1986

Comparison between Mycoplasma bovoculi strains by electrophoretic and immunoblotting techniques: identification of membrane proteins involved in adherence

Barik A. Salih
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd
Part of the Microbiology Commons

Recommended Citation
https://lib.dr.iastate.edu/rtd/8297
INFORMATION TO USERS

While the most advanced technology has been used to photograph and reproduce this manuscript, the quality of the reproduction is heavily dependent upon the quality of the material submitted. For example:

- Manuscript pages may have indistinct print. In such cases, the best available copy has been filmed.

- Manuscripts may not always be complete. In such cases, a note will indicate that it is not possible to obtain missing pages.

- Copyrighted material may have been removed from the manuscript. In such cases, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, and charts) are photographed by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is also filmed as one exposure and is available, for an additional charge, as a standard 35mm slide or as a 17"x23" black and white photographic print.

Most photographs reproduce acceptably on positive microfilm or microfiche but lack the clarity on xerographic copies made from the microfilm. For an additional charge, 35mm slides of 6"x 9" black and white photographic prints are available for any photographs or illustrations that cannot be reproduced satisfactorily by xerography.
Salih, Barik A.

COMPARISON BETWEEN MYCOPLASMA BOVOCULI STRAINS BY ELECTROPHORETIC AND IMMUNOBLOTTING TECHNIQUES: IDENTIFICATION OF MEMBRANE PROTEINS INVOLVED IN ADHERENCE

Iowa State University

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark √.

1. Glossy photographs or pages √
2. Colored illustrations, paper or print
3. Photographs with dark background √
4. Illustrations are poor copy
5. Pages with black marks, not original copy
6. Print shows through as there is text on both sides of page
7. Indistinct, broken or small print on several pages √
8. Print exceeds margin requirements
9. Tightly bound copy with print lost in spine
10. Computer printout pages with indistinct print
11. Page(s) lacking when material received, and not available from school or author.
12. Page(s) seem to be missing in numbering only as text follows.
13. Two pages numbered. Text follows.
14. Curling and wrinkled pages
15. Dissertation contains pages with print at a slant, filmed as received
16. Other

University Microfilms International
Comparison between *Mycoplasma bovocull* strains by electrophoretic and immunoblotting techniques: Identification of membrane proteins involved in adherence

by

Barik A. Salih

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Interdepartmental Program: Immunobiology
Major: Immunobiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Professor-in-Charge Program of Immunobiology

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa
1986
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXPLANATION OF THESIS FORMAT</td>
<td>iv</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td><strong>PART I. COMPARISON BETWEEN MYCOPLASMA BOVOCULI STRAINS</strong></td>
<td>16</td>
</tr>
<tr>
<td>BY ELECTROPHORETIC AND IMMUNOBLOTTING TECHNIQUES:</td>
<td></td>
</tr>
<tr>
<td>IDENTIFICATION OF MEMBRANE PROTEINS</td>
<td></td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>18</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>19</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>20</td>
</tr>
<tr>
<td>RESULTS</td>
<td>25</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>36</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>39</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>40</td>
</tr>
<tr>
<td><strong>PART II. INTERACTIONS OF MYCOPLASMA BOVOCULI STRAINS</strong></td>
<td>43</td>
</tr>
<tr>
<td>WITH ERYTHROCYTES</td>
<td></td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>45</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>46</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>48</td>
</tr>
<tr>
<td>RESULTS</td>
<td>52</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>56</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>60</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>61</td>
</tr>
</tbody>
</table>
# PART III. IMMUNOGOLD LABELING OF *MYCOPLASMA BOVOCULI* MEMBRANE PROTEINS INVOLVED IN ADHERENCE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>65</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>67</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>68</td>
</tr>
<tr>
<td>RESULTS</td>
<td>72</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>94</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>97</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>98</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>100</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>103</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>111</td>
</tr>
</tbody>
</table>
EXPLANATION OF THESIS FORMAT

This thesis consists of a general introduction, literature review, three separate manuscripts, a list of literature cited, conclusions and acknowledgements. The doctoral candidate Barik Abdul-kadir Salih, is the senior author and principal investigator for each of the manuscripts.
GENERAL INTRODUCTION

Several species of mycoplasmas have been isolated from eyes of cattle with cases of infectious bovine keratoconjunctivitis (IBK). These included *Mycoplasma bovirhinis*, *Acholeplasma laidlawii*, *Mycoplasma verecundum*, *Mycoplasma bovoculi*, *Ureaplasma* spp. (recently designated *U. diversum*), *Mycoplasma bovigenitalium* and *Acholeplasma oculi* (22,24,41). Of these, *M. bovoculi* was the most frequently isolated, together with the gram negative bacterium *Moraxella bovis* (17,41,49,69).

*Mycoplasma bovoculi* was first isolated in Canada (49). Its role in IBK was characterized by Rosenbusch and Knudtson (69) and Rosenbusch (68). Ocular instillation of calves with *M. bovoculi* suspension resulted in conjunctivitis (69). Prolonged ocular colonization of *Moraxella bovis* and *Moraxella ovis* was detected when these organisms were inoculated following exposure to *M. bovoculi* (68,70). Studies on the immune responses of calves to *M. bovoculi* infection revealed a predominant IgA response in tears and IgG and IgM in serum (76). Only convalescent sera but not tears from these animals had metabolic inhibiting activity (76). In another experiment cattle previously exposed to *M. bovoculi* were found resistant to infection when rechallenged with the organism by ocular instillation. These animals had detectable levels of IgA in tears by ELISA which increased significantly following challenge (Salih et al., manuscript for publication). This might suggest that the presence of local antibodies plays a significant role in protection against
reinfection. Attempts to provide protection against *M. bovoculi* infection by parenteral vaccination were unsuccessful despite the induction of a strong serum antibody response (Salih et al., manuscript for publication).

Little is known about the mycoplasma membrane antigens involved in eliciting an antibody response to the organism and in mediating the attachment to host cells. The development of monoclonal antibodies facilitated the study of such antigens. Monoclonal antibodies prepared against a membrane surface protein (P1) isolated from a terminal bleb-like structure of *M. pneumoniae* inhibited the attachment of the organism to respiratory epithelium (13). Unlike *M. pneumoniae*, *M. bovoculi* and most other mycoplasmas do not possess a terminal bleb-like structure so antigens involved in adherence may be widely distributed around the membrane surfaces.

Variation in antigenic composition among mycoplasma species and subspecies, and in particular the composition of the membrane surface is well recognized (1,56,61). Both species specific and strain specific antigens have been characterized in studies done on *M. pulmonis* and *M. arginini* (1,61).

The objectives of these studies were to examine and compare the protein profiles of *M. bovoculi* strains by SDS-polyacrylamide gel electrophoresis, determine the antigenic activities of these proteins by western blots and apply monoclonal antibodies for identification and characterization of membrane antigens shared among the different strains of *M. bovoculi*. 
An adherence assay was developed to determine if the identified membrane antigens were involved in attachment and to study the role of monoclonal antibodies and other inhibitors in blocking and characterizing the receptors involved in attachment. Interactions with erythrocytes were further used for comparison between *M. bovoculi* strains using hemagglutination, hemadsorption and hemolysis tests. Determination of capsule formation *in vivo* and *in vitro* was assessed by electron microscopy. Confirmation of membrane surface location of antigens involved in adherence was done by immunogold labeling and electron microscopy. Monoclonal antibodies or Protein A tagged with colloidal gold particles were used for this purpose.
Mycobacteria membrane proteins

Mycobacteria membrane proteins comprise about two-thirds of the total membrane mass by dry weight (2). These constitute enzymes, transport systems and antigenic proteins. As is the case for other membrane proteins, mycobacteria membrane proteins are of two kinds, peripheral proteins and integral proteins. The former are not tightly bound to the membrane and can be detached by treatment with EDTA or by changing the ionic strength of the pH of the medium. The integral proteins are more intimately associated with membrane lipids, most probably by combination of hydrophobic and ionic bonds and can only be solubilized effectively by detergents or organic solvents (63). Localization of surface proteins has been carried out using a variety of techniques such as lactoperoxidase-mediated iodination (2), crossed immunoelectrophoresis (35), freeze-fracturing (75), enzymatic digestion (15) and immunocytochemistry (72). Asymmetrical distribution of membrane proteins was demonstrated from the results obtained from the iodination technique where more proteins were found exposed on the inner surface than on the outer surface of the mycoplasma membrane (2). Similar observations were reported using crossed immunoelectrophoresis analysis of erythrocyte membranes (35).

Cell protein analysis of mycoplasma, acholeplasma, ureaplasma and spiroplasma species by two dimensional electrophoresis revealed a wide variation in the number of protein spots detected. Between 150 to 350 protein spots were detected when these species were compared by the above
method (67). These numbers are small when compared to those in eu-
bacteria where up to 1100 protein spots were detected (40). Of the 320
individual protein spots of *A. laidlawii* detected by two-dimensional
electrophoresis, 140 were associated with the plasma membrane (63).
However, each spot may not represent an individual protein since membrane
proteins exist in complex forms that tend to dissociate into subunits
during solubilization. According to Razin (63), membrane proteins
isolated from mycoplasma species have a molecular weight range from
15,000 to over 200,000, which is similar to the range seen for proteins
from other biological membranes (27).

Media contamination of mycoplasma membranes is a major problem in
antigen preparation. Medium containing high percentages of serum, yeast
extract and peptone, and small yield of mycoplasmas in such medium were
factors attributed to the problem (42). Growth medium composition and
the harvesting stage of the growth cycle had a profound effect on the
expression of antigenic determinants on the membrane surface of *A.
laidlawii*. Growth of the organism in medium containing unsaturated fatty
acids resulted in release of membrane proteins into the medium. In these
experiments, cells harvested at early growth phase exhibited greater
expression of antigenic determinants on the surface membrane than at later
stages as detected by immunoabsorption and surface iodination (36). The
use of a dialysate medium prepared from soy peptone and fresh yeast allevi­
viates the problem of medium contamination for many mycoplasmas (42).

