Comparative Gel-Electrophoresis Studies of Ribosomal Proteins of Erwinia Species and Members of the Enterobacteriaceae

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ABSTRACT

Proteins of 70 Svedbergs (S) ribosomes from *Erwinia amylovora, E. carotovora, E. herbicola, Escherichia coli*, and *Salmonella typhimurium* were analyzed by electrophoresis on acrylamide-urea gels, pH 4.3. The erwinias possess typical 70-S ribosomes which dissociate into 50-S and 30-S subunits at low magnesium concentration. The *Erwinia* ribosomal protein patterns are strikingly similar to those of *E. coli* and *S. typhimurium*. A characteristic feature of gel electropherograms of all these species is that the proteins cluster into two major electrophoretic groups of six to nine bands each. The gel electropherograms differ from that of *Agrobacterium tumefaciens*. The implications of the results are discussed.

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Additional key words: ribonuclease-1 deficient mutant of *Erwinia rubrifaciens*; linear transport.

The genus *Erwinia* contains species pathogenic to plants as well as nonphytopathogenic species that have been isolated from insects, fish, mammals, humans, and plant materials (1, 2, 8, 9, 11, 17, 20, 26, 37). Because of the ecological diversities of *Erwinia* species, it has been proposed that the genus be divided into at least three groups with the following representative type species: (i) *E. amylovora*; (ii) *E. carotovora*; and (iii) *E. herbicola* (3, 4, 5, 14, 24). The suggested group distinctions were based on phenotypic characters derived from biochemical and physiological tests and the ecological niches; i.e., plant-pathogenic types and saprophytic types. Although it has been questioned whether all of the *Erwinia* species should be placed in the Enterobacteriaceae (6, 10), no attempts have been made to relate these organisms by DNA-DNA hybridization and analysis of structural proteins.

Taxonomic assessments of phytopathogenic bacteria by molecular methods such as those employing DNA-DNA hybridization and analysis of structural proteins are superior to various physiological and biochemical tests in current use. The classical tests are often difficult to interpret; variable and single phenotypes are being tested which are prone to variation depending on the environment in which the bacteria have to grow. Thus, a test for a given enzyme may or may not be positive, since the enzyme (e.g., nitrate reductase in erwinias) could be repressed under certain growth conditions and derepressed under other conditions. Furthermore, classical tests have the inherent disadvantage of reflecting only a very small portion of the bacterial genome. The ideal approach to bacterial taxonomy is to compare the entire bacterial genome by direct base sequence analysis (DNA-DNA homologies) or by examining highly conserved and very stable direct products of structural genes. We have, therefore, chosen the analysis of proteins from ribosomes. Ribosomes are direct products of structural cistrons.

These cistrons are redundant in the chromosome of bacteria (27, 28). A single analysis of bacterial ribosomes is equivalent to at least 50 to 60 different biochemical tests.

Comparative studies of ribosomal proteins of different bacteria may be useful in elucidating the genetic relatedness and phyllogenetic relationships at the specific level. Such studies already have been made with several bacteria (21, 22, 35, 38), but not with any plant pathogenic species.

The present study reports some striking similarities between the ribosomal protein patterns of 70 Svedbergs (S) ribosomes of the type species of the *Erwinia* groups and those of *Escherichia coli* and *Salmonella typhimurium*.

MATERIALS AND METHODS.—*Bacterial cultures and media.*—The following cultures were employed: *E. amylovora* 1D32, 1D34; *E. carotovora* 3D31, 3D32; *E. nigrifluens* 5D33; *E. rubrifaciens* 6D321, 6D323, 6D318; *E. herbicola* ICPB 3161 (from M. P. Starr), *E. coli* AB295 (from A. J. Clark); and *S. typhimurium* LT-2 (from J. L. Ingraham). The ribonuclease-I deficient mutant of *E. rubrifaciens* 6D318 was obtained by the method of Gesteland (7); *E. coli* Q13 was obtained from A. J. Wahba. All bacteria were cultured in medium 523 (12) at room temperature in 2.4-liter Fernbach flasks for 18 hr on a rotary shaker (1.5 liters of medium/flask). The cells were harvested by centrifugation, washed once with 0.15 M NaCl, and stored frozen.

