Interaction of *Mycoplasma hyopneumoniae* with the Porcine Respiratory Epithelium as Observed by Electron Microscopy

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An in vivo-passaged strain of *Mycoplasma hyopneumoniae* attained viability titers of $10^6$ to $10^8$ color-changing units per mg of tissue in pig lungs and caused gross and histological pulmonary lesions. Mycoplasmas were readily located in the lumina of the respiratory tract by electron microscopy. In sections of tissue fixed in glutaraldehyde-osmium, the organisms were found to possess many radial fibrils on the outer surface of the limiting membrane. These fibrils appeared to interconnect adjacent mycoplasmas and to extend between the organism and epithelial cell. Ruthenium red staining demonstrated a thick, dark layer of capsular material enveloping the entire mycoplasma cell. The capsular material was seen to bridge the space between the mycoplasma and host cell. The general morphology of the in vitro-passaged strains grown in broth medium was essentially similar to that of the in vivo-passaged strain. In these organisms, however, no long fibrils were seen, although a fuzzy layer was present outside the cell membrane. The ruthenium red-positive capsule was stained less intensely, and its width was only about one-half that observed for the in vivo-passaged strain. In negatively stained preparations, the cells had an outer fringe of amorphous material apparently corresponding to the fuzzy layer seen in thin sections. The in vitro-passaged strain grew poorly in pig lungs and lost its ability to produce gross pulmonic lesions. The organisms in the respiratory tract had a capsule much thinner than that of the in vivo-passaged strain.

*Mycoplasma hyopneumoniae* is the causative agent of a worldwide, chronic pneumonia in pigs known as swine enzootic pneumonia (SEP) (21). Although there have been a number of publications on the histological and ultrastructural changes in SEP, little is known about the interaction of the organism with its host cell. In electron microscopic studies on the pneumatic lungs of pigs infected with *M. hyopneumoniae*, numerous mycoplasmas were located predominantly between the cilia and microvilli of the bronchiolar and bronchial epithelial cells (16, 19, 26). In these studies, mycoplasmas have never been found in contact with the cell surfaces. Similar results have been described by Baskerville (3) and Baskerville and Wright (4) in ultrastructural studies on the lungs of pigs infected with *M. hyorhinis*. These findings are in a striking contrast to those observed with the human pathogen *M. pneumoniae* (8) and the avian pathogen *M. galliseptica* (24). In these two species of mycoplasmas, it has been generally said that the adherence of the organisms to the respiratory epithelium is a prerequisite for pathogenicity.

Those electron microscopic findings reported by previous workers (16, 19, 26) on SEP raise questions as to how *M. hyopneumoniae* becomes established on the mucosal surface of the respiratory tract despite the mucociliary clearance mechanism, and how the organism may exert its effect on host cells from an extracellular location. To answer these questions, we examined the association between *M. hyopneumoniae* and the porcine respiratory epithelium by transmission electron microscopy of thin sections. Organisms grown in broth medium were also studied in the electron microscope by negative staining of whole cells and thin sectioning of cells.

**MATERIALS AND METHODS**

*Mycoplasma strains and media*. Two strains of *M. hyopneumoniae* were used. The MI-3 strain was isolated at our laboratory from the lungs of a pig with SEP. The strain was maintained by serial passage in pigs or in LGM-5 broth medium. When used for the present study, the in vivo-passaged strain was at passage 8, and the in vitro-passaged strain was at passage 9. The VPP 11 strain was derived from the original isolate of Mare and Switzer (18) and obtained from T. Fujikura,
This strain was chosen because it has been shown to be nonpathogenic in the respiratory tract of pigs at passage 6 in broth medium by repeated intranasal inoculations (T. Yagihashi, unpublished data). Cultured organisms from passage 6 were used for the present study.

The in vitro-passaged strains were grown in LGM-5 broth medium (29) consisting of Hanks balanced salt solution (50 ml), Hartley broth (30 ml), inactivated swine serum (20 ml), lactalbumin hydrolysate (0.5 g), 25% fresh yeast extract (5 ml), 2.5% thallium acetate (1 ml), and 100,000 U of penicillin G potassium. The solid medium, which was used for the disk growth inhibition test, was prepared by adding 0.6 g of Oxoid Ionagar no. 2 to the broth medium. The pH of both media was adjusted to 7.6 with 1 N sodium hydroxide.