One of the advantages of working with mycoplasmas is that they lack
cell wall. Investigators were encouraged by the fact that isolated
membranes contain few cellular contaminants since the organisms have no intracellular membranous structure (66). Lack of cell wall and internal membranous structures made the isolation of mycoplasma membranes much less complicated than for other bacteria. Several techniques were employed for this purpose. Osmotic lysis is the simplest and involves the transfer of the organism from high tonicity medium to low tonicity medium. Preloading of the organism with glycerol enhances susceptibility to lysis by increasing osmotic pressure in the cell (63). Aging of the culture and the presence of even traces of divalent cations reduces sensitivity to osmotic lysis (63). The resistance of some mycoplasmas (e.g., *M. gallisepticum*) to osmotic lysis was attributed to the presence of disaturated phospholipids in the membrane which induce the formation of segregated lipid domains and thus provides sites for increased ion permeability. This in turn reduces the internal pressure and minimizes cell swelling and lysis (75). Mycoplasmas resistant to osmotic lysis might be solubilized by digitonin treatment which binds cholesterol found in the membranes of sterol-requiring species. Digitonin forms a complex with cholesterol which results in rearrangement of the lipid bilayer towards an increased permeability and subsequent cell lysis (74). Aging and the presence of divalent cations has no effect on the sensitivity of mycoplasma to digitonin lysis. Isolated membranes obtained by this method retain the digitonin-cholesterol complex and this alters the membrane composition, structure and molecular weight of individual components (63). Other techniques reflect more serious drawbacks. Lysis at pH 10 or higher may cause dissociation of some membrane proteins.
Freezing and thawing may fail to lyse the bulk of the cells. Mechanical disruption of cells fragments the membrane into minute particles that can not be sedimented even at high gravitational forces (63). Recently, a new approach was described for the isolation of mycoplasma membranes from osmotically resistant organisms by the use of ATPase inhibitor (77). This enzyme is tightly associated with the mycoplasma membrane unlike other prokaryotic ATPases (64) and functions in the regulation of cell volume. Mycoplasma gallisepticum treated with dicyclohexylcarbodiimide in an isoosmotic NaCl solution were lysed as a result of inhibition of ATPase function (77).

Of the membrane proteins that have been isolated from mycoplasma species, PI protein isolated from M. pneumoniae was the most significant. Extensive studies have been conducted utilizing this protein which in turn made a great contribution to the understanding of its role in adherence and pathogenicity. This protein is located in a dense area at the tip-like structure found in one end of the organism (39). The protein was also found less frequently dispersed along the rest of the surface membrane (5). Other mycoplasma membrane proteins purified and described were reviewed by Razin (63). These include the spiralin protein isolated from Spiroplasma citri, and D_{12}, T_2, T_3, T_4a, and T_4b proteins of Acholeplasma laidlawii. The spiralin constitutes 22% of the total membrane protein fraction and is considered the most abundant protein in S. citri membranes. Its function is still unclear but its role in cell shape determination was suggested (85).

Recently it was found that D_{12} protein of A. laidlawii expresses
antigenic determinants on both the external and the internal surfaces of the membrane. Antigenic determinants of $T_2$ protein were expressed on the external surface while those of $T_{4a}$ and $T_{4b}$ proteins were on the internal surface. $T_3$ protein expressed no antigenic determinants on either surface (36). An actin-like protein has been extracted from M. pneumoniae by alkaline treatment (60) in analogy to rabbit muscle F-actin (54). In another study on Spiroplasma citri an actin-like protein was also described using anti-actin antibodies coupled to horseradish peroxidase and was suggested to be involved in mediating motility (83). Wise and Watson (84) described a surface protein P120 from M. hyorhinis that was expressed during colonization of murine T-lymphoblastoid cells. Metabolic inhibition proteins of M. arthritidis were characterized using monoclonal antibodies and the results obtained showed the involvement of more than one antigen that can elicit antibodies that inhibit metabolism (82). Unlike eukaryotes, membrane glycoproteins are rare in prokaryotes. Despite this fact investigators continued the search for such proteins. According to Razin (63) a glycoprotein was isolated from M. pneumoniae membrane that contains 7% carbohydrate. It is located on the external surface of the membrane as determined by lactoperoxidase iodination and was found to be involved in attachment to epithelial cells (37).

Membrane bound enzymes were extensively studied in Acholeplasma laidlawii. Of those characterized some were integral membrane proteins such as ATPase, P-nitrophenyl phosphatase, peptidase, NADH oxidase, lysophospholipase, acyl-CoA thioesterase, and others were mostly peripheral such as ribonuclease and deoxyribonuclease (63). Membrane
Localization of the electron transport system is well documented in prokaryotes (63). This system was found in the soluble fractions of the cytoplasm rather than the membrane in mycoplasmas. The flavin-terminated respiratory system of mycoplasmas lacks both quinones and cytochromes. Since quinones and cytochromes in other bacteria are membrane associated, this may explain why mycoplasmas have a free electron transport system in the cytoplasm that is independent of membrane structures required for organization. The NADH oxidase enzyme is membrane-bound in acholeplasma but not in mycoplasma or ureaplasma (63).

**Adherence properties of mycoplasmas**

Mycoplasmas are the smallest free-living organisms that are able to exist independently in nature. There are over 70 species of mycoplasmas that were identified and characterized as members of the class mollicutes. More than half of these species were associated with disease conditions. Generally they colonize the mucosal surfaces of their host. Studies conducted to examine the adherence properties of these organisms utilized tissue culture cells, organ culture cells and live animals. Several species of mycoplasmas were studied with more emphasis on *M. pneumoniae*, an organism that possesses a tip-like terminal structure that is involved in adherence.

The fact that in mycoplasmal respiratory infections the organism first comes in contact with a mucus layer, led researchers to examine this mechanism *in vitro* by adherence to inert surfaces such as glass or plastic. It was found that attachment of *M. pneumoniae* to glass is an
energy requiring process and inhibitors of energy metabolism or glucose analogues reduced attachment (8). Trypsin treatment or scraping of the adhered organisms resulted in detachment while adding EDTA had no effect suggesting no role for divalent cations (13). Attachment of \textit{M. pneumoniae} to host cells is mediated by the PI protein found in the terminal tip-like structure. Monoclonal antibodies developed against PI protein blocked attachment (13). Adherence to host cells was energy-independent since nonviable organisms and isolated membranes can adhere similarly. These observations were done studying \textit{M. pneumoniae}, \textit{M. gallisepticum} and \textit{M. pulmonis} attachment to erythrocytes (4,38). However, attachment of \textit{M. pneumoniae} to hamster tracheal organ cultures required viable organisms. The fact that tracheal epithelium cells are covered with densely packed cilia and a mucus layer may suggest that a motile organism is required for adherence (19).

Host cell receptor sites for mycoplasmas varied in chemical nature. Some were neuraminidase sensitive, others were protease sensitive and still others had undefined chemical natures (8). In several mycoplasmas, the binding sites were found to be proteinaceous (e.g., \textit{M. dispar}, \textit{M. pneumoniae} and \textit{M. gallisepticum}) (15,30,38), however, binding sites on some mycoplasmas were protease insensitive (e.g., \textit{M. pulmonis}) (57). It was suggested that these sites were either nonprotein in nature or proteins that resist proteolytic digestion (38). Great variability among erythrocytes receptor sites involved in attachment to \textit{M. pneumoniae} was shown. Neuraminidase-sensitive receptors were described for sheep erythrocytes that bound surface protein on the organism, while receptors on
human erythrocytes were partially sensitive to neuraminidase treatment and those on rabbit erythrocytes were insensitive. This indicated that receptors other than sialic acid moieties play a role in this process (15). Similarly, neither neuraminidase nor proteolytic treatment of erythrocytes had any effect on attachment of *M. pulmonis* (38). The role of sialic acid in attachment of mycoplasma to cultured cells was studied. *M. pneumoniae* attachment to human lung fibroblasts was reduced following the removal of sialic acid from the later tissue (18). It was suggested that two steps are involved in the attachment of mycoplasmas to erythrocytes when receptors in addition to sialoglycoprotein participated. In the first step, the organism attached to sialic acid receptors, this is followed by the 2nd step where other linkages occur possibly via other carbohydrate, proteins or membrane lipids. This was demonstrated in experiments where glycophorin addition prior or with the addition of erythrocytes inhibited attachment. However, addition of glycophorin following attachment detached only 10% of the mycoplasmas from erythrocytes (38).

The distribution of the receptor binding sites on the mycoplasma was first studied on *M. pneumoniae*. The P1 protein was found densely clustered at the tip-like structure (16). Although the P1 protein was also shown to be distributed in less dense foci along the membrane surface of the organism (5), it appears that *M. pneumoniae* utilize P1 protein located at the tip structure primarily for attachment to ciliated respiratory epithelium of hamster tracheal organ culture and other host cells as shown by electron microscopy (13). Membrane receptors of *M.*
gallisepticum involved in attachment to human erythrocytes were found to be unequally distributed among cells within a given mycoplasma population. Results of a study have shown that unattached M. gallisepticum following first exposure to erythrocytes when reincubated with erythrocytes for the second time had very low attachment capacity (3). It was suggested that there are two different mycoplasma populations in a suspension, one with higher attachment avidity to erythrocytes than the other. It is also possible that the nonadherent mycoplasmas possess fewer binding sites or the distribution of these sites on the cell surface is not optimal (4).

Electron microscopic studies showed no real linkage between the attached mycoplasmas and the host cells. Electrostatic bonds are more likely to be involved although other bonds might also participate. This was deduced from experiments where increases in ionic strength or pH of the attachment mixture decreased M. gallisepticum attachment to human erythrocytes (3). In contrast to these findings, increased ionic strength was found to increase attachment of M. pneumoniae to sheep erythrocytes (15). Differences in the experimental approach and in the species of mycoplasma and erythrocytes used were attributed as a cause for this discrepancy. Another condition that affects attachment is temperature. Incubation of the attachment mixtures at temperatures below 37°C resulted in less attached mycoplasmas. Longer incubations at 37°C increased attachment and reached maximum after 35 min. This did not change significantly following extended incubation times (3).
Hemagglutination, hemadsorption and hemolysis

These three phenomena involve the interactions between mycoplasmas and erythrocytes. They have been utilized earlier for identification and characterization of different mycoplasmas. Hemagglutination tests revealed strain variation among *M. dispar* strains and some of those strains were differentiated by the erythrocyte species agglutinated (30). The correlation between these three properties is still unclear, although earlier assumptions considered hemadsorption and hemagglutination to be a similar property (20). These studies showed that some mycoplasma strains are capable of one activity but not the other.

In addition, receptor sites involved varied among the mycoplasma and erythrocyte species used. Sialic acid receptors found on erythrocytes were the attachment site for some mycoplasmas (53), but were not involved in attachment of *M. dispar* (30). Trypsin treatment of erythrocytes was required for eliciting hemagglutinating property. This was found in a recent study when *M. pulmonis* did not agglutinate untreated erythrocytes (57). The description of a cryptic receptor on the erythrocytes was suggested in that study. Trypsin treatment of *M. dispar* reduced hemagglutination titers for bovine and sheep erythrocytes but not for rabbit erythrocytes (30). Earlier work distinguishing *M. pneumoniae* from other human mycoplasmas utilized the hemadsorption activity. However, later work with other mycoplasmas found that hemadsorbing properties were varied when using erythrocytes from different species (20). The involvement of sialic acid residues on the erythrocytes in binding to some mycoplasmas but not to others in this test was also reported (53).
Hemolysin activities of several species and strains of mycoplasmas were studied using erythrocytes from guinea pig, sheep, rabbit, duck and chicken. All mycoplasmas tested possess such activity. Susceptibility of erythrocytes to lysis varied however, where guinea pig erythrocytes were the most susceptible while rabbit erythrocytes were the least susceptible (11).

