Differentiation of Postharvest Soft Rotting Bacteria with Two-Dimensional Polyacrylamide Gel Electrophoresis

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ABSTRACT

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Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of acidic ribosome-enriched proteins was used to differentiate several strains of *Erwinia* and *Pseudomonas*. Thirty-five erwinias including *E. carotovora* pv. *carotovora*, *E. carotovora* pv. *atroseptica*, *E. chrysanthemi*, *E. rhapontici*, *E. amylovora*, *E. herbicola*, and unknown soft rotting strains were compared to strains of *Pseudomonas fluorescens*. Soft rotting bacterial strains could be readily differentiated with this technique; *E.c. carotovora* and *E.c. atroseptica* were consistently distinct enough to be separated into two species and unknown soft rotting isolates could be identified.

Identification of postharvest soft rotting bacteria can be time-consuming and inexact. Recent taxonomic treatments have not produced agreement as to the nomenclature of this group of pathogens (3,9,12). It was demonstrated in an earlier study (5) that analysis of acidic ribosomal proteins may provide a way of characterizing strains of *Erwinia*.

This report describes the use of two-dimensional electrophoresis of acidic ribosomal proteins to differentiate a number of *Erwinia* spp., to distinguish them from soft-rotting *Pseudomonas* strains, and to identify unknown soft-rotting bacteria.

MATERIALS AND METHODS

Bacterial ribosomes were extracted from cells of 37 bacterial strains (Table 1) according to the procedure of Tissieres et al (11) and Schaad (8) as outlined previously (5) with the following modifications. Prior to disruption, pelleted bacterial cells were washed overnight in 0.15 M NaCl on a rotary shaker at 0 C. The extraction medium contained 0.5 M RNase-free sucrose, 10 mM tris, 5 mM $MgCl_2$, and 6 mM 2-mercaptoethanol. Ribonuclease inhibitor was not added, as intact ribosomal RNA was not required.

Cells were disrupted by sonication for 3×15 sec with a microtip on a Fisher model 320 Sonic Dismembrator operated at 35% of total power with an output reading of 0.5 (relative output 50%). Ribosome-enriched fractions were obtained by centrifugation at 100,000 *g* for 2 hr in a 70 Ti rotor in a Beckman L8-55 ultracentrifuge. Ribosome-enriched fractions were resuspended in the extraction medium by shaking on a rotary shaker overnight at 0 C and clarified by centrifuging at 10,000 *g* for 30 min.

Ribosome-enriched proteins (500 μl) were concentrated by freeze-drying. Freeze-dried proteins were solubilized in 100 μl of lysis buffer (5). Protein content of samples was determined according to the procedure of Bradford (1) with the following modifications: protein was placed directly in a filtered Bio-Rad protein assay mixture that had been diluted 4:1 with glass distilled water, and the absorbance was measured at 595 nm with a Beckman DU spectrophotometer.

Isoelectric focusing electrophoresis (IEF) was carried out overnight for an equivalent of 4,800 volt-hours, then at 800 V for 1 hr according to the procedure of O'Farrell (7) in a Bio-Rad model 150A gel electrophoresis cell.

Immediately following IEF, gels were removed from glass tubes and attached to the stacking gel of the second dimension with hot 1% agarose (5,7). Second-dimension 8% SDS-PAGE slab gels were run in a BRL model V162 vertical gel electrophoresis at 20 ma/gel according to the procedures of O'Farrell (7).

Molecular weight protein standards were co-electrophoresed in an outside track of polyacrylamide gels. The molecular weights of these standards (myosin, β-galactosidase, phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme) ranged from 10,000 to 250,000 (hereafter, their approximate molecular masses will be given in kdaltons).

