immune response (Conlon et al., 1991). In that study, it was observed that vaccination of calves with culture supernatant and recombinant Lkt provided significant protection; however, recombinant Lkt alone failed to protect calves against experimental *P. haemolytica* challenge (Conlon et al., 1991). Transthoracic vaccination with agar beads impregnated with lipopolysaccharide or Lkt failed to induce protection against transthoracic challenge with live *P. haemolytica*, while transthoracic vaccination with agar beads impregnated with capsule antigen could only induce partial protection (Purdy et al., 1993). Harland et al., 1992, studied the effect of a subunit or modified live bovine herpes virus-1 (BHV-1) vaccine on the efficacy of a vaccine containing OMPs of *P. haemolytica* and genetically attenuated Lkt. They observed that subunit vaccine comprised of BHV-1 glycoproteins, when administered along with *P. haemolytica* vaccine, conferred a significant protection, while vaccination with modified live BHV-1 and parainfluenza-3 interfered with protection conferred by the *P. haemolytica* vaccine. Another subunit vaccine containing genetically altered Lkt and bacterial extracts of both *P. haemolytica* and *Haemophilus somnus* significantly reduced bovine respiratory disease morbidity (Donkersgoed et al.,

1993). Vaccination with two commercial vaccines, Septimune®PH-K (Fort Dodge laboratories, Fort Dodge, IA) or One Shot™ (SmithKline Beecham Animal Health, Lincoln, NB) significantly increased antibodies against surface antigens and Lkt and also induced significant resistance to experimental *P. haemolytica* infection (Confer and Panciera, 1994).

Recently, the protective abilities of different experimental subunit vaccines, which contained different combinations of *P. haemolytica* immunogens, were studied (Sreevatsan et al., 1996). This study concluded that serum antibodies against Lkt, IROMPs, and capsular polysaccharide correlated with low lung lesions scores, upon experimental *P. haemolytica* challenge. The effect of route of vaccination on its protective ability was also studied, and it was demonstrated that aerosol exposure with a virulent *P. haemolytica* strain provided better protection against homologous challenge than subcutaneous vaccination with the same strain (Newman et al., 1982). However, subcutaneous vaccination with an *H. somnus* and *P. haemolytica* vaccine containing attenuated Lkt was able to provide significant protection to feedlot cattle (Donkersgoed et al., 1993).

A meta-analysis has been conducted to compare the efficacy of different experimental and commercial vaccines used in the past 12 years (Srinand et al., 1995). This statistical analysis revealed that cattle vaccinated with live *P. haemolytica* had the lowest lung-lesion scores, suggesting that live *P. haemolytica* provide the most significant protection against pneumonic pasteurellosis. However as mentioned above, vaccination with live *P.*

haemolytica can lead to complications. Culture supernatant and KSCN (potassium thiocynate) extract vaccines, which contain numerous OMPs, also conferred significantly better protection than bacterins (Srinand et al., 1995). These vaccines were not as efficacious as live vaccines.

These vaccine trials suggest that immunity to *P. haemolytica* is likely to be multifactorial and associated with numerous cell-surface and extracellular molecules including OMPs. To determine the exact role played by these molecules in immunity to pneumonic pasteurellosis, it is important that each factor be examined individually. One approach to this problem would include isolating the genes which encode these *P. haemolytica* immunogenic factors, characterizing the individual molecules, their genes, and the immune response against these molecules, by using recombinant clones and recombinant gene products in *in vitro* and *in vivo* analyses. As discussed above, mutagenesis could be another useful tool; however, mutations in certain genes can prove to be lethal, thus limiting the application of this powerful technique.

Host Defense Mechanisms

Because *P. haemolytica* remains extracellular in bovine pneumonic pasteurellosis, humoral immunity is probably most important in protection against infection. Purdy et al. (Purdy et al., 1991) reported a decrease in the serum complement levels of calves exposed to stress by travel and overcrowding. Also, calves showing morbidity due to *P. haemolytica*

infections, had lower levels of serum complement than healthy calves (Purdy et al., 1991). *P. haemolytica* cells are susceptible to complement-mediated killing, by bovine serum (MacDonald et al., 1983) (Chae et al., 1990). It has been demonstrated that antibodies are required to initiate the complement cascade (classical pathway), whereas direct activation of the complement in an antibody-independent fashion (alternate pathway) did not induce killing of *P. haemolytica* (MacDonald et al., 1983). These observations suggest a protective role for complement in pneumonic pasteurellosis. Which bacterial molecules elicit antibodies that mediate complement-mediated killing is however unknown and needs to be investigated. Possible targets of complement-fixing antibodies could be the surface-exposed domains of OMPs of *P. haemolytica*.

IgG has been demonstrated to be the host serum antibody isotype most important in protection against *P. haemolytica* infection (Mosier et al., 1989b). Although IgM levels in serum and bronchioalveolar lavage fluid are high in the early phase of infection, IgG seems to be more important subsequently (McBride et al., 1992). McBride et al., 1992, failed to detect any significant levels of IgA in the bronchioalveolar lavage fluid after experimental exposure to *P. haemolytica*. However, a surfactant-associated anionic peptide of low molecular weight has been demonstrated in ovine pulmonary secretions that is bactericidal to *P. haemolytica* (Brodgen et al., 1996). This and similar proteins are expected to play an important role in innate immunity against *P. haemolytica* especially in the absence of significant levels of IgA

Neutrophils are important in the pathogenesis of *P. haemolytica*-induced fibrinous pleuropneumonia. Neutrophils are extravasated into the lungs in large numbers when the lungs are colonized by *P. haemolytica* (McBride et al., 1992), and their degeneration, which is partly due to Lkt-mediated lysis, results in the release of inflammatory mediators and lysosomal enzymes which cause tissue damage (Slocombe et al., 1985) (Vandenbroucke-Grauls et al., 1987). *P. haemolytica* culture fluid has been demonstrated to induce chemotaxis, oxygen burst, and superoxide anion release from bovine neutrophils (Mdurvwa and Brunner, 1994). Lkt also causes an increased secretion of a proinflammatory eicosanoid, leucotriene B_4 (LTB_4), from bovine leukocytes (Clinkenbeard et al., 1994). A protective effect of neutrophil depletion against *P. haemolytica* infection has been suggested (Slocombe et al., 1985); however, Breider et al., 1988, demonstrated that *P. haemolytica* can also induce lung injury through neutrophil-independent mechanisms. In the latter study the intensity of lung injury was less in neutrophil-depleted than in normal calves. Neutrophils are also important in host defense against *P. haemolytica*; they have been demonstrated to kill high concentrations of the organism before Lkt causes their lysis (Czuprynski et al., 1987). In the presence of immune serum, neutrophils significantly reduced the endothelial cell damage caused by direct application of *P. haemolytica* to endothelial cells (Sharma et al., 1992). It appears that the role of neutrophils in pathogenesis of pneumonic pasteurellosis is rather complex. On the one hand they release cytotoxic and proinflammatory factors

that cause exacerbation of the disease, while on the other hand, they are important in killing *P. haemolytica* and to some extent protect against the direct cytotoxicity caused by the organism.

Alveolar macrophages are a component of the innate immune mechanism of the lungs. They are, therefore, expected to play a role in resistance to the colonization of the bovine lung by *P. haemolytica*. The bactericidal effects of alveolar macrophages is dependent on the concentration of *P. haemolytica* (Morales et al., 1994). Experimental intrabronchial administration of *P. haemolytica* in mice led to an increase in the number of macrophages in interalveolar septa and also in alveoli (Pace et al., 1994). However, Czuprynski et al., 1991a, found that the phagocytic activity of bovine macrophages is significantly reduced by the capsular polysaccharide of *P. haemolytica*. These authors argue that because of the inhibitory effects of factors such as capsular polysaccharide, Lkt, stress, and viral infection on phagocytosis, alveolar macrophages may only have a limited antibacterial activity against *P. haemolytica* during pneumonic pasteurellosis (Czuprynski et al., 1991a) (Himmel et al., 1982) (Walker et al., 1984).

Alveolar macrophages may, however, play a considerable role in the pathogenesis of pneumonic pasteurellosis. Morphological studies show that exposure of alveolar macrophages to LPS causes changes that are consistent with activation (Whiteley et al., 1991). *P. haemolytica* endotoxin causes the release of proinflammatory cytokines, such as tumor necrosis factor (TNF) α and interleukin-1β (IL-1β) from bovine alveolar macrophages (Yoo et al.,

1995) (Bienhoff et al., 1992). When stimulated by LPS, alveolar macrophages also release nitric oxide, a mediator that modulates hemodynamics in the immediate vicinity of its production (Yoo et al., 1996). The release of TNF α and interleukin-1β from the macrophages subsequently enhances the endotoxin-mediated injury to endothelial cells (Sharma et al., 1992). The studies discussed above suggest that the role of alveolar macrophages in development of resistance to *P. haemolytica* infection may be limited; however, they are important players in the induction of an acute inflammatory response that is associated with pneumonic pasteurellosis.

Summary and statement of research problem

The review of relevant literature suggests that *P. haemolytica* induced fibrinous pleuropneumonia in calves is a complex disease process. The stage is set by one or more stress-inducing factors that have a detrimental effect on the host immune system. This is followed by proliferation of *P. haemolytica* in the URT of these stressed calves and subsequently their aerosolization into the lungs. In the lungs, *P. haemolytica* proliferate rapidly and interact with the host immune components to induce an acute and invasive fibrinous bronchopneumonia and pleuritis. It appears that an excessive and indiscriminate host immune response plays a significant role in the exacerbation of the lung lesion associated with the disease. Nevertheless, it is possible to induce a protective immune response against pneumonic pasteurellosis as is best seen in the cases of vaccination with live *P.*

haemolytica. Live vaccines, however, have several undesirable side effects. Development of an attenuated *P. haemolytica* strain for use as a vaccine may help in alleviating complications of live vaccines; but there is always a chance of an avirulent strain reverting to its virulent form. A better approach would be to develop an efficient sub-unit vaccine that provides the protection of a live vaccine without the untoward side effects.

In order to develop an efficacious sub-unit vaccine, it is important to characterize an effective host immune response. For this purpose, the immune response of cattle that have been found resistant to experimental *P. haemolytica* infection should be comprehensively studied. Specific OMPs of *P. haemolytica,* especially those with surface-exposed domains, are expected to be crucial components of an efficacious subunit vaccine. To determine the potential of specific OMPs as vaccine candidates, OMPs should be studied individually and the roles of antibodies against these OMPs in host defense mechanisms should be clearly and unequivocally established. Molecular cloning and recombinant DNA technology are powerful tools that can be successfully used in achieving the above mentioned goals.

One of the objectives of my research project was to identify the repertoire of surface-exposed OMPs of *P. haemolytica* S1 by analysis of susceptibility of OMPs, when present on intact *P. haemolytica,* to extracellular proteases. Surface exposure was also determined by assaying for the ability of intact *P. haemolytica* to adsorb out antibodies against these immunogenic and surface

exposed OMPs from immune sera of cattle that were resistant to experimental *P. haemolytica* infection.

Two OMPs namely, Lpp38 and PlpE, that have surface-exposed domains were examined. The genes encoding these proteins were cloned and analyzed. The recombinant *E. coli* clones that expressed these proteins were used to study the bovine immune response against these proteins. We determined that Lpp38, although very immunogenic, was only minimally surface exposed. On the other hand, PlpE, which is also the target of a significant immune response, had numerous surface-exposed regions. We decided to determine the role of antibodies against PlpE in complement-mediated killing and found that absorbing out anti-PlpE antibodies from bovine immune serum caused a significant decrease in complement-mediated killing of *P. haemolytica*.

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Chapter II

IDENTIFICATION OF IMMUNOGENIC, SURFACE-EXPOSED OUTER MEMBRANE PROTEINS OF *Pasteurella haemolytica* SEROTYPE 1

Abstract

Pasteurella haemolytica serotype 1 (S1) is the bacterium most frequently recovered from the lungs of cattle that have succumbed to shipping fever pneumonia. *P. haemolytica* outer membrane proteins (OMPs) are important immunogens in the development of resistance to pneumonic pasteurellosis. The purpose of this study was to identify the repertoire of immunogenic, surface-exposed *P. haemolytica* (S1) OMPs, that could be important in the development of protective immunity. We determined surface-exposure of OMPs by (1) their susceptibility to protease treatment and (2) their ability to adsorb out antibodies from bovine immune sera. For a comprehensive identification of immunogenic, surface-exposed OMPs, we used bovine antisera from calves that were resistant to experimental *P. haemolytica* challenge after (1) natural exposure to *P. haemolytica*, (2) vaccination with live *P. haemolytica*, or (3) vaccination with *P. haemolytica* OMPs. We identified 18 immunogenic, surface-exposed *P. haemolytica* OMPs. Most were recognized by all three immune sera. However, some were recognized by one or two of the three antisera. Our analyses identified surface-exposed, immunogenic proteins that were not identified in previous studies.

Introduction

Pasteurella haemolytica serotype 1 is the bacterium most commonly associated with shipping fever, a disease of beef cattle characterized by fibrinous pleuropneumonia (Frank, 1986; Frank, 1989; Whiteley et al., 1992). Conditions that lead to stress in cattle, such as shipping, viral infections, and inclement weather may cause immunosuppression and reduction of bacterial clearance, thus predisposing the lungs to colonization by *P. haemolytica* (Confer et al., 1988; Confer et al., 1990). Numerous *P. haemolytica* immunogens have been studied, and their roles in development of resistance to *P. haemolytica* infection have been evaluated (reviewed in (Confer, 1993)).

Outer membrane proteins (OMPs) have been proposed to be important in the pathogenesis of pneumonic pasteurellosis and in development of a protective immune response against the disease (Confer et al., 1990; Confer, 1993; Morton et al., 1995; Squire et al., 1984b; Sreevatsan et al., 1996). Vaccination with *P. haemolytica* outer membranes protected cattle against experimental challenge with the homologous serotype (Morton et al., 1995). Additionally, results of a recent meta-analysis (Srinand et al., 1995), in which twenty-seven vaccine efficacy studies were evaluated, revealed that vaccines delivering OMPs and other surface antigens, extracted from cells with different media, provided the best protection against *P. haemolytica* challenge. Protection afforded by those vaccines was comparable to that afforded by live vaccines.

In addition to providing protection against *P. haemolytica* strains of the homologous serotype, OMPs extracted from *P. haemolytica* serotype 1 also have potential for providing immunity against pneumonia caused by heterologous serotypes. Antibodies, stimulated by vaccination of cattle with *P. haemolytica* serotype 1 OMPs, recognized numerous OMPs from untypeable *P. haemolytica* strains, and the antibody response against several of those correlated with resistance to experimental *P. haemolytica* challenge (Simons et al., 1992). Similarly, when cattle are vaccinated with outer membranes from *P. haemolytica* serotypes 6 or 9, some protection is afforded against experimental challenge with a serotype 1 strain (Morton et al., 1995). Antibodies against surface-exposed *P. haemolytica* OMPs and other surface antigens are likely to enhance opsonization of the bacterium and contribute to (1) inhibition of colonization (Frank et al., 1996), (2) phagocytosis and killing by neutrophils and macrophages (Breider et al., 1991; Chae et al., 1990; Czuprynski et al., 1987; Sharma et al., 1992), and (3) complement-mediated lysis (Chae et al., 1990; MacDonald et al., 1983; Penaredondo et al., 1988; Sutherland et al., 1989) .