**Pigs and method of infection.** Specific pathogen-free miniature pigs of the Pitman-Moore strain, known to be free of mycoplasma, were obtained from the Nippon Institute for Biological Science Laboratory Animal Research Station, Kobuchizawa, Japan, and used throughout the study. For inoculation with the in vivo-passaged strain, portions of the consolidated lung lobes from pigs inoculated intranasally with the strain were ground up in tryptose phosphate broth (Difco Laboratories) and suspended in the same broth to give a 10% (wt/vol) suspension. The inoculum contained 10⁶ color-changing units (CCU) of *M. hyopneumoniae* per ml. It was bacteriologically sterile, and no viruses were demonstrated by cell culture and chicken embryo inoculation tests. Each of five pigs, 7 weeks old, was inoculated intranasally with 5 ml of the inoculum under sedation with ketamine hydrochloride (15 mg/kg, given intramuscularly). The inoculation was repeated three times at 4-day intervals. All inoculated pigs were housed separately in a cage. One pig was killed at 3 weeks, and the remaining four pigs were killed at 5 weeks after the first inoculation; their lungs were then subjected to titration of mycoplasmas and examination by light microscopy and electron microscopy. For inoculation with the in vitro-passaged MI-3 strain, three pigs, 8 weeks old, were each inoculated similarly with 5 ml of broth culture containing 10⁶ CCU/ml. One pig each was killed at 2, 3, and 5 weeks after the first inoculation, and their lungs were processed as described for the in vivo-passaged strain.

**Titration and identification of mycoplasma.** Lung tissues from consolidated areas in pigs inoculated with the in vivo-passaged strain and from the anterior lobes in pigs inoculated with the in vitro-passaged strain were homogenized in LGM-5 medium to make a 10% (wt/vol) suspension. CCU present in the suspensions were determined by a method described by Denny et al. (9). Isolates were identified as *M. hyopneumoniae* by the disk growth inhibition technique (7).

**Light microscopy.** Specimens for histological examination were collected from the anterior lobes of the lungs of all animals. The tissue was fixed in 10% Formalin solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**Electron microscopy.** The lung tissue was taken after local irrigation with cold 2.5% phosphate-buffered glutaraldehyde. Small tissue blocks were fixed in 2.5% glutaraldehyde for 2 h at 4°C. After being washed with phosphate buffer, the tissue blocks were postfixed in 1% osmium tetroxide for 1 h at 4°C, dehydrated in an ascending series of ethanol, and embedded in epoxy resin. The other portions of the lung tissue were fixed in the presence of ruthenium red (RR) essentially as described by Springer and Roth (23). In brief, small tissue blocks less than 1 mm³ were fixed in a mixture of equal volumes of 3.6% (wt/vol) glutaraldehyde, 0.2 M cacodylate buffer, pH 6.5, and RR (1.5 mg/ml of water) for 1 h at 4°C. After being washed with three changes of cacodylate buffer, the tissue blocks were postfixed by adding a mixture of equal volumes of 4% (wt/vol) osmium tetroxide, 0.2 M cacodylate buffer, pH 6.5, and RR (1.5 mg/ml of water) and keeping them for 3 h at room temperature. The specimens were dehydrated and embedded as before.

The in vitro-passaged MI-3 and VPP 11 strains were grown in LGM-5 broth medium for 41 h at 37°C. The cultures were harvested by centrifugation at 17,000 × g for 20 min. Pellets were suspended in fixative with or without RR and further processed as described above for tissue blocks. Thin sections were cut by using glass knives on a Porter-Blum ultramicrotome and stained with uranyl acetate followed by lead citrate. Negative staining was applied to the in vitro-passaged strains grown in broth medium for 41 h at 37°C, either unfixed or after fixing with formaldehyde. Fixation of organisms was done by adding formaldehyde directly to the broth cultures containing 10⁶ CCU/ml to a final concentration of 0.74%. The cultures were incubated with the fixative for 8 h at room temperature. In either case, 1 drop of the culture was spread on a Formvar-carbon-coated grid, and then the grid was inverted and floated on the surface of 1% ammonium acetate for 5 min to remove salts. The grid was blotted from the edge with filter paper and floated on 1 drop of 1% uranyl acetate for about 1 min. The excess fluid was drawn off with filter paper.

Thin sections and negatively stained preparations were examined with a JEM-100B electron microscope, using an accelerating voltage of 80 kV.

**RESULTS**

**Ultrastructural features of *M. hyopneumoniae* grown in broth medium.** The dimensions and general morphology of the in vitro-passaged MI-3 and VPP 11 strains grown in broth medium were similar to each other. The predominant cells appeared round or oval with a diameter of from 400 to 1,200 nm. The organisms were bounded by a unit membrane about 10 nm thick, and fibrillar nuclear material and ribosomes could be identified in the cytoplasm (Fig. 1). In thin sections of organisms of both strains not treated with RR, a fuzzy layer approximately 16 nm in width was observed outside the limiting membrane of the cells. In preparations treated with RR, both strains had an RR-positive capsule external to the limiting membrane. The capsule was about 20 nm in thickness and appeared to be fibrillar or amorphous (Fig. 2).

