

Mycoplasma Taxonomy Studied by Electrophoresis of Cell Proteins

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The electrophoretic patterns of cell proteins in polyacrylamide gels were used for the study of several taxonomic problems in the *Mycoplasmatales*. The patterns of five *Mycoplasma hominis* strains showed marked differences that corresponded with their known serological and nucleic acid heterogeneity. The patterns of three *M. mycoides* var. *mycoides* strains isolated in different countries were essentially identical. The electrophoretic patterns of several caprine strains resembled those of *M. mycoides* var. *mycoides*, supporting their classification as *M. mycoides* var. *capri*. Strain B3, a swine isolate, accordingly was tentatively identified as *M. mycoides* var. *capri*. The bovine mastitis strain *M. agalactiae* var. *bovis* possessed a pattern basically similar to that of the goat mastitis strain *M. agalactiae*, supporting the inclusion of both strains in one species. Three *M. pulmonis* strains isolated from rats or tissue cultures showed nearly identical patterns. The pattern of the toxigenic *M. neurolyticum* (Sabin A) strain resembled but was not identical with that of the nontoxigenic PG28 strain. The avian *Mycoplasma* species, *M. gallisepticum*, *M. meleagridis*, *M. synoviae*, *M. gallinarum*, and *M. iners* showed easily distinguishable and specific patterns, supporting their present classification in different species. Several improvements in the electrophoretic technique are described, and its advantages and limitations as a taxonomic tool are discussed.

Identification of *Mycoplasma* by the electrophoretic patterns of cell proteins has been recently suggested (24, 27). The aim of the present study was to investigate with the new technique several taxonomic problems in the *Mycoplasmatales*.

MATERIALS AND METHODS

Organisms and growth conditions. The designation and source of the *Mycoplasma* strains examined are given in Table 1. The organisms were grown in 100 to 200 ml of broth (10) for 24 to 48 hr at 37 C. The cells were harvested by centrifugation at $13,000 \times g$ for 10 min, washed twice in 0.25 M NaCl, and suspended in 1 ml of the NaCl solution. The amount of cell protein in the suspension was determined according to Lowry et al. (18). The cells were sedimented, and 0.2 ml of phenol-acetic acid-water (2:1:0.5, w/v/v) was added to each milligram of cell protein in the pellet. The insoluble material was removed by centrifugation at $30,000 \times g$ for 15 min.

Polyacrylamide gel electrophoresis. Polyacrylamide gels containing 35% acetic acid and 5 M urea were prepared as described before (24). The clear solution of cell material in phenol-acetic acid-water (50 to 100 μ liters containing about 400 μ g of protein) was placed on top of the gel and was mixed with 50 μ liters of water and 50 μ liters of a 40% (w/v) sucrose solution in 50% (v/v) acetic acid. A 75% (v/v) acetic acid solution (0.5 ml) was then carefully layered

over the sample-sucrose mixture, and the tube was filled up with a solution of 10% (v/v) acetic acid. Both upper and lower reservoirs of the electrophoresis apparatus were filled with 10% (v/v) acetic acid. The lower electrode served as a cathode, and electrophoresis was carried out at room temperature for 2.5 hr at a constant current of 5 ma per tube. The gels were stained with 1% (w/v) Amido Black 10B in 7% (v/v) acetic acid for 30 min and were then rinsed well with tap water. The gels were transferred to a destaining apparatus made according to Farmer, Turano, and Turner (8) by E. Yechezkel (The Mechanical Workshop, The Hebrew University-Hadassah Medical School, Jerusalem, Israel.) The apparatus was filled up with 7% (v/v) acetic acid and left overnight. In the morning, the acetic acid solution was replaced with a fresh one, and destaining was completed within 30 min by applying a direct current of 200 ma at right angles to the long axis of the gels.

Growth inhibitor by antisera. The method described by Clyde (1) was used. Zones of growth inhibition were measured from the edge of the filter-paper discs.

