Evaluation of Two-Dimensional Polyacrylamide Gel Electrophoresis of Acidic Proteins of Ribosome Preparations for Identifying Plant Pathogenic Soft-Rotting Bacteria

H. E. Moline, K. S. Johnson, and J. D. Anderson

Research plant pathologist and biological laboratory technician, Horticultural Science Institute; and plant physiologist, Plant Physiology Institute, U.S. Department of Agriculture, S&E, ARS, Beltsville, MD 20705, respectively.

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Reference to a specific brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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ABSTRACT

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Two-dimensional polyacrylamide gel electrophoretic separation of proteins was successfully used for identifying cultures of plant pathogenic bacteria. Profiles of soluble proteins were very complex; however, profiles of ribosomal protein-enriched fractions contained only 5–10 major proteins and could be used to distinguish strains of *Erwinia carotovora*, *E. atroseptica*, and *E. chrysanthemi*. Most significant differences in profiles of ribosomal-enriched protein of *Erwinia* spp. were in those of the acidic proteins in the 20–50 Kdalton molecular weight range. Although several protein clusters were similar on all strains of *E. carotovora* and *E. atroseptica* examined, significantly different clusters were observed between these strains and those of *E. chrysanthemi*.

The soft-rotting bacteria are a heterogeneous group of related genera (9) that include the *Erwinia* species. Numerous investigators have attempted to improve the taxonomic characterization of these genera (7,9,12); however, a good deal of disagreement remains as to their status. A simple method of distinguishing these soft-rot bacteria based on separation of cellular proteins would be of great help in characterizing strains of *Erwinia*.

The O'Farrell (15) method for obtaining high-resolution, two-dimensional (2-D) electrophoretic separation of soluble proteins in polyacrylamide gels and staining them with silver (14) is potentially valuable for identifying bacteria isolated from diseased plant tissues. A single 2-D profile, which may reveal as many as 100 silver-stained proteins, may be as effective as the series of 50 or more conventional biochemical tests currently needed for identifying these bacteria (3,6,12). However, these profiles of soluble proteins are quite complex, whereas profiles of the acidic ribosomal-enriched proteins contain only a few proteins and are much easier to analyze. Since ribosomal proteins are structural proteins they do not vary with the environment as may be the case with other soluble proteins (11). The purpose of this study was to compare the ribosomal-enriched protein profiles of a number of soft-rotting *Erwinia* strains (Table 1) using the O'Farrell (15) 2-D electrophoretic technique.

MATERIALS AND METHODS

Growth of cells and extraction of ribosomes. Ribosomes were extracted from bacterial cells according to the procedures of Tissieres et al (20) and Schaad (17) with the following modifications. A 24- to 48-hr-old bacterial liquid culture was used to seed 150 ml of sterile Difco nutrient broth in 250-ml Erylenmeyer flasks. Suspensions seeded with *E. carotovora* and *E. atroseptica* were shaken at 20 C for 18–24 hr and those seeded with *E. chrysanthemi* were shaken at 37 C for 24 hr (Table 1).

Cells were harvested by centrifugation at 10,000 *g* for 15 min in a JA14 rotor in a refrigerated Beckman J-21C preparative centrifuge at 0 C. The pelleted cells were washed in 0.15 M NaCl, centrifuged as above, and resuspended in 10 ml of the following extraction medium: 0.5 M RNAse-free sucrose (Bethesda Research Laboratories [BRL]), 5 mM tris, 5 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 5% ribonuclease inhibitor (BRL). All extractions were carried out on ice at 0 C. Cells were disrupted by sonication with a Sorvall sonicator for 3 × 30 sec at setting 5. Cellular debris was removed by centrifuging at 10,000 *g* for 30 min. Desoxycholic acid was added to the supernatant at a final concentration of 0.5% to solubilize membrane fragments. Ribosomes were pelleted by centrifuging at 100,000 *g* for 90 min with a model 40 rotor in a model L Spinco ultracentrifuge at 0 C. Pelleted ribosome-enriched fractions were resuspended in the extraction media and clarified by centrifugation at 10,000 *g* for 30 min. The ultraviolet absorption spectrum (260–235 nm ratio) of a portion of the ribosome-enriched fraction was determined from a sample resuspended in lysis buffer (in which Cleland's reagent replaced 2-mercaptoethanol as the antioxidant) using a Beckman model 35 spectrophotometer.

