Studies on *Mycoplasma dispar* capsular material

by

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INTRODUCTION

Several mycoplasma species were isolated from lungs of pneumonic calves, but only a few have been proven to participate in respiratory disease, apart from Mycoplasma mycoides subsp. mycoides, was proven only in a few of those isolated. Among these, M. dispar was one of the most frequently isolated mycoplasma from pneumonic calves (Tanskanen, 1987; Viring et al., 1986; Knudtson et al., 1985).

Experimental infections of bovine respiratory tissue, using "in vitro" and "in vivo" approaches were used to demostrate the pathologic effect caused by M. dispar (Thomas and Howard 1974, Howard et al., 1976; Gourlay et al., 1976). The most striking pathologic effect noticed in bovine tracheal ring explants was ciliostasis. No specific virulence factor could be linked to this effect. A toxic mechanism, was however postulated as responsible for the ciliary damage (Thomas and Howard, 1974).

Mycoplasma dispar was reported as being surrounded by ruthenium red-stainable capsular material which, in natural infections, was shown to bein close association with ciliary membranes (Howard and Gourlay, 1974; Allan and Pirie, 1977). Based on this observation, it was suggested that M. dispar capsular material could participate in attachment by serving as filling material to cover the gap existing between the mycoplasma and host cell membrane (Howard and Gourlay, 1974).

However, no information was provided to validate this assumption, or to explain any other pathogenic role for this capsular material.

The objectives of this work were to determine conditions for the reproducible and efficient production of M. dispar capsular material "in vitro" and to characterize the "in vitro" produced M. dispar capsular material.

LITERATURE REVIEW

General Characteristics of the Class Mollicutes

Mollicutes are the smallest non-parasitic procaryotes capable of self-replication. They posses a genome size of 5-10 x 10 (8) which is believed to be the minimum required for independent replication (Razin, 1978). Their phospholipid-bilayer cell membrane is similar to cell membranes of eucaryotes and unlike those of other procaryotes, they lack cell wall. This wall-less characteristic was used to separate these procaryotes from eubacteria into a new class named Mollicutes (Molli: soft, Cutes: skin) (Razin, 1978). The common name "mycoplasmas" will be used in this review to denote any species included in the class Mollicutes, whereas the trivial name ureaplasma, acholeplasma, anaeroplasma, spiroplasma, or thermoplasma will be used to refer the corresponding genus.

Based on morphologic and nutritional criteria, the class Mollicutes is subdivided into three families designated Acholeplasmataceae, Mycoplasmataceae, and Spiroplasmataceae. Mycoplasmataceae and Spiroplasmataceae members are cholesterol dependent, and this compound is incorporated without modifications into the membrane, providing the sufficient membrane fluidity for survival under physiologic conditions. Acholeplasmataceae are not sterol dependent and this characteristic plus their genome size of 1 x 10.9 make them resemble bacteria (Razin 1978).

Spiroplasmataceae members, which receive their name because of their helical shape, are primarily associated with plant diseases such as "stubborn" disease in citrus and corn stunt disease, as well as multiple insect habitats (Marmorosch, 1973). A spiroplasma was described as the etiological agent of the suckling mouse cataract disease and this has remained as the only reported spiroplasma species to date found capable of causing disease in animals (Tully et al., 1976).

Based on metabolic and nutritional characteristics the family Mycoplasmataceae was further divided into Ureaplasmas and Mycoplasmas. Ureaplasmas differ from all other member of Mycoplasmataceae in possessing urease. Based on their metabolic requirement and DNA base composition, two other genera, Thermoplasma and Anaeroplasma were included in the class Mollicutes (Razin, 1978). Thermoplasma receives its name because of the high temperature (59C optimum) and low pH (1.0 to 1.2) that characterizes its ecological niche. Anaeroplasma, as its name indicates, is a genus comprising obligate anaerobic mycoplasmas isolated from the rumens of cattle and sheep. At least one these anaerobic microorganisms have the unique characteristic of digesting bacteria, ability that is reflected in its name, Anaeroplasma bactoclasticum (Robinson et al., 1975).

Nutritional requirements determine a close association between mycoplasmas and cells of the host mucosa and consequently this leads to surface mucosal parasitism. This, in turn, explains the frequent colonization of mucosal surfaces of several animal body systems, in which

the respiratory tract is not an exception. That also explains why some mycoplasmas species were found as members of the normal flora of the respiratory tract (Gourlay and Howard, 1982).

Pathogenic Mycoplasmas of the Bovine Respiratory Tract

Thirteen members of this genus have been isolated from the bovine respiratory system, but the association of these mycoplasmas with respiratory disease has not been proven. Experimental inoculations in susceptible animals did not demonstrate association between infection with 8 of these mycoplasma species and calf pneumonic processes (Howard et al., 1980). Among the mycoplasmas with pathogenic capabilities for the respiratory tract, M. mycoides var. mycoides was the species which best fulfilled Koch's postulates (Howard, 1980).

Mycoplasma bovis, Mycoplasma dispar, and Ureaplasma diversum were commonly isolated from housed calves suffering respiratory disease and their pathogenicity was demonstrated under experimental conditions. Isolation of these species from healthy calves have been reported (St. George et al., 1973; Viring et al., 1986). Pathogenic and non-pathogenic mycoplasmas isolated from bovine respiratory tract are listed in table 1.

Table 1. Pathogenicity of Mollicutes isolated from the bovine respiratory tract (Howard et al., 1980)

Pathogen for respiratory tract.	Pathogenicity for respiratory tract not proven.
M. mycoides var. mycoides	M. bovirhinis
M. bovis	M. canadense
M. dispar	Mycoplasmas sp. group 7
Ureaplasma diversum	M. alkalescens
M. bovigenitalium	M. arginini
	A. laidlawii
	A. axanthum
	A. modicum
	M. bovoculi
	M. gallisepticum
	.....
	.....

Mycoplasma dispar, Mycoplasma bovis, and Ureaplasmas sp. were frequently associated with bovine respiratory processes such as enzootic calf pneumonia and "shipping fever" (Bryson, 1985). The first isolation of Mycoplasmas sp. from cattle suffering "shipping fever" was reported by Carter (1954). However, Jarret et al. (1954), described a specific respiratory disease in young calves in which peribronchial lymphocytic hyperplasia was the constant histopathological finding. This feature was used to designate "cuffing pneumonia" to those respiratory processes presenting similar histopathological lesions, and was used as histopathological feature of mycoplasmal respiratory infections in several species of domestic animals.

Mycoplasma dispar was first isolated from pneumonic lungs of calves without clinical signs of respiratory disease (Gourlay and Leach, 1970). This microorganism was also detected in lungs of calves that died or were killed "in extremis" showing symptoms of respiratory disease (Gourlay et al., 1970). Isolation of M. dispar was subsequently reported from healthy and naturally diseased calves with subclinical pneumonia (St. George et al., 1973; Pirie and Allan, 1975; Bitsch et al., 1976; Tinant et al., 1979). In several of these reports, in which pathological studies of diseased and non-diseased animals were included, a positive correlation between the presence of pneumonic lesions and high rates of isolation of M. dispar could be demonstrated. Mycoplasma dispar was isolated less frequently from non-diseased than from pneumonic lungs.

Since association between M. dispar presence and pneumonic lesions does not imply causal relationship, and because there were several reported isolations of M. dispar from healthy cattle (Thomas and Smith, 1972; St. George et al., 1973; Viring et al., 1986), demonstration of the pathogenicity of this species was required for the definition of its role in respiratory disease.

Initially, models for experimental reproduction of respiratory disease were used to fulfill Koch's postulates. Subclinical pneumonia was seen in conventionally reared calves subsequent to endobronchial inoculation of M. dispar and Ureaplasmas. However, presence of pathogenic bacteria in lung tissues and consequently, coexistence of possible preexisting subclinical pneumonia, led the authors to question the validity of the results (Gourlay et al., 1969). Coexistence of subclinical pneumonic processes was avoided by using gnotobiotic calves. A subclinical pneumonia with histologic characteristic of cuffing pneumonia was produced following intratracheal inoculation of gnotobiotic calves with M. dispar. These results, in the author's opinion, constituted a fulfillment of Koch's postulates, yet the experiment demonstrated the inability of M. dispar to produce clinical disease (Howard et al., 1976). Clinical manifestation of respiratory disease however, occurred as a result of synergistic effect of mycoplasmas and other microorganism pathogenic for the respiratory tract (Gourlay and Howard, 1978).

In a comparative study of pathogenesis using M. bovis and M. dispar it was reported that the infection caused by M. dispar was limited to the surface of respiratory epithelial cells, whereas M. bovis was found apparently penetrating between the ciliated epithelial cells and multiplying on the submucosal space (Howard et al., 1987). In a separate study, where mycoplasma isolation techniques were supplemented with electron microscopy examination of diseased lungs, Mycoplasma dispar was isolated in 47% of the examinated lungs and no other capsulated mycoplasma was detected. Pathological cellular changes observed in the bronchial epithelium included loss of cilia, protrusion of cell cytoplasm, distention and disruption of mitochondria, and formation of intracytoplasmic vacuoles. Electron microscopic examination of these lung tissues revealed the presence of ruthenium red (RR)-stainable capsulated mycoplasmas, a feature associated with M. dispar, in close association with ciliated epithelial cells. Based on these observations, the authors suggested participation of capsular material in the attachment process (Allan and Pirie, 1977).

The use of explant of bovine tracheas to study the mechanisms by which M. dispar and other mycoplasmas caused pathogenic effect on the respiratory epithelium was another approach used. Rapid ciliostatic effect and epithelial sloughing were detected in early phases of the infection. No effect was observed when the tracheal explant was exposed

to filtered M. dispar broth supernatant, and to heated or washed M. dispar (Thomas and Howard, 1974). In a similar approach, a correlation was found between the presence of ciliostatic effect and number of viable mycoplasmas (Thomas and Howard, 1974). A second element was also found to correlate with ciliostasis, and this was the concentration of fetal bovine serum (FBS) used in the maintenance medium. The authors speculated that FCS promoted rapid growth, and rapid overwhelming increase in the numbers of viable mycoplasmas that, in some unknown way, was the cause of ciliostasis. No toxic effect caused by hydrogen peroxide metabolites or by other toxic means could be detected in this study. It was concluded that the ciliostatic effect exerted by M. dispar might be a local toxic effect requiring very close mycoplasma-target cell interaction (Thomas and Howard, 1974).

