

Characterization of Membrane and Cytoplasmic Antigens of *Mycoplasma arginini* by Two-Dimensional (Crossed) Immunoelectrophoresis

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Two-dimensional immunoelectrophoresis was employed to electrophoretically identify membrane and cytoplasmic antigens of *Mycoplasma arginini* G-230. Five distinct cytoplasmic antigens were observed in soluble fractions prepared by digitonin lysis with electrophoretic mobilities (relative to bovine albumin) ranging from 0.36 to 0.86; four of these were common to other *M. arginini* strains: leonis and 23243. Five membrane antigens were identified, two of which (0.4 and 0.2) were common to the other *M. arginini* strains. The most prominent antigenic component of the membrane fraction (the complex membrane antigen) was electrophoretically heterogeneous, showing four antigenically related components with electrophoretic mobilities of 1.2, 0.95 to 0.76, and 0.05. The complex membrane antigen was exposed on the outside of the mycoplasmic cell because absorption of antiserum with live organisms removed antibody to this component. Antibodies to two other membrane components (0.6 and 0.2) were removed by absorption with Triton-solubilized membranes, but not by untreated membranes, indicating that these components were, at best, little exposed on either membrane surface. Antiserum was prepared against the complex membrane antigen using precipitin lines from two-dimensional electropherograms as the immunogen. This antiserum reacted only with the complex membrane antigen and did not react with the other *M. arginini* strains, indicating that the complex membrane antigen was unique to strain G-230.

As organisms bounded only by a unit membrane and with no internal membranes, the *Mycoplasmatales* have proven to be highly useful model organisms for study of structure and function of membranes (22). Few studies have been carried out on the antigenic structure of membranes (14); these have concerned primarily *Mycoplasma hominis* (9), *Acholeplasma laidlawii* (2, 11, 20), and *Mycoplasma gallisepticum* (18, 20). Interestingly, the number of antigenic components that can be demonstrated is relatively few in comparison with the abundance of components resolved on polyacrylamide electrophoresis of mycoplasmic cell membranes (1, 25). Previous studies from this laboratory using two-dimensional (crossed) immunoelectrophoresis have shown that as many as 20 components may be recognized in lysed whole *Mycoplasma arginini* organisms and that large variation in antigenic components was observed between strains of this species (28, 29). This report is concerned with the determination of the source of immunoelectrophoretically identified antigens of *M. arginini* in

terms of membrane, cytoplasm, and surface location.

MATERIALS AND METHODS

Antigens. The organisms studied were *M. arginini* G-230 obtained from M. F. Barile (3), *M. arginini* strain leonis obtained from W. Dowdle (30), and *M. arginini* 23243 (7) obtained from the American Type Culture Collection.

The medium used for preparing serological test antigens was soy peptone, yeast extract dialysate broth (21) or soy peptone, fresh yeast dialysate broth (12) supplemented with 5 to 6% "agamma" horse serum, 100 µg of penicillin per ml, 0.33 mg of thallium acetate per ml, and 10 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer at a final pH of 7.3. Immunogens were grown in soy peptone-yeast extract dialysate broth with 10% agamma rabbit serum substituted for horse serum for strains G-230 and leonis. Ten percent agamma calf serum was used for the 23243 strain. Agamma sera were obtained from North American Biologicals. A 1% inoculum of an actively growing culture was employed, and cultures were incubated at 37°C until a faint haze was observed. Cells were harvested by centrifugation at 8,000 × g for 15 min,

washed three times, and resuspended in 0.15 M NaCl buffered with 10 mM TES at pH 7.3. Protein concentration of antigens and immunogens was measured by the method of Lowry et al. (19) with crystalline bovine albumin as standard. The protein concentration of each serological test antigen was adjusted to 1 mg per ml. All antigens and immunogens were stored at -20°C until used.

