JOURNAL OF BACTERIOLOGY, Oct. 1969, p. 71-77
Vol. 100, No. 1

Printed in U.S.A.

Generic Identification of L-Forms by Polyacrylamide Gel Electrophoretic Comparison of Extracts from Parent Strains and Their Derived L-Forms

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Received for publication 11 July 1969

A polyacrylamide gel electrophoretic method was used for identification of L-forms in the genera *Streptococcus*, *Staphylococcus*, and *Proteus*, by comparing phenol-acetic acid-water extracts of homologous parent L-form pairs to one another and to other pairs. The method requires only milligram quantities of material for analysis. The standard patterns for the strains used in this study are shown as pictures of the gels and as densitometric tracings of appropriate gels. They show that, despite occasional minor differences in some organisms, the gel electrophoretic patterns of homologous L-forms and bacterial pairs are sufficiently similar—as well as sufficiently dissimilar from patterns of other genera—to permit generic identification of an unknown L-form by reference to patterns derived from possible parental bacteria.

There has been general interest in the relationships between bacteria and their progeny growing in the L state. Nucleic acid hybridizations have proved useful for elucidation of the extent of relationship. Direct-binding deoxyribonucleic acid-deoxyribonucleic acid hybridizations in agar columns (8) have shown the close relationships between gram-negative bacteria and their homologous L-type growth. In addition, deoxyribonucleic acid-ribonucleic acid hybridizations performed on filter membranes (9) have given similar results with the streptococcal GL8 system. A more rapid method has been described for identification of some L-forms by the pattern of protein bands obtained from extracts of crude membrane fractions subjected to polyacrylamide gel electrophoresis (10). It was, therefore, of further interest to determine the similarity of protein distributions in extracts of the parent strains of the respective L-forms of several representative bacteria. If these patterns were sufficiently similar, it was hoped that a quick identification scheme could be developed for unknown L-forms by comparing them with known bacterial strains.

MATERIALS AND METHODS

Organisms and culture methods. Table 1 lists the parent strains and L-forms and their sources. All parent strains and L-forms, except *Streptococcus faecalis* strain F24 and its L-form, were grown for 3 days at 37 C in Trypticase Soy Broth (BBL) containing 3% (w/v) additional NaCl and 10% (v/v) horse serum (inactivated at 56 C for 30 min). These were grown for 18 to 24 hr at 37 C in Brucella broth (Albimi Laboratories) containing 0.43 M NH_4Cl and 0.5% (w/v) glucose. All organisms were harvested by centrifugation and washed three times in distilled water. This converted the osmotically sensitive L-forms to crude membrane preparations. All specimens were lyophilized as pellets.

Mechanical disruption of bacteria. The parent strain bacteria in distilled water were disrupted essentially by the method of Cummins and Harris (2). Suspensions of bacteria containing 15 to 20 mg (dry weight)/ml were disrupted in 6-ml portions by shaking the suspension with an equal volume of type 380-5005 Superbrite glass beads (Minnesota Mining and Manufacturing Co.) for 30 min at maximum amplitude and frequency on a Mickle apparatus. Glass beads and undisrupted bacteria were removed by centrifugation. The suspension of disintegrated bacteria was lyophilized.

Protoplast formation. Protoplasts of the gram-positive cocci were prepared with the aid of muralytic enzymes. A crude phage-associated lysin was used for the group A streptococcal strains by the method of Gooder and Maxted (4); lysozyme was used for strain F24 by the method of Bibb and Straughn (1); a staphylococcal lytic enzyme (courtesy of Archibald C. Scott, University of Glasgow) active against some staphylococci was used for the staphylococcal strain. Crude membrane preparations were made by washing protoplast suspensions three times in distilled water. The crude membranes were lyophilized.

Reversion of strain F24 L-forms to the parent strain.