Colonies of *M. pneumoniae* and *A. laidlawii* produced complete (8) hemolysis when incubated with guinea pig erythrocytes (81). Results of experiments which attempted to characterize the mycoplasma hemolysin showed that colonies that were rendered nonviable by ultraviolet irradiation or heating at 56°C did not produce hemolysis. Reduced oxygen tension and incubation with an old culture of mycoplasma suppressed the hemolytic activity (81). These results indicated at least that the hemolysin is not protein in nature. Further characterization of the hemolysin was done by incubating the erythrocyte-agar overlay with catalase or peroxidase. No hemolysis was seen and the specificity of inhibition was confirmed by heating the enzymes before addition to the mixture which abolished their effect (78). These observations indicated that the hemolysin of *M. pneumoniae* and *A. laidlawii* is a soluble factor (peroxide) that is produced and released in the environment. When the hemolysin of several other species of mycoplasmas were examined, they were also found to be peroxides (11). It was concluded that mycoplasma hemolysin is unique to these organisms since other bacterial hemolysins described were either protein or lipid in nature. Recently, a different mechanism for hemolytic activity of *M. pulmonis* was described. Intimate contact between *M.*
pulmonis and trypsin treated erythrocytes was required to exert lysis (56). A protein nature for the hemolysin was proposed since trypsin treatment of the mycoplasma abolished hemolysis.

Immunocytochemistry

Antigenic localization using immunoglobulins, lectins and enzymes has been successfully applied in electron microscopic studies (6). Since its introduction into the field of immunocytochemistry, protein A tagged with different markers (peroxidase, ferritin, gold particles) has been used to improve the ability to localize antigenic sites at the light and electron microscopic level (6). Protein A is a cell wall protein produced by most strains of *Staphylococcus aureus*. It consists of a single polypeptide of 42,000 molecular weight that has the ability to bind to the Fc region of IgG antibodies from several animal species (71). Colloidal gold is a negatively charged hydrophobic sol which under appropriate conditions binds to macromolecules by noncovalent electrostatic adsorption (6). The tagged gold particles remain stable and had no effect on the biological activity of the molecule. Another advantage of the use of gold particle is that the size of the particle can be adjusted as desired. This can help in localization of several antigenic sites by double or triple labeling techniques (28,72).
PART I. COMPARISON BETWEEN MYCOPLASMA BOVOCULI STRAINS BY ELECTROPHORETIC AND IMMUNOBLOTTING TECHNIQUES: IDENTIFICATION OF MEMBRANE PROTEINS

This manuscript has been submitted for publication to Infection and Immunity.
COMPARISON BETWEEN *MYCOPLASMA BOVOCULI* STRAINS
BY ELECTROPHORETIC AND IMMUNOBLOTTING TECHNIQUES:
IDENTIFICATION OF MEMBRANE PROTEINS

BARIK A. SALIH
RICARDO F. ROSENBUSCH

From the Veterinary Medical Research Institute, Iowa State University,
Ames, Iowa 50011.
ABSTRACT

Six isolates of *Mycoplasma bovoculi* from cattle herds with infectious bovine keratoconjunctivitis were analyzed by gel electrophoresis and immunoblotting techniques. All six strains showed similarity in their protein profiles although no two patterns were identical. A monoclonal antibody, hyperimmune rabbit serum, and post-exposure sera from three mycoplasma-free calves experimentally infected with one strain were used for immunoblotting analysis. Monoclonal antibody recognized a band of 94,000 molecular-weight designated p94 common to all strains. This p94 was recognized by post-exposure antisera from all three calves. Hyperimmune rabbit serum detected antigenic differences between these strains ranging from 15k-136k. Trypsin treatment of intact mycoplasma cells resulted in the removal of p94 when tested with monoclonal antibody and calf sera. Trypsin treated cells reacted with hyperimmune rabbit serum revealed the presence of strain-specific and conserved antigens between these strains. These studies identify the presence of a conserved trypsin-sensitive surface protein (p94) of *M. bovoculi* involved in eliciting antibody response in the infected host.
INTRODUCTION

Mycoplasma protein analysis using polyacrylamide gel electrophoresis has been used earlier for taxonomic purposes (20,22). More recently, immunoblotting techniques of separated proteins were used for identification and characterization of membrane proteins (9,16,26). The extensive studies conducted following the identification of P1 protein of M. pneumoniae on a bleb-like terminus structure on the organism have provided a significant amount of information for understanding the role of this surface protein in pathogenesis (2,5,6,7,11). Little is known about other mycoplasmas which lack such surface protein. Antigenic variations among species and strains within a species of mycoplasmas particularly with regard to membrane surface antigens has been reported (1,16,19). In this study, several strains of Mycoplasma bovoculi, a bovine pathogen frequently found associated with cases of infectious bovine keratoconjunctivitis (24) were compared by SDS-PAGE and immunoblotting techniques. We describe the identification of a conserved surface antigen by immunoblot detection with monoclonal antibody, post-exposure calf sera and hyperimmune rabbit serum, and the presence of several other strain-specific and conserved antigens detected by the above sera.
MATERIALS AND METHODS

**Mycoplasma** Six strains of *Mycoplasma bovoculi* were compared in this study (FS8-7, Cs101, Cs239, C181, Cs117, M165/69). The first five strains were isolated from the eyes of cattle with infectious bovine keratoconjunctivitis (IBK) from several farms in Iowa. The 6th strain was obtained from the American Type Culture Collection and was isolated in Canada (13). All strains were cloned once by filtering through a sterile 0.45 μm Millipore membrane (Millipore Corp., Bedford, Mass.) and plating onto modified Friis agar (10). Single colonies were isolated, grown in modified Friis broth (10) then adapted to dialysate medium (8) with 10% fetal calf serum (FCS) (GIBCO laboratories, Grand Island, N.Y.) and used at the 9th or 10th subculture.

**Membrane preparation** *Mycoplasma bovoculi* grown in dialysate medium containing 10% FCS for 24 h at 37°C was washed twice with phosphate buffer saline (PBS) (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2) and resuspended to contain 5 mg cell protein per ml (14). Since preliminary trials showed that *M. bovoculi* cells were sensitive to hypotonic lysis, membranes from the six strains of *M. bovoculi* were isolated by glycerol-assisted lysis as previously described (23). Briefly, mycoplasmas grown and washed as above were resuspended in 2 ml warmed (37°C) 2 M glycerol and incubated for 15 min at 37°C. The suspension was loaded into a 5 ml syringe and injected rapidly into 250 ml of prewarmed deionized water and incubated for 10 min at 37°C. Lysed cells were sedimented by centrifugation (48,000 X g for 30 min) then washed three times alternately with 25 ml of cold (4°C) deionized
water, 25 ml of cold 0.05 M NaCl in 0.01 M phosphate buffer pH 7.5 and finally with 25 ml of cold deionized water. The pellet was resuspended to the original volume with 1:20 dilution of beta-buffer (0.15 M NaCl, 0.05 M Tris-HCl and 0.01 M 2-mercaptoethanol pH 7.4). The suspension was layered on a 30-60% sucrose gradient and centrifuged at 27,000 X g for 30 min in a SW65 rotor. The layer formed at 2/3 from the top was collected, resuspended in beta-buffer and centrifuged at 27,000 X g for 1 h in a 50Ti rotor. Finally, the pellet was resuspended in beta-buffer and frozen at -70°C. Frozen M. bovoculi membranes isolated by osmotic lysis were thawed and centrifuged in a microcentrifuge (13,500 X g for 5 min). Pellets were resuspended in 1 ml of 0.02 M MgCl₂ containing 10 μg bovine pancreatic deoxyribonuclease per ml (Sigma Chemical Co., St. Louis, Mo.) and incubated for 15 min at 37°C with intermittent shaking. The suspension was centrifuged as above, washed alternately with PBS and deionized water, then frozen at -70°C.

Preparation of hyperimmune serum Mycoplasma bovoculi strain FS8-7 grown in dialysate medium containing 10% rabbit serum (Dutchland Laboratories, Inc., Denver, Penn.) for 24 h at 37°C was washed twice with PBS and resuspended in Tris-Tricine buffer pH 8.6 containing 0.5% Triton X-100. The suspension was mixed with equal volume of Freund's complete adjuvant. One ml of this suspension was injected into a rabbit intramuscularly and boosted twice at 10 day intervals. The animal was bled 11 days after the third injection. The serum collected was treated with 50% saturated ammonium sulphate pH 7.8 and the precipitated globulins were dialyzed against Tris-Tricine buffer pH 8.6 with 0.85% NaCl for 72 hr. The dialyzed
fluid was then stored at 4°C.

**Calf sera**  Sera from three mycoplasma-free calves experimentally exposed to *M. bovoculi* strain FS8-7 by ocular inoculation were collected four weeks after exposure. Sera were heat-inactivated at 56°C for 30 min and stored at -20°C.

**Monoclonal antibodies**  Hybridomas and mouse monoclonal antibodies were developed in our laboratory as described previously (Wannemuehler and Rosenbusch, manuscript for publication). Briefly, hybridomas were made by fusing spleen cells from a BALB/c mouse (injected with *M. bovoculi* FS8-7 strain) with myeloma cells SP2/0. One monoclonal antibody (M25.5) that recognized a protein of 94,000 molecular-weight in western blots of strain FS8-7 was used.

**Trypsin treatment**  Six strains of *M. bovoculi* were grown and washed as mentioned above. Trypsin (Difco Laboratories, Detroit, Mich.) at 100 µg per ml was added and the mycoplasma cells were incubated for 1 h at 37°C. The cells were then centrifuged in a microcentrifuge for 5 min, washed once with PBS and used for SDS-PAGE (4).

**SDS-Polyacrylamide gel electrophoresis**  Discontinuous SDS-PAGE was performed as described previously (12) using a 10% separating gel and 4% stacking gel. Whole cells and membrane preparations of the 6 *M. bovoculi* strains were treated with 2X treatment buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), boiled for 3 min and 0.01% bromophenol blue was added. A 50 µl sample containing 10-20 µg of protein was placed in each slot and electrophoresed in a vertical slab electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.)
at a current of 40 mA per gel. The run was stopped when the tracking dye reached the end of the separating gel and the gel was stained with Coomassie brilliant blue dye. A set of known molecular weight standards (Pharmacia Fine Chemicals, Piscataway, N.J.) was used to estimate the molecular weights of the separated protein bands.

