APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1976, p. 603-609 Vol. 32, No. 4
 Printed in U.S.A.

# Isoelectric Focusing of Mycoplasma Proteins

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Received for publication 25 February 1976

Polyacrylamide gel isoelectric focusing (PAGIF) in thin layer was used to resolve proteins of *Mycoplasma* spp., *Acholeplasma* spp., and eight strains of *Ureaplasma urealyticum* (T-strain). A mixture of urea, Triton X-100, and dithioerythritol was used to solubilize sonically disrupted cells. PAGIF was performed in the range of pH 3 to 10. Protein patterns were carefully compared, demonstrating resolved and distinguishable species-specific protein bands. The eight serotypes of *U. urealyticum* (T-strain) gave identical protein patterns in the pH 3 to 10 range. The characteristic "fingerprints" of a species appeared to correlate with the biochemical nature and not the habitat in each case. Arginine-hydrolyzing species seemed to show more diverse focusing than those that ferment glucose, or prefer an acid environment. Characterization and identification of highly resolved species-specific proteins, ease of performance, and reproducibility of this method suggest that PAGIF might be employed as a taxonomic aid.

Although mycoplasmas possess many distinctive characteristics (8), they are more difficult than most other bacteria to classify by means of customary physiological-biochemical criteria, in part because of their highly complex nutritional requirements. Electrophoresis has been extensively used for speciating these microorganisms (1, 3, 6, 7, 9-11, 14-17, 19, 20, 22, 24, 25). The first such attempt, with a starch gel system, revealed a considerable number of patterns in the soluble fractions of mycoplasma proteins (10). The use of polyacrylamide, as a disc gel (1, 3, 6, 7, 9, 11, 14-17, 19, 20, 22), or direct comparison, as a flat gel (24, 25), requires solubilization of the cells, either in a phenol-acetic acid-water mixture (14) or in sodium dodecyl sulfate (6). It has been difficult to distinguish species-strain-specific antigens in such preparations (9). Polyacrylamide gel isoelectric focusing (PAGIF) enables high-resolution separation of proteins as first demonstrated by Awdeh et al. (2), Leaback and Rutter (13), and Dale and Latner (5) with immunoglobulins, hemoglobins, and serum proteins, respectively. Briefly, separation is based on differences in the pK values of proteins. A fine pH gradient, achieved through "carrier" ampholytes, permits protein molecules to be focused at their specific pI. Proteins whose pK values differ by as little as 0.02 units can thus be differentiated (18).

This report introduces the use of thin-layer PAGIF as a means of obtaining highly resolved "fingerprints" of mycoplasma proteins.

## MATERIALS AND METHODS

**Growth and maintenance of mycoplasmas.** Various species of mycoplasmas used for PAGIF analysis are listed in Table 1. Stock cultures were maintained in Hayflick medium (12) by transferring 10% (vol/vol) of a growing culture to fresh medium at weekly intervals. Organisms were stored at −70°C in a So-Low refrigerator (So-Low Environmental Equipment Co., Cincinnati, Ohio).

*Acholeplasma* spp. and *Mycoplasma* spp. were grown in 1-liter Erlenmeyer flasks containing 500 ml of Hayflick broth without inhibitors (12). Final concentrations of the constituents were 2.1% (wt/vol) PPLO broth (Difco), 20% (vol/vol) horse serum (Flow Laboratories, Rockville, Md.), and 10% (vol/vol) of a 25% (wt/vol) freshly prepared yeast extract solution (Standard Brands Inc., New York). The final pH of this medium was 7.6.

*Ureaplasma urealyticum* (T-strain) serotypes I to VIII (21) were cultivated similarly in modified Shepard A3B fluid medium (personal communication), which contained 30% (wt/vol) Trypticase soy broth (pH adjusted to 5.5), 20% (vol/vol) normal horse serum (Flow Laboratories, Rockville, Md.), 0.02% urea (wt/vol), 0.01% (wt/vol) L-cysteine-hydrochloride, and 5% (vol/vol) of freshly prepared 25% (wt/vol) Fleishmann's yeast extract (pH 6.0). The final pH of the medium was approximately 6.5.

All strains were serially transferred five times before preparation of the final culture. Mycoplasmas were checked for purity periodically by means of growth inhibition tests (4) utilizing homologous antiserum inbibed in a paper disk which, upon incubation at 37°C, provided a zone of inhibition only with a specific strain under investigation. All the cultures were incubated at 37°C under aerobic conditions.