Extraction and solubilization of protein sample. Samples of

TABLE 1. List of *Erwinia* spp. and strains included in this study of biochemical taxonomy

Erwinia spp. and strains[a]	Host of origin	Source
E. carotovora		
C7	Iris rhizome	W. L. Smith, Jr. Collection, Beltsville, MD
C7s	Iris rhizome	
E31	Iris leaf	
E. atroseptica		
E18	Potato tuber	W. L. Smith, Jr. Collection, Beltsville, MD
E25	Potato stalk	
E. chrysanthemi		
M 80-1	Sweet potato root	Salisbury, MD; H. E. Moline, 1980
A17	Sweet potato root	Experiment, GA; N. W. Schaad

[a] As identified by Burkholder and Smith, 1949.

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ribosome-enriched proteins (200 μg) were concentrated by lyophilization. Lyophilized proteins were solubilized in 200 μl of lysis buffer containing 9.5 M urea; 2% w/v NP40; 2% ampholins, 1.6% pH range 5 to 7 and 0.4% pH range 3 to 10; and 5% 2-mercaptoethanol (15). Protein content of 10% TCA precipitates was determined according to the procedure of Bradford (4). Standard curves were prepared from data obtained by measuring known concentrations of ovalbumin.

Isoelectric focusing of ribosomal proteins. Solubilized ribosomal protein-enriched samples were either frozen at −76 C for storage or layered onto tubes containing the first-dimension isoelectric focusing (IEF) gels. Electrophoretic separations of the IEF-separated proteins was according to the nonequilibrated gel system of O'Farrell (15) except that stacking gels were 38 mm. Separation gels contained 10% acrylamide. As many as 12 gels were run simultaneously. Just before the samples were layered onto the IEF gels, the proteins were diluted so that 10–30 μl of sample contained approximately 10 μg of protein. IEF electrophoresis was carried out for 18,000 V-hr according to O'Farrell (15).

Polyacryamide gel electrophoresis. Immediately after IEF was completed, the gel was removed from the glass tube and placed on the stacking gel of the second dimension (15). The IEF gel was attached to the stacking gel with hot 1% agarose, which in most cases contained molecular weight protein standards (phosphorylase b, ovalbumin, carbonic anhydrase, and trypsinogen of molecular weights 92, 45, 31, and 24 Kdaltons, respectively). Single-dimensional SDS polyacrylamide gel electrophoresis on 10% acrylamide separation slab gels were also run.

Following SDS-PAGE, the gels were removed and fixed for 30 min in a solution of 50% methanol and 10% acetic acid (375 ml per six gels) and then overnight in 5% methanol and 7% acetic acid (500 ml per six gels). Staining was done using the sensitive silver stain method of Switzer et al (19), as simplified by Oakley et al (14), and modified by us. We obtained the best results by thoroughly washing the gels (after gluteraldehyde treatment) in glass distilled water and changing the water several times within 3–4 hr. The extended wash seems to help prevent dark backgrounds. Routinely, six gels were placed in 375 ml of freshly prepared ammonical silver solution for 15 min with shaking. Gels (up to 12) were then removed and soaked in at least 1 L of water for at least 30 min prior to developing them with formaldehyde-citric acid solution. Gels were developed individually to the desired intensity and development was stopped by placing gels in 10% (v/v) acetic acid. Generally, this gave proteins that stained brown to dark yellow on a clear to slightly yellowed background. The longer water wash we employed after the ammonical silver solution treatment seemed to prevent much of the surface silver deposits that were frequently obtained with shorter washes. This longer water wash also helped by lengthening the development time of the proteins.

Completed, stained gels were photographed to record data on 10.2 × 12.7-cm (4 × 5 in.) negatives. The gels were stored by drying them between two cellophane dialysis tubing sheets.