The identification of envelope proteins, OMPs, and surface-exposed OMPs that are produced by various serotypes of *P. haemolytica* have been active areas of research (Davies et al., 1992; Donachie et al., 1984; Knights et al., 1990; McCluskey et al., 1994; Morton et al., 1996; Rossmanith et al., 1991; Squire et al., 1984a). Using ^{125}I-labeling of whole cells, others have identified surface proteins from a *P. haemolytica* serotype 1 strain (Craven et al., 1991; Morton et

al., 1996). However, those studies did not examine the immunogenicity of the ^{125}I-labeled OMPs. Similarly, several studies have examined the host antibody response to OMPs from various *P. haemolytica* serotypes, but the presence of surface-exposed regions on those OMPs was not evaluated (McCluskey et al., 1994; Simons et al., 1992). Mosier et al. (1989) evaluated the bovine immune response to antigens present in a saline extract of whole *P. haemolytica* serotype 1. That study identified 13 saline-extracted antigens that are recognized by immune bovine sera. Knights et. al (Knights et al., 1990) performed a comprehensive analysis of potentially immunogenic and surface-exposed OMPs produced by a serotype 2 strain. The proteins were identified by assaying for the ability of intact *P. haemolytica* to absorb out antibodies against OMPs from hyperimmune rabbit sera and by determining the susceptibility of OMPs on intact cells to proteinase K treatment. However, serotype 2 strains are not commonly associated with bovine shipping fever pneumonia. Furthermore, others have shown that serotype 1 and serotype 2 *P. haemolytica* strains are significantly different in their OMP profiles (Davies and Donachie, 1996), and that serotype 2 strains are phylogenetically distinct from all other *P. haemolytica* serotypes (Davies et al., 1996). A comprehensive analysis of *P. haemolytica* serotype 1 OMPs, that are both surface-exposed and immunogenic, is lacking.

Therefore, the purpose of our study was to identify OMPs, produced by a bovine isolate of *P. haemolytica* serotype 1, that are both immunogenic in cattle and exposed on the bacterial surface. To identify these OMPs, we

utilized a virulent *P. haemolytica* strain that was isolated from a calf which had succumbed to pneumonic pasteurellosis and immune sera from cattle that were shown to be resistant to experimental challenge after natural exposure to *P. haemolytica* or after vaccination with *P. haemolytica* OMPs or with live *P. haemolytica*. We identified surface-exposed, immunogenic OMPs that are recognized by all three immune sera and others that are recognized by only one or two of these sera.

Materials and Methods

Bacterial strain and culture conditions

P. haemolytica serotype 1, strain 89010807N (Murphy and Whitworth, 1994) was used in all experiments. Bacteria were cultured in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI, USA), to the mid-logarithmic phase, on a rotary shaker (250 rpm) at 37°C, unless otherwise noted.

Bovine antisera

Bovine antisera were collected from calves following natural exposure to *P. haemolytica* (Gentry et al., 1985; Pandher and Murphy, 1996) or after subcutaneous vaccination on day 0 and day 21 with (i) live *P. haemolytica* (2 ml of 1 x 10^9/ml of 18 hr cultures) or (ii) OMPs (2 mg in 1 ml of phosphate buffered saline (PBS) and 1 ml of aluminum hydroxide-DDA-bromide adjuvant). This adjuvant was described in Confer et al., 1997. All calves were found to be resistant to experimental *P. haemolytica* challenge (Gentry et al.,

1985; this study), using a lesion score system previously described by Panciera et al., 1984. The calf that was naturally exposed to *P. haemolytica* exhibited a high indirect immunofluorescence titer (>90) to *P. haemolytica* whole cell antigens. The calf was experimentally challenged on day 21 of that experiment. The other two calves (OMP and live *P. haemolytica* vaccinates) were part of a separate study and were challenged on day 36. All sera used in the study were collected from calves on the day of experimental challenge, except the serum from the naturally exposed calf that was used in the absorption experiment. Day of challenge (day 21) serum from that calf was exhausted during immunoblot analysis of protease treated cells. However, serum from the naturally exposed calf, collected on day -4 of that experiment was available and was used in the absorption experiment.

Absorption of antisera with *P. haemolytica*

Bovine immune sera were absorbed with intact *P. haemolytica* using a modification of a previously described method (Hansen et al., 1988). *P. haemolytica*, from one liter of culture were pelleted by centrifugation, washed once in PBS, and resuspended in immune serum, diluted 1:100 in Tris-HCl (10 mM, pH 7.4), NaCl (0.9%), and nonfat dry milk (1%) (TSM). Cells resuspended in serum were incubated at 4°C for 3 hrs on a rocking platform. Following incubation, cells were pelleted by centrifugation at 11,000 x g. The supernatant was carefully removed and stored at -20°C following the addition of sodium azide to 0.02%. Unabsorbed immune sera were used as controls and were diluted 1:100 in TSM before use.

Protease treatment of intact *P. haemolytica*

P. haemolytica (1 ml of mid-log phase culture) were pelleted in a microcentrifuge, washed once with 1 ml of suitable enzyme buffer, and pelleted again. Cells were resuspended in 250 µl of enzyme buffer containing 50 µg of the appropriate protease (trypsin, chymotrypsin, or staphylococcal V8 protease; Sigma Chemical Co., St. Louis, MO, USA) and incubated for 1 hr at 37°C in a shaking incubator. The cells were then pelleted in a microcentrifuge, washed once with PBS, and resuspended in 50 µl PBS. For controls, cells were incubated in 250 µl of enzyme buffer without protease and processed in the same manner. Enzyme buffers consisted of: trypsin buffer, 0.041 M Tris-HCl (pH 8.0), 0.012 M $CaCl_2$; chymotrypsin buffer, 0.08 M Tris-HCl (pH 8.0), 0.1 M $CaCl_2$; Staphylococcal V8 protease buffer, 50 mM NH_4HCO_3 (pH 7.8).

For protease treatment of decapsulated *P. haemolytica*, capsular polysaccharide was removed by incubating cells in PBS at 42°C for 1 hr, as described by Gentry et. al (1982). Cells were pelleted in a microcentrifuge and processed as described above.

Western immunoblot analyses

P. haemolytica whole cell lysates were prepared by incubating the cell suspensions in an equal volume of final sample buffer (10% glycerol, 2.5% SDS, 0.1% bromophenol blue, 5% merceptoethanol) at 95°C for 10 minutes. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) (10%) as previously described (Dabo et al., 1994) and transferred to nitrocellulose (MSI, Westboro, MA, USA) using the Trans-Blot apparatus (BioRad, Richmond, CA, USA), according to the manufacturer's instructions. Western blots were probed with bovine antisera and bovine IgG was detected as previously described (Dabo et al., 1994). Protein relative molecular masses (M_r) were estimated by comparison with wide-range protein standards (Novex, San Diego, CA, USA) stained with Ponceau S (Sigma).

Results

Identification of surface-exposed OMPs by protease treatment

Surface exposure of *P. haemolytica* proteins was determined by comparing western immunoblots of whole cell lysates from protease-treated cells with those from respective buffer-treated controls. Immunoblots were probed with antisera from cattle that were resistant to experimental challenge after vaccination with *P. haemolytica* OMPs (Fig. 1), vaccination with live *P. haemolytica* (Fig. 2), or natural exposure to *P. haemolytica* (Fig. 3). Proteins were determined to be surface-exposed if the corresponding band was missing or reduced in intensity after protease treatment of intact cells (Table 1). Using these methods, we identified 17 distinct proteins as surface-exposed and immunogenic. Ten proteins were recognized by all three immune sera. Antisera from the live vaccinate recognized two proteins (60 kDa, 76 kDa) that were not detected by the other two antisera. The same antisera did not

recognize two other proteins (23-24 kDa and 33-34 kDa) that were recognized by antisera from both the OMP vaccinate and the naturally-exposed animal. Immunogenic proteins with M_rs of 31, 38, and 62 kDa showed only a slight decrease or no decrease in intensity after protease treatment of encapsulated *P. haemolytica* but showed a much greater decrease in intensity after protease treatment of decapsulated *P. haemolytica* .

Identification of surface-exposed OMPs with immune sera absorbed against intact *P. haemolytica*.

Next, we compared the reactivity of bovine antisera with whole cell antigens, before and after adsorption of the immune sera with intact *P. haemolytica*. Antibodies binding to surface-exposed domains on the OMPs are depleted from immune sera adsorbed in this manner (Knights et al., 1990). When adsorbed sera were used to probe western immunoblots, we observed a loss of reactivity or a reduction in reactivity with several *P. haemolytica* antigens (Fig. 4, Table 1). Adsorbed immune sera displayed a complete absence of reactivity with some antigens that were detected by unabsorbed sera and only a reduction in reactivity with other antigens (25, 28, 32, 33-34, and 37 kDa). Some antigens that were identified as surface-exposed by this technique (32, 37, and 86 kDa), were not recognized by all three immune antisera.

☺☺Discussion

To maximize the number of surface-exposed and immunogenic *P. haemolytica* proteins that we could identify, we (1) performed both protease cleavage and antibody absorption assays, (2) used three different proteases for analysis, (3) evaluated protease susceptibility in the presence and absence of capsular polysaccharide, and (4) probed immunoblots with three different bovine immune sera. The immune sera were from (1) a calf vaccinated with live *P. haemolytica*, (2) a calf vaccinated with *P. haemolytica* OMPs, and (3) a calf naturally exposed to *P. haemolytica*. We chose the first two antisera because vaccination of cattle with live *P. haemolytica* consistently affords the greatest protection against *P. haemolytica* challenge (Srinand et al., 1995) and because vaccination with OMPs (or surface extracts) confers resistance to challenge comparable to that observed for vaccination with live *P. haemolytica* (Morton et al., 1995; Srinand et al., 1995). We chose the third serum because the immune response that develops from natural exposure to *P. haemolytica*, followed by recovery and resistance to subsequent challenge, may differ from that elicited by vaccination and may be superior (Confer, et al., 1984. This comprehensive analysis identified 18 *P. haemolytica* proteins that are immunogenic and contain surface-exposed domains.

Overall, the repertoires of immunoreactive and protease-sensitive bands were similar for all three antisera. Likewise, the repertoire of antigens that adsorbed out antibodies from the three immune sera were also similar. However, differences in surface protein recognition among the three antisera

were evident. The intensity of the immunoreactive band at ~38 kDa was much stronger for sera from the OMP and live vaccinates than for the serum from the naturally-exposed calf. However, antibodies against this protein in the natural exposure serum were more effectively adsorbed out by whole cells. In contrast, the intensities of protease susceptible and immunoreactive bands at 24 and 28 kDa were greater for the serum from the naturally-exposed animal than for the other two sera.

When we compare the two methods for assessing surface exposure, protease susceptibility and absorption of antibodies, differences are apparent. Protease treatment identified 17 of the 18 surface-exposed proteins; whereas, antibody absorption with whole cells identified 11 proteins as surface-exposed, including one (54 kDa) not identified by protease-treatment. Although cells were washed afterward, we have observed that the specific enzyme buffers used for protease treatment may affect protein migration. It is probable that the 54 kDa protein co-migrated with the non-surface-exposed 55 kDa protein on those gels. Several proteins $\geq$ 60 kDa, were cleaved by extracellular proteases but did not absorb out antibodies from immune sera. We only performed serum absorption studies on logarithmic phase cells, which would have been encapsulated (Gentry et al., 1982). It is possible that regions of these higher M_r proteins, posses protease cleavage sites extending beyond the capsule but lack exposed epitopes that could be recognized by antibodies present in the immune sera.

The serum concentrations used for the two techniques may also account for observed differences in antigen detection. For serum absorption studies, it was necessary to dilute the immune sera 1:100 to deplete the surface-reactive antibodies with a workable amount of *P. haemolytica*; whereas, in the protease treatment studies the sera were used at a 1:25 dilution. This may also explain the lack of reaction with some higher molecular weight proteins (110, 104, 97, and 76 kDa) in the immunoblots from absorption studies (Fig. 4), that are represented by faint bands in the western immunoblots of protease treated cells (Figs. 1-3).

We also observed that the western immunoblot antigen recognition profile for the serum from the naturally exposed calf was different in the protease treatment studies and serum absorption studies (Figs. 3, 4). The western immunoblot for serum absorption analysis revealed a 55 kDa non-surface-exposed protein that was not present in protease treatment blots. This is likely because the serum used for the protease treatment study was collected on day 21 of that experiment, the day of experimental challenge (see Materials and Methods). A limited amount of that serum sample was available requiring the use of a different serum samples from the same calf (day -4) for the serum absorption study. Interestingly, the loss of an antibody response to the 55 kDa antigen in the serum of that calf, which occurred over the subsequent 25 days, did not compromise the resistance of the calf to experimental *P. haemolytica* challenge.

Our analysis has identified *P. haemolytica* serotype 1 surface proteins that likely correspond to some identified in previous studies, as well as other surface proteins not recognized in previous studies. As mentioned earlier, Mosier et al. (1989) identified 13 antigens (16-86 kDa) that were present in a saline extract of *P. haemolytica* and were recognized by at least one of ten bovine immune sera that were analyzed. Extract antigens of 31, 34, 38, 49, 51, 62, 66, and 86 kDa identified in that study are probably analogous to the 31, 34, 37-38, 47, 54, 62, 66, and 86 kDa proteins that we found to be surface-exposed in this study. Using radioiodination, Morton et. al (1996) identified surface-exposed *P. haemolytica* serotype 1 proteins of 16, 29.5, 41, 43, 49, 53.5, 84, and 94 kDa. Our study identified surface-exposed proteins with similar M_rs to all of those except the 16 kDa protein, and further demonstrated that they are immunogenic in cattle. We identified several surface-exposed proteins that were not detected by Morton et. al (1996). This is probably because radioiodination is dependent upon the presence of a surface-exposed tyrosine. Consequently, a surface-exposed protein without such a residue would go undetected.

Our results include new information that extends the results of previous studies. We have identified the repertoire of *P. haemolytica* serotype 1 proteins that are both surface-exposed and recognized by immune sera from cattle resistant to *P. haemolytica* challenge. Our analysis identified additional such proteins that were not previously recognized in studies which focused on identifying surface-exposed proteins (Morton et al., 1996) or immunogenic

surface extract proteins (Mosier et al., 1989). Furthermore, our results discriminate surface-exposed, immunogenic proteins from cellular proteins that elicit an antibody response (Mosier et al., 1989), but may not contain surface-exposed regions. Identification of these proteins will allow for more detailed analyses on the contribution of the antibody response against specific antigens in mechanisms of host defense.

Acknowledgments

This research was supported by grant 95-37204-1999 from the National Research Initiative Competitive Grants Program of the USDA, the Oklahoma Agricultural Experiment station (Project OKL02179), and the Oklahoma State University College of Veterinary Medicine. K. Pandher was supported by an OSU-CVM graduate research assistantship award.

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Fig. 1. Western immunoblots of *P. haemolytica* whole cell lysates, prepared from protease-treated, encapsulated (capsule +) and decapsulated (capsule –) cells. Immunoblots were probed with serum from a calf that was resistant to experimental *P. haemolytica* challenge after vaccination with *P. haemolytica* OMPs. Lanes: T, C, and V are whole cell lysates of *P. haemolytica* treated with trypsin, chymotrypsin, and staphylococcal V8 protease respectively. Lanes: B, whole cell lysates of *P. haemolytica* treated with respective enzyme buffers (negative controls).

