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Serological Cross-Reactions Between *Mycoplasma genitalium* and *Mycoplasma pneumoniae*

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The recently discovered mycoplasma species *Mycoplasma genitalium* was isolated from urethral specimens from men with nongonococcal urethritis (Tully et al., Lancet i:1288–1291, 1981). In a previous report (K. Lind, Lancet ii:1158–1159, 1982), prominent serological cross-reactions were demonstrated between this mycoplasma and *M. pneumoniae*. In the present study, the two mycoplasma species were compared more extensively. In classical mycoplasma medium without thallium acetate, *M. genitalium* grew more slowly than *M. pneumoniae* did but finally formed similar amounts of acetic acid and lactic acid from glucose. Although their colonies on solid medium were indistinguishable, transmission electron microscopy showed that the flask-formed cells of *M. genitalium* (especially their necks) were shorter than those of *M. pneumoniae*. The two species were distinct since DNA hybridization showed only 1.8% homology in base sequences, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed significantly different profiles of the two strains. However, considerable similarities were found in their antigenic reactions in various serological tests. The presence of common or closely related antigens was demonstrated in the two species with rabbit immune sera in complement fixation test with chloroform-methanol-extracted antigens by an indirect immunofluorescence test on microcolonies, and by metabolism inhibition and growth inhibition tests. Cross-reactions were also demonstrated by crossed immunoelectrophoresis. The role of *M. genitalium* as a human pathogen in the genital tract has not been assessed. If serological tests are to be used in this assessment, caution must be exercised due to the extensive cross-reactions demonstrated. Some of the species-specific antigens which we have demonstrated would be appropriate for use in such tests and would help to circumvent problems caused by cross-reactions.

In 1981, Tully and co-workers (27) described a new mycoplasma which was isolated from urethral specimens from men with nongonococcal urethritis. Although this mycoplasma had several properties and characteristics similar to those of *Mycoplasma pneumoniae*, extensive studies, especially by growth inhibition tests, showed it to be distinct from all other known mycoplasmas; *Mycoplasma genitalium* is the name proposed for this new species (28).

In an attempt to discover complement-fixing antibodies to *M. genitalium* in patients with sexually transmitted disease, we found prominent cross-reactions between this mycoplasma and *M. pneumoniae* (19). The cross-reactions were detected by several serological techniques and, to some degree, by growth inhibition tests. The finding of a close antigenic relationship between the two species in addition to both cultural and morphological similarities (19, 27, 28) prompted us to compare more extensively *M. genitalium* and *M. pneumoniae* with the aim of describing differences and similarities.

MATERIALS AND METHODS

Strains of mycoplasmas. Strain Mac of *M. pneumoniae* was received from E. A. Freundt in 1962 (17) and was used between passages 56 and 58 in our study. Strain M1121 of *M. pneumoniae* was recovered from a Danish patient and used uncloned at passage 9. Strain G37 (type strain) of *M. genitalium* (19) was received in 1982 from D. Taylor-Robinson, who had cloned it three times; it was used between passages 1 and 12 in our media.

Culture media. *M. pneumoniae* was grown in classical Hayflick medium (11) consisting of seven parts of PPLO broth (Difco Laboratories, Detroit, Mich.), two parts of heat-treated, filtered horse serum (56°C for 60 min), one part of 10% (wt/vol) yeast extract (Fleischmann, Standard Brand Inc., New York, or Difco), 1% (wt/vol) glucose, 0.002% phenol red, 0.02% thallium acetate, and 500 U of penicillin G per ml. Solid medium was prepared by adding 1.4% (wt/vol) agar (Difco) to this medium without glucose. Rubber-stoppered vials and plates were incubated at 36°C, the latter in a humid atmosphere. For the production of antigen used in serological tests, polyacrylamide gel electrophoresis, crossed immunoelectrophoresis (CIE), and DNA hybridization experiments, *M. pneumoniae* was grown in Roux flasks, each containing 100 ml of the liquid medium supplemented with 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Sigma). Colonies attached to the glass were washed three times in phosphate-buffered saline (pH 7.2), scraped off, washed thrice again, and stored in small volumes at −80°C (20).

M. genitalium was grown in the same medium as *M. pneumoniae*, except that thallium acetate was omitted and carbenicillin (25 μg/ml), nystatin (25 U/ml), and polymixin B (50 U/ml) were added. The solid medium was prepared by adding 0.5% (wt/vol) agarose (HSA; Litex, Denmark) instead of agar, and plated cultures were incubated at 36°C in N_2 plus 10% CO_2 in a humid atmosphere. Antigens were prepared from cells grown in the liquid medium with HEPES as described for *M. pneumoniae*. In preliminary experiments, SP-4 medium (29) modified with carbenicillin (25 μg/ml) was used.

Gas chromatography. Gas chromatography as described by Holdeman et al. (12) was kindly performed by T. Justesen, Institute for Medical Microbiology, University of Copenhagen.

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Preparation for electron microscopy. The two test strains were grown in culture flasks (Nunclon Delta; Nunc, Roskilde, Denmark), each containing 100 ml of broth medium with HEPES buffer. After incubation for seven days, colonies were scraped off the bottom with a rubber policeman. The suspensions were centrifuged at 12,000 × *g* for 20 min, the sediment from each sample was suspended in 5 ml of PBS (pH 7.2), and an equal volume of 3% glutaraldehyde was added to each buffer sample. The material was incubated at 4°C for 22 h for fixation and then centrifuged. The pellets were resuspended in 20% bovine serum albumin in phosphate-buffered saline (pH 7.4), centrifuged, and solidified by cross-linking the remaining bovine serum albumin with 2 ml of 6% glutaraldehyde in phosphate-buffered saline. At this stage, the pellets were cut into small cubes and treated as previously described (4) before being cut into sections and examined with a Philips EM 300 or EM 201 electron microscope.