RESULTS

Improvements in the electrophoretic technique. Several modifications in the electrophoretic procedure of Razin and Rottem (24) were introduced and have been described in Materials and

TABLE 1. *Source of Mycoplasma strains*

Type or species	Strain	Recovered from	Obtained from
<i>M. hominis</i>	PG21	Man	D. G. ff. Edward ^a
	PG23	Man	D. G. ff. Edward ^a
	PG25	Man	D. G. ff. Edward ^a
	PG26	Man	D. G. ff. Edward ^a
	15056	Man	A. T. C. C. ^b
<i>M. mycoides</i> var. <i>mycoides</i>	PG1	Cattle	D. G. ff. Edward ^a
	PG46(T3)	Cattle	D. G. ff. Edward ^a
	V5	Cattle	A. W. Rodwell ^c
<i>M. mycoides</i> var. <i>capri</i>	PG3	Goat	D. G. ff. Edward ^a
	pp. goat	Goat	E. Klieneberger-Nobel ^d
<i>Mycoplasma</i> sp.	Y	Goat	A. W. Rodwell ^c
<i>Mycoplasma</i> sp.	14	Goat	M. E. Tourtellotte ^e
<i>Mycoplasma</i> sp.	B3	Swine	J. G. Tully ^f
<i>M. agalactiae</i>	PG2	Goat, sheep	D. G. ff. Edward ^a
<i>M. agalactiae</i> var. <i>bovis</i>	Donetta	Cattle	M. E. Tourtellotte ^e
<i>M. pulmonis</i>	PG22	Mice, rats	D. G. ff. Edward ^a
	PG34	Mice, rats	D. G. ff. Edward ^a
	PG53(Negroni)	Tissue culture	D. G. ff. Edward ^a
	PG28(L5)	Mice	D. G. ff. Edward ^a
<i>M. neurolyticum</i>	PG39(Sabin TypeA)	Mice	D. G. ff. Edward ^a
<i>M. gallinarum</i>	PG16	Chicken	D. G. ff. Edward ^a
	J	Chicken	P. F. Smith ^g
<i>M. gallisepticum</i>	PG31	Chicken	D. G. ff. Edward ^a
	S6	Chicken	H. E. Adler ^h
	A5969	Chicken	M. E. Tourtellotte ^e
<i>M. synoviae</i>	WVU1853	Chicken	N. O. Olson ⁱ
<i>M. iners</i>	PG30	Chicken	D. G. ff. Edward ^a
<i>M. meleagridis</i>	N	Turkey	R. Yamamoto ^h

^a Wellcome Research Laboratories, Beckenham, Kent, England.

^b American Type Culture Collection, Rockville, Md.

^c C.S.I.R.O. Animal Health Research Laboratory, Parkville, Victoria, Australia.

^d The Lister Institute of Preventive Medicine, London, S.W.1.

^e University of Connecticut, Storrs.

^f National Institutes of Health, Bethesda, Md.

^g University of South Dakota, Vermillion.

^h School of Veterinary Medicine, University of California, Davis.

ⁱ West Virginia University, Morgantown.

Methods. The addition of the phenol-acetic acid-water mixture directly to the washed cell pellet permitted the analysis of minute quantities of cells (containing less than 1 mg of protein) and improved the solubilization of cell proteins. The longer electrophoretic run (2.5 hr instead of 2 hr) resulted in a better separation of the protein bands over almost the entire gel length. Cell protein solutions in phenol-acetic acid-water could be stored at 4 C for long periods (thus far over 1 year) with no apparent change in their electrophoretic properties. Hence, protein solutions of reference strains can be conveniently stored and used when needed for comparison with unknown isolates. Freeze-drying did not affect the solubilization of the cells in phenol-acetic acid-water. The electrophoretic patterns of the dried cells were identical with those of nondried cells, facilitating

the transportation of material to be analyzed from one laboratory to another. According to our experience, the type of proteins solubilized in the phenol-acetic acid mixture is a constant characteristic of each strain, as the electrophoretic pattern is exactly the same with different batches of cells of the same organism.

