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Identification of L Forms by Polyacrylamide-Gel Electrophoresis

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Crude membranes were obtained from L forms by allowing the cells to lyse in distilled water. The crude membranes were washed several times in distilled water, lyophilized, and extracted with phenol-acetic acid-water. The membrane proteins were separated electrophoretically in polyacrylamide gels run at *p*H 4.5. Electrophoretic patterns and densitometric tracings of the gels showed distinct, reproducible intergeneric differences among L forms of *Proteus*, *Streptobacillus*, *Staphylococcus*, and *Streptococcus*. Differences within a genus could not be detected except between the group A streptococcal L forms and the one group D F-24 L form. This electrophoretic method offers possibilities for ready identification of L forms through the use of standard reference strains.

The lack of a rigid cell wall has made the identification of bacterial L forms difficult. Taxonomic criteria (i.e., Gram reaction, cellular and colonial morphology, serological typing) normally used for the classification of bacteria are not applicable to L forms. Biochemical data for the identification of L forms are either lacking or insufficient, and serological data, if available, are often equivocal (9). Recently, deoxyribonucleic acid base composition and nucleic acid hybridization techniques have been used with some success in classifying *Mycoplasma* species and a few L forms (5). However, these procedures are time-consuming and complex, and in some cases, especially with gram-positive microorganisms, the hybridization data were inconclusive (9). Thus, there exists a need for a simple method of identifying L forms. Such a method could also be used to determine the relationship between freshly induced or isolated L forms and their progeny after extended serial passage.

Recently, Rottem and Razin (6) reported on the identification of *Mycoplasma* species by use of polyacrylamide-gel electrophoresis. Their work was an extension of the observations of Fowler et al. on starch-gel (1) and polyacrylamide-gel (2) electrophoresis of both *Mycoplasma* and a few L forms. Rottem and Razin (6) showed characteristic band patterns obtained when purified membrane proteins from each species examined were dissolved in phenol-acetic acid-water (2:1:0.5, w/v/v) and run on polyacrylamide gels containing 7.5% acrylamide, 5 M urea, and 35% acetic acid. Later, Razin and Rottem (4) showed that whole-cell protein extracts rather than purified membrane proteins could be used, and they stated that L forms of *Streptobacillus* and *Proteus* could also be distinguished by this method. These findings led us to investigate further the applicability of such a method for the characterization of bacterial L forms.

MATERIALS AND METHODS

Organisms and growth conditions. The sources of L forms used in these studies are shown in Table 1. Each strain was grown statically in 1 liter of Trypticase Soy Broth (BBL) containing 3% (w/v) added NaCl and 10% (v/v) horse serum. For the growth of *S. moniliformis* L1, the medium was enriched with 0.5% (w/v) Oxoid yeast extract. After 3 days of growth at 37 C, the cells were harvested by centrifugation at 10,000 × *g* for 30 min and washed three times with distilled water. The resulting crude membrane fractions were lyophilized and stored in a desiccator at −10 C. Approximately 50 to 75% of the lyophilized L-form material was protein (3).

Preparation of extracts. For electrophoretic analysis, 2 mg of dried L-form material was suspended in 0.1 ml of distilled water. To this was added 0.2 ml of phenol-acetic acid-water (2:1:0.5, w/v/v), and this preparation was incubated in a water bath at 37 C with occasional mixing until an even dispersion was obtained. The insoluble material was removed by centrifugation at 4,500 × *g* for 20 min at room temperature, and to the clear supernatant fluid containing the phenol-acetic acid-water extracted proteins was added an equal volume of 40% (w/v) sucrose in 7% (v/v) acetic acid. A 0.1-ml amount of this mixture containing 175 to 250 μg of protein was subjected to electrophoresis. Fresh extracts were prepared daily.

