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NOTES

Capsular Material of *Mycoplasma gallisepticum* and Its Possible Relevance to the Pathogenic Process

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A ruthenium red-staining capsule was observed on two pathogenic strains, but not on one nonpathogenic strain, of *Mycoplasma gallisepticum*. The capsule appeared to mediate cytadsorption of mycoplasmas to the chicken tracheal epithelium without evidence of membrane fusion. No relationship was seen between the presence of capsule and hemagglutination titers of the strains examined.

Mycoplasma gallisepticum is an avian pathogen and predominantly causes disease of the respiratory system in chickens and turkeys. The organism has been shown by electron microscopy to adhere to cultured mammalian cells (20, 21), erythrocytes (3, 12), and chicken tracheal epithelial cells in vitro (1) and in vivo (16, 18). It has generally been accepted that such an association between the adhering mycoplasmas and host cells is a prerequisite for development of the respiratory disease caused by this mycoplasma (1, 16). A ruthenium red (RR)-staining capsule has been reported to occur on *M. meleagridis* (6), *M. mycoides* subsp. *mycoides* and *M. dispar* (7), *Ureaplasma urealyticum* (13), and *M. pneumoniae* (19). It has been suggested that the capsule is associated with the adhesive properties (6, 19) or virulence of mycoplasmas (7). More recently, Ajufo and Whithear (2) described an RR-staining capsule on *M. synoviae* and *M. gallisepticum* and suggested that the capsule on *M. synoviae* may be responsible for the hemagglutinating properties of this mycoplasma. These reports prompted us to examine, by the RR technique, *M. gallisepticum* for the presence of a capsule and its role in adherence of the organisms to host cells.

M. gallisepticum strains SAS, 12T, and KP-13 were used. Strains SAS and 12T were isolated at the authors' laboratory and have been proven to be pathogenic for chickens to almost the same extent (16; T. Yagihashi, unpublished data). Strain KP-13 was isolated by Sato et al. (14) from a chicken with chronic respiratory disease and was chosen because it has been shown to be nonpathogenic for chickens (9). All strains were grown in a modified Chanock broth (4). Seven-week-old specific-pathogen-free White Leghorn chickens, known to be free of mycoplasma, were used throughout the study. Conventional methods for preparation of the chicken tracheas for transmission electron microscopy have been described (16). Fixation of the tissue blocks and mycoplasma cells in the presence of RR was essentially the same as described by Springer and Roth (15). Negative staining with 1% uranyl acetate was applied to unfixed organisms or organisms that had been fixed by adding formaldehyde directly to the broth cultures to a final concentration of 0.74%. Color-changing units were determined according to a method described by Taylor-Robinson et al. (17). The hemagglutination test was carried out essentially as described by Manchee and Taylor-Robinson (11).

In thin sections of organisms grown in broth medium for 16 h at 37°C and not treated with RR, all three strains had a layer of fuzzy material on the surface of the limiting membrane of the cells. In preparations treated with RR, strains SAS and 12T had a dense-staining capsule external to the limiting membrane. The capsule extended for about 13 nm outside the membrane and appeared to be diffuse with no obvious structure (Fig. 1). Strain KP-13 did not possess a definite capsule equivalent to that observed for the other two strains. This strain had only a small amount of amorphous material outside the limiting membrane, similar to that seen in the organisms fixed by the ordinary method (Fig. 2). The examination of negatively stained whole cells revealed an outer fringe of amorphous material approximately 10 nm thick around the cells of all three strains (Fig. 3). No uniform

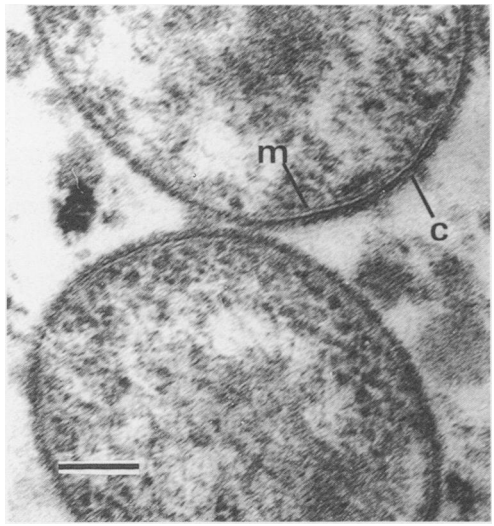

FIG. 1. *M. gallisepticum* SAS, 16-h culture. The organisms were fixed in the presence of RR and the section was stained with uranyl acetate and lead citrate. Capsular material (c) is evident outside the limiting membrane (m). Bar, 100 nm.

surface projections such as those described by Chu and Horne (5) on *M. gallisepticum* were seen in our preparations.

