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Comparative Zone Electrophoresis of Enzymes of *Pseudomonas solanacearum* and *Pseudomonas cepacia*

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The technique of starch-gel electrophoresis with specific staining for a series of enzymes was used to compare 21 *Pseudomonas* strains representing both *P. cepacia* and *P. solanacearum*. These experiments produced no evidence for close similarity of the two species. Twelve strains of *P. solanacearum* were compared by means of data obtained from nine different enzymes, and the data indicate that these strains belong in two biotypes. Except for the assignment of two strains, these groups are the same as the two major groups previously derived from nutritional properties and from deoxyribonucleic acid hybridization experiments. Eleven enzymes were available for comparisons of the *P. cepacia* strains. Eight of these strains form a homogeneous group, but the last strain, number 249, differs considerably from the other representatives of the species.

The two species, *Pseudomonas solanacearum* and *P. cepacia* (*P. multivorans* is a synonym) have been intensively and extensively characterized by biochemical, nutritional, and deoxyribonucleic acid (DNA) associations (1, 6). These studies demonstrated that each of the above species is distinct from the other and from all other *Pseudomonas* species that have been so characterized by these means by the Berkeley group (1, 6, 7, 9). Yet members of each of these species display a larger degree of heterogeneity of phenotype and apparent DNA sequence homology than do the representatives of such tidy species as *P. aeruginosa* (9) or *P. mendocina* (7). We have previously shown that zone electrophoresis of enzymes in starch gels (zymograms) has utility in the delineation of species within the *Enterobacteriaceae* (2), providing an estimate of genetic proximity between pairs of strains or species. This tool has been applied to an examination of 8 strains of *P. cepacia* and to 12 strains of *P. solanacearum* (representing the four Hayward biotypes; reference 4).

MATERIALS AND METHODS

The bacterial strains were provided by M. Doudoroff and N. J. Palleroni and correspond to the collection numbers of the Department of Bacteriology, University of California, Berkeley. The properties and histories of these cultures have been described (1, 6). Cultures were grown in 1-liter amounts in nutrient broth (Difco) supplemented with 0.2% yeast extract (Difco) and 0.2% L-asparagine (replaced by a like amount of sodium succinate for strains 769 and 776) on a rotary shaker at 22 C. Cultures were harvested at the end of logarithmic growth by centrifugation as previously described (2) and streaked on plates (1.5% agar in the medium in which grown) to check clonal purity.

Small samples of each cell paste [about 200 mg (wet weight)] were used for extraction of DNA and analysis by CsCl analytical ultracentrifugation (5).

Methods for preparation of cell extracts and for starch-gel electrophoresis were described previously (2, 8).

Stain methods for the following enzymes: malate dehydrogenase (EC 1.1.1.37; MDH), isocitrate dehydrogenase (EC 1.1.1.41; IDH), 6-phosphogluconate dehydrogenase (EC 1.1.1.43; 6PGD), glucose-6-phosphate dehydrogenase (EC 1.1.1.49; G6PD), tetrazolium oxidase (TO), glutamate dehydrogenase (EC 1.4.1.2; GDH), xanthine dehydrogenase activity (XDH), fumarase (EC 4.2.1.2), and esterase activities are described elsewhere (8). The stain solution for glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; G3PD) has been described by Shaw and Prasad (8), and the starch gel was modified by the addition of 5×10^{-5} M nicotinamide adenine dinucleotide (NAD) and 10^{-3} M β-mercaptoethanol to the hot starch solution just before the gel was made. The stain solution for quinone reductase was: NAD, 50 mg; 2,6-dichlorophenolindophenol, 5 mg; flavin mononucleotide, 2 mg; 1.0 M sodium-L-malate (*p*H 7.0), 10 ml; MDH, 200 units; 0.1 M phosphate (*p*H 7.0), 40 ml; 0.1 M NaCN, 10 ml; water, 50 ml. The electrophoresis period was 18 hr in all cases, and the migration distances are expressed rel-

ative to the migration distance of a standard. The corresponding principal enzyme activity in a rat liver extract was used as the standard, except in the cases of fumarase and glyceraldehyde-3-phosphate dehydrogenase where the migration distances were calculated relative to the "tetrazolium oxidase" activity of the rat liver extracts. A minimum of two electrophoretic analyses of each extract was performed.

RESULTS

Table 1 presents the data on relative electrophoretic mobilities of 11 enzymes for 9 strains of *Pseudomonas cepacia* and Table 2 shows similar data for 12 strains of *P. solanacearum* (9 enzymes). The electrophoretic migrations of the enzymes are designated in these tables by numbers which represent the average migration distance relative to the reference protein. This number is then used to identify the enzyme type. Enzymes of the same type are assumed to be identical in structure, although this is not proved and in certain cases may not be true. The guanine plus cytosine (GC) content in the DNA of each culture, calculated from the buoyant densities in CsCl, is also given in each table.