Isolation of ribosomes and ribosomal proteins.—Ribosomes were obtained from 40-60 g (packed wet weight) of cells. The cells were disrupted with acid-washed glass beads (0.1-0.11 μ, 2.9 g beads/g cells) (Potter Industries, Inc., Carlstadt, N.J.) in a chilled Sorvall OmniMixer and blended for 15 min at maximum speed in 3 volumes of TM buffer (0.05 M Tris [tris (hydroxymethyl) amino methane] (Sigma Chemical Co., St. Louis, Mo.), pH 7.5, containing 0.01 M $MgCl_2$). Pancreatic deoxyribonuclease (ribonuclease-free) (Worthington Biochemical Corp., Freehold, N.J.) was added to a final concentration of 20 μg/ml. The glass beads were re-extracted twice with TM buffer, and the extracts were pooled and centrifuged for 5 min at 2,000 *g*. The supernatant was then centrifuged for 20 min at 12,000 *g*. The ribosomes in the 12,000-*g* supernatant were concentrated by centrifugation for 2.5 hr at 105,000 *g*. The sedimented ribosomes were dissolved in 0.5 M ammonium chloride, 0.01 M Tris-Cl, pH 7.4, containing 10 mM $MgCl_2$ and purified according to the method of Spitnik-Elson & Atsmon (30). Ribosomal proteins were prepared by the chilled acetic acid method (36) with slight modifications. The purified ribosomes were suspended in 6 mM 2-mercaptoethanol, and 2 volumes of glacial acetic acid were added. The precipitated ribosomal RNA was removed by centrifugation, and the supernatant was dialyzed exhaustively against 6 mM 2-mercaptoethanol. The dialyzed protein solution was adjusted to pH 8.0 with NH_4OH to dissolve the precipitated proteins, and dried in the frozen state under vacuum.

Chemical analyses.—Protein was determined by the method of Lowry et al. (16) with crystalline bovine serum albumin (Cal Biochemical Corp., Los Angeles, Calif.) as standard. Ribosomal RNA was determined by the orcinol method (25) with purified yeast RNA as standard.

Analytical centrifugation.—Isolated ribosomes were analyzed in a Spinco analytical ultracentrifuge, Model E, equipped with a photoelectric scanner. Sedimentation analyses were made in TM buffer, pH 7.4, and in low magnesium buffer, pH 7.4 (1 mM $MgCl_2$).

Polyacrylamide gel electrophoresis.—The modified procedure of Reisfeld et al. (23) using deionized 8M urea was employed. Analytical disc electrophoresis was performed at pH 4.3 using the gel concentrations of Moore et al. (19). The lower gel (prepared in deionized 8M urea) concentration was 7.5% (w/v) acrylamide, 0.8% (w/v) bisacrylamide, and 0.2% N,N,N',N'-tetramethylethylenediamine. Gels with this degree of cross-linkage gave the best resolution. Electrophoresis was performed in glass tubes, 80 mm long and 5 mm in diam. The volume of the lower gel mixture was 0.85 ml; and the upper spacer gel, 0.20 ml. The current for electrophoresis was 3 ma/tube at 2 C with the anode at the upper end of the gel. Pyronine Y was employed as the tracking dye to determine the length of the run, which usually required about 2.3 hr. The proteins in the gel were stained for 30 min with a 1% solution of amido schwarz in 7.5% acetic acid. The gels were rinsed and destained with 7.5% acetic acid.

Quantitative assessment of protein bands.—Polyacrylamide gels containing stained protein bands were scanned at 560 nm using a 0.2-nm slit width in a Beckman ACTA III spectrophotometer equipped with a specially designed linear transport. The scan patterns were recorded on unscribed chart paper and photographed directly for direct comparison with the gels.

RESULTS.—*Sedimentation coefficients.*—Like other bacterial ribosomes, the ribosomes of *Erwinia* species showed a sedimentation coefficient of 70 S in high Mg buffer (50 mM Tris-Cl, pH 7.4, 10 mm $MgCl_2$). Two characteristic peaks of 50 S and 30 S were always observed in low magnesium buffer (50 mM Tris-Cl, pH 7.4, 1 mM $MgCl_2$) during velocity sedimentation analyses. The ribosomes of *E. rubrifaciens*, for example, have sedimentation coefficients ($S_{20,w}$) of 32.9 S and 52.5 S when corrected for viscosity and density differences in the low magnesium buffer and extrapolated to infinite dilution (Fig. 1). The ribosomes at high magnesium (10-mM) concentration sedimented mostly as 70-S particles with smaller amounts of 50-S and 30-S subunits.

Chemical analysis.—The ribosomes of the erwinias contained 63.8-65.2% RNA and 34.8-36.2% protein. The RNA/protein weight ratios were about 1.8, and are similar in RNA and protein content to other bacterial ribosomes (29, 32).