TABLE 2. Comparison of protein clusters from ribosomal-enriched protein profiles on two-dimensional polyacrylamide gel electrophoresis plates[a]

Protein or protein cluster	Approximate mol. wt. (Kdaltons)	Species comparisons[b]			Strain comparisons		
		E. car.[b]	*E. as.*	*E. ch.*	*E. car.*	*E. as.*	*E. ch.*
1	33	+[c]	+	+	+	+	0
2	54	+	+	+	+	−	−
3	83	+	+	+	+	−	−
4	45	−	+	+	−	−	0
5	46	−	−	+	−	−	−
6	60	−	−	+	−	−	0
7	73	−	−	−	−	−	−

[a] This table refers to and summarizes information in Fig. 1.

[b] *E. car.* = *Erwinia carotovora*, *E. as.* = *Erwinia atroseptica*, and *E. ch.* = *Erwinia chrysanthemi*.

[c] + = protein cluster with unique characteristics, − = protein cluster without unique characteristics, and 0 = absence of protein in the corresponding area of the electrophoresis profile.

RESULTS AND DISCUSSION

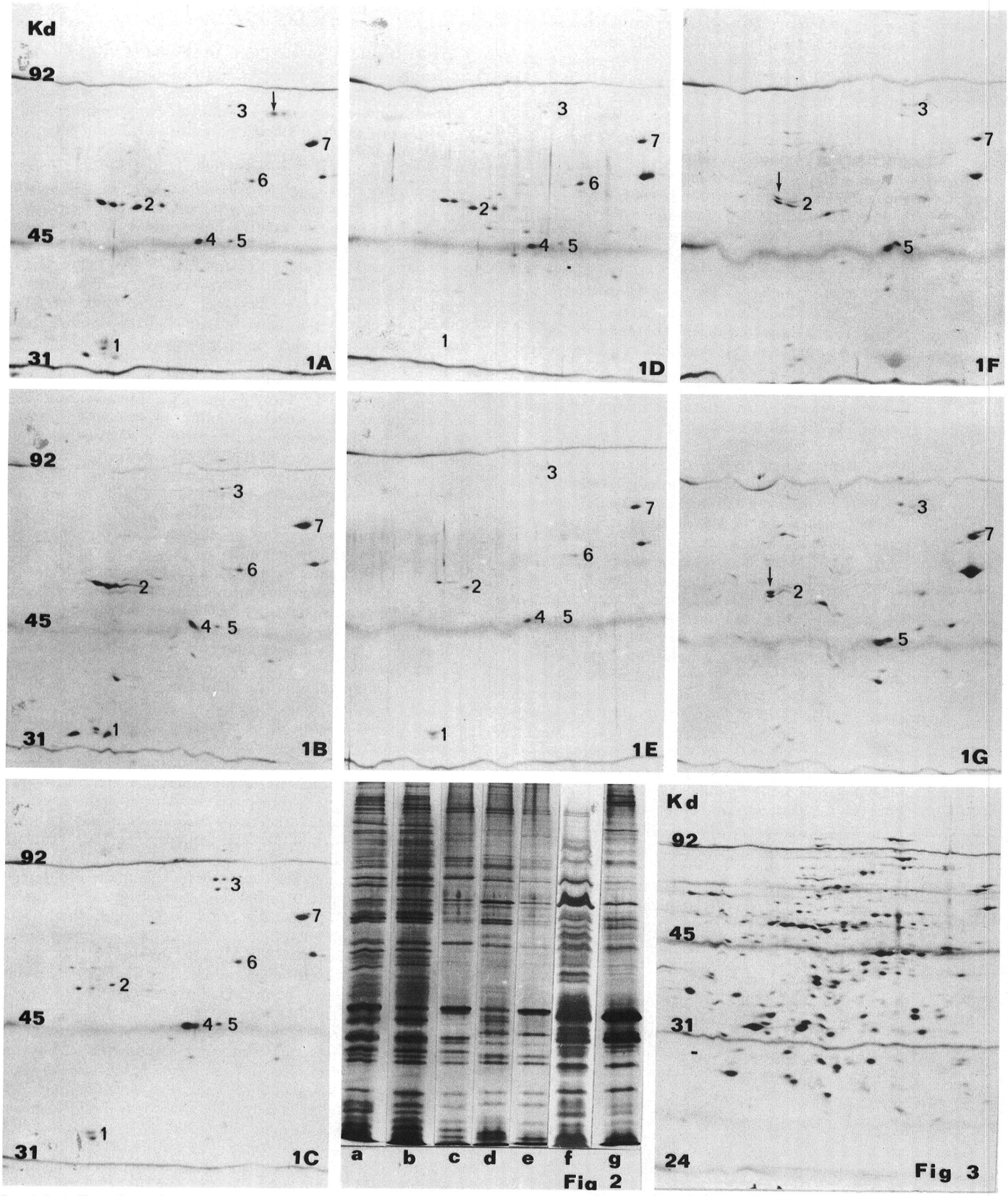
Protein patterns typical for strains of *E. carotovora*, *E. atroseptica*, and *E. chrysanthemi* are shown in Fig. 1. Several proteins or protein clusters are similar for all strains of *E. carotovora* and *E. atroseptica* examined. *E. chrysanthemi* differs from the other species in having a doublet 54-Kdalton polypeptide (Fig. 1F and G, No. 2, arrow) and a different isoelectric point (P.I.) for the 83-Kdalton polypeptides (No. 3). It also lacks a protein comparable to 45 Kdalton (No. 4) and 60 Kdalton (No. 6), which are present in all isolates of *E. carotovora* and *E. atroseptica* that were examined. The 33-Kdalton polypeptides (No. 1) vary with each species, being absent in *E. chrysanthemi* (Fig. 1F and G), present as a doublet in one isolate of *E. atroseptica* (Fig. 1E), and present as either two, three, or four separable components in the strains of *E. carotovora* that were examined (Fig. 1A–C). An 80-Kdalton polypeptide band is visible to the right of the 83-Kdalton polypeptides (No. 3) in Fig. 1A only.

