Characterization of *Pseudomonas solanacearum* Biovars Based on Membrane Protein Patterns

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ABSTRACT

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Membrane proteins were extracted from 65 isolates representing biovars 1, 2, and 3 of *Pseudomonas solanacearum*. The isolates were from five regions of Brazil and seven host species. The proteins were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All strains of biovars 1 and 3 had protein bands with approximate molecular masses of 35–37 kDa, which were absent in biovar 2 strains. A similarity matrix, based on the presence, absence, or intensity of an average of 29 different bands, revealed the relationships between strains classified in all three biovars. Comparisons of *P. solanacearum* to three other nonfluorescent pseudomonads and to four pathovars of *Xanthomonas campestris* showed close similarity within the first group, contrasting to clear separation from xanthomonads. Virulent, extracellular polysaccharide-producing isolates of *P. solanacearum* and nonfluidal forms of those same strains had similar protein patterns.

Additional keywords: fluorescent pseudomonads.

Intraspecific grouping of *Pseudomonas solanacearum* remains a complex subject. Arrangements based on host specificity, which resulted in five races (2,3,6), did not coincide with grouping based on physiological criteria (5,6), which lead to the recognition of five biovars. Protein fractions of the cell envelopes have been used to characterize *Xanthomonas* and *Pseudomonas* species (4,12,15) and also pathovars of *X. campestris* (12,14). To supply additional data which may help to understand the complex species

P. solanacearum, we compared the membrane proteins of 65 isolates of this bacterium by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We found that membrane protein patterns may be useful for taxonomic studies of *P. solanacearum*.

MATERIALS AND METHODS

Bacterial isolate and biovar determination. Isolates of *P. solanacearum* were collected from seven plant species growing in 14 Brazilian states during 1981–1986. The hosts included potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.), eggplant (*Solanum melongena* L.), eucalyptus (*Eucalyptus grandis* Hill ex Maiden, *E. pellita* F. Muell., and *E. urophylla* S.T. Blake), banana (*Musa* sp.), pepper (*Capsicum annuum* L.), and *Solanum gilo* Raddi. Isolates were preserved in sterile tap water (9) and lyophilized or desiccated in filter paper (16). All isolates were tested for pathogenicity in their original hosts. Isolates of *X. campestris, P. cichorii, P. syringae* pv. *tabaci, P. syringae* pv. *glycinea, P. viridiflava*, and *P. caryophylli* also were compared to *P. solanacearum*.

Fluidal colonies of *P. solanacearum* were selected from Kelman's tetrazolium medium (8) before growing each isolate for biovar determination (5). Sugar alcohols (sorbitol, mannitol, or dulcitol) or carbohydrates (maltose, lactose, or cellobiose) were used as carbon sources in a basal medium containing 1 g of $NH_4H_2PO_4$, 0.2 g of KCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1 g of peptone, in 1,000 ml of distilled water, pH 6.8–7.0 adjusted with 1 N KOH. Cultures were incubated at 30 C for 10 days to determine changes in color of the indicator dye, bromothymol blue.

Membrane isolation. Colonies of *P. solanacearum* selected for fluidity on Kelman's medium were grown in liquid 523 medium (7) at 30 C and 150 rpm. Logarithmic phase cells ($A_{550nm} = 0.6–0.8$) were harvested by centrifugation at 13,200 *g* for 20 min at 4 C and washed twice in cold 3.3 mM Tris-HCl, pH 7.4 (Buffer 1). The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, containing 0.75 M sucrose and 100 μg of lysozyme per milliliter, followed by a 10-min incubation on ice. Cells were ruptured by adding two volumes of Buffer 1. This ice-cooled suspension was then sonicated in a Biosonik Model Bio II apparatus (Bronwill Scientific, Rochester, NY) at maximum power. Eight 30-sec sonication cycles separated by identical resting intervals were applied to the suspensions. The resulting membrane suspension was incubated for 30 min at 5 C before centrifuging at 4,080 *g* for 15 min to remove whole cells present in the pellet. The supernatant containing the total membrane fraction was centrifuged at 36,900 *g* for 60 min. The pellet, which was designated the total membrane fraction, was resuspended and washed twice in Buffer 1 before it was suspended in minimum volume of Buffer 1 containing 0.25 M sucrose. Membrane fractions were stored at −20 C. Nonfluidal variants of isolates 577 (biovar 1), 147 (biovar 2), and 139 (biovar 3) also were selected in Kelman's medium and their membranes extracted.