Production of antisera. One group of five rabbits (no. 6762 through 6766) was given sonically killed *M. genitalium* with Freund incomplete adjuvant either subcutaneously or intramuscularly in four courses, each consisting of two injections. Intervals between the two injections were 1 to 3 weeks; the animals were bled 1 week after every second vaccination, and revaccination was resumed 1 to 2 weeks later. Another five rabbits (6767-6771) were given intravenous injections of live *M. genitalium* cells suspended in RPMI 1640 medium. They were vaccinated and bled on the same days as the first group. For CIE, a pool of sera from the fourth bleeding of rabbits vaccinated with killed *M. genitalium* cells was used without any purification or concentration.

Other groups of rabbits were immunized with sonically killed *M. pneumoniae* by alternating subcutaneous, intramuscular, and intravenous routes. Pool 16+17 refers to sera pooled from bleedings 16 and 17 after immunization over a 1.5-year period. For CIE, the antiserum was purified by ammonium sulphate precipitation and DEAE ion-exchange chromatography (1).

Serological tests. Complement fixation (CF) tests were performed as previously described (20) or by a similar method in a microtiter system. The antigens were prepared from both *M. pneumoniae* and *M. genitalium* by chloroform-methanol (CM) extraction (15, 20). Indirect hemagglutination was performed as described by Cho et al. (6) with a sonicate of *M. genitalium* cells to sensitize formaldehyde-treated sheep erythrocytes. In the indirect immunofluorescence test, the antigens were microcolonies of each mycoplasma strain that were grown on glass in Roux flasks, washed, and acetone fixed on microscope slides (15 Multiwell; Teknunc, Roskilde, Denmark). When testing human sera a fluorescein-conjugated horse anti-human globulin (Roboz Surgical Instrument Co., Inc., Washington, D.C.) diluted in phosphate-buffered saline containing 10% normal horse serum was used. For rabbit sera, a sheep anti-rabbit conjugate (Statens Seruminstitut) diluted in phosphate-buffered saline containing 10% normal sheep serum was applied. The metabolism inhibition (MI) test was performed by the technique described by Taylor-Robinson et al. (26). Disk growth inhibition (DGI) was performed as described by Clyde (8).

HAD inhibition. For hemadsorption (HAD) inhibition, 1.5-cm squares of agar blocks with well-spaced growing colonies (diameter, 100 to 200 μm) were placed on slides; 25 μl of serum and 25 μl of a 1% suspension of human group O erythrocytes in saline was then added on top. After incubation at 37°C for 15 min, most of the nonadherent erythrocytes were washed off by gently dropping saline over the surface. HAD was graded from 0 to 4^+ according to the percentage of the colony periphery covered with erythrocytes.

Peroxide secretion. Peroxide secretion from the colonies was read after the further addition of 1 drop of 0.01% methylene blue which after 10 min stained erythrocytes in contact with H_2O_2-secreting colonies (18).

CIE. The technique for demonstration of *M. pneumoniae* antigens as previously described (24) was used for both strains. This was the case also when CM extracts of the strains were used as antigens.

In some of the experiments before starting the second-dimension electrophoresis, 5 μl of growth medium was placed in a well at the right-hand end of the first-dimension gels to identify any precipitation line not totally absorbed by the 200 μl of medium incorporated in all intermediate gels.

SDS-polyacrylamide gel electrophoresis. Discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on Protean Double Slab Electrophoresis Cell (Bio-Rad Laboratories, Richmond, Calif.) with the Laemmli buffer system (16). The mycoplasma cells were suspended in 500-μl volumes containing 1% SDS and sonicated at maximal effect with an MSE 100-W sonicator (Scientific Instruments, Sussex, England) in four 30-s cycles at 0°C. After centrifugation at 39,000 × *g* for 5 min at 4°C, the supernatant phase was resonicated in two 30-s cycles in the presence of 1% SDS. The sonicate was mixed with equal volumes of 0.5 M Tris-hydrochloride (pH 6.8) containing 0.1 M dithiotreitol, 2% SDS, and 0.002% bromophenol blue. After boiling for 2 min, samples of 20 to 50 μl (depending on protein content) were added to a 4% acrylamide stacking gel. Electrophoresis was carried out at 25 mA. The current was increased to 40 mA when the tracing dye reached the separating gel containing 10% acrylamide and 4 M urea. Both gels contained 0.1% SDS. The total running time for a gel slab (1.5 by 140 by 140 mm) was ca. 4 h. Immediately after electrophoresis, the gel was immersed in 0.06% Coomassie blue in 3.5% perchloric acid for 24 h at room temperature. Destaining was performed for another 24 h in 5% acetic acid. The gel was photographed and dried between two sheets of polyvinyl chloride foil held in brackets. Molecular weight of protein was estimated by coelectrophoresis of high-molecular-weight and low-molecular-weight protein standards (Bio-Rad) diluted to a ratio of 1:20 in the buffer. For identification of protein bands originating from the growth medium, a sample of a 20-fold dilution of growth medium was included in each run.

DNA-DNA hybridization experiments. DNA extraction was from frozen pellets of the two strains G37 and Mac, and DNA labeling and filter hybridization were done as described previously (7, 23).

