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Serological Comparison of Five Arginine-Utilizing *Mycoplasma* Species by Two-Dimensional Immunoelectrophoresis

CHARLES E. THIRKILL AND GEORGE E. KENNY

Department of Pathobiology, School of Public Health and Community Medicine, University of Washington, Seattle, Washington 98195

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Five arginine-utilizing, nonglycolytic *Mycoplasma* species were compared serologically by two dimensional immunoelectrophoresis. The survey included: *Mycoplasma hominis*, *Mycoplasma arthritidis*, *Mycoplasma gateae*, *Mycoplasma gallinarum*, and three strains of *Mycoplasma arginini*. Although the *M. arginini* strains showed strong cross-reactions, each strain produced a different antigenic profile which was distinct and which indicated significant antigenic differences between strains. The *M. arginini* strains showed strong relationships with *M. gateae*; lesser cross-reactions were observed with other strains. Common antigens were demonstrable in the entire group. When all these species were compared with a glycolytic species, *Mycoplasma gallisepticum*, no cross-reactions were observed. Two-dimensional immunoelectrophoresis was a far more sensitive technique than double-immunodiffusion since as many as 20 components could be resolved.

The organisms classified in the *Mycoplasmatales* are strikingly heterogeneous antigenically (12, 17). The present evidence indicates that the species can be divided into six or seven groups when compared by double immunodiffusion with potent antiserum (14). Essentially no cross-reactions or indications of common antigens are observed between groups, but common components are readily demonstrable inside a group. In this study we compared the antigenic structure of five arginine-utilizing, nonglycolytic *Mycoplasma* species, a group which appears to be closely related antigenically (12, 14, 21). The two-dimensional immunoelectrophoretic method of Laurell (16) was chosen for analysis because of its great sensitivity and resolving power. The goals of our study were to characterize electrophoretically and quantitatively both the common and specific components of this group of *Mycoplasma* species and to describe the antigenic similarities both within strains of a species and between closely related species.

MATERIALS AND METHODS

Organisms. Seven strains representing five mycoplasmic species were employed in this study. Four strains were obtained from the American Type Culture Collection: *Mycoplasma arginini* (ATCC 23243) (7), *Mycoplasma gallinarum* (ATCC 15319), *Mycoplasma hominis* (ATCC 14027), and *Mycoplasma gallisepticum* (ATCC 15302). *Mycoplasma gateae* (Siam) was obtained from B.C. Cole, *Mycoplasma arginini* (*leonis*) (22) was obtained from W. Dowdle, *Mycoplasma arginini* (G-230) was obtained from M. F. Barile, and *Mycoplasma arthritidis* (PG-6) was obtained from the Reference Reagent Branch of the National Institutes of Health.

Serological test antigens. Each strain was grown in 10 liters of "hot" yeast dialysate, soy peptone broth (19), supplemented with 10% agamma horse serum, 100 U of penicillin per ml, and 0.33 mg of thallium acetate per ml. A 2-ml volume of an actively growing culture was used as an inoculum. These cultures were incubated at 37 C for 1 to 3 days until the medium displayed a fine haze indicative of growth. The organisms were then concentrated by centrifugation at 8,000 × *g* for 20 min, washed three times in 200 ml of TES saline—0.15 M NaCl with 5 mM TES [*N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.0]—before being resuspended in 20 ml of distilled water in which they were disrupted by sonic treatment in an ice bath at maximum power with a Bronwill sonicator for 2 min with interruptions to prevent overheating. Their protein concentration was measured by the Lowry method (18) and adjusted to 10 mg/ml.

Immunogens. Organisms were grown in 2.5 liters of fresh yeast dialysate broth (11) with 10% agamma rabbit serum except for the 23243 strain of *M. arginini*, which was grown in the same broth with agamma calf serum (12). The risk of contamination by foreign species was minimized by using organisms which had been subcultured no more than six times since their arrival in this laboratory. The organisms were then concentrated, washed three times, and resuspended in 12 ml of TES saline. Each immunogen contained 4 to 10 mg of protein per ml as measured by the Lowry method.

Immunization procedure. New Zealand white female rabbits were immunized as previously described

(13). Briefly, 2 to 5 mg of mycoplasmic protein was emulsified in an equal volume of Freund incomplete adjuvant and injected intramuscularly. Three weeks later, four intravenous injections were administered at 5-day intervals by using volumes of 0.1, 0.2, 0.3, and 0.4 ml, respectively, of the immunogens. Sera were obtained within 7 days of the last immunization. Antisera to bovine serum albumin and agamma horse serum were prepared in rabbits by using the same immunization scheme.