Membranes also were extracted from the following reference strains all of which are deposited in the culture collection of the Universidade de Brasília: *X. c. cassavae* 59, *X. c. manihotis* 184, *X. c. vesicatoria* 545, *X. c. campestris* 33, *Pseudomonas caryophilli* 17, *P. cichorii* 492, *P. syringae* pv. *tabaci* 647, *P. syringae* pv. *glycinea* 670, and *P. viridiflava* 681.

Electron microscopy. Total membranes suspended in Buffer 1 were centrifuged for 3 hr at 90,000 *g*. The pellet was fixed for 16 hr in 3% glutaraldehyde, contained in 0.05 M phosphate buffer, pH 7.4. Fixed pellets were washed three times in 0.05 M cacodylate buffer, pH 7.2, before treatment for 2 hr with 1% osmium tetroxide dissolved in the same buffer. The pellets then were washed in distilled water and prestained in 0.5% uranyl acetate for 16 hr at 10 C. Dehydration of the fixed membranes was done in an acetone series before embedding in Spurr's medium. Ultrathin sections were stained in 4% uranyl acetate and 1% lead citrate before they were examined in a JEOL-JEM-100 C electron microscope.

SDS-polyacrylamide gel electrophoresis. SDS-PAGE with 0.1% SDS was conducted according to Laemmli (10). A stacking gel with 5% acrylamide was layered on top of a 16% acrylamide gel in a Hoeffer slab gel apparatus. Thirty micrograms of protein (11) was applied to each sample well. Proteins were released from membranes by incubating for 5 min at 100 C in sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue). Molecular weight standards (Sigma, St. Louis, MO) were run in each slab gel. Electrophoresis was initiated with 5 mA current until the tracking dye migrated beyond the stacking gel; the current then was increased to a constant 10 mA for approximately 18 hr. Then gels were fixed for 60 min in 30% methanol with 7% acetic acid and stained with 1% Coomassie Brilliant Blue G for 12 hr. Destaining was performed in fixing solution. Approximate

TABLE 1. Values attributed to the comparisons between pairs of reference bands showing color intensities I–V in the gel

Band color comparisons[a]	Values[b]
I vs. II	5
I vs. III	5
I vs. IV	5
I vs. V	5
II vs. III	1
II vs. IV	2
II vs. V	3
III vs. IV	1
III vs. V	2
IV vs. V	1

[a] I = absent, II = incipient, III = weak, IV = intense, and V = very intense.

[b] These values were used to calculate the Similarity Coefficient. They express quantitative differences between isolates of each pair for the same reference band.