TABLE 1. *Sources of strains*

Organism	Obtained from:
Group A streptococcus, ADA parent	L. Dienes[a]
Group A streptococcus, ADA L-form	M. F. Barile[b]
Group A streptococcus, GL-8 parent	N. L. Somerson[c]
Group A streptococcus, GL-8 L-form	M. F. Barile
Group D streptococcus, F 24 parent	J. R. King[c]
Group D streptococcus, F 24 L-form	J. R. King
Staphylococcus aureus ATCC 6538P, L-form	M. Boris[d]
S. aureus ATCC 6538P, parent	ATCC[e]
Proteus 52, parent	L. Dienes
Proteus 52, B type, L-form	L. Dienes
Proteus 9, ATCC 14273, parent	ATCC
Proteus L9, A type, L-form, ATCC 14273	ATCC

[a] Massachusetts General Hospital, Boston, Mass.
[b] Division of Biologics Standards, NIH, Bethesda, Md.
[c] National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md.
[d] North Shore Hospital, Manhasset, N.Y.
[e] American Type Culture Collection, Rockville, Md.

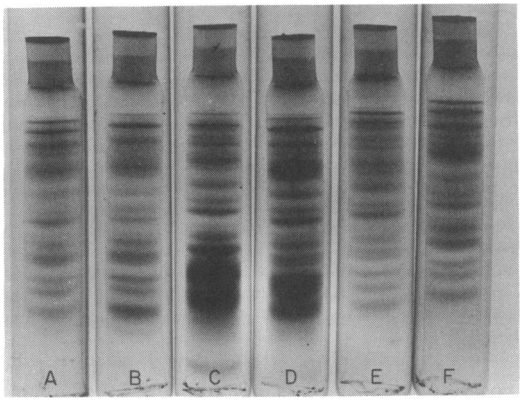

FIG. 1. *Polyacrylamide gel protein patterns from extracts of Streptococcus faecalis strain F 24. (A) Parent whole cells, (B) revertant parent whole cells, (C) parent protoplasts, (D) revertant parent protoplasts, (E) L-forms, and (F) disintegrated bacteria.*

L-forms were induced from strain F24 by the action of lysozyme by using the method of H. Gooder and J. R. King (Bacteriol. Proc., p. 71, 1964). A large number of freshly induced L colonies were subcultured three times on Trypticase Soy Agar containing 0.43 M NH_4Cl, 0.5% (w/v) glucose, and 2% (v/v) horse serum. Spontaneous reversion occurred in a few colonies after two subcultures in the L state. The progeny from such a revertant streptococcal colony were used in this study as whole cells or protoplasts.

Preparation of extracts and electrophoretic methods. Phenol-acetic acid-water extracts of the lyophilized materials described above, including whole bacteria, were subjected to acid polyacrylamide gel electrophoresis as previously described (10). The protein content, as determined by the method of Lowry et al. (7), was adjusted to 200 to 300 μg/0.1 ml of extract. Protein bands were stained with 1% amido black and destained with 7.5% acetic acid by simple diffusion.

Densitometry. Densitometric tracings of gel electrophoretic patterns were made with a model 542A Photovolt densitometer.

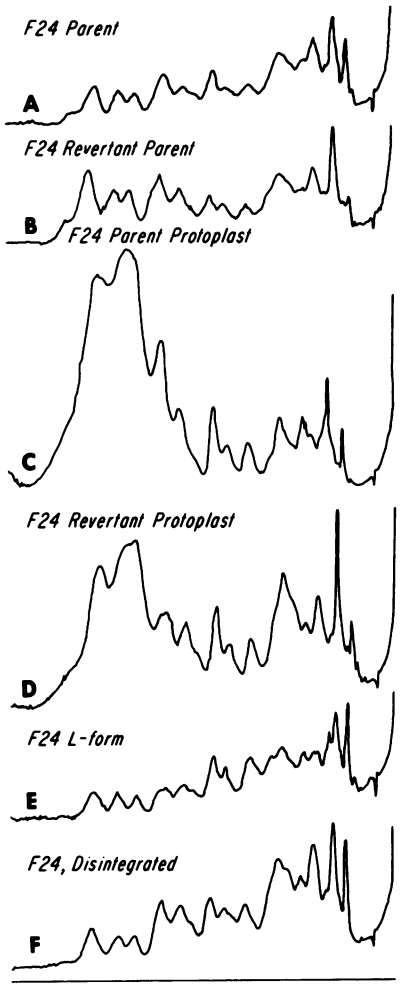

FIG. 2. *Densitometric tracings of the same polyacrylamide gels shown in Fig. 1.*