Electrophoretic blots The western blot technique described previously (25) was applied to transfer proteins separated on SDS-PAGE gels to nitrocellulose sheets in an electrophoretic transfer apparatus (Bio-Rad). The gels received three rinses of 10 min in transfer buffer (containing 25 mM sodium phosphate buffer, pH 6.8, 20% methanol) before transfer. Transfer was performed at 20 mA overnight. Strips of nitrocellulose were cut at widths of 0.5 cm and placed in tubes containing 10 ml washing buffer (0.15 NaCl in 0.01 M Tris buffer pH 8.6, 0.05% Tween 20). Strips were washed 3 times for a total of 30 min with continuous shaking. Blocking was performed by adding 8 ml of washing buffer with 1% gelatin. Hyperimmune rabbit anti-M. bovoculi (1%) was incubated for 2 h with shaking, then washed 3 times. This was followed by the addition of $^{125}$I-Protein A (1 μCi/ml) (Amersham Corp., Arlington Heights, Ill.). After a 1 hr incubation, the tubes were washed as above. Strips tested with calf sera (10%) were first treated with these sera for 2 h, washed and then a secondary unlabeled goat anti-bovine IgG (0.5%) (Nordic Laboratories, ElToro, Calif.) was added for 1 h. After washing, $^{125}$I-Protein A (1 μCi/ml) was added for 1 h and washed. Strips tested with monoclonal antibodies (10%) were treated for 2 h then labeled with $^{125}$I-rabbit anti-mouse antibody (1 μCi/ml) (Amersham) for 1 h followed by washes. Autoradiography was
done using Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) at 
-70°C.
RESULTS

Membrane and intact cell profiles Figure 1 shows the protein profiles of isolated membranes as compared to those of intact cells of the six M. bovoculi strains. No differences were seen between profiles obtained with extracts of membranes and cells. All six strains showed similarity although no two patterns were identical. Between 40 and 45 bands ranging from 15k to over 220k were separated on the gel.

Immunoblot analysis Hyperimmune rabbit serum developed against strain FS8-7 detected antigenic differences between the six strains of M. bovoculi ranging from 15k to 136k (Fig. 2). The hyperimmune serum detected 22 bands of strain FS8-7, 17 bands of strain M165/69, 18 bands of strain Cs101, 15 bands of strain Cs239, 15 bands of strain C181 and 17 bands of strain Cs117. The major differences in recognition were seen in 43k to 51k and 60k to 136k protein bands. Calf antisera to strain FS8-7 also varied in its recognition patterns. As shown in Table 1, serum from calf 91 detected 5 bands of strain FS8-7, 3 bands of strains M165/69 and C181, 2 bands of strains Cs101 and Cs239 and one band of strain Cs117. Calf 108 recognized 7 bands of strain FS8-7, no bands of strain M165/69, 6 bands of strain Cs101, 4 bands of strain Cs239, 2 bands of strain C181 and one band of strain Cs117. Serum from calf 84 detected 3 bands of strain FS8-7 and Cs101 and one band of strain Cs239. No bands were detected from the other strains. Of the common protein bands detected by these sera, 94k appeared to be prominent. The band was detected in all 6 M. bovoculi strains by calf 91, in 5 strains by calf 108 and in 3 strains by calf 84.
FIG. 1. SDS-PAGE profiles of intact cells (X) and isolated membranes (X') of *M. bovoculi* strains. Strain FS8-7 (A,A'); strain ML65/69 (B,B'); strain Cs101 (C,C'); strain Cs239 (D,D'); strain Cl81 (E,E'); strain Cs117 (F,F'). Molecular weight standards are shown on the left.
FIG. 2. Immunoblot of *M. bovoculi* proteins separated on a 10% SDS-gel, transferred to nitrocellulose filters, reacted with hyper-immune rabbit anti-FS8-7 antiserum and $^{125}$I-labeled Protein A. Strain FS8-7 (lane A); strain M165/69 (lane B); strain Cs101 (lane C); strain Cs239 (lane D); strain C181 (lane E); strain Cs117 (lane F). Molecular weight standards are shown on the left.
Table 1. Molecular weights of protein bands of *M. bovoculi* strains recognized by sera from mycoplasma-free calves experimentally exposed to strain FS8-7a

<table>
<thead>
<tr>
<th>Calf</th>
<th>Mycoplasma strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FS8-7</td>
</tr>
<tr>
<td>91</td>
<td>118k</td>
</tr>
<tr>
<td>94k</td>
<td>94k</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>83k</td>
<td>83k</td>
</tr>
<tr>
<td>78k</td>
<td>-</td>
</tr>
<tr>
<td>68k</td>
<td>68k</td>
</tr>
<tr>
<td>108</td>
<td>118k</td>
</tr>
<tr>
<td>106k</td>
<td>-</td>
</tr>
<tr>
<td>94k</td>
<td>-</td>
</tr>
<tr>
<td>83k</td>
<td>-</td>
</tr>
<tr>
<td>68k</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>50k</td>
<td>-</td>
</tr>
<tr>
<td>43k</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>84</td>
<td>118k</td>
</tr>
<tr>
<td>94k</td>
<td>-</td>
</tr>
<tr>
<td>43k</td>
<td>-</td>
</tr>
</tbody>
</table>

aData obtained from immunoblot analysis of *M. bovoculi* proteins separated on 10% SDS-PAGE, transferred to nitrocellulose filters then reacted with unlabeled goat anti-bovine IgG and ^125I^-labeled Protein A. Molecular weights were estimated using molecular weight standards.

Another common band was one of 118k, which was recognized by all 3 calf sera but not in all strains. All protein bands detected with calf sera were also detected by rabbit serum. Monoclonal antibody M25.5 developed against strain FS8-7 recognized this 94k protein band (designated p94) in all
6 strains (Fig. 3). To test the possibility of growth medium contamination of these strains, dialysate medium with 10% FCS was prepared for SDS-PAGE and transferred to nitrocellulose. When these transfers were reacted with hyperimmune rabbit serum no bands were detected even when growth medium protein concentrations were used in excess of the amount used for mycoplasma, indicating that these protein bands were not of growth medium origin.

**Trypsin sensitive antigen common to M. bovoculi strains**  
Trypsin treatment of intact M. bovoculi cells resulted in the loss of p94 from SDS-PAGE gels (data not shown), suggesting that this protein has an external surface location. Similarly, monoclonal antibody M25.5 reacted with no bands on electroblots from trypsin treated cells, which further confirms the above results. When electroblots from trypsin treated cells of strains FS8-7, M165/69, Cs101, Cs239 were reacted with hyperimmune rabbit serum, loss of p94 was evident. It could also be shown that these strains share 5 major trypsin insensitive bands of 91k, 81k, 64k, 34k and 29k and two minor bands of 106k and 14k which were less intensely stained (Fig. 4).
FIG. 3. Immunoblot of *M. bovoculi* proteins separated on a 10% SDS-gel, transferred to nitrocellulose filters, reacted with anti-FS8-7 monoclonal antibody M25.5 and ^125^I-labeled rabbit anti-mouse IgG. Strain FS8-7 (lane A); strain M165/69 (lane B); strain Cs101 (lane C); strain Cs239 (lane D); strain Cl81 (lane E); strain Cs117 (lane F)
FIG. 4. Immunoblot of trypsin-treated *M. bovoculi* proteins separated on a 10% SDS-gel, transferred to nitrocellulose filters, reacted with hyperimmune rabbit anti-FS8-7 antiserum and \(^{125}\text{I}\)-labeled Protein A. Strain FS8-7 (lane A); strain M165/69 (lane B); strain Cs101 (lane C); strain Cs239 (lane D). Molecular weight standards are shown on the left.
DISCUSSION

Although several methods had been described for the isolation of mycoplasma membranes, glycerol-assisted osmotic lysis is commonly used since it provides membrane preparations free of detergent contamination (21). Membrane purity was judged by SDS-PAGE following centrifugation of the isolated membranes on sucrose density gradients and treatment with deoxyribonuclease. Inability to remove unlysed cells was attributed to aggregate formation, and centrifugation of the suspension at a low g force did not eliminate this problem. Since SDS-PAGE profiles of isolated membranes and whole cell preparations were identical, presence of a protein band in SDS-PAGE profiles from isolated membranes could not be taken as the sole criterion for membrane localization of the protein.

The one-dimensional gel electrophoresis technique applied in our study was efficient enough to make comparisons between M. bovoculi strains. With this technique, overall protein profiles of these strains were similar, which indicates their close relatedness. Strain relatedness was also shown for other mycoplasmas such as M. ovipneumoniae, M. hominis, and ureaplasma (15,17,20).

Serological and genetic heterogeneity among mycoplasma strains was reported (20). In studies done on M. pulmonis and M. arginini both strain specific and species-specific antigens were demonstrated (1,19). Marked heterogeneity was observed among M. ovipneumoniae strains using restriction endonuclease DNA analysis (15). Hyperimmune rabbit serum prepared against one strain of M. bovoculi contained antibodies to most of the
antigenic determinants that the organism possesses (both membrane and cytoplasmic), making possible the evaluation of the degree of heterogeneity between these strains using the immunoblotting technique. It is important however, to review the limitations of this technique. Proteins separated on the gel might not renature and not all proteins might transfer to nitrocellulose filters. Even so, both strain specific and conserved antigens were detected. Although detected antigenic determinants may not be endowed with similar biological functions, two of these determinants (94k and 29k) detected with monoclonal antibodies were found to block the attachment of M. bovoculi to bovine erythrocytes (Salih and Rosenbusch, Part II, herein).

Of several protein bands detected by post-exposure calf sera, p94 was common to all strains. This antigen was shown to be removed by trypsin treatment of intact mycoplasma cells confirming its proteinaceous nature and membrane surface location. It appears that this is a major protein found on the surface of M. bovoculi, and antibody responses are easily mounted against this antigen. Other proteins shared between some strains possible exhibit surface location since trypsin treatment resulted in their removal. Trypsin resistant bands might represent inaccessible proteins that are either integral membrane proteins or cytoplasmic proteins.

Monoclonal antibodies are a powerful tool for identification of membrane proteins. Wise and Watson (27) were able to identify a p120 protein on the surface of M. hyorhinis using a monoclonal antibody. Possession of p94 protein by all M. bovoculi strains examined could be shown by immunoblotting analysis and its trypsin sensitivity was
indicated by its loss following enzyme treatment. We found that M25.5 is responsible for blocking adherence of *M. bovoculi* to bovine erythrocytes, and immunogold labeling using this monoclonal antibody confirmed the external location of p94 protein (Salih and Rosenbusch, Part III, herein).

Sharing of antigenic determinants between mycoplasma species was explored recently (3). Monoclonal and monospecific antibodies prepared against Pl protein of *M. pneumoniae* reacted with proteins from *M. genitalium* and *M. gallisepticum*. This provided evidence for antigenic conservation among species of mycoplasmas. We have demonstrated such antigenic conservation within strains of *M. bovoculi* using monoclonal antibodies towards surface proteins.

