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Pathogenicity and Cytadherence of *Mycoplasma imitans* in Chicken and Duck Embryo Tracheal Organ Cultures

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Two strains of the avian organism *Mycoplasma imitans* were examined for pathogenicity and cytadherence in chicken and duck embryo tracheal organ cultures, and a virulent strain of the related pathogen *Mycoplasma gallisepticum* was included for comparison. All consistently caused ciliostasis in tracheal explants from both hosts, and examination of infected tissues by immunofluorescence and transmission electron microscopy demonstrated that *M. imitans* proliferated on the epithelial surface and adhered to the respiratory epithelium by means of its terminal tip structure in the same manner as *M. gallisepticum*. These observations endorse the striking phenotypic similarities between *M. imitans* and *M. gallisepticum* and suggest that *M. imitans* may have pathogenic potential in vivo.

Tracheal organ cultures (TOCs) derived from various animal hosts have proved very useful for studying the pathogenesis of respiratory mycoplasma infections in vitro. Ciliary activity, which has been shown to correlate with tissue viability, is used as a measure of injury (18), and TOCs prepared from chicks or chicken embryos have been used by a number of workers to assess pathogenicity of avian and mammalian mycoplasmas (1, 4, 12, 14–16, 26, 31, 35, 42).

Mycoplasma gallisepticum is a well-known respiratory pathogen of poultry, and its pathogenicity for chicken TOC provides a useful indicator of its pathogenicity in vivo in the host (31, 35). In contrast, *Mycoplasma gallinarum*, which is not considered pathogenic for chickens, does not impair the viability of chicken embryo TOCs and may even enhance it (14, 35, 43).

Thus, we chose to use the avian embryo TOC system for preliminary pathogenicity studies on a new avian *Mycoplasma* species, *Mycoplasma imitans* (7). This mycoplasma has been isolated from ducks, geese, and partridges and has been shown to share a significant (40 to 46%) genomic relationship with *M. gallisepticum* (19, 20). Little is known about the pathogenicity of *M. imitans*, but its in vitro phenotypic properties are remarkably similar to those of *M. gallisepticum* and include possession of a tip-like structure (7) which is similar in appearance to the attachment organelle of *M. gallisepticum*. Therefore, it was of interest to see if *M. imitans* also caused ciliostasis in TOCs and, if so, to examine the host-parasite relationship in more detail using immunofluorescence (IF) and transmission electron microscopy (TEM) for evidence of adherence and to see if the tip-like structure played a similar role to that of *M. gallisepticum.*

Two strains of *M. imitans* were used, and the well-characterized virulent strain S6 of *M. gallisepticum* was included for comparison. Both chicken and duck embryo TOCs were used because *M. imitans* was first isolated from a duck and because Dupiellet (19) had compared its pathogenicity with that of *M. gallisepticum* S6 in chicken and duck embryos and in chicken and duck embryo fibroblasts and found that *M. imitans* was more pathogenic for the duck than for the chicken tissues, while the reverse was true for *M. gallisepticum.*

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MATERIALS AND METHODS

Mycoplasma strains. *M. imitans* 4229 was isolated in France from the turbinate of a duck with airsacculitis and peritonitis. Strain B2/85 was isolated in the United Kingdom from the eye of a partridge with sinusitis. Both were cloned three times by filtration. The in vitro passage level of 4229 on arrival in this laboratory was not known, but it underwent 12 further passages, while strain B2/85 had undergone a total of 12 in vitro passages. *M. gallisepticum* S6 had been passaged through specific-pathogen-free turkeys (35) and had undergone four in vitro passages thereafter. Several in vivo experiments have confirmed that this particular S6 culture is a primary pathogen for turkeys (9, 33) and can act synergistically with *Escherichia coli* (3) to cause disease in chickens. The identity and purity of cultures were confirmed by indirect IF tests on colony-bearing agar blocks (37). Stocks were frozen in aliquots at −70°C, and the number of CFU per milliliter after one freeze-thaw cycle was established (8).

Rabbit antiserum. Antisera to *M. imitans* 4229 and B2/85 were prepared in rabbits (7), and antisera to *M. gallisepticum* PG31 was from the former Food and Agriculture Organization-World Health Organization Collaborating Centre for Animal Mycoplasmas, University of Aarhus, Aarhus, Denmark.

Mycoplasma broth (MB). The medium used for mycoplasma isolation, routine subculture, and viable counts has been described (6).