RESULTS

The similarity of band patterns among the extracts of *S. faecalis* F24 growth forms is readily apparent (Fig. 1 and 2). The patterns from the parent streptococcus and the L-form are identical, and their respective densitometric tracings are virtually indistinguishable. Disintegration of the streptococci did not change the pattern extensively. Extracts from revertant cocci and homologous protoplasts contained proteins with the same distribution as the parent strain. In the bottom of the patterns from protoplasts, there was an unusual broad band, which could be produced by subjecting a sample of purified lysozyme to electrophoresis. The densitometric tracing shows that the usual peaks seen in the absence of lysozyme may be present in addition to the broad band of lysozyme. The region of the gel containing lysozyme is obscured to observations by the unaided eye, but the densitometer can resolve these peaks even in the presence of such a high background absorbance.

Similarly, two strains of group A streptococci were subjected to electrophoretic analysis, and gel patterns typical of group A streptococcal L-forms were found. Patterns obtained with the parent strain GL8, protoplasts of GL8, GL8 L-forms, and the disrupted cocci are shown in Fig. 3 and 4. When comparable amounts of protein were initially added to each column, there were a few bands present in the parent streptococcus pattern which were not as prominent in the L-form. These bands were also present in disrupted cocci and protoplasts. Comparable results were obtained with strain ADA (Fig. 5 and 6). A typical band pattern is seen in each of these gels, but the same type of minor differences in the band pattern exist within the strain ADA as with GL8. Parent streptococci, protoplasts, and mechanically disrupted cells produced a pattern which contained a few bands more intense than the corresponding bands in the L-form.

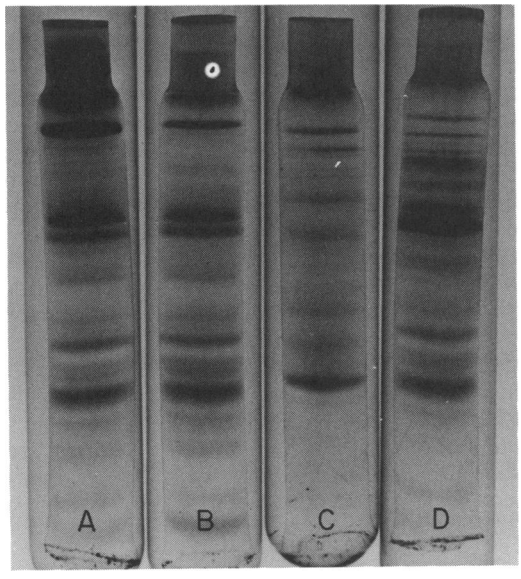

FIG. 3. *Polyacrylamide gel protein patterns from extracts of Streptococcus pyogenes strain GL-8. (A) Parent whole cells, (B) protoplasts, (C) L-forms, and (D) disintegrated bacteria.*