The destained gels could be kept in test tubes filled with 7% acetic acid for over 1 year, with no apparent loss in the color intensity of the protein bands. Drying the gels provided a more convenient way for storage and transportation. The gels were placed in open petri dishes covered with Parafilm M (Marathon Products Co., Neenah, Wis.). The dishes were transferred to a 37 C incubator, and the gels were completely dried within 10 to 15 hr. The dried gels could be restored by transferring them into test tubes filled

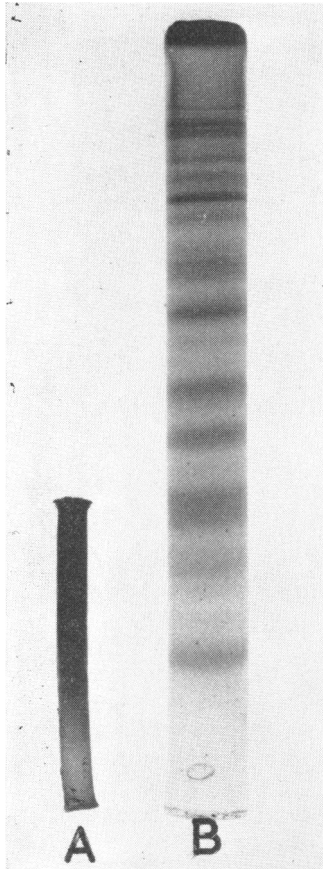


FIG. 1. Polyacrylamide gels used for the electrophoresis of *M. gallisepticum* cell proteins. (A) A dried gel; (B) the same gel after rehydration in 7% acetic acid. $\times 1.5$ of the natural size.

with 7% acetic acid. Figure 1 shows a dried and restored gel of *M. gallisepticum* A5969 proteins. The electrophoretic pattern did not change even after several repeated dryings and restorals.

Mycoplasma hominis strains. Although, the five *M. hominis* strains tested have a common basic pattern, none of the patterns is identical with any of the others (Fig. 2). Growth inhibition tests with antisera against *M. hominis* obtained from various sources (Table 2) confirmed the heterogeneity of the strains, and their results correlated well with those obtained by the electrophoretic method; this showed that *M. hominis* PG26, which was not inhibited by any of the antisera, is the most unrelated strain in this group.

Bovine and caprine strains. The three *M. mycoides* var. *mycoides* strains, the representative strain PG1, the Australian strain V5, and the African strain PG46 (T3) exhibited essentially identical electrophoretic patterns (Fig. 3). The

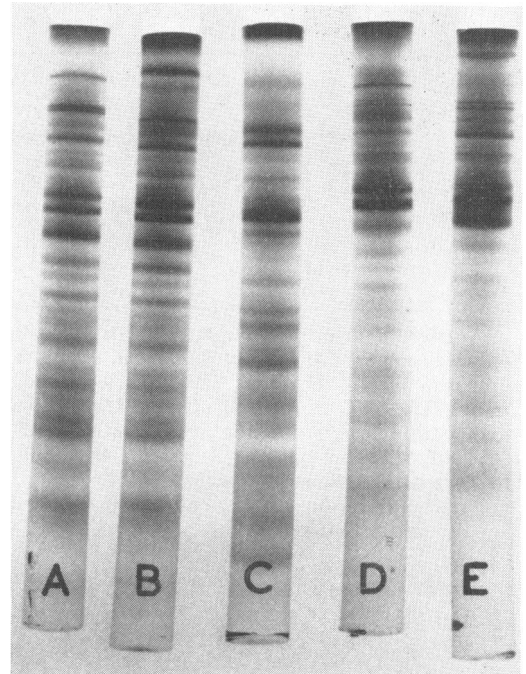


FIG. 2. Electrophoretic patterns of cell proteins of *M. hominis* strains. (A) ATCC 15056; (B) PG25; (C) PG26; (D) PG21; (E) PG23.

pattern of *M. mycoides* var. *mycoides* resembled but was not identical with that of *M. mycoides* var. *capri* PG3. Figure 4 shows the similarity of the patterns of the three caprine strains PG3, Y, and 14. The patterns of strains PG3 and Y were nearly identical, as were those of strains pp. goat and 14. Slight, but consistent differences, especially at the upper part of the gels, were found between the PG3 and the pp. goat patterns (Fig. 4). The B3 strain, originally isolated from swine by Dinter, Danielsson, and Bakos (5), exhibited an electrophoretic pattern practically identical with that of the caprine PG3 strain (Fig. 4). The B3 strain resembled the caprine strains also in its heavy growth and colonial morphology. The electrophoretic patterns of the goat mastitis strain *M. agalactiae* PG2 and the bovine mastitis strain *M. agalactiae* var. *bovis* (Donetta) differed only in several bands (Fig. 5).