Organ culture medium (OCM). The maintenance medium was similar to that of Cherry and Taylor-Robinson (14). It contained 10 ml of Eagle's 10× minimal essential medium (Gibco Ltd, Paisley, Scotland) supplemented with Earle's salts and L-glutamine, 85 ml of 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, and 20,000 U of sodium benzyl penicillin. This was used with or without 5 ml of inactivated newborn calf serum (Gibco Ltd), and the pH was adjusted to 7.3.

Mycoplasma inocula. Mycoplasma inocula were grown in MB, and dilutions were made to give a dose containing "low" or "high" numbers of CFU (approximately 10^3 and 10^6, respectively) per tracheal ring. Dilutions were made in MB without thallium acetate except for the final one, which was prepared in OCM.

TOCs. TOCs were prepared from 19-day-old chicken embryos and from 25-day-old duck embryos. The chicken eggs were from our department's specific-pathogen-free flock, but the duck eggs were from a commercial company. No mycoplasmas were recovered from repeated monitoring of duck embryos from this source, nor were any isolated from the uninoculated control organ cultures.

The methods of preparation, selection, and incubation of tracheal rings were as described by Cherry and Taylor-Robinson (14) except that rings of 0.6 mm were cut with a mechanized McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, United Kingdom). Each was placed in a separate tube in 0.9 ml of OCM, and after 24 h of incubation at 37°C only those showing 100% vigorous ciliary activity were selected. Twenty replicate rings were assigned to each group for inoculation with 0.1 ml of the appropriate inoculum or with OCM in the case of the controls. For the ciliostasis experiments, the high and low doses of mycoplasma inocula were investigated, using OCM with or without added serum, while for the subsequent studies by IF and TEM, only the low dose (approximately 10^3 CFU) of inoculum and OCM without serum were used.

Examination of organ cultures. TOCs were examined daily for ciliary activity (14). Mycoplasma viable counts were determined for the inocula at the start of each experiment and then every other day for pooled 50-μl aliquots of supernatant fluid from five replicate infected rings. Control uninfected organ cultures were similarly sampled for attempted mycoplasma culture. The identity of a random selection of reisolated mycoplasmas was confirmed by indirect IF (37).

