Polyacrylamide Gel Identification of Bacterial L-Forms and *Mycoplasma* Species of Human Origin

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Polyacrylamide gel electrophoretic patterns of acidified phenol extracts prepared from whole cells can be used for the identification of bacterial L-forms and *Mycoplasma* species of human origin. Ten human *Mycoplasma* serotypes and eight L-forms belonging to five different genera were studied. The gel patterns were sufficiently distinct and reproducible that it was possible not only to identify L-forms at the genus level (group with streptococci) and different *Mycoplasma* serotypes but also to differentiate between the two of them. The parentage of L-forms of *Streptobacillus moniliformis* L1, *Listeria monocytogenes*, *Streptococcus MG*, and *Staphylococcus aureus* Smith strain was established by relating their gel patterns directly to parent bacteria. It was found that an L-form designated *S. moniliformis* An (ATCC 14220) was actually an L-form of *Proteus*. In addition, it was shown electrophoretically that no relationship existed between the *Streptococcus MG* L-form and *M. pneumoniae*. The applicability of this method as a diagnostic and taxonomic tool for the differentiation of L-forms and mycoplasmas is discussed.

In addition to the established role of *Mycoplasma pneumoniae* in the etiology of primary atypical pneumonia, there is increasing evidence that other mycoplasmas are capable of inducing disease in man (4, 21). The role that stable L-phase bacterial variants (L-forms) play in disease is still of some dispute, but the recovery of these microbial forms from human would seem to necessitate continued efforts to establish their role in certain infectious states (7, 22). Although some distinctions can be made between mycoplasmas and bacterial L-forms (2), there is considerable similarity in their morphology and biochemical properties. These similarities and the fact that both of these agents can be recovered from the same tissue sites in man indicate that their differentiation still poses a problem for the clinical laboratory.

Recently, electrophoretic analysis of the extracted membranes and cellular proteins of whole cells on cationic polyacrylamide gels has been used successfully for the identification of various *Mycoplasma* serotypes (12, 15, 18, 19, 20) and selected L-forms (5, 13, 17, 18). Stable L-phase variants could be identified to the genus level by comparing the electrophoretic pattern of the unknown strain to the patterns obtained on a number of known L-forms or, more specifically, to the parent bacterial species. The purpose of this report is to show that electrophoretic analysis can be of diagnostic and taxonomic value in the identification and differentiation of L-forms and mycoplasmas of human origin.

**MATERIALS AND METHODS**

*Organisms.* *Mycoplasma* strains were *M. orale* type 1, CH 19299; *M. orale* type 2, CH 20247; *M. orale* type 3, DC 333; *M. fermentans* PG-18; *M. hominis* PG-21; *M. salivarium* PG-20; *M. lipophilum* MABy; *Mycoplasma* species Navel; *M. pneumoniae* FH; and *Acholeplasma laidlawii* A, PG-8 (1).

Bacterial strains were *Streptococcus faecium* group D, F-24 L-form (ATCC 19635); *S. pyogenes* group A, ADA L-form; *Streptococcus MG* parent and L-form (S. Madoff, Massachusetts General Hospital, Boston); *Staphylococcus aureus* L-form (ATCC 6538P); *S. aureus* Smith parent (ATCC 19636) and L-form (ATCC 19640); *Streptobacillus moniliformis* (ATCC 14647), L-form (LI Rat 30; ATCC 14075) and L-form (An; ATCC 14220); *Proteus mirabilis* L-form (L9; ATCC 14168); *Proteus* 52, B-type L-form; and *Listeria monocytogenes* parent (W. C. Eveland, University of Michigan, Ann Arbor) and L-form.

*Cultural conditions.* All mycoplasmas except *M. pneumoniae* were grown in Mycoplasma broth base
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FIG. 1. Gel patterns of Mycoplasma and Acholeplasma species. (A) M. orale type 1, (B) M. orale type 2, (C) M. orale type 3, (D) M. fermentans, (E) M. hominis, (F) M. salivarium, (G) M. lipophilum, (H) Mycoplasma species Navel, (I) A. laidlawii A, and (J) M. pneumoniae.