RESULTS

Cultural properties. After a few passages, strain G37 grew well in the thallium acetate-free classical medium, changing the color of phenol red from red to yellow-orange in ca. 1 week; the medium with the multiply passaged Mac strain reached this color a few days earlier. In cultures in which the color turned bright to lemon yellow, thereby showing slight turbidity, the G37 strain survived better than the Mac strain did; however, the difference was not quantitated. Thallium acetate at a 1:2,000 dilution totally inhibited the growth of G37; at a 1:4,000 dilution, it demonstrated only partial inhibition.

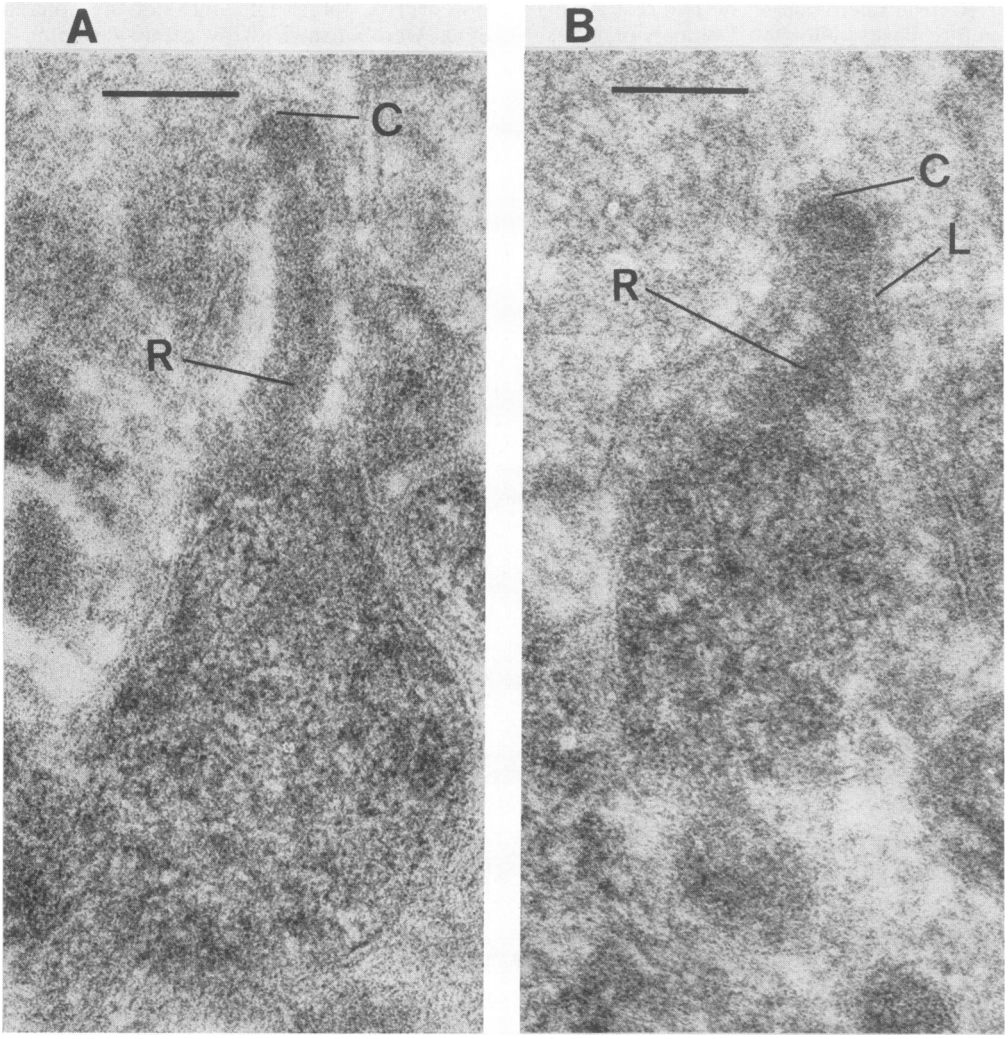

FIG. 1. Electron micrographs of sections of 7-day-old cultures of an *M. pneumoniae* cell (A) and an *M. genitalium* cell (B). Note the flask shape with a neck which is elongated in A and truncated in B. The rodlike structure (R) of the neck and the surrounding translucent zone is more prominent in A than in B. In both strains, the rod ends in a tip surmounted by a cap (C). Note the electron-dense layer (L) coating the membrane of the tip and neck in *M. genitalium* (B). Bar: 0.1 μm.

On solid medium, strain G37 formed colonies a few days later than did the Mac strain; when fully developed, however, the colonies of the two strains were indistinguishable, the larger ones showing the typical "fried egg" appearance. The growth of strain G37 was more dependent on an as-yet-undefined quality of the growth medium than was the Mac strain. The classical media, both liquid and solid, supported the growth of strain G37 better than our SP-4 medium did. In liquid medium with glucose, both strains formed ca. 10 meq of acetic acid and ca. 3 meq of lactic acid per liter as measured by gas chromatography.

Morphology and ultrastructure. Electron microscopy of sectioned material from 7-day-old cultures (Fig. 1) showed flask-shaped cells of both strains, but they were much fewer in the G37 than in the Mac strain culture. *M. pneumoniae* cells of this type had an elongated neck with a central electron-dense rod surrounded by a translucent zone. The rod ended in a tip surmounted by a cap (Fig. 1A). The ultrastructure of *M. genitalium* cells was similar, but the neck was short and broad and looked truncated and the translucent zone around the rod was less prominent (Fig. 1B). Dimensions of these cell types were as follows for *M. pneumoniae*: length, 0.3 to 0.8 μm; largest width, 0.2 to 0.3 μm. For *M. genitalium*, the cell dimensions were as follows: length, 0.3 to 0.5 μm; width, 0.1 to 0.3 μm. In some *M. genitalium* cells, the cytoplasmic membrane of the tip and neck was coated by an electron-dense layer, but this was difficult to resolve due to the intimate contact between the cells and due to the structure of the surrounding bovine serum albumin (Fig. 1B).