Comparison of ribosomal proteins in acrylamide gels.—The band patterns of 70-S ribosomal proteins in

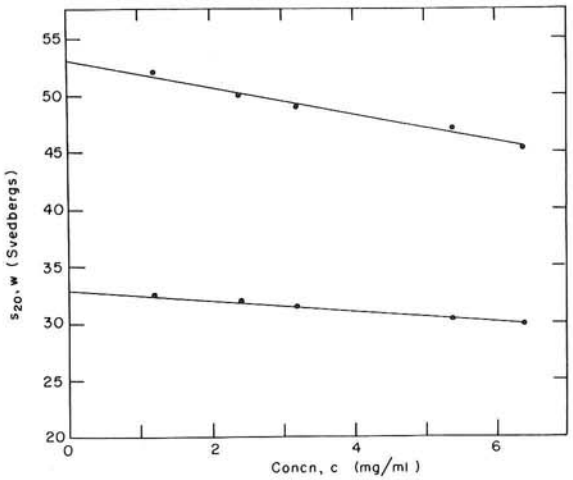

Fig. 1. Sedimentation coefficients as a function of ribosome subunit concentrations. The sedimentation coefficients were obtained with the Schlieren optical system. The concentration of the ribosome (as mixtures of both subunits) was based on a specific extinction coefficient of 157 for a 1% solution at 260 nm (30, 32).

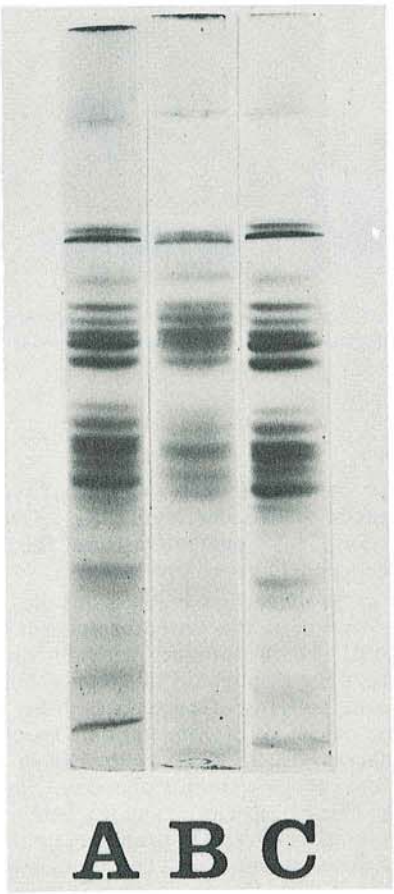

Fig. 2. 70-S ribosomal protein patterns in acrylamide-urea gels of *Erwinia rubrifaciens* strains. Strains: A, 6D321; B, 6D323; and C, 6D318 (ribonuclease-I deficient mutant).

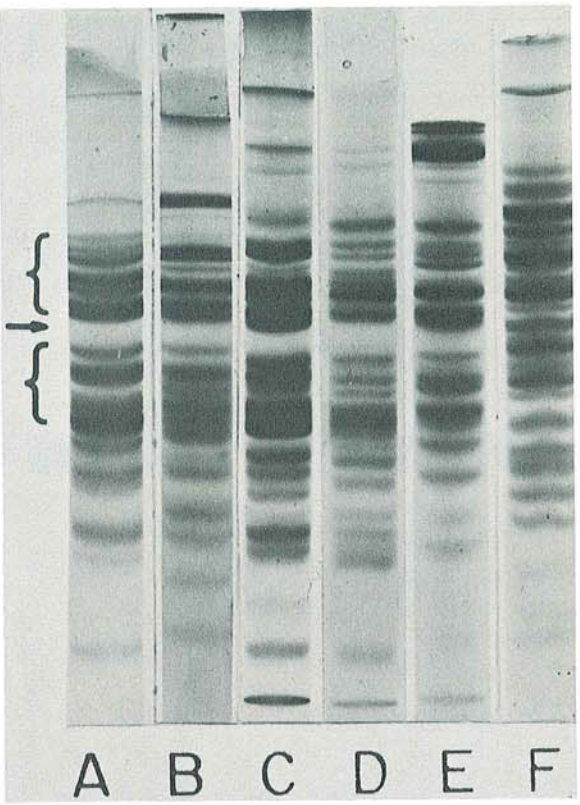

Fig. 3. Comparative 70-S ribosomal protein patterns in acrylamide-urea gels of: A, *Erwinia amylovora* 1D32; B, *E. carotovora* 3D31; C, *E. herbicola* ICPB3161; D, *Escherichia coli* Q13; E, *Salmonella typhimurium* LT-2; and F, *Agrobacterium tumefaciens* 1D135. Band groups are designated by brackets characteristic of the *Erwinia* species and *E. coli* Q13 and *S. typhimurium* LT-2. Arrow designates a clear zone.