TABLE 1. *Various mycoplasmas used for PAGIF*

| Genus and species | Culture collection no. | Source | Habitat |
|---|---|---|---|
| *Acholeplasma axanthum* | 25176 | ATCC[a] | Murine leukemic tissue cultures |
| *A. oculusi* | 27350 | ATCC | Eyes of goat |
| *A. granularum* | BTS-39 | NIH[b] | Swine nasal cavities |
| *A. laidlawii* | PG-9 | NIH | Sewage, soil, animals |
| *Mycoplasma anatis* | 1340 | NIH | Duck sinus, air sacs |
| *M. pulmonis* | Ash | NIH | Mouse respiratory tract |
| *M. salivarium* | PG-20 | NIH | Human oropharynx |
| *M. maculosum* | PG-15 | NIH | Dog urogenital tract |
| *M. arginini* | G-230 | NIH | Wide mammalian host range |
| *M. arthritidis* | PG-6 | NIH | Rat leg joints |
| *M. meleagridis* | 17529 | NIH | Turkey respiratory tract |
| *M. iners* | PG-30 | NIH | Chicken respiratory tract |
| *M. fermentans* | PG-18 | NIH | Human genital tract |
| *M. hominis* | 14027 | ATCC | Human lower urogenital tract |
| *M. orale* | Type 1 | NIH | Human oropharynx |
| *Ureaplasma urealyticum* (T-strain) | Types I-VIII | M. Shepard[c] | Human urogenital tract |

[a] ATCC, American Type Culture Collection, Rockville, Md.
[b] NIH, National Institutes of Health, Bethesda, Md.
[c] Maurice C. Shepard, Chief, Microbiology Division, Camp Lejeune, N.C.

**Preparation of mycoplasma extracts.** Cells were harvested from 24-h cultures by centrifugation for 1.5 h in a refrigerated (4°C) centrifuge (Sorvall RC2-B) at 10,000 rpm (16,300 × *g*), washed seven times in Dulbecco phosphate-buffered saline (without $Mg^{2+}$ or $Ca^{2+}$), and sonicated for a total of 5 min at 30-s intervals in a 4°C water bath employing a Sonifier (Branson model W 185 D) at 50 W. Equal volumes of the sonicates were mixed with a "solubilizing mixture" containing 12 M urea (denaturant), 4% Triton X-100 (vol/vol) (nonionic detergent), and 0.2% (wt/vol) dithioerythritol (reducing agent). The preparation was concentrated, if necessary, by dialyzing against polyvinyl pyrrolidione K-90 (Matheson-Cole & Bell, Norwood, Ohio) to obtain concentrations of approximately 5.0 mg/ml. Insoluble debris was removed by centrifugation at a low speed for 10 min, and the supernatant fluid was stored at 4°C until used, but not longer than a month.

**Preparation of thin-layer polyacrylamide gels.** The basic procedure was essentially that described by Vesterberg (23); however, modifications were deemed necessary due to the nature of proteins under investigation. All stock solutions were prepared in distilled water and clarified by filtration through a membrane filter (0.50-μm pore size, Millipore Corp.). To each glass plate (125 by 260 by 1 mm) was added: 10.0 ml of 30.5% acrylamide, 10.0 ml of 1% *N*, *N'*-methylene bisacrylamide, 30.0 ml of 10 M urea, 0.2 ml of 5% dithioerythritol, and 4.5 ml of ampholyte solution. The different ampholytes (Ampholine, LKB Bromma, Chicago, Ill.) were mixed to prepare a pH 3.0 to 10.0 stock as follows: 15 ml of ampholine of pH 3 to 10, 2 ml of pH 2.5 to 4, 1 ml of pH 4 to 6, 1 ml of pH 5 to 7, 1 ml pH 8 to 9.5, and 2 ml of pH 9 to 11. The mixture for the gel was placed in vacuo (to remove entrapped air bubbles) for 10 min, and finally 3 ml of 10% Triton X-100 and 3.5 ml of 0.004% riboflavin were added. The preparation was stored in the dark until it could be poured with the help of a syringe and a needle to cast the gel.