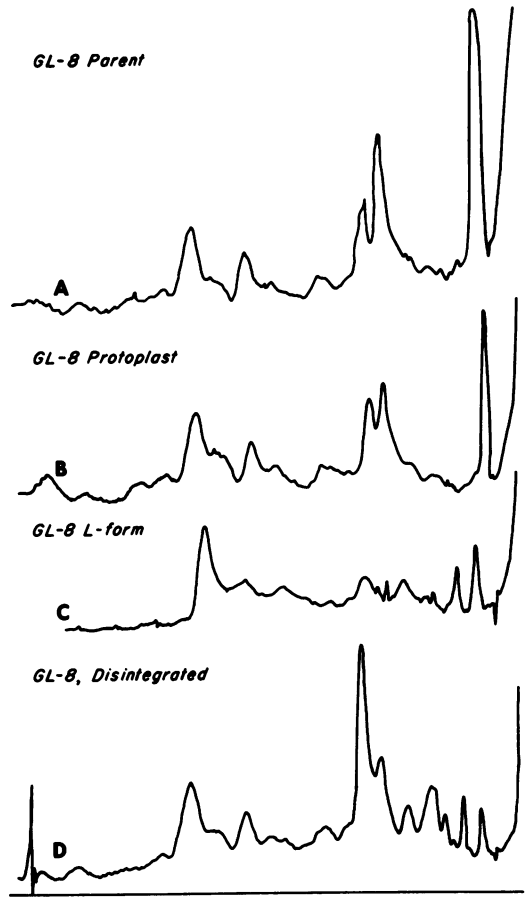

FIG. 4. *Densitometric tracings of the same polyacrylamide gels shown in Fig. 3.*

Similarly, a strain of *Staphylococcus aureus* was subjected to analysis (Fig. 7 and 8). When samples containing equivalent amounts of protein were applied to the gel columns, a few bands were much darker in the gels containing parent strain extracts. Whole cells, protoplasts, and mechanically disintegrated staphylococci possessed essentially the same pattern, which differed only slightly from the L-form patterns.

Analyses of this nature were also applied to

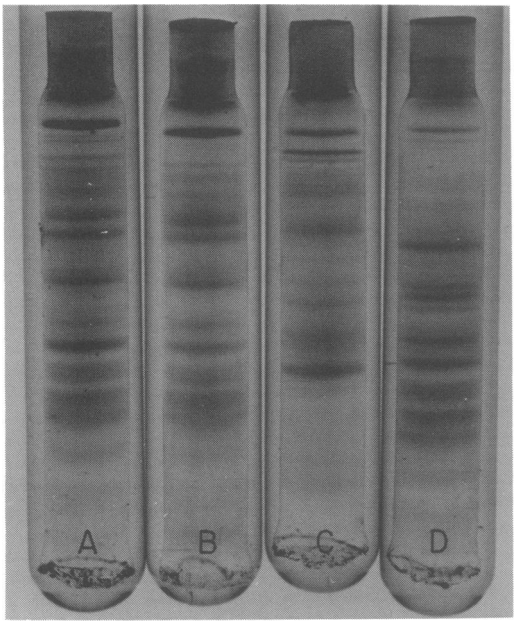

FIG. 5. *Polyacrylamide gel protein patterns from extracts of Streptococcus pyogenes strain ADA. (A) Parent whole cells, (B) protoplasts, (C) L-forms, and (D) disintegrated bacteria.*

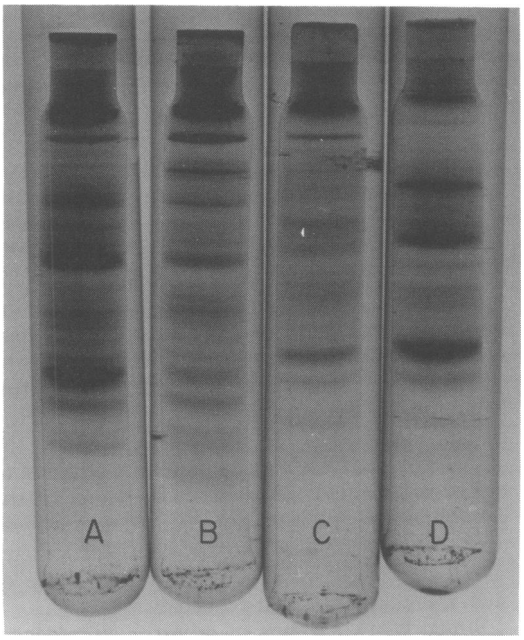

FIG. 7. *Polyacrylamide gel protein patterns from extracts of Staphylococcus aureus. (A) Parent whole cells, (B) protoplasts, (C) L-forms, and (D) disintegrated bacteria.*