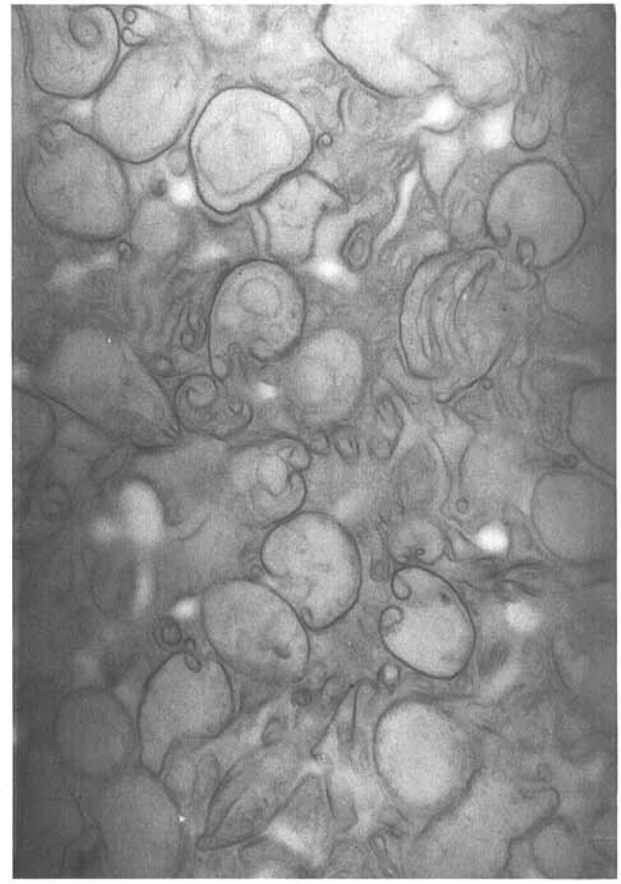

Fig. 1. Total membranes of biovar 1 isolate of *Pseudomonas solanacearum* from *Eucalyptus urophylla* as viewed in electron microscopy ×28,000.

molecular masses, in kilodaltons (kDa), were estimated according to Weber and Osborn (17).

Similarity matrix. A similarity matrix was based on the intensity of seven major bands selected as reference bands (A–G, 85, 83, 74, 39, 35–37, 24–26, and 23 kDa, respectively). The two band complexes (E and F) were included. The E complex contained three bands of 35, 36, and 37 kDa, and the F complex contained only one wide band of 24–26 kDa. The reference bands were assigned to five classes according to their visual intensity (Table 1). Pairwise comparisons were made and values were attributed to differences in intensity. The sum of these values for seven reference bands (SV) and the difference in number of secondary bands of the two isolates being compared (IDsI) were used to calculate a similarity coefficient (SC):

$$SC = \frac{RB}{RB + SV} \times \frac{RB}{RB + IDsI} \times 100,$$

where RB = 7, the number of reference bands.

The SC was calcuated for all possible pairwise combinations.

RESULTS

Electron microscopy. Membrane preparations, when viewed by electron microscopy (Fig. 1), consisted of concentrated packets of circular and C-shaped membraneous elements, as is characteristic of preparations described for other gram-negative bacteria (14). These sections verified the purity and nature of the preparations used for SDS-PAGE gels.

Biovar designations. Among the 65 isolates studied, 36 were identified as biovar 1, 12 as biovar 2, and 17 as biovar 3. Biovar 1 isolates had been obtained from two solanaceous (tomato and potato) and two nonsolanaceous (banana and eucalyptus) hosts, whereas biovars 2 (from tomato, eggplant, and potato) and 3 (from tomato, *S. gilo*, eggplant, and pepper) had been isolated only from species belonging to the family Solanaceae.

