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Serological Comparison of Ten Glycolytic *Mycoplasma* Species

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Seventeen strains of mycoplasmata representing 11 named species were compared serologically by three parameters: growth inhibition on agar, double immunodiffusion, and complement fixation. In growth-inhibition studies, a strain labeled *Mycoplasma histotropicum* was found related to and perhaps best classified as *M. pulmonis*, a relationship confirmed by double immuno-diffusion studies. A comparison of the remaining 10 species demonstrated that two pairs of species could be shown to be closely related by complement fixation and double immunodiffusion but not by growth inhibition; these were: *M. granularum-M. laidlawii* and *M. felis-M. canis*. *M. pneumoniae* and *M. gallisepticum* were the most serologically unique organisms in this study, showing very few cross-reactions with each other or other species. Overall, taxonomic groupings obtained by comparative serology appeared to correlate with the groupings obtained when the guanine plus cytosine contents of the deoxyribonucleic acid of mycoplasmata were employed as classification criteria. The group of organisms having a guanine plus cytosine content of 23 to 28% (*M. canis*, *M. fermentans*, *M. hyorhinis*, *M. neurolyticum*, and *M. pulmonis*) appeared to be generally serologically related. Thus the remarkable heterogeneity observed in the base composition of the deoxyribonucleic acid of order *Mycoplasmatales* is also reflected and apparently paralleled by a corresponding serological heterogeneity.

The comparative serology of the *Mycoplasmataceae* is of considerable theoretical interest because mycoplasmata are the smallest and simplest free-living organisms known. Their simplicity is revealed by their small deoxyribonucleic acid (DNA) content: 500×10^6 to $1,200 \times 10^6$ daltons, sufficient perhaps for 1,500 genes (16). Their synthetic capabilities are correspondingly limited as evidenced by their extremely fastidious and complex growth requirements. The current classification system (6) places these minute organisms, which have no cell wall, form characteristic microcolonies, and occur in nature, into one genus only: *Mycoplasma*. This implies, at least semantically, that *Mycoplasma* species are closely related. Although a variety of criteria were originally employed for classification (i.e., host range, pathogenicity), the presently known *Mycoplasma* species thus far have been shown to be clearly distinguishable on the basis of a unique species-specific growth inhibition by antisera (2). This property may not be peculiar to mycoplasmata but may be common to cells without cell walls whose membranes are directly exposed to the environment. On the other hand, several noteworthy differences between species are known. The base composition [expressed as percent guanine plus cytosine (% GC)] of the DNA of the species studied is not only low but also shows a remarkable range for organisms in a single genus: 23 to 39% (14, 17). Approximately half of the known 30+ named species utilize glucose with the formation of acid, whereas the others utilize arginine with the formation of ornithine and ammonia; these properties appear to be mutually exclusive for most species (1). The apparent simplicity of genus *Mycoplasma* combined with genetic heterogeneity as shown by variation in GC content is of considerable interest to comparative serology. As the number of possible antigens are minimized, the antigenic differences between species showing the greatest genetic differences logically should be greatest.

The present study was undertaken to determine the antigenic relationships between species in genus *Mycoplasma* as a prelude for the selection of representative species for antigenic analysis. Additionally, if certain antigens were common to a number of *Mycoplasma* species,

these might be very useful for diagnosis of infections of man and animals in which the *Mycoplasma* species involved might be extremely difficult to cultivate. Since it was impractical to attempt to report a total comparison of the presently known 30+ species at one time, I am reporting the results of a serological comparison of 10 of the 15+ known glucose-utilizing species. These were chosen rather than the arginine-utilizing species because they are easier to cultivate and contain more pathogens.

MATERIALS AND METHODS

Mycoplasmataceae. The species employed were strains from the American Type Culture Collection or were obtained from individual investigators (Table 1). The organisms were stored at −70 C.

Cultivation of organisms. Organisms were cultivated in the soy peptone dialysate broth previously described (10) containing: serum, 10 to 12%; 0.05% phenol red; penicillin, 100 units/ml; and thallium acetate, 0.33 mg/ml. Agamma calf serum was used as serum supplement for cultivation of immunizing antigens, whereas agamma horse serum was employed for cultivation of serological test antigens (10). A 2-ml amount of an actively growing culture was used as inoculum for each 800 ml of culture. Cultures were incubated at 37 C (32 C for *M. laidlawii* A and B) with a magnetic spin bar rotating slowly in the bottom. Cultures were incubated until a haze representing growth was observed and the *p*H decreased (3 to 5 days). Organisms were concentrated from 12 liters of culture by sedimentation of broth cultures at 10,000 × *g* for 0.5 hr. The sedimented organisms were washed three times with 100 ml of TES saline {NaCl, 150 mM; TES [*n* tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid; Calbiochem], 5 mM at *p*H 7.0} and suspended either in a volume of TES saline 1/160 the original broth volume (for animal immunization) or in 1/400th volume of distilled water (for serological testing). The yields of organism ranged from 12 to 30 mg of protein per liter of culture or 5 to 13 mg per ml (average, 9 mg/ml) of the serological test antigen. Since the dialysate broth was free of sediment and the agamma horse serum and agamma calf serum were selected for ability to remain clear during incubation, a collectible residue was not obtained upon sedimentation of incubated uninoculated cultures. Antigens were stored at −20 C until used. Serological test antigens were frozen and thawed at least five times prior to testing; for double immunodiffusion, antigens were additionally disrupted by sonic oscillation for the optimal time of 6 min with the maximum power allowable for the microprobe on a Bronwill sonic oscillator (Biosonik III).

Immunizing antigens were prepared from organisms cultivated less than five serial transfers from the source to minimize possibilities of contamination and replacement of strains with foreign species. Immunizing antigens were also prepared from three times colony-cloned organisms representative of each species as indicated on Table 1.