Staining and fixation of proteins in gels was done according to the methods of Steck et al (10) with the following modifications: After SDS-PAGE, the gels were removed and simultaneously fixed and stained in a solution containing 180 ml of 95% ethanol, 420 ml of deionized distilled water, 100 ml of 35% formaldehyde, and 0.8 g of Coomassie Brilliant Blue R-250 for 2–3 hr. Gels were then destained in 250 ml of 95% ethanol, 750 ml of deionized distilled water, and 10 ml of 35% formaldehyde until a clear background was achieved (approximately 3 hr with several changes). Gels were washed overnight in deionized distilled water and restained in silver nitrate (5). After silver staining, gels were stored in a solution containing 5% acetic acid and 2% glycerol until photographed and dried.

RESULTS AND DISCUSSION

Electrophoretic profiles of acidic ribosome-enriched proteins of selected strains of *Erwinia carotovora* pv. *atroseptica*, *E.c. carotovora*, *E. chrysanthemi*, *E. rhapontici*, *E. amylovora*, *E. herbicola*, and a soft rotting strain of *Pseudomonas fluorescens* are shown in Fig. 1. Seven major protein clusters have been identified (5) that were used to differentiate these strains. The approximate molecular weights of these proteins are shown and differences between profiles of strains are summarized in Table 2. Although several protein clusters appeared that were similar in strains of *E.c. atroseptica*, *E.c. carotovora*, and *E. chrysanthemi*, there were

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differences in the presence of the 33 kdalton, 45 kdalton, and 54 kdalton polypeptide bands (Fig. 1A–C, clusters 1, 4, and 2) (5). The use of silver nitrate and Coomassie Blue to stain the gels gave a greater degree of sensitivity than afforded by the silver stain used in an earlier study (5).

Ribosome-enriched protein profiles of two strains of *E. rhapontici* were compared to other strains of *Erwinia* (Table 2). It was easy to distinguish the 2-D PAGE protein profiles of these strains (Fig. 1D) from those of the other three soft rotting *Erwinia* spp. (Fig. 1A–C) by the presence in *E. rhapontici* of an additional major acidic polypeptide band of approximately 46 kdaltons not present on any other gels, directly beneath the 73 kdalton polypeptide band (Fig. 1D, cluster 7). The 73 kdalton polypeptide in *E. rhapontici* is similar to that in strains of *E.c. carotovora* (Fig. 1B). Other similarities between ribosome-enriched protein fractions from the *E.c. carotovora* (Fig. 1B) and *E. rhapontici* (Fig. 1D) strains examined included two 54 kdalton polypeptides (cluster 2), a single 83 kdalton band (cluster 3), two 45–46 kdalton polypeptides (clusters 4 and 5) and a major 33 kdalton polypeptide component (cluster 1).

A soft rotting strain of *P. fluorescens* (Fig. 1E) and a previously unidentified soft rotting pseudomonad isolated from seed coats of lima bean were also distinguishable based on their 2-D PAGE polypeptide profiles by the almost total absence of acidic polypeptides. One diffuse, moderately acidic, 73 kdalton polypeptide band was present approximately in the center of the gel. Two less acidic polypeptide bands of slightly lower molecular weight (70–72 kdaltons) and another major (32 kdaltons) polypeptide band also occurred in this strain. These strains could also be differentiated from the soft rotting *Erwinia* spp. on the basis of the purification characteristics of the ribosomal fraction. An exopolysaccharide slime was present that did not separate from the ribosome fraction after the cell contents were lysed and the cell walls removed. A portion of this exopolysaccharide sedimented with the ribosomal fraction resulting in a ribosome pellet of abnormally high volume. However, this pellet contained a low specific volume of protein relative to purified ribosomal fractions of the strains of *Erwinia* that were studied.