Photopolymerization was carried out for 1 h with Polylite 2114 (LKB Instruments Inc., Rockville, Md.). After removal of the top plate by careful injection of air with two flat spatulas, the gel plates were wrapped in thin plastic sheets (e.g., Saran Wrap) and stored at 4°C in the dark until used.

**Sample application and isoelectric focusing.** Samples were carefully and evenly soaked in Whatman no. 3 filter paper (6 by 12 mm) and applied near the anode, directly on the surface of the gel.

The electrolyte solutions for pH ranges between 3 and 10 consisted of 1 M phosphoric acid (anode solution) and 1 M sodium hydroxide (cathode solution). They were applied with the help of paper electrofocusing strips (LKB Instruments Inc., Rockville, Md.).

Electrofocusing was carried out for approximately 2 h with a power supply (model 3371) in a Multiphor 2117 (both from LKB Instruments Inc., Rockville, Md.). The initial current was 45 mA, and the final current was 25 mA. A starting voltage of 200 V was increased slowly to 1,000 V to maintain the decreasing amperage.

**Staining and destaining.** Focused gels were stained directly, without prefixing in trichloroacetic acid, with a solution of 0.2% (wt/vol) Coomasie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, Calif.) in 9 parts ethyl alcohol to 9 parts water to 1 part glacial acetic acid solution for 30 min at room temperature. Destaining was carried out with ethyl alcohol-water-glacial acetic acid (3:8:1), until the background became clear. Several quick changes of detaining solution were required to hasten the process.

For preservation, stained gels were immersed in a 10% aqueous solution of glycerol for 1 to 2 h, and swollen edges were trimmed if necessary; they were

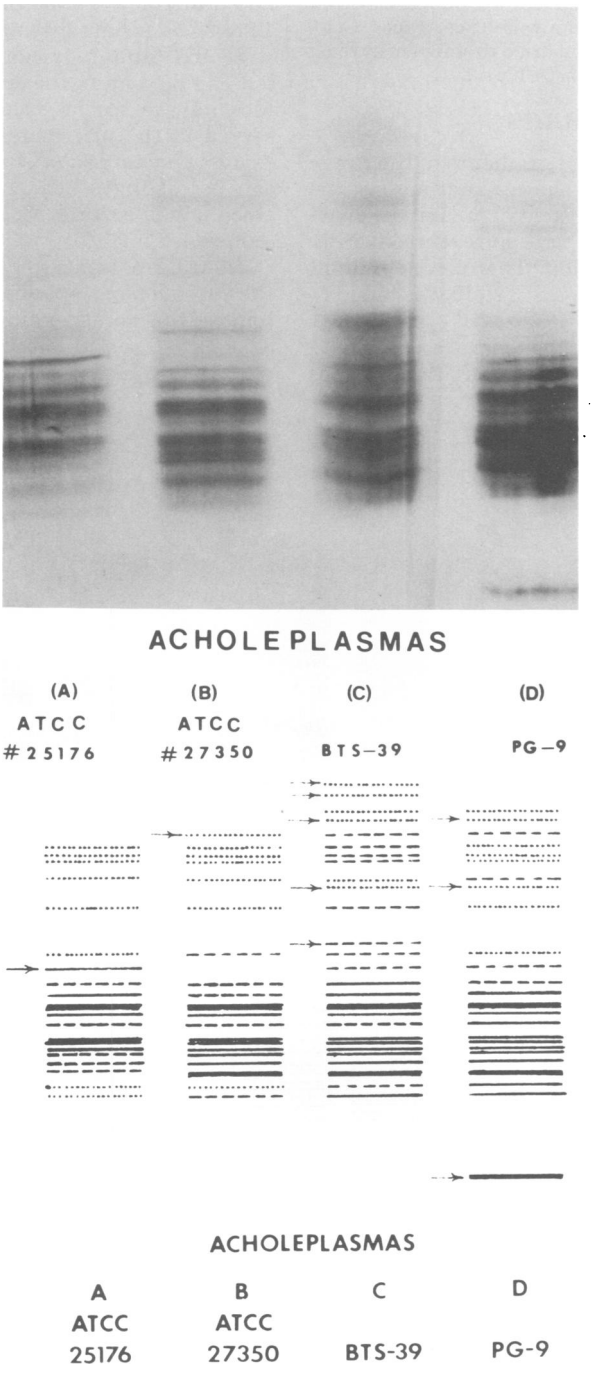


FIG. 1. *PAGIF patterns of the four species of genus Acholeplasma in a pH gradient of 3 to 10. Upper end of the picture corresponds to pH 10, the lower approximately pH 3. Nomenclature is presented in Table 1. Arrows in the diagrammatic representation of patterns shown in the photograph indicate species-specific bands or those shared with related strains.*

covered, in a manner to avoid air bubbles, with a dialysis membrane 120 mm wide (Spectrapor; Cole-Parmer, Chicago, Ill.), and dried first for 8 h at room temperature, and then for 24 h at 27°C.