Immunization of animals. Female New Zealand white rabbits (about 2.7 kg) were immunized with a divided dosage scheme, as described previously (10), by using an intramuscular injection of adjuvanted vaccine followed by an intravenous series of injections with fluid vaccine. Each rabbit received approximately 6 to 15 mg of mycoplasmic protein in the sequence of injections. Control antisera were prepared by immunizing rabbits with dialysate broth supplemented with either 20% agamma horse serum or 20% agamma calf serum using the same immunization schedule and antigen volumes.

Growth inhibition. The antisera were tested by the

TABLE 1. *Description of Mycoplasma species employed*

Species	Strain designation	Source	Clone
M. canis[a]	PG 14 C.55	ATCC 19525	X[b]
M. felis[a]	B2	Cole et al. (3)	X
M. felis	Co	Cole et al. (3)	X
M. fermentans[a]	PG-18	R. M. Chanock	
M. gallisepticum[a]	S-6	ATCC 15302	
M. granularum[a]	BTS 39	ATCC 19168	X
M. histotropicum		ATCC 23115	
M. hyorhinis[a]	BTS 7	ATCC 17981	X
M. hyorhinis		W. P. Switzer	
M. laidlawii[a]	Laidlaw A	ATCC 14089	
M. laidlawii[a]	Laidlaw B	ATCC 14192	
M. neurolyticum[a]	KSA	ATCC 15049	X
M. pneumoniae	AP-164	This laboratory (11)	X
M. pneumoniae[a]	AP-1046	This laboratory	X
M. pulmonis[a]	63	This laboratory (4)	X
M. pulmonis	Kon	Fallon & Jackson (7)	X
M. pulmonis	Negroni	Fallon & Jackson (7)	X

[a] Strain used for serological comparison in Tables 3, 4, and 5.

[b] Colony-cloned strain also used for preparation of antigens and antisera.

method of Clyde (2) for ability to inhibit the formation of colonies around a paper disc (6 mm, Whatman no. 3 paper) saturated with undiluted antiserum. The medium employed was composed of: soy peptone-agar base, 70%; fresh yeast extract dialysate, 10%; unheated horse serum, 20%; penicillin, 100 units/ml; and thallium acetate, 0.033 mg/ml. Soy peptone-agar base was composed of: soy peptone, 2%; NaCl, 0.5%; Noble agar (Difco), 1%); this mixture was adjusted to *p*H 7.4 with 1 N NaOH. [Fresh yeast dialysate was prepared by dialyzing an autoclaved (110 C for 10 min) yeast suspension (900 g in 2.5 liters of water) against 2 liters of water in the cold for 48 hr. The dialysate was autoclaved and stored frozen.] The inoculum levels of organisms employed were calculated to yield discrete colonies at levels of approximately 500 to 5,000 colonies per 30-mm plate. The degree of inhibition was estimated by measuring the distance of total inhibition of colony formation from the edge of the disc to the margin of the zone of inhibition. The best antisera for inhibition were usually obtained after the complete immunization schedule.

Micro-double-immunodiffusion. A plastic matrix [supplied by Mann Research Laboratories to the specifications described by Sharpless and LoGrippo (20)] was employed on agarose films. Slides were precoated with a dried agarose film by applying 1 ml of 0.5% agarose (Bausch & Lomb) in distilled water to a microscope slide (75 by 25 mm); they were allowed to harden and were baked for 30 min at 100 C. Agar films were prepared by applying 0.7 ml of melted agarose (agarose, 0.5%; NaCl, 0.5%; merthiolate, 0.01%; and 10 mM TES, *p*H 7.0) onto an area (2.5 by 4.5 cm) on a precoated slide. This resulted in an agarose film 0.6 mm thick. The agarose was permitted to harden and a matrix was placed upon the agar film. Slides were used the same day they were prepared. The wells in the matrix were filled with approximately 25 μliters of antigen (about 120 to 500 μg of protein) or antibody by use of a 1-ml syringe. All the bleedings from each rabbit were surveyed in the homologous system, and that serum yielding the most precipitin lines was employed for comparison. At least three comparisons were made: (i) antigens in outer wells, antiserum in the inner well; (ii) antisera in outer wells, antigen in inner well; and (iii) antigen in only one outer well, homologous antiserum in the inner well. Two preparations of antigen and antisera from two individual rabbits were compared for each *Mycoplasma* species. Slides were incubated for 48 hr at room temperature in a humidified chamber. Templates were then removed, and slides were soaked in 0.85% saline for at least 4 hr and then soaked in distilled water overnight. Slides were stained wet for 5 min in amido-schwarz 10B (0.2% in 2% acetic acid, dye obtained from Schmid and Co., Stuttgart, Germany). Agar films were rinsed with distilled water to remove excess stain and destained in 2% acid for 5 hr or until the background stain was removed. The number of precipitin lines were counted by viewing on a projector (National Instrument Laboratories) at 8× magnification. For presentation and illustrative purposes, slides were cut to size, mounted in 35-mm holders, and used as transparencies for projection or negatives for printing black and white prints.

Complement fixation. Block titrations were performed by testing twofold serial dilutions (1:16 through 1:4,096) of antigens against the same dilutions of antibody. Two full units of complement were employed with overnight fixation at 4 C. End points for both antigens and antibody were expressed as the reciprocal of the highest initial dilution of antigen (or antibody) which gave complete fixation of complement. This system is essentially the micro-modification of the Kolmer procedure described previously (11).

Protein. Protein was measured by the Folin method (9) with crystalline bovine albumin (Calbiochem A grade) used as a standard.

RESULTS

All of the *Mycoplasma* species employed in this study produced an acid reaction in dialysate medium, whereas known non-glycolytic, arginine deiminase (1)-containing species (e.g., *M. hominis*) eventually produced a slight alkaline reaction. The acid reaction was enhanced when the medium contained 5 mM glucose. One of the species, *M. granularum*, was found to grow and to be serially transferrable in the serum-free Trypticase soy-yeast extract medium used for sterility testing for bacteria. *M. laidlawii* strains A and B, which are well-known saprophytes, also grew in this serum-free medium.

