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# Disposition of Exposed Antigens on the Faces of Isolated *Mycoplasma gallisepticum* Membranes

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The transverse disposition of exposed protein antigens on the two faces of isolated *Mycoplasma gallisepticum* membranes have been investigated by using indirect immunoferritin labeling to accomplish visualization of the antigens at the ultrastructural level. Comparison between the labeling patterns obtained with unabsorbed specific mycoplasma antiserum and antiserum from which antibodies directed against outer side determinants had been removed revealed that the majority of protein antigens were the same on the opposed membrane faces or at least displayed extensive interside cross-reactivity. The relatively scarce tagging of isolated *Acholeplasma laidlawii* membranes, contrary to membranes on intact organisms observed in this investigation, precluded conclusions regarding the disposition of membrane antigens of this species. The advantages and limitations of the employed method in disposition studies and the factors influencing the transverse distribution of membrane proteins in mycoplasmas are discussed.

An asymmetric transverse distribution of mycoplasma membrane proteins with more proteins facing the cytoplasmic side of the membrane than exposed on the outer face has been inferred from several studies (2-4, 9, 10). The membranes used as models in these investigations of membrane architecture have been derived most often from *Acholeplasma laidlawii* and to some extent from *Mycoplasma hominis* (2, 3) and *Mycoplasma pneumoniae* (10). The methods, which have been used to reveal dispositional asymmetry of the membrane proteins, have been based on radioactive labeling or proteolytic digestion of exposed proteins or on immunoelectrophoretic assay of detergent-solubilized membranes (9). The predominance of proteins on the cytoplasmic side of the lipid bilayer is illustrated also by the higher number of intramembranous particles on the convex fracture faces than on the concave faces showing up in freeze-etching experiments (15).

In the present paper, the results of an electron microscopic study of the transverse disposition of membrane proteins of *Mycoplasma gallisepticum* are presented, applying indirect immunoferritin labeling of membrane antigens (18) as a means of visualizing the proteins. *M. gallisepticum* membranes show a higher density and a greater protein-to-lipid ratio than other mycoplasmas (13, 17), possibly indicating a different protein organization. For comparison, membranes of *A. laidlawii* were included in the investigation.

## MATERIALS AND METHODS

**Organisms.** The type strains of *M. gallisepticum* PG 31 and of *A. laidlawii* PG 8 were used.

**Culture conditions.** The organisms were grown in equal amounts of liquid B medium (8) for the production of membranes and for the preparation of immunization antigens and antiserum absorption antigens. Cells were harvested at the late exponential growth phase in yields of $10^7$ to $10^8$ colony-forming units per ml. Growth on solid B medium agar took place as previously described (18).

**Preparation of membranes.** Membranes of the two organisms were isolated by the methods of Rottem et al. (17), i.e., by osmotic lysis of a washed cell sediment of *A. laidlawii* in distilled water and by osmotic lysis of glycerol-loaded *M. gallisepticum* organisms. The collected membranes were washed in deionized water. Membranes were suspended in dilute $\beta$-buffer (17) at a concentration of 0.25 mg/ml of protein (Lowry) and stored at −70°C until used.

**Indirect immunoferritin labeling.** Indirect immunological labeling of the mycoplasma membranes was carried out by a previously published procedure (18). Membranes were fixed with 0.3% glutaraldehyde for 1 h at room temperature before agar embedding and reaction with the first-step antibody, which was specific rabbit-antiserum diluted 1:100 (1:200 in some experiments) or the same antiserum absorbed with a preparation of intact cells. Control labelings in which preimmune rabbit serum or buffer was substituted for hyperimmune serum were always carried out in parallel. The ferritin label consisted of ferritin-conjugated gamma globulin fraction of goat anti-rabbit immunoglobulin G (IgG) (Miles-Yeda, Ltd, Israel) and was utilized in the dilution 1:60. Ferritin labeling of agar-grown mycoplasma colonies prefixed with 0.3% glutaraldehyde also proceeded as previously described.