Methods for detection and study of extracellular polysaccharides of microorganisms

Specific agglutination and/or labeling with lectins and specific staining of carbohydrate with RR and periodate-Schiff reagent was used to show that sugar residues of lipoglycans were located in the surface of the mycoplasma cell (Smith, 1984). It has been proposed that the reaction of RR with carbohydrate residues was based on the linkage of the stain with acidic polysaccharide of the capsular material (Luft, 1971). Specific staining with RR was used for the ultrastructural study of the

carbohydrate capsule of Klebsiella pneumoniae and Diplococcus pneumoniae (Springer and Roth, 1973). Identification of extracellular polysaccharides was also accomplished by using iron dextran-labelled concanavalin A, which specifically reacts with D-glucose, D-mannose and sterically related carbohydrates (Mayberry-Carson et al., 1978; Robertson and Smook, 1976). Polycationic ferritin was also used in the study of negatively charged surface cell structures (Danon et al., 1972). This methodology was used to describe capsular material of P. multocida (Jacques and Foiry, 1987), P. hemolytica (Gilmour et al., 1985), Klebsiella sp. (Weiss et al., 1979) and C. neoformans (Weiss et al., 1987). In all of these reports, positive labeling with polycationic ferritin as well as staining with RR was used as indication of the acidic carbohydrate nature of the capsule of the forementioned microorganism. Treatment of P. hemolytica with sodium salicylate was effective in removing the anionic site to which RR and polycationic ferritin bind, indicating a parallelism between the materials detected by both techniques (Gilmour et al., 1985).

Characterization of bacterial proteins and lipopolisaccharides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly applied method in comparative and structural studies. A modification of the silver stain method used for protein detection (Tsai and Frasch, 1982), allows the preferential detection of polysaccharide chains in bacterial lipopolysaccharide (LPS), analyzed by SDS-PAGE

separations. This procedure and later modifications (Hitchock and Brown 1983), have been used to detect the polysaccharide fraction rather than the lipid A portion of LPS preparations, and is dependent upon the presence of cis-hydroxils in the core and side polysaccharide chains. A yellow-brown stain of the carbohydrate bands can then be observed (Koprinsky et al., 1986). Information available about the silver staining of lipids is contradictory. Hitchock and Brown (1982), reported grey-red staining of bands, which may depend on the fatty acid constituents of lipid A or on the presence of phosphate groups and carbohydrate constituents in the outer core of the analyzed LPS. Koprinsky et al. (1986), reported charcoal-grey staining of lipid A, which could be the result of small quantities of non-hydrolyzed LPS in the preparation, or be due to silver binding sites present in the lipid A moiety. In contrast, Tsai and Frasch (1982), reported no staining of lipid A obtained by acid hydrolysis of LPS preparations.

The periodic acid-Schiff method was also used for detection of macromolecular carbohydrate material in polyacrylamide gels (Russell and Johnson, 1975; Jann and Westphal, 1975). The principle of this method involved an initial step of fixation followed by oxidation of bound carbohydrate by periodic acid which resulted in the cleavage of C-C bonds between adjacent alpha glycols. The resulting dyaldehydes then react with Schiff's reagent to form a red colored aldehyde (Kapitany and Zebrowski, 1973). Alcian blue staining techniques were also used for detection of

carbohydrates and,similarly this technique involves an initial oxidation with periodate, a subsequent reduction of excess periodate and iodate with metabisulfite and staining of the oxidized carbohydrate with Alcian blue reagent (Wardi and Michos, 1972). The relative mobility pattern of LPS preparations in polyacrylamide gels, which increases in positive correlation with the shortening of polysaccharide chain in conjunction with the preferential LPS silver stain method, has been used as a tool for examining LPS heterogeneity based on the length of the carbohydrate chains (Hitchock and Brown, 1983; Darveau and Hancock, 1983; Progulske and Holt, 1984). A recently described methodology for the characterization of capsular polysaccharide involves polyacrylamide gel electrophoresis and detection by a Alcian blue-silver staining method (Min and Cowman, 1986; Pelkonen et al., 1988). This staining technique was compared with fluorographic detection of sodium borotritiide-labeled polysaccharide after gel electrophoresis (Pelkonen et al., 1988). Less than 1 ug of borotritiide-reduced polysaccharide and 5 to 10 ug of unlabeled polysaccharide were detected by fluorography and alcian blue-silver staining, respectively (Pelkonen et al., 1988).

Mycoplasmal lipoglycans and capsules

Polysaccharides covalently attached to lipids were found in several species of Mollicutes. These polymeric surface structures were designated as lipoglycans to distinguish them from Gram negative lipopolysaccharide,

from which mycoplasmal lipoglycans differ not only in their biological effect on host cells, but also in their molecular structures (Smith, 1984). The first description of these polymers was the galactan of M. mycoides. Lipoglycans were described in other species of mycoplasmas, acholeplasmas, and anaeroplasmas (Smith, 1984). The carbohydrate chain of mycoplasmal lipoglycans differ from those described in acholeplasmas and anaeroplasmas, in that the former was composed by homopolysaccharides instead of heteropolysaccharides. The carbohydrate structure detected in the galactan of M. mycoides was identifiable as D-galactofuranosyl-(beta 1 6)-D-galactopyranose which was covalently linked to glycerol, and the corresponding glucan of Mycoplasmas sp. (bovine arthritis strain) was recognized as glucopiranosyl-(beta 1 2) glucopiranose (Buttery and Plackett, 1960; Plackett et al., 1963). No information was published concerning the lipid composition of this lipoglycan. Specific carbohydrate staining and exposure of M. mycoides cells to specific antibodies revealed a visible precipitate that was interpreted as a capsular layer made of carbohydrates (Gourlay and Thrower, 1968).

Capsular material that was stainable with RR and visualized by electron microscopy was reported in M. dispar, M. hyopneumoniae, M. pulmonis (Howard et al., 1974; Tajima and Yagihashi, 1982; Taylor-Robinson et al., 1981) and Ureaplasma urealyticum (Robertson and Smook, 1976). Presence of carbohydrate residues on the capsular envelope of U. urealyticum was also shown by specific reaction with concanavalin A-

Iron dextran (Robertson and Smook, 1976; Whitescarver et al., 1975). The existence of mannose residues on the surface of Thermoplasma acidophilum was demonstrated by the specific binding of concanavalin A.

The chemical composition of a capsular polysaccharide of the F-38 strain was determined using thin layer chromatography. Neutral sugars, such as glucose, mannose and fucose, joined with glucosamine and galactosamine were the main components detected in this capsular material (Rurangirwa et al., 1987).

The insertion of the lipoglycans into the membranes was studied using the production of specific antibodies directed against terminal sugar residues, specific staining and lectin binding. Results from these experiments were interpreted as indication of free exposed sugar residues (Gourlay and Thrower, 1968; Buttery and Plackett, 1960; Schiefer et al., 1974). Based on these results, it has been speculated that polysaccharide chains were held in position by acyl chains which may form part of the lipid bilayer of the membrane (Smith, 1984).

Mycoplasmas and attachment

Limited de novo synthetic capabilities determine the dependence of mycoplasmas on host cells and surrounding milieu. A close interaction between host cells and mycoplasmas has been shown, which allowed mycoplasmas to acquire material such as fatty acids, cholesterol and nucleic acid precursors (Razin, 1978).

The success of the mycoplasmal surface parasitism requires an adequate attachment mechanism to be expressed by the mycoplasma so as to overcome clearance systems present in normal mucosal surfaces (Gourlay and Howard, 1979). Mycoplasmas which effectively surmount these first defense lines, have better chances of colonization, and those with pathogenic capabilities, increased opportunity for invasion and induction of pathogenic changes. Therefore, the attachment of pathogenic mycoplasmas to cells plays a fundamental role in the initiation of colonization and induction of cellular damage (Collier, 1985). The characteristic of the mycoplasma cell surface determines the capacity for adhesion and for evasion of the host defense mechanisms, including phagocytosis and lysis by complement (Gourlay and Howard, 1982).

Since the surface of the mycoplasma and the eucaryotic cell are both negatively charged, a significant repulsive force must be overcome for adhesion to occur (Razin, 1978).

The DLVO theory attempts to explain the attachment between two rigid bodies with like charge (Jones and Isaacson, 1983). This model was based in that bodies approaching one to another are subjected to attractive and repulsive forces which are additive in effect and independent of the distance of separation. At relative long distances of separation, (more than 10 nm), the bodies are held in a state of mutual reversible attraction which is held by weak and easily reversed forces. At shorter distances,(5-8 nm), diffuse ion clouds absorb on each interacting surface

causing repulsion between the bodies. At this point, if these repulsive forces are overcome, and smaller separation distances can be achieved, (1 nm or less), the attraction forces become greater than the repulsive forces again. As a result, the bodies could be maintained in a irreversible attachment state (Jones and Isaacson, 1983). The same authors stated that bacteria do not possess sufficient kinetic energy to overcome this repulsion state therefore, in theory, they would not able to reach the close distance that mediates irreversible attachment. Repulsive and attractive forces between bodies with same charge are directly proportional to the radial curvature of the interacting surfaces. Repulsion and attraction forces decrease as the curvature radii of the surface decrease but, those of repulsion are more affected (Jones and Isaacson, 1983). Changes of shape that reduce radial curvature, such as those experienced by mycoplasmas, or pseudopodia observed in lymphocytes were mechanisms that could overcome the intermediate range of repulsive forces, thus reaching close distances between host cells and microorganism surfaces. The close approach between the adhesin and its receptor enable molecular recognition and specific interaction, creating forces that reinforce the attachment and make it irreversible (Jones and Isaacson, 1983).

Minion et al. (1984) proposed a multistep model of attachment for Mycoplasma pulmonis. This model includes a first step of recognition, followed by exposure of additional masked receptors in the eukaryotic cell

membrane and finally attachment with implied intimate association between membranes. This interaction would allow the mycoplasma to compete for nutrients with the host cell and induce cell damage as a consequence of toxic end products of the mycoplasma metabolism (Razin, 1978). The initial recognition step seems to be mediated by long-range hydrophobic forces (Minion et al., 1984). No specificity was present at this point, as was shown by the variety of cell types to which M. pulmonis attached. Once contact between host cell membrane and mycoplasma is made, a molecular rearrangement in the host surface cell topography is induced by electrostatic pressure, as a result of repulsive forces. Thus the rearranged membrane shows a new conformation with additional binding sites and reduced repulsive forces near those new attachment areas. The reduced electrostatic pressure allows final recognition of receptors and a close association between opposite membranes occurs (Minion et al., 1984). This model proposed for the attachment of M. pulmonis seems to occur through generalized interaction of the mycoplasma membrane, which is different to the specialized tip-like organelle-mediated attachment process observed with M. gallisepticum and M. pneumoniae (Hu et al., 1977; Cassell et al., 1978; Kahane, 1984).