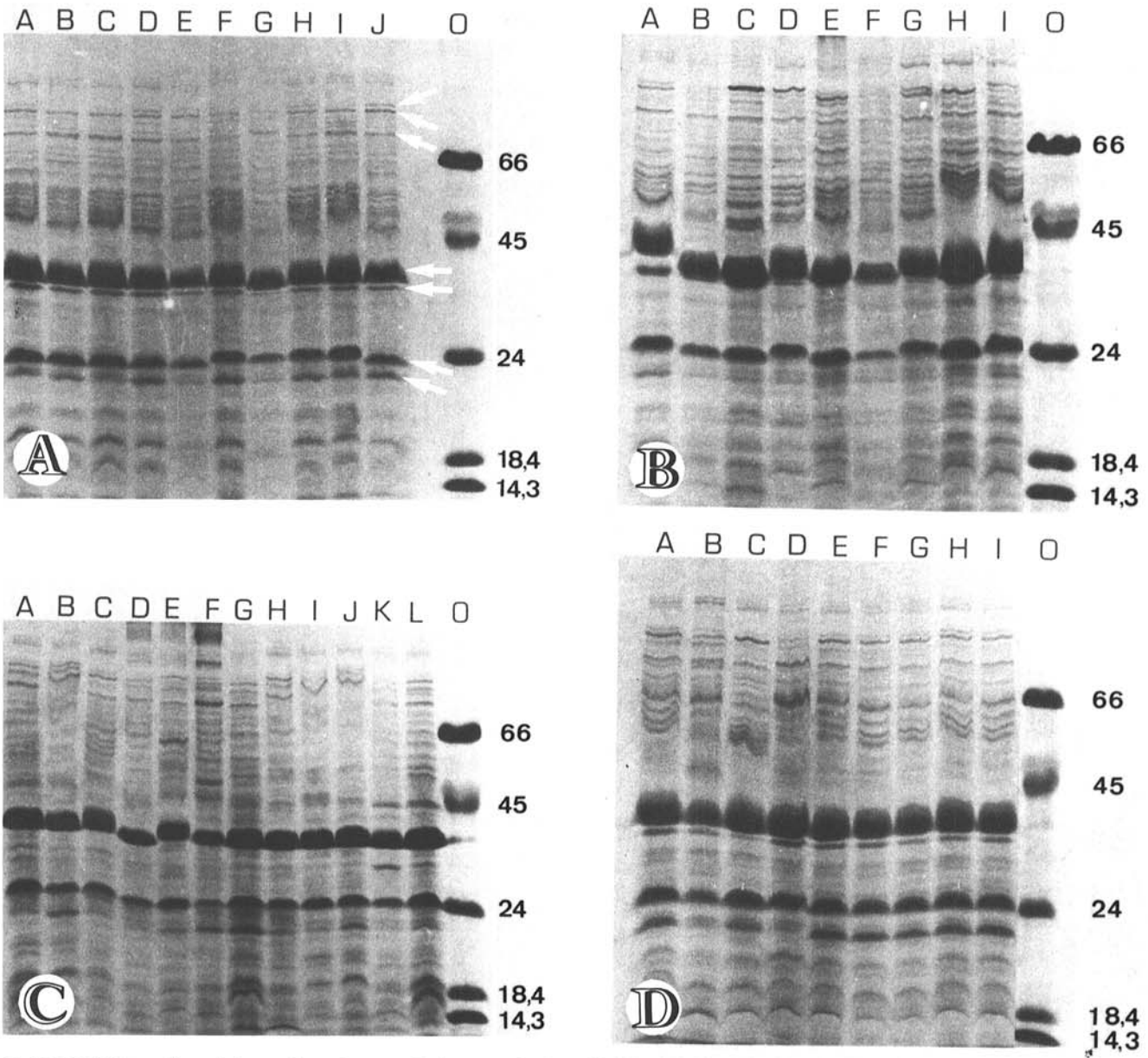

Fig. 2. SDS-PAGE profiles of the total membranes of: **A,** potato isolates 38 (A), 612 (B), 578 (C), 470 (D), 486 (E), 115 (F), 134 (G), 158 (H), 126 (I), and 93 (J) belonging to biovar 1 of *Pseudomonas solanacearum*; **B,** Eucalyptus isolates 603 (A), 539 (B), 540 (C), 573 (D), 574 (E), 575 (F), 576 (G), 577 (H), and 579 (I) belonging to biovar 1 of *P. solanacearum*; **C,** isolates 80, 210, and 147 from tomato (A–C), 88 from eggplant (D); 116, 60, 68, 41, 40, 107, 102, and 171 from potato (E–L); all belonging to biovar 2 of *P. solanacearum*; **D,** isolates 75 (A) from *Solanum gilo*; 76, 163, and 534 (B–D) from eggplant; 77, 142, 582, 468, and 625 (E–I) from pepper; all belonging to biovar 3 of *P. solanacearum*. Columns labeled O show the molecular weight standards and their respective values in kilodaltons. Arrows from top to bottom indicate reference bands A–G with approximate molecular masses of 85, 83, 74, 39, 35–37, 24–26, and 23 kilodaltons, respectively.

Membrane protein patterns. The initial comparisons in each gel involved isolates of the same biovar. Isolates from the same hosts within each biovar showed uniform patterns (Fig. 2A–D), except for those isolated from *Eucalyptus* species (Fig. 2B).