Antisera. The immunization procedure for preparing antiserum to complete organisms was as previously described (13). Briefly, 10 to 15 mg of protein of cells was used as immunogen for each rabbit. Half of the immunogen dosage was given with Freund incomplete adjuvant intramuscularly. This was followed 3 weeks later by five injections of fluid antigen intravenously at 3- to 4-day intervals. Animals were bled 7 days after the last injection. Antiserum to the 0.95 precipitin line was prepared by immunizing a rabbit with immunoprecipitate material obtained from two-dimensional electropherograms as adapted from the method of Crowle et al. (4) by Hackman and Kenny (unpublished data). Specific precipitin line material was excised from 20 two-dimensional electropherograms and homogenized by repeated ejections from a syringe equipped with a 27-gauge needle. Half the material was injected intradermally in Freund complete adjuvant. After 14 days, the animal was given an intramuscular booster injection with the remaining material in Freund incomplete adjuvant. The animal was bled 2 weeks later, and the serum was concentrated two times with membrane cones (Diaflo, Amicon Corp., Lexington, Mass.). The amount of immunogen is unknown, but would be substantially less than the 200 μg of cell protein fractionated on the 20 slides.

Lysis of cells. Digitonin lysis was initially performed as generally described for *M. hominis* by Rottem and Razin (26). Specifically, varying amounts of digitonin (20 to 60 μg per ml) were added to suspensions of cells in 0.25 M NaCl containing 35 to 60 μg of cell protein per ml. Incubation was at 37°C ; lysis was monitored by measurement of absorbance at 500 nm with a spectrophotometer (Gilford model 2400-S). In subsequent experiments, the concentration of digitonin used was 40 μg per ml, and incubation was for 20 to 30 min. Sonication of 1 to 3 ml of cell suspension at 7 to 10 mg of protein per ml was for 10 s at maximum power with a Bronwill sonicator. The samples were cooled in an ice-water bath during treatment.

Separation of membrane and soluble fractions. Digitonin-lysed cells were centrifuged at $8,000 \times g$ for 15 min to remove unbroken cells. Membranes were sedimented at $34,000 \times g$ for 60 min and washed three times in 0.25 M NaCl. The supernatant fraction was concentrated by centrifugation in Diaflo membrane cones. The fractions were adjusted to contain 1 mg of protein per ml in 0.25 M NaCl.

Absorption of antisera. Antiserum samples were absorbed by intact cells, washed membranes, or Triton-solubilized membranes. For absorption by intact cells, a 700-ml batch of cells was grown to late-exponential phase. The culture was divided into two aliquots, and the cells were harvested by centrifugation at $8,000 \times g$ for 15 min. The pellet from one

aliquot (2 to 3 mg of cell protein) was resuspended into 4 ml of antiserum at 4°C (to avoid lysis) and held for 30 min. The cells were removed by centrifugation at $10,000 \times g$ for 30 min. For some experiments, as cited in the text, a second absorption of the serum was carried out at 4°C overnight. Absorption with membranes was carried out by resuspending a washed membrane pellet (4 to 6 mg of protein) into 5 ml of antiserum, holding the mixture on ice for 90 min, and recentrifuging at $34,000 \times g$ for 60 min. Solubilized membranes (0.2 ml, containing 0.4 mg of protein with 0.1% Triton) were mixed with 1.0 ml of antiserum, incubated at 37°C for 60 min, and kept overnight at 4°C . The precipitate that formed was removed by centrifugation at $1,000 \times g$.

Immuno-electrophoresis. The two-dimensional immuno-electrophoresis technique of Laurell (16) was used as adapted by Thirkill and Kenny (28). Briefly, glass slides (4 by 4 cm) were covered with 3 ml of 0.5% agarose (Matheson Scientific, Inc.) in barbital buffer at pH 8.6 and ionic strength 0.05, which also contained 0.5% Triton X-100. Ten to twenty micrograms of antigen (Triton solubilized) was employed in 10 μl of sample. Electrophoresis for the first dimension was for 120 min at 3 V per cm as measured directly on the agarose. Second-phase electrophoresis was carried out for 6 h at 3 V per cm or 12 h at 1.5 V per cm with 0.1 to 0.6 ml of antiserum in the second phase gel (2 ml total volume). The peaks in the two-dimensional profiles were identified by the relative migration distances with respect to bovine serum albumin (29).

Rocket electrophoresis was done as adapted from the technique described by Weeke (31). Six milliliters of agarose, as described above, was applied to a slide (5 by 7.5 cm). Wells of 3-mm diameter were cut approximately 5 mm from the narrow edge of the slide. All but a 1.5-cm-wide strip of agarose containing the wells was removed and replaced by 5 ml of agarose containing 0.1 ml of antiserum. Electrophoresis was for 5 h at 1.5 V per cm per slide, which was sufficient for equilibrium.