These studies identify a surface protein (p94) on *M. bovoculi* membrane that is trypsin sensitive and possessed by all the strains examined. Close relatedness was noticed among strains, however, they varied in their antigenic activities.
ACKNOWLEDGEMENTS

This work was supported in part by a grant from USDA Farm Bill Section 1433. The authors would like to thank Ms. Y. Wannemuehler for her technical assistance.
LITERATURE CITED

strain-specific and common surface antigens of *Mycoplasma arginini.*

mediate *Mycoplasma pneumoniae* attachment to human and sheep

attachment protein of *Mycoplasma pneumoniae* shared by other patho-

4. Feldner, J., W. Bredt, and I. Kahane. 1979. Adherence of erythro-

A. M. Collier, and W. A. Clyde, Jr. 1982. *Mycoplasma pneumoniae*
infection: Role of a surface protein in the attachment organelle.
Science 216:313-315.

6. Hu, P. C., C. H. Huang, Y. S. Huang, A. M. Collier, and W. A. Clyde,
Jr. 1985. Demonstration of multiple antigenic determinants on
*Mycoplasma pneumoniae* attachment protein by monoclonal antibodies.

Baseman. 1985. Detection of the major adhesin P1 in triton
shells of virulent *Mycoplasma pneumoniae.* Infect. Immun. 50:944-
946.

8. Kenny, G. E., and F. D. Cartwright. 1977. Effect of urea concen-
tration on growth of *Ureaplasma urealyticum* (T-strain mycoplasma).

of *Mycoplasma hyopneumoniae* identified from an *Escherichia coli*

of mycoplasmatales in pneumonic calf lungs. Vet. Microbiol. 11:
79-91.

11. Krause, D. C., and J. B. Baseman. 1983. Inhibition of *Mycoplasma*
*pneumoniae* hemadsorption and adherence to respiratory epithelium


PART II. INTERACTIONS OF *Mycoplasma bovoculi* STRAINS WITH ERYTHROCYTES

This manuscript has been submitted for publication to Infection and Immunity.
INTERACTIONS OF *MYCOPLASMA BOVOCULI* STRAINS WITH ERYTHROCYTES

BARIK A. SALIH

RICARDO F. ROSENBUSCH

From the Veterinary Medical Research Institute, Iowa State University, Ames, Iowa 50011.
ABSTRACT

Adherence of two strains of *M. bovoculi* to bovine erythrocytes was measured using \(^{35}\)S methionine labeled organisms. Trypsin treatment of the organisms abolished this process completely, while trypsin treatment of erythrocytes had no effect. Similarly, pretreatment of erythrocytes with increasing concentrations of neuraminidase resulted in no measurable effect on adherence. Monoclonal antibodies (M25.5 and M7.3) directed against two mycoplasmal surface antigens of 94,000 and 29,000 molecular-weight (termed p94 and p29, respectively) blocked adherence. Blocking was decreased by increasing the dilutions of the antibodies. Other properties of *M. bovoculi* were tested using six strains of the mycoplasma and erythrocytes from several animal species; calf, sheep, horse, rabbit, chicken and turkey. None of the strains showed hemagglutinating or hemadsorbing activities with these erythrocytes. Hemolytic activity varied among both the strains and erythrocytes used. Clear zones of \(\beta\)-hemolysis were seen with sheep, horse and rabbit erythrocytes. None of the strains lysed chicken or turkey erythrocytes. We conclude that the receptor sites of *M. bovoculi* involved in attachment to bovine erythrocytes are trypsin sensitive. Blocking with monoclonal antibodies indicated that two or more mycoplasmal receptors are involved in this process.
INTRODUCTION

Studies on the adherence of mycoplasmas to eukaryotic cells have shown that mycoplasmas can adhere to a variety of cells including organ culture cells, tissue culture cells, erythrocytes and lymphocytes (3, 9, 15). At the molecular level, receptors involved in this process were affected by both protease and neuraminidase treatments (15). A surface protein of \textit{M. pneumoniae} designated P1 is a good example. Removal of this protein with trypsin decreased the adherence of the organism to tracheal rings (6). Neuraminidase treatment of the host cell also decreased attachment of \textit{M. pneumoniae} to sheep erythrocytes (3). \textit{Mycoplasma bovoculi}, an important predisposing factor in infectious bovine keratoconjunctivitis (16) does attach to tissue culture cells \textit{in vitro} and to conjunctival cells both \textit{in vivo} and \textit{in vitro} (Salih and Rosenbusch, Part III, herein). Unlike \textit{M. pneumoniae}, \textit{M. bovoculi} possess no specialized structure for attachment.

Hemadsorptive, hemagglutinative and hemolytic properties of mycoplasmas are used for identification and characterization of different species and strains within species (4). Attempts to describe the mechanistic basis and interrelationships between these three phenomena have been unsuccessful. Correlation of these processes with pathogenicity is also unclear.

We have recognized earlier a trypsin sensitive protein (termed p94) on the surface of \textit{M. bovoculi} by western blotting using monoclonal antibodies (Salih and Rosenbusch, Part I, herein). This study
was conducted to examine the possible involvement of p94 and another surface protein of 29,000 molecular weight (termed p29), which had been detected previously (Wannemuehler and Rosenbusch, manuscript for publication), in adherence to erythrocytes and to evaluate other properties of the organism following interactions with erythrocytes.
MATERIALS AND METHODS

Mycoplasma Mycoplasma bovoculi strains (FS8-7, Cs101, Cs239, C181, Cs117, and M165/69) were used in this study. The first 5 strains were isolated from the eyes of cattle with infectious bovine keratoconjunctivitis (IBK) from several Iowa farms. Strain M165/69 was obtained from the American Type Culture Collection. Cloning of these strains was done by filtering through a sterile 0.45 μm Millipore membrane (Millipore Corp., Bedford, Ma.) then plating onto modified Friis agar (8). Single colonies were removed, grown in modified Friis broth (8) then adapted to dialysate medium (7) with 10% fetal calf serum (FCS) (GIBCO Laboratories, Grand Island, N.Y.) and used at the 9th or 10th subculture.

For adherence assays early log-phase culture (18 h pH 7.0) were incubated for 4 h at 37°C in Hanks balanced salt solution containing 10% FCS and 2.5 μCi of (35S) methionine per ml (Amersham Corp., Arlington Heights, Ill.). Equal volume of fresh prewarmed dialysate medium was added and incubated for an additional 1 h at 37°C, then the cultures were washed twice with PBS and resuspended to a standard concentration as determined by reading optical density at 420 nm. Removal of mycoplasmal aggregates was carried out by centrifugation at 500 X g for 4 min prior to use in the assay. Measurement of total mycoplasma protein concentrations were done by Lowry's method (12). At the same time the amount of radioactivity was determined for each mycoplasma culture.

Erythrocytes Erythrocytes from several species of animals (cow, sheep, horse, rabbit, chicken, turkey) were collected in Alsever's
solution, then washed three times with phosphate buffer saline (PBS) (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2), resuspended in the same buffer and stored at 4°C. For adherence assays, bovine erythrocytes collected and washed as above were separated by sedimentation through Ficoll-Hypaque 1077 (Sigma Chemical Co., St. Louis, Mo.) at 400 X g for 40 min at room temperature. The pelleted erythrocytes were then washed twice with PBS and resuspended in the same buffer to 4% (vol/vol).

**Enzyme treatments** Washed suspensions of *M. bovoculi* cells were treated with trypsin (Difco Laboratories, Detroit, Mich.) at a concentration of 100 μg per ml, incubated for 1 h at 37°C, then centrifuged at 13,500 X g for 5 min and washed once with PBS. Bovine erythrocytes were treated with trypsin as mentioned above or with neuraminidase (Sigma). Several concentrations of neuraminidase (0.01, 0.02, 0.05, 0.1, 0.2 U/ml) were added to 10% erythrocyte suspensions and incubated for 60 min at 37°C. The erythrocytes were washed and resuspended in PBS as above (15).

**Monoclonal antibodies** Hybridomas and mouse monoclonal antibodies were developed in our laboratory as described previously (Wannemuehler and Rosenbusch, manuscript for publication). Briefly, hybridomas were made by fusing spleen cells from a BALB/c mouse (injected with *M. bovoculi* strain FS8-7) with myeloma cells SP2/0. Two monoclonal antibodies (M25.5 and M7.3) were used. These recognized respectively 94,000 and 29,000 molecular weight protein bands of the above strain.

**Attachment of mycoplasmas to erythrocytes** Bovine erythrocytes, collected in Alsever's solution and separated on Ficoll-Hypaque were used within one week for the adherence assay (9). A 50 μl aliquot of 4%
erythrocyte suspensions were added to 100 μl of untreated, trypsin treated or monoclonal antibody-treated (35S) methionine radiolabeled M. bovoculi suspensions and incubated with intermittent mixing for 30 min at 37°C. Controls received 50 μl of PBS with no erythrocytes. Similarly, 50 μl of trypsin-treated or neuraminidase-treated erythrocytes were added to radiolabeled M. bovoculi suspensions. The mixtures of M. bovoculi and erythrocytes were layered on 150 μl of 40% sucrose in 1.5 ml microfuge tubes and centrifuged at 1000 X g for 4 min. The fluid above the erythrocyte pellet (upper two third) was aspirated, and the tubes were frozen on dry ice. The tip of each tube was sliced, placed in a scintillation vial containing 0.6 ml of 1% SDS and left overnight. Five ml of Ready-Solve scintillation fluid (Beckman Instruments, Inc., Palo Alto, Calif.) was added and the samples were counted in a liquid scintillation counter.

Hemadsorption Six strains of M. bovoculi were grown on Friis agar plates for 3 days at 37°C with 5% CO₂. Colonies of each strain were flooded with washed 1% (vol/vol) erythrocytes in PBS from several species as indicated above. The plates were incubated for 30 min at 37°C and excess erythrocyte suspensions were poured off gently. Plates were then washed carefully with PBS to remove unattached red cells and examined under 100 X magnification (4).

Hemagglutination The test was performed in U-bottom microtitration plates (Flow Laboratories, Inc., Mclean, Va.). M. bovoculi strains were grown in dialysate medium with 10% FCS, harvested by centrifugation, washed twice and resuspended to 1 mg per ml in PBS. Serial 2-fold dilutions of this suspension were made in PBS (0.025 ml in each well). Equal
volumes (0.025 ml) of 0.5% erythrocytes and PBS were then added to each well (4). Each test was run in duplicate. The plates were shaken, sealed with tape, and incubated for 1 h at 37°C. The hemagglutination titer was read as the highest dilution of mycoplasma suspension that agglutinated the red cells.