Examination of the protein profiles of these representative samples from the three *Erwinia* species shows a considerable degree of homology among protein profiles. However, differences between species are sufficiently significant to allow separation of strains of the species examined based on profiles of ribosomal-enriched protein samples (Table 2). Whether differences within species are sufficient to allow subgrouping of strains remains to be determined. During this study we found strains we previously identified as members of the *E. carotovora* group (5) that fit better in the *E. atroseptica* group on the basis of protein profiles. Examples of intermediate profiles are those shown in Fig. 1C and E. These profiles appear to share some proteins with each other that they do not share with other members of their respective groups. While single-dimensional SDS-PAGE ribosomal-enriched protein separations (Fig. 2) can also reveal major differences between groups, they do not always allow for positive differentiation of species. This is consistent with the observations of others (11,17,18) who observed no quantitative differences. Archer and Stevens (2) separated *Erwinia* isolates into species based on one-dimensional paper chromatography of cytoplasmic proteins; however, they used only widely divergent species.

Comparisons of total soluble protein fractions and ribosomal protein-enriched fractions were made to determine ease of separation of selected strains as well as to see if there was some major protein segment other than ribosomal that could be used for identification. The total soluble protein profiles (Fig. 3) contained too many individual stained polypeptides to allow for easy separation. Although we could obtain quantitative differences, sorting through more than 100 stained spots on a gel was so time consuming that little benefit could be gained from the study. Computer-assisted analysis of these gels may yet prove to make total soluble protein the desired sample for study (1,10).

Ribosomal purification procedures are modified from those previously reported (17). We elected to use the sonication method employed by O'Farrell (15) rather than a French pressure cell to fracture bacterial cells because of its availability. While this method may have somewhat reduced the purity and quantity of ribosomes in our preparations it gave quite adequate and reproducible results and satisfied a major goal of the study—to identify a polypeptide fraction that could offer a relatively rapid method of screening soft-rotting *Erwinia* strains. We found it essential to complete purification of the ribosomal fraction once cells had been sonicated. Experiments in which the soluble proteins were washed overnight before ribosomal enriched samples were pelleted gave extremely low yields, and gels of these preparations indicated that ribosomal integrity was not maintained following this procedure. We recognize that our purification procedure may permit the inclusion of contaminants in our ribosomal enriched preparations because we did not separate the fraction on a density gradient. However, UV scans indicated that our preparations were quite clean with an $A_{260-235\ nm}$ ratio of 1.7. The number of major polypeptide bands and reproducibility of our purification procedure was our major concern.