Serological cross-reactions. Prominent cross-reactions were observed when CM-extracted antigens of strains G37 and Mac were used in CF tests (Table 1). Both antigens detected rabbit antibodies against both the homologous and heterologous strains, and this was the case also when the IF test was applied. When a whole-cell sonicate of *M. genitalium* was used as the antigen in both the CF test (19) and the IHA test, both tests reacted at significant titer levels with both anti-strain G37 and anti-strain Mac rabbit sera. Human sera with high titers of antibodies to *M. pneumoniae* also reacted with *M. genitalium* antigens in these tests.

Less prominent but significant cross-reactions were often observed when both rabbit and human sera were tested by growth inhibition measured by MI and DGI (Table 1). Sera

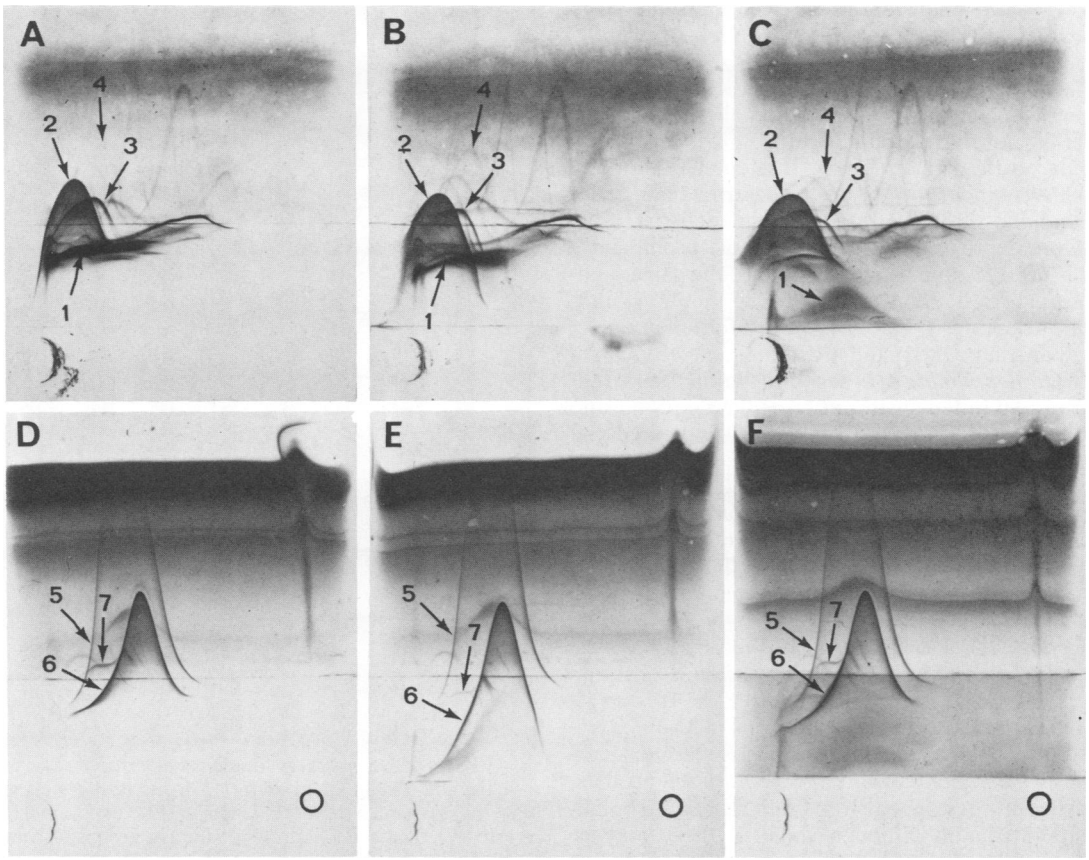

FIG. 2. CIE gels. (A to C) First-dimension gels contained 25 μl of soluble *M. pneumoniae* antigens. Intermediate gels contained the following: 200 μl of growth medium (A); 200 μl of medium plus 50 μl of rabbit anti-*M. genitalium* serum (B); and 200 μl of medium plus 50 μl of human anti-*M. pneumoniae* serum (CF titer of ≥4,000) (C). Second-dimension gels contained 1 ml of rabbit anti-*M. pneumoniae* globulin. (D to F) First-dimension gels contained 25 μl of soluble *M. genitalium* antigens. Intermediate gels contained the following: 200 μl of growth medium (D); 200 μl of medium plus 50 μl of rabbit anti-*M. pneumoniae* globulin (E); and 200 μl of medium plus 50 μl of human anti-*M. pneumoniae* serum (CF titer of ca. 4,000) (F). Second-dimension gels contained 1 ml of rabbit anti-*M. genitalium* serum. Round wells on right-hand side of D to F contained 5 μl of growth medium.

from rabbits immunized with live *M. genitalium* cells generally inhibited cultures of both strains more markedly than did sera from rabbits immunized with killed *M. genitalium* cells.