Other models of interactions between mycoplasmas and host cell described in recent years include, a) interaction of mycoplasmal lectin with their specific syaloconjugate receptors exposed in the outer surface of the host cell, b) interaction of protein exposed in the mycoplasma

surface with host cell receptors and, c) hydrophobic interactions between host and mycoplasma membrane constituents (Kahane et al., 1984; Minion et al., 1984).

Mycoplasma gallisepticum is an example of mycoplasma lectin-mediated attachment. The microorganism binds to high molecular weight mucin-like structures with high sialic acid contents, among which N-acetyneuraminic acid seems to be the most prevalent (Glasgow and Hill, 1980).

Mycoplasma pneumoniae attachment was described as a process mediated by interactions between mycoplasma lectin and specific cell syaloconjugate. This mycoplasma species attached to cellular receptor sites that contain N-acetilneuraminic acid using their tip-like organelle. Two membrane proteins were described, designated P1 and P2, and it was demonstrated that they were involved in the attachment of M. pneumoniae (Hu et al., 1977). A parallelism between the lack of attachment capabilities and the absence of P1 was observed. Briefly trypsinated strains of Mycoplasma pneumoniae lost their attachment capabilities as well as protein (P1) band in SDS-PAGE separations. In other studies it was found that P1 is present on the entire membrane surface, and presence of P1 was not exclusive of the adherent tip region, but augmented density of P1 on the mycoplasma polar adhesive area compared with other regions of the mycoplasmal membrane was detected (Kahane, 1984). It was also shown that some of these adhesins were phosphorylated and bound to the cytoskeleton, while others were free on the membrane (Kahane, 1984).

Based on these observations this author linked the phosphorilization of the adhesins with the somewhat selective mobilization of these toward the attachment area. It was therefore suggested that the attachment process involves a changing configuration of the mycoplasmal surface and host cell membrane, which includes rearrangement of the mycoplasmal surface in response to outside stimuli. This changing configuration also might actively adapt the mycoplasma cell shape to the host mucosal surface. Mycoplasmas have often been seen in the respiratory mucosa between cilia and adjacent to microvilli, adopting an elongated shape while coccoidal-shaped mycoplasmas were free in airway lumen (Tajima et al., 1979; Gabridge, 1983). Shift in shape could provide advantages in the attachment process by allowing more intimate contact with the target cell surface, increasing the attachment ability by reducing the curvature radii of the interacting end of the mycoplasma and allowing contact between specific host cell receptors and attachment tip structures in those mycoplasmas species that present such organelles. Also this would permit the mycoplasmas to accommodate between cilia and avoid the mechanical clearance mechanisms of the mucociliary blanket as well as the direct contact with host macrophagic cells (Bredt et al., 1981). Dynamic adaptation to the surface of host cells, which is possible in mycoplasma cells due to the lack of rigid membrane structures, was described in experimental infections with Mycoplasma gallisepticum (Tajima et al., 1979). Attachment of M. gallisepticum to host cells seems to involve

their terminal tip structure, which was always present in attached M. gallisepticum but only rarely in non-attached organisms which were free in the airway's lumen.

A different kind of receptor than those mentioned previously was shown to be expressed by M. dispar (Howard et al., 1974). Red blood cells (RBC) exposed to receptor-destroying enzyme, which inactivated sialic-acid-containing receptors, were as competent for the attachment of M. dispar as untreated RBC. Furthermore, M. dispar seems to have two different receptor sites for RBC. Treatment of mycoplasma cells with pronase or trypsin abolished the capacity to attach to sheep and bovine but not to rabbit RBC. Results from these experiments were taken as indication of the protein nature of the receptors and the affinity of M. dispar for two different RBC receptors.

Participation of the mycoplasmal capsular material in the pathogenic process

Capsule and attachment Studies on microbial adhesion and aggregation have shown the latch effect of polymeric capsules (i.e., polysaccharides) in the adhesion process (Robb, 1984). Bacterial capsules, are in most of the cases polymers built up with repetitive CH2O units, conforming chains with backbones of these units. Practically all bacterial capsules consist of negatively charged polysaccharides with a

high proportion of acid components such as hexuronic, neuraminic acid or pyruvate substitutes (Jann and Westphal, 1975).

Polymers can adhere onto negatively charged solid surfaces in an apparently irreversible but non-specific fashion as a result of large amounts of very weak, polymer-surface, polymer-polymer bonds. This large number of weak bonds, results from the segmental characteristic of polymers, and could provide sufficient energy to overcome electrical repulsion present between interacting surfaces. Polymers may have 10(5) units in their chains, which have a very small probability of coming in contact with the solid surface all at once. A common situation would be that 30 to 60% of these segments contact with the adsorbed surface. Thus, 10(4) units may have contact, which implies that, even when each segment energy bond is small, about 0.01 kT, the total energy bond value of a 10(3)units polymer was 10 kT. This kind of attachment is unlikely to be broken, since cleavage of all bonds at once would be implausible, and even when this occurs, it would certainly be possible to form new bonds.

In addition to extracellular polysaccharide, enzymes and proteins buried into the membranes and arising from extracellular carbohydrate layers (capsule) may provide important binding/adsorption sites in negative to negative-charged surface attachment. This observation is based on the fact that proteins contain anionic and cationic groups which would interact with sites with different charge than the extracellular polysaccharide (Robb, 1984).

Association between attachment and virulence of mycoplasma species was reported by several authors (Gabridge, 1983; Bredt et al., 1981; Yayoshi, 1983) and participation of the mycoplasma capsule in the attachment process was suggested (Green and Hanson, 1973; Wilson and Collier, 1976; Howard et al., 1974).

Electron microscopy pictures of M. dispar attached to RBC shown presence of RR stainable capsular material and fine threads of extracellular material bridging gaps between membranes. The author suggested that this was evidence of participation of M. dispar capsule in the attachment process (Howard et al., 1974). Similar observations and suggestion were made by Tajima and Yagihashi (1982), in their study of the interaction of Mycoplasma hyopneumoniae with porcine respiratory epithelium as observed by transmission electron microscopy. In this experiment, comparative studies in ultrastructure and pathogenic properties were carried with strains of M. hyopneumomiae that had been passed several times under "in vitro" or "in vivo" conditions. More severe pneumonic lesions were produced after experimental intratracheal inoculation of susceptible pigs with the "in vivo" strain than those produced in pigs inoculated with the "in vitro" strain. Observation of the ultrastructure of these mycoplasma strains revealed fibrillar appendages of 5nm in diameter and up to 200mn in length which were present in the "in vivo" but not with "in vitro" strains. These fibrils were seen extending between the mycoplasma and host cell membranes.

Dense RR-stainable carbohydrate capsular material of about 40nm in thickness were also seen in the more virulent ("in vivo") strain. The less virulent strain also showed the same kind of extracellular material, but in this case, the capsule was less thick, about 20nm, and less densely stainable by RR. As part of the same study, electron microscopic examination of the "in vivo" strain attached to ciliated respiratory porcine epithelium showed capsular material filling gaps between the mycoplasma and the host cell membrane. The authors concluded that correlation existed between pathogenicity and amount (thickness) of the capsular material and presence of fibrillar structures. They also proposed that part of the capsule and fibrillar material was lost after several "in vitro" passages of the mycoplasma strain (Tajima and Yagihashi, 1982). Similar results were reported with Mycoplasma pulmonis. After multiple passages in mycoplasma broth medium, the strain lost mouse virulence and hemoadsorbing capacity. Electron microscopy of the avirulent high passage strain revealed amorphous RR-stainable material slimmer than that which was present in the virulent strain. Avirulent strains also were deficient in three protein bands in SDS-PAGE electrophoresis profiles when compared with virulent M. pulmonis.

Capsule and inhibition of phagocytosis A classic dogma regarding capsular polysaccharides indicates that these structures exert antiphagocytic effect and this effect is produced because the negative

electrostatic charge of the capsule repels electronegatively charged phagocytic cells (Kasper, 1986). However, other mechanisms triggered by polysaccharide capsules which result in resistance to phagocytosis were recently described. Clearance of bacteria by polymorphonuclear lymphocytes (PMN) is primarily dependent upon complement deposition on the surface of the microorganism (Kasper, 1986). Complement fraction C3b was identified as the major serum opsonin for most encapsulated bacteria, and the interaction with specific receptors on the surface of PMN mediated the phagocytosis of encapsulated microorganisms. Non-encapsulated microorganisms activated the complement cascade most frequently by the alternative pathway, which resulted in deposition of C3b on their surface, even in the absence of antibody. Encapsulated microorganism were resistant to direct complement activation and required immune complex formation on their surface to activate complement (Kasper, 1986). Immune complex formation resulted in activation of the classic complement pathway which converts C3 to C3b through the classic C4b2a convertase pathway. A steady turnover of C3 to C3b by the alternative pathway also took place, but such turnover occurred with low frequency and did not allow deposition of sufficient C3b on the surface of microorganism to stimulate effective phagocytosis. However, there was an amplification mechanism which assured adequate C3b deposition for effective opsonization. This mechanism was described as a feed-back loop after small amounts of C3b were deposited on the surface of microorganisms (Kasper, 1986). This feed-back loop,

through increased recruitment of factors B, D, and P, results in the formation of alternative pathway convertase C3bBd. Two factors (H and I) block the amplification loop by competition with factors B, D, and P for deposition on C3b complement fraction (Kasper, 1986).

It has been shown that capsular polysaccharides inactivate the amplification loop by at least two different mechanisms. One described mechanism was the affinity of capsular polysaccharides for factor H which then results in deposition of H-C3b rather than the converting enzyme C3BbP on the bacterial cell surface (Edwards et al., 1980). The other described mechanism involved a decrease in the affinity binding for factor B, which again resulted in a increased affinity for the formation of H-C3b and blockage of the amplification loop of the alternative complement pathway (Brown et al., 1983).

Hydrophobicity (surface tension) of the microorganismal surfaces also plays a role in opsonophagocytosis (van Oss and Gillman, 1972; Absolom, 1988). In biological fluids containing IgG most non-encapsulated microorganisms, because of their hydrophobic surface (increased aqueous surface tension) non specifically adsorb IgG. It was also found that an IgG coat renders microorganism more hydrophobic and that IgG and subsequent complement activation with deposition of C1423 enhances their surface hydrophobicity even further (van Oss and Gilman, 1973; Absolom 1988). Phagocytic engulfment by PMNs was increased with more hydrophobic particles, in accordance with surface thermodynamic principles.

Encapsulated microorganisms, which were hydrophilic because of their extracellular coat, did not adsorb IgG at their periphery to any significant extent, and failed to induce phagocytic ingestion (Stinson and van Oss, 1971). In contrast, anticapsular antibody of IgG class specifically coated the outer periphery of capsulated microorganisms, which effectively opsonized them, and caused phagocytic engulfment of the microorganism. Complement activation subsequent to specific interaction with IgG provided further opsonization (Absolom, 1988).