The two-dimensional immuno-electrophoresis procedure was modified for tandem separation of two samples (15) as follows. Two sample wells with 1.0-cm distance between centers were punched at the origin. The reference sample (unfractionated cells) was placed in the outer well. Either the supernatant or the membrane sample was placed in the inner well. Other details for these runs were as above. Related or identical antigens showed confluence of peaks with reactions of identity.

RESULTS

Lysis and separation of fractions. Lysis of cell suspensions of *M. arginini* at various concentrations of digitonin appeared as a rapid decrease in turbidity at each digitonin concentration tested and resulted in similar degrees of turbidity loss over the range of digitonin concentrations (Fig. 1). After centrifugation of cells lysed at 40 μg of digitonin per ml, the relative distribution of antigens between pellet

and supernatant was assessed by rocket electrophoresis (Fig. 2A). Digitonin-treated uncentrifuged cells were used as controls. The four tallest peaks in the cell samples appeared to be supernatant material; the low, densely staining peak appeared to be membrane (the distribution of these components was verified in two-dimensional profiles [see below]). The membrane was clearly enriched for the densely staining component and devoid of supernatant components, whereas the supernatant contained little membrane material. In contrast, although membranes prepared by sonication (Fig. 2B) appeared not to contain supernatant material, the supernatant contained abundant

membrane presumably in small fragments that do not sediment at $34,000 \times g$.

Antigenic profiles. The antigenic profile produced by disrupted whole cells (Fig. 3) differed from that previously published (29) for two rea-

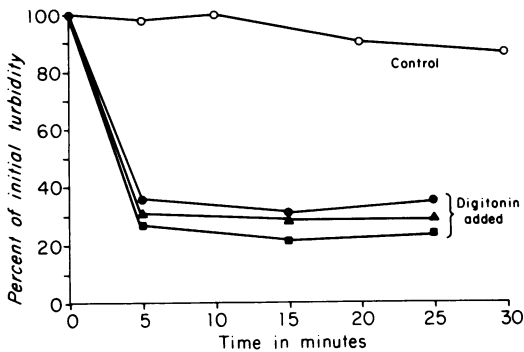


FIG. 1. Lysis of cells of *Mycoplasma arginini* G-230 by digitonin. The reaction mixture contained 35 to 60 µg of cell protein per ml and digitonin concentrations as follows: (●) 20 µg per ml, (▲) 40 µg per ml, and (■) 60 µg per ml. Turbidity is reported as absorbance at 500 nm.

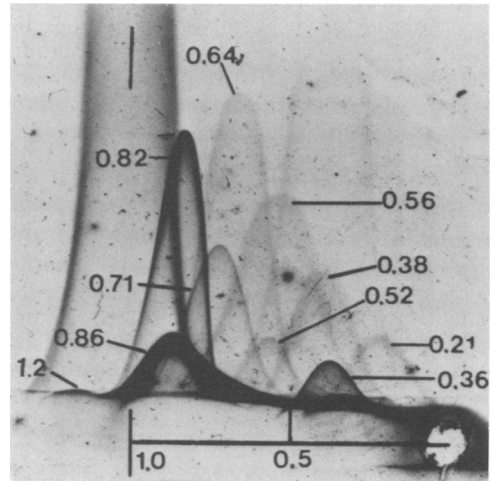


FIG. 3. Two-dimensional immunoelectrophoresis profile of cells of *Mycoplasma arginini* G-230. Ten micrograms of cell protein was subjected to electrophoresis in the first phase (anode to the left of the figure) and developed against 0.1 ml of antiserum to G-230 cells in the second phase (anode to the top of the figure). The scale at the bottom of the figure indicates the relative electrophoretic mobilities of the peaks: 1.0 equals the mobility of bovine serum albumin, which peak is identified by the vertical bar at the top of the figure. Optical magnification of print, $\times 2$.