Two-dimensional electrophoresis. The supporting agar matrix used throughout this study consisted of 0.5% agarose in pH 8.6 Barbital buffer, ionic strength 0.05, with 0.5% Triton X-100 detergent and 0.01% sodium azide. The baths contained this buffer at ionic strength 0.1. A miniaturized version of two-dimensional electrophoresis (4, 16) was developed. Glass mounting slides (4 by 4 cm Perrocolor precision slide binders, Switzerland) were covered with 3 ml of the agarose mixture. A 5-μliter amount (50 μg of protein) of the standardized antigen mixed with 2 μliters (2 μg of protein) of a 1 mg/ml solution of bovine serum albumin (Calbiochem, Los Angeles), which acted as a tracer of the migration rate of each fraction, was added to a well 2 mm in diameter cut in the corner of the slide (Fig. 1) of the cathode side.

First-phase electrophoresis was carried out for 90 min at 6 V per cm. Agar, except for a 1-cm strip in the protein migration path, was removed (Fig. 1). A total of 1.8 ml of antibody-containing, agarose mixture

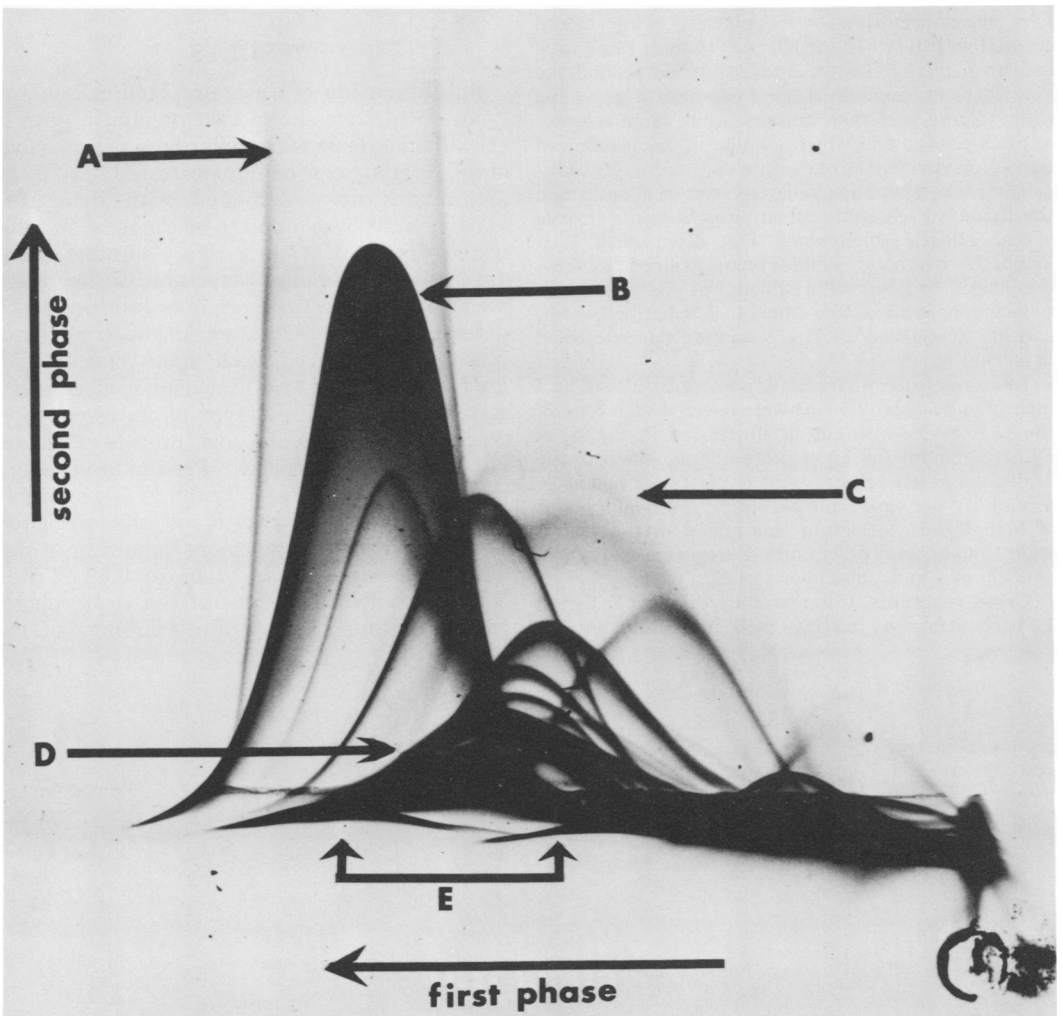

FIG. 1. *Two-dimensional immunoelectrophoretic profile of M. arginini (leonis) against homologous antiserum (0.15 ml). (A) Bovine albumin tracer. (B) Lead peak having approximately the same migration rate as the bovine albumin tracer; moderate intensity of precipitation line indicates presence of significant quantity of antigen which has reacted with high levels of antibody. (C) Ghost peak, formed by small amount of antibody. (D) A collection of electrophoretically related antigens. (E) These highly intense reactions indicate presence of large amount of antibodies to these fractions, antigenic concentration may be high. The anode is at the left side of the slide during first-phase electrophoresis and at the top of the slide for second-phase electrophoresis.*

(from 0.15 ml to 1.0 ml of mycoplasmic antiserum plus 0.1 ml of anti-bovine albumin) was then poured over the cleared area, and the second phase was run at right angles to the electrophoretic direction of the first phase for 6 h at 6 V per cm. Precipitin lines were allowed to further intensify in a moist chamber at 4 C for 24 h.