For the IF and TEM studies duplicate infected or uninfected TOCs were

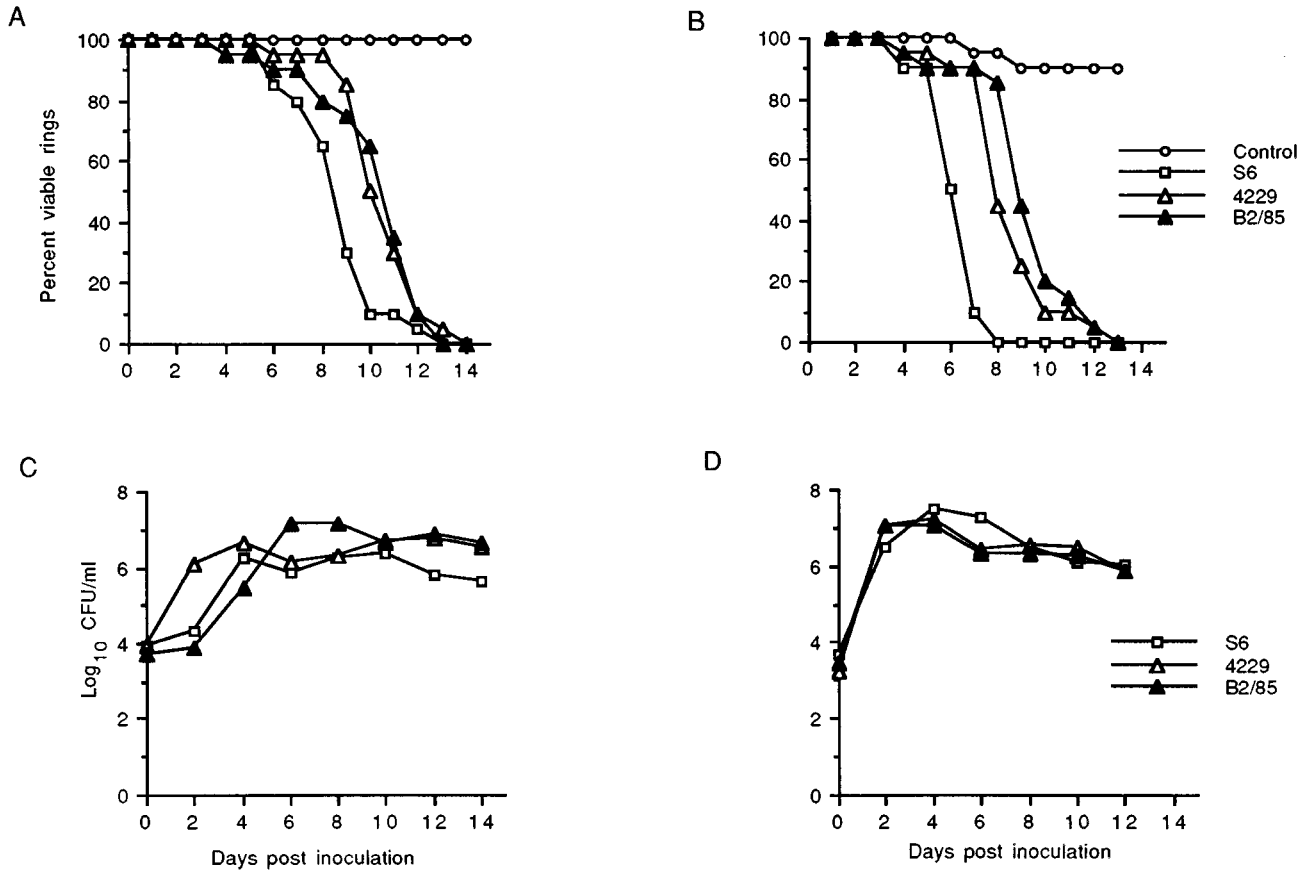

FIG. 1. Chicken embryo organ culture: ciliostatic effects (A and B) and growth curves (C and D) of *M. gallisepticum* S6 and *M. imitans* 4229 and B2/85 in OCM without serum (A and C) and with serum (B and D), using a low dose of inoculum.

removed at 0 (before inoculation), 1, 2, 3, and 5 days post inoculation (p.i.) and processed as described below.

IF staining. Tracheal rings were snap-frozen in OCT (Lab-Tek Products, Miles Laboratories, Inc., Naperville, Ill.) in liquid nitrogen. Sections of 4 to 5 μm in thickness were cut on a cryostat and fixed in acetone for 10 min. They were stained by the indirect method using an appropriate dilution of rabbit antiserum followed by fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (F-9262; Sigma Chemical Co., St. Louis, Mo.). The sections were mounted in nonfade mountant (28) and examined for specific fluorescence. The following controls were included with all tests: uninfected TOCs reacted with test antiserum and conjugate, infected TOCs reacted with normal rabbit serum and conjugate, and infected TOCs reacted with conjugate only.

TEM. TEM was done as described by Dykstra et al. (21). Tracheal rings were placed directly into McDowell's and Trump's fixative (32) and kept for 24 h at 4°C. Tissues were washed in phosphate buffer (0.1 M, pH 7.2) and then postfixed in 1% osmium tetroxide in phosphate buffer at room temperature for 30 to 60 min. They were washed in deionized water, dehydrated, embedded in Spurr's resin, and prepared as ultrathin sections as described by Cole (17). Sections were examined on an electron microscope (H-600; Hitachi, Tokyo, Japan) with an accelerating voltage of 80 kV. Electron micrographs were taken with EM film (Ilford Ltd., Mobberley, United Kingdom).

RESULTS

Growth curves and ciliary activity. Growth curves were first determined for the two strains of *M. imitans* and for *M. gallisepticum* S6 in OCM with or without calf serum, without tracheal rings, and using either the high or low dose of inoculum. Cultures were set up as for organ cultures and incubated at 37°C on a roller drum. The mycoplasmas gave no indication of growth in OCM either without or with calf serum, irrespective of the inoculum dose. There were no increases in viable counts and no color changes in the pH indicator. Growth curves in MB were also determined under these conditions using the same inocula, and proliferation was recorded for all cultures.

The three mycoplasma strains were then examined for their ciliostatic effect in a series of experiments involving the two dose levels of inocula in chicken and duck TOCs with and without calf serum.

The ciliostatic effects of the organisms in chicken embryo TOCs, using the low dose of inoculum without and with added calf serum, are shown in Fig. 1A and B, respectively, and Fig. 1C and D show the corresponding growth curves. All strains caused ciliostasis, although with *M. gallisepticum* it occurred 2 to 3 days earlier than with *M. imitans*. Ciliostasis also tended to occur earlier in the presence of serum. The growth curves indicated that proliferation of mycoplasmas had occurred in all cases, although slightly lower peak titers were attained in the absence of serum. Results (not shown) were similar with the higher dose of inoculum.