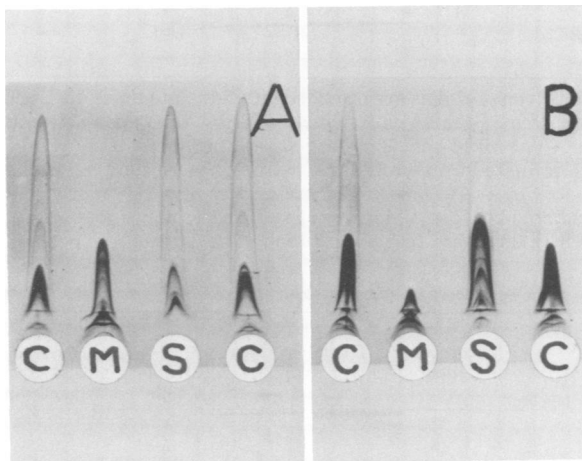


FIG. 2. (A) Rocket electrophoresis of preparations from digitonin-lysed cells of *Mycoplasma arginini* G230. Five micrograms (protein) each of unfractionated cells (C), membrane pellet (M), supernatant (S), and unfractionated cells (C) was subjected to electrophoresis into 0.1 ml of antiserum to G-230 cells until equilibrium was achieved (1.5 V/cm per slide for 5 h). The anode is at the top of the figure. (B). Rocket electrophoresis of preparations from sonicated cells of *M. arginini* G-230. Conditions identical to (A).

sons: a different antiserum was employed and 10 μg of antigen was used rather than 50 μg . Nevertheless, it was possible to identify most of the components previously recognized, even though some peaks differed in relative heights (Tables 1 and 2). Profiles produced by the membrane fraction were sharply different from disrupted whole cells (Fig. 4). For example, prominent peaks 0.82, 0.71, and 0.64 were clearly missing. The major component (the membrane complex) appeared as a series of intensely stained peaks extending from 0.91 to 0.18. These peaks appeared to show reactions of partial identity which, however, were obscured by the intense precipitate. Accordingly, at this stage of the analysis it was not clear whether the membrane complex represented different or related antigens. The area of this component was increased in the membrane fraction, suggesting enrichment, and this component was absent from the supernatant profile (Fig. 5). The membrane complex and two other distinct antigens with mobilities of 0.43 and 0.33 appeared with joined peaks in profiles of solubilized whole cells and membranes subjected to electrophoresis in tandem (Table 1). Five antigens (with mobilities of 0.82, 0.71, 0.64, 0.56, and 0.36 as defined for the cell profile, Fig. 3)

were assigned to the soluble fraction. Tandem runs of unfractionated cells and supernatant verified this classification (Table 2). Several antigens in the region between 0.6 and 0.3 could not be assigned to either fraction by this analysis because of variability in electrophoretic mobilities of identical components in different samples.

Antiserum absorption. Johansson and Hjerten (10) located external components of *A. laidlawii* by absorbing antisera to membranes with whole organisms and evaluating the absorption by two-dimensional immunoelectrophoresis. The quantitative nature of this technique is highly advantageous for studying serum absorption, because peak height is inversely proportional to antibody concentration. Thus, the removal of some of the antibody will yield a detectable change; e.g., if half the antibody is removed then the affected peak is doubled in height. More extensive absorption will result in the affected peak being eliminated from the slide, because the antibody content will be so reduced that the affected antigen will migrate off the top of the slide. When antiserum was absorbed once with live organisms, only peak 0.95 (a subcomponent of the membrane complex) was dramatically increased in height

TABLE 1. Description of membrane antigens of *Mycoplasma arginini*

Relative mobility		Letter designation ^a	Tandem (identity) ^b	Absorption		Cross-reactions	
Cell profile	Membrane			Whole cells	Dissociated membrane	Leonis	23243
0.86	0.93-0.70	E	+	++ ^c	+	-	-
0.52 (faint)	0.53		- ^d	-	+	-	-
0.38	0.43	I	+	+ ^e	+	+	+
Not visible	0.33		+	+ ^e	+	-	-
0.21	0.18		- ^d	-	+	+	+

^a Letter designations for given antigens are provided only to relate these data to the previous study (29).

^b Antigenic comparison of membrane with whole cells by tandem electrophoresis (15) showed clear identity with cell profile (+) or could not be identified (-).

^c ++, Antigenic peak eliminated from profile (i.e., strong absorption); +, peak significantly increased in height.

^d Not clearly distinguishable because of density of staining of major membrane antigen.

^e Peak height significantly increased by second round of absorption.

TABLE 2. Description of supernatant antigens of *Mycoplasma arginini*

Relative mobility		Letter designation ^a	Tandem (identity) ^b	Absorption		Cross-reactions	
Cell profile	Supernatant			Whole cells	Dissociated membrane	Leonis	23243
0.82	0.86	B	+	-	-	+	+
0.71	0.78	F	+	-	-	-	-
0.64	0.69	G	+	-	-	+	+
0.56	0.54		+	-	-	+	+
0.36	0.36	H	+	-	-	+	+

^a Letter designations for given antigens are provided only to relate these data to the previous study (29).