Growth inhibition. The 12 organisms were compared by growth inhibition (Table 2). *M. laidlawii* strains A and B appeared related: *M. laidlawii* A antisera inhibited *M. laidlawii* B; however, *M. laidlawii* B antisera did not inhibit *M. laidlawii* A, an organism which showed the smallest zone of inhibition in the homologous system. *M. histotropicum* showed reciprocal inhibition with *M. pulmonis*. *M. histotropicum* was excluded from further survey because this cross-relationship and hence species identity with *M. pulmonis* was confirmed by double immunodiffusion (Fig. 1). The antiserum reagents thus were able to distinguish 10 entities or species among this group of organisms. This indicates that both the organisms and the immunizing antigens used to make the antisera were pure cultures and not gross mixtures of each other (particularly since most antisera still gave significant inhibition at a 1:8 dilution in the homologous system). The same serological relationships were observed with antisera against parental (uncloned) strains and with antisera against colony-cloned strains.

Double immunodiffusion. The remaining 10 distinct species were further compared by double immunodiffusion (Table 3). The number of

TABLE 2. *Comparison of 11 Mycoplasma species by inhibition of colony formation*

Antigen	Antiserum											
	1	2	3	4	5	6	7	8	9	10	11	12
1. *M. canis*	3.5[a]	—	—	—	—	—	—	—	—	—	—	—
2. *M. felis*	—	4.7	—	—	—	—	—	—	—	—	—	—
3. *M. hyorhinis*	—	—	6.1	—	—	—	—	—	—	—	—	—
4. *M. pulmonis*	—	—	—	5.7	6.0	—	—	—	—	—	—	—
5. *M. histotropicum*	—	—	—	5.7	5.3	—	—	—	—	—	—	—
6. *M. neurolyticum*	—	(1.8)[b]	—	—	—	10.0	—	—	—	—	—	—
7. *M. fermentans*	—	—	—	—	—	—	5.2	—	—	—	—	—
8. *M. pneumoniae*	—	—	—	—	—	—	—	5.0	—	—	—	—
9. *M. gallisepticum*	—	—	—	—	—	—	—	—	5.7	—	—	—
10. *M. granularum*	—	—	—	—	—	—	—	—	—	9.0	—	—
11. *M. laidlawii* A	—	—	—	—	—	—	—	—	—	—	2.6	—
12. *M. laidlawii* B	—	—	—	—	—	—	—	—	—	—	4.5	3.4

[a] Zone of inhibition, measured in millimeters from edge of disc to first appearance of colonies; (—) equals zone of inhibition less than 1 mm.

[b] Indicates inhibition by preimmune sera.

immunodiffusion lines observed in the homologous systems ranged from 5 to 11 [a minimal estimate, since substantial overlapping of lines sometimes occurred in the microsystem used (Fig. 2)]. In all cases, the number of precipitin lines was most in the homologous system. The most striking relationship was seen between *M. granularum* and *M. laidlawii*. These species appeared very closely related. Figures 3 and 4 show the reciprocal nature of the cross-reactions observed. At least three common precipitin lines were observed.

Another striking cross-reaction was between *M. canis* and *M. felis*. Common antigens were clearly demonstrable on a reciprocal basis (Fig. 5 and 6). This cross-reaction also was observed when cloned *M. canis*, cloned *M. felis* B2, or *M. felis* Co were used as antigens; similarly, antisera produced against the clone of either species showed the same cross-reactions as antisera against the parental strains.

A lesser though clearly significant cross-reaction was observed between *M. hyorhinis* and *M. pulmonis*. *M. hyorhinis* antisera were found to cross-react with antigens prepared from *M. pulmonis* strains 63, Kon, and Negroni (Fig. 7). However, the *M. hyorhinis* antiserum employed was the strongest antiserum prepared among all species tested, and this cross-reaction was most marked when this particular antiserum was tested against *M. pulmonis* antigens. *M. pulmonis* antisera were comparatively weaker against both homologous antigens and *M. hyorhinis* antigen. A number of minor reactions were seen between various species in the 23 to 28% GC group, indicating that this entire group might be related. The serological test antigens prepared from organisms in this group did not show any significant reactions with the higher GC organisms. *M. pneumoniae* and *M. gallisepticum* were remarkably serologically distinct from each other and from all other species. *M. granularum* and *M. laidlawii* A antigens did not react with the other species tested and their antisera did not show significant cross-reactions with other species. However, *M. laidlawii* B antigen showed

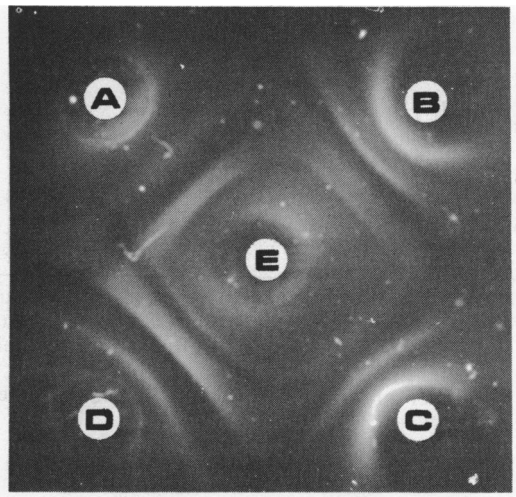

FIG. 1. *Microimmunodiffusion patterns obtained with: (A) M. histotropicum antigen; (B) M. pulmonis (Kon) antigen; (C) M. pulmonis (63) antigen; (D) M. pulmonis (Negroni) antigen; (E) M. histotropicum antiserum.* × 8.