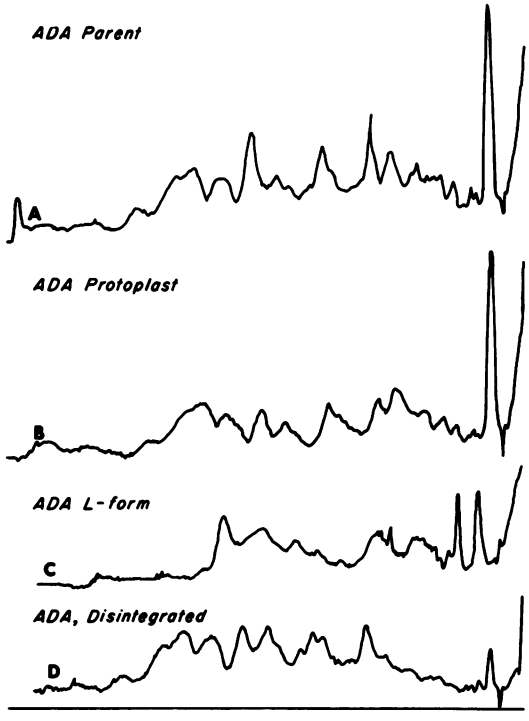

FIG. 6. *Densitometric tracings of the same polyacrylamide gels shown in Fig. 5.*

bacteria and their L-forms in the genus *Proteus*. Mechanical disruption had no effect on typical *Proteus* patterns from bacillary growth. Figures 9 and 10 show that *Proteus* 52 and its L-form have identical patterns. The distinguishing intense double bands are apparent in the L-type growth as well as the bacillary form. Similarly, *Proteus* 9 and its L-form closely resemble each other, but the typical intense double band region is not as strong as in *Proteus* 52. The region of the double bands seen in *Proteus* 52 can be resolved into three weaker bands in *Proteus* 9 and its homologous L-form.

The results reveal that the bacteria and L-forms within the same genus possess similar electrophoretic patterns. For comparison, the standard (10) patterns from L-form strains used in this study are shown in Fig. 11. With these gels in juxtaposition, the generic differences in the patterns appear quite clearly with mere visual inspection. Similarly, the parent strain patterns are shown in Fig. 12. The same typical generic patterns were found in the parent strains tested.

DISCUSSION

Identification of L-forms to the generic level by comparing their polyacrylamide gel patterns with patterns of the homologous parent strains has been made with the limited number of genera

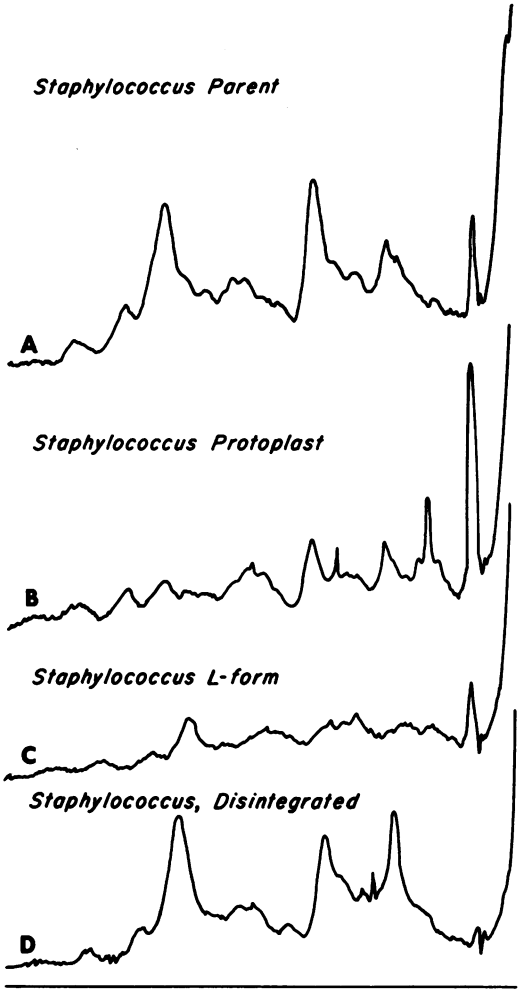

FIG. 8. *Densitometric tracings of the same polyacrylamide gels shown in Fig. 7.*