Some sera from patients with pneumonia and with serological evidence of *M. pneumoniae* infection inhibited the metabolism of *M. genitalium* cultures. In some of these sera, a DGI zone was observed, but it was generally smaller and less distinct than in *M. pneumoniae* cultures. It should be noted that serum 10,887/80, showing such cross-reactions in the MI and DGI tests, originated from a patient who had received penicillin only (Table 1). In the case of serum 10,660/80, erythromycin was administered, but we have no information on the dosage.

HAD to colonies of strain G37 was less pronounced than that to colonies of strain Mac, and so was the methylene blue test for H_2O_2 secretion. Rabbit antisera to the homologous strain abolished HAD, but although rabbit anti-*M. genitalium* serum inhibited HAD to Mac strain colonies in the cross test, the hyperimmune rabbit anti-Mac strain did not inhibit HAD to strain G37 colonies. Human sera with high-level antibodies to *M. pneumoniae* in other tests diminished HAD to colonies of the homologous but not the heterologous species.

CIE. The reference precipitation pattern in CIE gels with *M. pneumoniae* antigens and antibodies is shown in Fig. 2A. When *M. genitalium* antiserum was incorporated in the intermediate gel, depression of some of the precipitation arcs (no. 1 to 3) was seen (Fig. 2B). This indicates that some antibodies raised in rabbits to *M. genitalium* cross-react with antigens of *M. pneumoniae*. The precipitates which were not depressed in this experiment may represent antigens specific for *M. pneumoniae*.

The reference precipitation pattern in CIE gels of *M. genitalium* antigens and antiserum is shown in Fig. 2D. The incorporation of rabbit antibodies to *M. pneumoniae* in the intermediate gel resulted in a depression of precipitates no. 6 and 7 (Fig. 2E), indicating that rabbit *M. pneumoniae* antibodies cross-reacted with at least two antigens of *M. genitalium*. Precipitate no. 5 (Fig. 2E) was not depressed and might represent an antigen specific for *M. genitalium*.

Figure 3 shows the CIE pattern of *M. pneumoniae* antigens precipitating with a reduced amount of rabbit antibodies to this species to identify the precipitate (arrow) earlier described as CM precipitate (24). In Fig. 3B, a CM extract of *M. pneumoniae* was incorporated in the intermediate gel; in addition, a CM extract of *M. genitalium* was placed in a square well at the right-hand end of the first-dimension gel before starting the second-dimension electrophoresis. The precipitation line formed by the CM extract of *M. pneumoniae* was elevated in the area of the CM precipitate (see Fig.

3A), thus showing antigenic identity between the CM precipitate and the CM extract of *M. pneumoniae*. Above the square well in which the CM extract of *M. genitalium* was placed, the line was elevated, too. This indicates that CM extracts of the two mycoplasma species contain immunologically identical or closely related antigens.

When human serum with an *M. pneumoniae* CF antibody titer of ≥4,000 was incorporated in the intermediate gel of the *M. pneumoniae* CIE reference system, at least three precipitates were depressed as evidenced most prominently by precipitates no. 1, 3, and 4 (Fig. 2C). With the same high-titer human serum in the intermediate gel of the *M. genitalium* CIE reference pattern (Fig. 2F), the left foot of arc no. 6 was depressed and elongated, and arcs no. 5 and 7 remained unchanged. *M. pneumoniae* CF-negative human sera (CF titer of ≤16) incorporated in the intermediate gels had no effect on these patterns (data not shown). This indicated that antibodies formed during an *M. pneumoniae* infection may react with *M. genitalium* antigens. The fact that precipitate no. 5 in the *M. genitalium* CIE reference pattern was not changed, either by rabbit *M. pneumoniae* antibodies or by the human immune serum, might indicate that the patient had produced antibodies against *M. pneumoniae* rather than against *M. genitalium*.

SDS-polyacrylamide gel electrophoresis. Figure 4 shows the electrophoretic profiles of *M. pneumoniae* strain M1121 and *M. genitalium* strain G37 as representative of 10 experiments, including various strains and passages of *M. pneumoniae* and other mycoplasmas. About 50 bands were discernible in both *M. pneumoniae* and *M. genitalium* profiles. Overall, the two profiles appear to be different, but similarities can be pointed out, although some of these may be missed because of the obviously lower protein concentration in the *M. pneumoniae* than in *M. genitalium* material. Three major groups of bands (no. 3, 4, and 5 [horizontal arrows]) of these two strains had similar molecular weights. On the other hand, several bands distinct for each species were easily distinguished (oblique arrows). *M. genitalium* had at least seven major protein bands (no. 6 to 12 on Fig. 4, oblique arrows) which were absent or present only as very faint bands in *M. pneumoniae*.

In the profile of *M. pneumoniae*, a band of molecular weight 190,000 was seen (no. 1 in Fig. 4; oblique arrow). This protein, which could not be seen in the *M. genitalium* profile, may be similar to the attachment protein designated

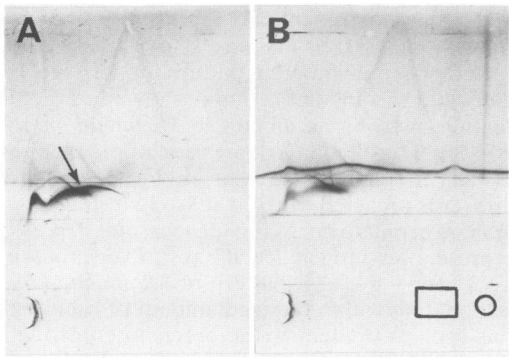

FIG. 3. CIE gels. First-dimension gels contained 25 μl of soluble *M. pneumoniae* antigens. Intermediate gels contained the following: 200 μl of growth medium (A) and 200 μl of medium plus 500 μl of CM extract of *M. pneumoniae* (B). Second-dimension gels contained 200 μl of rabbit anti-*M. pneumoniae* globulin. Square well contained 40 μl of CM extract of *M. genitalium*. Round right-hand well contained 5 μl of growth medium.