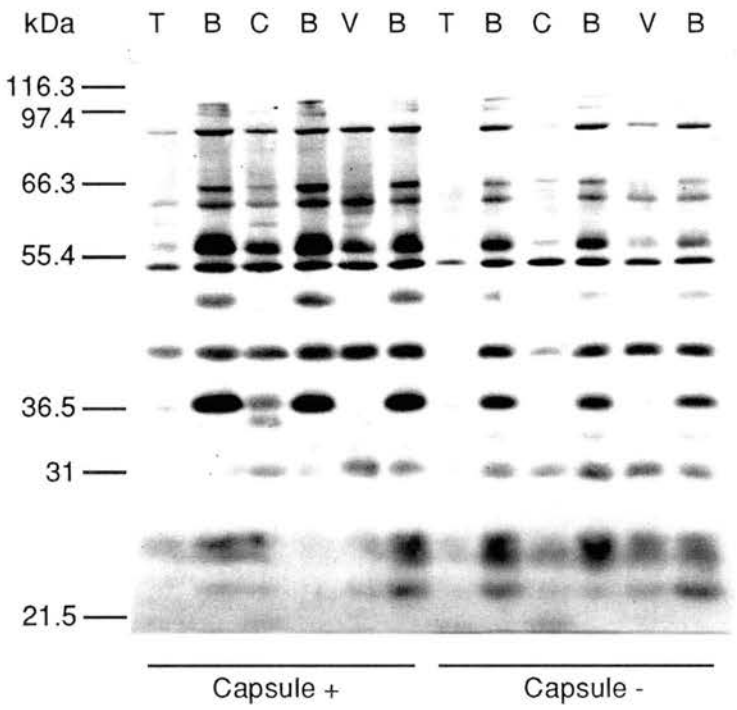
kDa
T B C B V B T B C B V B
116.3
97.4
66.3
55.4
36.5
31
21.5
Capsule +
Capsule -

Fig. 2. Western immunoblots of *P. haemolytica* whole cell lysates, prepared from protease-treated, encapsulated (capsule +) and decapsulated (capsule –) cells. Immunoblots were probed with serum from a calf that was resistant to experimental *P. haemolytica* challenge after vaccination with live *P. haemolytica*. Lanes: T, C, and V are whole cell lysates of *P. haemolytica* treated with trypsin, chymotrypsin, and staphylococcal V8 protease respectively. Lanes: B, whole cell lysates of *P. haemolytica* treated with respective enzyme buffers (negative controls).

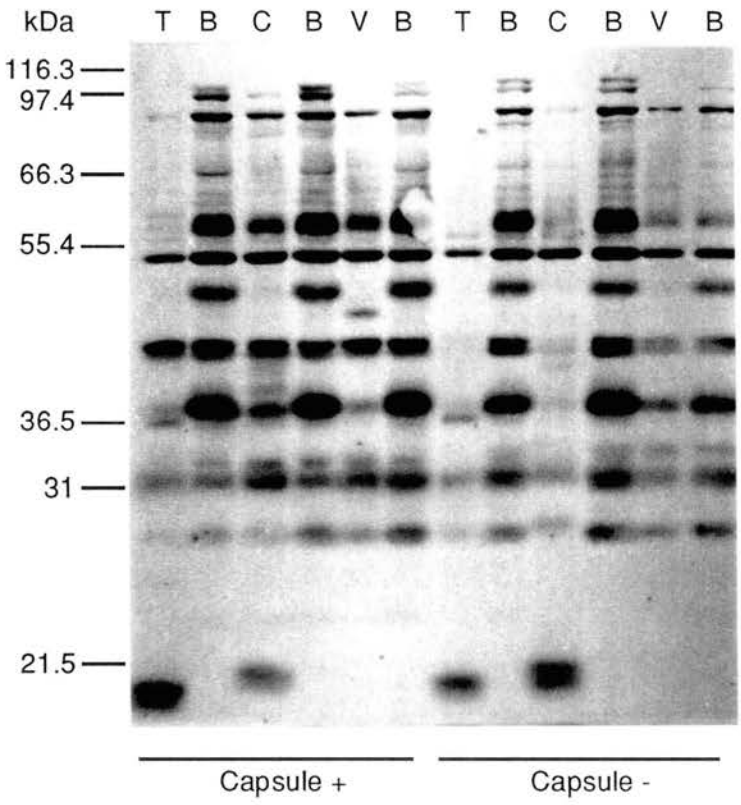
kDa
T B C B V B T B C B V B
116.3
97.4
66.3
55.4
36.5
31
21.5
Capsule +
Capsule -

Fig. 3. Western immunoblots of *P. haemolytica* whole cell lysates, prepared from protease-treated, encapsulated (capsule +) and decapsulated (capsule –) cells. Immunoblots were probed with serum from a calf that was resistant to experimental *P. haemolytica* challenge after natural exposure to *P. haemolytica* infection. Lanes: T, C, and V are whole cell lysates of *P. haemolytica* treated with trypsin, chymotrypsin, and staphylococcal V8 protease respectively. Lanes: B, whole cell lysates of *P. haemolytica* treated with respective enzyme buffers (negative controls).

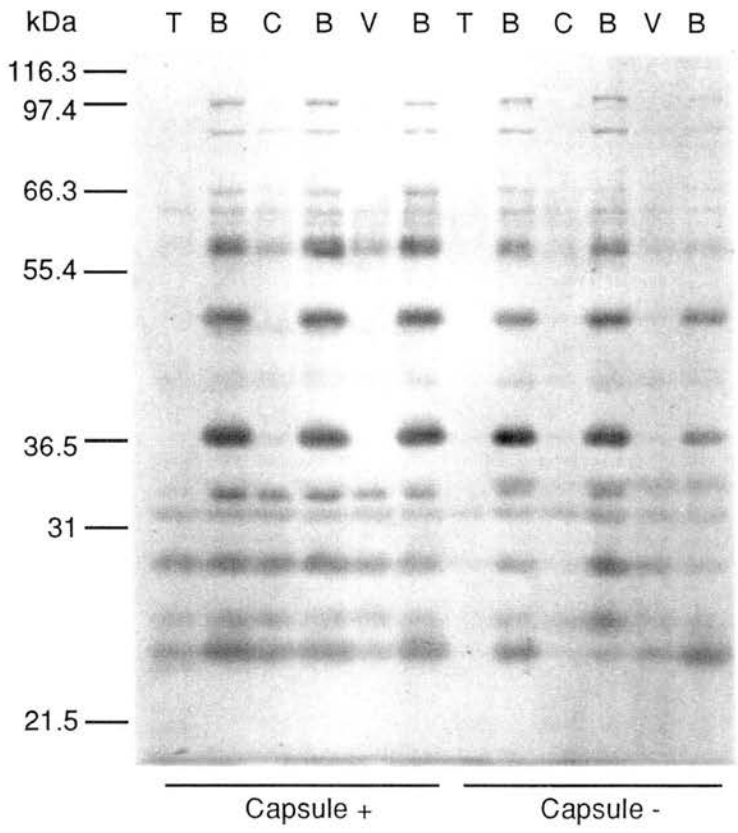
kDa
T B C B V B T B C B V B
116.3
97.4
66.3
55.4
36.5
31
21.5
Capsule +
Capsule -

Fig. 4. Western immunoblots of *P. haemolytica* whole cell lysates probed with; OMP, serum from a calf resistant to experimental *P. haemolytica* challenge after vaccination with *P. haemolytica* OMPs ; Live, serum from a calf resistant to experimental *P. haemolytica* challenge after vaccination with live *P. haemolytica;* Natural, serum from a calf resistant to experimental *P. haemolytica* challenge after natural exposure to *P. haemolytica*. Lanes: A were probed with the immune serum after absorption with intact, logarithmic phase *P. haemolytica;* U were probed with the unabsorbed serum (negative control).

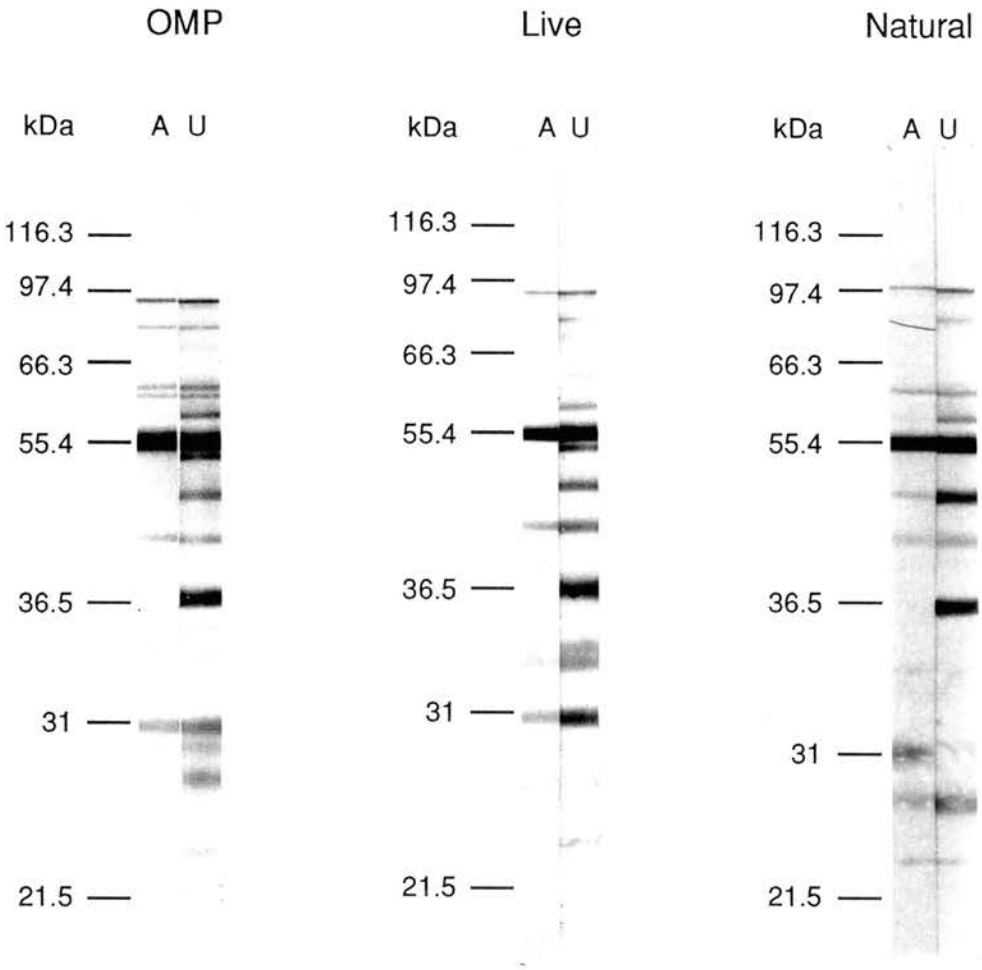
OMP
Live
Natural
kDa
A U
116.3
97.4
66.3
55.4
36.5
31
21.5

Table 1. *P. haemolytica* serotype 1 proteins, recognized by bovine immune sera, and identified as surface-exposed after protease treatment analysis and immune sera absorption analysis.

[a] Whole cell lysates of protease-treated intact *P. haemolytica* were probed with immune sera from calves that were resistant to *P. haemolytica* challenge (Figs. 1-3)

[b] Immune sera from cattle resistant to *P. haemolytica* challenge were absorbed with intact *P. haemolytica* and used to probe *P. haemolytica* whole cell lysates (Fig. 4)

[c] M_rs of proteins identified as surface-exposed from Fig. 1.

[d] M_rs of proteins identified as surface-exposed from Fig. 2

[e] M_rs of proteins identified as surface-exposed from Fig. 3

* Proteins susceptible to proteases only after removal of capsular polysaccharide

R Proteins that showed a reduction in reactivity with absorbed sera when compared to the unabsorbed controls

Table 1. *P. haemolytica* proteins, recognized by bovine immune sera, and identified as surface-exposed by protease treatment of intact *P. haemolytica* and immune sera absorption analysis.

	Protease Treatment[a]			Serum Absorption[b]		
	Serum			Serum		
	OMP Vaccinate[c]	Live Vaccinate[d]	Natural Exposure[e]	OMP Vaccinate	Live Vaccinate	Natural Exposure
1	110 kDa					
2	104 kDa	105 kDa	104 kDa			
3	97 kDa	97 kDa				
4	90 kDa	87 kDa	90 kDa			86 kDa
5		76 kDa				
6	66 kDa	65 kDa	66 kDa			
7	62 kDa*	62 kDa	62 kDa*			
8		60 kDa				
9	56 kDa	56 kDa	57 kDa	58 kDa	58 kDa	58 kDa
10				54 kDa	54 kDa	
11	46 kDa	47 kDa	47 kDa	48 kDa	48 kDa	47 kDa
12	38 kDa*	38 kDa*				37 kDa^R
13	36.5 kDa	36 kDa	36 kDa*	36 kDa	36 kDa	36 kDa
14	34 kDa		33 kDa	34 kDa	33 kDa^R	
15	32 kDa	32 kDa	32 kDa*		32 kDa^R	
16	31 kDa*	31 kDa*	28 kDa*	28 kDa^R	31 kDa^R	28 kDa^R
17	26 kDa	26 kDa*	26 kDa	25 kDa^R	25 kDa	
18	23 kDa		24 kDa*	23 kDa	23 kDa	

CHAPTER III

GENETIC AND IMMUNOLOGICAL ANALYSES OF A 38 KDA SURFACE-EXPOSED LIPOPROTEIN OF *Pasteurella haemolytica* S1

Abstract

Pasteurella haemolytica serotype A1 is the bacterial pathogen most frequently isolated from the lungs of cattle with bovine respiratory disease. As part of a study to characterize *P. haemolytica* antigens which are important in eliciting resistance to pneumonic pasteurellosis, we have cloned and sequenced the gene encoding a 38 kDa lipoprotein, Lpp38. The deduced amino acid sequence of Lpp38 is similar to those of the *Escherichia coli* polyamine transport proteins PotD (70%) and PotF (33%). *P. haemolytica* Lpp38 is present in both inner membrane and outer membrane fractions of the cell envelope. Susceptibility of Lpp38 to cleavage by extracellular proteases indicates that portions of the protein are surface-exposed. A protein of similar molecular mass in *P. haemolytica* strains from all 12 serotypes of biotype A and in an untypeable strain was detected by an anti-Lpp38 monoclonal antibody. Lpp38 is recognized by sera from calves resistant to infection after natural exposure to *P. haemolytica* and by sera from calves protected against infection by vaccination with *P. haemolytica* A1 outer membranes or with live bacteria. These data suggest a role for this protein in the development of immunity to *P. haemolytica* infection.

Introduction

Pasteurella haemolytica biotype A serotype 1 is the bacterium most commonly associated with shipping fever or pneumonic pasteurellosis, a disease of beef cattle characterized by a fibrinous pleuropneumonia (reviewed in Frank, 1989). Currently available commercial vaccines for the prevention of bovine pneumonic pasteurellosis consist of *P. haemolytica* bacterins, attenuated live *P. haemolytica*, cell extracts, or culture supernatant preparations (Confer, 1993). Many experimental vaccine formulations and those currently available commercially have proven to be largely ineffective or of limited effectiveness in eliciting protection.

Several studies indicate that *P. haemolytica* outer membrane proteins (OMPs) are among the most important antigens in stimulating resistance to pneumonic pasteurellosis (Morton et al., 1990, 1994, 1995; Mosier et al., 1989; reviewed in Confer, 1993). Mosier et al. (1989) demonstrated a statistically significant correlation between resistance to pneumonic pasteurellosis and the presence of serum antibodies (Abs) directed against several proteins present in a saline extract of whole *P. haemolytica*. More recently, cattle were shown to be resistant to experimental *P. haemolytica* challenge after immunization with sarkosyl insoluble OMPs (Morton et al., 1995). In that study, antibodies against OMPs with relative molecular masses (M_r) of 16.5, 37, 45.5, 50.5, and 84.5 kDa were shown to correlate significantly with this resistance (Morton et al., 1994).