Acidic and basic protein profiles were run on the second-



Figs. 1–3. 1. Two-dimensional polyacrylamide gel electrophoresis profiles of silver stained ribosomal-enriched proteins from strains of three *Erwinia* spp. Molecular weight markers appear as horizontal streaks across the gel and are labeled on the left margin of **A–C**, at the top of 92 Kdaltons, middle 45 Kdaltons, and bottom 31 Kdaltons. Approximate molecular weights of correspondingly numbered polypeptide clusters: 1 = 33 Kdaltons; 2 = 54 Kdaltons; 3 = 83 Kdaltons; 4 = 45 Kdaltons; 5 = 46 Kdaltons; 6 = 60 Kdaltons; and 7 = 73 Kdaltons. In each gel, the slightly basic to neutral proteins appear to the left and the acidic proteins to the right. **A–C.** Strains of *E. carotovora* grown at 20 C. **A** = strain C7; **B** = strain C7s, a variant of C7; **C** = strain E31. **D–E.** *E. atroseptica* strains grown at 20 C. **D** = strain E18; **E** = strain E25. **F–G.** *E. chrysanthemi* strains grown at 37 C. **F** = strain M80-1; **G** = strain A17. While several similarities exist in these patterns, differences are identifiable at markers 1, 2, 3, 4, and 6; arrows also highlight differences. **2.** A single dimensional SDS-polyacrylamide gel electrophoresis pattern of silver-stained ribosome enriched proteins. Separation of strains C-7, C-7s, E-31 of *Erwinia carotovora* (ABC), strains E18 and E25 of *E. atroseptica* (DE), and strains M80-1 and A17 of *E. chrysanthemi* (FG). The figure represents lanes A–G of a single slab gel from which lanes were cut and compressed for mounting. **3.** Two-dimensional polyacrylamide gel electrophoresis pattern of total soluble protein fraction from *Erwinia carotovora* C7. Molecular weight markers from the top to bottom are 92, 45, 31, and 24 Kdaltons. Basic proteins appear on the left and acidic proteins on the right.

dimensional gels. It is well documented that the majority of ribosomal proteins are basic, with no more than four or five acidic proteins present in purified ribosomal preparations (11,17,21). We chose acidic protein profiles for our comparisons as there were relatively few polypeptide spots on the gels, and preliminary tests revealed significant differences in these spots among the bacterial isolates studied (Fig. 1). Basic protein fractions contained more polypeptides, but few of these spots were different enough to allow strain differentiation. Use of ovalbumin as a standard for protein determination produced better reproducibility of gels than the BSA that was used initially.

Electrophoretic patterns of ribosomal protein-enriched samples are potentially useful for comparing bacterial strains (8,11,17). While the full potential of 2-D electrophoretic separation of ribosomal proteins of *Erwinia* spp. remains to be determined, results of this study indicate the potential for further investigation. Other studies have proven the usefulness of this tool for separation of bacterial strains (13,16).

The major differences between profiles of ribosomal protein-enriched strains of *E. carotovora* and *E. chrysanthemi* may make it possible to separate these species on the basis of one-dimensional electrophoretic patterns (Fig. 3). Kado et al (11) found it possible to identify widely divergent *Erwinia* spp. by subjecting basic proteins of 70S ribosomes to one-dimensional polyacrylamide gel electrophoresis (PAGE). There may be little to justify the use of 2-D PAGE analysis where the single-dimensional PAGE separation will show clear differences. However, the wide divergence of biochemical responses of strains within these three species used in this study (6) can benefit from a closer analysis of structural proteins such as ribosomes. A study of this kind is currently underway, which may lead to more objective methods of grouping bacterial strains and thus help reduce the current confusion about taxonomic criteria.

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