Masking of microorganism binding receptors for opsonic factors by capsular layer has also been suggested. Capsule may be permeable to opsonic factors that recognizes sites beneath the capsular surface, but this structure may present a physical barrier that prevents the opsonic ligand from contacting the phagocytic cell (Horowitz and Silverstein, 1980; Horowitz, 1982).

Microorganisms have been found in host systems forming microcolonies immersed in a glycocalix which creates a protective niche where individual microorganism survive host defense mechanisms. The protective halo in which microorganism were immersed was made of joined individual exopolysaccharides which cannot be penetrated by antibiotic agents. Moreover, mammalian enzymes were incapable of breaking the beta-glycosidic bonds that link many of the microbial carbohydrates. Consequently, phagocytes were also unable to break the exopolysaccharide barrier to engulf these microcolonies (Isenberg, 1988).

Ability to evade phagocytosis was described for some pathogenic mycoplasmas. All but one of fifteen mycoplasma strains tested were resistant to killing by gnotobiotic-calf serum and this was attributed to lack of activation of the alternative complement pathway (Howard, 1980). Also it was suggested that since all these mycoplasmas species were pathogenic for cattle, the ability to avoid activating the alternative pathway was a virulence determinant (Howard and Taylor, 1985).

Ability to attach and multiply on the surface of cultured macrophages and neutrophils without stimulation of ingestion was also described for mycoplasma strains pathogenic for the respiratory tract, including M. dispar and M. bovis (Howard, 1980). Insufficient density of receptors for engulfment on the leukocyte surface or lack of stimulation of the appropriate ones was proposed as an explanation for this phenomenon. However, active inhibitory mechanisms were also considered, since M. dispar and M. bovis have been reported to have an inhibitory effect on the ability of neutrophils to phagocytose E. coli (Howard, 1980). It was also suggested that the ability to evade phagocytosis in the absence of added Ig may be affected by the extra-membranous capsular material demonstrated in several mycoplasma species.

In conclusion, the role of bacterial capsules in the pathogenic process has been proven by several authors. Resistance to phagocytosis and attachment are some of the mechanisms in which participation of bacterial capsule have been consistently shown. Mycoplasmal capsules

share some characteristics with those present in pathogenic bacteria. These characteristics plus indirect evidence of their pathogenic properties have led to the suggestion that they participate in the pathologic process.

MATERIALS AND METHODS

Cell Cultures and Mycoplasmas

Bovine lung fibroblasts (BLF) derived from adult bovine lung tissues by trypsin digestion were grown in Eagles's minimal essential medium (MEM) supplemented with 10% FBS (Gibco Laboratories, Grand Island, NY), and used below the 15th passage level.

Strain SD-0 of Mycoplasma dispar (Tinant et al., 1979) was cloned twice and used at passage level 7 and 50.

Culture Conditions

High and low level passage of M. dispar SD-0 were grown in Friis modified broth medium (FMBM) as previously described (Knudtson et al., 1985). Microorganisms were harvested by centrifugation when cultures reached titers of 10 (6) color forming units (cfu), and were washed three times with phosphate buffer saline solution (PBS) pH 7.2. Mycoplasma suspensions were then aliquoted in 1 ml volumes in tightly sealed tubes, placed in a mixture of dry ice and ethanol until the suspension was frozen, and kept at -70C. High and low passage levels of the microorganism were co-cultured with BLF. For this procedure, roller bottles of 490 cm2 with confluent monolayer were infected with 10 (6) cfu of M. dispar SD-0. Mycoplasmas were previously washed once in PBS (pH 7.2) and resuspended in the same volume of MEM with 10 % of FBS.

A modification of this procedure, which consisted in resuspending M. dispar inside a dialysis bag and then introducing the bag into the cell culture flask was also performed. In this case MEM used to resuspend mycoplasmas was supplemented with 10% FBS whereas the medium used in the cell culture flask was with 20 % FBS.

To determine the effect of FBS on capsule production, mycoplasmas were co-cultured with BLF using alternatively, MEM supplemented with 10%, 20% or 40% of FBS. Microorganism were harvested by centrifugation at 22 hr of growth. Wash and storage procedures were identical to those previously described.

SDS-PAGE

Discontinuous SDS-PAGE was performed with 4% stacking and 12.5% separating gels using the two buffer system described by Laemmli (1970). A vertical slab gel unit (Hoefer SE 600) was used and the procedures were basically as outlined in the Hoefer catalogue (pp 89-93, 1983 Hoefer Scientific Instruments, San Francisco, CA). Mycoplasma dispar grown in FMBM and co-cultured with BLF was washed three times with PBS and resuspended in the same solution at a protein concentration of 3 mg/ml (Lowry et al., 1951). Samples were predigested with Proteinase K (PK), (E. Merck, Darmstadt, Germany), following the procedure described by Hitchock and Brown (1983).

Mycoplasma samples were also treated with several glycosidases and with lipase. For this procedure, samples were incubated with beta galactosidase and beta glucosidase (Sigma, St. Louis, MO) at final concentrations of 500 U/ml, and lipase (Sigma) at final concentration of 10 mg/ml for 30 min at 37 C, and with alpha galactosidase and alpha glucosidase (Sigma) at final concentrations of 500 U/ml, for 30 min at 25 C. Mycoplasma samples were then solubilized with treatment buffer containing 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, and 10% glycerol in 0.063 M Tris-hydrochloride buffer at 100 C for 90 sec. Predigested and non predigested samples were loaded at a concentration of 20 mg/ml of protein per lane and run at a constant current of 40 mA until tracking dye was approximately at 0.5 cm from the bottom of the gel. Bands were visualized by a silver stain procedure (Hitchock and Brown, 1983), and by Commasie Blue staining (Russell and Johnson, 1975). Carbohydrate bands were also visualized by staining with Schiff reagent and with Alcian blue (Kapitany and Zebrowski, 1973; Wardi and Michos, 1972). Apparent molecular weight of the visualized bands were calculated by comparison to known molecular weight standards, (Amersham Corp.), (Sigma, St. Louis, MO).

Experimental Calves

Four colostrum-deprived calves caught at birth in sterile bags were raised in individual isolation rooms, and fed twice a day with antibiotic-

free milk replacement. Mixed grain feed and alfalfa hay were given in increasing amounts beginning in the 2nd week of life. Nasal and ocular samples were taken twice before experimental infection and cultured for mycoplasmas. After inoculation, calves were sampled every two weeks and samples were processed for M. dispar isolation. All calves were inoculated by intratracheal cannulation with 10 (10) cfu of M. dispar SD-0 suspended in 10 ml of sterile PBS pH 7.2. Two calves were inoculated at 15 day intervals, whereas the other two received a single dose of the mycoplasma.

Lung Lavage Fluid

Calves were killed 20 days after the last inoculation and lung lavage fluid (LLF) was obtained by instillation of 500 ml of PBS with 1mM EDTA. After lung massage, 150 to 200 ml of LLF were collected in sterile bottles. At the laboratory, LLF was treated with 1:100 Sputolysin (Calbiochem, San Diego, CA), centrifuged for mucus and cells removal, and kept at -20C. Globulins from the lavage fluid were then precipitated by addition of 50% (v/v) of saturated Ammonium Sulfate solution (pH 7.8). Precipitated immunoglobulins were resuspended in Tris-Tricine with 0.85% of ClNa, Sodium Azide, and 1mM of EDTA and dialyzed against the same solution at 4C for 72 hrs.

Rabbit Antiserum Antigen preparation

A Mycoplasma dispar SD-O low molecular weight silver-stainable band obtained in SDS-PAGE separations was sliced from the gel and macerated by forcing the gel slice through a 20 gauge needle. The sample containing the band was them stored at -70C. Aliquot of this material were mixed 1:10 with hemocyanin (Sigma) and with 1 ml of sterile PBS and emulsified with a equal volume of incomplete Freund adjuvant (Miles Scientific, Naperville. IL). Two New Zealand White rabbits were subcutaneously inoculated with 2 ml of the antigen preparation and boosted every 10 days. Three booster inoculations were given and 10 days after of the last injection rabbits were bled out. Globulins were separated from serum by ammonium sulfate precipitation followed by dialysis as described before and kept at 4C.

Preparations of Fab antibody fractions

Preparation of Fab fragments from rabbit serum antibodies were performed using a kit of prepacked enzyme and affinity columns (Pierce Chemical Company, Rockford, IL). Briefly, rabbit antibodies were treated with immobilized papain during 5 hr at 37 C, centrifuged and the supernatant passed through a Protein A affinity column. Retained Fab were them eluted by washing the column with 10mM Tris buffer (pH 7.5). After collection, the eluted fraction was keep at 5C.

Transmission Electron Microscopy

Aliquots of the microorganisms used for SDS-PAGE, from BFL cell fractions and from cultures in dialysis bags were sedimented by centrifugation and pellets fixed in 3% glutaraldehyde-cacodylate buffer with and without 1% RR (Sigma, St. Louis, MO) for 3 hr at 4C, then washed three times of 30 min each with 0.1 M cacodylate buffer. 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 followed by washes as above. Pellets were dehydrated through acetone series and embedded in Epon 812 resin.

Mycoplasmas harvested from BLF co-cultures and from FMBM were labeled with polycationic ferritin (Sigma) following the procedures described by Jacques and Foiry (1987). Briefly, mycoplasmas were harvested by centrifugation, washed once in PBS and fixed in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 hr at 22C. Fixed mycoplasmas were suspended in 0.1 M sodium cacodylate buffer for 30 min and allowed to react with polycationic ferritin. The reaction was stopped by 10-fold dilution in PBS. Organisms were then sedimented by centrifugation, washed three times in the same buffer, immobilized in 4% agar, washed five times in the same buffer, postfixed in 2% osmium tetroxide for 2 hr, and washed again. Samples were dehydrated as before and embedded in Epon 812 resin.

Estimates of the ratio of encapsulated to non-encapsulated mycoplasmas were obtaining by direct counting under transmission electron microscopy (TEM). The ratio of encapsulated/non-encapsulated

microorganism of the co-cultured supernatant samples were compared against the ratio obtained from mycoplasmas grown in FMBM. Also, the ratio obtained from the cellular fraction of co-cultured mycoplasmas was compared against that obtained from the supernatant fraction of the same co-cultured system. The obtained numbers were analyzed using a chi-square test for a 2 x 2 contingency table.

Effect of increased percentage of FBS on capsular production was determined by measuring the capsular thickness of co-cultured M. dispar with the addition of 10, 20, and 40% FBS.