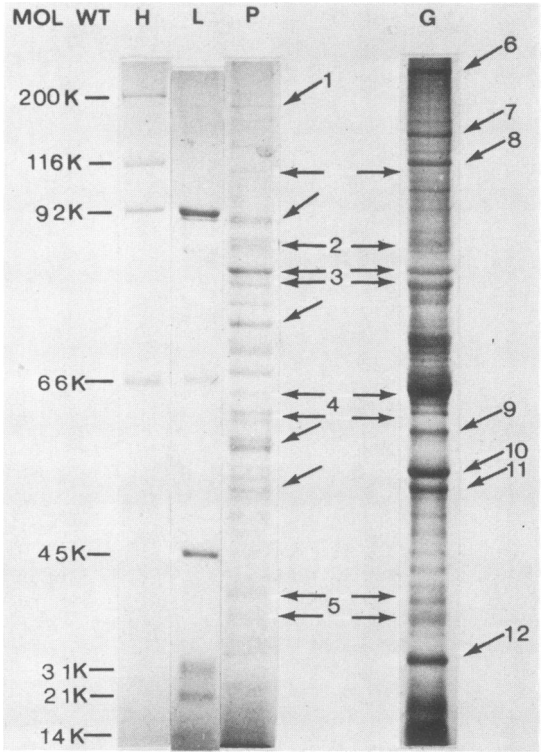

FIG. 4. SDS-polyacrylamide gel electrophoresis profiles of high-molecular-weight standard (H), low-molecular-weight standard (L), *M. pneumoniae* strain M1121 (P), and *M. genitalium* (G). Horizontal arrows indicate single protein bands or groups of bands of the same molecular weight in the two mycoplasmas; e.g., no. 3 with two bands of molecular weights 83,000 and 80,000; no. 4 with two bands of molecular weights 64,000 and 61,000; and no. 5 with two bands of molecular weights 40,000 and 37,000. Band no. 2 was found to be a protein of the growth medium. Oblique arrows indicate bands that are distinct for each strain of mycoplasma. Band no. 1 has a molecular weight of 190,000 similar to the P_1 protein of *M. pneumoniae* described by Hu et al. (13). Band nos. 6 to 12 are proteins distinct for *M. genitalium* with molecular weights as follows: >200,000 (no. 6), 150,000 (no. 7), 116,000 (no. 8), 59,000 (no. 9), 53,000 (no. 10), 51,000 (no. 11), and 31,000 (no. 12). Lanes H, L, P, and G were cut from a photograph of the same gel.

P_1 by Hu et al. (13). It was only occasionally discernible in profiles of the Mac strain.

DNA-DNA hybridization experiments. DNA from *M. genitalium* strain G37 and *M. pneumoniae* strain Mac showed 1.8% homology in base sequences.

DISCUSSION

When *M. genitalium* and *M. pneumoniae* were compared by culture conditions, similarities were more apparent than differences. Both strains grew in classical Hayflick medium in which the main difference seemed to be due to *M. genitalium* sensitivity to thallium acetate. The latter grew more slowly than the Mac strain did, but they could not be distinguished accurately by the morphology of their outgrown colonies on solid media.

By electron microscopy, the morphological differences were evident. The appearance of strain Mac cells was similar to previous descriptions of *M. pneumoniae* (3). Strain G37 displayed the same main characteristics, but strain Mac cells were generally somewhat larger, especially their necks, which were longer than those of strain G37.

The presence of identical or closely related antigens in the

TABLE 1. Serological cross-reactions between *M. genitalium* strain G37 and *M. pneumoniae* strain Mac[a]

Serum and source	CF titer with glycolipid antigen		IF titer with microcolonies		IHA titer with sonicate of *M. genitalium*	MI titer with cultures		DGI zone (mm) in cultures	
	M. genitalium	*M. pneumoniae*	*M. genitalium*	*M. pneumoniae*		*M. genitalium*	*M. pneumoniae*	*M. genitalium*	*M. pneumoniae*
Rabbit 6762 I, prevaccination	<8	<8	<50	<50	<20	<2	2	0	0
Rabbit 6762 III, vaccinated with killed G37	512	512	4,000	4,000	2,560	32	64	5 P	1.5 P
Rabbit 6762 V, vaccinated with killed G37	16	<16	4,000	2,000	5,120	128	64	4	<1
Rabbit 6764 I, prevaccination	<8	<8	<50	<50	<20	<2	2	0	0
Rabbit 6764 III, vaccinated with killed G37	128	64	2,000	2,000	640	16	8	3 P	0
Rabbit 6764 V, vaccinated with killed G37	≦8	<16	1,000	500	2,560	128	128	4	0
Rabbit 6767 I, prevaccination	<8	<8	<50	<50	<20	<2	4	0	0
Rabbit 6767 III, vaccinated with live G37	512	512	2,000	2,000	2,560	64	32	5	3
Rabbit 6767 V, vaccinated with live G37	512	128	1,000	500	1,280	1,024	128	6	2.5
Rabbit 6769 I, prevaccination	<8	<8	<50	<50	<20	<2	<2	0	0
Rabbit 6769 III, vaccinated with live G37	1,024	1,024	8,000	2,000	2,560	256	128	10	7
Rabbit 6769 V, vaccinated with live G37	1,024	2,048	2,000	2,000	1,280	512	1,024	7	7.5
Rabbit pool 16+17, vaccinated with killed *M. pneumoniae* strain Mac	128	≧2,048	2,000	2,000	1,280	64	256	4	9
Human 9100/80, negative anti-*M. pneumoniae* reference	<8	<8	20	40	<20	<2	8	1–3 P	1–2 P
Human pool 1C, positive anti-*M. pneumoniae* reference	1,280	2,560	640	640	160	64	128	4 P	7–9
Human 7682/82, respiratory tract infection	1,024	≧4,096	1,280	1,280	160	2	8	1 P	2 P
Human 10,660/80, respiratory tract infection	256	512	2,560	1,280	640	2	32	2–4 P	0
Human 10,887/80, respiratory tract infection	64	256	2,560	2,560	2,560	32	32	7	8