**Electron microscopy.** Ultrathin sections of Vestopal-W-embedded mycoplasma cells or membranes, fixed with 3% glutaraldehyde and 1% $OsO_4$ fixatives after tagging with ferritin (18), were examined in a JEOL JEM 100B electron microscope either unstained or after section staining with lead citrate alone or lead

citrate and magnesium uranyl acetate.

**Immunization procedure.** Rabbit hyperimmune sera against cells of *M. gallisepticum* and *A. laidlawii* were produced as outlined by Black (5). The sera were heat inactivated before use, and their activity was tested by the metabolism inhibition method (14). Two different antisera against each of the two mycoplasma species were used in the experiments with identical results.

**Absorption of antisera.** To effect removal of antibody directed against outer membrane face antigenic determinants on cells of *M. gallisepticum* and *A. laidlawii*, about $10^{10}$ mycoplasma cells were suspended in 1 ml of 0.1 M Tris buffer, pH 7.4. One milliliter of the respective antiserum diluted 1:50 was added, and the mixture was held at 4°C for 3 h with frequent shaking. After separation of cells by centrifugation at 27,000 × *g*, the pellets were fixed in 0.3% glutaraldehyde and processed for electron microscopy by the thin-sectioning method and examined for the presence of disintegrated cells. The efficiency of the absorption procedure was monitored by comparing the metabolism-inhibiting activity (14) of the supernatant to that of unabsorbed serum diluted 1:100. In typical experiments an 8- to 10-fold decrease in serological activity resulted. The supernatants from absorption mixtures were used directly for labeling purposes.

**Immunodiffusion tests.** Ouchterlony double-diffusion precipitation in agar was employed to assess the chemical nature of the membrane antigens of *M. gallisepticum* detected by the antisera used in the present study. Precipitation lines resulting from the reaction of undiluted specific antiserum with membranes solubilized in 5% Triton X-100, or membranes heated at 65°C for 1 h and then solubilized, were compared.

Double-diffusion precipitation was likewise used to test the rabbit hyperimmune sera for the presence of media-directed antibody derived from the growth medium utilized for preparing immunization antigens. The antisera were allowed to react with solubilized membranes of their homologous mycoplasma species, with liquid growth medium (B), and with undiluted horse serum, which is the main component of B medium.

Finally, single radial immunodiffusion (Mancini) was carried out to determine the amount of unspecific absorption of rabbit IgG from hyperimmune sera raised against *M. gallisepticum*, as a result of the absorption of these sera with suspensions of cells described in the above. Agar plates (10 by 10 cm) were prepared containing 0.05 mg of IgG fraction of goat anti-rabbit IgG (Miles-Yeda, Ltd) per ml. Absorbed and unabsorbed antiserum and preimmune serum diffused radially for 3 days at 4°C, after which time the areas of the developed precipitation zones were compared.

## RESULTS

Indirect immunoferritin labeling of agar-grown *M. gallisepticum* or *A. laidlawii* organisms with antiserum absorbed with whole cells, as the first step reagent in the composite labeling procedure, resulted in only spurious attachment of ferritin particles to the outer side of the cytoplasmic membrane on the cells. Cells of both species treated with unabsorbed specific antiserum, on the other hand, displayed a uniform antibody coating tagged with numerous evenly scattered ferritin particles, as illustrated for *A. laidlawii* in Fig. 1. Replacement of specific antisera with preimmune sera led to a labeling equally as scarce as that seen with absorbed antisera.