Non-soft rotting *Erwinia* spp. that were studied were easily differentiated from soft rotting *Erwinia* spp. on the basis of the ribosomal-protein profiles. The strains of *E. amylovora* (Fig. 1F) and *E. herbicola* (Fig. 1G) contained few acidic ribosomal proteins. Two acidic polypeptides of molecular weight 73 (no. 7) and 45 kdaltons (nos. 4 and 5) and several slightly basic polypeptides appearing toward the left margin of the gel were characteristic of the strains of *E. amylovora* that were studied (Fig. 1F). The presence of one doublet and another very large 35 kdalton polypeptide band was characteristic of the strains of *E. herbicola* examined (Fig. 1G) distinguishing it from all other gels. No comparable polypeptide patterns were observed in any of the pathogenic soft rotting bacterial strains that were studied (Fig. 1A–E).

The 2-D PAGE profiles of ribosomal proteins from type strains of *E.c. carotovora* and *E.c. atroseptica* obtained from the American Type Culture Collection, Rockville, MD, appeared to be nearly identical (Fig. 2). These protein profiles were similar enough to other strains of *E.c. carotovora* that were studied to allow inclusion of these two strains in the '*carotovora*' group on the basis of 2-D PAGE ribosomal-protein analysis, while the protein profiles from the remaining 12 strains of *E.c. atroseptica* and 14 strains of *E.c. carotovora* compared in this study (Table 2) were consistently different enough to separate them into two distinct groups. These results reinforce the necessity to exercise caution when evaluating material classified by others, and further indicate the confusion that exists in the taxonomy of the genus (9). A careful evaluation of strains of *Erwinia* used by investigators employing the subspecies designation to differentiate between the '*carotovora*' and '*atroseptica*' groups may reveal similar problems. The 12 strains of

TABLE 1. Bacterial strains used in this biochemical taxonomy study and their sources

Strain	Host	Source
Erwinia carotovora		
pv. *atroseptica*		
E1, E3, E4, E6, E8, E15, E25, E26, E27	Potato stalk	W. L. Smith, Jr. Collection[a]
E18, E24, C2	Potato tuber	W. L. Smith, Jr. Collection
33260	Potato tuber	ATCC (Type strain)
pv. *carotovora*		
C3, C6, E40	Celery stalk	W. L. Smith, Jr. Collection
C7, C9, E32	Iris rhizome	W. L. Smith, Jr. Collection
C14	Calla rhizome	W. L. Smith, Jr. Collection
E9, E11	Lettuce leaf	W. L. Smith, Jr. Collection
E21	Carrot root	W. L. Smith, Jr. Collection
E22, E60	Potato tuber	W. L. Smith, Jr. Collection
E31	Iris rhizome	W. L. Smith, Jr. Collection
12312	Tobacco leaves	ATCC
15713	Potato	ATCC (Type strain)
E. rhapontici		
1025, 1026	Rhubarb	Ange, France; J. Louisetti
E. chrysanthemi		
11663	Chrysanthemum	ATCC (Type strain)
A17	Sweet potato root	Experiment, GA; N. W. Schaad
M80-1	Sweet potato root	Salisbury, MD; H. E. Moline
E. amylovora		
15580	Pear twig	Kearneysville, WV; T. VanderZwet
E. herbicola		
33243	Type strain	ATCC
Pseudomonas fluorescens	Potato tuber	W. L. Smith, Jr. Collection
Pseudomonas sp.	Lima bean seed	Beltsville, MD; H. E. Moline

[a] As identified by Burkholder and Smith (2) and maintained at Beltsville, MD.