TABLE 3. *Comparison of 10 Mycoplasma species by double immunodiffusion*

Antigen	Antiserum													Protein[c] (mg/ml)	%GC in DNA[d]
	1	2	3	4	5	6	7	8	9	10	11	12[a]	13[b]		
1. *M. canis*....	**6**[e]	2	(2)	1	1	2	0	0	0	0	0	1	0	9.7	28
2. *M. felis*....	4	**6**	2	(1)	2	2	(1)	0	0	0	0	0	(1)	6.2	—
3. *M. hyorhinis*.....	1	0	**11**	3	1	(1)	0	0	0	0	0	1	0	7.5	27
4. *M. pulmonis*....	(1)	0	3	**9**	(1)	(1)	0	0	0	0	0	1	(1)	12.5	27
5. *M. neurolyticum*...	(2)	(1)	(2)	(2)	**7**	(1)	0	0	0	0	(1)	1	(1)	9.0	23
6. *M. fermentans*.....	(1)	1	(1)	0	(2)	**6**	0	0	0	0	0	1	0	12.0	28
7. *M. pneumoniae*...	0	0	(1)	0	0	0	**6**	0	(1)	(1)	(1)	1	(1)	6.8	39
8. *M. gallisepticum*.....	0	0	0	0	(1)	(1)	(1)	**7**	0	0	0	1	0	10.5	32
9. *M. granularum*....	0	0	0	0	0	0	0	0	**5**	3	4	(1)	0	6.7	30
10. *M. laidlawii A*...	0	0	0	0	0	0	0	0	3	**5**	4	1	1	13.0	32
11. *M. laidlawii B*...	(1)	2	(2)	0	2	(1)	0	0	4	9	**9**	2	0	5.3	32
12. 1:100 HoS[b].....	(1)	0	(1)	0	(1)	(1)	0	0	(1)	(1)	0	**7**	2	0.7	
13. 1:100 CaS[f].....	2	2	3	1	3	2	2	1	2	3	2	2	**7**	0.7	
14. 1:1000 HoS.....	0	0	0	0	0	0	0	0	0	0	0	**5–6**	0	0.07	
15. 1:1000 CaS.....	1	2	4	2	3	2	2	1	1	3	2	0	**2**	0.07	

[a] Rabbit antiserum against dialysate medium supplemented with 20% agamma horse serum.
[b] Rabbit antiserum against dialysate medium supplemented with 20% agamma calf serum.
[c] Milligrams of protein per milliliter in serological test antigen. One lot of antigen used for Tables 3–5.
[d] Per cent GC in mycoplasmic DNA (14, 17).
[e] Number of precipitin lines; boldface indicates homologous system; parentheses indicate weak reactions.
[f] HoS = agamma horse serum.
[g] CaS = agamma calf serum.

fairly extensive crossing against otherwise unrelated species: a result without an apparent explanation, since reciprocal reactions were not seen when *M. laidlawii* B antiserum was tested against heterologous antigens.

The relationships observed among species are necessarily dependent on the relative strengths of the serological test antigens and the mycoplasmic antisera employed. The serological test antigens used in this study were reasonably consistent in the protein content of the 400-fold concentrates of broth cultures: the values obtained ranged from 5.0 to 13 mg/ml (Table 3), averaging about 9 mg/ml. Essentially the same cross-reactions were observed between parental strains as were observed with the clonal strains. On the other hand, it is much more difficult to standardize antisera. The antigenic relationships observed in a serological survey will be determined more by the number of antigenic components which the test antisera can recognize than by the titer to any one component. The antisera used in this study usually gave six or seven clearly recognizable immunoprecipitin lines in the homologous system.

Complement fixation. The best antiserum from the various bleedings of each rabbit had been selected by double immunodiffusion, the criterion being the maximum number and intensity of precipitin lines. However, the optimum serum for complement fixation was frequently obtained from an earlier bleeding than for double immunodiffusion.

The patterns of serological relationships seen

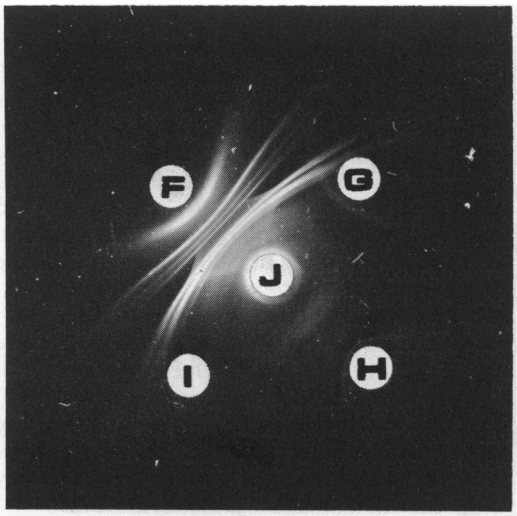

FIG. 2. *Microimmunodiffusion patterns obtained with: (F) M. hyorhinis antigen; (G) M. pulmonis (63) antigen; (H) M. neurolyticum antigen; (I) M. pneumoniae (1046) antigen; (J) M. hyorhinis (Switzer) antiserum.* ×5.

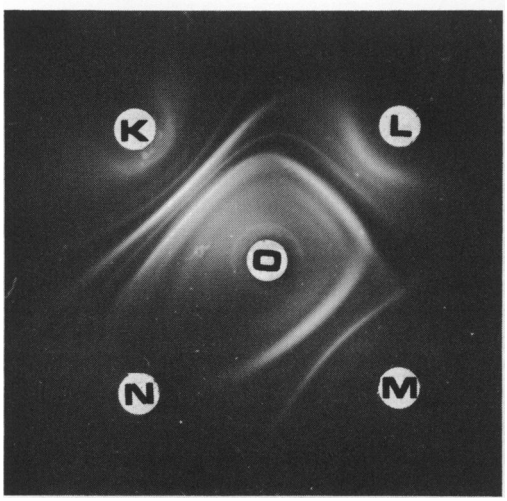

FIG. 3. *Microimmunodiffusion patterns obtained with: (K) M. laidlawii (A) antigen; (L) M. laidlawii (B) antigen; (M) M. granularum (19168) antigen; (N) M. hyorhinis (17981) antigen; (O) M. laidlawii (B) antiserum.* ×6.