The purpose of this study was to examine the specific nature of the immunogenic *P. haemolytica* OMP of approximately 37 kDa. We have cloned and sequenced the gene which encodes a surface-exposed, immunogenic OMP with a predicted M_r of 38 kDa. Using sera from cattle vaccinated with various *P. haemolytica* antigen preparations, we also demonstrate that this protein is the major immunogenic *P. haemolytica* OMP in the M_r range of 35-40 kDa.

Materials and methods

Bacterial strains, bacteriophage, and culture media

Escherichia coli DH5α and *P. haemolytica* serotype A1 Oklahoma strain (OK) (Newman et al., 1982) and strain 89010807N (Murphy and Whitworth, 1994) were grown as previously described (Murphy and Whitworth, 1994). *P. haemolytica* biotype A type strains were a gift of Glynn H. Frank (USDA, NADC, Ames, Iowa) and were grown as were serotype A1 strains. *E. coli* BB4 and XL1-Blue and bacteriophage λZAPII and R408 were supplied with a chromosomal library (Clontech Laboratories, Inc., Palo Alto, Calif.) and were grown according to the manufacturer's instructions. When appropriate, ampicillin (50 μg/ml) was added to media.

P. haemolytica genomic library, gene cloning, and DNA analyses

Chromosomal DNA was isolated from *P. haemolytica* 89010807N as described (Murphy and Whitworth, 1994), and a genomic library was prepared at Clontech Laboratories, using the bacteriophage vector λZAPII. The *P. haemolytica* chromosomal library was screened immunologically with

pooled monoclonal antibodies (produced as described below), and reactive phage were purified according to the manufacturer's instructions. Plasmid DNA (pBluescript) carrying the cloned DNA insert was excised from recombinant λZAPII bacteriophages according to the manufacturer's instructions. Plasmids were purified with the Magic system (Promega Corp., Madison, Wisc.) or the Qiagen system (Qiagen Inc., Chatsworth, Calif.). Cloned DNA inserts in recombinant plasmids were sequentially deleted from each end with the Erase-A-Base System (Promega). The subsequent recombinant plasmids were purified (Qiagen) and both strands of insert DNA were sequenced at the OSU Recombinant DNA/Protein Resource Facility, on an Applied Biosystems Inc. (Foster City, Calif.) 373A automated DNA sequencer. DNA sequences were assembled and analyzed with Assemblylign and MacVector DNA analysis software (Eastman Kodak Co., New Haven, Conn.).

Antigen preparation and analysis

P. haemolytica cell envelopes were prepared as previously described (Simons et al., 1989). Envelopes were extracted with an equal volume of 0.5% sarkosyl (*N*-lauryl sarcosine (Sigma Chemical Co., St. Louis, Mo.)) for 30 min at room temperature. Sarkosyl insoluble, OMPs were collected by centrifugation at 200,000 x g for 70 min. The supernatant contained sarkosyl soluble, inner membrane proteins. Protein concentrations were estimated with BioRad (Richmond, Calif.) or BCA (Pierce, Rockford, Ill.) protein assay kits. Whole cell lysates were prepared by resuspending 2 ml of cells grown to

stationary phase in 0.2 ml PBS and 0.2 ml final sample buffer (10% glycerol, 2.5% SDS, 0.1% bromophenol blue, 5% b-mercaptoethanol) and heating at 95°C for 5 min. Proteins in whole cell lysates, cell envelopes, and membrane fractions were separated by SDS-PAGE (10% or 12.5%) as previously described (Dabo et al., 1994).

Monoclonal Antibody Production

Monoclonal antibodies (MAbs) were prepared by the Oklahoma State University Hybridoma Center for Agricultural and Biological Sciences (Stillwater, Okla.) according to published methods (Bandla et al., 1993; Sherwood et al., 1987), by injection of mice with gel-purified protein as previously described for MAb 6A6C11 (Murphy and Whitworth, 1994).

Bovine antisera

Bovine antisera were kindly provided by Anthony W. Confer (OSU, Stillwater, Okla.). Sera were collected from calves shown to be resistant to experimental challenge with *P. haemolytica* A1 and were from three sources (i) animals naturally exposed to *P. haemolytica* [determined by high indirect immunofluorescence titers {>90}] (Gentry et al., 1985); (ii) animals vaccinated with live *P. haemolytica* A1 (Morton et al., 1995); and (iii) animals vaccinated with the sarkosyl insoluble outer membrane fraction of *P. haemolytica* A1 (Morton et al., 1995). Vaccinated calves received two subcutaneous injections, on day 0 and day 7, of either 2 ml of live *P. haemolytica* A1 (1×10^9/ml) or 2 ml of OMP preparations (2.0 mg/ml) mixed with an equal volume of

Freund's incomplete adjuvant (Morton et al., 1990, 1995). All sera used were collected on day 21, the day of experimental challenge.

Immunoblot analyses

For western immunoassays, proteins were transferred to nitrocellulose (MSI, Westboro, Mass. or BioRad Laboratories) using the Trans-blot apparatus (BioRad), according to the manufacturer's instructions. Western immunoblots with bovine sera were performed as previously described (Dabo et al., 1994). For MAb probing, blots were blocked with 1% non-fat dry milk (BioRad) in PBS with 0.02% sodium azide, washed in 0.01 M Tris-HCl, 0.2 M NaCl, pH 7.4 with 0.05% bovine serum albumin (BSA), incubated with MAb (hybridoma culture supernatant) diluted 1:25 in PBS/sodium azide. Immunoreactive bands were detected with alkaline phosphatase-conjugated, goat anti-mouse IgG (Sigma), and filters were developed in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (Sigma).

[^{3}H]-palmitic acid labeling of bacterial lipoproteins

Lipoproteins of logarithmic phase *P. haemolytica* A1 or *E. coli* harboring recombinant plasmids were radiolabeled with [9,10-^{3}H]-palmitic acid (Dupont, NEN, Boston, Mass.) and analyzed as described previously (Dabo et al., 1994).

Protease treatment of whole cells

P. haemolytica or *E. coli* cells (1 ml), in the mid-logarithmic phase of growth, were collected by centrifugation and washed in an equal volume of the appropriate enzyme buffer. Cells were resuspended in 0.25 ml of enzyme buffer, and 50 mg of enzyme in 0.005 ml was added. Negative controls

received an equal volume of enzyme buffer with no enzyme. Cells were incubated on a rotary shaker (120 rpm) for 1 hr at 37°C, collected by centrifugation and washed with 1 ml of PBS. Cells were resuspended in 0.05 ml PBS and 0.05 ml final sample buffer and heated at 95°C for 5 min before SDS-PAGE analysis. For protease treatment of de-capsulated *P. haemolytica*, capsule was removed by incubating cells in PBS for 1 hr at 42°C, as described previously (Gentry et al., 1982). Trypsin, chymotrypsin, and Staphylococcal V8 protease (Sigma) were used for protease treatment of whole cells. Trypsin digests were performed in 0.041 M Tris-HCl (pH 8.0), 0.012 M$CaCl_2$. Chymotrypsin digests were performed in 0.08 M Tris-HCl (pH 8.0), 0.1 M $CaCl_2$. Staphylococcal V8 protease digests were performed in PBS (pH 8.0).

GenBank accession number

The GenBank accession number for the nucleotide sequence of *lpp38* is U25682.

Results

Molecular cloning and DNA sequence of lpp38

We screened a λZAPII library of *P. haemolytica* chromosomal DNA with a panel of MAbs against a 38 kDa membrane protein and identified three recombinant phage which bound the MAbs. Recombinant plasmids were excised from phage DNA and transformed into *E. coli* (XL1-Blue). Restriction enzyme mapping revealed that the DNA inserts in all three plasmids were

identical. Therefore, one recombinant clone, *E. coli* (pKP420), was chosen for further study.

To compare the size of the recombinant protein expressed in *E. coli* with that expressed by *P. haemolytica*, we probed a western immunoblot of *E. coli* whole cell lysates and *P. haemolytica* outer membrane and inner membrane fractions with the MAb 6A6E5g (Fig. 5). The MAb recognized a protein of the same M_r in *E. coli* (pKP420) and in both *P. haemolytica* membrane fractions. A slightly larger immunoreactive band was also present in the recombinant clone.

DNA sequencing of the cloned insert revealed an open reading frame capable of encoding a protein of 364 amino acids (40.144 kDa) (Fig. 6). The deduced amino acid sequence of the protein contains a presumptive signal peptide and a consensus lipoprotein processing site. The M_r of the putative mature form of the lipoprotein, after cleavage of the signal peptide, is 38.3 kDa. We have designated this gene as *lpp38* and the protein encoded by the gene as Lpp38. A search of the GenBank DNA sequence databank revealed similarities between the deduced amino acid sequence of Lpp38 and two *E. coli* proteins, the polyamine transport protein PotD and the putrescine transport protein PotF. The sequence of Lpp38 is 50% identical to PotD and 17% identical to PotF. When conservative amino acid substitutions are included, Lpp38 is 70% similar to PotD and 33% similar to PotF (Fig. 7).

Lipid modification of the protein

To determine if this protein is actually lipid-modified, we compared total cellular lipoproteins from *P. haemolytica*, recombinant *E. coli* (pKP420), and non-recombinant *E. coli* after growth in the presence of [^{3}H]-palmitic acid. As shown in Fig. 8, whole cell lysates of *E. coli* (pKP420) contain a lipoprotein which migrates at the same M_r as one produced by *P. haemolytica* (~38 kDa). This lipoprotein is absent in non-recombinant *E. coli.*

Reactivity of bovine immune sera with Lpp38

Morton et al., (1994, 1995) have shown that sera from calves resistant to *P. haemolytica* challenge react with a protein of similar M_r as Lpp38. Those studies examined sera from (1) calves vaccinated with live *P. haemolytica* and (2) calves vaccinated with *P. haemolytica* OMPs. Here, we used those same sera to examine the immunological response of calves to Lpp38. We also used sera from calves that were found to be resistant to *P. haemolytica* infection after natural exposure to *P. haemolytica* (Gentry et al., 1985). By western immunoblot analysis, sera from these calves all recognized a *P. haemolytica* membrane protein of approximately 38 kDa, which migrated at the same M_r as recombinant Lpp38 produced by *E. coli* (pKP420) (Fig. 9). All the sera also recognized rLpp38 (Fig. 5 and data not shown).

Conservation of the protein among biotype A strains of *P. haemolytica*

To determine if Lpp38 is produced by other *P. haemolytica* serotypes, we examined whole cell lysates of all biotype A strains and one untypeable strain

by western immunoblot analysis. The anti-Lpp38 MAb 6A6C11 recognized a band of similar M_r in all these strains (data not shown).

Analysis of surface exposure of Lpp38

Regions of Lpp38 which are exposed on the surface of *P. haemolytica* may be targets for Ab-dependent immune mechanisms of the bovine host. To determine the presence of surface-exposed regions of Lpp38, we treated *P. haemolytica*, with and without intact capsule, and *E. coli* (pKP420) with three proteases and examined treated cells for alterations in the size of the anti-Lpp38 MAb-reactive band. Whole cell lysates of protease-treated cells were examined by western immunoblot analysis with the anti-Lpp38 MAb 6A6E5γ (Fig. 10). Trypsin treatment of encapsulated and de-capsulated *P. haemolytica* resulted in the appearance of two MAb-reactive bands, one faint band with the same M_r as Lpp38 and a more strongly reactive band with a slightly smaller M_r. Chymotrypsin and Staphylococcal V8 protease treatment of encapsulated *P. haemolytica* resulted in no change to Lpp38. However, treatment of de-capsulated *P. haemolytica* with those enzymes resulted in a marked reduction of the intensity of the Lpp38 band and the appearance of fainter bands of lower M_r. Treatment of recombinant *E. coli* with trypsin lead to changes in Lpp38 similar to those observed with *P. haemolytica*, but chymotrypsin and Staphylococcal V8 protease treatment of *E. coli* (pKP420) did not result in cleavage of rLpp38 (data not shown).

Discussion

In this study, we have cloned and sequenced a gene encoding an antigenic 38 kDa lipoprotein of *P. haemolytica* A1. The deduced amino acid sequence of this lipoprotein, Lpp38, is similar to that of the *E. coli* polyamine binding protein PotD (Furuchi et al., 1991) and to a lesser extent that of the *E. coli* putrescine transport protein PotF (Pistocchi et al., 1993). However, significant differences between Lpp38 and the *E. coli* proteins are apparent. Unlike Lpp38, the PotD and PotF amino acid sequences do not appear to possess the typical Gram-negative lipoprotein processing site. Analyses of the N-terminal amino acid sequences of PotD and PotF reveled that these proteins are apparently not lipid-modified in *E. coli* (Furuchi et al., 1991), and cell fractionation studies revealed that both are present in the periplasm of that organism. The *potD* gene is the terminal gene in an operon of four genes (*potA*, *potB*, *potC*, *potD*) all of which encode proteins involved in the uptake of polyamines. Here, we did not observe open reading frames 5' of *lpp38* which would correspond to *E. coli potA*, *potB*, or *potC*.

By western immunoblot analysis, we detected Lpp38 in both sarkosyl soluble and sarkosyl insoluble fractions of the *P. haemolytica* cell envelope. This result is consistent with our previous observations of other *P. haemolytica* membrane lipoproteins. We have found that an individual *P. haemolytica* lipoprotein may be present in both inner and outer membrane fractions purified by sarkosyl solubilization or on sucrose density gradients. Others have reported similar observations with lipoproteins in membrane

fractions purified on sucrose density gradients (Cooney and Lo, 1993). Preparation of *P. haemolytica* membrane fractions by sarkosyl solubilization results in an almost identical protein profile by SDS-PAGE and distribution of membrane markers (2-keto-3-deoxyoctonate (KDO), succinate dehydrogenase) as does preparation by sucrose density gradient centrifugation (Confer et al., 1995; Squire et al., 1984; data not shown). Neither method appears capable of determining the exact membrane location of *P. haemolytica* lipoproteins. Recent studies with other Gram-negative bacteria have indicated that lipoproteins may be associated with intermediate density membrane material which contains both inner membrane and outer membrane marker proteins and may be derived from a cell envelope region in which the two membranes are in intimate contact (Poquet et al., 1993). Others have also suggested numerous potential conformations for lipoproteins in the Gram-negative bacterial membrane (Pugsley, 1993).

Nevertheless, the accessibility of Lpp38 to proteases, upon treatment of intact *P. haemolytica*, suggests that at least a portion of this protein is exposed on the bacterial cell surface. Similar results with the recombinant form of the protein in *E. coli* indicate that Lpp38 assumes the same or a very similar conformation in the *E. coli* cell membrane as it does in the *P. haemolytica* membrane. We also found that the anti-Lpp38 MAb detected a protein of slightly larger M_r in *E. coli* than the band which represented the mature form of Lpp38 in *P. haemolytica* and *E. coli*. We believe that this band represents the precursor form of Lpp38 which is incompletely processed in *E. coli*. This

hypothesis is supported by the observation that the band of larger M_r is not labeled with [^{3}H]-palmitic acid (Fig. 7, lane 2).

The western immunoblot assays with sera from calves naturally exposed to *P. haemolytica* or exposed by vaccination demonstrated that Lpp38 is immunogenic. Similar assays performed on *E. coli* with and without pKP420 verified that Lpp38 is indeed the protein recognized by those sera (Fig. 9, lanes 1-2, and data not shown). It is of interest that the calves, whose sera were used in our experiments, were shown to be resistant to transthoracic experimental challenge with *P. haemolytica* (Morton et al., 1995). As part of that study, Morton et al. (1995) also demonstrated that vaccination with *P. haemolytica* OMPs elicited an antibody response, against a 37 kDa OMP, which correlated statistically with resistance to experimental challenge. Our data (Fig. 9) indicate that Lpp38 is the major immunogenic *P. haemolytica* A1 membrane protein migrating at an M_r of 37-38 kDa. Therefore, it is probable that Lpp38 represents the 37 kDa protein identified by Morton et al. (1995). The conservation of this protein among different *P. haemolytica* serotypes may also aid in resistance to infection with heterologous strains.