Immunogold Transmission Electron Microscopy

Co-cultured Mycoplasma dispar were incubated with different dilutions (1:2, 1:5, and 1:10) of LLF or rabbit globulins for 1 h at 22C and washed twice with 1% bovine serum albumin (BSA) in PBS. The mixture was allowed to react with 1.5 mg/ml of Protein A gold (Sigma, St Louis, MO). The preparation was centrifuged at 13,500 xg for 5 min and washed twice with 1% BSA in PBS. The pellet was fixed with 3% glutaraldheide-cacodylate buffer and processed for TEM. No osmium tetroxide post fixation was done on these preparations. Exclusion of LLF or rabbit antiserum from the preparation was used as control of non-specific Protein A attachment. Mycoplasmas grown in FMBM, allowed to react with LLF or rabbit antibodies followed by incubation with Protein A gold as described above, were used as negative control. Specific decoration was defined as the

antigen/antibody-dependent deposition of gold particles outside of the mycoplasmal plasma membrane.

Lectin Agglutination

Concanavalin A (Con A), Soybean agglutinin (SBA), Wheat germ agglutinin (WGA), Dolichos biflorus agglutinin (DBA), Ulex europeus agglutinin I (UEA I), Peanut agglutinin (PNA), and Ricinus communis agglutinin I (RCA I) were assayed by slide agglutination test and agglutination in microtiter plates following the procedures described by Schiefer et al. (1974). Briefly, serial two-fold dilutions of each lectin in 10 mM HEPES-buffered saline solution (pH 7.8) were performed in microtiter plates with U-shaped bottoms. Agglutination in microtiter plates was performed using M. dispar SD-O at passage level 7 grown in FMBM, co-cultured with BLF, and co-cultured-suspended in dialysis bags. These were suspended at 1X concentration in the same HEPES buffer solution, and 25 ul of this suspension was added to each well. After thorough mixing, microplates were covered, incubated for 2 h at 37 C and read using a microtiter mirror. A positive reaction was seen as visible aggregation of particles. A negative reaction appeared as cloudy suspensions.

The titer was given as the reciprocal of the highest lectin dilution causing visible agglutination. Slide agglutination test was performed using one drop (25 ul) of similar mycoplasma suspensions, that were

carefully mixed on a slide with one drop of the lectin solution and the reaction was read 2 min later. The intensity of the agglutination was visually recorded as: 0 no agglutination, (- +) barely visible incomplete agglutination, (+) small definite agglutination, (++) strong agglutination.

Radioimmunoprecipitation

Using information obtained from studies with lectins, a protocol of radioimmunoprecipitation (RIP), using M. dispar capsular material and agarose lectin beads (ALB), was developed. Briefly, M. dispar SD-O co-cultured with BLF were digested with PK as previously described. The obtained capsular material was then treated with phenylmethylsufonylchloride at a final concentration of 10 mM, and allowed to react for 1 h at 25 C with ALB at a final concentration of 0.5 mg ALB/ml of PK-digested M. dispar SD-O. After 5 washes with 10 mM HEPES-buffered saline (pH 8.5), ALB with adhered capsular material were allowed to react with several dilutions of 50 X concentrated LLF globulins obtained from calves experimentally infected with M. dispar SD-O, and with serum globulins from rabbits inoculated with a band sliced from SDS-PAGE separations of PK-treated M. dispar SD-O. Serum and LLF globulins from non-infected animals (rabbit and calf) were used as a negative control. This reaction was performed for 90 min at 4C. After 5 washes in the buffered solution previously described, 0.1 uCi of 125-I protein G

(Amersham, Arlington Heights, IL), was added to each dilution and incubated 1 h at 25 C in a plate shaker. The ALB were then washed five times in the same buffer and radioactivity as counts per minute (CPM) was detected in a gamma counter (Beckmann, Palo Alto, CA).

The same preparation with the exclusion of capsular material was used as a control, and 10 mM HEPES buffered saline (pH 8.5) was used as background sample.

A blocking reaction of the RIP assay was performed by using Fab fractions of rabbit anti band globulins. The Fabs were incubated with capsular material attached to ALB for 1h at room temperature previous to the incubation with LLF calf globulins. Subsequent steps were similar to those described above. As a control, a similar reaction was performed using Fab fractions of non-inoculated rabbits. Non-specific reaction of globulins, Fab fractions, and [125] I protein G with ALB were also tested.

RESULTS

SDS-PAGE

A silver-stainable proteinase K-resistant band with a approximate molecular weight of 4 kilodaltons (kD) was detected in electrophoretic studies of co-cultured M. dispar S-DO. This band was not present in M. dispar grown in FMBM (Fig. 1). Similar preparations of M. dispar revealed a analogous molecular weight band that was stained with silver stain, alcian blue and with periodic acid-Schiff reagent (Fig. 2). The band was not present in silver stained separations of PK digested M. dispar SD-O grown in FMBM at passage level 50 (Fig. 1). Treatment of co-cultured M. dispar SD-O with alpha and beta galactosidase or lipase previous to SDS-PAGE separations, resulted in digestion, or partial digestion in the case of lipase treatment, of the low molecular weight band. No effect was seen when alpha or beta glucosidase were used instead (Fig. 3).

Transmission Electron Microscopy

Very few mycoplasmas with RR-stainable capsular material were detected by transmission electron microscopy on preparations of M. dispar SD-O passage level 7 grown in FMBM. When similar studies were carried out with M. dispar SD-O at passage level 50, no significant difference in the number of capsulated mycoplasmas was detected when compared with M. dispar passage level 7 (Table 2).

FIG. 1. SDS-PAGE profiles of M. dispar SD-O co-cultured with bovine lung fibroblasts (lanes B and C) grown in Friis modified broth medium (lanes E and D). Molecular weight standards given are run on lane A and sizes are shown on the left. Arrow shows a 4kD molecular weight, silver stainable, proteinase-K resistant band.

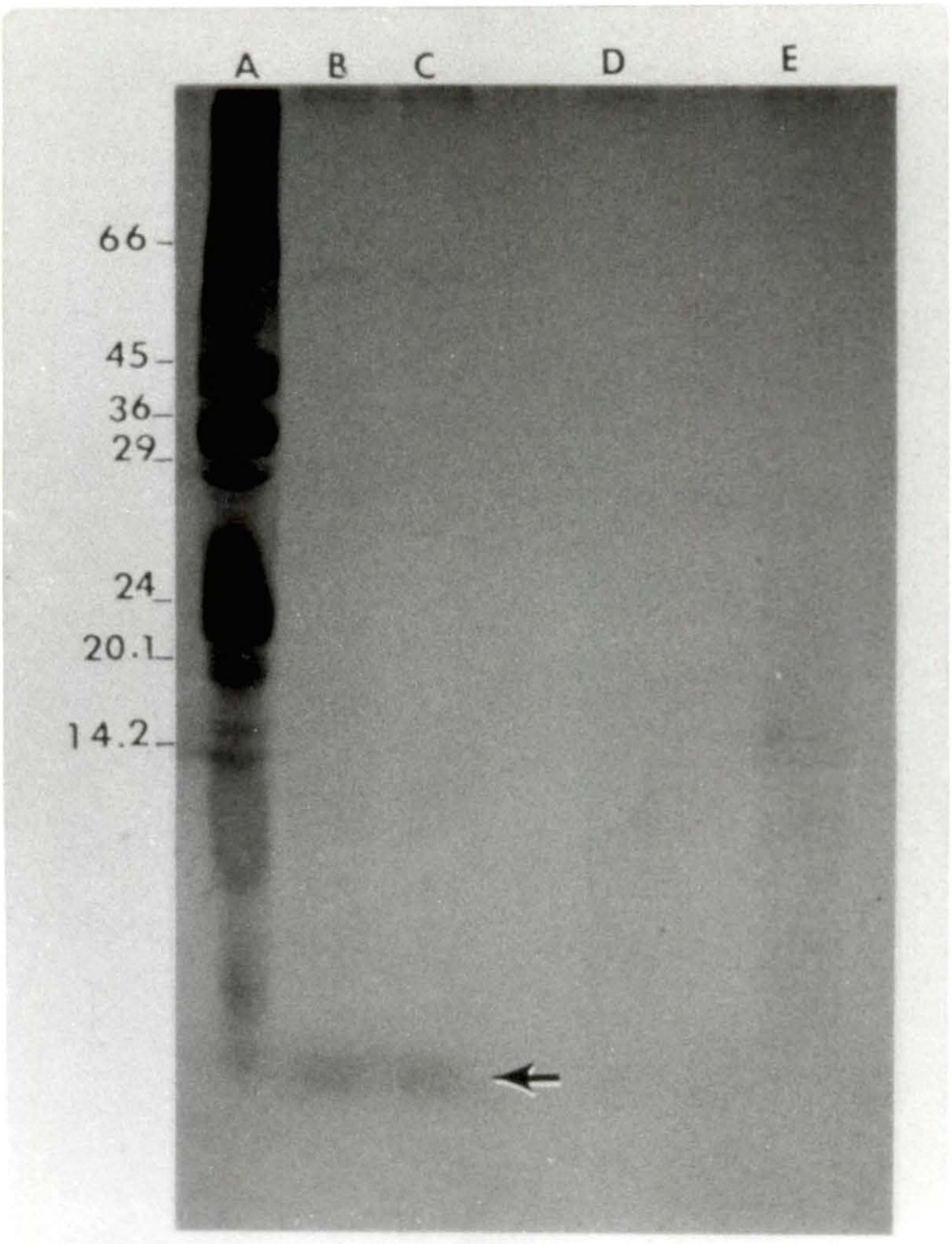
A
B
C
D
E
66
45
36
29
24
20.1
14.2

FIG. 2. SDS-PAGE of M. dispar SD-O co-cultured with bovine lung fibroblasts. Proteinase-K resistant band stained with Alcian blue (A); periodic acid-Schiff (B); and silver stain (C). Molecular weight standards in kD are shown in the left.