To correlate the presence of a capsule on mycoplasma organisms with their adhesive properties or virulence, pathogenic strain SAS and nonpathogenic strain KP-13 were inoculated into chickens via tracheal route. Two chickens were inoculated with 0.5 ml of broth culture of strain SAS containing 3×10^6 colony-forming units per ml. The viable titers in these chickens were 10^7 and 10^8 color-changing units per ml of tracheal washing collected on day 14 after inoculation. In thin sections of the tracheas, mycoplasmas were seen in large numbers in the tracheal lumen, mostly in close apposition to the luminal surface. An example of the intimate association between the adhering mycoplasma and host cell, seen in the trachea fixed in the absence of RR, is illustrated in Fig. 4. In this case, the unit membranes of both organism and host cell are nearly perpendicular to the plane of section in most parts of the apposed areas, and both membranes are clearly discernible, being separated from each other by a gap about 7 nm wide. The organism appeared to be surrounded by a fuzzy surface layer. In the tracheal tissue fixed and stained by the RR technique, dark-staining capsular material was clearly seen around the mycoplasma cells. The capsule extended for approximately 20 nm outside the limiting membrane and stained more intensely than that of organisms grown in vitro. A 7-nm gap, existing between the membranes of the organism and host cell at the site of adherence, was filled with capsular material, and the gap was no longer recognizable (Fig. 5). The mycoplasmas frequently appeared to attach to the adjacent microvilli or cilia not only by the bleb but also by other membrane sites through capsular material, suggesting that this capsular materi-

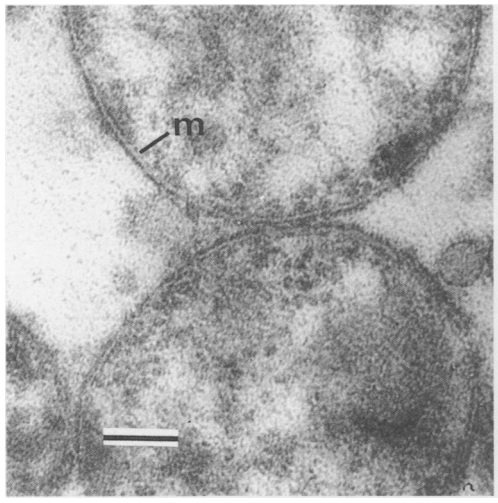

FIG. 2. *M. gallisepticum* KP-13, 16-h culture. The organisms were fixed and stained as for Fig. 1. No capsular material is present, but some amorphous material is seen outside the limiting membrane (m). Bar, 100 nm.

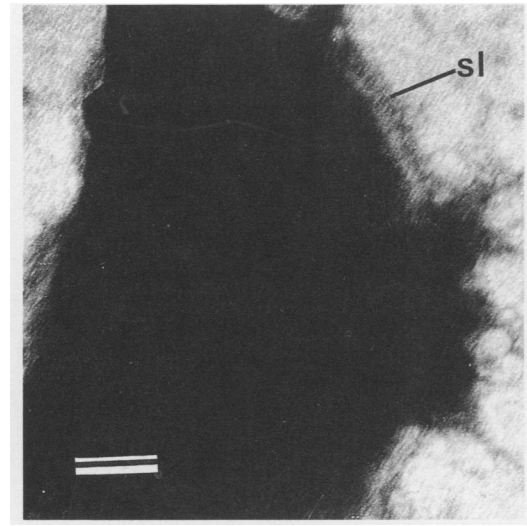

FIG. 3. *M. gallisepticum* SAS, 16-h culture. The organism was unfixed and negatively stained with uranyl acetate. A surface layer (sl) of amorphous material is seen outside the membrane. Bar, 100 nm.