**Hemolysis**  
*M. bovoculi* strains grown on Friis agar plates for 3 days at 37°C with 5% CO₂ were overlaid with 4% (vol/vol) blood agar and incubated for 5 additional days at 37°C. Colonies of each strain grown on six plates received erythrocytes from the six species of animals. The plates were observed daily for hemolytic plaques around colonies (4).
RESULTS

Adherence of mycoplasmas to erythrocytes

M. bovoculi strains FS8-7 and M165/69 to bovine erythrocytes is shown in Table 1. Only a low percentage of the total population of mycoplasmas attached to erythrocytes. Increasing the concentration of mycoplasmas while keeping that of the erythrocytes constant resulted in an increase in the number of mycoplasmas attached, although it did not change the percentage significantly. In preliminary studies, strain M165/69 attached to bovine erythrocytes at a much higher percentage than strain FS8-7.

Table 1. Attachment percentages of various concentrations of radiolabeled Mycoplasma bovoculi to bovine erythrocytes

| M. bovoculi strain | Total mycoplasmas (as µg of protein) | % of attached mycoplasmas
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>FS8-7</td>
<td>40</td>
<td>13.0±3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>13.4±2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>15.6±2</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>15.0±3</td>
</tr>
<tr>
<td>M165/69</td>
<td>60</td>
<td>19.1±2</td>
</tr>
</tbody>
</table>

^Mycoplasmas were attached to a standard concentration of erythrocytes (1X10^9 cells/ml). Measurements of mycoplasma protein were derived from radioactivity counts.

^bPercentage of attached mycoplasmas as compared to the total population. The values are the means of duplicates ± standard deviations.
Centrifugation of the mycoplasma suspension at 500 X g for 40 min before use in the assay eliminated this problem. Another complication observed was that bovine erythrocytes were agglutinated when incubated with fetal calf serum, therefore, hybridomas producing monoclonal antibodies used for blocking adherence were grown in serum-free medium supplemented with endothelial cell growth supplement (Collaborative Research Inc., Lexington, Ma.).

**Effect of trypsin on mycoplasma** Trypsin treatment at 100 μg per ml for 1 h at 37°C abolished the attachment of two *M. bovoculi* strains to bovine erythrocytes (Table 2). This treatment released about 30% of mycoplasma protein as determined by counting radioactivity of the supernatant and comparing to the total present in the tube (data not shown).

### Table 2. Effect of enzymatic treatments on the adherence of radiolabeled *Mycoplasma bovoculi* to bovine erythrocytes

<table>
<thead>
<tr>
<th>M. bovoculi strain</th>
<th>Trypsin treated mycoplasmas (%)</th>
<th>Trypsin treated erythrocytes (%)</th>
<th>Neur. treated erythrocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS8-7</td>
<td>4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>M165/69</td>
<td>5</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

^aPercentage of mycoplasmas attached as compared to untreated cells. Mycoplasmas at 0.5 mg/ml and erythrocytes at 1x10^9 cells/ml were used.

^b,^cTrypsin at 100 μg/ml.

^dNeuraminidase at 0.2 U/ml.
Treatment of erythrocytes with trypsin and neuraminidase

Pretreatment of erythrocytes with trypsin (100 μg/ml) did not affect the attachment properties of both strains of *M. bovoculi* (Table 2). Similarly, neuraminidase pretreatment using several concentrations of the enzyme had no effect on attachment to either strain. Increasing the concentration of neuraminidase beyond 0.2 U per ml resulted in erythrocyte lysis.

Blocking of adherence

Monoclonal antibodies M25.5 and M7.3 blocked the attachment of both strains of *M. bovoculi* to bovine erythrocytes (Table 3). Neither of the antibodies blocked the attachment

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Strain FS8-7</th>
<th>Strain M165/69</th>
</tr>
</thead>
<tbody>
<tr>
<td>M25.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undiluted</td>
<td>60±3</td>
<td>65±4</td>
</tr>
<tr>
<td>1:100</td>
<td>62±2</td>
<td>68±4</td>
</tr>
<tr>
<td>1:1000</td>
<td>77±5</td>
<td>79±2</td>
</tr>
<tr>
<td>M7.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undiluted</td>
<td>50±2</td>
<td>62±3</td>
</tr>
<tr>
<td>1:100</td>
<td>50±3</td>
<td>77±2</td>
</tr>
<tr>
<td>1:1000</td>
<td>78±3</td>
<td>89±2</td>
</tr>
</tbody>
</table>

*Mycoplasmas were pretreated with antibodies for 1 h at room temperature.*

*Percentage of mycoplasmas attached as compared to untreated cells. Values are the means and standard deviations of triplicate experiments.*
completely. The percentage attached was dependent on the antibody
dilution. Higher dilutions had less blocking effects.

**Hemadsorption and hemagglutination** None of the six *M. bovoculi*
strains hemadsorbed to erythrocytes of the six animal species tested. In
addition, no hemagglutinating properties were detected.

**Hemolysis** As shown in Table 4, all strains could lyse sheep,
horse and rabbit erythrocytes, but varied in ability to lyse bovine
erythrocytes. None of the strains lysed chicken or turkey erythrocytes.
Single colonies were first surrounded with a greenish zone (incomplete
hemolysis) which later became clear indicating complete lysis of erythro-
cytes.

Table 4. Hemolytic activity of *Mycoplasma bovoculi* strains

<table>
<thead>
<tr>
<th><em>M. bovoculi</em> strain</th>
<th>Bovine</th>
<th>Sheep</th>
<th>Horse</th>
<th>Rabbit</th>
<th>Chicken</th>
<th>Turkey</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS8-7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M165/69</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cs101</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cs239</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cl81</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cs117</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*+(+) indicates a clear zone of hemolysis; (±) indicates a greenish
zone of hemolysis; (-) no hemolysis.*
DISCUSSION

Our finding that only a low percentage of the *M. bovoculi* population attached to erythrocytes is in agreement with an earlier report with *Mycoplasma gallisepticum* (1). As suggested by the above group the presence of a population with high avidity for erythrocytes within the mycoplasma suspension might explain this phenomenon since reincubation of a pre-exposed suspension with erythrocytes resulted in very low attachment percentage. This was also supported in our experiment as increasing the amount of mycoplasmas maintained a certain attachment percentage. The binding capacity of the two strains of *M. bovoculi* was comparable, the slight difference in percentage attached suggesting that strain M165/69 suspensions still had a residual amount of small aggregates.

The trypsin-sensitive nature of the receptor site of *M. pneumoniae* involved in attachment to erythrocytes has been reported (3). Similarly, trypsin treatment of two *M. bovoculi* strains inhibited their attachment to bovine erythrocytes. However, trypsin treatment of *M. pulmonis* had no effect on its binding capacity to human erythrocytes (15). In contrast to other studies with *M. pulmonis* (15), trypsin treatment of bovine erythrocytes did not increase their attachment capacity to *M. bovoculi*. This indicates that the bovine erythrocyte receptors involved in attachment to *M. bovoculi* were resistant to trypsin.

The sensitivity of erythrocyte receptor sites to neuraminidase treatment was found by others to be dependent upon the species of erythrocytes. Neuraminidase treated sheep erythrocytes did not attach to *M. pneumoniae*
while treatment of human or rabbit erythrocytes with neuraminidase had no effect on adherence (3). Treatment of human erythrocytes with neuraminidase decreased the binding of these erythrocytes to \textit{M. gallisepticum} (1). In our assay, bovine erythrocytes treated with increasing concentrations of neuraminidase did not exhibit increased binding to \textit{M. bovoculi} strains.

Mycoplasmas are usually noninvasive organisms that colonize the mucosal surfaces of their hosts and the attachment to host cell surfaces is considered an initial step in the establishment of pathogenicity. The antigens on the mycoplasma membranes must have a great impact on their interactions with those cells and blocking of attachment by antibodies directed towards these antigens will stop such interactions and thus eliminate infection with these organisms. Blocking of attachment of \textit{M. pneumoniae} to chicken erythrocytes and to sheep erythrocytes was done successfully using monospecific anti-Pl antiserum (3,9). The two monoclonal antibodies that recognized two different antigenic determinants on the membrane surface of \textit{M. bovoculi} blocked the adherence to bovine erythrocytes. Blocking was found to be dose dependent and neither of the two antibodies blocked the attachment completely. This might suggest that two or more mycoplasmal antigens were involved in adherence and the process might be blocked similarly \textit{in vivo}.

Tests run on 6 strains of \textit{M. bovoculi} showed no hemagglutinating or hemadsorbing activities with the several erythrocytes tested. Langford and Leach (11) similarly reported no hemadsorption activity for strain M165/69 in spite of testing erythrocytes from two additional species
(guinea pig and human). Adherence to erythrocytes, hemadsorption and hemagglutination are all processes that require interaction with erythrocyte membranes. The first two processes were reported to be related based on their susceptibility to enzymatic treatment (10,15). Others found no correlation between the above processes and hemagglutination (13,15).

The ability of *M. bovoculi* to adhere but not to hemadsorb or to hemagglutinate might be explained as suggested earlier for *M. pulmonis* (15). It was hypothesized that mycoplasma-erythrocyte interactions might not be strong enough to cause agglutination, or the receptors involved might be univalent in nature or binding could result in loss of affinity for other erythrocytes.

Unlike other bacterial hemolysins, mycoplasmal hemolysin has been described as being a peroxide rather than protein in nature (17). This peroxide activity was detected in hemolysins from a wide range of mycoplasma species (2). Recently, Minion and Goguen (14) described a different mechanism for hemolytic activity of *M. pulmonis*. Intimate contact between mycoplasma and host cell rather than a diffusible substance was required together with altered erythrocyte membranes (trypsin treatment) to exert lysis. Trypsin treatment of the mycoplasma on the other hand prevented lysis. Hemolytic activity of *M. bovoculi* strains was in agreement with previous results (11). These authors also noticed no hemolysis of guinea pig or human erythrocytes with strain M165/69. It is most probable that *M. bovoculi* produces a diffusible substance that resulted in erythrocyte lysis since no alterations of erythrocyte membranes were required.
Trypsin sensitivity of the receptor sites of *M. bovoculi* involved in attachment to bovine erythrocytes was demonstrated. Blocking with monoclonal antibodies showed that at least 2 receptors were involved in attachment.
ACKNOWLEDGEMENTS

This work was supported in part by a grant from USDA Farm Bill Section 1433. The authors would like to thank Ms. Y. Wannemuehler for her technical assistance.
LITERATURE CITED


PART III. IMMUNOGOLD LABELING OF MYCOPLASMA BOVOCULI MEMBRANE PROTEINS INVOLVED IN ADHERENCE

This manuscript has been submitted for publication to Infection and Immunity.
IMMUNOGOLD LABELING OF MYCOPLASMA BOVOCULI
MEMBRANE PROTEINS INVOLVED IN ADHERENCE

BARIK A. SALIH
RICARDO F. ROSENBUSCH

From the Veterinary Medical Research Institute, Iowa State University,
Ames, Iowa 50011.
ABSTRACT