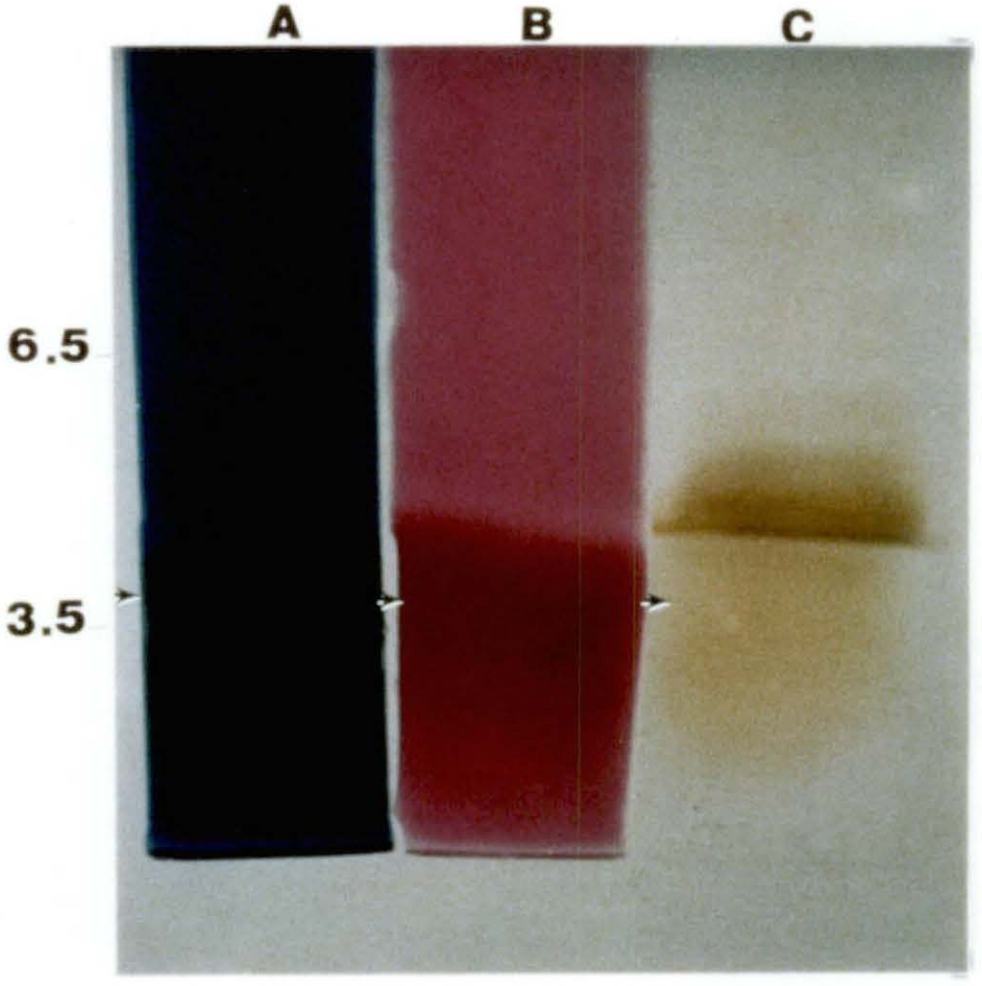
A
B
C
6.5
3.5

FIG. 3. SDS-PAGE profiles of M. dispar SD-O co-cultured with bovine lung fibroblasts and digested with glycolytic and lipolytic enzymes; with alpha glucosidase (lanes A and B); with beta glucosidase (lanes C and D); with alpha galactosidase (lanes E and F); with beta galactosidase (lanes G and H); with lipase (lane I). Arrows indicate disappearance of the 4kD band. Molecular weight standards given in kD are shown in the left.

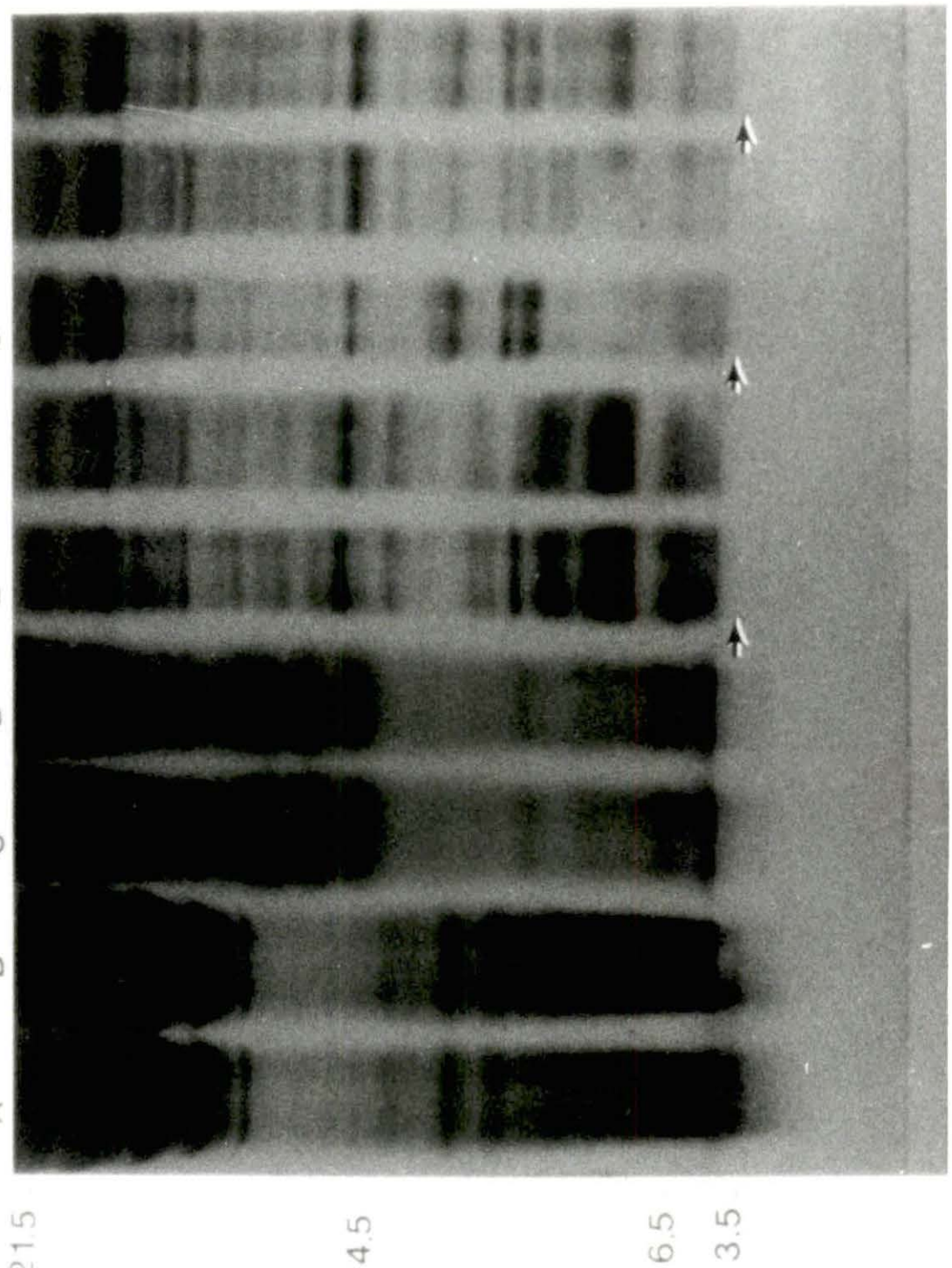
A
B
C
D
E
F
G
H
I
21.5
14.5
6.5
3.5

Table 2. Effect of passage level 7 and 50 in modified Friis broth medium on the percentage of capsulated and non-capsulated mycoplasmas[a]

Passage level	Capsulated *M. dispar*	Non-capsulated *M. dispar*
7	2.8	97.2
50	2.6[b]	97.4
		Chi-square value= 0.0148

[a]Capsular material detected by ruthenium red staining and transmission electron microscopy examination.

[b]Not significant at $p \leq 0.05$.

Increased percentage of mycoplasmas surrounded by RR stainable capsular material were noticed when *M. dispar* SD-O was co-cultured with BLF than those detected on *M. dispar* grown in FMBM (Fig. 4 and 5). The difference in numbers of capsulated microorganism between mycoplasmas co-cultured with BLF or grown in FMBM was highly significant (Table 3). A feature found in the latter preparations was fusion of capsular material among mycoplasma cells that presented close interaction.

FIG. 4. Electron micrograph of Mycoplasma dispar SD-0 grown in Friis modified broth medium and stained with ruthenium red. X 57510.

FIG. 5. Electron micrograph of Mycoplasma dispar co-cultured with bovine lung fibroblasts and stained with ruthenium red. X 49200.

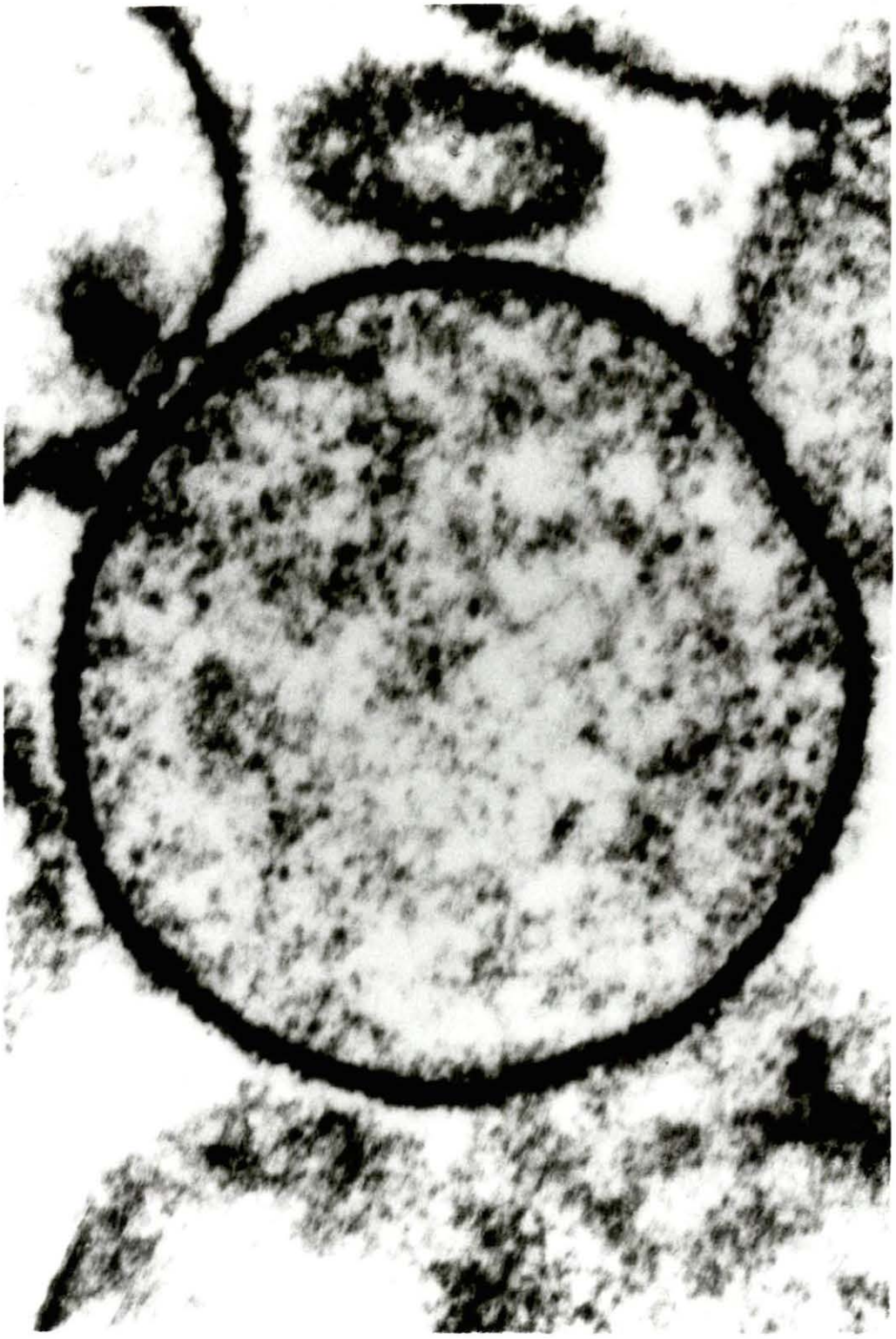

Table 3. Effect of culture in modified Friis broth medium and co-culture with bovine lung fibroblast on the percentage of M. dispar expressing capsular material[a]

	Capsulated M. dispar	Non-capsulated M. dispar
Cultured in FMBM[b]	4	96
Co-cultured with BLF[c]	29*	71
	Chi-square value = 34.84	

[a] Capsular material detected by ruthenium red stainig and transmission electron microscopy examination.