listed in Table 1. When minor intrageneric pattern differences were found, they did not affect the general appearance of the generic pattern as determined by the method of Theodore, King, and Cole (10). Mechanical disintegration or protoplasting had no major effect on the gel patterns from appropriate parent strains, although residual lysozyme partially obscured a few bands in the lower region of strain F24 protoplast gels.

There is an important difference between the present method for identification of an L-form genus and the initial method proposed by us (10). The initial method requires a library of patterns from broth-grown L-forms with known parental relationships, or the careful maintenance of a collection of L-forms. By using the method

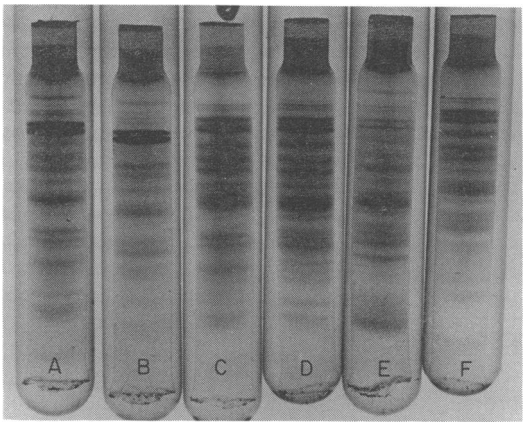

FIG. 9. *Polyacrylamide gel protein patterns from extracts of two strains of Proteus. (A) Proteus 52 whole cells, (B) Proteus 52 L-forms, (C) Proteus 52 disintegrated, (D) Proteus 9 whole cells, (E) Proteus 9 L-forms, and (F) Proteus 9 disintegrated.*

described here, unknown L-form-like growth can be compared directly to possible parent strains. Thus, it is possible to use patterns from bacteria with well-defined cultural and taxonomic characteristics as a set of standards that can be easily expanded to include previously untested genera from which L-forms have not yet been induced in the laboratory. Ideally, nucleic acid hybridization techniques (5, 8, 9) should be used, but these techniques require lengthy procedures and considerably more material for analysis. Electrophoretic comparison of unknown L-forms to potential parent strain bacteria could be extremely useful as a screening method for nucleic acid hybridizations required for definitive L-form identification.

Polyacrylamide gel electrophoretic identification is rapid and therefore useful, because crude protein mixtures extracted from untreated bacteria may be used as a basis for comparison. Washed whole cells produce patterns as specific as patterns from washed crude membrane preparations of either L-forms or protoplasts. The presence of intact cell wall apparently does not interfere with extraction of the membrane proteins from gram-positive organisms.

Group A streptococcal parents show some bands that are less intense in the L-form patterns. The nature of these bands is to be investigated. It is possible that they represent cell wall or periplasmic enzymes, or other protein antigens associated with surface structures. Indeed, a recent publication (6) has now indicated characteristic differences of gel patterns among cell wall extracts from some different group A serotypes. It would not be surprising to find, as testing of

parent L-form pairs is extended and improved, that distinguishable bands representing known surface proteins of the parent are commonly found superimposed on a common generic pattern. Such proteins, though probably produced by some L-form growth, may not remain localized to the cells but diffuse into the medium as shown for streptococcal M protein (3), and are therefore not extractable from crude washed cell membranes. Nor may they necessarily be extracted, even from parents, by the particular solvent system used. In addition, it is possible that even the production of such proteins is reduced in stable L-forms, whether stability be the result of selection during prolonged and repeated passage in media not optimal for such protein production or induction (as enzymes) or whether it be caused by an actual genomal defect (5). In the only known example of the latter, a 4 to 6% deletion of the genome of the stable L-form is identical and superimposable.