Similar results were obtained in duck embryo TOCs, and those for the low dose of inoculum are shown in Fig. 2. As with the chicken TOCs, the ciliostatic effects in duck TOCs occurred earlier in the presence of serum and maximum viable counts tended to be higher. Also, as before, *M. gallisepticum* caused slightly earlier ciliostasis than the *M. imitans* strains. Results (not shown) of the experiments using the higher inoculum dose were essentially the same and, as with chicken embryo TOCs, ciliostasis was not enhanced with the larger dose of mycoplasmas.

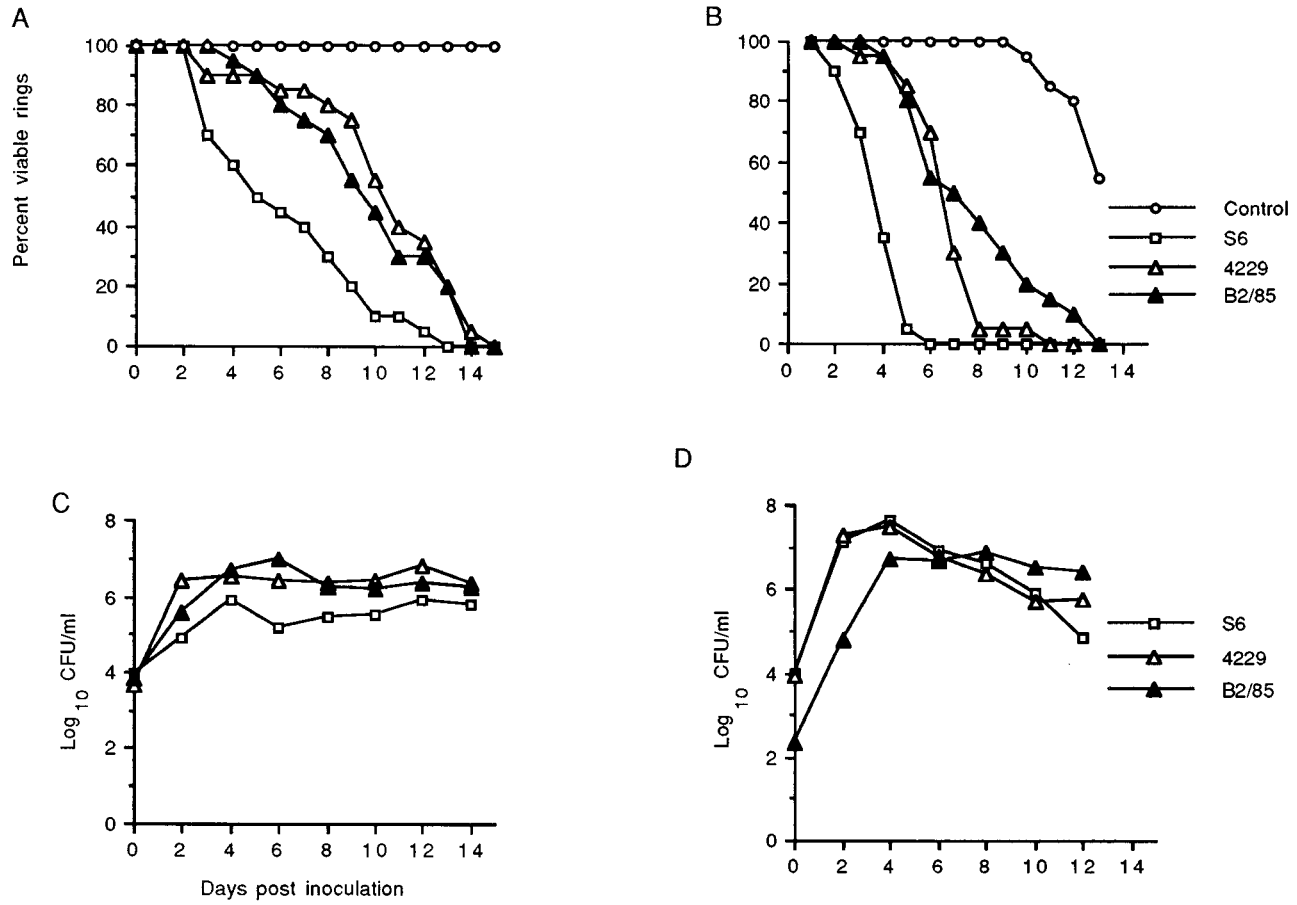

FIG. 2. Duck embryo organ culture: ciliostatic effects (A and B) and growth curves (C and D) of *M. gallisepticum* S6 and *M. imitans* 4229 and B2/85 in OCM without serum (A and C) and with serum (B and D), using a low dose of inoculum.