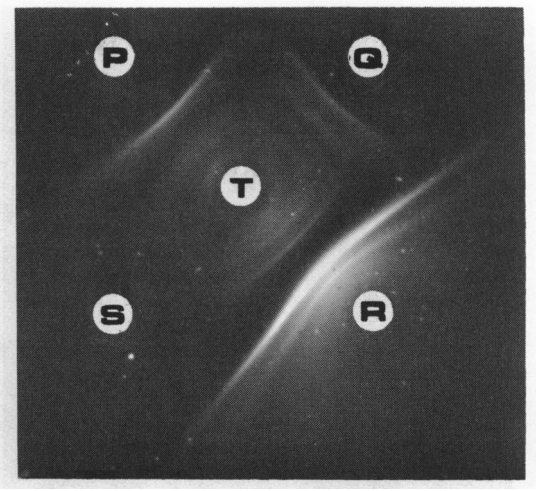

FIG. 4. *Microimmunodiffusion patterns obtained with: (P) M. laidlawii A antigen; (Q) M. laidlawii B antigen; (R) M. granularum antigen; (S) M. hyorhinis (17981) antigen; (T) M. granularum antiserum.* ×6.

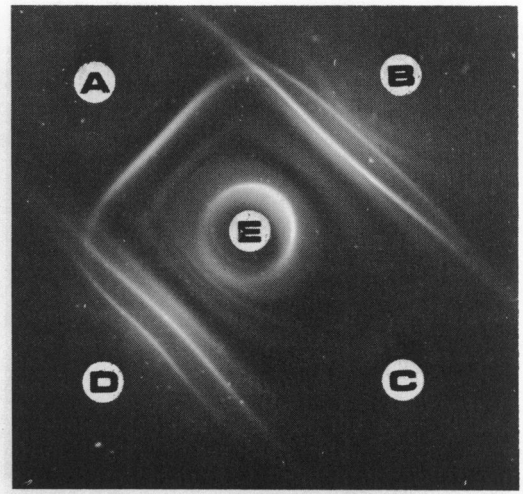

FIG. 5. *Microimmunodiffusion patterns obtained with: (A) M. canis antigen; (B) M. felis (B2) antigen; (C) M. hyorhinis antigen; (D) M. felis (Co) antigen; (E) M. felis (Co) antiserum.* ×7.

in complement-fixation block titrations were similar to those observed in double immunodiffusion. A comparison of the various species by maximum antigen titer against homologous and heterologous antisera (Table 4) showed the following relationships between species: (i) *M. granularum* was clearly and indistinguishably related to *M. laidlawii* strains A and B; (ii) *M. canis* and *M. felis* were clearly related, though one rabbit immunized with *M. canis* did not show a detectable antibody response against *M. felis* antigen; and (iii) *M. hyorhinis* antigen appeared to react with a number of antisera, including both antisera against *M. pneumoniae*. In an analogous manner, *M. felis* antisera showed a broad reactivity: all the species in the low GC group gave high antigen titers against these antisera.

When the species were compared by antibody

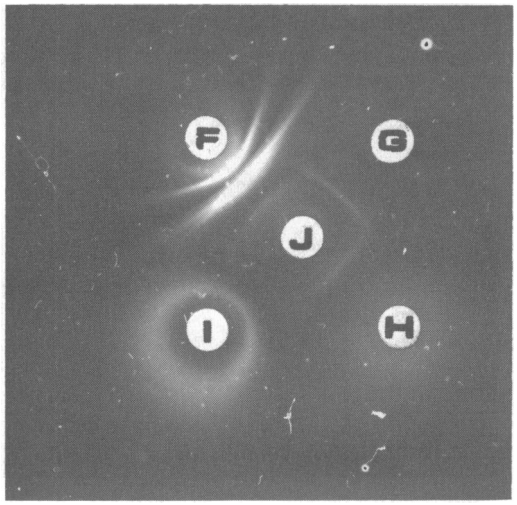

FIG. 6. *Microimmunodiffusion patterns obtained with: (F) M. canis antigen; (G) M. felis (B2) antigen; (H) M. fermentans antigen; (I) M. gallisepticum antigen; (J) M. canis antiserum.* ×4.

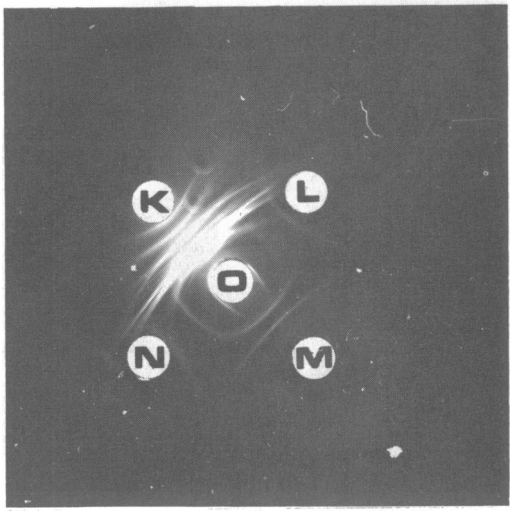

FIG. 7. *Microimmunodiffusion patterns obtained with: (K) M. hyorhinis antigen; (L) M. pulmonis (63) antigen; (M) M. pulmonis (Negroni) antigen; (N) M. pulmonis (Kon) antigen; (O) M. hyorhinis (Switzer) antiserum.* ×3.

titer from the block titrations (Table 5), the same general relationships had to occur by definition, since an antibody titer must appear where the antigen has shown a positive reaction. However, these reactions were differentiated in a more species-specific manner, most likely because the antibody response of the rabbit served as a mechanism for amplifying differences. *M. granularum* was distinguishable from *M. laidlawii* A and B, but it was not possible to distinguish the two strains of *M. laidlawii* with these antisera. Similarly, the distinction between *M. canis* and *M. felis* was enhanced. The numerous cross-reactions shown by *M. hyorhinis* antigen and *M. felis* antibody were shown to be relatively low-level reactions in terms of antibody titer. One of the antisera against *M. pneumoniae* showed a number of cross-reactions with other antigens. The preimmune serum did not react with the various antigens.

Two additional antisera against *M. pneumoniae* strain AP-1046 from other rabbits were titrated against all antigens. One antiserum cross-reacted with both *M. neurolyticum* antigen (1:16) and *M. granularum* antigen (1:64), whereas the homologous antiserum titer was 1:256. The other antiserum appeared monospecific, reacting only with the homologous antigen at 1:256. At present, the exact significance of these cross-reactions of *M. pneumoniae* antisera with various antigens is not clear, since the reactions were neither reciprocal nor demonstrable in double immunodiffusion. However, the cross-reaction with *M. granularum* antigen is of interest because it was seen with three of four antisera.