(BBL) containing 10% fresh yeast extract (Microbiological Associates), 1% serum fraction (Difco), and penicillin (500 units/ml). M. pneumoniae was grown in broth base containing 10% fresh yeast extract, 20% tissue culture select horse serum (BBL), thallium acetate (30 μg/ml), amphotericin (30 μg/ml), and penicillin (500 μg/ml).

S. moniliformis parent and L-forms were grown in
M. orale 1
M. orale 2
M. orale 3
M. fermentans
M. hominis
M. salivarium
M. lipophilum
M. species, navel
A. laidlawii A
M. pneumoniae
S. faecium, F-24L
S. pyogenes, ADAL
Streptococcus MgL
S. aureus, ATCC 6538 P,L
S. moniliformis, L1
Proteus 9L
Proteus 52L
L. monocytogenes L

Fig. 3. Densitometric tracings of gel patterns of (A) Mycoplasma strains shown in Fig. 1 and (B) L-forms shown in Fig. 2.

Broth base containing 10% fresh yeast extract and 1% serum fraction. *Streptococcus* MG parent and L-form were grown in the broth base supplemented with 10% sucrose and 10% horse serum. Penicillin was added to the *Streptococcus* MG L culture at a final concentration of 1,000 units/ml. *L. monocytogenes* parent and L-form were grown in the broth base supplemented with 3% NaCl, 0.5% glucose, and 10% horse serum. (Penicillin at 1,000 units/ml was added to the L-form culture medium.) *S. aureus* Smith parent and L-form were grown in Heart Infusion broth (Difco) containing 3% NaCl and 10% horse serum. Penicillin, (1,000 units/ml) was added to the L-culture. All other L-forms were grown in broth base containing 3% NaCl, 0.5% glucose, and 10% horse serum.

Cultures were grown at 37 °C for 3 to 5 days, depending on the amount of growth, harvested by centrifugation, and washed twice with 0.85% NaCl in 0.067 M phosphate buffer, pH 7.5. *M. salivarium*, *M. lipophilum*, and the three *M. orale* serotypes were grown in an atmosphere of 95% nitrogen and 5% CO₂. The pelleted material was resuspended in distilled water and stored at −10°C. Protein was estimated by the method of Lowry et al. (6).

**Electrophoretic analysis.** Extraction procedures, gel formulations and preparations, and staining and destaining procedures have been described previously (18). A 0.1-ml amount of the acidified phenol extract in sucrose, containing approximately 250 μg of protein, was subjected to electrophoresis for 75 min at a constant current of 4 mA/tube. Densitometric tracings of the gels were done on a Photovolt Densicord 542A electrophoresis densitometer with an electronic integrator attachment. The tracings of the gel patterns were converted to bar graph representations. The height, width, and distance between bars are directly proportional to the intensity, width, and distance between the bands in the gel.

**RESULTS AND DISCUSSION**

All microorganisms except *S. aureus* (Smith parent) and its L-form were grown in Mycoplasma broth basal medium and provided with supplements according to their specific growth requirements. Proteins present in various media formulations have not been shown to interfere with cell protein electrophoretic patterns (12, 14, 17). Figure 1 shows the gel patterns of nine serologically distinct human *Mycoplasma* strains and one strain of *A. laidlawii* A (PG-8). The latter serotype was included because it has been isolated occasionally from humans (8, 11). Figure 2 shows the gel patterns of eight L-forms belonging to five different genera. Each *Mycoplasma* and L-form is identifiable by its own distinct and characteristic gel pattern which is highly reproducible. For comparison, the densitometric tracings of the gels in Fig. 1 and 2 are shown as bar graph representations in Fig. 3. From these data, it is possible to determine, by the use of standard reference strains, whether a culture is one of the known stable L-phase strains or a *Mycoplasma* of human origin. Although no one particular band or general pattern can be used to distinguish between these two classes of microorganisms, each strain examined is sufficiently unique, electrophoretically, that it can be identified either as an L-form at the genus level (group level with the streptococci) or as a *Mycoplasma* species. These studies confirm and
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Fig. 4. Gel patterns of (A) Streptobacillus moniliformis parent, (B) S. moniliformis L1, (C) Listeria monocytogenes parent, (D) L. monocytogenes L, (E) Streptococcus MG parent, (F) Streptococcus MG L, and (G) Mycoplasma pneumoniae.