Rat and murine strains. The three *M. pulmonis* strains included in this study possessed nearly identical electrophoretic patterns (Fig. 6). The two *M. neurolyticum* strains showed a somewhat different pattern. The main difference was a heavy band in strain PG28 observed only faintly in strain PG39 (Fig. 7).

Avian strains. The electrophoretic pattern of *M.*

TABLE 2. Growth inhibition of *Mycoplasma hominis* strains by antisera^a

Strain	Growth inhibition zone (mm)		
	Antiserum 1	Antiserum 2	Antiserum 3
ATCC 15056	4 ^b	4 ^b	3 ^b
PG21	8	5	7
PG23	0	1	3
PG25	0	3	0
PG26	0	0	0

^a Antiserum 1, prepared in our laboratory by Hava Haas against *M. hominis* PG21. Antiserum 2, purchased from Robbin Laboratories, Inc., Chapel Hill, N.C., prepared against *M. hominis* type 1 (strain designation not given). Antiserum 3, a gift of Microbiological Associates, Bethesda, Md., prepared against *M. hominis* PG21.

^b Partial inhibition, minute colonies present all over the inhibition zone.

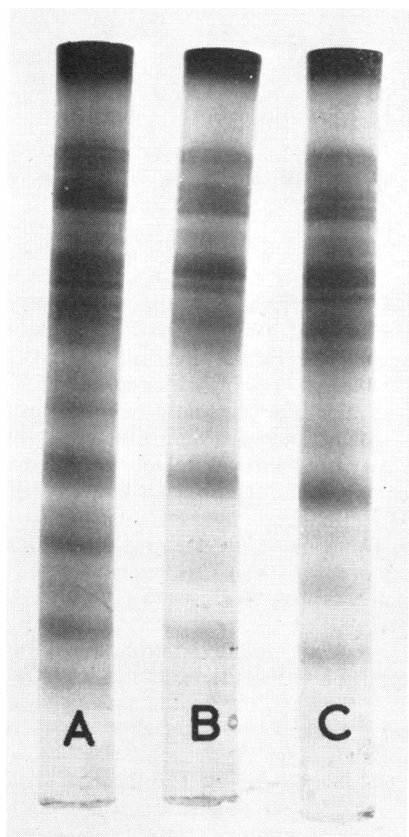


FIG. 3. Electrophoretic patterns of cell proteins of *M. mycoides* var. *mycoides* strains. (A) PG1; (B) PG46; (C) V5.

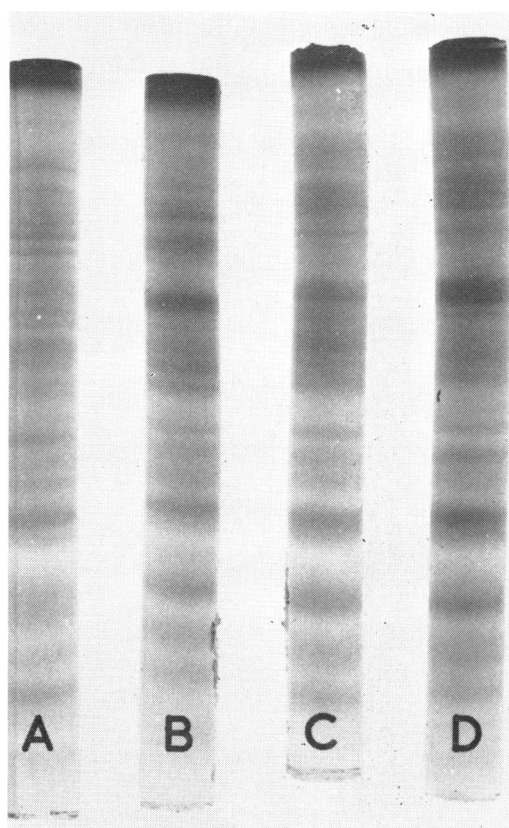


FIG. 4. Electrophoretic patterns of cell proteins of caprine strains. (A) *Mycoplasma* sp. strain 14; (B) *Mycoplasma* sp. strain Y; (C) *M. mycoides* var. *capri* PG3; (D) *Mycoplasma* sp. strain B3 (isolated from swine).

gallinarum strain J differed from that of the representative strain of this species, strain PG16 (Fig. 8). The electrophoretic patterns of the three *M. gallisepticum* strains, A5969, S6, and PG31, were identical. The patterns of *M. meleagridis*, *M. synoviae*, and *M. iners* differed markedly from each other.