In a few cases, enzymes have identical or nearly identical average migration rates but are distinguishable when they are in adjacent positions on the same gel. Figure 1 shows some IDH types which illustrate this point. IDH bands from *P. cepacia* consistently appear quite narrow in contrast to the elongated area stained by *P. solanacearum* IDH enzymes. This must represent some difference in the structure of the enzyme molecule even though the nature of the difference is not understood. In Fig. 1, the IDH types from strains 771 and 424 are clearly different even though they migrate at indistinguishable rates.

Another example of this differentiation is the fumarase in most strains of *P. cepacia* as compared to the fumarase in *P. solanacearum* strains 768, 776, 777, 730, and 780. These enzymes appear nearly equal in average migration rate, but, when they are examined side by side, the *P. cepacia* enzyme consistently moves a little further than the other type. Similarly, the G6PD in *P. solanacearum* is different from that found in the fast migrating bands of *P. cepacia* strains 382 or 383. In addition, the "XDH" type in *P. cepacia* strain 249 differs from any type found in *P. solanacearum*.

When the above apparent exceptions are considered in Tables 1 and 2, then there is no case where a *P. solanacearum* enzyme is not distinguishable from the corresponding *P. cepacia* enzyme. This strengthens the conclusion of Palleroni and Doudoroff that these two species are not closely related (6). As a contrasting example, certain strains of *Escherichia coli* and species of *Salmonella* have the same enzyme types in three out of eight enzymes studied (2). Likewise, some strains of *Bacillus subtilis* and of *B. cereus* have common types in 3 enzymes out of 11 (*unpublished data*).

TABLE 1. *Relative electrophoretic migration distances of enzymes and guanine plus cytosine contents of the deoxyribonucleic acids of strains of Pseudomonas cepacia*

Enzyme	Migration distances of enzymes and DNA of strain[a]								
	249	104	382	424	725	727	383	385	396
Malate dehydrogenase	1.27	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20
Isocitrate dehydrogenase	1.73	1.34	1.34	1.34	1.34	1.34	1.34	1.34	1.34
	2.10	2.00	1.89	1.89	1.89	1.89	1.89	1.73	1.73
6-Phosphogluconate dehydrogenase	1.41	1.62	1.62	1.62	1.62	1.62	1.62	1.62	1.62
Glucose-6-phosphate dehydrogenase	2.2	1.76	1.76	1.76	1.76	1.76	1.76	1.76	1.76
			2.10				2.10	2.50	2.50
"Tetrazolium oxidase"	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09
L-Glutamate dehydrogenase	0.8	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13
"Xanthine dehydrogenase"	2.6	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1
Fumarase	0.92	0.92	0.92	0.92	0.92	0.92	1.01	1.01	1.01
Glyceraldehyde-3-phosphate dehydrogenase	1.57	1.91	1.91	1.91	1.91	1.91	1.57	1.91	1.91
Quinone reductase"	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Esterase	—[c]	3.6	3.1	3.1	3.6	3.1	3.8	3.6	3.6
		3.8	3.8			3.8			
DNA guanine + cytosine[b] (moles%)	68.1	68.4	67.3	67.3	66.6	66.6	67.3	67.7	68.4

[a] Values expressed as ratios of distance of bacterial enzyme to distance of the reference standard.
[b] Includes individual data from earlier observations (1) averaged with present analyses.
[c] Not detected.

TABLE 2. *Relative electrophoretic migration distances of enzymes and the deoxyribonucleic acid base compositions of strains of Pseudomonas solanacearum*

Enzyme	Migration distances of enzymes and DNA of strains[a]											
	Biotype 1[b]					Biotype 2		Biotype 3		Biotype 4		
	768	771	735	769	782	773	772	776	777	730	780	781
Malate dehydrogenase	0.87	0.87	0.56	0.56	0.56	0.56	0.56	0.87	0.87	0.87	1.04	0.87
Isocitrate dehydrogenase	1.33	1.33	1.01	1.20	1.20	1.20	1.20	1.20	1.20	1.33	1.33	1.20
Glucose-6-phosphate dehydrogenase	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1
"Tetrazolium oxidase"	0.76	0.84	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76
L-Glutamate dehydrogenase	—[c]	0.87	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]	0.87	—[c]	—[c]	—[c]
"Xanthine dehydrogenase"	2.6	2.6	2.5	2.5	2.5	2.3	2.3	2.5	2.5	2.6	2.5	2.3
Fumarase	0.93	0.79	—[c]	0.66	0.85	0.62	0.62	0.93	0.93	0.93	0.93	0.66
Glyceraldehyde-3-phosphate dehydrogenase	0.85	0.47	0.85	1.18	1.18	1.18	1.18	0.47	0.47	0.47	0.47	0.47
"Quinone reductase"	—[c]	1.18	0.41	0.41	0.41	0.41	0.41	1.18	1.18	1.18	1.18	1.18
	2.8	—[c]					2.5	2.5	2.5	2.5		2.5
	3.5	3.5							4.3	3.5		
DNA guanine + cytosine[d] (moles %)	67.3	67.3	66.5	66.6	67.0	67.7	67.7	67.0	67.7	67.7	68.1	67.3

[a] Values expressed as ratios of distance from origin of bacterial enzyme to distance of the reference standard.
[b] Classification of Hayward (4).
[c] Not detected.
[d] Mean of at least two determinations, calculated from buoyant density in CsCl.