Several distinct cross-reactions were shown by *M. granularum* antigen against *M. pneumoniae*, *M. neurolyticum*, and *M. canis*; however, these reactions were not reciprocal reactions. Of all the species tested with these reagents, *M. gallisepticum* could be distinguished most specifically by cross-complement-fixation testing. *M. pneumoniae* antigen did not react with any of the antisera to the other species.

Serological specificity. The complex media used for cultivation of mycoplasmata are particularly immunogenic to the animals immunized when heterologous serum is used. Antibodies to medium components may well obscure serological distinctions and suggest antigenic relationships between strains and species which reflect only contamination of serological test antigens with medium components.

My methodology differed considerably from previous studies by others (5, 12, 13, 23), since I employed soy peptone-yeast dialysate broth supplemented with agamma calf serum—heterologous to the animals (rabbits) injected—for cultivation of the immunizing antigen and agamma horse serum for cultivation of the serological test antigen (10). This procedure was chosen because most of the species grew poorly in the homologous serum (rabbit serum) with low yields of mycoplasmata, whereas all species readily grew in "agamma" calf serum without apparent "adaptation" and produced yields of

TABLE 4. *Comparison of 10 Mycoplasma species by antigen titers from block titrations by complement fixation*

Antigen	Antiserum											Anti-medium[a]
	1	2	3	4	5	6	7	8	9	10	11	
1. *M. canis*	**256**	256[b]	—[c]	—	—	—	—	—	—	—	—	16
2. *M. felis*	(128)	**256**	—	—	—	—	—	—	—	—	—	8
3. *M. hyorhinis*	(128)	128	**1,024**	64	64	(16)	32	—	—	16	—	4
4. *M. pulmonis*	—	256	(256)	**256**	—	—	(32)	—	—	—	—	32
5. *M. neurolyticum*	—	(128)	(256)	(32)	**256**	—	(32)	—	—	—	—	8
6. *M. fermentans*	(32)	32	—	(64)	—	**256**	—	—	—	(64)	—	4
7. *M. pneumoniae*	—	—	—	—	—	—	**256**	—	—	—	—	4
8. *M. gallisepticum*	—	—	—	—	—	—	(64)	**128**	—	—	—	4
9. *M. granularum*	(256)	—	—	(128)	64	—	128	—	**512**	512	512	8
10. *M. laidlawii A*	—	—	—	(64)	—	—	(64)	—	512	**1,024**	512	8
11. *M. laidlawii B*	—	—	—	(64)	—	—	(32)	—	256	1,024	**512**	8

[a] Antibody against dialysate broth plus 20% agamma calf serum; agamma horse serum as antigen titers 1:3,200 against this antibody at optimal dilution.

[b] Parentheses indicate that only one of two rabbits reacted; boldface indicates homologous system; titers are expressed as the reciprocal of the greatest antigen dilution giving complete fixation of complement.

[c] Minus (—) indicates titer less than 1:16.

TABLE 5. *Comparison of 10 Mycoplasma species by Antibody titers from block titrations by complement-fixation*

Antigen	Antiserum										
	1	2	3	4	5	6	7	8	9	10	11
1. *M. canis*	**512**	128	—[a]	—	—	—	—	—	—	—	—
2. *M. felis*	(32)[b]	**256**	—	—	—	—	—	—	—	—	—
3. *M. hyorhinis*	(16)	32	**1,024**	64	16	(16)	64	—	—	16	—
4. *M. pulmonis*	—	16	(32)	**512**	—	—	(32)	—	—	—	—
5. *M. neurolyticum*	—	(16)	(32)	(32)	**256**	—	(64)	—	—	—	—
6. *M. fermentans*	(32)	32	—	(16)	—	**2,048**	—	—	—	(16)	—
7. *M. pneumoniae*	—	—	—	—	—	—	**512**	—	—	—	—
8. *M. gallisepticum*	—	—	—	—	—	—	(16)	**256**	—	—	—
9. *M. granularum*	(16)	—	—	(16)	64	—	64	—	**512**	64	128
10. *M. laidlawii A*	—	—	—	(128)	—	—	(64)	—	32	**512**	256
11. *M. laidlawii B*	—	—	—	(32)	—	—	(64)	—	64	256	**256**
12. Agamma horse serum[c] (1:100)	<2	4	2	4	4	<2	16	16	4	2	2

[a] Indicates titer less than 1:16.

[b] Parentheses indicate that only one of two rabbits reacted; boldface indicates homologous system; titers are expressed as the reciprocal of the highest serum dilution giving complete fixation of complement.

[c] Anti-calf dialysate broth serum antibody titered 1:640 against horse serum antigen in a block titration.

organism equivalent to those in horse serum-supplemented dialysate broth.

Horse serum and calf serum antigens and antisera were found to cross-react in the micro-immunodiffusion system (Table 3). The optimum dilution of horse serum or calf serum for the homologous system was between 1:100 and 1:1,000, whereas the heterologous system gave the maximal number of lines at 1:1 to 1:10 antigen dilution with no lines at 1:1,000 dilution of heterologous antigen. Accordingly, if the horse serum content of the mycoplasmic antigen

could be reduced to the equivalent of a 1:1,000 dilution (ca. 0.07 mg of protein/ml), reactions due to anticalf serum antibodies in the antiserum would be eliminated. When the mycoplasmic antigens were tested with antisera to horse serum-dialysate medium, one immunodiffusion line was observed (Table 3), indicating that the horse serum content of the antigens was equivalent to a 1:1,000 dilution of agamma horse serum or less. When the mycoplasmic antisera were tested against the optimal agamma horse (1:100) serum antigen dilution, no lines or only one faint line was observed. The mycoplasmic antisera had substantial activity measurable with agamma calf serum antigen (homologous system), but this appeared to be less than that observed with specific hyperimmune serum against agamma calf serum-dialysate medium (Table 3). This last result suggests that the medium serum supplement (heterologous to the animal immunized) cannot be sufficiently washed out of the immunizing antigen to avoid antimedium antibody. However, in this system, although the serological test antigens were found to contain detectable horse serum antigen when measured with antiserum to horse serum, only slight activity was detected in the heterologous system with antisera to calf serum. The mycoplasmic antisera had only small amounts of heterologous antibody against horse serum, an amount which appears to be insufficient to cause immunoprecipitin lines in the mycoplasmic system. The controls used in this study are more rigorous than the actual test system in that the optimal dilution of horse serum antigen was employed for evaluation of the anti-horse antibody status of the mycoplasmic antisera and hyperimmune anti-horse and anti-calf sera were employed for testing contamination levels of the antigens.