Polyacrylamide-gel electrophoresis. Electrophoresis

was carried out in glass columns (5 × 65 mm) containing 1.0 ml of separating gel (7.5% acrylamide and 8 M urea) and 0.2 ml of stacking gel (2.5% acrylamide and 8 M urea). For the preparation of the gels, all but one of the following stock solutions were prepared fresh every 2 months. Solution A contained (in 100 ml): urea, 48 g; *N,N,N',N'*-tetramethylenediamine (TEMED), 2.0 ml; and 1 N KOH, 48 ml; adjusted to *p*H 4.5 with acetic acid. Solution B contained (in 100 ml): urea, 48 g; TEMED, 0.46 ml; and 1 N KOH, 48 ml; adjusted to *p*H 6.7 with acetic acid. Solution C contained (in 200 ml): urea, 96 g; *N,N'*-methylenebisacrylamide (BIS), 0.4 g; and acrylamide, 5 g. The above solutions were made to volume with distilled water. Solution E contained 1 mg of riboflavine in 50 ml of 8 M urea; solution F, 1 g of ammonium persulfate in 89 ml of 8 M urea (prepared fresh each week); solution G, 1.0 ml of dimethylaminopropionitrile in 9.0 ml of distilled water; and solution H, 8 M urea. All solutions were filtered through Whatman no. 42 filter paper and stored in dark bottles in the cold. Prior to use, stock solutions were mixed in the following proportions: separating gel, A-C-F-H (1:4.5:0.7:1.8); stacking gel, B-D-E-G-H (1:4:1:0.01:2). Each glass column was loaded with 1.0 ml of separating gel solution, overlaid with water, and allowed to polymerize for 30 min at room temperature with the aid of a fluorescent light. After polymerization, the excess water was drained off, and the inner walls of the column were rinsed with stacking-gel solution. To the top of the separating gel was added 0.2 ml of stacking-gel solution; the column was overlaid with water and allowed to polymerize as before. Then the excess water was drained off and 0.1 ml of the sample-sucrose mixture was placed on top of the stacking gel. Without disturbing the samples, the columns were filled with 0.07 M β-alanine buffer adjusted to *p*H 4.5 with acetic acid. Both upper and lower reservoirs of the electrophoresis apparatus (Canalco model 66 disc electrophoresis) contained 0.07 M β-alanine buffer, *p*H 4.5. The lower electrode served as the cathode. Electrophoresis was carried out at a constant current of 4 ma per tube for 90 min at room temperature. Gels were stained for 90 min with 1% (w/v) Amido Black in 7% (v/v) acetic acid, rinsed in tap water, and destained by simple diffusion in frequent changes of 7.5% acetic acid. Tracings of the band patterns were made with a Photovolt Densicord 542A electrophoresis densitometer (W. H. Curtin & Co., Rockville, Md.).

TABLE 1. *Sources of L-form cultures*

Organism	Obtained from
Group A streptococcus, types 13L and 1L	Roger M. Cole, National Institutes of Health, Bethesda, Md.
Group A streptococcus, ADL-L and GL8-L	M. F. Barile, National Institutes of Health
Group D streptococcus, F-24-L	James R. King, National Institutes of Health
Staphylococcus aureus ATCC 6538-L and 212-L	M. Boris, North Shore Hospital, Manhasset, N.Y.
Streptobacillus moniliformis L1, ATCC 14075	American Type Culture Collection, Rockville, Md.
Proteus XKL and 18L, and *P. mirabilis* 52L[a]	L. Dienes, Massachusetts General Hospital, Boston, Mass.

[a] All B-type L forms.

RESULTS

The electrophoretic patterns of three B-type *Proteus* L forms are shown in Fig. 1. *Proteus* 18L, XKL, and 52L were virtually indistinguishable from one another. The two intense broad bands located near the top of the separating gel are characteristic of all of the B-type *Proteus* L forms examined. The gel patterns of each L form examined were run several times. They were reproducible and did not vary significantly between experiments or with different batches of the same organism. In cases where L forms could be cultivated in the absence of horse serum, the omission of this component from the growth medium did not alter the electrophoretic pattern.

Electrophoretic patterns of *S. moniliformis* L1 and two strains of staphylococcal L forms are presented in Fig. 2. Strain L1 had a band distribution quite distinct from *Proteus* and staphylococcal patterns, and could be characterized by

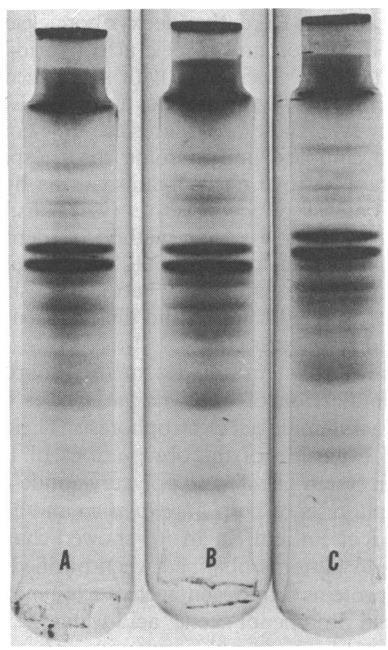

FIG. 1. *Electrophoretic patterns of crude membrane proteins of Proteus L forms. (A) Proteus 18L; (B) Proteus XKL; and (C) Proteus 52L.*

the presence of a heavy band at the top and bottom of the separating gel and by the intense double band in the center. As was the case with *Proteus* L forms, staphylococcal intrageneric similarity was evident. These latter L forms could be easily identified by the absence of any broad intense bands and by the presence of a series of evenly distributed bands of equal intensity.