acrylamide gels were the same for three different *E. rubrifaciens* isolates whether the ribosomes were prepared from cells in logarithmic or stationary phase of growth in rich or minimal medium (Fig. 2). Similar results were obtained with ribosomes of *E. coli* MRE600 (33). There were no obvious differences in ribosomal protein patterns of the ribonuclease-I deficient mutant and the wild type (Fig. 2). The electrophoretic patterns of the five *Erwinia* species were similar in that the protein bands were clustered into two major groups (Fig. 3). The first group had five to seven distinct bands, and the second group had 9 to 12 bands, separated from the first by a conspicuous clear space. These clusters and the clear region, designated by the arrow in Fig. 3, seem characteristic of the type *Erwinia* species, *E. coli*, and *S. typhimurium*. No such clusterings were observed with *Agrobacterium tumefaciens* (Fig. 3-F). Spectrophotometric scans of the gels reveal differences in the detailed patterns superimposed in over-all similarity (Fig. 4-A, B, C, D). In these analyses, some 20 to 25 bands were resolved. However, the darker bands probably consist of more

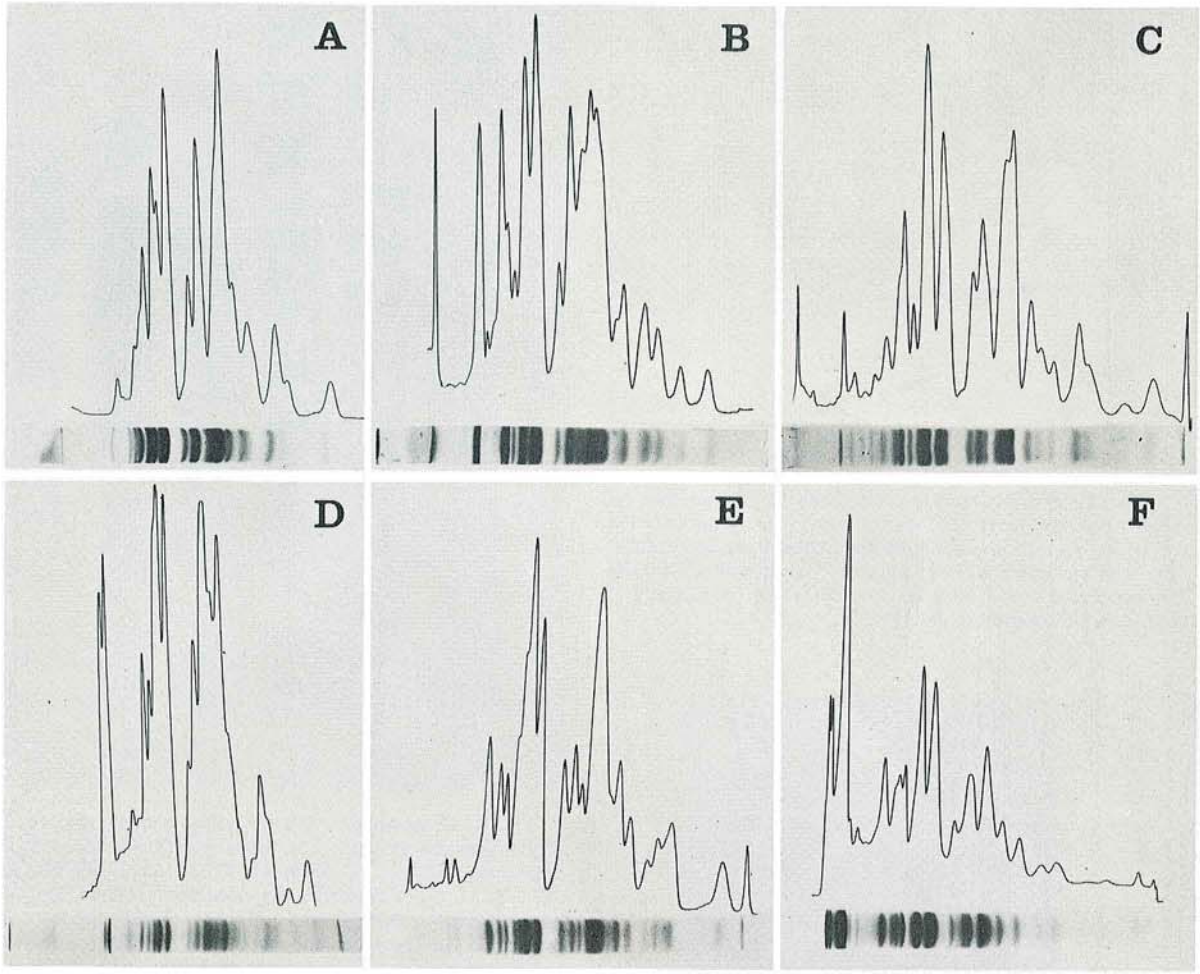

Fig. 4. Comparative gel scans and corresponding acrylamide-urea gels of 70-S ribosomal proteins of: **A)** *Erwinia amylovora* 1D32; **B)** *E. carotovora* 3D31; **C)** *E. herbicola* ICPB3161; **D)** *E. rubrifaciens* 6D321; **E)** *Escherichia coli* AB295; and **F)** *Salmonella typhimurium* LT-2.

than one protein, since ribosomes of *E. coli* contain 55 distinct proteins (13, 34) and acrylamide gels have been shown to have overlapping protein bands. By far the most striking similarity was found between the band pattern distribution of the ribosomal proteins of *E. herbicola* and *E. rubrifaciens* with those of *E. coli* (Fig. 4-C, D, E). As a check for any electrophoretic anomalies which might have appeared, the band patterns of the erwinias were compared with published photographs of *E. coli* ribosomal protein band (e.g., 15, 18, 33). Again, the same striking similarities were evident.

DISCUSSION.—The band patterns of ribosomal proteins were reproducible in different electrophoretic runs, and were similar for different strains of each *Erwinia* species. There was an over-all similarity among the *Erwinia* species, yet the band patterns for each species were distinctive. The ribosomal proteins of these erwinias distributed into definitive over-all patterns, and do not seem to exist in species of a different family; e.g., *Agrobacterium tumefaciens*. This would suggest that the proteins are very much alike in net charge and size.