Similar conclusions concerning medium component antigens and antibodies can be drawn from the serological comparisons by complement fixation. All of the mycoplasmic serological test antigens contained complement-fixing activity measurable by anti-calf serum antibody: the titers ranged from 1:4 to 1:32 (Table 4). When these values are compared to the titer of horse serum antigen (1:3,200) against anti-calf serum antibody, the level of horse serum antigen contamination in the mycoplasmic antigen appears to be equivalent to, at most, a 1:100 dilution and more likely to a 1:1,000 dilution of agamma horse serum. These results parallel the estimations of horse serum contamination provided by the double-immunodiffusion tests. The serological test antigens were found to contain horse serum antigen detectable by anti-calf serum antibody; however, the mycoplasmic antisera contained only small amounts of heterologous antibody measurable by horse serum antigen (Table 5). Therefore, the simple expedient of beginning block titrations at 1:16 dilutions for both antigens and antisera served to eliminate false-positive reactions due to horse serum.

These data further permit an estimate of the amount of contaminating foreign protein in these three times washed antigens. It appears that the serological test antigens which contained an average of 9 mg of protein per ml were contaminated with about 1% horse serum antigen (i.e., 0.1 mg of horse serum protein per ml).

An additional and simple means of determining whether medium component antigens or antibodies are involved in the serological test system is to determine whether a serologically unrelated *Mycoplasma* species cross-reacts in the test system. The fact that *M. gallisepticum* and *M. pneumoniae* show very few cross-reactions with the other species suggests that the mycoplasmic serological test antigens were not significantly contaminated with medium components. Similarly, the failure of the low GC group to cross-react with *M. laidlawii-M. granularum* group is strong evidence that the cross-reactions observed inside these groups are specific and not due to medium components. An unrelated organism was used as a control for Figs. 2, 3, 4, and 6 (e.g., *M. pneumoniae* antigen was the unrelated control for Fig. 2).

DISCUSSION

All species in this study were determined to be fermentative, a conclusion which is at variance with some previous studies concerning *M. hyorhinis*, *M. granularum*, *M. canis*, and *M. felis*. *M. canis* and *M. hyorhinis* were reported by Freundt (8) to be nonfermentative. Neither *M. canis* nor *M. hyorhinis* has been found to possess arginine deiminase activity [produce ammonia from arginine (1)], facts which are relevant because carbohydrate fermentation and the possession of arginine deiminase are usually mutually exclusive properties for *Mycoplasma* species. Switzer (21) pointed out that the results concerning the fermentative abilities of *M. granularum* and *M. hyorhinis* varied between laboratories. The initial report describing the properties of *M. felis* indicated that this was a nonfermentative species which did not produce ammonia from arginine (3), but later findings (B. C. Cole, *personal communication*) indicate that this species is fermentative. Since demonstrable carbohydrate fermentation may be readily observed in one medium but not in another and early-passage strains may not grow well enough

in laboratory media to produce detectable acid from glucose, caution is advised in using the lack of fermentation as a criterion for classification of mycoplasmata.

The finding that the organism labeled *M. histotropicum* (24, 26) appears to belong to the species *M. pulmonis* does not establish whether this is the original strain that Sabin described (19). The origin of the strain in the American Type Culture Collection was described by Tully and Ruchman (26). This organism did not show pathogenicity for mice (26), an important criterion for this species, and was distinguished from a number of *Mycoplasma* species found in rodents, including *M. pulmonis* strains with the fluorescent-antibody reagents employed by Tully (24). It is conceivable that the original *M. histotropicum* strain was in fact a variant of *M. pulmonis* and that the strain now in existence is attenuated. In a previous study, Deeb and Kenny (4) described a method for distinguishing the antigenic variants which exist in species *M. pulmonis*. Therefore, it should be possible to determine whether *M. histotropicum* is a specific subtype of *M. pulmonis* and whether similar variants exist. The results reported here confirm and amplify Razin's demonstration that *M. histotropicum* appeared closely related to *M. pulmonis* by electrophoretic patterns on polyacrylamide gel (18).

Tully and Razin (25) recently showed that *M. granularum* is closely related to *M. laidlawii* culturally and by patterns on polyacrylamide gel electrophoresis. I confirm their finding that *M. granularum* can be grown in serum-free medium. The results with the biological reagents used in this study indicate that *M. granularum* is very closely related serologically to *M. laidlawii* but the two species can be distinguished by growth-inhibition. Tully and Razin (25) reported that *M. granularum* showed only a one-way cross-reaction with *M. laidlawii* and that the two species could be distinguished by antibody titer by using an indirect fluorescent-antibody method. They also showed that, whereas *M. granularum* showed slight growth inhibition on agar with *M. laidlawii* antiserum, the two species could be totally distinguished when serum-free agar-grown colonies were used as antigen in a direct fluorescent-antibody technique. Since their methods for preparing both serological test antigens and antisera differed from mine, it is difficult to compare the results of the two studies. Additionally, since different serological tests were used, it is not known whether the same or different antigen-antibody systems were demonstrated. However, it is clear from both studies that these two species are closely related and possibly should be classified as variants of a single species.