The seven reference bands selected for qualitative and quantitative differentiation of the isolates were clearly separated on the gels. The band E complex (35–37 kDa) was a major character present in all biovar 1 isolates from potato, tomato, and eucalyptus (Fig. 2A shows results from potato isolates). In all banana isolates, however, it was impossible to separate band E from band D, whereas three *Eucalyptus* isolates out of nine showed the bands D and E well developed (Fig. 2B). In all biovar 3 isolates, it was present as a strong 35 kDa band (Fig. 2D). Finally, the E band complex was absent in all biovar 2 isolates from tomato, eggplant, and potato (Fig. 2C).

When representative isolates of the three biovars of *P. solanacearum* were compared to four different pathovars of *X. campestris* (Fig. 3), the two species could be easily separated on the basis of the main bands. *X. c.* pvs. *campestris, cassavae, manihotis,* and *vesicatoria* had bands at 44 and 20 kDa, whereas *P. solanacearum* had main proteins at 39 and 24–26 kDa.

Comparisons of *P. solanacearum* with fluorescent and another nonfluorescent *Pseudomonas* species (Fig. 4) showed clear differences. The fluorescent species had a 44 kDa major band that was absent in all isolates of *P. solanacearum*. On the other hand, the 24–25 kDa major band of *P. solanacearum* was not present in any of the fluorescent isolates tested. Only the isolate of *P. cichorii* showed an intense but narrow 39 kDa band that was similar to the main membrane protein of *P. solanacearum*. The nonfluorescent *P. caryophylli* showed protein patterns similar to those of the three biovars of *P. solanacearum* including two main bands with sizes identical to those of *P. solanacearum*. Major differences between the two nonfluorescent species were detected only when comparisons were based on bands ranging from 45 to 63 kDa. Avirulent colonies selected in Kelman's medium showed protein patterns identical to virulent forms of the same isolates grown either in solid or in liquid medium.

A similarity matrix was built taking into account differences in the number of secondary bands and the intensity of seven reference bands (Fig. 5). It presents a general view of the population and reflects major differences between groups of isolates, mainly due to the lack of a band in the 35–37 kDa range in biovar 2.

DISCUSSION

Baptist et al (1) separated strains from four different biovars of *P. solanacearum* into two groups based on starch gel electrophoresis of nine enzymes. These two groups were similar to those previously described by Palleroni and Doudoroff (13), who used DNA homology and nutritional characteristics to separate 23 strains of *P. solanacearum* belonging to four biovars. In both cases biovars 1 and 2 were placed in one group and biovars 3 and 4 in another. This arrangement does not agree with the present data, where three biovars of *P. solanacearum* from Brazil were distributed in two groups, based on the presence or absence of membrane proteins with 35 and 37 kDa. In the first group are the biovar 2 isolates; the second includes biovars 1 and 3.

Few conclusions concerning race relationships can be drawn from our SDS-PAGE data. All banana isolates were biovar 1 and should correspond to race 2. These isolates have a 37 kDa band which was different from the 35 kDa band detected in all biovar 3 isolates. Biovar 1 and 3 strains differ from biovar 2 strains, which had no E band. Buddenhagen et al (3) indicated that race 1 has a wide variety of hosts, comprising a large number of strains distributed among biovars 1, 2, and 3. Race 3 includes strains from potato and is found at higher elevations in the American tropics (3) and is equivalent to biovar 2. Biovar 2 could be separated from biovar 1 isolates from potato because of the lack of an E protein.

Intraspecific comparisons of four pathovars of *X. campestris* with three fluorescent pseudomonads revealed a common main band at 44 kDa. This clearly separated these two groups from *P. solanacearum* and from another nonfluorescent pseudomonad (*P. caryophylli*), which have a 39–40 kDa main band. A similar comparison, on more limited scale, was made by Minsavage and Schaad (12), but these data are not comparable to ours because of differences in methods used to prepare the proteins.