## RESULTS

Preliminary studies of gradients in the range of pH 2 to 10 or 3 to 10 showed that most of the proteins focused were acidic in nature; however, some distinctive antigens were also focused in the basic region. Therefore, throughout the study a pH range of 3 to 10 was found to be satisfactory.

Results with the various species of *Acholeplasma* seen in Fig. 1 show that each species had a distinctive protein pattern. As many as 19 to 28 bands are distinguishable (some bands were too faint to visualize photographically, but can be seen in the graphic representation). Most of the species-specific bands can be observed in the pH range 3.0 to 6.0, as seen in *Acholeplasma laidlawii* in particular, which can be distinguished readily from the others since it has several proteins with different pI values.

Figure 2 presents the complete spectrum of the *Mycoplasma* species examined. Most proteins of this genus exhibited pI values between 3.0 and 7.0, as illustrated by certain species, e.g., *Mycoplasma arginini*, *Mycoplasma ar-*

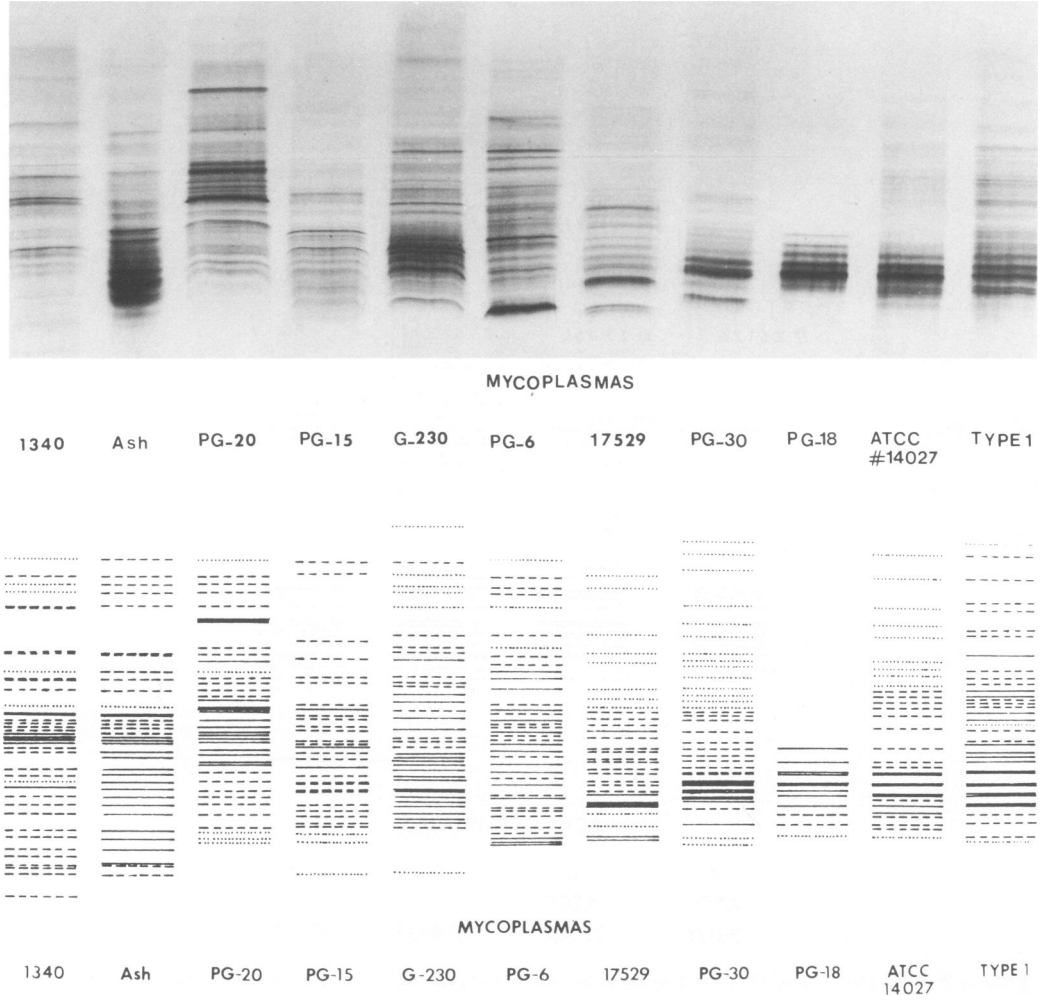


FIG. 2. *Polyacrylamide gel isoelectric focusing of various species of Mycoplasma in a pH range of 3 to 10. Upper end of the picture represents pH 10 and the lower approximately pH 3. Nomenclature is presented in Table 1. The diagrammatic representation illustrates the multiplicity of bands and emphasizes the diversity observed among the various Mycoplasma spp.*

*thritidis*, and *Mycoplasma salivarium*. However, each *Mycoplasma* species, like the *Acholeplasma* species, revealed a completely unique pattern.

Thirty-two distinct lines can be seen in preparations from *U. urealyticum* (T-strain) in Fig. 3. All eight serotypes were found to have similar protein patterns. These results agree with the findings of Razin et al. (16), who employed classical disc gel electrophoresis.

The PAGIF patterns of the mycoplasma proteins appear to be independent of the species habitat, but biochemical differences between the various species may be reflected in their constituent proteins. It is perhaps significant that those species of *Acholeplasma*, *Mycoplasma*, and *Ureaplasma* (T-strain) that have the ability to ferment glucose, e.g., *Acholeplasma* spp., *Mycoplasma pulmonis*, and *Mycoplasma fermentans*, were found to possess an