Application of the labeling procedure to isolated membranes of *M. gallisepticum*, with unabsorbed antisera in the first step, yielded mostly open membranes or membrane fragments covered with amorphous material, which was tagged with ferritin particles on both membrane faces (Fig. 2). Counting of particles on each side of open membrane fragments did not reveal any asymmetry in the amount of ferritin, and also the amorphous layer of antibody appeared rather evenly distributed between the two sides. A minor proportion of the membranes in these preparations appeared sealed or belonging to unbroken mycoplasma cells. Such membranes were tagged on the exterior side only. Identical results were obtained with 1:100 and 1:200 dilutions of the first-step antibody. In contrast, use of absorbed antisera gave membranes with very little attachment of adhering material and ferritin (Fig. 3), this incidental labeling being observable on both membrane faces of unsealed membranes. Substitution of preimmune serum (Fig. 4) or buffer for antiserum also did not result in any consistent labeling of the membranes. Similar experiments with isolated *A. laidlawii* membranes revealed considerably less immunological tagging, compared to that of *M. gallisepticum* membranes, when unabsorbed sera were employed (Fig. 5) and also much less la-

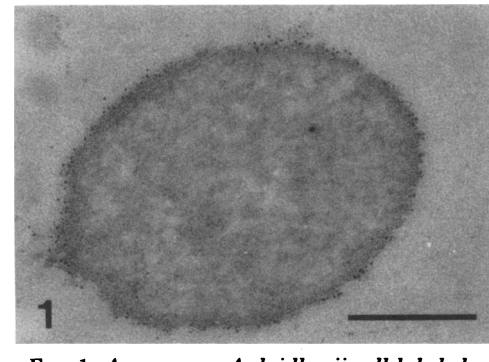


FIG. 1. *Agar-grown A. laidlawii cell labeled with specific mycoplasma antiserum and ferritin-conjugated anti-IgG. The cytoplasmic membrane is uniformly coated with a layer of antibody which is tagged with numerous ferritin particles. Section staining omitted. Bar represents 250 nm.* ×67,500.

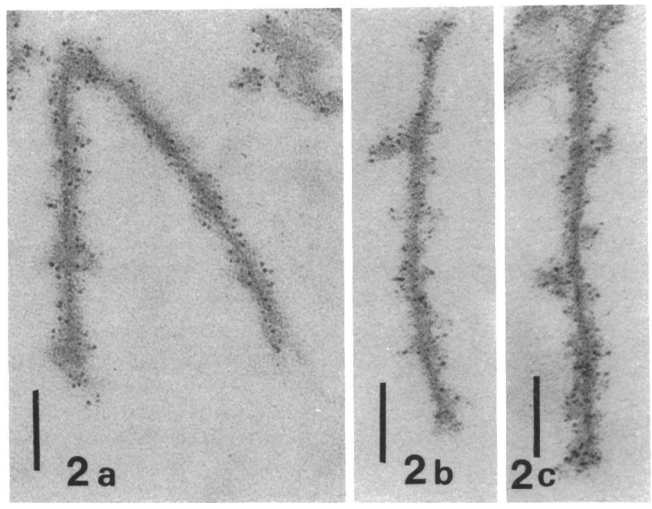


FIG. 2. *Fragments of isolated M. gallisepticum cell membranes treated with unabsorbed antiserum and the ferritin label. The immunological labeling appears equally intense and relatively uniform on both sides of the fragments. Section staining omitted. Bars represent 100 nm. ×105,000.*

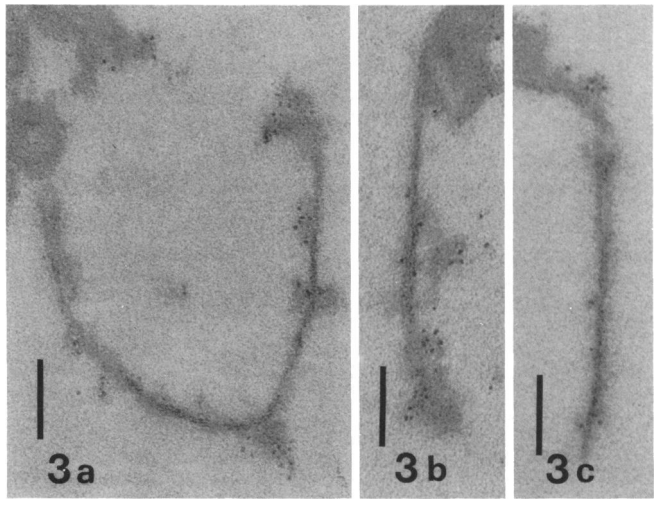


FIG. 3. *Fragments of M. gallisepticum membranes treated with specific antiserum, which has been absorbed with intact mycoplasma cells, and ferritin label. Only occasional adherence of material tagged with ferritin particles is observed on the membrane faces. Section-staining omitted. Bars represent 100 nm. ×105,000.*