IF staining. No fluorescence was seen in the negative controls prepared from either chicken or duck TOCs (Fig. 3A). Small fluorescent granules appeared on the epithelial surface of the lumens of chicken and duck TOCs infected with strain 4229 on day 1 p.i. which by day 2 had progressed into small accumulated masses of brightly fluorescent material (Fig. 3B). These increased in amount with time and persisted to the end of the experiment at 5 days. Similar changes were seen with strain B2/85. *M. gallisepticum* S6 demonstrated bright fluorescent masses on the luminal epithelial surface of both chicken and duck TOCs by day 1 p.i., and by days 3 and 5 p.i. it was more extensive, appearing as small masses and abundant large masses on the epithelial surface (Fig. 3C). Detachment of the epithelial cell layer was noted on days 3 and 5. All strains also showed fluorescence around the outside of the tracheal rings on the peritracheal adventitia. It was not seen in the uninfected controls but was present in all infected TOCs except at 1 day p.i. in chicken TOCs infected with *M. imitans*. The intensity did not appear to increase with time.

TEM. Ultrathin sections of control uninfected tracheal rings of both chicken and duck embryo origin had epithelial cells with cilia and microvilli and some nonciliated cells. There were no obvious differences between *M. imitans* 4229 and B2/85 either in their ultrastructure or in their effects on chicken and duck explants. In both chicken and duck TOCs there was loss of cilia and the microvilli were reduced in number. This was seen by day 2 p.i. with strain 4229 but not with B2/85. Three and 5 days after infection there was further loss of cilia and the microvilli were fewer in number and shorter in length. Many mycoplasmas were in close proximity to the epithelial surface between the cilia or microvilli, and others were lying free in the lumen. The organisms were rounded when more distant from the epithelial surface but when closer were pearshaped with their narrow ends towards the epitheleum. Many appeared to be attached to the epithelial cell surface, and some appeared to be attached to the microvilli or cilia by means of the terminal tip-like structures (Fig. 4) which were pushed down onto the epithelial surface. This tip consisted of a dense central core surrounded by moderately dense material covered externally by the triple-layered membrane. Attached organisms were seen on both ciliated and nonciliated cell surfaces, but no mycoplasmas could be seen within the epithelial cells or between them.

Similar changes were seen with *M. gallisepticum* S6, but the damage to the epithelium was more severe and deciliation was followed by detachment of fragments of epithelium, with attached mycoplasmas, into the lumen. As with *M. imitans* most of the *M. gallisepticum* organisms were in close proximity to the epithelial surfaces of both ciliated and nonciliated cells and were attached via their terminal tips. This was seen in both chicken and duck TOCs.