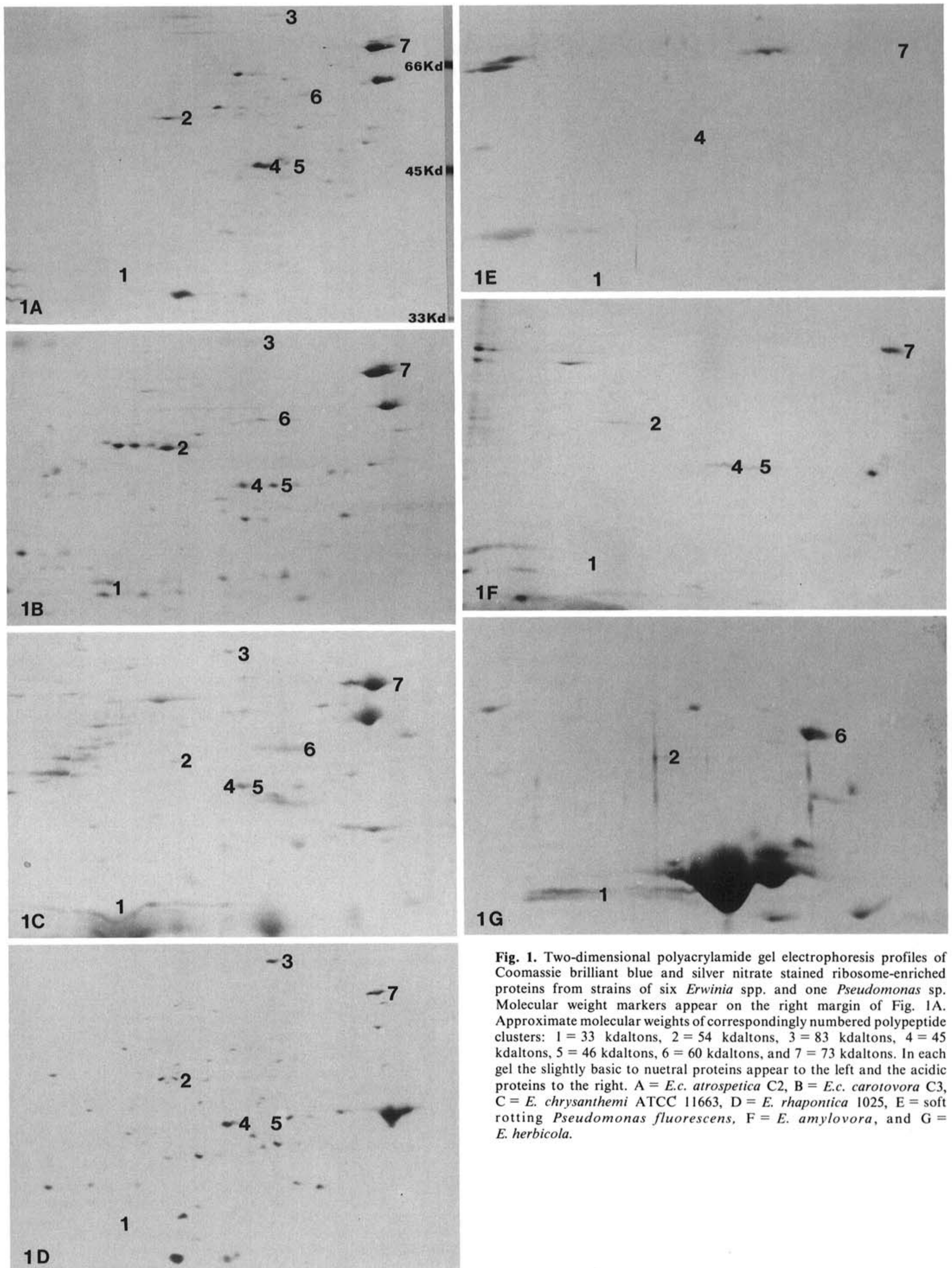

Fig. 1. Two-dimensional polyacrylamide gel electrophoresis profiles of Coomassie brilliant blue and silver nitrate stained ribosome-enriched proteins from strains of six *Erwinia* spp. and one *Pseudomonas* sp. Molecular weight markers appear on the right margin of Fig. 1A. Approximate molecular weights 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 nuetral proteins appear to the left and the acidic proteins to the right. A = *E.c. atrospetica* C2, B = *E.c. carotovora* C3, C = *E. chrysanthemi* ATCC 11663, D = *E. rhapontica* 1025, E = soft rotting *Pseudomonas fluorescens,* F = *E. amylovora*, and G = *E. herbicola.*