^b Antigenic comparison of supernatant fraction with whole cells by tandem electrophoresis (15) showed clear identities of indicated components in all cases.

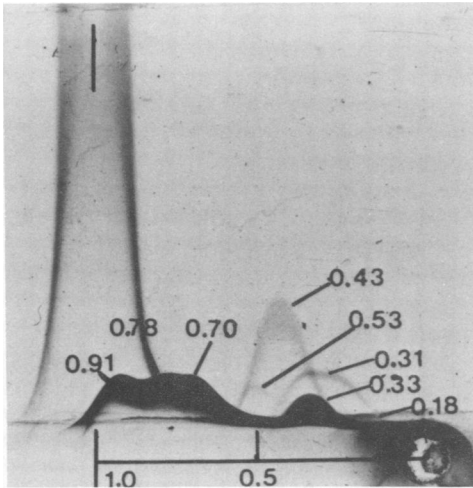


FIG. 4. Two-dimensional immunoelectrophoresis profile of membranes of *Mycoplasma arginini* G-230. Ten micrograms of protein of membrane from digitonin-lysed cells was subjected to electrophoresis in the first phase (anode to the left of the figure) and developed against 0.1 ml of antiserum to G-230 cells in the second phase (anode to the top of the figure). The scale at the bottom of the figure indicates the relative electrophoretic mobilities of the peaks: 1.0 equals the mobility of bovine serum albumin, which peak is identified by the vertical bar at the top of the figure. Optical magnification of print, $\times 2$.

(Fig. 6), indicating that this subcomponent was prominently exposed on the outside of the cell. This result would not exclude the possibility that this component transects the membrane and may also be a surface component on the interior of the cell. Slight elevation of the other peaks was probably due to dilution of the antiserum by water in the absorbing pellet. Absorption with membranes gave the same result (Fig. 7), with the suggestion of the elevation of an additional peak at 0.35, but the observed enhancement was not large enough to be conclusive. When antiserum was absorbed twice with live organisms (see Materials and Methods), absorption was more complete. The entire membrane complex was absent from the profile, as well as two antigens in the 0.3 to 0.35 region, probably identical to those seen at 0.3 and 0.4 in the cell membrane tandem profiles. The peaks labeled as cytoplasm (Table 2) were clearly unaffected by any of the absorbents. Absorption with membranes dissolved in Triton under conditions permitting antigen-antibody precipitate formation was more effective in removing antibody, even though cytoplasmic peaks were increased because fluid had to be included with the solubilized membranes (Fig. 8). The membrane complex disappeared as well as components 0.63 and 0.37 seen in the control

profile. A peak at 0.35 also appeared to be increased significantly. The determinants for the latter three components could react with antibody most readily when the membranes were dissolved in Triton.

Monospecific antiserum. To further characterize the membrane complex, antiserum was prepared to subcomponent 0.93 of the complex with the leading side of that peak as immunogen, because it was the most free of contaminating antigens of any peak on the profile. When the resulting antiserum was reacted with Triton-solubilized whole cells, a series of peaks at 1.1, 0.93, 0.64, and 0.05 was seen, which all appeared serologically related (Fig. 9). Spurs indicative of partial identity appeared on both sides of peak 0.93. This result indicates that the antigenic determinant found in the leading edge of peak 0.93 is related to those of the slower components, and thus the membrane complex is a collection of related antigens or possibly different pieces of the same membrane component (termed the "complex membrane antigen" for the remaining discussion). This antiserum did not produce any peaks when tested against the supernatant fraction.