DISCUSSION

The serological heterogeneity of the *Mycoplasma* strains included in the species *M. hominis* type 1 was first observed by Nicol and Edward (22) and recently confirmed by the sensitive metabolic inhibition test (23). Nucleic acid homology analysis also revealed the genetic heterogeneity of the strains of this species (25, 26, 29). The marked differences in the electrophoretic patterns of the *M. hominis* strains included in our study correspond well with these findings. This phenom-

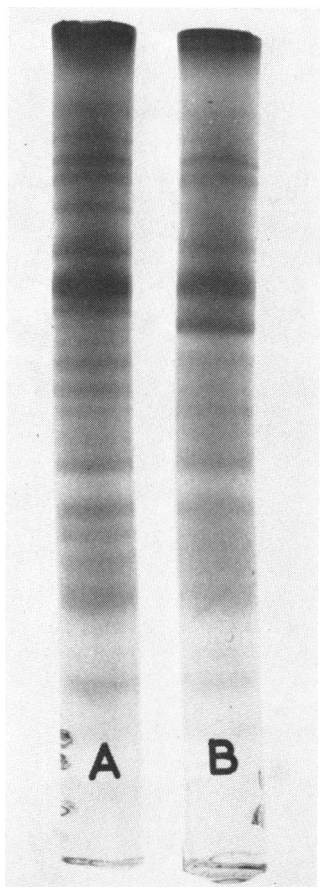


FIG. 5. Electrophoretic patterns of cell proteins of mastitis strains. (A) *M. agalactiae* var. *bovis* (Donetta); (B) *M. agalactiae* PG2.

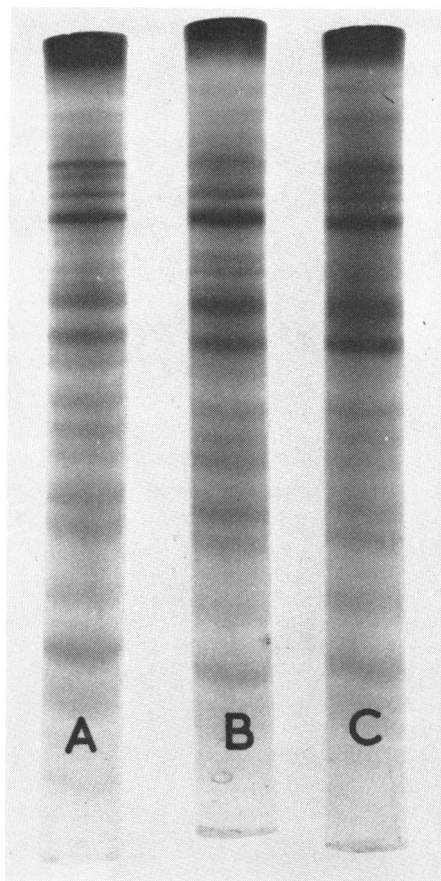


FIG. 6. Electrophoretic patterns of cell proteins of *M. pulmonis* strains. (A) PG53 (Negroni); (B) PG22; (C) PG34.