beling than seen on the membranes of intact *A. laidlawii* cells (Fig. 1). However, definite and specific attachment of ferritin to both sides of these membranes could be established. Due to the low number of particles along the membranes, it was difficult to decide whether the two membrane sides bound the ferritin particles with equal affinity. Ferritin was practically absent on the isolated membranes in labeling experiments with whole-cell-absorbed *A. laidlawii* sera and in control preparations with preimmune serum.

Electron microscopic examination of centrifugal pellets of mycoplasma cells of both species, having been used to absorb away antibody directed against surface determinants, disclosed that the vast majority of the cells had resisted the treatment with specific antibody without disintegration of their limiting membrane (Fig. 6), although the interior of cells sometimes appeared disorganized.

Double radial immunodiffusion tests of the antisera, with the purpose of detecting the presence of antibodies directed against growth medium components, showed that none of the *M.*

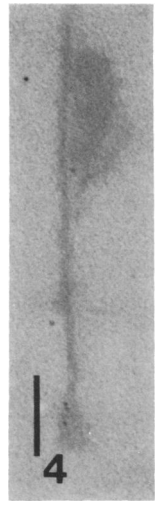


FIG. 4. *M. gallisepticum membrane fragment incubated with preimmune serum and ferritin label. Only a few attached ferritin particles and some adhering material is seen. Section staining omitted. Bar represents 100 nm. ×105,000.*

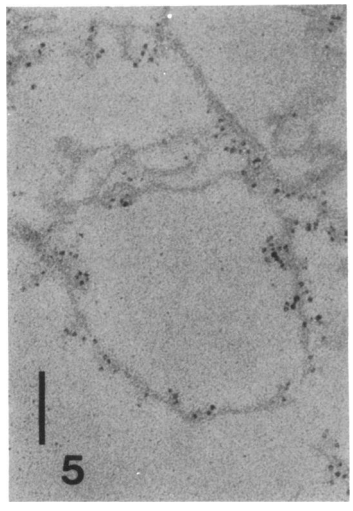


FIG. 5. *Isolated A. laidlawii cell membranes incubated with specific antiserum and ferritin label. Tagging is scarce on both sides of the membranes. Section staining omitted. Bar represents 100 nm. ×94,500.*

*gallisepticum* antisera contained such antibody, and only a single strong precipitation line emerged between wells containing specific antiserum and detergent-solubilized membranes. One strong line and one faint line could be detected in the agar between wells filled with *A. laidlawii* antiserum and solubilized membranes and three weak lines between the former well and the reservoir holding undiluted horse serum. Thus, the *A. laidlawii*-specific rabbit antisera did contain minor medium antibody components. These components were, however, effectively removed by absorption with growth medium (6), the medium-absorbed sera giving the same results in labeling experiments as unabsorbed sera. Agar immunodiffusion also demonstrated the proteinaceous nature of *M. gallisepticum* antigens binding the antibody and ferritin, since precipitation lines were completely absent when the first step antibody was allowed to react with membranes heated at 65°C as opposed to the single strong line developing from unheated solubilized membranes. Comparison of the circular precipitation zones forming on single radial immunodiffusion plates (Fig. 7) revealed that the absorption of *M. gallisepticum* antiserum with suspensions of intact organisms caused a decrease in the concentration of total IgG to about one-half the original amount, to a level corresponding roughly to that observed in preimmune serum.