Proteins exposed on the bacterial cell surface are potential targets of the host immune response. Two important mechanisms of bovine immunity to *P. haemolytica*, complement-mediated killing and neutrophil-mediated phagocytosis, are further enhanced by opsonization of the bacteria (Chae et al., 1990; Czuprynski et al., 1987) and by removal of capsule (Chae et al., 1990). The antigenic nature of Lpp38 in cattle naturally exposed to *P. haemolytica* or

cattle vaccinated with various antigen preparations suggests a possible role for Abs against this protein in immunity. Although both complement-mediated killing and neutrophil phagocytosis of *P. haemolytica* have been shown to be more effective in the absence of capsule, the cleavage of Lpp38 by extracellular protease even in the presence of the bacterial capsule, indicates that regions of the protein are accessible to antibody binding. The role of Abs against Lpp38 in these immune processes is currently under investigation.

Acknowledgments

This work was supported by the Oklahoma Agricultural Experiment Station (Project OKL02181), the Oklahoma State University College of Veterinary Medicine, and grant 92-37204-7773 from the National Research Initiative Competitive Grants Office of the USDA.

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Mosier, D. A., Simons, K. R., Confer, A. W., Panciera, R. J. and Clinkenbeard, K. D., 1989. *Pasteurella haemolytica* antigens associated with resistance to pneumonic pasteurellosis. Infect. Immun., 57: 711-716.

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Fig. 5 Western immunoblot of *P. haemolytica* outer membrane and inner membrane fractions and *E. coli* whole cell lysates probed with the anti-Lpp38 MAb, 6A6E5γ. Lanes: 1, *E. coli* XL1-Blue (pBluescript), non recombinant; 2, *E. coli* XL1-Blue (pKP420); 3, *P. haemolytica* A1 outer membrane; 4, *P. haemolytica* A1 inner membrane.

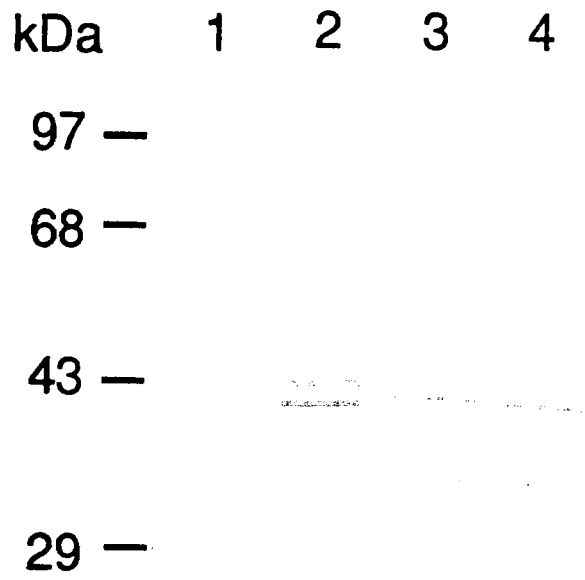

kDa
1
2
3
4
97
68
43
29

Fig. 6 Nucleotide sequence of the gene encoding Lpp38. The deduced amino acid sequence is shown beneath the nucleotide sequence. The location of putative promoter sequences (-35 and -10) are underlined. The putative ribosome binding site is also underlined. Putative sequence motifs were determined based upon comparison with consensus sequences and the presence of appropriate nucleotide spacing, using the MacVector (Eastman Kodak Co., New Haven, Conn.) subsequence analysis feature.


1 AATTCACTTTTTAATCAGAAAAGGCTTGCAAATACGGGGGGGGCTTAAAATGCCCTAGTCTTTTCCGCCCTTAGGCAAGGCTATATTTTTTAATCACTT
101 TTCTTTAACGGAGAAAACTTAAATGAAAAAATTAGCGGGTTTATTTGCAGCAGGTTTAGCAACAGTTGCATTAACAGCGTGTAATGAAGAAAAAGCGGCA
M K K L A G L F A A G L A T V A L T A C N E E K A A
201 GAGCCAAAAGCAACTGCGGCTGAAGCAAAACCTCAAGCAACAAATCAAACAGTTCACCTTTATACTTGGACTGAATATGTGCCTGAAGGTTTGTTAGAT
E P K A T A A E A K P Q A T N Q T V H L Y T W T E Y V P E G L L D
300 GAATTTACGAAAGAAACCGGCATTAAAGTTGAAGTTTCAAGTCTTGAATCTAACGAAACAATGTACGCTAAATTAAAATTACAAGGCAAAGATGGCGGT
E F T K E T G I K V E V S S L E S N E T M Y A K L K L Q G K D G G
399 TATGATGTAATTGCTCCATCAAACTACTTTGTATCTAAAATGGCGAAAGAAGGAATGTTAGCGGAATTAGATCACGCACAACTTCCGGTTATTAAAGAA
Y D V I A P S N Y F V S K M A K E G M L A E L D H A Q L P V I K E
498 TTAAACCAAGATTGGTTAAATAAACCTTACGATCAAGGAAACAAATATTCATTACCTCAATTATTAGGTGCGCCGGGTATTGCATTTAATACGGCAGAT
L N Q D W L N K P Y D Q G N K Y S L P Q L L G A P G I A F N T A D
597 TACAAAGGTGAAACATTCACTTCTTGGGGTGATTTGTGGAAACCTGAATTTGCAGGTAAAGTACAATTTATTAGATGATGCTCGTGAAGTATTCAACATT
Y K G E T F T S W G D L W K P E F A G K V Q L L D D A R E V F N I
696 GCGTTATTAAAATTAGGTAAAAACCCAAATACAACTAATCCGGATGAAATCAAAGAAGCCTTTGAAGAGTTGAAAAAATTACGTCCAAATGTGCTTTCA
A L L K L G K N P N T T N P D E I K E A F E E L K K L R P N V L S
795 TTTACCTCTGATAACCCGGCAAACTCATTTATTGCTGGTGAGGTTTCTGTAGGTCAGTTATGGAACGGTTCTGTACGTATTGCGAAGAAAGAAAACGCA
F T S D N P A N S F I A G E V S V G Q L W N G S V R I A K K E N A
894 CCGGTTGATATGGTATTCCCGAAAGAAGGTCCTGTGCTTTGGGTCGATACTTTAGCCATTCCGGCAAATGCGAAAAACAAAGAAAATGCACATAAATTA
P V D M V F P K E G P V L W V D T L A I P A N A K N K E N A H K L
993 ATCAACTACTTATTAAGTGCAAAAGTAGCTGAAAAATTAACGTTAGCGATTGGTTACCCAACTTCAAACGTAGAAGCATTAAAAGTATTACCGAAAGAA
I N Y L L S A K V A E K L T L A I G Y P T S N V E A L K V L P K E
1092 ATTACTGAAGATACAGCAATTTACCCATCTGCAGAGGTTTTACAAAAATCTCAATGGCAAGATGATGTTGGTGATGCAATTGAGCTTTACGAAAAATAC
I T E D T A I Y P S A E V L Q K S Q W Q D D V G D A I E L Y E K Y
1191 TACCAAGAGTTAAAAGCAGCAAAATAATGCGTGATT
Y Q E L K A A K

Fig. 7 Alignment of the deduced amino acid sequences of *P. haemolytica* Lpp38 and the *E. coli* proteins PotD and PotF. Numbering corresponds to the Lpp38 sequence. The location of the presumptive signal peptide of Lpp38 is shown. The * indicates the amino terminal, lipid-modified cysteine of the mature lipoprotein, Lpp38. Amino acids in bold type are those in Lpp38 which are identical with either PotD or PotF.

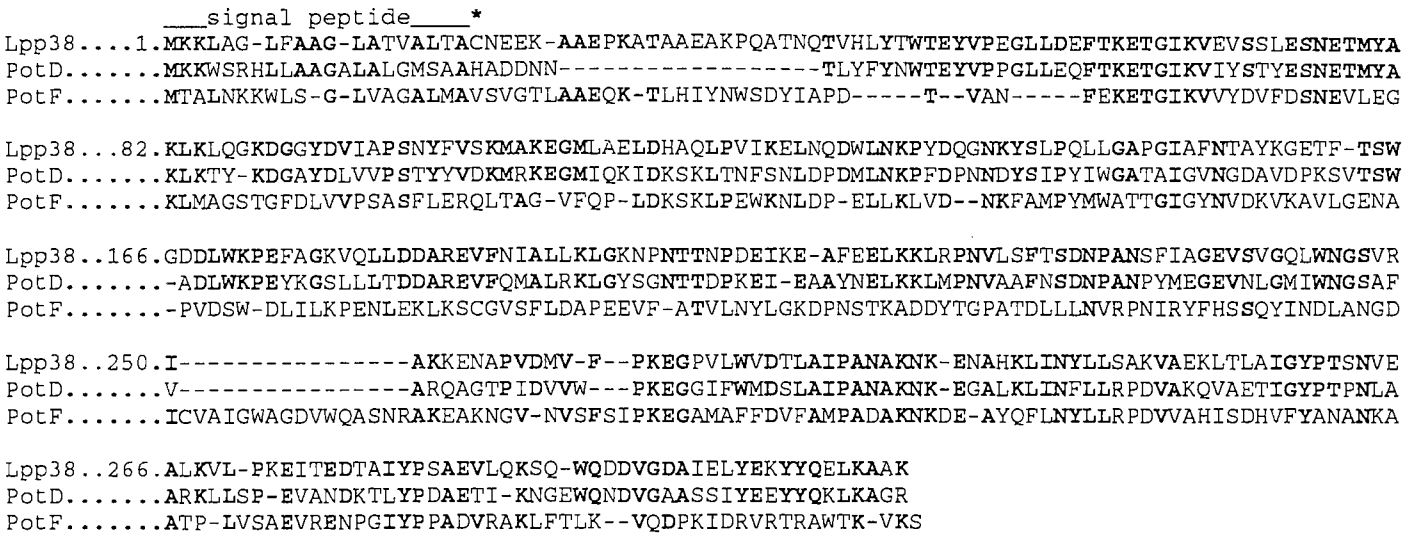

___signal peptide____*
Lpp38....1.MKKLAG-LFAAG-LATVALTACNEEK-AAEPKATAAEAKPQATNQTVHLYTWTEYVPEGLLDEFTKETGIKVEVSSLESNETMYA
PotD.....MKKWSRHLLAAGALALGMSAAHADDNN----------TLYFYNWTEYVPPGLLEQFTKETGIKVIYSTYESNETMYA
PotF.....MTALNKKWLS-G-LVAGALMAVSVGTLAAEQK-TLHIYNWSDYIAPD-----T--VAN-----FEKETGIKVVYDVFDSNEVLEG
Lpp38...82.KLKLQGKDGGYDVIAPSNYFVSKMAKEGMLAELDHAQLPVIKELNQDWLNKPYDQGNKYSLPQLLGAPGIAFNTAYKGETF-TSW
PotD.....KLKTY-KDGAYDLVVPSTYYVDKMRKEGMIQKIDKSKLTNFSNLDPDMLNKPFDPNNDYSIPYIWGATAIGVNGDAVDPKSVTSW
PotF.....KLMAGSTGFDLVVPSASFLERQLTAG-VFQP-LDKSKLPEWKNLDP-ELLKLVD--NKFAMPYMWATTGIGYNVDKVKAVLGENA
Lpp38..166.GDDLWKPEFAGKVQLLDDAREVFNIALLKLGKNPNTTNPDEIKE-AFEELKKLRPNVLSFTSDNPANSFIAGEVSVGQLWNGSVR
PotD.....-ADLWKPEYKGSLLLTDDAREVFQMALRKLGYSGNTTDPKEI-EAAYNELKKLMPNVAAFNSDNPANPYMEGEVNLGMIWNGSAF
PotF.....-PVDSW-DLILKPENLEKLKSCGVSFLDAPEEVF-ATVLNYLGKDPNSTKADDYTGPATDLLLNVRPNIRYFHSSQYINDLANGD
Lpp38..250.I----------AKKENAPVDMV-F--PKEGPVLWVDTLAIPANAKNK-ENAHKLINYLLSAKVAEKLTLAIGYPTSNVE
PotD.....V----------ARQAGTPIDVVW---PKEGGIFWMDSLAIPANAKNK-EGALKLINFLLRPDVAKQVAETIGYPTPNLA
PotF.....ICVAIGWAGDVWQASNRAKEAKNGV-NVSFSIPKEGAMAFFDVFAMPADAKNKDE-AYQFLNYLLRPDVVAHISDHVFYANANKA
Lpp38..266.ALKVL-PKEITEDTAIYPSAEVLQKSQ-WQDDVGDAIELYEKYYQELKAAK
PotD.....ARKLLSP-EVANDKTLYPDAETI-KNGEWQNDVGAASSIYEEYYQKLKAGR
PotF.....ATP-LVSAEVRENPGIYPPADVRAKLFTLK--VQDPKIDRVRTRAWTK-VKS

Fig. 8 Autoradiogram of SDS-polyacrylamide gel with ^{3}H-palmitic acid-labeled total cellular lipoproteins. Lanes: 1, *E. coli* DH5a (pBluescript), a non-recombinant strain; 2, *E. coli* DH5a (pKP420), a recombinant carrying the *P. haemolytica* lpp3 gene; 3, *P. haemolytica* A1 89010807N. Lpp38 is indicated with an arrowhead.

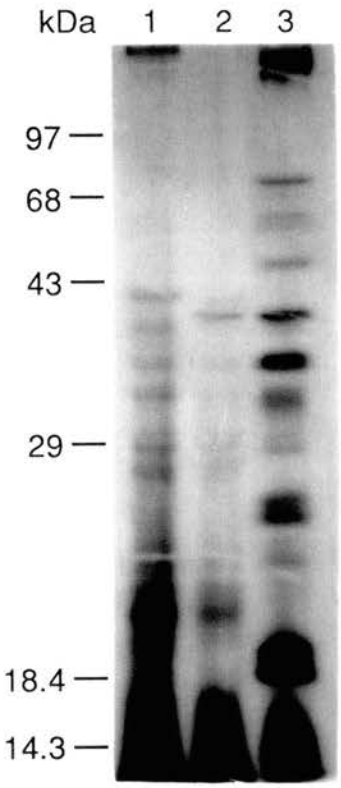

kDa
1
2
3
97
68
43
29
18.4
14.3

Fig. 9 Western immunoblot of *P. haemolytica* cell envelope fractions and *E. coli* whole cell lysates probed with bovine sera. Lane: 1, non recombinant *E. coli* XL1-Blue; 2, *E. coli* XL1-Blue (pKP420); 3 - 7, *P. haemolytica* cell envelopes. Lanes 1 - 3 were probed with serum from a calf vaccinated with live *P. haemolytica*. Lanes 4 - 5 were probed with sera from calves vaccinated with outer membranes of *P. haemolytica*. Lanes 6 - 7 were probed with sera from calves naturally exposed to *P. haemolytica*.