[b] Mycoplasmas grown in Friis modified broth medium.

[c] Mycoplasmas co-cultured with bovine lung fibroblast.

* Significant at $P \leq 0.05$.

Also was detected that the number of capsulated mycoplasmas was higher in the cell fraction than in the supernatant fraction of the co-cultured system, and this difference was statistically significant (Table No 4).

Mycoplasma dispar enclosed in dialysis bag and co-cultured with BLF also exhibit capsular material producction in similar proportions than above described for co-cultured M. dispar. The proportion of capsulated

mycoplasmas observed in mycoplasmas grown in MEM with 10% of FBS inside of dialysis bags and these bags suspended in MEM with 20% FBS and co-cultured with BLF, were higher than the observed in M. dispar grown under similar conditions but without co-culture with BLF. These differences were highly significant (Table 5).

Table 4. Percentage of capsulated and non-capsulated M. dispar detected in supernatant and cell fraction of inoculated bovine lung fibroblast cell cultures[a]

	Capsulated M. dispar	Non-capsulated M. dispar
CF[b]	66*	34
SF[c]	33	67
		Chi-square value = 21.8

[a]Capsular material detected by ruthenium red staining and transmission electron microscopy examination.

[b]Capsulated and non-capsulated mycoplasmas detected in the cellular fraction of bovine lung fibroblast cell cultures.

[c]Capsulated and non-capsulated mycoplasmas detected in the supernatant fraction of bovine lung fibroblast cell cultures.

*Significant at $P \leq 0.05$.

Table 5. Effect of culturing M. dispar in dialysis bags[a]

	Capsulated M. dispar	Non-capsulated M. dispar
Co-cultured with BLF[b]	38*	62
Cultured in MEM[c]	9	91
		Chi-square value = 23.3

[a]Mycoplasmas were suspended in Eagle's minimal essential medium with 10% bovine fetal serum in dialysis bags and cultured with and without bovine lung fibroblast in Eagle's minimal essential medium with 20% FBS.

[b]Mycoplasmas included in dialysis bags and co-cultured with bovine lung fibroblast.

[c]Mycoplasmas included in dialysis bags and cultured in Eagle's minimal essential medium with 20% fetal bovine serum.

*Significant at $P \leq 0.05$.

Mycoplasma dispar SD-0 capsular material was also visualized by labelling it with polycationic ferritin (Fig. 6). Greater number of capsulated mycoplasmas were found among those microorganism co-cultured with BLF compared with those grown in FMBM, differences that were statistically significant. The ratio of capsulated to non-capsulated mycoplasmas grown in FMBM or co-cultured with BLF was determined for these preparations were similar to those detected previously using staining with RR (Table 6).

FIG. 6. Electron micrograph of Mycoplasma dispar co-cultured with bovine lung fibroblasts and labelled with polycationic ferritin. X 57510.

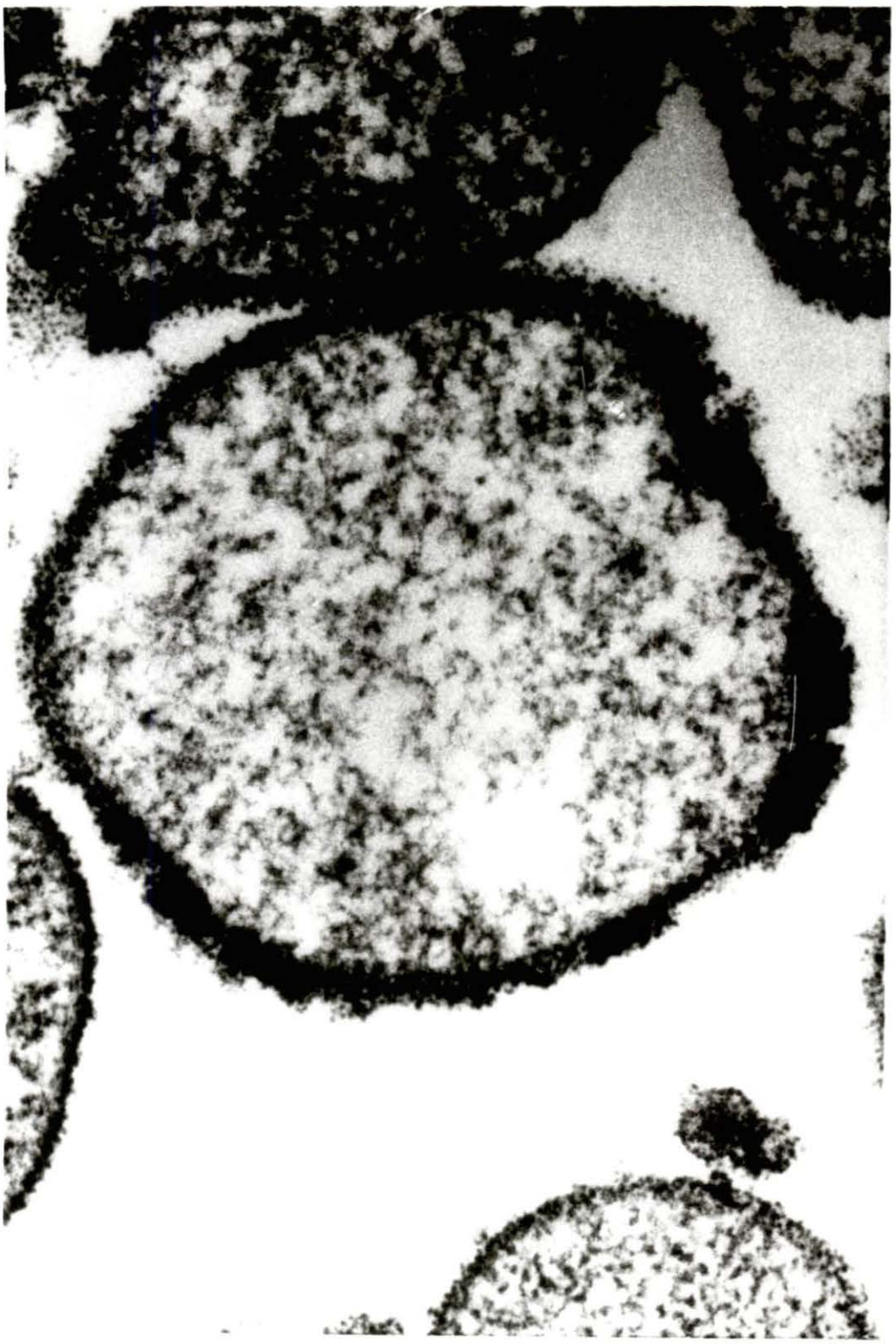

Table 6. Effect of culture in modified Friis broth medium or co-culture with bovine lung fibroblasts on the percentage of M. dispar expressing capsular material[a]

	Capsulated M. dispar	Non-capsulated M. dispar
Cultured in FMBM[b]	7	93
Co-cultured with BLF[c]	30*	70
	Chi-square value = 17.5	

[a]Capsular material detected by polycationic ferritin labeling and transmission electron microscopy examination.

[b]Mycoplasmas grown in modified Friis broth medium.

[c]Mycoplasmas co-cultured with bovine lung fibroblasts.

*Significant at $P \leq 0.05$.

No significant differences were founded in the capsular thickness of co-cultured M. dispar SD-0 when increasing percentages of FBS were added to BLF cell cultures (Table 7).

Immunogold TEM

Specific decoration of BLF-co-cultured M. dispar SD-0 was detected in experiments using globulins from LLF of calves inoculated with these mycoplasma (Fig. 7). This specific decoration was not present in all

FIG. 7. Immunogold reaction of co-cultured Mycoplasma dispar following with 1:10 diluted lung lavage fluid-derived globulins from convalescent calf. X 49200.

capsulated mycoplasmas, but was dependent on the antibody concentration,

Table 7. Effect of addition of fetal calf serum to the cell culture medium on capsular material thickness of co-cultured mycoplasmas[a]

Experiment number	Percentage of fetal bovine serum		
	10	20	40
1	236[b]	254	270
2	236	228	200
3	220	200	218
4	227	200	218
5	212	216	191
Mean	226	220	219
SEM[c]	3	5	5

[a] M. dispar was co-cultured with bovine lung fibroblasts and with addition of 10%, 20%, and 40% of fetal bovine serum.

[b] Average of 5 capsular thickness measurements given in Å. Mycoplasmas capsules were stained with RR.

[c] Overall Mean thickness and standard error of the mean M. dispar capsular thickness.

since more decorated mycoplasmas were seen when undiluted LLF was used (data not shown). No specific gold decoration was present when M. dispar

SD-O grown in FMBM was used. A specific gold decoration was also detected when co-cultured M. dispar were incubated with anti-4K rabbit antibodies (Fig. 8).

Lectin studies

Agglutination of Mycoplasma dispar

Mycoplasma dispar SD-O grown in FMBM, co-cultured with BLF or suspended into dialysis bags and co-cultured with BLF were strongly agglutinated by lectins which react with galactose residues and to a lesser degree by lectins which react with glucose residues (Table 8). Ricinus communis agglutinin and Dolichos biflorus agglutinin, which react with D-galactosyl and N-acetylgalactosaminyl residues respectively, were lectins with the highest titre of agglutination with M. dispar capsular material.