In general, strains of the same biovar from the same host showed high similarity levels among themselves. But when biovars from

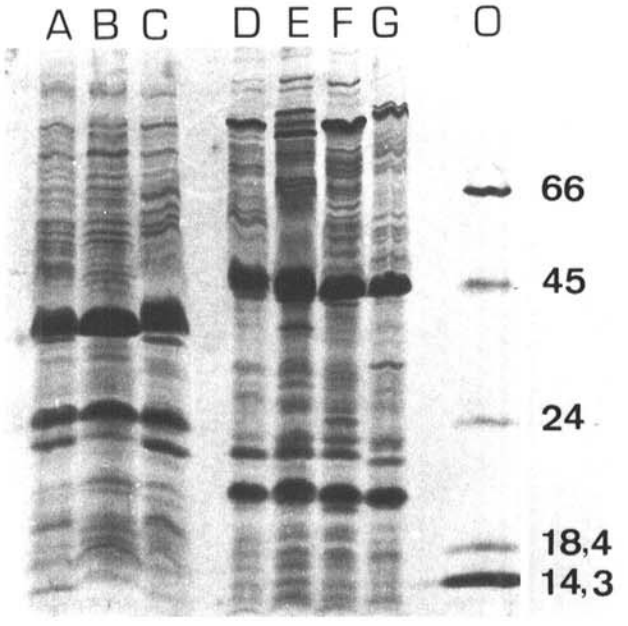

Fig. 3. *Pseudomonas solanacearum* biovars 1, 2, and 3 (A–C), compared to *Xanthomonas campestris* pvs. *cassavae* (D), *manihotis* (E), *vesicatoria* (F), and *campestris* (G) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Column O contains the molecular weight standards and their values in kilodaltons.

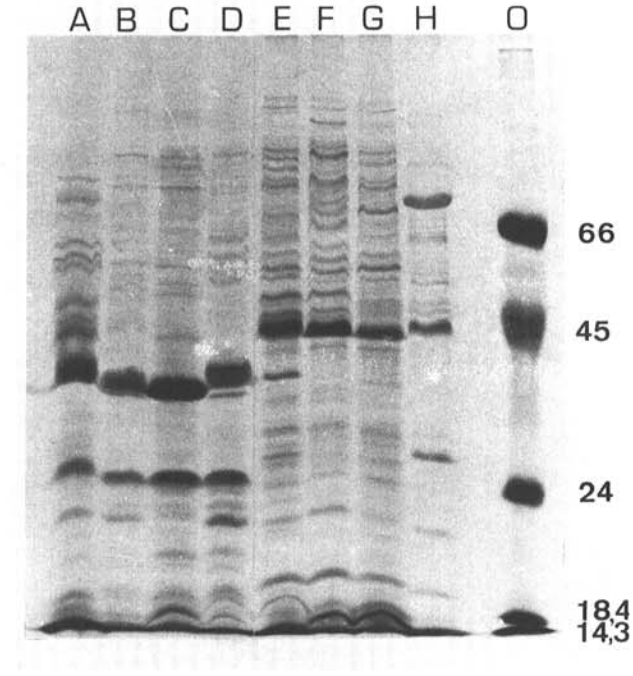

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of *Pseudomonas solanacearum* biovars 1 (B), 2 (C), and 3 (D) compared to *P. caryophylli* (A), *P. cichorii* (E), *P. syringae* pv. *tabaci* (F), *P. syringae* pv. *glycinea* (G), and *P. viridiflava* (H). Column O contains the molecular weight standards and their values in kilodaltons.