When L forms derived from group A and group D streptococci were subjected to electrophoretic analysis, the results shown in Fig. 3 were obtained. Note that all four group A types exhibited the same basic pattern. The distinguishing characteristics were (i) the sparse and irregular distribution of bands of even intensity in the upper portion of the gel, (ii) the absence of any intense broad bands, and (iii) the presence of relatively few bands in the lower region of the gel. The pattern for the group D F-24 L form resembled the group A pattern; however, it could be distinguished from the others on the basis of the position of bands in the middle and lower region of the gel.

For comparison, electrophoretic patterns and densitometric tracings of the gels from each of the four genera studied are shown in Fig. 4 and 5, respectively. Clearly, these data show the specificity of band patterns for each genus, and they show that there are indeed intergeneric differences among L forms of *Streptococcus* (group A), *Staphylococcus*, *Streptobacillus* and *Proteus*.

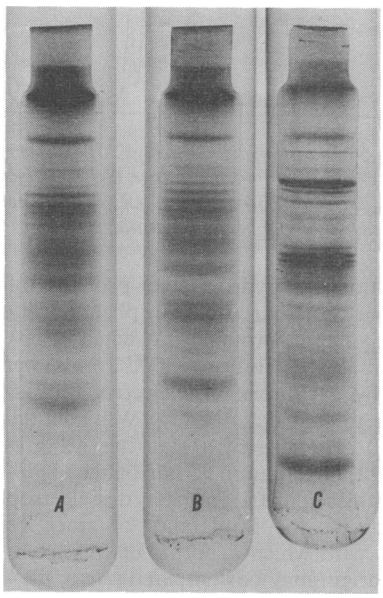

FIG. 2. *Electrophoretic patterns of crude membrane proteins of Staphylococcus and Streptobacillus L forms. (A) S. aureus ATCC 6538-L; (B) S. aureus 212-L; and (C) S. moniliformis strain L1.*

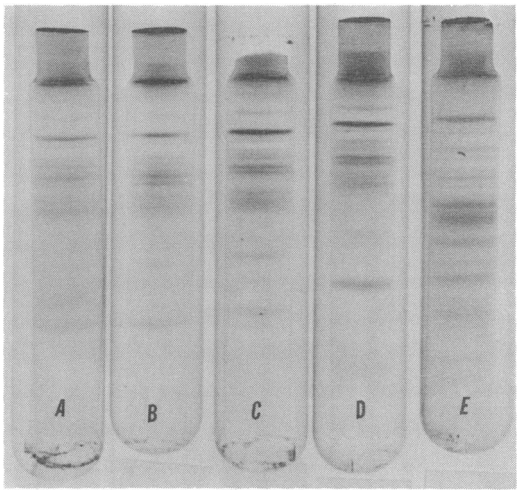

FIG. 3. *Electrophoretic patterns of crude membrane proteins of streptococcal L forms. (A) Group A streptococcus type 13L; (B) group A streptococcus type 1L; (C) group A streptococcus ADA-L; (D) group A streptococcus GL8-L; and (E) group D streptococcus F-24-L.*

DISCUSSION

The results of limited testing indicate that polyacrylamide-gel electrophoresis of crude membrane proteins can be used to differentiate bacterial L forms at least at the genus level. L forms of *Proteus*, *Streptobacillus*, *Staphylococcus*, and *Streptococcus* showed distinct, reproducible differences in electrophoretic patterns. A change in the growth medium did not alter the electrophoretic patterns, since L forms grown in the presence and absence of horse serum yielded similar results.

Strain specificity could not be established, as shown by the similarity of L forms within the genera *Proteus*, *Staphylococcus*, and *Streptococcus*. *Proteus* 18L, XKL, and 52L had identical gel patterns, as did the two staphylococcal L forms. In the streptococcal L forms, groups could be distinguished, as shown by the differences in electrophoretic patterns between the four types of group A and the one group D F-24 L form. Apparently, either the relationship among types is sufficiently close that minute differences were not detected or the specificity is on the cell wall and is lost upon conversion to the L form. Differentiation might be achieved if purified rather than crude membrane material were used. However, such procedures are tedious, require large amounts of starting material, and