The presence of a specialized structure (tip) in some mycoplasmas facilitates their attachment to host cells. Virulence of some other mycoplasmas was found to be associated with the presence of extracellular material (capsule). *Mycoplasma bovoculi* strain FS8-7 examined by transmission electron microscopy (TEM) showed no specialized membrane structure and organisms grown both *in vivo* and *in vitro* did not exhibit capsule when stained with ruthenium red. The organisms attached to bovine lung fibroblasts *in vitro*. No mycoplasma was seen on these cells when the organism was pretreated with trypsin or with anti *M. bovoculi* antibodies. *In vivo* attachment to conjunctival epithelium was demonstrated by *in situ* fixation. A 5 month old mycoplasma-free calf inoculated with *M. bovoculi* by ocular instillation was sacrificed 5 days later, the eyes were fixed with 3% glutaraldehyde and the corneal and conjunctival epithelium were removed and processed for TEM. To demonstrate the distribution of attachment receptor sites on the membrane surface of the organism, hyperimmune rabbit serum and monoclonal antibodies were used. Mycoplasma cells pretreated with these antibodies were labeled with protein A-gold and showed random distribution of these receptors around the membrane. Gold labeled monoclonal antibodies M25.5 and M7.3, directed against two different antigens on the surface of the membrane also showed random distribution of these surface receptors in double labeling experiments. *M. bovoculi* appeared to attach to a variety of cells without the aid of a specialized structure. Attachment appeared to be inhibited by trypsin or antibody treatment.
The receptors involved appeared to be randomly distributed on the surface of the mycoplasma and the two monoclonal antibodies recognized two different receptor sites.
Electron microscopy is a useful and powerful technique to study the ultrastructural morphology and interactions between different cells. Recently, the use of immunocytochemistry for protein and enzyme characterization has added a new dimension to electron microscopic studies (1). Immunogold labeling is one example that was applied successfully for double and triple labeling of cell surface receptors (10,17). *Mycoplasma bovoculi*, a bovine pathogen known to cause conjunctivitis in calves (16) has been studied with these techniques. Attachment of this mycoplasma to host cells is a prerequisite for the establishment of its pathogenicity. The purpose of this study was to examine the ultrastructural morphology of *M. bovoculi*, its interaction with host cells both *in vivo* and *in vitro* and the localization of membrane antigen receptors by the use of monoclonal antibodies or protein A tagged with colloidal gold particles.
MATERIALS AND METHODS

Mycoplasma Mycoplasma bovoculi strain FS8-7 isolated from the eyes of cattle with infectious bovine keratoconjunctivitis in Iowa was used in this study. The organism was grown in dialysate medium (14) with 10% fetal calf serum (FCS) (GIBCO Laboratories, Grand Island, N.Y.), harvested by centrifugation at 27,000 X g for 20 min, washed twice with phosphate buffer saline (PBS) and further processed for transmission electron microscopy (TEM).

Tissue culture Bovine lung fibroblast cells were grown on chambered glass slides (Miles Scientific, Naperville, Ill.) with minimal essential medium (MEM) and 10% FCS for 24 h and 37°C with 5% CO₂. Just before testing, the confluent monolayers of cells were washed once with fresh MEM, then incubated with a 24 h old culture of washed M. bovoculi suspension for 1 h at 37°C with 5% CO₂. Cells were then washed gently three times with PBS. One ml of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) was added to the cells in each chamber and kept at 4°C for 3 h. The buffer was aspirated, the cells were scraped off the glass with a razor blade while still wet and pelleted by centrifugation at 500 X g for 5 min. To test blocking of attachment, M. bovoculi suspensions were treated with trypsin (100 µg/ml) or with hyperimmune rabbit anti-M. bovoculi serum prior to attachment (Salih and Rosenbusch, Part II, herein).

Corneal and conjunctival epithelium A 5 month old mycoplasma-free calf was inoculated by ocular instillation of 1.5 ml of M. bovoculi strain FS8-7 suspension. The animal was sacrificed 6 days later, the eyes were
fixed in situ with 3% glutaraldehyde-cacodylate buffer and the corneal and conjunctival epithelium were then dissected with a sterile surgical blade and processed for TEM.

Transmission electron microscopy (TEM) The technique described by Cole (4) was applied for processing the tissue for TEM with some modifications. Tissues fixed with 3% glutaraldehyde-cacodylate buffer were dehydrated into a series of acetone concentrations (no osmium post-fixation) and then embedded into Epon 812 resin. Thin sections were cut with a diamond knife (Electron Microscopy Science, Fort Washington, Penn.), stained with 2% uranyl acetate and Reynold's lead citrate and examined under the transmission electron microscope. For capsule study M. bovoculi pellets were fixed with 3% glutaraldehyde-cacodylate buffer with or without 1% ruthenium red (Sigma Chemical Co., St. Louis, Mo.) for 3 h at 4°C, then washed three times with 0.1 M cacodylate buffer for 30 min each (11). Post-fixation was done in 1% osmium tetroxide (Sigma) in 0.1 M sodium cacodylate buffer pH 7.2 for 1 h at room temperature, then washed as above. The pellets were dehydrated through an acetone series and embedded in Epon 812 resin.

Monoclonal antibodies Hybridomas and mouse monoclonal antibodies were developed in our laboratory as described previously (Wannemuehler and Rosenbusch, manuscript for publication). Briefly, hybridomas were made by fusing spleen cells from a BALB/c mouse (injected with M. bovoculi strain FS8-7) with myeloma cells SP2/0. Two monoclonal antibodies (M25.5, M7.3) were used, recognizing respectively, 94k and 29k protein bands of strain FS8-7.
Protein A-gold labeling A prefixation method described previously (6) was applied with some modifications. M. bovoculi suspensions (0.5 mg/ml) were incubated with hyperimmune rabbit serum for 1 h at room temperature, washed once with PBS, then 1.5 μg per ml of Protein A-gold (15 nm) (E. Y. Laboratories Inc., San Mateo, Calif.) was added and incubated for 30 min at room temperature. The mixture was centrifuged at 13,500 X g for 5 min, then washed once with PBS. The supernatant was aspirated, the pellet was fixed with 3% glutaraldehyde-cacodylate buffer and processed for TEM. No osmium post-fixation was applied. For labeling with monoclonal antibodies, suspensions of M. bovoculi were first incubated with monoclonal antibodies for 1 h at room temperature, washed once with PBS, then incubated with unlabeled goat anti-mouse IgG (Cappel Laboratories, Cochranville, Penn.) for 30 min at room temperature and washed. Protein A-gold labeling and processing for TEM were as above. Controls received no hyperimmune rabbit serum or monoclonal antibodies.

Monoclonal antibody-gold complex The two monoclonal antibodies were adsorbed to two different sizes of colloidal-gold particles (15 nm, 5 nm) (E. Y. Laboratories). The technique applied was described previously (1). Briefly, the minimal amount of antibodies required for stabilization of the colloidal gold was determined by adding a constant 1 ml volume of colloidal gold to 0.1 ml of serial 2-fold dilutions of the antibodies. Following a 10 min incubation at room temperature, 0.1 ml of 10% NaCl solution was added. Visualization of color change from wine-red to purple-blue was used as criterion of flocculation. A 10 ml volume of the colloidal gold suspension (15 nm) was added to 0.5 mg of the monoclonal antibody
M25.5. After 2 min, 1 ml of polyethylene glycol (PEG) (MW 20,000) was added and the mixture was centrifuged at 25,000 rpm in a Ti50 rotor (Beckman Instruments, Inc., Palo Alto, California) for 30 min at 4°C. The clear supernatant containing free antibodies was aspirated carefully. The dark red sediment was resuspended in 1.5 ml PBS pH 7.2 containing 0.02% PEG and stored at 4°C. Monoclonal antibody M7.3 was adsorbed to 5 nm gold particles similarly, except that higher centrifugation speed (35,000 rpm) was applied.

**Double labeling with monoclonal antibodies-gold complexes**

A modification of a previously reported technique (17) was applied. The two complexes were mixed together to contain equal amount of protein concentration. This was incubated with *M. bovoculi* suspension for 30 min, then centrifuged at 13,500 X g for 3.5 min, washed once with PBS and fixed in 3% glutaraldehyde-cacodylate buffer (no osmium post-fixation was used).
RESULTS

Staining of *M. bovoculii* strain FS8-7 with (Fig. 1) or without ruthenium red (Fig. 2) showed no difference in the extracellular morphology of the organism indicating that capsule was absent. Although capsulated bacteria tend to lose their capsules when grown *in vitro*, *M. bovoculii* strain FS8-7 grown *in vivo* (ocular colonization) were also negative when stained with ruthenium red.

**Attachment to tissue culture, corneal and conjunctival epithelium**

Attachment of *M. bovoculii* to bovine lung fibroblasts is demonstrated in Fig. 3. There was no apparent involvement of any specialized structure in this process and no alteration of cellular membranes were seen at this stage. Pretreatment of *M. bovoculii* with trypsin or with hyperimmune rabbit serum blocked the attachment to lung cells. The organism was also seen attached *in vivo* to conjunctival epithelium (Fig. 4). No mycoplasmas were found on corneal epithelium in this study.

**Immunogold labeling**

*Mycoplasma bovoculii* incubated with hyperimmune rabbit serum and Protein A-gold were labeled randomly (Fig. 5). The distribution of gold particles appeared all around the mycoplasma membranes. Monoclonal antibodies M25.5 (Fig. 6) and M7.3 (Fig. 7) labeled mycoplasma cells less intensely than hyperimmune serum. This is expected since these antibodies recognize a single epitope on the membranes. To determine the prevalence of these antigenic receptors on the membrane of the mycoplasma, each monoclonal antibody was tagged with a different size of gold particles, M25.5 with 15 nm and M7.3 with 5 nm
FIG. 1. Electron micrograph of *Mycoplasma bovoculi* strain FS8-7 stained with ruthenium red. Bar = 0.1 μm
FIG. 2. Electron micrograph of *Mycoplasma bovoculi* strain FS8-7
without ruthenium red. Bar = 0.1 μm
FIG. 3. *Mycoplasma bovoculi* attached to bovine lung fibroblasts cell *in vitro*. Bar = 0.2 μm
FIG. 4. *Mycoplasma bovoculi* attached to bovine conjunctival epithelium *in vivo*. Bar = 0.2 μm
FIG. 5. Protein A-gold labeling of *Mycoplasma bovoculi* following incubation with hyperimmune rabbit serum. Bar = 0.1 μm
FIG. 6. Protein A-gold labeling of *Mycoplasma bovoculi* following incubation with monoclonal antibodies M25.5. Bar = 0.1 μm
FIG. 7. Protein A-gold labeling of *Mycoplasma bovoculi* following incubation with monoclonal antibodies M7.3. Bar = 0.1 μm
gold particles and a mixture of both labeled monoclonal antibodies was used. It could be shown that each of these antibodies recognize a different epitope on the mycoplasma membrane (Fig. 8).