The practice of precoating slides, in which a small volume of agar is allowed to dry on the glass surface to improve adhesion of the gel used in the experiments, was not utilized in the two-dimensional electrophoresis experiments since this inhibited the removal of agar after the first phase and background staining was more intense. A method was therefore devised which prevented the agarose gel from floating from the surface of the slide during the washing procedure. The fully developed slides were placed on racks and covered with Kimwipe or Kleenex tissues; these were held in place by elastic bands stretched around the long edges of the racks, taking care not to touch the slides. These were then immersed into large volumes of distilled water in which simple diffusion removed unprecipitated antiserum within approximately 24 h.

The racks were then removed from the water and the tissue was carefully rolled away from the surface of the slides, which were then dried in a 37-C incubator overnight before being stained in 0.5% Coomassie brilliant blue which was dissolved in a solvent consisting of 45% ethanol, 45% distilled water, and 10% acetic acid (2). Decolorization was performed in solvent alone.

Immunoelectrophoresis. Microscope slides (3 by 1 inch; about 7.5 by 2.5 cm) were covered with 3 ml of the agarose gel and cut as illustrated in Fig. 2. A 5-μliter amount (50 μg of protein) was added to the 2-mm diameter well and was electrophoresed for 30 min at 5 V per cm (5 mA per slide). A 0.1-ml amount of homologous antiserum was added to the 2-mm-wide troughs, and precipitation arcs were allowed to develop in a moist chamber at 4 C.

Cross-reactions. Common antigens were detected in each strain by testing each individual antigen against each heterologous antiserum in volumes which would produce a complete profile within the dimensions of the slide. These volumes were derived by trial and error and ranged from 0.2 to 1.0 ml. The electrophoretic cell used in this study was capable of running 12 slides simultaneously, thereby enabling each individual strain to be run against all antisera at the same time under the same conditions. The quantities of a given antigen can be determined by comparing the areas of peaks (2, 4) provided that the same antiserum is employed with an antigen preparation of known content. Since fractionation of mycoplasmic antigens has not been carried to the point where specific components are available, it was only possible to relatively compare the amounts of antigens between various strains.

RESULTS

Solubilization of antigens. Hollingdale and Lemcke (9) have shown that precipitin lines in gel diffusion tests with membrane preparations of *M. hominis* can be demonstrated only if the membranes are solubilized with detergents (Triton X-100 was found to be the most suitable tested). Since it is likely that a number of the antigenic components detectable in our organisms are of membrane origin, solubilization by detergents was investigated. Although good profiles were produced with some species (*M. arginini, leonis*) by two-dimensional electrophoresis without solubilization of antigens other than sonic treatment, poor profiles were obtained with most species. Preliminary experiments showed that 0.5% Triton X-100 produces reasonable solubilization of the sonically treated suspensions, but more important, it was found necessary to incorporate 0.5% Triton X-100 in both dimensions of the electrophorogram in order to maintain solubility of the

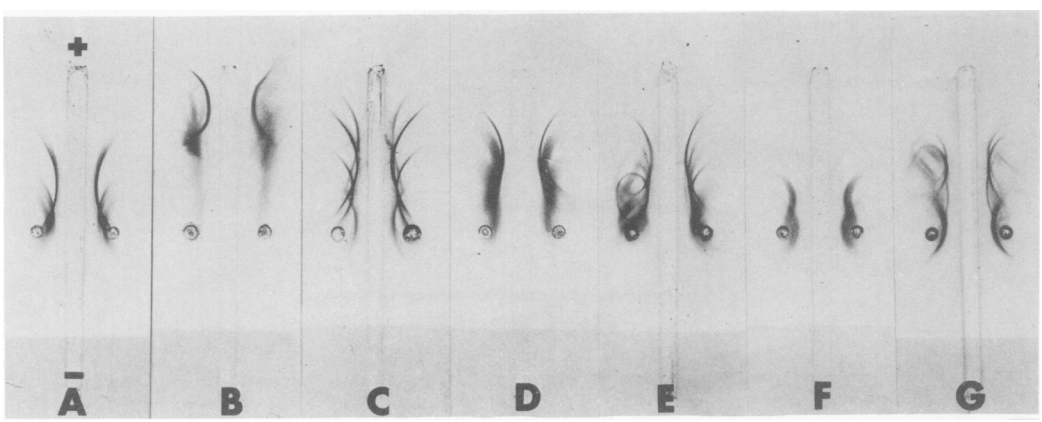

FIG. 2. *Immunoelectrophoretic separation of mycoplasmic antigens in agarose gel. Troughs contain homologous antiserum only. Antigens: (A) M. hominis; (B) M. gateae; (C) M. arginini, 23243; (D) M. arginini, leonis; (E) M. arginini, G-230; (F) M. arthritidis, and (G) M. gallinarum.*