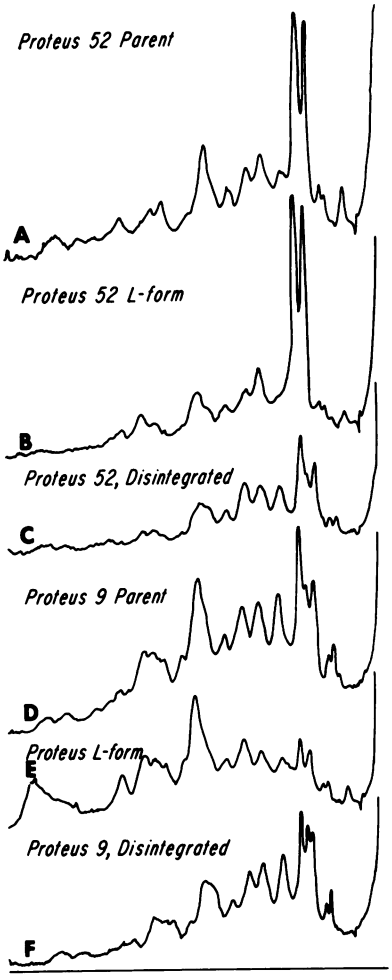

FIG. 10. *Densitometric tracings of the same gels shown in Fig. 9.*

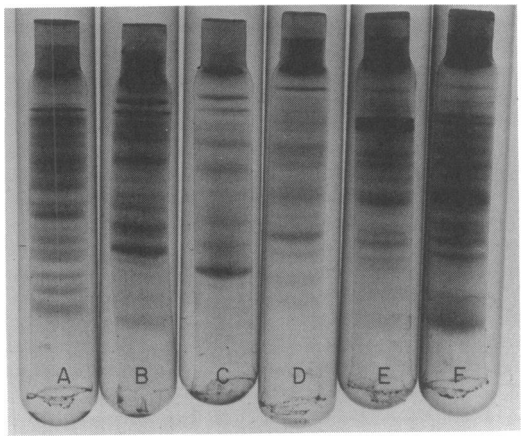

FIG. 11. *Polyacrylamide gel protein patterns from extracts of the representative L-forms used in this study. (A) Streptococcus faecalis F24,[1] (B) S. pyogenes ADA, (C) S. pyogenes GL-8, (D) Staphylococcus aureus, (E) Proteus 52, and (F) Proteus 9.*

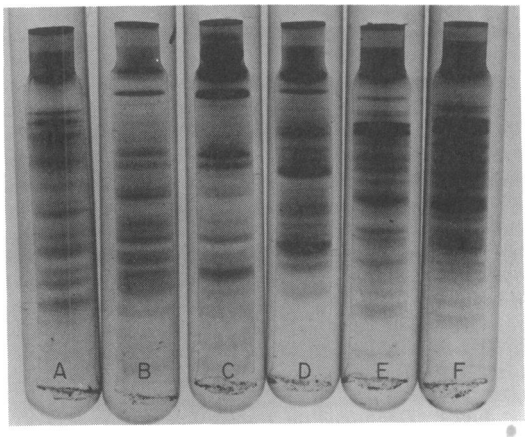

FIG. 12. *Polyacrylamide gel protein patterns from extracts of the representative parent strains used in this study. (A) Streptococcus faecalis F 24, (B) S. pyogenes ADA, (C) S. pyogenes GL-8, (D) Staphylococcus aureus, (E) Proteus 52, and (F) Proteus 9.*

It is anticipated that the described method for comparison of gel electrophoretic patterns may prove of value not only in determining or confirming generic origins of L-forms, but also in clarifying differences among L-forms of a given origin, caused by different modes of induction, by differences in growth media, and by passage histories which may lead to L-form stability.

ACKNOWLEDGMENTS

We are grateful to Richard Whitt and Willard Davis for their able technical assistance.

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