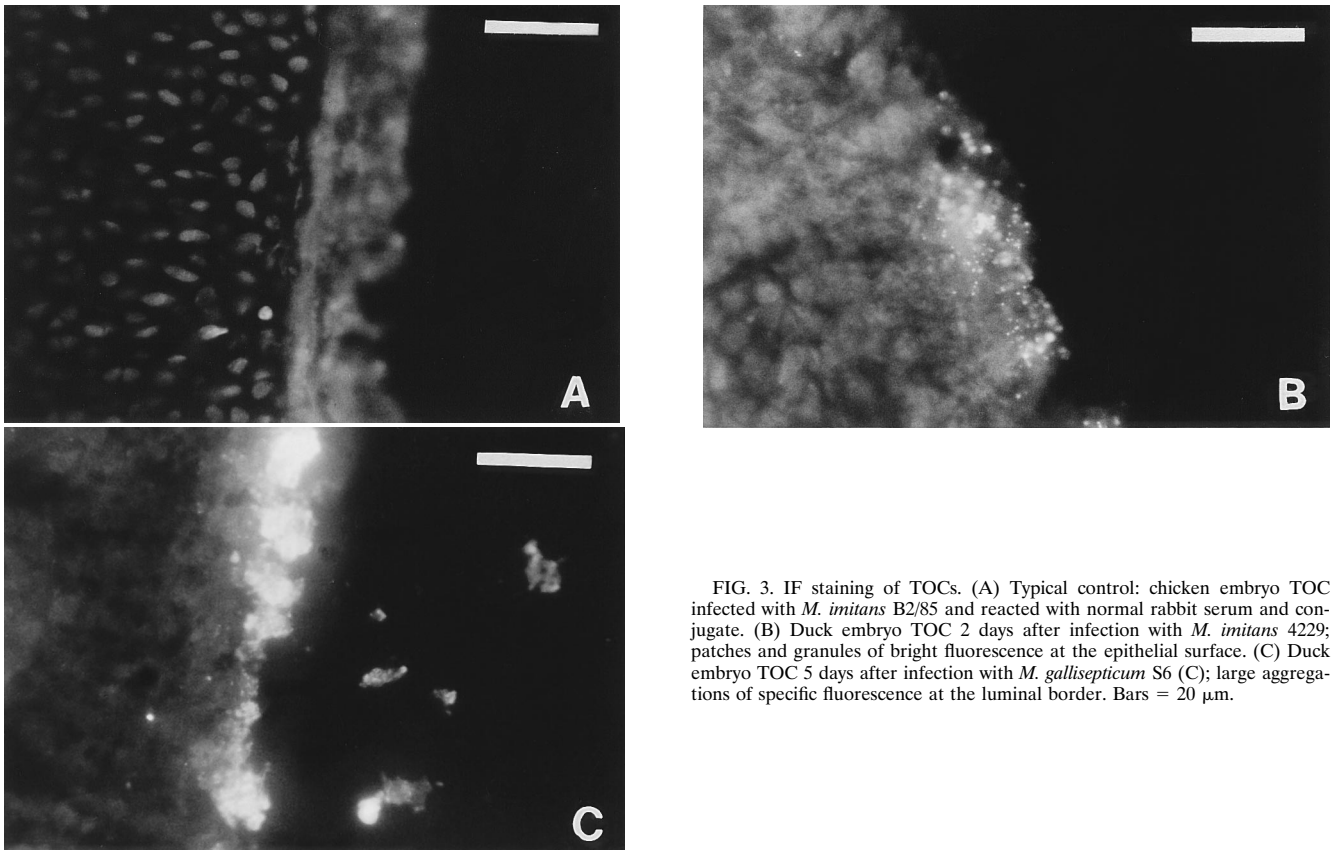

FIG. 3. IF staining of TOCs. (A) Typical control: chicken embryo TOC infected with *M. imitans* B2/85 and reacted with normal rabbit serum and conjugate. (B) Duck embryo TOC 2 days after infection with *M. imitans* 4229; patches and granules of bright fluorescence at the epithelial surface. (C) Duck embryo TOC 5 days after infection with *M. gallisepticum* S6 (C); large aggregations of specific fluorescence at the luminal border. Bars = 20 μm.

DISCUSSION

A nonpermissive maintenance medium which did not support mycoplasma growth (11, 14) was used for the chicken and duck embryo explants because under permissive conditions cell damage might not represent the processes that occur in the actual host-parasite cellular interaction (23). A nonpermissive medium forces the mycoplasmas to rely on host cells for their nutrients (13). OCM with or without calf serum was not permissive for *M. imitans*, as shown earlier with *M. gallisepticum* (1, 16, 42).

Since the pathogenicity of *M. imitans* had not been investigated before in chicken TOCs and neither *M. imitans* nor *M. gallisepticum* had been used in duck embryo organ culture, comprehensive studies were performed in medium with or without calf serum and using high and low doses of inoculum.