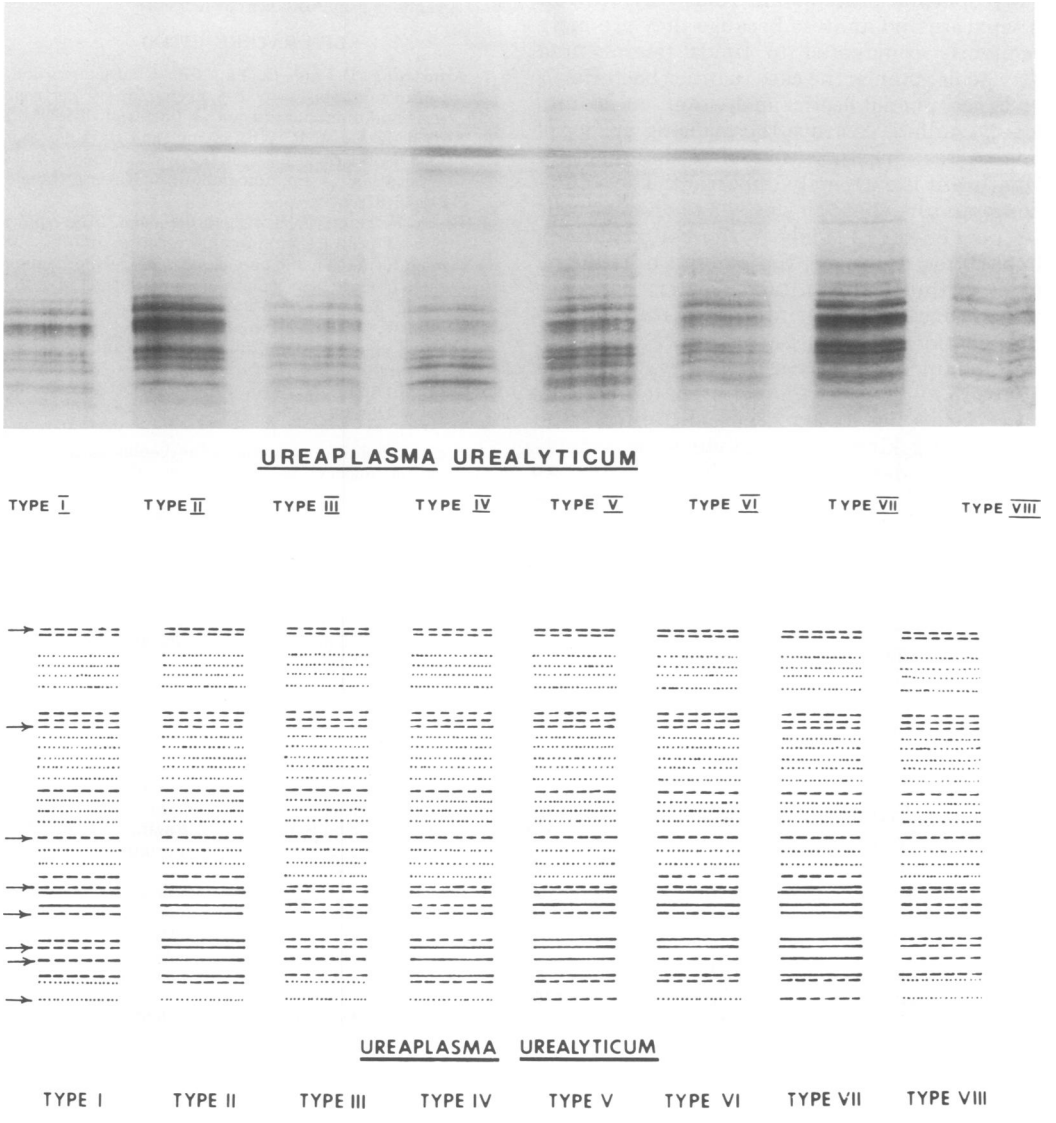


FIG. 3. *Isoelectric focusing of eight serotypes of U. urealyticum (T-strain) in thin-layer polyacrylamide gel, pH range 3 to 10. Upper end of the photograph corresponds to pH 10; the lower end corresponds to approximately pH 3. Arrows in the diagrammatic representation of patterns shown in the photograph indicate species-specific bands or those shared with related strains.*

abundance of acidic proteins (focusing below pH 7). Similarly, all the strains of *U. urealyticum*, which prefer an acidic environment for their growth, contained most of the proteins shown to be acidic in nature, whereas arginine-hydrolyzing species, e.g., *M. arginini*, *M. arthritidis*, *M. orale*, and *M. salivarium*, had diverse proteins focusing over a broad pH range.

## DISCUSSION

Mycoplasma proteins are relatively difficult to separate and analyze because they are more frequently conjugated to lipids (sterols and fatty acids) than is the case in other bacteria (7, 9). Since phenol-acetic acid-water or sodium dodecyl sulfate destroys the focusing ability of ampholytes, incorporation of a detergent or denaturant is extremely important. The solubilizing mixture that was devised to dissolve cells can also be added safely to the gel containing ampholytes. However, the amount of reducing agent (dithioerythritol) added is critical since higher concentrations interfere with photopolymerization. If a reducing agent is not present, the amount of riboflavin can be reduced from 3.0 to 1.5 ml/60 ml of the gel preparation.

In achieving good reproducibility, the amount and kind of ampholyte is important. Because the density gradient is a limiting factor, slight changes in combination of ampholytes appreciably alter the specific patterns. However, if the conditions are kept constant, one can obtain two electrofocused plates with identical patterns.