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

Bacterial strains examined[a]	Protein clusters with approximate mol. wt.						
	1 (33 kd)[b]	2 (54 kd)	3 (83 kd)	4 (45 kd)	5 (46 kd)	6 (60 kd)	7 (73 kd)
Erwinia carotovora							
pv. *atroseptica*							
E1, E3, E4, E6, E8, E15, E25	0[c]	+	+	+	−	−	−
E26, E27	+	+	+	+	−	−	−
E18	0	+	+	+	−	−	−
E24	+	+	+	+	−	−	−
C2	0	+	0	+	−	−	−
ATCC 33260	+	+	+	+	−	−	−
pv. *carotovora*							
C3, C6, E40, C7, C9, E32	+	+	+	−	−	−	−
C14, E9, E11, E21, E22, E60	+	+	+	−	−	−	−
E31, ATCC 12312, ATCC 15713	+	+	+	−	−	−	−
Erwinia chrysanthemi							
ATCC 11663, A17, M80-1	+	+	+	+	+	+	−
Erwinia rhapontici							
1025, 1026	0	+	+	−	0	0	−
Erwinia amylovora							
ATCC 15580	0	+	0	+	−	0	−
Erwinia herbicola							
ATCC 33243	+	+	0	0	0	+	0
Pseudomonas fluorescens							
Pf-1, Psp	0	0	0	+	0	0	0

[a] This table summarizes data obtained from the 37 bacterial strains that were examined and are listed in Table 1.
[b] The abbreviation "kd" = kilodaltons (approximate molecular mass).
[c] 0 = absence of protein in corresponding area of the electrophoresis profile; + = protein cluster with unique characteristics; and − = protein cluster without unique characteristics.

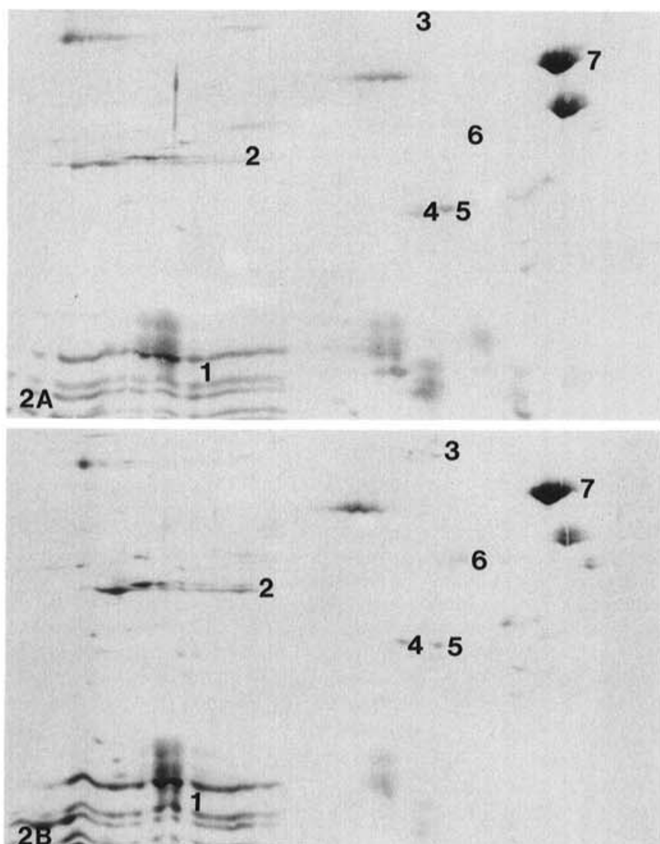

Fig. 2. Comparative two-dimensional polyacrylamide gel electrophoresis of profiles of ATCC type strains of **A,** *Erwinia carotovora* pv. *atroseptica* 33260 and **B,** *E.c.* pv. *carotovora* 15713.