The gross similarities of the ribosomal patterns of other members of the Enterobacteriaceae should be compared to determine whether similar band groupings exist throughout this family. On examining gross band patterns of bacteria in other taxonomic families, such as *Agrobacterium* species of the Rhizobiaceae (C. I. Kado, *unpublished data*), the same band groups were not observed. Thus, it seems that the *Erwinia* species examined herein at least fit the ribosomal genotype of other recognized members of the Enterobacteriaceae. The closeness of fit should be expected with the *E. carotovora* group in which species are phenotypically similar to other Enterobacteriaceae members (10) and possess a guanine:cytosine (GC) content similar to that of *E.*

coli (51-52% GC for *E. carotovora* versus 51% GC for *E. coli*) (31). This is reflected in the over-all similarity of ribosomal patterns in acrylamide gels depicted in our studies. *Erwinia rubrifaciens* strains also have an average GC content of 51-52% (J. M. Gardner & C. I. Kado, *unpublished data*). A GC content ranging from 52 to 52.6% was reported for this bacterium (31). Again, the similarities in ribosomal band patterns of *E. rubrifaciens* to those of *E. coli* are quite apparent.

In the above comparisons of electropherograms of ribosomal proteins in a polyacrylamide gel, it was assumed that charge and size differences between these proteins were sufficient to resolve differences between the enterobacteria. Such assumptions seemed validated by the studies of other bacterial ribosomal proteins (15, 22). Thus, although there are limitations in using polyacrylamide electropherograms such as overlapping protein bands, the comparison of over-all protein distribution appears to serve as a fingerprint of a particular bacterial grouping, and thus obviates this inherent limitation. The use of two-dimensional gel electrophoresis of these structural proteins may support this contention (13).

Recent serological comparative studies with ribosomal proteins of members of the Enterobacteriaceae have shown that the ribosomes of *Salmonella, Serratia,* and *Proteus* react as strongly as those of the homologous system using antiserum to *E. coli* ribosomal proteins (38). This seems to lend further support to the genotypic similarities that may exist between members of the Enterobacteriaceae and to the inclusion of the *Erwinia* species (at least those tested herein) in this taxonomic family.

Analysis of electropherograms on polyacrylamide gels of undefined soluble bacterial proteins have been made in the past. Comparative analysis of these proteins may be difficult to reproduce because bacteria have relatively high turnovers of these proteins (enzymes). Hence, variations in the amount of protein will usually occur. Dependence on precise growth conditions and media are required to obtain standardized quantities of these proteins. Such requirements are obviated by utilizing stable structural proteins that reflect large segments of the genome such as those of ribosomes.

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