For best results it is also very important to analyze preparations of cells grown under identical conditions (such as same media, same age, same incubation conditions). The work with polyacrylamide gel separation has shown the importance of the medium and age of the cells in obtaining reproducible results (1, 19, 24).

Although PAGIF (pH 3 to 10) failed to reveal strain-specific antigens in *U. urealyticum* (T-strain), a number of species-specific proteins were encountered in the various *Mycoplasma* spp. and *Acholeplasma* spp. tested by this method.

In certain cases, when all the proteins were acidic in nature, the separation could be made in a narrow pH range to identify strain-specific proteins.

In conclusion, PAGIF is better able to resolve mycoplasma proteins than polyacrylamide gel electrophoresis systems employed earlier because it permits one to immobilize the individual proteins present in complex mixtures at their distinctive isoelectric points. The method is highly reproducible, simple, and precise and should provide an important tool for the characterization, identification, and speciation of the mycoplasma group of bacteria. Further studies on *Spiroplasma* spp. are under investigation in this laboratory.

## ACKNOWLEDGMENTS

We would like to thank Karl Johansson for his critical readings of the manuscripts and Gerard R. Vela for his helpful discussions and comments. Thanks are due also to Maurice Shepard for his generosity in providing strains of *U. urealyticum*. The help of Kenneth Foreman in taking photographs is gratefully acknowledged.

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