M. felis and *M. canis* also appear to be very closely related species. Similarly, *M. pulmonis* and *M. hyorhinis* reciprocally cross-react, though the relationship is not nearly so striking as for *M. canis* and *M. felis*. The low GC organisms in this study (23 to 28%) appear to comprise an overall serologically related group of *Mycoplasma* species. The frequency of reciprocal cross-reactions in this group and the correlation of this with a consistently low GC content suggest that these species may be phylogenetically related. On the other hand, *M. gallisepticum* has a reported GC content of 32%, very close to the values for *M. granularum* and *M. laidlawii*, but it did not show any serological relationship with these organisms. *M. laidlawii* and *M. gallisepticum* also were shown to be unrelated by DNA homology techniques (14). Of all the species tested, *M. gallisepticum* and *M. pneumoniae* appear most serologically unique.

One of the aims of this study was to determine whether the glycolytic *Mycoplasma* species possessed common or group antigen(s). Although common components among all species in the group were not demonstrable with the techniques used in this study, it is plausible that common components exist which perhaps are not recognized immunologically by rabbits or are not present in a high enough concentration to be observed in the serological assays. However, reciprocal cross-reactions were observed frequently enough in the low GC group to suggest that at least some members of this group possess common antigens. Although it appears unlikely that any general diagnostic test employing a common mycoplasmal antigen is possible for the glycolytic *Mycoplasma* species, such an approach would seem possible for closely related groups of species; i.e., *M. granularum* infections might show antibody responses measurable by *M. laidlawii* antigens. Marmion et al. (15) showed that persons infected with *M. pneumoniae* show antibody measurable by *M. mycoides* var. *mycoides* antigens. Common components frequently have been observed in the arginine-fermenting group. *M. hominis* cross-reacts with *M. salivarium*, *M. arthritidis*, and *M. pharyngis* (orale 1; 12, 13, 22, 23); similarly, a new isolate from cats, *M. gateae*, was found to share antigenic components with *M. hominis* (3). Whether these common components would be useful serodiagnostically has not yet been determined.

The degree of antigenic heterogeneity observed in these glycolytic species is remarkable and comparable to the striking heterogeneity ob-

served in their GC ratios (14, 17) and gel electrophoresis patterns (18). Lemcke's comparative study of various *Mycoplasma* species by double immunodiffusion (13) included five of the glycolytic species employed in my study. Her study also demonstrated striking antigenic heterogeneity among species. It is plausible that the antigenic heterogeneity displayed, even in the low GC group, is a reflection of the limited amount of genetic material available in these organisms, since the probabilities for random duplication of antigenic determinants must be far less than in more complex organisms. This degree of heterogeneity appears to be totally incompatible with the present classification of the pleuropneumonia-like organisms into one genus: *Mycoplasma*. Hopefully, all pleuropneumonia-like organisms can at least be grouped into the presently existing order *Mycoplasmatales*, so that the trivial name mycoplasmata (mycoplasmas) will still have meaning as a common term.

At present, the most reasonable approach to separation of mycoplasmata into distinct species appears to be by growth inhibition (2). This method is a very insensitive measure of antibody but this very insensitivity tends to mask subspecies differences. By this criterion, *M. histotropicum* was found related to and best classified as *M. pulmonis*, a relationship also observed as sharing most immunodiffusion lines. On the other hand, significant reciprocal growth inhibition was not observed between either pair of species which showed the most extensive cross-reactions by double immunodiffusion (*M. canis-M. felis*, *M. granularum-M. laidlawii*). Accordingly, if one were to use the criterion of growth inhibition for species separation, each organism of these two related pairs would be assigned to separate species. However, the sharing of antigens detectable by the other serological tests cannot be ignored in classification. The question is: Which property should determine species rank? A most reasonable approach for the further investigation of the significance of these serological relationships would be by DNA homology studies. Moreover, the existence of closely related organisms provides excellent opportunities for cross-adsorption studies for further antigenic analysis of these organisms. Such closely related strains will also provide material for studying the nature of neutralizing or growth-inhibiting antigens in *Mycoplasmataceae* since, logically, the shared antigens should not be specific growth-inhibiting antigens for those species unrelated by growth inhibition. The indication that the species in the low GC group appeared serologically related as well is a very encouraging corollary and suggests that this group should perhaps have family rank in some future reclassification effort. However, neither the characteristics nor the chemical nature of any of the antigens which cross-react in any of the groups of species are presently known. Since at least one remarkable cross-reaction between different chemical classes of antigens of two different *Mycoplasma* species is known [i.e., the cross-reaction of the lipid antigen of *M. pneumoniae* with a galactan preparation of *M. mycoides* (15)], caution is advised in designating any cross-reacting component as a common antigen. Further studies involving cross-adsorption of sera and characterization of cross-reacting antigens will be required to elucidate the significance of these cross-reactions.

The use of double-immunodiffusion techniques is a very rapid way of serologically comparing *Mycoplasma* species. A rapid method is particularly important for describing new species, since it is incumbent on those describing new species that they compare the new species with all known species. Potent antisera can readily be prepared and only small amounts of antigen or antisera are required in the microtest. It would be necessary only to concentrate several liters of culture, standardize this by protein content, and test against a reference file of antisera. Nearly all *Mycoplasma* species have been grown in the soy peptone-yeast extract dialysate broth supplemented with horse serum. The antigens thus produced show negligible cross-reactions with the rabbit immune sera prepared against organisms grown in calf serum. It is stressed that the results of immunodiffusion identification of *Mycoplasma* species will be different in some cases from those obtained by growth inhibition, since some strains distinguishable by growth inhibition were found to be closely related by double immunodiffusion. Furthermore, immunodiffusion classification will provide an important means of classifying strains or variants of a given species (4) since a number of antigen-antibody systems (6 to 11 immunodiffusion lines) may be observed. Lemcke (13) pointed out that double immunodiffusion had utility for classification: "Homologous reactions were complex and sufficiently distinctive for the technique [double immunodiffusion] to be used to identify unknown strains." However, it will be necessary to cross-compare all of the known species to determine interrelationships; such a project is now underway in this laboratory.

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