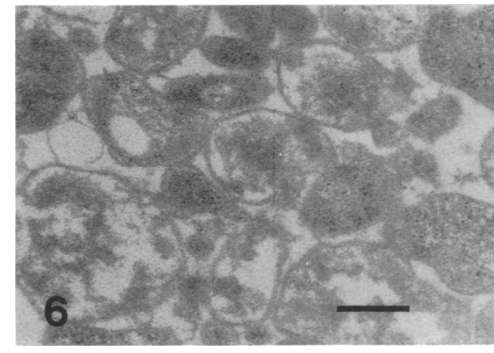


FIG. 6. *Section through M. gallisepticum cell pellet demonstrating the integrity of cells used to absorb away antibodies directed against outer-membrane-site antigenic determinants. Bar represents 250 nm. ×37,000.*

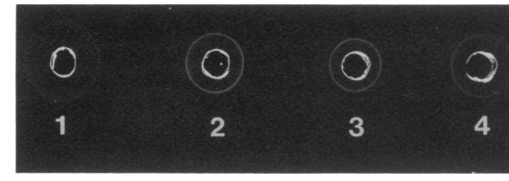


FIG. 7. *Single radial immunodiffusion plate. Antigens in the wells were (1) specific M. gallisepticum antiserum, (2) same antiserum absorbed with whole cells, (3) preimmune serum, (4) same antiserum as in (1) and (2), but absorbed with a different batch of whole cells. The absorption with whole cells resulted in a reduction of the diameters of precipitation zones of about 25%.*

## DISCUSSION

The efficient labeling of open membranes and membrane fragments of *M. gallisepticum* with unabsorbed specific antiserum and ferritin tag in the present investigation is at variance with observations made in a previous study (18), in which only sealed membranes showed any consistent labeling. The reason for this discrepancy is most likely found in the different ways in which membranes were isolated in the two investigations. In the initial experiments (18), *M. gallisepticum* membranes were obtained by osmotic lysis in distilled water followed by repeated freeze-thawing at −70°C, whereas, in the present study, membranes were liberated by osmotic lysis of glycerol-loaded organisms (17). Possibly, glycerol may have served to protect the membranes from damage during osmolysis or during the subsequent storage at low temperature. The difficulties encountered in preserving the membrane antigens throughout the composite labeling procedure are illustrated also by the relatively scarce labeling of the *A. laidlawii* membranes, obtained by direct osmotic lysis, as compared to the *M. gallisepticum* membranes and intact acholeplasma organisms in this study. It is noteworthy that solubilized *A. laidlawii* membranes retained a high antigenic potency as judged from the immunodiffusion tests carried out.

The primary object of the study undertaken has been to try to elucidate the disposition of exposed membrane antigenic determinants on the faces of isolated *M. gallisepticum* cytoplasmic membranes and, in particular, to investigate whether determinants common to the two sides of the membranes exist. The quite heavy antibody coating and ferritin tagging showing up upon intact cells, as also observed in a previous study (18), demonstrate the abundance of antigenic determinants on the outer face of these organisms. The labeling pattern seen on isolated open membranes of *M. gallisepticum*, on the other hand, revealed that the number of antigenic sites on the cytoplasmic side of the membranes is comparable to that on the external side. Furthermore, since removal of antibody directed against antigenic sites exposed on the outer side, by absorption with intact mycoplasma cells, resulted in the concurrent substantial reduction of label on both membrane sides, it can be concluded that most of the determinants detected by the specific antisera displayed specificities common to the two sides of the *M. gallisepticum* membranes or at least extensive interside cross-reactivity. This conclusion holds true whether or not antigens on the cytoplasmic side of the mycoplasma cells used to immunize rabbits have acted as immunogens. The possibility that the observed reduction of labeling by using absorbed sera could be due to unspecific removal of most of the IgG molecules in the sera by the absorbing cells (7, 18) also can be excluded since, as shown by immunodiffusion, about half of the total IgG was extracted by the absorption procedure, but doubling the dilution of the first-step antibody had no effect on the intensity of labeling with unabsorbed sera and ferritin. Likewise, the influence of antibodies directed against adsorbed media proteins (16) was ruled out by their absence in amounts detectable by immunodiffusion. Finally, it was noted that ferritin tagging was not present to any significant measure in clumps of cytoplasm sometimes found adhering to the inner side of the cell membranes of partially disintegrated organisms in the membrane preparations, in accordance with the known poor immunogenic potency of the *M. gallisepticum* cytoplasm (11, 13). Although specific indirect immunoferritin labeling of both membrane sides of isolated *A. laidlawii* membranes was observable, the scarcity of labeling in the present study did not permit any definite conclusions to be drawn regarding the relationship between the antigenic determinant specificities displayed by the two membrane faces.