[a] Abbreviations used in table are as follows: IF, indirect immunofluorescence; IHA, indirect hemagglutination; and P, partial. Roman numerals refer to number of bleeding.

glycolipid fraction extracted by CM was demonstrated by the CF test and confirmed by CIE. The extensive cross-reactions seen by IF tests may also be due to glycolipids present among membrane antigens which are presumably reacting by this technique (21).

In addition, the CIE patterns indicated that other closely related antigens might cause serological cross-reactions and might perhaps be responsible for some of the cross-reactions seen by MI and DGI tests. The MI titers and DGI zones (Table 1), which are results of several experiments, are in contrast to the serological results obtained by Tully et al. (28) which indicated that *M. genitalium* is unrelated to mycoplasmas described previously. In the report by Taylor-Robinson et al. (25), cross-reactions between this species and *M. pneumoniae* were insignificant when sera from various inoculated and vaccinated animals were tested by MI. They also found the G+C content of the DNA of the two mycoplasmas to be different and their protein patterns by polyacrylamide gel electrophoresis to be distinct.

Our SDS-polyacrylamide gel electrophoresis experiments also demonstrated that the majority of the proteins from the two species were different; however, some major protein bands were found to have the same, or a very similar, molecular weight. The 190,000-molecular-weight protein in strain M1121 may be similar to the P_1 protein found in the pathogenic *M. pneumoniae* strain M129 by Hu et al. (13). A similar protein has been described also by other groups (2, 9) to be an external membrane protein associated with the tip structure. The 190,000-molecular-weight protein was better visualized in our strain M1121 than in strain Mac, but it was not seen in strain G37. The absence or lower concentration of this high-molecular-weight protein in the Mac strain has

also been observed by others (5, 10). The protein may not be associated with adsorption to glass, plastic, or erythrocytes, since this property was shared by the strains in our study.

The one-way cross-reaction found in HAD inhibition experiments might indicate a test that discriminates between antibodies to the two species. The lack of inhibition of HAD to *M. genitalium* by human sera with high-level antibodies to *M. pneumoniae* might indicate that the patients had been infected by this organism rather than by *M. genitalium*.

The value of 1.8% for homology in base sequences which we found for strain G37 and strain Mac strongly indicates that the strains belong to two different species. This corresponds to the level for homology generally found when mycoplasmas are compared at the species level, although a few mycoplasma species exhibit a much higher level of DNA homology (7, 22).

Historically, species placed in the genus *Mycoplasma* have been defined as strains or groups of strains showing consistent and significant serological distinctness from other strains. Species differentiation in this genus is best afforded by the DGI, MI, or immunofluorescence tests (14). The validity of these techniques may be questioned when two mycoplasmas such as the present ones are described as belonging to distinct species which even colonize the same host. Although common or related antigens which cut across taxonomic lines are recognized in microbiology, the above serological techniques have generally been helpful and practical tools in mycoplasma taxonomy at the species level, in addition to analysis by culture, biochemical tests, and gel electrophoresis.

As pointed out by Tully et al. (28), there are not enough data to relate *M. genitalium* to urogenital infection in humans, but its isolation from urethral specimens, its specialized structure, and its capacity to adhere to cells and to produce cytopathic effects suggest that it may have a role in human disease. To this may be added its ability to produce H_2O_2.

It is stressed that the present demonstration of serological cross-reactions is based on sera from rabbits immunized with the test organisms. Results from testing human sera are included to demonstrate that they may give rise to similar cross-reactions, although possible past infections with *M. genitalium* in the patients cannot be excluded.

If serological tests are to be used in the assessment of the pathogenic role of *M. genitalium*, caution must be exercised due to the extensive cross-reactions demonstrated. This caution was advanced also by Taylor-Robinson et al. (25), who advocated the use of the MI test to reduce the chance of cross-reactions; however, our data indicate that this test may not be sufficiently specific for discrimination between the two species. Our results also dictate caution when serological tests, especially CF and immunofluorescence tests, are used to identify infections in patients with diseases that are not clinically compatible with *M. pneumoniae* infections.

It may, therefore, be relevant to look for different antigens which are specific for each species. We have demonstrated such antigens by both CIE and polyacrylamide gel electrophoresis; these might be candidates for use in specific serological tests. However, further experiments with selected sera, together with isolation and characterization of precipitated antigens, are needed before this can be achieved.