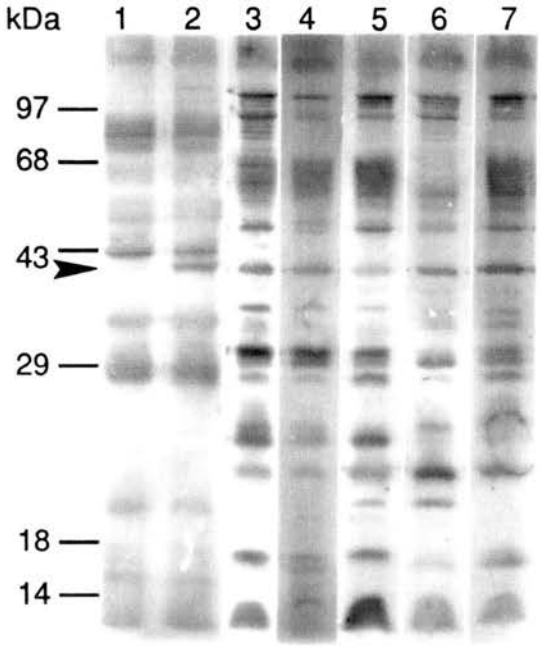
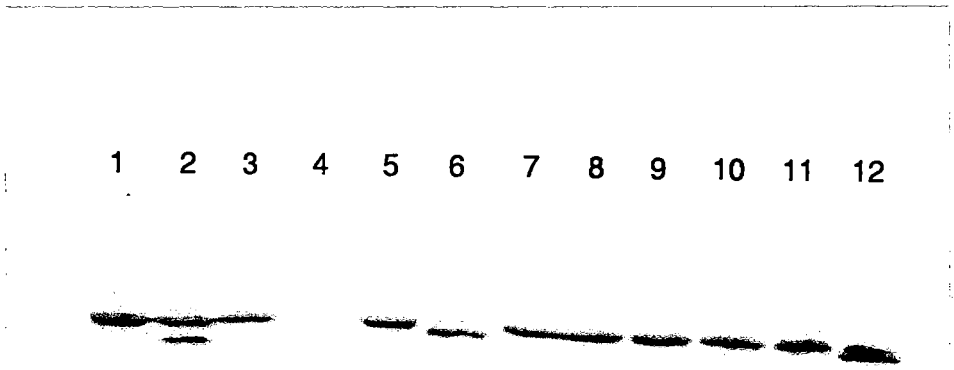

kDa
1
2
3
4
5
6
7
97
68
43
29
18
14

Fig. 10 Western immunoblots of *P. haemolytica* whole cell lysates prepared after treatment of intact cells with various proteases. The blot was probed with the anti-Lpp38 MAb 6A6C11. **Lanes: 1-6**, decapsulated *P. haemolytica* treated with; **1**, Staphylococcal V8 protease buffer; **2**, Staphylococcal V8 protease; **3**, chymotrypsin buffer; **4**, chymotrypsin; **5**, trypsin buffer; **6**, trypsin. **Lanes: 7-12** intact (encapsulated) *P. haemolytica* treated with; **7**, Staphylococcus V8 protease buffer; **8**, Staphylococcus V8 protease; **9**, chymotrypsin buffer; **10**, chymotrypsin; **11**, trypsin buffer; **12**, trypsin.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CHAPTER IV

GENETIC AND IMMUNOLOGIC ANALYSES OF PlpE, A LIPOPROTEIN IMPORTANT IN COMPLEMENT-MEDIATED KILLING OF *Pasteurella haemolytica* S1

Abstract

Pasteurella haemolytica S1 is the organism most commonly associated with bovine shipping fever pleuropneumonia. Antibodies against outer membrane proteins (OMPs) of *P. haemolytica* have been correlated with development of resistance to the disease. In this manuscript, we present research on an immunogenic, surface-exposed OMP of *P. haemolytica*, PlpE. We affinity-purified antibodies against PlpE from bovine immune serum and used these antibodies to clone the gene encoding PlpE. DNA sequencing and analysis of the deduced amino acid sequence revealed that PlpE is a lipoprotein and that it is similar to *Actinobacillus pleuropneumoniae* lipoprotein, OmlA. Vaccination of pigs with OmlA from serotype 1 has been shown to enhance protection of pigs against homologous *A. pleuropneumoniae* challenge. Anti-PlpE antibodies recognize a protein of same or different molecular weights in all serotypes of *P. haemolytica* except serotype 11. Our data suggest that PlpE and recombinant PlpE are surface-exposed in *P. haemolytica* and *E. coli*, respectively. We used intact recombinant *E. coli* that express PlpE, to adsorb out PlpE-reactive antibodies from bovine immune serum. In an assay measuring the complement-mediated killing of *P. haemolytica*, serum depleted of antibodies specific for PlpE, by absorption with intact recombinant *E. coli*, had a significant reduction in bacterial killing as compared to the same serum absorbed with non-recombinant *E. coli*. Our data indicates that PlpE is an important immunogen of *P. haemolytica* and a favorable candidate for a sub-unit vaccine.

Introduction

Pasteurella haemolytica serotype 1 (S1) is the organism most commonly associated with shipping fever, a disease of beef cattle characterized by fibrinous pleuropneumonia (Frank, 1989). The disease is of significant economic importance to the beef industry in the USA, amounting to annual losses approaching 1 billion dollars (Dee, 1997). Shipping fever pneumonia is precipitated by stress inducing conditions such as shipping, viral infections, inhalation of diesel fumes, overcrowding, and weaning (Confer et al., 1988). *P. haemolytica* S1 reside in low numbers in the upper respiratory tract of cattle and under stressful conditions they proliferate and are aerosolized, in large numbers, into lung alveoli where they cause the disease (Frank and Briggs, 1992).

Numerous surface and secreted molecules of *P. haemolytica* S1 have been studied to evaluate their roles in immunity to *P. haemolytica* infection (reviewed in (Confer, 1993)). Included among these are *P. haemolytica* outer membrane proteins (OMPs) (Squire et al., 1984; Sreevatsan et al., 1996; Confer et al., 1990). Vaccination of cattle with an OMP-enriched fraction of *P. haemolytica* cell envelopes significantly reduced lung damage following experimental challenge with *P. haemolytica* (Morton et al., 1995). Statistical correlations have been demonstrated between resistance to pneumonia and the bovine antibody response to proteins present in surface extracts of *P. haemolytica* (Confer et al., 1989; Sreevatsan et al., 1996). Several studies have

analyzed the bovine antibody response that develops after vaccination with *P. haemolytica* antigens or after *P. haemolytica* infection, to purified individual OMPs including a 94 kDa membrane protein (Nelson and Frank, 1989) and PomA, a protein belonging to the OmpA family, (Mahasreshti et al., 1996). We and others have cloned and sequenced genes encoding *P. haemolytica* OMPs and examined the bovine antibody response to the recombinant proteins, including several membrane lipoproteins (Craven et al., 1991; Cooney and Lo, 1993; Dabo et al., 1994; Pandher and Murphy, 1996). These studies suggest a role for outer membrane antigens in eliciting protective immunity. However, the capacity for *P. haemolytica* OMP-specific antibodies, to function in host defense mechanisms remains uncharacterized. For the development of more effective vaccines, it will be important to characterize individual OMPs and identify those that elicit host antibodies that enhance resistance to *P. haemolytica* infection.

Complement-mediated lysis is an important host-defense mechanism against microbial infection and is believed to play a role in controlling *P. haemolytica* pneumonia. Serum complement concentrations were found to be lower in stressed cattle after transport to a feedlot (Purdy et al., 1991). Lower complement concentrations were associated with higher morbidity in the feedlot, and morbid calves had significantly lower complement levels than did healthy calves in the same feedlots (Purdy et al., 1991). These data suggest that a decrease in serum complement levels might facilitate *P. haemolytica* infection. However, complement-mediated killing of *P*

haemolytica requires sensitization with antibodies (MacDonald et al., 1983). Antibodies against surface-exposed epitopes of OMPs can function as opsonins as well as they are likely to play an important role in complement-mediated lysis of *P. haemolytica.*

Cattle that are resistant to *P. haemolytica*-induced pneumonia develop antibodies to a surface-exposed, ~45kDa OMP (chapter 1). The purpose of this study was to determine, through genetic cloning and DNA sequencing, the specific identity of the immunogenic 45 kDa protein and to evaluate the contribution of antibodies against this protein, to complement-mediated killing of *P. haemolytica.* We have found that the 45 kDa protein is a lipoprotein, designated PlpE, and that antibodies against PlpE, present in bovine immune sera, contribute to complement-mediated killing of *P. haemolytica.*

Materials and Methods

Bacteria, bacteriophage, culture media, and genomic library

P. haemolytica (89010807N) serotype 1 was grown in BHI broth or on BHI agar (Difco Laboratories, Detroit, MI, USA) as previously described (Murphy and Whitworth, 1994). *Escherichia coli* strains BB4 and XL1-Blue and bacteriophages lambda ZAPII and R408 were supplied with a *P. haemolytica* genomic DNA library (Clontech Laboratories, Palo Alto, Calif.) (Pandher and Murphy, 1996) and were grown according to the manufacturer's instructions.

Recombinant *E. coli* were always grown in the presence of ampicillin (50 μg/ml).

Bovine immune sera and purification of antibodies

Two bovine immune sera were used, one from a calf hyperimmunized with live *P. haemolytica* (Lessley et al., 1985) and one from a calf that was vaccinated with *P. haemolytica* OMPs and was resistant to experimental *P. haemolytica* challenge (Confer et al., unpublished data). Antibodies against *P. haemolytica* ~45 kDa OMPs were purified from the bovine hyperimmune serum by an immunoaffinity procedure (Harlow and Lane, 1988). *P. haemolytica* outer membranes were purified as described previously (Simons et al., 1992), separated by preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Dabo et al., 1994), and transferred to nitrocellulose membranes (Pandher and Murphy, 1996). The region of the nitrocellulose membrane containing ~45 kDa proteins was identified by comparison with pre-stained molecular weight standards (BioRad Laboratories, Richmond, Calif.) on the membrane. The 45 kDa region of the nitrocellulose was excised and incubated for 1 hr with the bovine hyperimmune serum (1:25) in 0.01M Tris-HCl (pH 7.4), 0.2M NaCl, (TS) containing 0.05% gelatin and 0.05% Tween (TSGT). Nitrocellulose strips were washed with TS, 3 x 5 min. Anti-45 kDa antibodies bound to antigen on the nitrocellulose strips were eluted as described (Harlow and Lane, 1988). The eluted antibodies were concentrated using a Centriprep 10 concentrator (Amicon Inc., Beverly, Mass.).

Gene cloning, DNA sequencing, site-directed mutagenesis, and sequence analysis

The *plpE* gene was cloned by immunoscreening a *P. haemolytica* expression library (described above) with affinity purified anti-45 kDa antibodies according to the manufacturer's instructions. Additional DNA cloning was performed as described previously (Pandher and Murphy, 1996). Site-directed mutagenesis was performed using the Gene Editor™ *in vitro* Mutagenesis System (Promega Corp., Madison, Wis.). Mutations were confirmed by DNA sequence analysis. Plasmid inserts were progressively deleted from both ends using the Erase A Base kit (Promega), and progressively smaller plasmid inserts were sequenced using the universal and reverse primers. Both DNA strands were sequenced. DNA sequencing was performed at the OSU Recombinant DNA/Protein Resource Facility, on an Applied Biosystems (Foster City, Calif.) 373A automated DNA sequencer. Sequences were analyzed with MacVector/Assemblylign (Oxford Molecular Group, Inc., Campbell, Calif.). The deduced amino acid sequence of PlpE was compared with other sequences in GenBank using BLAST 2.0 (Altschul et al., 1997), and alignments were generated with CLUSTALW 1.7 at the Baylor College of Medicine Search Launcher.

Antigen preparation and western immunoblots

Whole cell lysates were prepared and western immunoblots were performed as described previously (Pandher and Murphy, 1996) (Dabo et al., 1994). Primary antibodies used for each western immunoblot experiment are

described in the Results section. Alkaline phosphatase-conjugated mouse monoclonal, anti-bovine IgG, (Sigma Immunochemicals, St. Louis, Mo.), (1:20,000) in TSGT, was always used as the secondary antibody in western immunoblots.

[^{3}H]-Palmitic acid labeling of bacterial lipoproteins

Labeling and analysis of logarithmic-phase *P. haemolytica*, non-recombinant *E. coli*, and recombinant *E. coli* that express PlpE, were performed using [9, 10-^{3}H]-palmitic acid (Dupont, NEN, Boston, Mass.) as described previously (Dabo et al., 1994).

Absorption of immune serum with intact cells

Bovine immune serum was absorbed with intact *P. haemolytica* using a modification of a previously described method (Hansen et al., 1988). Logarithmic phase *P. haemolytica*, from one liter of culture (A_{600} of 0.5), were pelleted by centrifugation, washed once in PBS, and resuspended in immune serum diluted 1:100 in Tris-saline-nonfat dry milk (TSM, 10mM Tris pH 7.4, 0.9% (w/v) NaCl, 1% non fat dried milk). Cells resuspended in serum were incubated at 4°C for 3 hrs on a rocking platform. Following incubation, cells were pelleted by centrifugation at 11,000 x g. The supernatant was carefully removed and stored at -20°C following the addition of sodium azide at 0.02%. Unabsorbed immune serum was used as control and was diluted 1:100 in TSM before use. Absorption with recombinant *E. coli* (pB4522) and non-recombinant *E. coli* (pBluescript SK-) was performed similarly except stationary phase organisms were used.

Complement-mediated killing assay

Serum from a calf with a low ELISA antibody titer against *P. haemolytica* was used as a complement source. The complement-source serum was depleted of any existing antibodies against *P. haemolytica* by incubation with excess, stationary phase *P. haemolytica*, at 4°C, on a rocking platform, for 1 hr. Before incubation with complement-source serum, *P. haemolytica* were washed once with cold PBS (4°C).

Serum from an OMP-vaccinated calf (described above) was used as the source of antibodies. The antibody-source serum was heat-inactivated at 56°C for 30 min (to inactivate compliment) and was used in two different forms: (1) Anti-PlpE antibodies were removed from antibody-source serum by adsorbing the serum with recombinant *E. coli* expressing PlpE. This process would also adsorb out any anti-*E. coli* antibodies that were present in the serum. (2) Therefore a control serum from which only anti-*E. coli* antibodies were removed and was prepared by adsorbing the antibody-source serum with non-recombinant *E. coli* (pBluescript SK-) (Stratagene, La Jolla, CA, USA). Adsorption of serum with recombinant and non-recombinant *E. coli* was performed as follows. Recombinant and non-recombinant *E. coli* were grown overnight in 100ml of Luria broth (GIBCO-BRL, New York, USA) and harvested by centrifugation at 4°C. The cells were washed once with PBS and subsequently used to adsorb out antibodies from antibody-source serum that had been diluted 1:1 with PBS. Two ml of this diluted antibody-source serum was incubated with recombinant *E. coli*, at 4°C for 3 hrs, on a rocking

platform (# 1). At the same time another 2 ml of diluted antibody-source serum was also incubated with non-recombinant *E. coli* (# 2). After incubation, cells were removed from serum by centrifugation. This process was repeated until the serum incubated with recombinant *E.coli*, did not recognize PlpE in a western immunoblot (data not shown).