The examination of negatively stained whole cells revealed a layer of amorphous material about 16 nm wide external to the limiting membrane (Fig. 3). This layer was observed on all cells examined, whether they were unfixed or...
fixed with formaldehyde before negative staining, and apparently corresponded to the fuzzy surface layer observed in thin sections of organisms.

**Pathological changes and mycoplasmal growth in the lung.** All five pigs which were inoculated with the in vivo-passaged MI-3 strain and killed 3 or 5 weeks after the first inoculation had gross consolidated lesions of various sizes in the anterior lobes of their lungs. Histologically, the most conspicuous feature of lesions was massive peribronchiolar, peribronchial, and perivascular lymphoid hyperplasia. There was a considerable amount of cellular exudate in the respiratory tract and alveoli in some sections. In consolidated areas, the alveoli were collapsed and partly filled with inflammatory cells and edema fluid (Fig. 4). Viability titers of mycoplasmas in consolidated areas of the lungs of these pigs ranged from $10^6$ to $10^9$ CCU/mg of tissue. All isolates were identified as *M. hyopneumoniae* by the disk growth inhibition technique. No bacteria were demonstrated in any of the lungs examined.

Three pigs inoculated with the in vitro-passaged MI-3 strain had no gross pneumatic lesions. In pig 1 killed at 2 weeks and pig 2 killed at 3 weeks after the first inoculation, a slight peribronchiolar and perivascular lymphocytic infiltration was seen in some sections prepared from the anterior lobes of their lungs. There was little intraluminal exudate in the respiratory tract and alveoli (Fig. 5). In the lungs of pig 3 killed at 5 weeks after inoculation, there was a slight increase in peribronchiolar and interalveolar connective tissue which contained a small number of lymphocytes. CCU/mg of lung tissue in pigs 1 and 2 were $10^2$ and $10^5$, respectively, and in pig 3, no mycoplasma was recovered from the lungs.

**FIG. 1.** In vitro-passaged VPP 11 strain of *M. hyopneumoniae*; 41-h culture. The organisms were fixed in glutaraldehyde-osmium, and the section was stained with uranyl acetate and lead citrate. The predominant cells are round with a diameter of between 500 and 800 nm. A fuzzy layer can be seen outside the limiting membrane of the cells. Bar, 1,000 nm.

**FIG. 2.** In vitro-passaged MI-3 strain of *M. hyopneumoniae*; 41-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.

**FIG. 3.** In vitro-passaged MI-3 strain of *M. hyopneumoniae*; 41-h culture. The organism was fixed in formaldehyde and negatively stained with uranyl acetate. A surface layer (sl) of amorphous material is seen outside the membrane. Bar, 100 nm.
Ultrastructure of *M. hyopneumoniae* grown in vivo and its interaction with the respiratory epithelium. In thin sections prepared from grossly affected lobes of five pigs inoculated with the in vivo-passaged MI-3 strain, numerous mycoplasmas were present in the lumina of bronchioles and bronchi. They were found predominantly between the cilia and on the tips of the microvilli of the epithelial cells (Fig. 6), but others were lying free in the lumen. Most of the mycoplasma cells were oval or round and varied in diameter between approximately 500 and 1,000 nm. When the organisms were between the cilia, they tended to assume the elongated form of the shapes of spaces, suggesting the plasticity of the cell body. Occasional mycoplasmas were elongated up to 1,300 nm in length and constricted in the middle portion of the body, giving a dumbbell-like appearance. They seemed to be in the process of binary fission (Fig. 6, inset). The mycoplasmas in the lungs had the same basic ultrastructural features as those grown in broth medium. When viewed at higher magnification, however, all mycoplasma cells in the lumina of the respiratory tract were seen to possess many fine fibrils radiating from the outer layer of the unit membrane (Fig. 6, inset, and Fig. 7). The fibrils measured about 5 nm in diameter and up to 250 nm in length and extended between the mycoplasma and the plasma membrane of adjacent microvilli or cilia. Adjacent mycoplasma cells were also interconnected by the fibrils (Fig. 7).