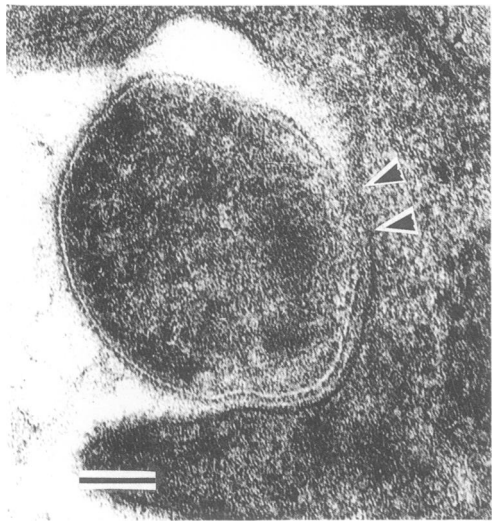

FIG. 4. A portion of the tracheal epithelium of a chicken inoculated intratracheally with *M. gallisepticum* SAS and killed 14 days after inoculation. The tissue was fixed in the absence of RR, and the section was stained with uranyl acetate and lead citrate. A mycoplasma is seen attaching to the plasma membrane of the epithelial cell. In most of the apposed areas, the limiting membrane of the mycoplasma is separated from the host cell membrane by a gap of about 7 nm wide, and both membranes are clearly discernible. In some parts, however, the identity of both membranes is lost, resulting from oblique sectioning (arrows). Bar, 100 nm.

al may be an additional means of holding the organisms close to the host cells. Broth culture of strain KP-13 containing 3×10^8 colony-forming units per ml was inoculated into three chickens. They were killed 5 days after inoculation, because it has been shown that this strain grows poorly in the chicken trachea and organisms disappear gradually on and after postinoculation day 7 (9; Yagihashi, unpublished data). In one of these chickens, no mycoplasmas were recovered from tracheal washing, and in the remaining two chickens, only 10^2 and 10^3 color-changing units per ml of tracheal washings were recovered. No mycoplasmas could be located in any of the tracheas by transmission electron microscopy despite an extensive search.

These findings seem to suggest a possible correlation between the presence of capsular material and the virulence of mycoplasma strains. The capsule on strains SAS and 12T grown in vitro was thinner and stained less intensely than that observed for strain SAS grown in vivo. This may be attributable to conditions of growth, since in several bacterial species it has been shown that the amount of capsular polysaccharide decreases significantly with in vitro passage (8, 10). It is also possible that a heavier capsule on the organisms grown in vivo may be due in part to adsorption on the mycoplasmal surface of glycocalyx shed by the epithelial cells. Nonpathogenic strains might be eliminated from the chicken trachea by the mucociliary clearance mechanism for lack of the adhesive properties. The fusion of the cell membrane of *M. gallisepticum* with that of erythrocytes has been suggested by electron microscopy (3). In our preparations processed by the ordinary method, however, a gap of about 7 nm separating the mycoplasma membrane from that of the host cell was always seen, when sections had appropriately been cut. This gap was filled with capsular material after RR treatment. The existence of the extracellular capsular material between the cell membranes of the mycoplasma and host cell may be cited as evidence to show that the fusion of both membranes has not occurred.

Strains SAS, 12T, and KP-13 grown in broth medium for 23 h at 37°C were harvested by centrifugation at 17,000 × *g* for 20 min. Pellets were suspended in phosphate-buffered saline (pH 7.2) to give a 10× concentration of organ-

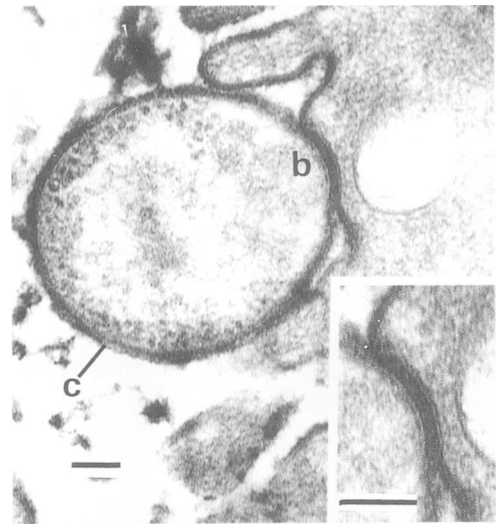

FIG. 5. A portion of the tracheal epithelium of a chicken treated as described for Fig. 4. The tissue was processed as for the mycoplasmas in Fig. 1. A mycoplasma is attached to the plasma membrane of the epithelial cell. The gap between the membranes of the bleb (b) of the organism and host cell is filled with the capsular material (c). The capsular material also appears to bridge the interspace between membrane sites other than the bleb and the adjacent microvilli. Bar, 100 nm. Inset shows the attachment site at higher magnification. The unit membranes of both mycoplasma and host cell are clearly visible. Bar, 100 nm.

isms. Antigen prepared in this way hemagglutinated 0.25% suspensions of chicken erythrocytes to a titer of 1:64 for strain SAS, 1:16 for strain 12T, and 1:256 for strain KP-13. Thus, the presence of capsular material on the strains of *M. gallisepticum* could not be correlated with their hemagglutinating activity.

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