enon gives rise to the question of whether it is justified to include the heterogeneous *M. hominis* strains in one species. It is difficult to answer this question because the concept of a species in bacterial taxonomy is vague and is based on relative (subjective) rather than on absolute (objective) criteria. Electrophoretic analysis of the *Enterobacteriaceae* cell proteins showed that the patterns of the different genera of this family were more similar than the patterns of the strains included in the single *M. hominis* species (Haas, Sacks, and Razin, *in preparation*). Furthermore, the electrophoretic patterns of strains belonging to different genera, such as *Escherichia* and *Shigella*, were nearly identical, corresponding with the close relationship found between the deoxyribonucleic acids of these species (19, 28). McGee, Rogul, and Wittler (20) stress that there is less genetic homology among some *Mycoplasma* species than

among various genera of bacteria. Hence, in comparing the present classifications of *Mycoplasma* and *Enterobacteriaceae*, we are more than justified in dividing the single *Mycoplasma* genus into several genera or into higher taxa. However, since the classification of the *Enterobacteriaceae* is artificial and seems to be genetically ill-founded (14), this may not be a good example on which to base further classifications. It seems worthwhile to consider the separation of the *M. hominis* strains into more than one species. Our results indicate that strain PG26 is the first candidate for exclusion because of its different electrophoretic pattern and resistance to growth inhibition by antisera against the representative strain PG21.

The three *M. mycoides* var. *mycoides* strains included in our study showed identical electrophoretic patterns. This corresponded with the

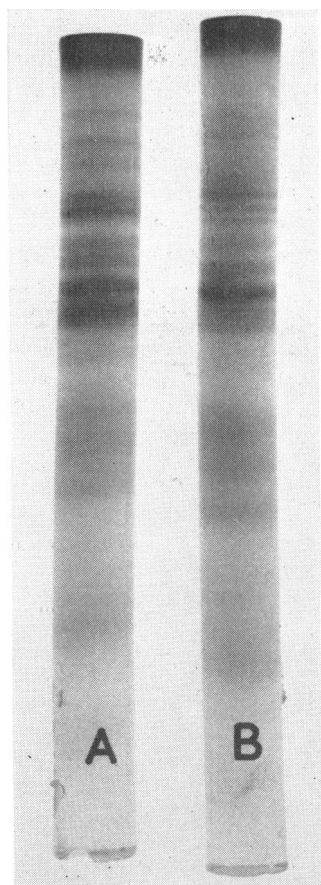


FIG. 7. Electrophoretic patterns of cell proteins of *M. neurolyticum* strains. (A) PG28; (B) PG39 (Sabin A).

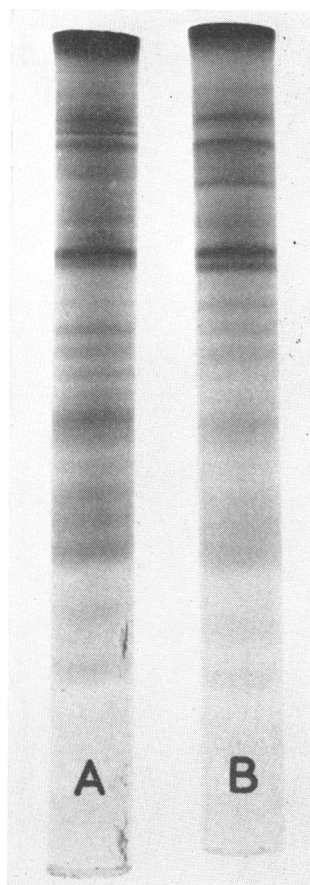


FIG. 8. Electrophoretic patterns of cell proteins of *M. gallinarum* strains. (A) strain J; (B) PG16.

finding of Leach (15) that the African strain PG46(T3) is serologically indistinguishable from the representative strain PG1 and with our findings (Argaman and Razin, *to be published*) that showed the close serological relationship of the Australian strain V5 with strain PG1. Our previous results showing the V5 to differ from PG1 (obtained by comparison of the electrophoretic patterns of membrane proteins (27)) were proven wrong. The PG1 culture used in the previous study was found to be mixed with another *Mycoplasma*. This points to the advantage that the electrophoretic technique has over serological methods for determining the purity of a microbial culture. Serological techniques, apart from the direct fluorescent-antibody technique on colonies (4), usually fail to indicate whether a culture is mixed or not, but the electrophoretic technique easily detects it, since the electrophoretic pattern of a mixed culture is

different. The electrophoretic technique is, therefore, a most valuable tool for checking the purity of clones. Identical electrophoretic patterns were shown by four *M. hominis* DC-63 clones sent to us by R. H. Purcell, which proved that they were identical and pure. Indeed, he demonstrated that they were serologically indistinguishable.