The complement-mediated killing assay was developed by modifying the techniques described by Chae et al. (Chae et al., 1990) and Murphy et al. (Murphy et al., 1986). To ensure that the assay was capable of detecting a change in the amount of bactericidal antibody, numbers of bacteria and concentration of complement were evaluated in preliminary experiments (Murphy et al., 1986). For complement-mediated killing assays, bacteria were grown in BHI broth for 18 hours at 37°C, on a rotary shaker (200 rpm). Cells were washed once with PBS and resuspended in PBS to an A_{600} of 0.5. Complement-source serum (50 µl) and antibody-source serum (#1 or #2 described above) (50 µl) were added to 150 µl of PBS. *P. haemolytica* (9000-18,000 cfu in 40 µl PBS) were then added. Immediately after the addition of *P. haemolytica* (t = 0) and after incubating for 30 min in a 37°C water bath (t = 30), 100 µl samples were removed and diluted 1:100 in PBS. Dilutions were prepared and plated on BHI agar plates for determination of cfu. At t = 30, undiluted reaction mix was also plated for determining cfu. To monitor the killing activity of the complement-source serum alone, a control with only complement-source serum and no antibody-source serum was evaluated in an identical manner. We also determined that the antibody-source serum

alone had no killing activity (data not shown). Percent killing was calculated by the following formula: $\{(CFU_{t=0} - CFU_{t=30}) / CFU_{t=0}\}$ 100. Percent survival was calculated as 100-percent killing.

Statistical analysis

Complement-mediated killing assays were done in triplicate for each antibody source serum and the complement-source serum control. Three separate experiments were done on different days. Statistically significant differences between killing by antibody-source sera 1 and 2 were determined within experiments by Student's *t* test (Bailey, 1981).

Results

Cloning of *plpE*

To isolate a clone expressing the immunogenic 45 kDa protein, we screened a genomic library of *P. haemolytica* S1 with anti-45 kDa antibodies that were affinity purified from bovine immune serum. We isolated recombinant lambda ZAPII phage that reacted with the affinity-purified antibodies. A recombinant plasmid containing a 4.5 kbp insert was excised from one phage clone and transformed into *E. coli* (XL1-Blue). We subcloned a 2.2 kbp fragment from this insert into pBluescript SK(-) and named this plasmid pB4522. *E. coli* (pB4522) expressed a 45 kDa protein that was recognized by the affinity purified antibodies (Fig. 11a). We named this protein PlpE.

Surface exposure of PlpE. As mentioned in the Introduction, we previously demonstrated the presence of an immunogenic 45 kDa *P.*

haemolytica protein that is surface exposed (chapter 1). To determine if PlpE expressed by the recombinant *E. coli* strain, corresponds to the strongly immunogenic *P. haemolytica* surface protein, we examined surface exposure of the protein on intact *P. haemolytica*. Adsorption of bovine immune serum with intact *P. haemolytica* resulted in a loss of antibody reactivity on western immunoblots with recombinant PlpE and a protein of the same M_r in *P. haemolytica* whole cell lysates (Fig. 11a, b), suggesting that PlpE is surface-exposed in *P. haemolytica*. Similarly, absorption of the same bovine immune sera with intact recombinant *E. coli* (pB4522) expressing PlpE resulted in a loss of reactivity to rPlpE and to a 45 kDa protein in *P. haemolytica* (Fig. 11c, d). These data suggest that PlpE is also exposed on the surface of recombinant *E. coli* and that PlpE is the primary 45 kDa surface-exposed immunogen of *P. haemolytica*.

Sequence analysis of PlpE. DNA sequencing of the cloned insert in pB4522 revealed an open reading frame of 1064 nucleotides that begins with a GTG codon and encodes a protein with a calculated molecular weight (MW) of 39.1 kDa (Fig. 12). The deduced amino acid sequence contains a putative hydrophobic signal peptide followed by a consensus lipoprotein processing site (LSAC) (Fig. 12). The calculated MW of the putative mature form of PlpE is 37.03 kDa. The N-terminal region of the putative mature PlpE contains eight copies of a six amino acid repeat (Fig. 12) that are predicted to form a hydrophilic domain (Fig. 13). PlpE also has numerous other hydrophilic domains that correspond with regions of high surface probability (Fig. 13). A

search of GenBank sequences and subsequent sequence alignments revealed that the deduced amino acid sequence of PlpE has 18% identity and 32% similarity to an outer membrane lipoprotein (OmlA) produced by *Actinobacillus pleuropneumoniae* serotype 1 and 20% identity and 35% similarity to the OmlA protein from *A. pleuropneumoniae* serotype 5 (Fig. 14).

Site-directed mutagenesis of the putative GTG start codon. To verify that GTG functions as a translational start codon for PlpE, we performed site-directed mutagenesis and converted the codon to GGG. Western immunoblots of whole cell lysates from the wild-type and mutant *E. coli* (pB4522) strains, probed with anti-45 kDa antibodies, revealed that the mutant strain no longer produced a 45 kDa immunoreactive protein, suggesting that GTG functions as the translational start codon (Fig. 15).

Lipid Modification of PlpE. Because the deduced amino acid sequence contained a consensus lipoprotein processing site, we examined *P. haemolytica* and *E. coli* (pB4522) for the presence of 45 kDa lipid-modified proteins. *P. haemolytica*, *E. coli* (pB4522), and non-recombinant *E. coli* (pBluescript) were grown in the presence of ^{3}H-palmitic acid. A 45 kDa, ^{3}H-labeled lipoprotein is present in whole cell lysates of *P. haemolytica* and *E. coli* (pB4522) but absent from the non-recombinant *E. coli* strain (Fig. 16).

Conservation of PlpE among serotypes of *P. haemolytica*. To determine if PlpE is expressed by other *P. haemolytica* serotypes, we examined whole cell lysates of *P. haemolytica* serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, and an

untypeable strain of *P. haemolytica*, by western immunoblot analysis, for reactivity with the anti-45 kDa antibodies (Fig. 17). The antibodies recognized a 45 kDa protein in serotypes 1, 5, 6, 7, 8, 12, and 14, an approximately 35 kDa protein in serotypes 2 and 13, and a ~65 kDa protein in serotype 9 and the untypeable strain. The purified antibodies did not recognize a protein in the serotype 11 strain.

Role of anti-PlpE antibodies in complement-mediated killing of *P. haemolytica*. We sought to determine if anti-PlpE antibodies contribute to complement-mediated killing of *P. haemolytica*. Since we had observed that *E. coli* (pB4522) have the capacity to remove anti-PlpE antibodies from bovine serum, we used the recombinant strain to deplete those antibodies from bovine immune serum. We then compared complement-mediated killing of *P. haemolytica* using, as an antibody source, bovine immune serum or immune serum depleted of anti-PlpE antibodies. As shown in Fig 18, immune serum that was depleted of anti-PlpE antibodies caused less killing of *P. haemolytica* than did immune serum that was not depleted of those antibodies. The difference in killing activity was statistically significant ($p<0.003$).

Discussion

We have cloned, sequenced, and analyzed an immunogenic lipoprotein (PlpE) of *P. haemolytica*. In our previous studies, we had noted an intense

immunogenic band at 45 kDa level, in western blots of *P. haemolytica* OMPs (sarkosyl insoluble fraction), probed with sera from immune cattle (data not shown). Our studies directed at identifying surface-exposed OMPs of *P. haemolytica* revealed that this immunogen at 45 kDa is surface-exposed (chapter 1). We hypothesized that antibodies against the surface-exposed regions of this protein are important in host defense mechanisms such as complement-mediated killing.

Although PlpE migrates to 45 kDa level on an SDS polyacrylamide gel, we found that the calculated molecular weight of the deduced amino acid sequence of the mature form of this protein is 37 kDa. This discrepancy could possibly be due to the fact that PlpE is rich in proline (5.93%), a turn inducing amino acid residue that causes the protein to migrate slower on SDS-PAGE (Postle, 1990). Analysis of the deduced amino acid sequence of PlpE revealed a positively charged amino terminal region, a central hydrophobic region followed by a consensus lipoprotein processing site LSAC (Hayashi and Wu, 1990). We were able to prove lipid modification of PlpE by ^{3}H-palmitate labeling of the mature form of PlpE in recombinant *E. coli* (pB4522). The N-terminal region of the protein is composed of eight copies of 6 amino acid repeats. We noted four of these to be exact copies while the other four contain substitutions with similar amino acid residues. It might be of interest to study these repeats in different strains of *P. haemolytica* in order to determine their candidacy as a site of antigenic variation.

A data base search revealed that PlpE is similar to an *Actinobacillus pleuropneumoniae* lipoprotein OmlA. Gerlach et al. (Gerlach et al., 1993) have demonstrated that vaccination of pigs with purified OmlA from serotype 1, significantly reduces lung damage and death of pigs upon subsequent experimental challenge with homologous *A. pleuropneumoniae* serotype.

Western immunoblot analysis revealed that antibodies reactive against PlpE from *P. haemolytica* serotype A 1 recognize proteins of similar molecular weight in serotypes 5, 6, 7, 8, and 14. These antibodies recognize a protein of ~65 kDa in serotype 9 and an untypeable strain of *P. haemolytica* and a proteins around 35 kDa in serotypes 2 and 13. Thus PlpE could be a significant cross-protective antigen making it a favorable candidate for inclusion into a sub-unit vaccine.

Another interesting feature of the gene *plpE* is that it utilizes GUG as a translational start codon. GUG, AUG, and UUG fall in the group of class I initiation codons that support efficient translation (Sussman et al., 1996). In *E. coli* the intrinsic activity of GUG is 12-15% of AUG (Ringquist et al., 1992). About 8% of known bacterial genes from *E. coli* and other organisms utilize GUG as the start codon (Schneider et al., 1986). To our knowledge *plpE* is the first example of a *P. haemolytica* gene utilizing GUG as a start codon.

From our previous studies it was unclear as to how many proteins contributed towards the major immunogenic band seen at 45 kDa level in western blots of *P. haemolytica* probed with bovine immune serum. Our

surface-exposure studies on PlpE helped answer that question. Absorption of bovine immune serum with intact recombinant *E. coli* (pB4522) virtually abolished the reactivity of this serum at 45 kDa level of *P. haemolytica* whole cell lysates. This result indicates that PlpE is the major immunogen responsible for the significant, immunogenic band at 45 kDa level. Further, the ability of intact recombinant *E. coli* (pB4522) to absorb out anti-PlpE antibodies suggest that the signal peptide of PlpE is functional in *E. coli* so that PlpE is trafficked to the *E. coli* cell membrane and is exposed on the cell surface. The surface-exposure of recombinant PlpE and its ability to absorb out anti-PlpE antibodies suggests that recombinant PlpE assumes a conformation quite similar to the native form in *P. haemolytica*. Hence, recombinant *E. coli* (pB4522) could be used in vaccination trials to deliver PlpE in a folded, membrane associated form that resembles closely, if not completely, the native *P. haemolytica* form.

According to our hypothesis, antibodies directed against surface-exposed epitopes of PlpE should help in opsonization of *P. haemolytica* and aid in host defense mechanisms like complement-mediated killing and phagocytosis by neutrophils and macrophages. We were able to demonstrate that depletion of antibodies against surface-exposed epitopes of PlpE, by absorption of immune serum with intact recombinant *E. coli* (pB4522), caused a significant reduction in complement-mediated killing of *P. haemolytica*. Our negative controls that had compliment source alone and no source of antibody failed to kill *P. haemolytica* (we actually saw growth).

These data suggest that complement activation through the alternative pathway does not play any significant role in the killing of the organism. This result is congruent with a previous study that demonstrated that the classical pathway is the only complement cascade important in killing of *P. haemolytica* (MacDonald et al., 1983).

The serum used as antibody source in complement-mediated killing assay was collected on day 36, after three immunizations with *P. haemolytica* OMPs (sarkosyl-insoluble fraction). Therefore, we expect IgG to be the predominant circulating isotype and to be the predominant isotype involved in complement-mediated killing. Although IgM is the most potent activator of the classical complement cascade, its concentration in circulation is relatively low in an anamnestic immune response. This observation has *in vivo* relevance. Most beef cattle develop a titer to *P. haemolytica* early in their life and continue to encounter *P. haemolytica* through natural exposure (Frank, 1985) and vaccination. Hence, it can be assumed that *P. haemolytica* infection of the lung encounters a secondary immune response, which consists of IgG as the predominant circulatory antibody. The role of IgM-mediated killing mechanisms in this scenario should be rather minimal. In addition, Mosier et al., 1989, found that anti-*P. haemolytica* IgM antibodies did not seem to play a major role in development of resistance to experimental pneumonic pasteurellosis.

This study has demonstrated that PlpE is a significant immunogen of *P. haemolytica* and that antibodies against this protein aids complement-

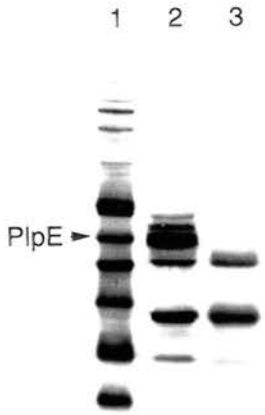
mediated killing and presumably other antibody-dependent host defense mechanisms. Our data justify the use of PlpE as an experimental vaccine to study the protection it may confer to cattle against experimental *P. haemolytica* challenge. We believe that PlpE is a strong candidate as a component of a sub-unit vaccine developed to induce immunity against *P. haemolytica* infection.

Acknowledgments

We thank Dr. A.W. Confer for providing us with immune sera and complement source serum and for performing the statistical analysis on the data from the complement-mediated killing assay . This research was supported by grant 95-37204-1999 from the National Research Initiative Competitive Grants Program of the USDA, the Oklahoma Agricultural Experiment station (Project OKL02179), and the Oklahoma State University College of Veterinary Medicine. KP was supported by an OSU-CVM graduate research assistantship award.

Fig. 11

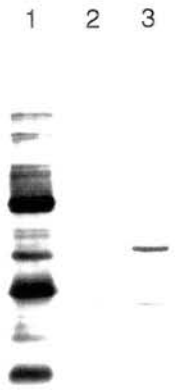
Western immunoblots demonstrating surface exposure of PlpE in *P. haemolytica* and in recombinant *E. coli*. Lanes: Whole cell lysates of (1) *P. haemolytica*; (2) *E. coli* (pB4522) expressing PlpE; (3) non-recombinant *E. coli* (pBluescript SK-). Blots were probed with (a) bovine immune serum, (b) immune serum absorbed with *P. haemolytica*, (c) immune serum absorbed with non-recombinant *E. coli* (pBluescript), and (d) immune serum absorbed with *E. coli* (pB4522).