components and avoid distortion of peaks. It should be noted that the 0.5% Triton concentration has not been found universally satisfactory for all *Mycoplasma* species.

Immunoelectrophoresis. For the technique of two-dimensional electrophoresis to function, the antigens of the organisms must be negatively charged so that they will migrate towards the anode in both phases. When each antigen was tested by immunoelectrophoresis using ordinary agar (Difco) or purified agar (Difco Noble) some antigens moved towards the cathode, a result caused or enhanced by electroendosmotic flow. Antigens of *M. arthritidis* migrated most strongly towards the cathode. The use of agarose gels minimized the effect of electroendosmosis: the greater part of the antigens of *M. arthritidis* migrated towards the anode although one fraction remained very close to the origin (Fig. 2); the antigens demonstrable in the other species migrated clearly towards the anode in agarose gel. Accordingly, we concluded that the use of agarose gel would provide suitable conditions for the detection of the greatest number of antigens in two-dimensional electrophoresis.

Antigenic profiles by two-dimensional electrophoresis. When each strain was tested against its homologous antiserum, 9 to 20 precipitin peaks were observed. Most antigenic components were revealed as single peaks or symmetrical curves. However, in several cases, for example, *M. hominis* (Fig. 3, frame D4), the presence of double peaks showing reactions of serological identity indicated that the fractions were electrophoretically heterogenous but immunologically identical. When second-phase migration ceases the area of each peak is proportional to the amount of antigen in that fraction and inversely proportional to the amount of antibody against it (4). The intensity of the precipitation lines is also a function of the concentration of these reactants in the gel; thus the tallest peak in the profile is not necessarily the most antigenically significant since its height is a function of both antigen and antibody concentration. Smaller, more intense peaks may contain more antigen but the peak height is suppressed by large antibody content. Antigens in which small amounts of antibody are present are frequently faint peaks which are quite tall (Fig. 1).

Interspecies antigenic variation. All antigens were compared with all antisera and the results were evaluated by a comparison of the number of precipitin arcs (peaks) formed (Table 1). Cross-reactions were evident between all of the arginine-utilizing nonglycolytic mycoplasmic species. The strongest antiserum was against *M. arginini* (*leonis*) and the weakest was against *M. arthritidis*, though the poor electrophoretic mobility of this species also contributed to the poor profiles produced. Although the *M. arginini* strains showed substantial cross-reactivity among themselves, highly significant differences were observed. In addition, these strains showed strong reciprocal cross-reactions with *M. gateae* and their antisera detected a large number of components in *M. arthritidis*, although this latter reaction was not reciprocal, perhaps because of the weakness of the *M. arthritidis* antisera.

Although the *M. hominis* antiserum recognized nine components in *M. arginini* (*leonis*), far fewer were detected in the two other *M. arginini* strains. *M. gallinarum* appeared to be the most serologically distinct organism in the group though several components could be observed when its antigen was tested with antisera to all species but *M. arthritidis*. Much more information can be obtained from examination of the electrophorograms of each species in cross-reaction and normal profile (Fig. 3).

Antiserum produced against *M. arginini* (G-230) was highly reactive with *M. gateae*, suggesting this species may possess a strong relationship to the arginini group. Although *M. gateae* appeared to be clearly distinct from *M. arginini* (G-230) when tested by disk inhibition on 20% serum agar medium, a suggestion of a cross-reaction appeared when medium with 5% serum was employed. Small zones of inhibition were observed with the three *M. arginini* strains when tested with *M. gateae* antiserum. The taxonomic significance of this cross-reaction is under further study. The *M. gallinarum* antigen showed a small dense fraction with this antiserum which had almost the same migration rate as the bovine albumin tracer; this particular fraction was also detected by *M. hominis* and *M. gateae* antiserum; in addition, two other larger but fainter peaks were revealed. Four fractions were detected in both *M. hominis* and *M. arthritidis* antigens with G-230 antiserum, the highest number of cross-reacting antigens found with these two species in this interspecies investigation.