The classification of some of the caprine mycoplasmas is still uncertain. Several reports claim that the contagious caprine pleuropneumonia organism *M. mycoides* var. *capri* has no serological relationship with *M. mycoides* var. *mycoides*. It has, therefore, been suggested that this variety be regarded as a separate species, *M. caprae* (32), or *M. capri* (6, 11). The present electrophoretic results show that the four caprine strains, PG3, pp. goat, Y, and strain 14, form one group, closely related to *M. mycoides* var. *mycoides*. These results also agree with the serological findings based on agglutination and gel-diffusion tests showing common antigens to all

the tested bovine and caprine strains (Argaman and Razin, *to be published*). Lemcke (17), in agar-gel diffusion tests, also noted several antigens common to *M. mycoides* var. *mycoides* (PG1) and *M. mycoides* var. *capri* (pp. goat). The deoxyribonucleic acid base composition of these *Mycoplasma* and several other caprine strains has also been found to be very similar (13, 21), indicating the close taxonomic relationship of these strains. Nevertheless, the caprine strains are not identical, and strain PG3 differs somewhat from pp. goat in its electrophoretic pattern. The tentative identification of the B3 strain isolated from swine (5) with *M. mycoides* var. *capri* is of interest. Thus far no serological identification of B3 could be made (D. Taylor-Robinson and Z. Dinter, Abstr. Meeting Soc. Gen. Microbiol., 1968). Our tentative identification will be ultimately proven or disproven by a serological comparison of the B3 strain with PG3. Thus, the isolation of a goat *Mycoplasma* from swine is not surprising; Cordy and Adler (2) showed that the K strain of goat arthritis was also pathogenic to swine.

Another taxonomic problem involves the two mastitis strains, *M. agalactiae* that causes mastitis in goats and sheep and *M. agalactiae* var. *bovis* (Donetta) that produces mastitis in cattle. Leach (15) agrees that the tentative name *M. agalactiae* var. *bovis*, suggested by Hale et al. (9), may be justified in view of some biological properties common to the two strains. However, Jain, Jasper, and Dellinger (12) claimed that, since a close relationship between the two strains has not been demonstrated, the Donetta strain should be established as a separate species, *Mycoplasma bovimmastitis*. Comparison of the electrophoretic patterns of the two mastitis strains favors their inclusion in one species.

Serological variations among *M. pulmonis* strains have been frequently encountered (3, 7, 16), and may account for the slight, but consistent, differences in the electrophoretic patterns of the three *M. pulmonis* strains included in the present study. Nevertheless, comparison of the patterns leaves little doubt that they all belong to the same species. The same applies to the two *M. neurolyticum* strains. The electrophoretic patterns of these strains differ in several bands but essentially show the same basic pattern. The two strains differ in toxigenicity; the PG39 (Sab in A) strain produces a potent neurotoxin, whereas PG28 does not (31). Theoretically, the production of the protein neurotoxin (30) should result in an additional band, but even in the best separation obtainable in polyacrylamide gels, some cell proteins undoubtedly migrate together as one band. Hence, small variations in the pro-

tein composition of the cells, which might be easily detected serologically, enzymatically, or in pathogenicity tests, might not be revealed by the electrophoretic pattern. This limitation of the electrophoretic method may be regarded as an advantage in species identification because, being less sensitive, it prevents the formation of highly complex patterns that are difficult to compare and which might accentuate minor genetic differences between strains.

The avian *Mycoplasma* species included in this study showed easily distinguishable electrophoretic patterns. The patterns of the two *M. gallinarum* strains PG16 and J differed to a considerable extent. This finding alone, however, cannot justify the exclusion of the J strain from *M. gallinarum*. Since the concept of the bacterial species is not defined by constant characteristics, the electrophoretic findings should be combined with other biochemical, serological, and nucleic acid analyses before such a step can be taken.

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ADDENDUM IN PROOF

The electrophoretic pattern of the cell proteins of *Mycoplasma histotropicum* strain PG 40 (obtained from D. G. ff. Edward) was found to resemble very much the patterns of the *M. pulmonis* strains. This finding supports the suggestion of J. G. Tully (*personal communication*) that *M. histotropicum* should be reclassified as *M. pulmonis*.

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