a

b

c



d

Fig. 12

The nucleotide sequence of *plpE* and the deduced amino acid sequence of PlpE. Sequences corresponding to *E. coli* consensus -35, -10 and ribosome binding sites (SD seq.) sites, 5' of the gene, are underlined. The consensus lipoprotein processing site is underlined with a dashed line. The eight imperfect, hydrophilic repeats in the amino terminal region of PlpE are also underlined.

```
1     AGGGCTAATCTACTACAGCCCCAAAAATTTTCATAAGGGAAACGTTTACGTAAAACTCCT   60
      -35                  -10
61    CAGACCACTCATTCTTATTTTATATAAAAAATGTGATAGACTTCTCGCAGTTTCGTTTTA  120
            SD seq.
121   TATATTTAAGGAATAACTAAGTGAAATTCAATAAAAAATTAATTTTAACATTTGCTGCAA  180
1                          V  K  F  N  K  K  L  I  L  T  F  A  A    13

181   CCTTAGTTTTAAGTGCTTGCGGAGGAAGCGGTAGCGGAGGTTCGTCTTCAACACCGAATC  240
14    T  L  V  L  S  A  C  G  G  S  G  S  G  G  S  S  S  T  P  N    33

241   ACCCCAAACCAGTACTAGTACCAAAAACACAAAATAATCTTCAAGCACAAAATGTTCCTC  300
34    H  P  K  P  V  L  V  P  K  T  Q  N  N  L  Q  A  Q  N  V  P    53

301   AGGCACAAAATGCCTCTCAGGCACAAAATGCCCCTCAGGCACAAAATGCTCCTCAGGCAC  360
54    Q  A  Q  N  A  S  Q  A  Q  N  A  P  Q  A  Q  N  A  P  Q  A    73

361   AAAATGCTCCTCAGGTGGAAAATGCTCCTCAGGCACAAAATGCTCCTCAGGTAGAAAATG  420
74    Q  N  A  P  Q  V  E  N  A  P  Q  A  Q  N  A  P  Q  V  E  N    93

421   CTCCTCAAGCAGAGGTTACTCCGCCTGTACCACAGCCACAATCACAAAAAATTGACGGTT  480
94    A  P  Q  A  E  V  T  P  P  V  P  Q  P  Q  S  Q  K  I  D  G   113

481   CTTTTGATAAAATTGGTTCAGTAAAACTCAATAAAGAGGCTCAAACTCTTGAGCTTAGTA  540
114   S  F  D  K  I  G  S  V  K  L  N  K  E  A  Q  T  L  E  L  S   133

541   GATTCACTTTGGTGGATAAATTAGGCACACCACCGAAGTTTGATAAAGTAAGCGGTAAAA  600
134   R  F  T  L  V  D  K  L  G  T  P  P  K  F  D  K  V  S  G  K   153

601   AAATTATTGAAGAAAAAGATTTTCTCGTATTAAATTTGTCTGATATTAATGCTGAACAAC  660
154   K  I  I  E  E  K  D  F  L  V  L  N  L  S  D  I  N  A  E  Q   173

661   TCTCTGGCGATTTTCTTATTCGCCGTAGCGATGATCTATTCTATGGCTACTATCACGATA  720
174   L  S  G  D  F  L  I  R  R  S  D  D  L  F  Y  G  Y  Y  H  D   193

721   CAAATGGCAAAAATCTTGTCGATGCTGCCGATAAATTCAGTCAATATTTTGTCGTGTATG  780
194   T  N  G  K  N  L  V  D  A  A  D  K  F  S  Q  Y  F  V  V  Y   213

781   ATGAGAAACGGGTAAATGATAATATCTCTGATAAATTAACAGCAACTTACCGTAAAAAAG  840
214   D  E  K  R  V  N  D  N  I  S  D  K  L  T  A  T  Y  R  K  K   233

841   AAGGCTTTGTATATGGTTCAAATCCACATACTAAAGAATTTGCCGCACGGATCAGCAAAT  900
234   E  G  F  V  Y  G  S  N  P  H  T  K  E  F  A  A  R  I  S  K   253

901   TGGGGGATGTAGAAATTAAATTTGAAAATGGTCAAGCTCAAGGAAGTATAAAAGACGAAA  960
254   L  G  D  V  E  I  K  F  E  N  G  Q  A  Q  G  S  I  K  D  E   273

961   AAGATGGAAATGCTGAGATCTTTACTATTAAAGGTGATACAAAACAGTTAGAGATTACCC 1020
274   K  D  G  N  A  E  I  F  T  I  K  G  D  T  K  Q  L  E  I  T   293

1021  CAACGGAAAGTAACCGAATCATTATAGCAATTTTAGACCAAAATCAAAAAAGCTATACTC 1080
394   P  T  E  S  N  R  I  I  I  A  I  L  D  Q  N  Q  K  S  Y  T   313

1081  CAGGAATGGAAAAAGCAATTATGGAAACTAAGTTTATTGATTCAAAGGCTGGTAATTCCG 1140
314   P  G  M  E  K  A  I  M  E  T  K  F  I  D  S  K  A  G  N  S   333

1141  ACCAAAAATACTTAATCGGTGAAGCAAAAAGCGATAACTGGCAAGCAATAATGGTTAGCG 1200
334   D  Q  K  Y  L  I  G  E  A  K  S  D  N  W  Q  A  I  M  V  S   353

1201  AGAAAAAATAAAGTTATCTTTTGCTAAAAACTGAAATAAAAAGGCTGAGTCCGGGTAATA 1260
354   E  K  K                                                      356

1261  TCGGCCTCAGTCTTTTAAATTGTAGAAAATCATCTGTAGAAGATCAAACC            1310
```

Fig. 13

Hydrophilicity plot of the deduced amino acid sequence of PlpE. The hydrophobic signal peptide and the hydrophilic, hexapeptide repeats are indicated. Positive values represent hydrophilic regions. The plot was generated using the Kyte-Doolittle algorithm in MacVector, with a window size of 7.

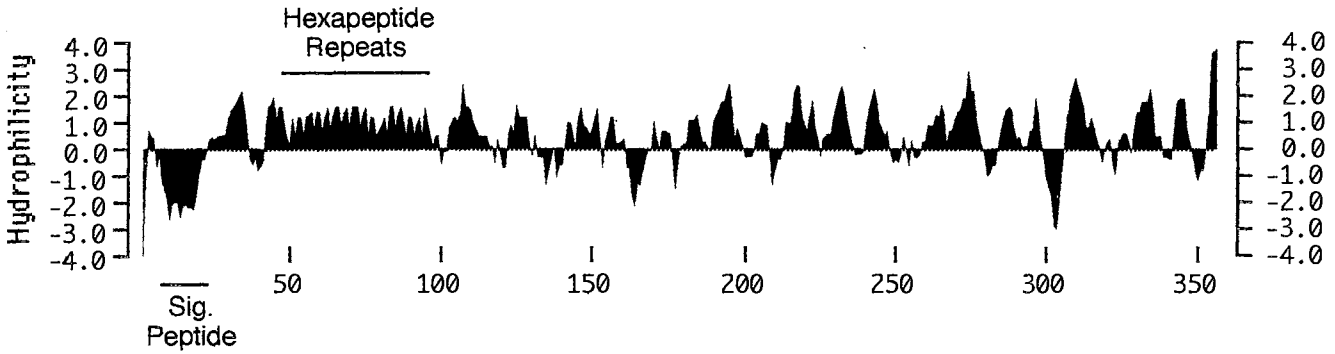

Hexapeptide
Repeats
Hydrophilicity
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
50
100
150
200
250
300
350
Sig.
Peptide

Fig. 14

Alignment of the deduced amino acid sequences of PlpE and OmlA proteins from *A. pleuropneumoniae* serotypes 1 (OmlA1) and 5 (OmlA5). The amino acids residues of OmlA1 and OmlA5 that are identical to PlpE have been capitalized.

```
        1              15 16              30 31              45 46              60 61              75 76              90
1 OmlA1 mniatKLmaslvAsv VLtACsGgGSsGSSS kPNseltpkVdmsap kaeqpkkeevpAdn skaeepkemAPQvd- sPkaEepknmapqmg     89
2 OmlA5 mniatKLiaglvAgL VLtACsGgGSsGSSp kPNsestpkVdmsap kaeqpkkeeApAds pkaekpksiAPlmme nPkVEkqkennlqek     90
3 PlpE  vkfnkKLiltfaAtL VLsACgGsGSgGSSS tPNhpkpvlVpktqn nlqaqnvpqAqAsq aqnapqaqnAPQaqn aPqVEnapqaqnapq     90

        91          105 106         120 121         135 136         150 151         165 166         180
1 OmlA1 npklndpqVmaPkmd nPQkdapKgeelskd ksnaeilKElgvkdi nSgiinnadvvlnlK iDekdhitvvldKgk inrNhlkvtntisaq    179
2 OmlA5 spkAdepqVmdPklg aPQkddQKleep-kn ksnaeilKElgikdi tSgtisisdielnlq lDsndnvKIsllnen LmrdnltINnkiags    179
3 PlpE  venApqaeVtpPvp- qPQ--sQKidgsfdk igsvklnKEaqtlel -Srftlvdklgtppk fDkvsgkKIieeKdf LvlNlsdINaeqlsg    176

        181         195 196         210 211         225 226         240 241         255 256         270
1 OmlA1 DiktlkdSsgkllgy YGYmqlnqvrqdeny sD-ekvslneYylls mNDadkirpTksisy KgdmfYsykdvgnqk lkAsveasyDdvtKk    268
2 OmlA5 DirtlkdSsgrllgy YGYvqlnqvtqdsrd pDnykhqFenhylls mNDaekilpeksley KgsmiYGyNtsgnek ltAevnakyDsstKk    269
3 PlpE  Dflirr-Sdd---lf YGYyhdtngknlvda aDkfsqyFvvYdekr vNDnisdklTatyrk KegfvYGsNp-htke faArisklgDveiKf    261

        271         285 286         300 301         315 316         330 331         345 346         360
1 OmlA1 vsmkvfGenndywKl Gefgrtn--llenQv tgakvgedgtIIngt Lyskidnfplkltpd anfsggIfgKnG--- -evLaGsAiSek--W    350
2 OmlA5 lsmkvyd-ndrywKl Gevmsnnvrlpeekv dgvkvdSdg-tInar Lylste-eplkltpd anfsggIfgKnG--- -evLaGkAeSikgeW    352
3 PlpE  engqaqG-sikdeKd Gnaeiftikgdtkql eitpteSnr-IIiai Ldqnqksytpgmeka imetkfIdsKaGnsd qkyLiGeAkSdn--W    347

        361         375
1 OmlA1 QgvIgatattkedKK    365
2 OmlA5 QgvIgatattkedKK    367
3 PlpE  Qa-Imvse-----KK    356
```

Fig. 15

Western immunoblot demonstrating the effect of mutagenesis of the GTG codon on production of PlpE. The blot was probed with affinity purified anti-45 kDa antibodies. Lanes: 1, *E. coli* (pB4522) with GTG and expressing PlpE; 2, *E. coli* (pB4522) clone in which GTG was changed to GGG by site-directed mutagenesis.

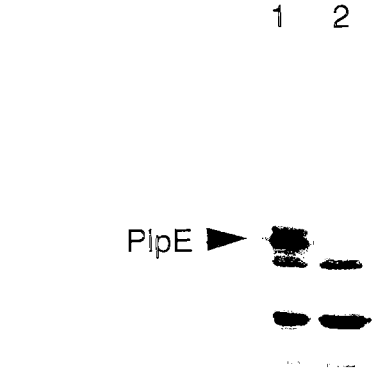

1 2
PlpE

Fig. 16

Autoradiograph of SDS-polyacrylamide gel of ^{3}H-palmitate-labeled total cellular proteins. Lanes: 1, *P. haemolytica;* 2, *E. coli* (pB4522) expressing PlpE; 3, *E. coli* (pBluescript SK-).

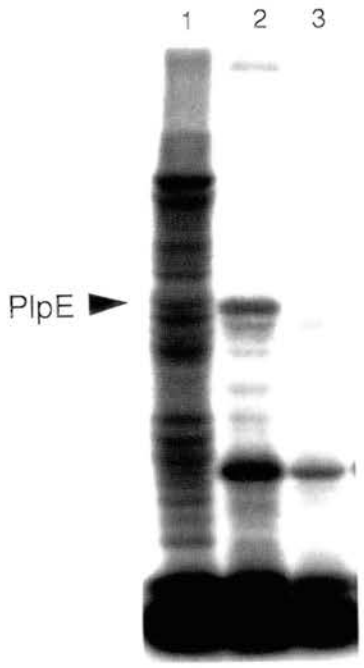

1
2
3
PlpE

Fig. 17

Western immunoblot of whole cell lysates of *P. haemolytica* serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, and an untypeable strain, probed with anti-45 kDa antibodies. The lane numbers represent the serotypes with UT representing the untypeable strain.

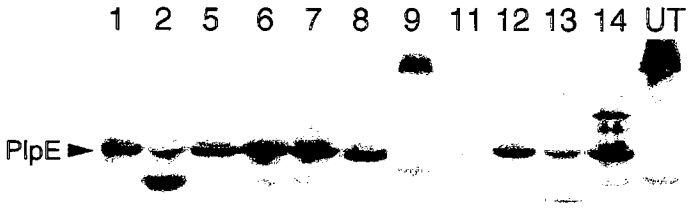
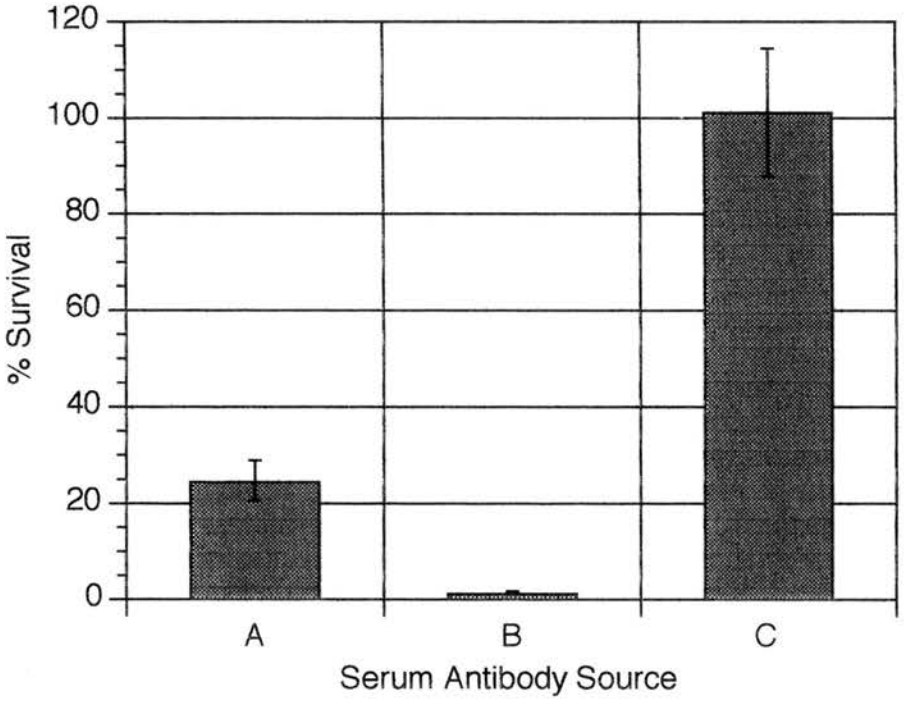

1 2 5 6 7 8 9 11 12 13 14 UT
PlpE

Fig. 18

Complement-mediated killing activity of anti-OMP bovine immune serum. Serum used as source of antibodies: A, bovine immune serum absorbed with *E. coli* (pB4522); B, bovine immune serum absorbed with non-recombinant *E. coli* (pBluescript SK-); C, complement source alone without anti-OMP serum. Values greater than 100% represent growth. Data shown are the mean and standard deviation of three replicates from a single experiment and are representative of the separate experiments. The differences between the means of A and B are statistically significant ($p<0.003$).


120
100
80
60
40
20
0
% Survival
A
B
C
Serum Antibody Source

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VITA

Karamjeet Pandher

Candidate for Degree of

Doctor of Philosophy

Thesis: MOLECULAR AND IMMUNOLOGICAL ANALYSES OF 38 kDA AND 45 kDA PROTEIN ANTIGENS OF *Pasteurella haemolytica* S1

Major Field: Veterinary Pathology

Biographical:

Personal Data: Born in Ludhiana, Punjab, India, On May 26, 1970

Education: Graduated from Sacred Heart Convent School, Ludhiana, Punjab in 1985; received Bachelor of Veterinary Science and Animal Husbandry from Punjab Agricultural University in 1992. Completed the requirements for the Doctor of Philosophy with a major in Veterinary Pathology at Oklahoma State University in May, 1998.

Experience: Did an Internship at College of Veterinary Sciences and at extension clinics run by the Government of Punjab, India, July to December 1992; Graduate Research Assistant in Dept. of Anatomy Pathology and Pharmacology, August 1993 to present.

Professional Memberships: American Society of Microbiology; New York, Phi Zeta, Nu Chapter, The Veterinary Medicine Honorary Society.