The *M. gallisepticum* antibodies active in the labeling experiments were apparently directed only against protein antigens of the membranes, as indicated by the complete absence of precipitation lines, when heated and solubilized membranes were reacted with the antisera in immunodiffusion tests. This is in agreement with previously recorded results, stating that the major membrane antigens of this species are proteins (11) and that extracted lipids are devoid of serological activity (12). Most likely the *A. laidlawii* membrane antigens, detected in a smaller proportion in this investigation, also were mainly proteins (11). The accordance between exposed membrane protein antigens on the two faces of isolated *M. gallisepticum* membranes both with respect to number and immunological specificity seems to deviate from the findings in other mycoplasma species. Using crossed immunoelectrophoresis of detergent-solubilized *A. laidlawii* membranes, Johansson and Hjertén (9) deduced that, of the soluble membrane proteins identified, three were exposed on the cytoplasmic face of the membranes and only one on the exterior face. However, as pointed out by the authors, the applied method did not permit exclusion of the possibility that the proteins with established inside determinants might also possess (different) determinants on the exterior membrane face. The present labeling experiments indicate

that major *M. gallisepticum* membrane proteins exist which, if they do not span the membrane, occupy positions on both membrane sides. Alternatively, the labeling results are compatible with proteins spanning the membrane but oriented in two opposite directions. Amar et al. observed that most of the membrane proteins in intact *A. laidlawii* and *M. hominis* cells were inaccessible to iodination and proteolytic digestion and consequently were located on the cytoplasmic side of the membranes (3), although one major protein seemed to span the *M. hominis* membrane. In *M. arginini*, one antigen was exposed on the exterior side, whereas two others were apparently buried within the membrane (1).

Several factors may influence the transverse distribution of membrane proteins as detected experimentally. The electron microscopic labeling method employed in the present investigation, by which it is visually ascertained that the labeling agents have had equal access to both membrane sides, circumvents the uncertainty regarding resealing of isolated membranes encountered in disposition studies based on nonultrastructural methods (1, 3). Also, it can be assumed that the use of antibody molecules as membrane labels ensures that the labels do not penetrate the membrane of cells as is sometimes observed with chemical labels (3). In contrast to the methods used in some of the previous disposition studies of mycoplasma membranes (1, 9), the present procedure allows analysis of insoluble membrane proteins, which may compose a considerable fraction of the total membrane proteins (9).

Aging of mycoplasma cells and the concurrent increase in protein-to-lipid ratio has been shown to affect the protein disposition of membranes of *A. laidlawii* and *M. hominis*, leading to a higher concentration of proteins on the cytoplasmic membrane faces of the aged cells (2, 4). From this point of view, the high protein content of *M. gallisepticum* membranes compared to that of other mycoplasma membranes (13, 17) would seem to contradict the symmetric distribution of membrane proteins inferred from the present investigation. The possibility that the protein architecture of isolated *M. gallisepticum* membranes differs from that of membranes of intact organisms cannot be excluded. In fact, the poor labeling of exposed antigens on isolated *A. laidlawii* membranes as opposed to the abundant tagging of intact cells noted, may be evidence of a differing organization as well.

ACKNOWLEDGMENT

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