Antiserum produced against *M. gateae* reacted more strongly with *M. arginini* (G-230) antigen than with any other species. As previously stated, it resolved the fraction in *M. gallinarum* antigen located within the bovine albumin tracer pillar and also one other which appeared to be in the same position as one of the fainter peaks produced in the cross-reaction with this antigen and *M. arginini* (G-230) anti-

serum. In reacting with *M. hominis* and *M. arthritidis* antigen this antiserum resolved, in addition to other fractions, a densely staining arc indicating a relatively high concentration of antibody against this entity.

Antiserum produced against *Mycoplasma gallinarum* resolved a greater number of fractions when reacted against *M. arginini* (G-230) and *M. gateae* antigen than either of the remaining two species, again demonstrating the strong relationship of these two closely related species.

Antiserum produced against *M. hominis* resolved the single fraction in *M. gallinarum* described as having a migration rate very close to that of the bovine albumin tracer, being located within the pillar, and which was also reactive with antisera to both *M. arginini* (G-230) and *M. gateae*. No other components were seen in this cross-reaction. The reactions

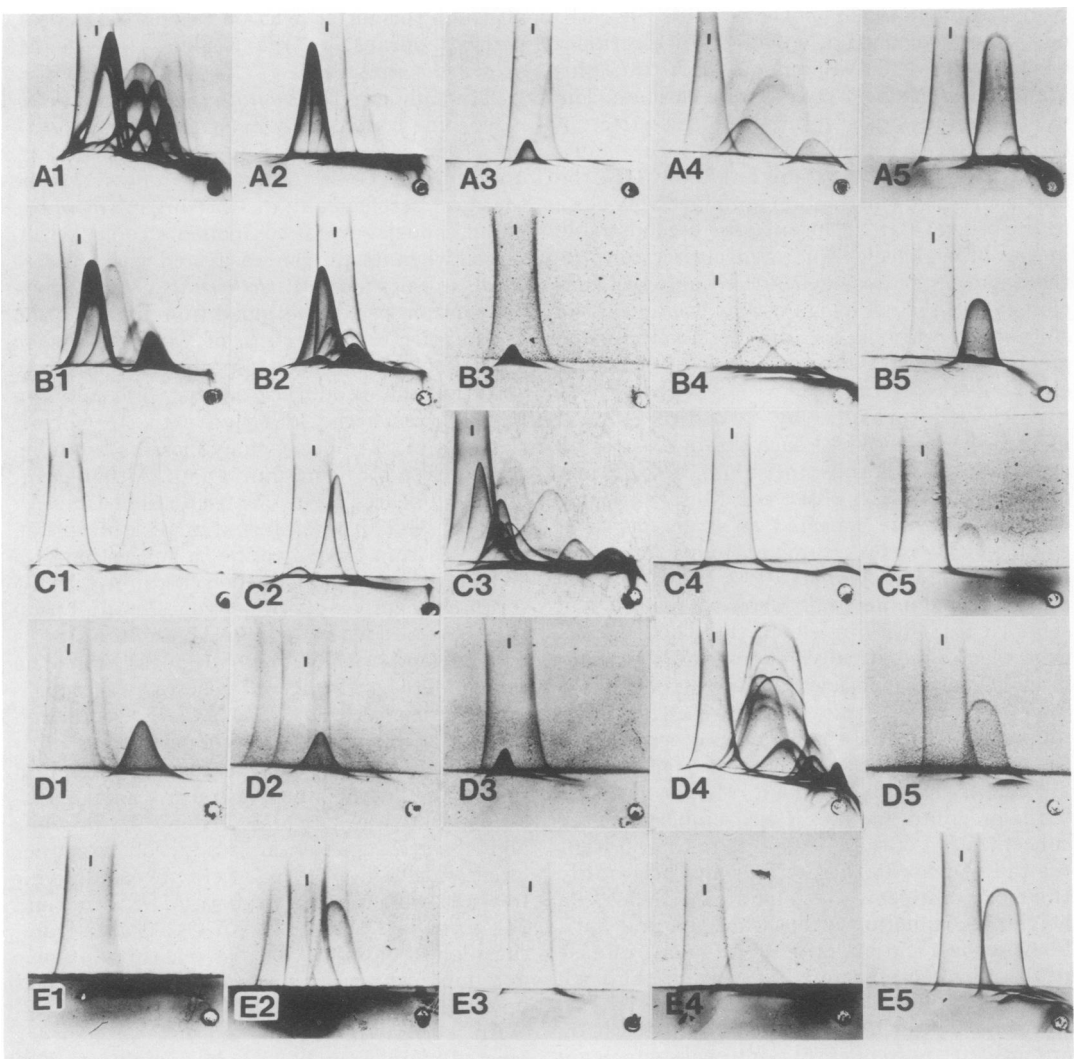

FIG. 3. *Serological comparison of five mycoplasmic species by two-dimensional immunoelectrophoresis. Antigens: (1) M. arginini, G-230; (2) M. gateae; (3) M. gallinarum; (4) M. hominis, and (5) M. arthritidis. Antisera: (A) M. arginini, G-230; column 1, 0.2 ml; 2, 0.6 ml; 3, 0.3 ml; 4, 0.3 ml; and 5, 0.5 ml. (B) M. gateae; column 1, 0.3 ml; 2, 0.3 ml; 3, 0.3 ml; 4, 0.5 ml; and 5, 0.7 ml. (C) M. gallinarum; column 1, 0.5 ml; 2, 0.5 ml; 3, 0.25 ml; 4, 0.5 ml; and 5, 1.0 ml. (D) M. hominis; column 1, 0.5 ml; 2, 0.5 ml; 3, 0.5 ml; 4, 0.3 ml; and 5, 1.0 ml. (E) M. arthritidis; column 1, 1.0 ml; 2, 1.0 ml; 3, 0.8 ml; 4, 1.0 ml; and 5, 0.6 ml. Vertical bar at top left of each frame indicates the center of the bovine albumin control pillar.*

TABLE 1. *Serological comparison of eight mycoplasmic species by two-dimensional immunoelectrophoresis*

Antiserum	Antigen[a]								
	M. arginini (*leonis*)	*M. arginini* (23243)	*M. arginini* (G-230)	*M. gateae*	*M. gallinarum*	*M. hominis*	*M. arthritidis*	*M. gallisepticum*	Agamma horse serum
M. arginini (*leonis*)	*20*	10	11	6	1	2	6	0	0
M. arginini (23243)	9	*11*	9	6	1	2	7	0	0
M. arginini (G-230)	12	9	*17*	6	3	4	4	0	0
M. gateae (Siam)	8	6	8	*11*	2	4	3	0	0
M. gallinarum	3	2	3	2	*12*	2	1	0	
M. hominis	9	2	2	2	1	*12*	1	0	0
M. arthritidis	1	1	1	3	0	1	*9*	0	0
M. gallisepticum	0	0	0	0	0	0	0	*15*	0
Agamma horse serum	2	2	2	2	2	2	2	2	*12*

[a] Antigens were employed at 50 μg of protein per slide and were tested against quantities of antibody which gave best profiles (see Fig. 3 and 4). The numbers given represent the number of precipitin lines which could be clearly distinguished on the two-dimensional electropherogram. Italics represent homologous reactions.