Since the presence of the RR-positive capsule on the organisms grown in vitro was suggested by the aforementioned findings, fixation and staining by the RR technique were applied to the consolidated lung tissue to demonstrate the polysaccharide capsule on the organisms. The RR technique demonstrated a thick, dark layer of stained material enveloping the entire mycoplasma cell. The capsule, extending about 40 nm from the cell surface, appeared diffuse with no obvious structure and had a jagged margin (Fig. 8). The mycoplasmas often appeared to be in close contact with the plasma membrane of neighboring microvilli or cilia through their capsule. The relationship between the mycoplasma cells and microvilli or cilia was examined by serial sections of the lung tissue stained with RR. Figure 9 illustrates serial sections. The mycoplasma is apparently lying free in the bronchial lumen in Fig. 9A, but in close contact with the tips of several microvilli in the adjacent section shown in Fig. 9B.

No mycoplasmas were identified in any of a large number of sections of the alveolar tissue from all the 5 pigs despite the presence of extensive reaction in alveoli.

In one (pig 2) of three pigs inoculated with the in vitro-passaged MI-3 strain, mycoplasmas were readily located in the bronchi and bronchioles of the anterior lobes of the lungs.
ultrastructural features seen in thin sections of tissues not treated with RR were similar to those observed in the lungs of pigs inoculated with the in vivo-passaged strain. In preparations treated with RR, however, the capsule of the organisms extended only for approximately 25 nm outside the limiting membrane (Fig. 10).

**DISCUSSION**

The morphological features of *M. hyopneumoniae* revealed in our study are essentially similar to those reported by others (16, 19, 26) in the respiratory tract of pigs with SEP. In the present study, however, the existence of extracellular structures was clearly demonstrated on the cell surface of the organisms. The organisms grown in vivo and processed by an ordinary method showed many delicate fibrils, measuring about 5 nm wide and up to 250 nm long and projecting outward from the cells. Such long radial fibrils have never been described in any mycoplasma studied so far, although extracellular structures, which were described as floccular material, fuzzy layer, nap, or surface projections, have been reported (2, 5, 6, 10, 25). The fibrils are similar in appearance to those filaments (antennulae microvilli) radiating from the microvilli of ciliated epithelial cells (11), but differ from the antennulae microvilli in that the former were much longer than the latter and they were also seen on the organisms far away from the epithelial surface.

A capsule staining with RR similar to that demonstrated in the present work has been shown in various mycoplasmas (1, 13, 14, 20, 28). It has been suggested that the capsule was associated with the adhesive properties (13, 28) or virulence of mycoplasmas (12, 14). The relationship between the RR-positive capsule and the radiating fibrils or a fuzzy layer seen on the mycoplasma cells not treated with RR remains to be determined. Howard and Gourlay (14) suggested that the outer fringe of amorphous material on bovine mycoplasmas not treated with RR could be the inner layer of the capsule, and the outer layer may be less tightly bound and lost during preparation unless treated with RR. It was also presumed that the hair-like fibrils on *Ureaplasma urealyticum* might provide a site of adherence for the material stained with RR (20).

As described by previous workers (26), *M. hyopneumoniae* cells were never seen in direct contact with the epithelial surface. However, fibrils radiating from the mycoplasma could be seen extending between the organism and the epithelial cell and among the organisms; in preparations treated with RR, a gap separating the mycoplasma membrane from that of the host cell appeared to be bridged by capsular material.
Our observations on serial sections suggest a possibility that mycoplasmas somewhat distant from the epithelial surface could have been associated with cells outside the plane of section. These findings indicate that the extracellular structures of *M. hyopneumoniae* may play an important role in the interaction of the organism with its host cell. The fibrils and RR-positive material on the mycoplasma cells may serve to hold the organisms close to the host cells, en-
a pig killed 3 weeks after inoculation with this strain did not differ in the basic morphological features from the in vivo-passaged strain. However, the RR-positive capsule on the organisms was much thinner than that of the in vivo-passaged strain. It has been noted that some strains of *M. hyopneumoniae* become less pathogenic in broth culture, and after serial broth passages they lose their ability to produce gross pneumonia in pigs (27). In several bacterial species, it has been reported that the amount of capsular polysaccharide is a major factor in their virulence (17, 22) and decreases significantly with in vitro passage (15, 17). Taking these observations into consideration, it is plausible that the in vitro-passaged strain was eliminated from the respiratory tract of pigs by the mucociliary clearance mechanism for the diminished adhesive properties resulting from the decrease in the amount of capsular material. It thus appears that the amount of capsular material on *M. hyopneumoniae* may be related to its pathogenicity.

**LITERATURE CITED**


**FIG. 10.** A portion of the bronchiolar epithelium of a pig inoculated intranasally with in vitro-passaged MI-3 strain and killed 3 weeks after inoculation. The tissue was processed as for Fig. 8. Capsular material (c) is evident outside the limiting membrane, but its width (about 25 nm) is much thinner than that of the in vivo-passaged strain. Bar, 200 nm.