extend the findings of Haas et al. (3), who, with a different gel system, showed that electrophoretic patterns of whole cells could be used to identify six of the known human Mycoplasma serotypes from numerous clinical isolates. We have extended the application of this method not only to the differentiation of L-forms and mycoplasmas of human origin but to a wide variety of mycoplasmas obtained from animals (18). The electrophoretic technique is, therefore, a valuable tool not only for practical diagnostic problems but in taxonomy questions (10, 19, 20). In the over 60 mycoplasmas examined in this laboratory, there has been excellent correlation between the serological and biochemical properties of the individual serotypes and the distinct characteristics of their electrophoretic patterns.

At present, gel electrophoresis is the only practical method for the identification of bacterial L-forms. However, one major limitation in the usefulness of this method is the admittedly small number of known L-forms tested to date. This is principally due to difficulties in adapting some L-forms to growth in liquid media. As new L-forms are successfully grown on artificial media, their gel patterns can be compared to those of other bacterial strains to provide an expanded list of reference strains. Figures 4 and 5 show the gel...
patterns and tracings, respectively, of three new bacterial parent L-form combinations not previously reported. *S. moniliformis* parent (ATCC 14647) and the L-form designated as L1 (ATCC 14075) were found to have almost identical patterns. However, another L-form originally designated as *S. moniliformis* An had an electrophoretic pattern identical to the *Proteus* L9 culture shown in Fig. 2 and 3. Biochemical tests on the L-phase An strain (R. Wittler, personal communication) verified our findings that this strain could not be the L-form of *S. moniliformis* but was in fact a *Proteus* L-phase culture. *L. monocytophages* and its derived L-form, except for a few differences in band intensity, are closely related. Of particular interest was the relationship between the gel pattern of *Streptococcus* MG parent, its L-form, and that of *M. pneumoniae*. Some question was raised earlier about the possibility of *M. pneumoniae* being a stable L-form of *Streptococcus* MG (9). Our results (Fig. 4) show that the *Streptococcus* MG parent and L-form possess similar patterns and appear to be closely related to each other but quite distinct from the pattern of *M. pneumoniae*. These data support the findings of Somerson et al. (16), who showed by nucleic acid hybridization techniques that no relationship exists between the *Streptococcus* MG parent and *M. pneumoniae*. However, our findings are even more direct since we were able to compare the *M. pneumoniae* pattern to both the *Streptococcus* MG parent and its derived L-form.

Figure 6 shows the gel patterns of *S. aureus* (Smith parent) and L-form strains obtained from the American Type Culture Collection (Rockville, Md.). Both patterns are the same and any differences between the two are quantitative rather than qualitative. These results are in contrast to those of Razin and Shafer (13), who indicated that the pattern of the *S. aureus* (Smith) L-form differs from that of its presumed parent. This result also indicates the value of the electrophoretic technique in checking the purity of cultures. Other advantages of the electrophoretic method for the identification of L-forms and mycoplasmas have been discussed extensively elsewhere (10, 18). In this paper, we have attempted to show the value of the method as it can be applied to problems in distinguishing mycoplasmas from bacterial L-phase organisms in the diagnostic laboratory.

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FIG. 6. Gel patterns of (A) *Staphylococcus aureus* Smith strain, ATCC 19636, and (B) *S. aureus* Smith L-form, ATCC 19640.
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