with *M. gateae* and *M. arginini* (G-230) antigen were very similar in that two fractions in each were resolved which had similar but not identical migration rates; however, it is known that immunologically identical fractions need not necessarily have the same migration rate in an electric field (15). The single peak produced in the cross-reaction with *M. arthritidis* antigen may possibly represent the large leading peak (fraction) seen in the homologous reaction with this organism.

Antiserum produced against *M. arthritidis* was the weakest in the cross-reactions and failed to react with *M. gallinarum*, even when large volumes, up to 1 ml, were used. It was weakly reactive against *M. arginini* (G-230) and *M. gateae* antigen and resolved a single faint fraction when reacted with *M. hominis*.

Intraspecies variation of M. arginini. Antigenic variation has been demonstrated in a number of species. In the case of double-immunodiffusion analysis which is most analogous to the present study, intraspecies variation has been clearly shown in *Mycoplasma pulmonis* (5, 6) and in *M. hominis* (9). We chose the *M. arginini* group for the study because we had the best antisera against these three strains. The three strains chosen are known to be related to each other by disk inhibition with specific antiserum (7, 22).

Antiserum to the prototype strain, *M. arginini* (G-230), produced a profile with the leonis strain which was quite similar to that obtained with the homologous antigen though fewer antigens were resolved even at greater serum concentrations (Fig. 4). Interestingly, the leonis strain antigen would appear to contain a higher concentration of the leading component located within the tracer pillar than is apparent in the homologous reaction since 0.4 ml of the *M. arginini* (G-230) antiserum was required to reduce this peak to a similar size to that seen in the homologous reaction using 0.2 ml of this antiserum. Similarly, fewer components were detected in strain 23243 antigen with this antiserum, but the leading component again appeared with an antigenic concentration greater than that evident in the homologous reaction.

The complexities in the antigenic relationships became apparent when we compared the results with antiserum to the leonis strain. Although this antiserum recognized a number of components in the homologous antigen, few components were detected in the G-230 strain. A fast component with similar electrophoretic mobility to that detected with the G-230 antiserum is observed in the 23243 antigen. Antiserum to the 23243 strain recognized components in the leonis and G-230 strains which resembled each other both in electrophoretic mobility and peak height.