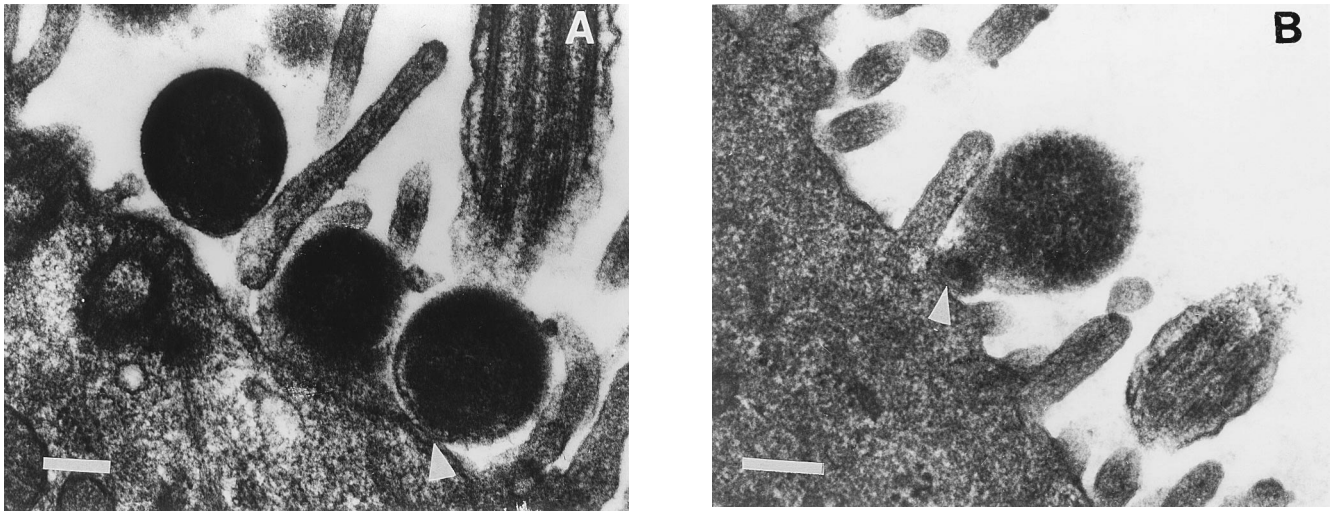

FIG. 4. Electron micrographs of TOCs 2 days after infection. (A) Ducks embryo TOC infected with *M. imitans* 4229. (B) Chicken embryo TOC infected with *M. imitans* B2/85. Terminal tip structures are present in close proximity to the epithelial surface, and some appear to be pushing down onto the surface. Bars = 200 nm.

The results clearly showed that the presence of either a chicken or a duck embryo tracheal ring allowed multiplication of *M. imitans* and *M. gallisepticum*. The two mycoplasma species also caused ciliostasis in both chicken and duck embryo TOCs, and in each case the effect was independent of the presence of serum, although ciliostasis tended to occur slightly earlier and the viable counts reached higher peak values in the presence of serum. This is in contrast to observations made with bovine fetal TOCs infected with *Mycoplasma dispar*, in which ciliostasis was dependent upon the presence of fetal calf serum in the maintenance medium and was related to the growth of the mycoplasmas (27).

Cherry and Taylor-Robinson (14, 16) reported that some mycoplasma species do not inhibit ciliary activity in TOCs despite their multiplication, indicating that ciliostasis is more likely to be related to pathogenicity. Certainly in our experiments maximum ciliostasis did not necessarily correspond with the peak titers of viable organisms but sometimes occurred when the growth curve was in decline. However, the viable counts measured only organisms which were free in the maintenance medium and excluded those on the luminal surface. Ciliostasis with *M. imitans* and *M. gallisepticum* was also independent of the inoculum dose, a phenomenon observed previously with *M. gallisepticum* (15), although it was suggested on the basis of other studies (31) that a much smaller dose of inoculum (5 to 50 CFU) might enable differences between strains to be observed.

A possible factor involved in ciliostasis is the production of acid by the mycoplasmas. In our studies, pH change was reduced by the addition of HEPES buffer and by omitting MB from the inoculum to minimize the amount of fermentable substrates. Despite this, slight acid color change was seen in all experiments. However, it is unlikely that this was responsible for the ciliostasis because in other experiments, not reported here, daily changing of the TOC medium completely prevented acid pH change but did not prevent the ciliostatic effect of *M. imitans* or *M. gallisepticum*. Furthermore, *Mycoplasma iowae*, which is an avian mycoplasma with no apparent pathogenicity for chicken or duck respiratory tissue, was found to multiply and produce acidity but no ciliostasis in our organ culture system. There was also no relationship between acid production and ciliostasis in rat or pig TOCs infected with *Mycoplasma mobile* (38).

Dupiellet (19) found that *M. imitans* 4229 was more pathogenic for duck fibroblasts and embryos than for chicken fibroblasts and embryos, but the reverse was true for *M. gallisepticum*. No such trend was seen in the TOCs, but strain 4229 had undergone further in vitro passages since the earlier study, which may have reduced its virulence. Ciliostasis always occurred earlier with the S6 strain of *M. gallisepticum* than with the *M. imitans* strains.

Good correlation has been found between the ciliostatic effect of *M. gallisepticum* strains on chicken TOCs and their pathogenicity for live chickens (31, 35), and changes in the tracheal epithelium seen in vitro and in vivo followed a similar pattern (21). If this correlation also applies to *M. imitans*, then our observations suggest that this mycoplasma is a potential pathogen for chickens in vivo.