Cross-reactions. The strain specificity of the antigen preparations was ascertained by testing cells, membranes, and supernatant with

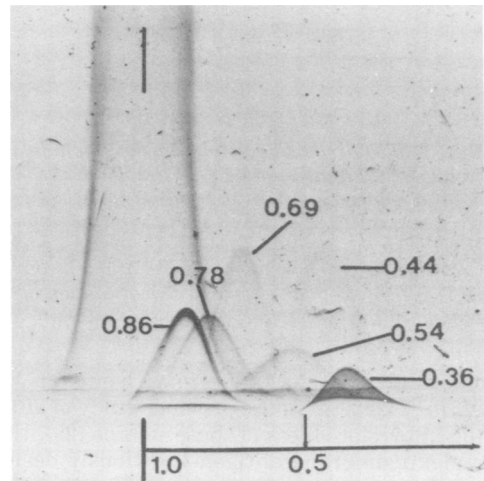


FIG. 5. Two-dimensional immunoelectrophoresis profile of supernatant fraction of *Mycoplasma arginini* G-230. Ten micrograms of protein of supernatant from digitonin-lysed cells was subjected to electrophoresis in the first phase (anode to the left of the figure) and developed against 0.1 ml of antiserum to G-230 cells in the second phase (anode to the top of the figure). The scale at the bottom of the figure indicates the relative electrophoretic mobilities of the peaks: 1.0 equals the mobility of bovine serum albumin, which peak is identified by the vertical bar at the top of the figure. Optical magnification of print, $\times 2$.

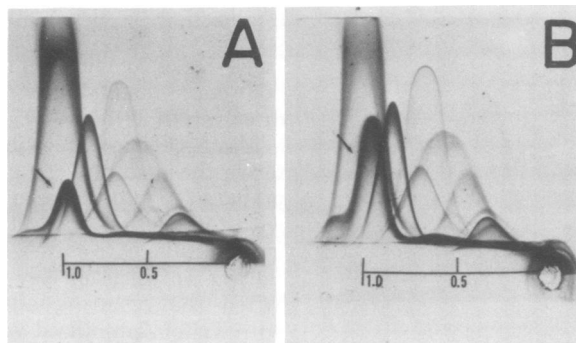


FIG. 6. Two-dimensional immunoelectrophoresis profiles of *Mycoplasma arginini* G-230 against (A) unabsorbed antiserum against G-230 cells and (B) the same antiserum absorbed with intact cells for 30 min at 4°C. Twenty micrograms of cell protein was subjected to electrophoresis in the first phase (anode to the left of the figure) and developed against 0.2 ml of antiserum in the second phase (anode to the top of the figure). The scale at the bottom of the figure indicates the relative electrophoretic mobilities of the peaks: 1.0 equals the mobility of bovine serum albumin, which peak is identified by the vertical bar at the top of the figure. The arrow indicates the peak most affected by treatment of the antiserum. Actual-size print.

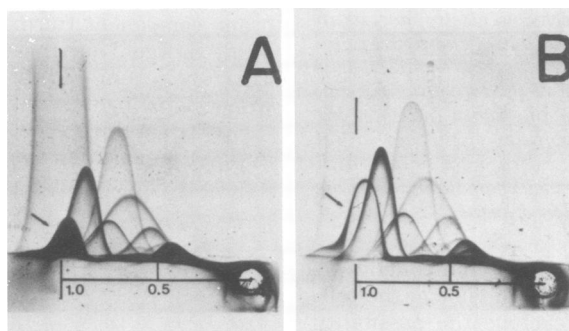


FIG. 7. Two-dimensional immunoelectrophoresis profiles of cells of *Mycoplasma arginini* G-230 against (A) unabsorbed antiserum against G-230 cells and (B) the same antiserum absorbed with washed undissolved membranes prepared from digitonin-lysed cells for 90 min at 4°C. Twenty micrograms of cell protein was subjected to electrophoresis in the first phase (anode to the left of the figure) and developed against 0.2 ml of antiserum in the second phase (anode to the top of the figure). The scale at the bottom of the figure indicates the relative electrophoretic mobilities of the peaks: 1.0 equals the mobility of bovine serum albumin, which peak is identified by the vertical bar at the top of the figure. The arrows indicate the peak most affected by treatment of the antiserum. Actual-size print.

antiserum either to *M. arginini* leonis or *M. arginini* 23243. As in the previous study of antigenic interrelatedness of these strains (29), the complex membrane antigen (previously designated E) was detected only by antiserum to G-230 and not by antiserum to leonis and 23243. The antiserum prepared against G-230 peak 0.93 did not react with leonis or 23243 antigens. The cross-reacting antisera (anti-leonis or anti-23243) both produced peaks at approximately 0.4 and 0.1 when reacted with G-230 membranes. This permitted verification of these antigens as membrane components since, on the cross-reaction slides, they were not

masked by the massive G-230-specific complex membrane antigen precipitate. When tested against the G-230 supernatant, the anti-leonis antiserum detected supernatant peaks at 0.87, 0.65, and 0.39 (B, G, and H, respectively, Table 1). An additional peak at 0.54 was clearly resolved. Anti-23243 also cross-reacted with antigens B, G, and H (peaks appeared at 0.82, 0.64, and 0.36).