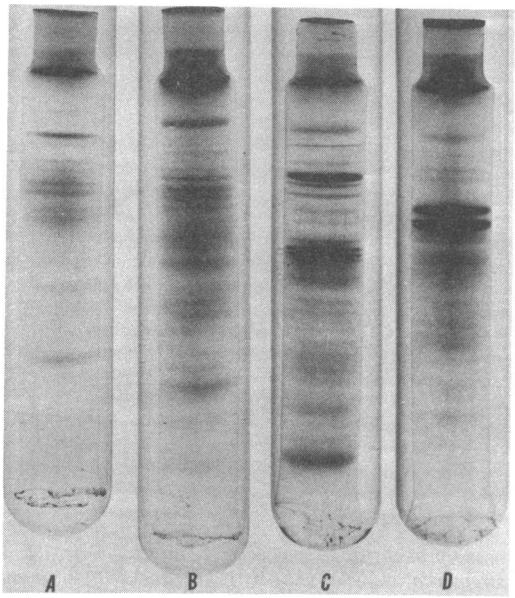

FIG. 4. *Comparison of electrophoretic patterns of crude membrane proteins of L forms of (A) group A streptococcus type 1L, (B) S. aureus ATCC 6538-L, (C) S. moniliformis strain L1, and (D) Proteus 18L.*

would be impractical for identification purposes. Another approach to this problem which is currently under investigation is comparison of the electrophoretic patterns of the parents of these L forms. Such studies will reveal whether differences in parental types, as determined previously by their morphological, biochemical, or serological characteristics, can be detected by gel electrophoresis. Also, a comparison can be made between the parent and its derived L form. Thus, it may be possible to characterize an L form not only by the use of standard reference strains but also by direct comparison with the parent bacterium. As yet, the number of different L forms in liquid culture available for testing is not sufficient to allow any conclusions on identification below the genus level, or the group level in the streptococci. Presently, L forms from genera other than those studied are being adapted to liquid medium.

The use of electrophoretic analysis for identification is not unique to bacterial L forms. Rottem and Razin (6) showed that electrophoretic patterns of purified membrane proteins could be used to identify the *Mycoplasma*. Later, it was reported that whole cells rather than extensively purified membranes were adequate for identification (4). The extraction procedure and separating-gel system used for the *Mycoplasma* were those described by Takayama et al. (7) for the

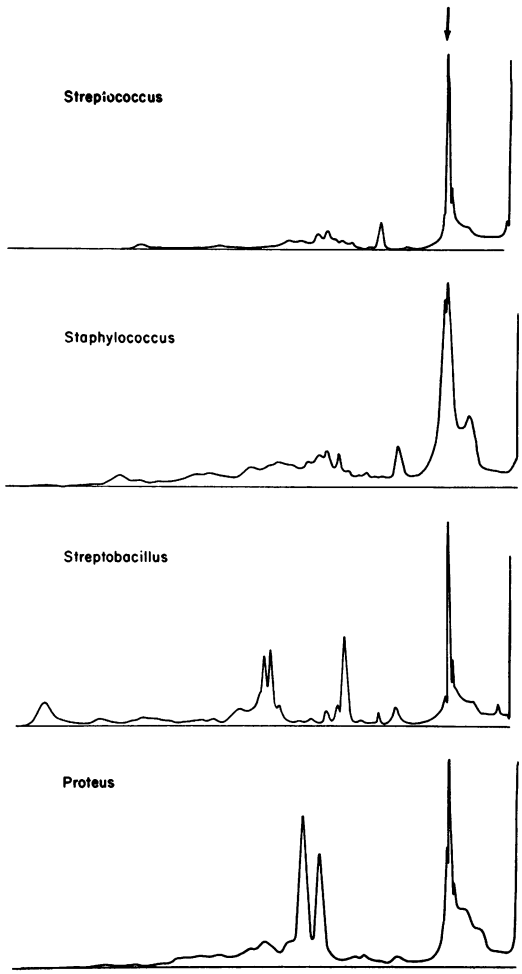

FIG. 5. *Densitometric tracings of polyacrylamide gels of group A streptococcus type 1L, S. aureus ATCC 6538-L, S. moniliformis strain L1, and Proteus 18L. Arrow indicates top of the separating gel.*

separation of hydrophobic proteins of mitochondria. In our studies, phenol-acetic acid-water soluble proteins extracted from crude membrane preparations of L forms were analyzed by a cationic disc electrophoresis system similar to that described by Williams and Reisfeld (8). The use of a stacking gel greatly increased the resolution of our bands.

Identification of L forms by disc electrophoresis depends on the availability of stock reference strains with which L forms of unknown or dubious origin may be compared. Also, the authenticity of a cell line can be readily determined. On the other hand, nucleic acid hybridization studies depend on the deoxyribonucleic acid homology between the L form and its parent.

The advantages of the electrophoretic method are that the procedures are simple and rapid, and relatively small amounts of material are needed.

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