LITERATURE CITED

1. **Axelsen, N. H., J. Krøll, and B. Weeke (ed.).** 1973. A manual of quantitative immunoelectrophoresis. Methods and applications. Universitetsforlaget, Oslo.
2. **Baseman, J. B., R. M. Cole, D. C. Krause, and D. K. Leith.** 1982. Molecular basis for cytadsorption of *Mycoplasma pneumoniae*. J. Bacteriol. **151**:1514–1522.
3. **Biberfeld, G., and P. Biberfeld.** 1970. Ultrastructural features of *Mycoplasma pneumoniae*. J. Bacteriol. **102**:855–861.
4. **Blom, J., B. Mansa, and A. Wiik.** 1976. A study of Russell bodies in human monoclonal plasma cells by means of immunofluorescence and electron microscopy. Acta Pathol. Microbiol. Scand. Sect. A Pathol. **84**:335–349.
5. **Chandler, D. K. F., S. Razin, E. B. Stephens, R. Harasawa, and M. F. Barile.** 1982. Genomic and phenotypic analyses of *Mycoplasma pneumoniae* strains. Infect. Immun. **38**:604–609.
6. **Cho, H. J., H. L. Ruhnke, and E. V. Langford.** 1976. The indirect hemagglutination test for the detection of antibodies in cattle naturally infected with mycoplasmas. Can. J. Comp. Med. **40**:20–29.
7. **Christiansen, C., and H. Ernø.** 1982. Classification of the F38 group of caprine mycoplasma strains by DNA hybridization. J. Gen. Microbiol. **128**:2523–2526.
8. **Clyde, W. A., Jr.** 1964. Mycoplasma species identification based upon growth inhibition by specific antisera. J. Immunol. **92**:958–965.
9. **Feldner, J., U. Göbel, and W. Bredt.** 1982. *Mycoplasma pneumoniae* adhesin localized to tip structure by monoclonal antibody. Nature (London) **298**:765–767.
10. **Gabridge, M. G., S. E. Singer, and R. A. Esposito.** 1976. Gradient, polyacrylamide gel electrophoresis of proteins from cytotoxic mycoplasma membranes. Biochem. Biophys. Res. Commun. **70**:271–279.
11. **Hayflick, L.** 1965. Tissue cultures and mycoplasmas. Tex. Rep. Biol. Med. **23**(Suppl. 1):285–303.
12. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
13. **Hu, P. C., R. M. Cole, Y. S. Huang, J. A. Graham, D. E. Gardner, A. M. Collier, and W. A. Clyde, Jr.** 1982. *Mycoplasma pneumoniae* infection: role of a surface protein in the attachment organelle. Science **216**:313–315.
14. **International Committee on Systematic Bacteriology. Subcommittee on the Taxonomy of *Mollicutes*.** 1979. Proposal of minimal standards for descriptions of new species of the class *Mollicutes*. Int. J. Syst. Bacteriol. **29**:172–180.
15. **Kenny, G. E., and J. T. Grayston.** 1965. Eaton pleuropneumonia-like organism (*Mycoplasma pneumoniae*) complement-fixing antigen: extraction with organic solvents. J. Immunol. **95**:19–25.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **Lind, K.** 1966. Isolation of *Mycoplasma pneumoniae* (Eaton agent) from patients with primary atypical pneumonia. Acta Pathol. Microbiol. Scand. **66**:124–134.
18. **Lind, K.** 1970. A simple test for peroxide secretion by *Mycoplasma*. Acta Pathol. Microbiol. Scand. Sect. B Microbiol. **78**:256–257.
19. **Lind, K.** 1982. Serological cross-reactions between "Mycoplasma genitalium" and M. pneumoniae. Lancet **ii**:1158–1159.
20. **Lind, K., and M. W. Bentzon.** 1976. Epidemics of *Mycoplasma pneumoniae* infection in Denmark from 1958 to 1974. Int. J. Epidemiol. **5**:267–277.
21. **Razin, S., B. Prescott, and R. M. Chanock.** 1970. Immunogenicity of *Mycoplasma pneumoniae* glycolipids: a novel approach to the production of antisera to membrane lipids. Proc. Natl. Acad. Sci. U.S.A. **67**:590–597.
22. **Reich, P. R., N. L. Somerson, J. A. Rose, and S. M. Weissman.** 1966. Genetic relatedness among mycoplasmas as determined by nucleic acid homology. J. Bacteriol. **91**:153–160.
23. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
24. **Schütten, H. J., H. H. Mogensen, and K. Lind.** 1980. *Mycoplasma pneumoniae* antigens demonstrated by crossed immunoelec-

trophoresis. Acta Pathol. Microbiol. Scand. Sect. C Immunol. **88:**137–143.

25. **Taylor-Robinson, D., P. M. Furr, and J. G. Tully.** 1983. Serological cross-reactions between Mycoplasma genitalium and M. pneumoniae. Lancet **i:**527.
26. **Taylor-Robinson, D., R. H. Purcell, D. C. Wong, and R. M. Chanock.** 1966. A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. J. Hyg. Camb. **64:**91–104.
27. **Tully, J. G., D. Taylor-Robinson, R. M. Cole, and D. L. Rose.** 1981. A newly discovered mycoplasma in the human urogenital tract. Lancet **i:**1288–1291.
28. **Tully, J. G., D. Taylor-Robinson, D. L. Rose, R. M. Cole, and J. M. Bové.** 1983. *Mycoplasma genitalium*, a new species from the human urogenital tract. Int. J. Syst. Bacteriol. **33:**387–396.
29. **Tully, J. G., R. F. Whitcomb, H. F. Clark, and D. L. Williamson.** 1977. Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. Science **195:**892–894.