E.c. atroseptica from the Burkholder and Smith collection (2) that were studied consistently produce black leg symptoms on potato stems and can also be differentiated from the 14 strains of *E.c. carotovora* on the basis of biochemical tests (Table 1). The pathogenicity of these strains has been maintained since their isolation by Burkholder and Smith (2). A major reason for the current confusion is the fact that standard biochemical procedures are not specific enough to clearly differentiate strains of '*atroseptica*' from those of '*carotovora*'; consequently, all strains have been lumped into the '*carotovora*' group (3,9,12). The 2-D PAGE analysis of acidic ribosomal proteins shows that by using a very small proportion of the total ribosomal proteins (90% are basic proteins) not only can soft-rotting *Erwinia* strains be distinguished, but also they may be differentiated from other soft-rotting bacteria (in this instance *P. fluorescens*) and from other *Erwinia* spp. While all of the 2-D PAGE profiles of soft rotting *Erwinia* spp. contained similar polypeptides (Table 2), differences were sufficient to separate them on the basis of the seven polypeptide clusters selected as markers (Fig. 1).

E.c. carotovora and *E.c. atroseptica* should be considered species, not pathovars; the evidence presented herein supports this position. Unpublished data on fatty acid composition also support this view (M. Sasser, *personal communication*). Further subgrouping of bacterial strains on the basis of 2-D PAGE profiles (6) may be possible by using computer assisted mapping of protein patterns (4).

LITERATURE CITED

1. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
2. Burkholder, W. H., and Smith, W. L., Jr. 1949. *Erwinia atroseptica* (Van Hall) Jennison and *Erwinia carotovora* (Jones) Holland. Phytopathology 39:887-897.
3. Dye, D. W. 1969. A taxonomic study of the genus *Erwinia*. II. The

'*carotovora*' group. N.Z. J. Sci. 12:81-97.
4. Hruschka, W. R., Massie, D. R., and Anderson, J. D. 1981. High resolution two-dimensional electrophoresis of soybean embryonic axis proteins. Computerized Data Analysis Proc. 1981. HP 1000 Int. Users Group Conference, Washington, DC.
5. Moline, H. E., Johnson, K. S., and Anderson, J. D. 1983. Evaluation of two-dimensional polyacrylamide gel electrophoresis of acidic proteins of ribosome preparations for identifying plant pathogenic soft-rotting bacteria. Phytopathology 73:224-227.
6. Noel, K. N., and Brill, W. J. 1980. Diversity and dynamics of indigenous *Rhizobium japonicum* populations. Appl. Environ. Microbiol. 40:931-938.
7. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021.
8. Schaad, N. W. 1974. Comparative immunology of ribosomes and disc gel electrophoresis of ribosomal proteins from Erwiniae, plectobacteria, and other members of the family Enterobacteriaceae. Int. J. Syst. Bacteriol. 24:42-45.
9. Starr, M. P. 1981. The genus *Erwinia*. Pages 1260-1271 in: The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria. Vol. II. M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. E. Schlegel, eds., Springer-Verlag, New York. 2284 pp.
10. Steck, G., Leuthard, P., and Burk, R. R. 1980. Detection of basic proteins and low molecular weight peptides in polyacrylamide gels by formaldehyde fixation. Anal. Biochem. 107:21-24.
11. Tissieres, H., Watson, T. D., Schlessinger, D., and Hollingsworth, B. R. 1959. Ribonucleoprotein particles from *Escherichia coli*. J. Mol. Biol. 1:221-233.
12. Tsuchiya, K., Matsuyama, N., and Wakimoto, S. 1983. Studies on the relationship between virulence and bacteriological properties in *Erwinia carotovora* subsp. *carotovora*. J. Fac. Agric. Kyushu Univ. 27:197-207.