Controls. Neither the agamma horse serum used in the medium nor Freund complete adjuvant produced precipitin lines against the antisera used in this study. Since procedures for antigen preparation and antiserum production

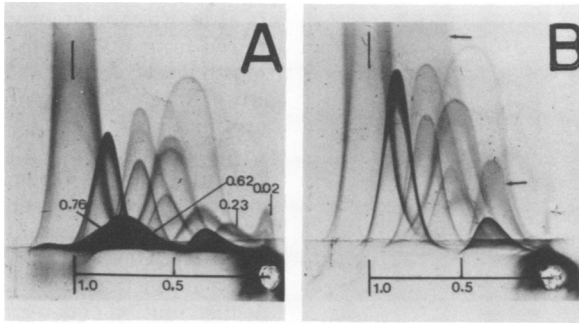


FIG. 8. Two-dimensional immunoelectrophoresis profiles of cells of *Mycoplasma arginini* G-230 against (A) unabsorbed antiserum against G-230 cells and (B) the same antiserum absorbed with Triton-dissolved washed membranes prepared from digitonin-lysed cells for 60 min at 37°C. Twenty micrograms of cell protein was subjected to electrophoresis in the first phase (anode to the left of the figure) and developed against 0.2 ml of antiserum in the second phase (anode to the top of the figure). The scale at the bottom of the figure indicates the relative electrophoretic mobilities of the peaks: 1.0 equals the mobility of bovine serum albumin, which peak is identified by the vertical bar at the top of the figure. The peaks missing from (B) are labeled with their relative electrophoretic mobility values in (A). The upper arrow indicates the complex membrane antigen that ran off the slide due to reduction in concentration of its specific antibodies. The lower arrow indicates a peak with a significant height increase. No correction was made for dilution of antiserum by the membrane suspension in (B). Actual-size print.

were the same as in previous studies from this laboratory, the controls described therein were also applicable (28).

DISCUSSION

Clear separation was shown between membrane and soluble fractions in these experiments. However, certain restrictions should not be ignored. Whether or not the supernatant fraction can be clearly defined as cytoplasm can be questioned. The soluble fraction might well contain loosely bound membrane components: ribonuclease is loosely bound in *A. laidlawii* (22). Additionally, small membrane fragments may be generated which do not sediment at $34,000 \times g$. It has been reported that the digitonin lysis method gave little contamination of supernatant fraction with membrane components (26). In addition, a number of arguments can be raised to support the contention that the soluble fraction largely represents cytoplasmic components. If generalized contamination with membrane material had occurred, one might expect that the slower subcomponents of the complex membrane antigen would have yielded some of this contamination. The failure of the antiserum specific to the complex membrane antigen to react with supernatant components indicates that the soluble fraction was not contaminated in this way. Similarly, peaks judged to be cytoplasmic (Table 1) were not elevated significantly when disrupted whole organisms were tested against antiserum absorbed with

intact membranes or membranes dissolved in Triton X-100. These data, then, argue that the cytoplasmic antigens have been effectively separated from the membrane antigen components. Rocket electrophoretic analysis proved to be an efficient means of preliminary verification of the degree of separation of membrane and cytoplasmic constituents.

It should also be noted that the membrane fraction could contain soluble components that have adsorbed to the membranes. Rottem et al. have indicated that soluble proteins such as lysozyme and cytochrome c bind to membranes (24). Two-dimensional profiles readily show that unwashed membranes are contaminated with soluble components, but these components disappear after washing of the membranes.