Controls. Both the serological test antigens and the immunogens were grown in dialysate broth, in the former case, supplemented with agamma horse serum and in the latter, with agamma rabbit serum. Although the dialysate broth by itself should contribute no foreign antigens, it has not proven possible to free concentrated mycoplasmic antigens of medium serum components. It has been estimated that mycoplasmic antigens prepared in this laboratory in the manner described contain about 1%

horse serum antigen (12), a result which is quite good when one considers that a liter of culture contains 6 to 7 g of horse serum protein versus 20 to 30 mg of organisms. Even though the rabbits should not have produced antibodies to horse serum because the immunogens were cultivated in agamma rabbit serum, it was possible that they might have had preexisting antibodies to horse antigens which might be detected by two-dimensional electrophoresis method because it is a more sensitive means of detecting antigens than double immunodiffusion. Each anti-mycoplasmic immune serum was tested against agamma horse serum (Table 1), the medium for cultivation of serological test antigens and a 10× concentrate of that medium; no precipitin lines were observed.

Each of the serological test antigens could be shown to contain horse serum antigens when tested against anti-horse serum. Two peaks were observed one of which migrated with the bovine albumin tracer, the other approximately midway between the tracer and the origin, whereas 12 peaks were observed with horse serum antigen. When anti-horse serum was mixed with mycoplasmic antiserum in the second phase of the test, no peaks were suppressed (2) which would indicate that none of the peaks shown in the normal pattern were of horse serum origin; the two fractions previously described appeared and were the only alteration to the normal result. The pre-immunization sera

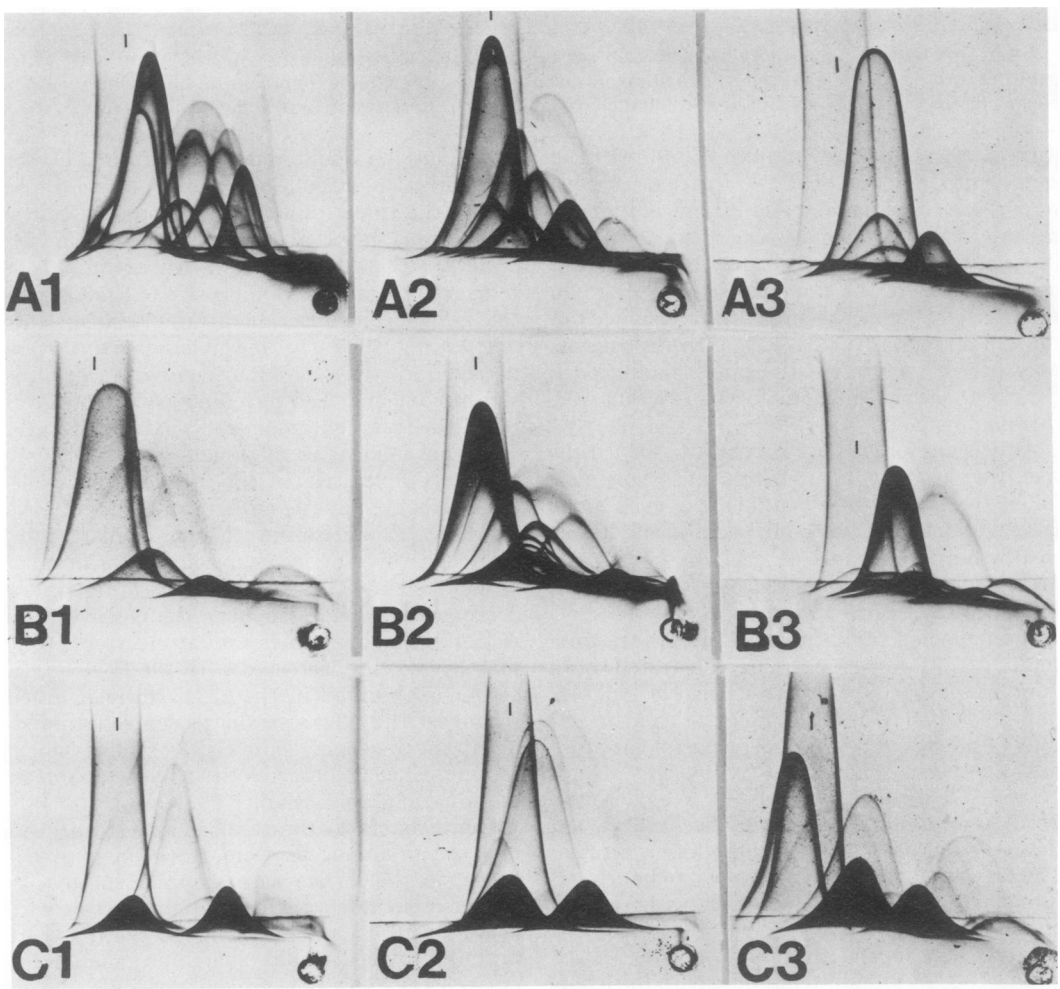

FIG. 4. *Antigenic heterogeneity in three strains of M. arginini. Antigens: 1, G-230; 2, leonis, and 3, 23243. Antisera: A, G-230: column 1, 0.2 ml; 2, 0.4 ml; and 3, 0.4 ml. B, leonis: column 1, 0.2 ml; 2, 0.15 ml; and 3, 0.3 ml. C, 23243: column 1, 0.3 ml; 2, 0.4 ml; and 3, 0.3 ml. Vertical bar at top left of each frame indicates the center of the bovine albumin control pillar.*