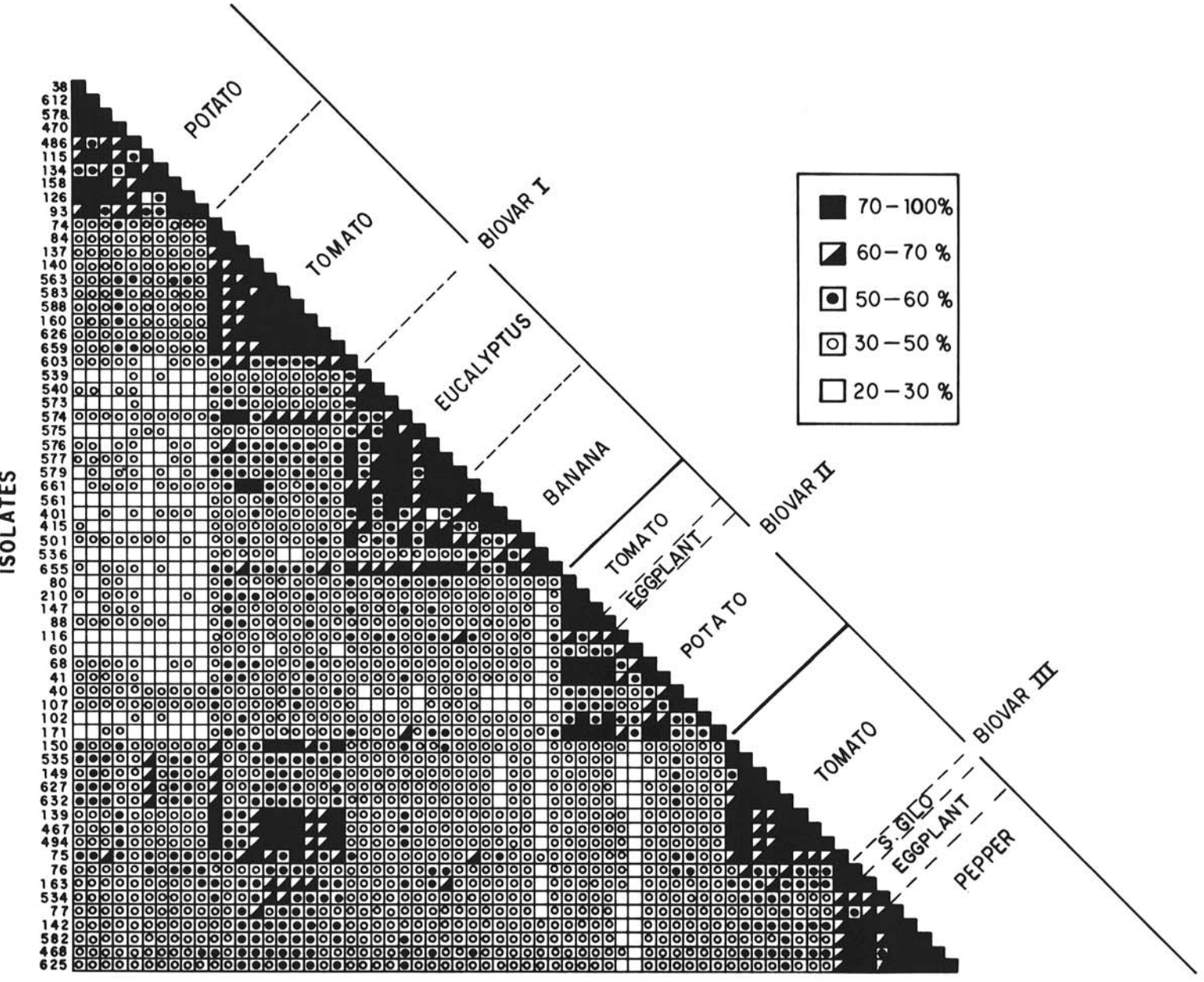

Fig. 5. Matrix of similarities of polypeptide bands produced by *Pseudomonas solanacearum* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The similarity of each pair of isolates was calculated on the basis of the percentage of secondary bands in common between the two isolates and also taking into account the differences in the intensity of seven selected main bands.

the same host were compared with each other, similarity was much lower. Examples are potato and tomato strains classified as biovars 1 and 2, which had more protein bands in common with isolates from other hosts than among themselves. Other examples of high similarity can be detected among groups of isolates from two to three different hosts. For example, biovar 1 isolates from eucalyptus and banana form an uniform group, as do the biovar 2 isolates from tomato, eggplant, and potato and the biovar 3 isolates from *S. gilo*, eggplant, and pepper.

Since these pairwise comparisons were based on more than 25 different bands, each representing at least one gene, the data may be useful for understanding phylogenetic relationships within the species. The system also offers a basis for future research in which selected polypeptides of *P. solanacearum* can be used as antigens for serological differentiation studies.

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