Antisera produced to immunogens that were whole organisms (at least initially) recognized both membrane and cytoplasmic components, a result similar to that reported by Lemcke for a variety of mycoplasmic species in double-immunodiffusion tests (17). In the present experiments, the organisms were stored frozen between immunizations; thus, some opportunity for lysis existed, even though mycoplasmas are quite stable to freezing and thawing. More important, however, is the fact that those mycoplasmic species tested are killed by antibody in a complement-mediated reaction closely resembling the lysis of sheep erythrocytes (6). This is a sensitive reaction, and the sequential immunogen doses would be quickly lysed once some antibody had been produced. Thus, it is

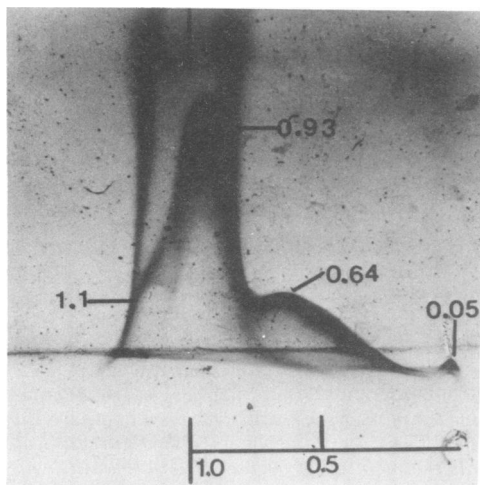


FIG. 9. Two-dimensional immunoelectrophoresis profiles of cells of *Mycoplasma arginini* G-230 against antiserum monospecific for subcomponent 0.93. Ten micrograms of cell protein was subjected to electrophoresis in the first phase (anode to the left of the figure) and developed against 0.5 ml of two-times-concentrated antiserum in the second phase (anode to the top of the figure). The scale at the bottom of the figure indicates the relative electrophoretic mobilities of the peaks: 1.0 equals the mobility of bovine serum albumin, which peak is identified by the vertical bar at the top of the figure. Optical magnification of the print, $\times 2$.

not surprising that the rabbits produced antibodies to internal cell components.

Several conditions are important in the interpretation of the absorption studies. Some conditions pertinent here have to do with the behavior of membranes upon purification: whether the membranes reseal as closed vesicles, perhaps preventing reaction with internal components, and whether the interactions between membrane components are "relaxed" upon purification, allowing increased accessibility of reactive sites to reagents (22). Both of these questions do not seem to apply to mycoplasmic membranes. Purified membranes do not form vesicles (27), and physical studies suggest that their structure remains "tight" (23).

Other conditions pertain to the antigen-antibody reactions. First, identification of the membrane components on the outer surface requires that the reagent not penetrate into the cell. This condition is readily met by antibody unless cell lysis occurs, a doubtful event if cell-antibody mixtures are kept at 4°C for a short period of time (6). A longer incubation time (as in the second round of depletion of antiserum by whole cells) might permit lysis and consequent reaction of antibody with internal components.

In this case, membrane components disappeared from the profile due to antibody depletion, but antigens of the soluble fraction were only moderately enhanced. Second, active determinant sites must be accessible for reaction with antibody. For high-affinity antibody species, binding sites need not be closely spaced for antibody depletion by binding to whole cells or purified membranes where precipitation is not necessary. However, if the affinity is low, then effective reaction may depend on the appropriate distribution of determinant sites on the surface. Furthermore, as argued by Johansson and Hjerten (10), presence of a given antigen on either surface does not preclude the penetration of that antigen through the membrane to the opposite surface. In our studies, at least a portion (the 0.93 peak) of the complex membrane antigen is present on the outer surface of the membrane. It may be strongly antigenic due to the specific configuration of its determinant site or because it is well exposed on the surface. Our studies do suggest that, whereas the complex antigen may project outwardly, it could be present in the hydrophobic region of the membrane (solubilization of most subcomponents occurred only with detergent). Two membrane components with mobilities at 0.3 to 0.4 may be external, but they may not extend as far. Easy identification of some external components by antibody depletion may be sterically blocked by high-avidity reaction of antibody toward another projecting antigen. In the case of *M. arginini*, the complex membrane antigen may be exerting such an effect. Such an observation has been made for mammalian cells (5).

Cross-reaction studies indicate the presence of a strain-specific membrane antigen (the complex membrane antigen) giving a very strong reaction. The importance of this antigen cannot be judged solely by staining intensity of the precipitin line, but requires additional information concerning its function in other serological tests such as growth inhibition and complement-mediated lysis. In addition, common antigens include both membrane and cytoplasmic components. Hollingdale and Lemcke have shown that *M. hominis* is similar in this respect (8). Studies to determine whether the leonis and 23243 strains present a similar antigenic map are in progress.

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