did not react with any of the antigens. A further control for specificity was the fact that none of the arginine-utilizing mycoplasmic antigens or antisera showed any reaction with antisera or antigens to an unrelated glycolytic species, *M. gallisepticum*. Although the immunogen used for the 23243 strain had been propagated in agamma calf serum, the resulting antiserum showed no cross-reactions against the antigens which could be attributed to horse serum antigens, a result similar to that described previously with double immunodiffusion (12). We believe it can be safely concluded that horse serum antigens were not part of the profiles observed because the mycoplasmic immune serum produced in this study did not contain horse serum antibodies and because none of the peaks could be diminished by anti-horse serum. The fact that the medium antigens can be clearly located and differentiated from the mycoplasmic antigens indicates that the two-dimensional electrophoresis method might be remarkably useful for antigenic analysis of those *Mycoplasma* species which cannot be grown in serum homologous to the animal to be immunized.

DISCUSSION

Common antigens were observed in the nonglycolytic arginine-utilizing *Mycoplasma* species, a result in sharp contrast to the heterogeneity observed in the glycolytic species (12). *M. gallinarum* was the most serologically different organism of these arginine-utilizing species tested. It is interesting to note that arginine deiminase of this species was serologically different from that of *M. hominis* and *M. arginini* which possessed serologically similar deiminases (7). It is not presently known which, if any, peak represents arginine deiminase.

An electrophoretically common antigenic component could be recognized in most species by antisera to any of the species. However, common components also were recognized between certain species that were not common to the group. It should be realized that the components recognized depend not only upon the ability of the rabbit to recognize them, but also upon their relative concentration between organisms. When the same antiserum was employed, common components appeared to be present in quite different concentrations between species. Further analysis of the relative quantities and identity of common components will be forthcoming. The nature of the antigens resolved is unknown though the fact that a nonionic detergent, Triton X-100, was necessary for the test suggests that at least some of the components are membrane associated.

The poorest antisera produced in this study were against *M. arthritidis*. Several attempts were made to make better antisera but none were more successful than the serum used in the study. Bergquist et al. (3) recently noted that *M. arthritidis* membrane preparations suppressed the immune response to the common antigen of gram-negative bacteria. Similarly, Kaklamanis and Pavlatos (10) demonstrated that concurrent injection of *M. arthritidis* and a pseudomonas bacteriophage resulted in suppression of the antibody response to the bacteriophage. Thus, the poor immune response to *M. arthritidis* may be a result of immunosuppression. *M. arthritidis* antigens showed the least negatively charged antigens in the present experiments, a factor which is certainly related to the poor performance of the antigen in two-dimensional electrophoresis and might have some relationship to the poor immunogenicity observed.

Both significant and marked serological differences were observed within strains of the species, *M. arginini*. Various common components were clearly recognized and each strain appeared to possess a number of specific components even though all could be typed by disk inhibition by any of the antisera. It is interesting to note that these same strains of *M. arginini* were also heterogeneous in their requirement for arginine for growth from small populations (7). Neither strain G-230 nor 23243 required arginine, whereas the leonis strain did.

The two-dimensional electrophoretic technique detected substantially more antigenic components (9 to 20 peaks) than are evident by micro-double-immunodiffusion, where 6 to 12 lines are the most which are resolved using antigens and antisera of similar strength to those used in this study (2). A number of reasons account for this rather remarkable increase in sensitivity: (i) the amount of antibody is large and its concentration is constant throughout the slide, whereas antibody concentration in both immunoelectrophoresis and double-immunodiffusion is in a decreasing gradient from the well; (ii) the electrophoretic separation and increased area on the slide improve the possibilities of visualizing the reactions; and (iii) all the antigen is used in the precipitin reaction because of its unidirectional flow. The same remarkable sensitivity of two-dimensional electrophoresis for assessment of antigens has been observed with a number of other microorganisms in recent reports: *Histoplasma capsulatum* (20), *Candida albicans* (1), and *Pseudomonas aeruginosa* (8).

Powerful antisera were required to demonstrate large numbers of components and the volume of antisera required was large even in this miniaturized method. Although it was not always possible to demonstrate common components by comparing a single antigen with the available antisera, a comparison of a variety of antigens against a single antiserum did permit such an evaluation within the limits of the animals' ability to respond to various components.

ACKNOWLEDGMENTS

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