Radioimmunoprecipitation

Capsular material from M. dispar SD-O co-cultured with BLF and treated with PK could be captured by ALB. These capsule-coated ALB react with globulins of LLF from calves infected with M. dispar as is shown in Table 9. Similarly, identical preparations of capsular material from M. dispar attached to ALB specifically react with serum globulins of a rabbit immunized against the PK-resistant band cut out of SDS-PAGE gels.

FIG. 8. Immunogold reaction of co-cultured Mycoplasma dispar SD-0 preincubated with anti-4kD band rabbit globulins used at 1:10 dilution. X 49200.

Table 8. Intensity and titer of agglutination of M. dispar with several lectins[a]

Mycoplasma culture conditions	Agglutination by lectins						
	Con A	SBA	WGA	DBA	RCA I	UEA I	PNA
M. dispar FMBM[b]	(+-)[c] (4)[d]	(+) (64)	(+-) (4)	(+) (64)	(+) (128)	(+) (32)	(+) (64)
M. dispar BLF[e]	(+-) (8)	(+) (68)	(+) (32)	(++) (128)	(+ +) (256)	(+) (64)	(+) (64)
M. dispar BLF db[f]	(+-) (4)	(+) (32)	(+-) (4)	(+) (64)	(++) (128)	(+-) (8)	(+) (128)

[a] M. dispar grown under several culture conditions and allowed to react with concanavalin A (CON A); soybean agglutinin (SBA); wheat germ agglutinin (WGA); dolichos biflorus agglutinin (DBA); ulex europaeus agglutinin (UEA I); peanut agglutinin (PNA); ricinus communis agglutinin I (RCA I).

[b] M. dispar grown in modified Friis broth medium.

[c] Intensity of agglutination was visually recorded in the lower dilutions as: no agglutination 0; barely visible agglutination (+-); small definite agglutination (+); strong agglutination (++).

[d] The numbers given in parentheses represent the reciprocal of the highest dilution of lectins causing visible aggluination of mycoplasmas.

[e] M. dispar co-cultured with bovine lung fibroblast.

[f] M. dispar co-cultured with bovine lung fibroblast within a dialysis bag.

Table 9. Radioimmunoprecipitation of purified M. dispar capsular material[a]

Experiment	LLI[b]	LLI	LLC[c]	RAG[d]	RCG[e]	Bkgr[f]
1	4197[g]	3150	904	1645	71	221
2	4548	3515	1054	1815	854	226
3	3826	2895	761	1779	731	230
Mean	4190	3187	907	1746	788	226
SEM	361	312	147	90	62	5

[a]Purified M. dispar capsular material was attached to agarose lectin beads and allowed to react globulins from LLF of calves and with globulins from rabbit, followed by reaction with [125] I-protein G.

[b]Lung lavage fluid of inoculated calf number.

[c]Lung lavage fluid control calf.

[d]Rabbit anti-band globulins.

[e]Rabbit control globulins.

[f]Background.

[g]Count per minute of [125] I.

The use of Fab fraction of rabbit anti-4 kD globulins resulted in blocking of the RIP of capsular material by calf LLF derived antibodies. In contrast, the use of Fab fraction of non-inoculated rabbit globulins did not block the reaction. These results are shown in Table 10.

Table 10. Blocking of M. dispar capsule radioimmuneprecipitation by Fab fraction of rabbit anti-4K rabbit globulins[a]

Experiment	FIR[b]	FNR[c]	NF[d]
1	2426[e]	9944	10426
2	1940	4473	7993
3	1276	6243	4992
4	2105	11377	4430
Mean	1937	8009	6960
SEM[f]	485	3199	2790

[a]Capsular material attached to agarose lectin beads was allowed to react with Fabs previous to reacting with calf LLF-derived antibodies.

[b]FIR = Fab fraction from rabbit anti-4K band rabbit antibodies.

[c]FNR = Fab fraction from non-inoculated rabbit antibodies.

[d]NF = No Fab fractions were added.

[e]Counts per minute of [125] I.

[f]Standard error of the mean

DISCUSSION

Capsular material of M. dispar could be produced under "in vitro" conditions as a result of the interaction between the microorganism and mammalian cells. A direct evidence was the significantly increased numbers of capsulated mycoplasmas detected in the co-culture system as compared with those present in FMBM. Increased expression of capsular material as a result of co-culture of mycoplasmas with animal cells was previously reported (Yagihashi et al., 1986). These authors described increase in thickness of the M. gallisepticum capsule as a result of serial passage in chick embryo cell cultures. While these data are in agreement with data presented here, the present data also show that high passsage level non-capsulated mycoplasmas can express capsule after a single passage in cell cultures. The positive reaction of the capsular material with RR indicated the acidic (negatively charged) nature of the product. The capsule could be composed of acidic polysaccharides, a supposition that is supported by the previously proposed specific reaction of RR with these anionic residues (Luft, 1971).

A parallelism between the extracellular material detected with RR and polycationic ferritin was previously indicated (Gilmour et al., 1985). In the present study, M. dispar SD-O capsular material was stained with ruthenium red and labeled with polycationic ferritin. Therefore, based on the similar proportions of capsulated mycoplasmas detected by both

methods, it could be suggested that ruthenium red and polycationic ferritin recognized the same capsular material.

Additional evidence of the "in vitro" expression of capsule comes from the results obtained in SDS-PAGE. A modification of the silver stain method (Tsai and Frasch, 1982) allows the preferential detection of polysaccharide chains in LPS analyzed by SDS-PAGE separations. The relative mobility pattern of LPS preparations in polyacrylamide gels, which increases in positive correlation with shortening of polysaccharide chains (Hitchock and Brown, 1983) has been used as a tool for examining LPS heterogeneity based on the length of the carbohydrate chains (Darveau and Hancock, 1983; Progulske and Holt, 1984). In this work we have utilized the same criterion for the study of mycoplasmal capsular material, and this constitutes a previously unreported approach. The 4 kD PK-resistant band detected in SDS-PAGE separations of co-cultured M. dispar was correlated with the presence of capsule, since no similar band was detected in SDS-PAGE separations of M. dispar grown in FMBM. This band could be visualized by staining with modified silver stain, periodic acid-Schiff or Alcian Blue, all of which indicate the presence of carbohydrate residues (Kapitany and Zebrowski, 1973; Koprinsky et al., 1986). Taken together, results from electron microscopy, SDS-PAGE, lectin agglutination, and glycosidase digestion of the 4 kD band allow us to conclude that co-cultured M. dispar produces capsule containing carbohydrate in which galactose residues are present.

In previous studies on mycoplasmal lipoglycans, it was reported that surface exposed carbohydrate chains held in position by acyl chains could form part of the lipid bilayer of the membrane (Smith, 1984). The digestion of the 4 kD band by lipase could be interpreted as presence of lipid on the capsule of M. dispar but we do not have evidence at this point of the lipid anchorage of the carbohydrate fraction to the mycoplasma cell membrane nor of the orientation of the lipid molecules. Alternatively, the lipase enzyme used had residual contaminating glycosidase activity, a possibility that can not be discounted.

Specific induction of capsule formation resulting from the interaction of the mycoplasma and the mammalian cell could be delineated from the results presented here. The reversion of non-capsulated high passage level M. dispar to the capsulated form after the first passage on cell culture, clearly indicates functional activation, which would depend on the activation of a gene or a family of genes that code for capsule expression. The fact that increased numbers of capsulated mycoplasmas were detected in the cellular fraction than in the supernatant fraction could indicate that expression of capsule was dependent on contact with mammalian cells or on a higher concentration of capsule-inducer triggering factor(s) present in this fraction. Results from TEM examination of co-cultured M. dispar kept within dialysis bags support the view that expression of capsule was not dependent on direct contact with mammalian cells, but induced by dialyzable factors from these cells. It is also

feasible that non-capsulated mycoplasmas detected on the supernatant fraction represent microorganism with defective capacity for adherence.

If mycoplasma capsule behaves in the same way as bacterial capsule does in the attachment process, then defectively capsulated mycoplasmas will have reduced attachment capacity and therefore diminished pathogenic capabilities. It was previously reported that pathogenic M. hyopneumoniae presented increased amount of capsular material when compared with non-pathogenic mycoplasmas of the same species. In addition, non-pathogenic mycoplasmas were commonly detected in the tracheal lumen in contrast with pathogenic mycoplasmas which were attached to the respiratory epithelium (Tajima and Yagihashi, 1984). Therefore it is possible to establish a correlation between capsule expression, attachment and virulence.

In the present study it was shown that increased amounts of fetal bovine serum added to BLF cell cultures at the time of M. dispar co-culture did not induced increase in the capsular thickness of the mycoplasmas, making unlikely the possibility that capsular material could result from deposition of fetal bovine serum components. It has been proposed that capsule enhances conditions for nutrient captation (Costerton and Irvin, 1981). Increased production of capsule would be a superfluous energy waste, since more nutrients would be available in the culture medium and less competition with other cells would be needed.

Slight difference in titer as well as in intensity of agglutination was observed when mycoplasmas grown in either MFBM or co-cultured with BLF were incubated with lectins. These results can be interpreted as that the agglutination was not only an indication of capsule expression but also presence of carbohydrates residues on the external surface of the mycoplasma cell, which in this case was galacatose and/or N-acetylgalactosamine.

Results of immunogold EM and RIP presented here can be taken as indication that the "in vitro" produced capsule are antigenically related to the capsule produced in natural infection by this mycoplasma. The recognition of "in vitro" produced M. dispar capsular material by globulins derived from LLF of convalescent calves and from anti-4 kD band rabbit antiserum not only is evidence of the antigenic relatedness but also confirms the identity of the 4 kD band. In addition, the blocking effect of rabbit's Fab fractions can be taken as indication that globulins derived from convalescent calves and from rabbit serum, both recognize similar capsular epitopes. The overall implications of these resuls are that the "in vitro" produced capsule was a mycoplasmal product rather than a contaminant from of the surrounding milieu, that this capsule material was very similar to the "in vivo" product, and that this material was immunogenic.

In conclusion, results obtained in the present study can be taken as indication of the carbohydrate composition of the capsular material of

M. dispar, in which galactose and /or N-acetylgalactosamine can be identified, and where lipids may also be present. Capsular material of M. dispar produced as a consequence of co-culture with bovine lung fibroblasts is antigenically related to the capsule exhibited by this mycoplasma under "in vivo" conditions. Consequently, the method proposed here for M. dispar capsule production allows the obtention of unlimited amounts of capsular material and is therefore applicable to structural and functional studies of the M. dispar capsule.

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