Controls that received Protein A-gold alone or unlabeled secondary antibody and Protein A-gold were negative. Adsorption of antibodies to the gold particles was confirmed by negative staining with 2% uranyl acetate. As noticed in Fig. 9, the presence of a clear halo around the gold particles indicated the presence of the antibody layer at their surfaces which was not seen when compared to the untagged ones (Fig. 10).
FIG. 8. Monoclonal antibodies-gold complex labeling of *Mycoplasma bovoculi* membranes. Monoclonal antibodies M25.5 tagged with 15 nm colloidal gold particle (closed arrow) and M7.3 tagged with 5 nm colloidal gold particle (open arrow) were seen. Bar = 0.1 μm.
FIG. 9. Negative staining of monoclonal antibody-colloidal gold complex (M25.5-15 nm gold) with 2% uranyl acetate. The halo visualized around the gold particle (arrow) indicates the presence of the antibody layer. Bar = 0.05 μm
FIG. 10. Negative staining of colloidal gold particles (15 nm) with 2% uranyl acetate. Bar = 0.05 μm
DISCUSSION

As early as 1960, Buttery and Plackett (3) described a polysaccharide (galactan) isolated from *Mycoplasma mycoides* var *mycoides*. Later on Hudson et al. (13) found that this material could act as a hapten and stimulate antibody production only when injected with Freund's complete adjuvant. The galactan induced a prolonged mycoplasemia and joint and kidney lesions when given prior to subcutaneous injection with the organism (15). Capsular material was also shown on some other mycoplasmas (9,11). *Mycoplasma bovoculi* possessed no capsule even when grown in vivo. Although virulence of *M. mycoides* var *mycoides* was attributed in part to the presence of galactan since avirulent strains lack this material, its role in pathogenicity must be limited since most other pathogenic mycoplasmas do not possess such material. This capsule detection step was undertaken to confirm that the antibodies used were developed towards cell surface proteins rather than capsular material.

The attachment of *M. pneumoniae* and *M. gallisepticum* to eukaryotic cells is by a specialized tip-like structure (2) and antibodies towards the structure of *M. pneumoniae* blocked the attachment of the organism (5). *M. bovoculi* attachment to animal cells exhibited an attachment pattern typical of other non-tipped mycoplasmas (7). Mycoplasmas in general have the capabilities to attach, grow and even acquire host cell antigens (8). This process requires intimate contact between the organism and the host cell. Following one hour incubation of *M. bovoculi* with lung fibroblasts no such contact was seen. We demonstrated earlier (Salih and Rosenbusch,
Part II, herein) that trypsin treatment abolished the attachment of *M. bovoculi* to bovine erythrocytes. Similarly, no organism was found attached to lung cells following the enzyme treatment. Hyperimmune rabbit serum reacted with *M. bovoculi* also blocked its adherence to lung cells.

Protein A-gold labeling has been used successfully for localization and characterization of cellular antigens (1,18,19). Labeling with these methods was applied on post-fixed thin sections. Advantages of the pre-fixation method when specifically applied for localization of antigens on the membrane surface of the cells are that (i) it decreases the possibilities of altering the antigenic structures that might occur following fixation, (ii) it does not limit the number of exposed surface antigens following thin sectioning, (iii) it eliminates the masking effect of osmium tetroxide post-fixation and (iv) it eliminates any possible non-specific binding to the resin. The prevalence of small size gold particles on the cell surface in double immunogold labeling was seen previously (6). The smaller the particle, the larger the number found on the surface. As hypothesized earlier (6) larger size gold particles can cover a wider surface area rendering the binding sites inaccessible. This could also be the case in our experiment. For this reason quantitative evaluation of the number of receptor sites can not be made due to the steric hindrance applied by these particles.

The studies that we conducted earlier using hyperimmune rabbit serum and monoclonal antibodies against *M. bovoculi* revealed that these antibodies recognize membrane surface antigens that were sensitive to trypsin
treatment and were involved in adherence to eukaryotic cells (Salih and Rosenbusch, manuscripts for publication). Labeling with Protein A- or monoclonal antibody-gold complexes allowed us to further confirm the membrane localization of these antigens.
ACKNOWLEDGEMENTS

This work was supported in part by a grant from USDA Farm Bill Section 1433. The authors would like to thank Ms. Jean Olson and Ms. Y. Wannemuehler for their technical assistance.
LITERATURE CITED


CONCLUSIONS

*Mycoplasma bovoculi* strains FS8-7, M165/69, Cs101, Cs239, C181 and Cs117 were used in this study. Electrophoretic analysis (SDS-PAGE) of whole cells revealed similar protein profiles among these strains although no two profiles were identical. Between 40-45 bands were detected ranging from 15,000 to over 220,000 molecular weight. Antigenic heterogeneity among strains and species has been reported (Alexander and Kenny, 1; Nichols and Kenny, 61). Hyperimmune rabbit serum developed against strain FS8-7 detected antigenic differences among *M. bovoculi* strains by western blot analysis. Bands detected were in the range of 15k to 136k and the major differences in recognition were seen in 43k to 51k and 60k to 136k protein bands. Trypsin treatment of whole cells of strain FS8-7, M165/69, Cs101, Cs239 resulted in the removal of few bands and it appeared that these strains share 5 major trypsin insensitive bands of 91k, 81k, 64k, 34k and 29k and two minor bands of 106k and 14k which were less intensely stained. Monoclonal antibodies M25.5 developed against strain FS8-7 recognized a protein band of 94,000 molecular weight designated p94 that was found in all strains examined. The proteinaceous nature and membrane surface location of p94 was shown by its degradation when mycoplasma cells were treated with trypsin. Sera obtained from three colostrum-deprived mycoplasma-free calves four weeks following inoculation with *M. bovoculi* strain FS8-7 recognized several bands including p94.

The functional activities of p94 and another protein of 29,000 molecular weight recognized earlier by monoclonal antibodies M7.3 and designated
p29 were tested in an in vitro adherence assay. Blocking of radiolabeled \textit{M. bovoculi} attachment to bovine erythrocytes was tested with the above monoclonal antibodies. Blocking activities were determined by counting radioactivity of attached mycoplasmas and compared to the total radioactivity. Up to 50% of inhibition was detected, however neither of these antibodies blocked the attachment completely indicating that at least two receptors were involved in this process. Characterization of the receptor sites participating in adherence was done by enzymatic treatment. Trypsin treatment of intact \textit{M. bovoculi} before use in the assay abolished attachment indicating that the receptors involved on the part of the mycoplasma are protein in nature. Conversely, trypsin or neuraminidase treatment of erythrocytes had no effect on attachment.

The hemadsorptive, hemagglutinative and hemolytic properties of the six \textit{M. bovoculi} strains were examined with erythrocytes from several animal species: calf, sheep, horse, rabbit, chicken and turkey. None of the strains demonstrated any hemadsorptive or hemagglutinative properties, however they varied in their hemolytic activities. Hemolysis was seen with mammalian erythrocytes but not with avian erythrocytes.

Adherence to bovine lung fibroblasts in vitro and to bovine conjunctival epithelium in vivo was examined by electron microscopy. \textit{M. bovoculi} attached to these cells without the aid of a specialized tip-like structure. \textit{M. bovoculi} stained with ruthenium red both in vitro and in vivo showed no such capsule.

The distribution of the antigenic receptors on the surface of the organism was tested with hyperimmune rabbit serum followed by protein A-
gold. Random distribution of gold particles around the membrane surface was noticed. Confirmation of the surface location of p94 and p29 proteins that were involved in adherence was done by immunogold labeling using monoclonal antibodies or protein A tagged with colloidal gold particles. Monoclonal antibody-gold complexes showed few widely distributed gold particles around the membrane surface.

These studies indicate that p94 is a trypsin sensitive protein located on the membrane surface of *M. bovoculi* that is involved in eliciting an antibody response to the organism. The biological function of this p94 and p29 protein as tested by the adherence assay showed that both of these proteins are involved in attachment to erythrocytes since monoclonal antibodies and trypsin treatment blocked this process. Random distribution of p94 and p29 on the membrane surface was observed by immunogold electron microscopy. Attachment of *M. bovoculi* to bovine lung fibroblasts and to bovine conjunctival epithelium occurred with no specialized structure and the organism possessed no capsular material when stained with ruthenium red. *Mycoplasma bovoculi* strains possessed no hemagglutinative or hemadsorptive activities but varied in their hemolytic activity. Hemolysis was seen with mammalian erythrocytes but not with avian erythrocytes.
LITERATURE CITED


Multiphasic interactions of Mycoplasma pulmonis with erythrocytes 
defined by adherence and hemagglutination. Infect. Immun. 44: 
394-400.

58. Mouches, C., A. Menara, J. G. Tully, and J. M. Bove. 1982. Poly-
acrylamide gel analysis of spiroplasmal proteins and its contri­
bution to the taxonomy of spiroplasmas. Rev. Infect. Dis. 4: 
S141-S146.

1981. Comparison of human and animal ureaplasmas by one- and two-
dimensional protein analysis on polyacrylamide slab gel. Ann. 
Microbiol. 132B:171-196.

60. Neimark, H. C. 1977. Extraction of an actin-like protein from the 
prokaryote Mycoplasma pneumoniae. Proc. Natl. Acad. Sci. 74:4041-
4045.

rization of a heat-stable surface antigen of Mycoplasma pulmonis ex­
pressing both species-specific and strain-specific determinants. 

62. Razin, S. 1968. Mycoplasma taxonomy studied by electrophoresis of 

63. Razin, S. 1979. Isolation and characterization of mycoplasma mem­
branes. Pages 213-229 in M. F. Barile and S. Razin, eds. The 
York.


65. Razin, S., and S. Rottem. 1967. Identification of mycoplasma and 
other microorganisms by polyacrylamide-gel electrophoresis of cell 

mycoplasma membranes. Pages 3-25 in A. H. Maddy, ed. Biochemical 


ACKNOWLEDGEMENTS

This dissertation is dedicated to my wife Shatha and our children Tara and Aiman who made it possible for me to finish this work through their sacrifices and inspiration during the last few years.

I wish to thank my major professor, Dr. Ricardo F. Rosenbusch for introducing me to the field of mycoplasmology, for his excellent guidance, patience and for letting me do things my own way.

A special thanks goes to Dr. Charles O. Thoen for his endless support, encouragement, valuable advice, and for serving on my committee.

For my committee members, Dr. Richard Ross, Dr. John Holt and Dr. Bernard White, their valuable advice is gratefully acknowledged.

To Jean Olson a special thanks for her technical assistance.

My thanks go to Yvonne, Chris, Julie, John and Raul for their continuous help, encouragement and most of all their friendship.

Finally, I am grateful to my government for giving me the opportunity to continue my higher education.