Since cytadherence of *M. gallisepticum* to chicken tracheal epithelial cells is known to occur via its tip-like structure (1, 41, 45) and is considered to be the first step in the pathogenic process (36), the IF and TEM studies were conducted to see if a similar phenomenon occurred with *M. imitans*. IF demonstrated that *M. imitans* organisms accumulated along the epithelial cell surface of the tracheal lumen in chicken and duck embryo TOCs. This was similar to the picture seen with the virulent S6 strain of *M. gallisepticum*, although with this mycoplasma the staining was more intense and extensive and there was also destruction and detachment of the epithelial cell layer. The IF results with *M. imitans* suggested the possibility of cytadherence to the tracheal epithelium, but this could be resolved only by the TEM studies. All three strains also showed fluorescence on the peritracheal ring adventitia, although it did not appear to increase with time. This fluorescence was almost certainly specific for the mycoplasmas because none was seen in negative control preparations, including uninfected tracheal rings reacted with antiserum and conjugate. A similar observation was made in an earlier study using chicken embryo TOCs and *M. gallisepticum* or *Mycoplasma synoviae* (34) and may have been due to the lack of serum in the medium. It has been reported that mycoplasmas can bind nonspecifically to the outer surface of the trachea in organ cultures (22, 24).

In ultrathin sections deciliation was the most prominent feature seen with all three strains. Although, as reported earlier (21), some epithelial cells in uninfected TOCs did not have cilia, most possessed intact cilia and microvilli and there was an obvious difference between uninfected and infected rings. The *M. imitans* strains apparently caused less damage to the tracheal epithelium of both chicken and duck embryo TOCs than the virulent S6 strain of *M. gallisepticum*, which caused deciliation of the epithelium followed by exfoliation. Nevertheless, the damage caused by the *M. imitans* strains indicated their pathogenicity for both chicken and duck embryo tissue and correlated with the ciliostasis seen earlier. TEM also demonstrated that both the *M. imitans* strains possessed a tip-like organelle which appeared to mediate attachment to the epithelial surface of chicken and duck cells in a manner similar to that of *M. gallisepticum* (46). It was also shown here for the first time that *M. gallisepticum* attaches to duck tracheal epithelium. This observation, taken together with the ciliostatic effect of *M. gallisepticum* S6 in duck TOCs, indicates that the duck has tissue receptors for this pathogen and that *M. gallisepticum* may have the potential to cause disease in duck respiratory tissue under some circumstances. Limited in vivo studies with live ducks support this suggestion (29).

The changing appearance of the mycoplasmas from round to pear-shaped with their increasing proximity to the epithelial surface has been observed before with *M. gallisepticum* (2, 41) and also with *M. mobile* (39). Correlation of specialized terminal organelles with attachment is well recognized for other mycoplasma species such as *M. pneumoniae* (5), *M. genitalium* (44), and *M. mobile* (30, 39), although cytadherence is not restricted to those species with terminal tip organelles (36). Although attachment of the organism to the respiratory epithelium is considered a prerequisite for cytopathogenicity of some mycoplasmas, including *M. gallisepticum* (1, 41, 45), the mechanisms by which they then cause damage to the host are far from clear. In our studies there was no evidence that *M. imitans* was able to penetrate the epithelial surface.

Since our studies with *M. imitans* have shown that infection of chicken and duck embryo TOCs results in ciliostasis, deciliation, and accumulation of attached organisms on the epithelial cells, it is important to know if pathological changes occur in vivo in chickens or ducks or in other avian hosts. Preliminary studies with geese (10) and with commercial ducks and turkeys (19) have suggested that this may be so, and we have recently demonstrated that experimental infection of 1-day-old mycoplasma-free turkeys with strain B2/85 causes mild but protracted upper respiratory disease (25).

Stådtländer et al. (40), when reporting the effects of *Mycoplasma pulmonis* in rat TOCs, commented that the cytopathic potential could not be fully assessed in the organ culture sys-

tem because host factors not present in vitro may be important in the disease process. The same may well apply to *M. imitans*, and its pathogenicity obviously requires more investigation. If the remarkable similarities between *M. imitans* and *M. gallisepticum* exist also in vivo, then it is possible that, as with *M. gallisepticum*, pathogenicity of *M. imitans* may not be fully expressed unless the host is exposed to environmental stress or to concurrent infection with other agents (29).

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