Eight of the *P. cepacia* strains are quite similar to each other. However, strain 249 has a high proportion of enzymes which differ from those in the other strains. In addition to those clearly indicated in Table 1 as different, the quinone reductase band can be distinguished from the bands of the other strains. Therefore, strain 249 has at least 7 unique enzyme types out of 11 available for comparison. This agrees with the earlier data (1) indicating a relatively low degree of DNA homology between this strain and the other strains of *P. cepacia*. The only other case where we were able to distinguish enzyme types not indicated in Table 1 is in "xanthine dehydrogenase" which produces two narrow bands in strain 104. The band migrating more slowly matched those of *P. cepacia* strains 382, 383, 385, and 396. The band migrating more rapidly in 104 matched bands observed in strains 424, 725, and 727.

The 12 strains of *P. solanacearum* are relatively heterogeneous as compared to other bacterial species. Of the eight enzymes available for comparison, only G6PD has the same enzyme type in every strain although "TO" is the same in all the strains except 771. Four other enzymes, MDH, G3PD, fumarase, and quinone reductase support the division of these strains into two well defined groups. However, IDH and "XDH" are present as types which do not distribute with these groupings. In addition to the "XDH" types indicated in Table 2, the enzyme type in strain 780 is distinguishable from that in strains 735, 769, 782, 776, and 777. In addition, the "XDH" in strain 781 is probably distinguishable from the type found in strains 772 and 773. These additional "XDH" types were used in construction of the similarity matrix of Fig. 2 which illustrates the two major varieties of *P. solanacearum*. One includes strains 730, 768, 771, 776, 777, 780, and 781. The other group includes strains 735, 769, 772, 773, and 782.

DISCUSSION

Palleroni and Doudoroff (6) initially found by competitive DNA-DNA binding what appeared to be a significant amount of homology between strain 382 of *P. cepacia* and two reference strains of *P. solanacearum* (769 and 776). In actuality, it was this observation that prompted the initiation of our zymographic comparison of the representatives of the two species. Whereas our colleagues were able to show that this degree of presumed relation was more apparent than real, that is, an artifact of the competition method not produced by direct binding assays (6), we have been able to provide confirmation of most of their general taxonomic conclusions concerning the relations between these aerobic pseudo-

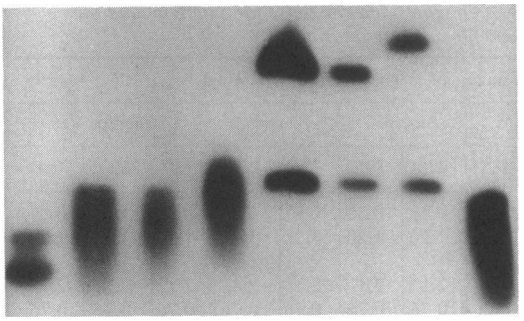

FIG. 1. *Starch gel stained for isocitric dehydrogenase. Samples, from left to right, are: (1) rat liver, (2) P. solanacearum strain 777, (3) P. solanacearum strain 776, (4) P. solanacearum strain 771, (5) P. cepacia strain 424, (6) P. cepacia strain 383, (7) P. cepacia strain 104, and (8) P. solanacearum strain 777. The anode is at the top.*

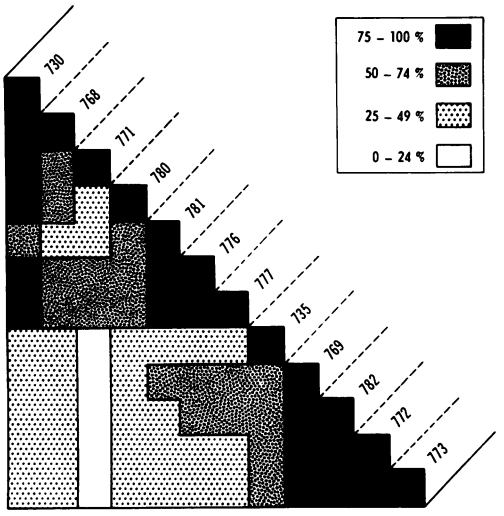

FIG. 2. *Matrix of similarities of enzyme types of P. solanacearum. The per cent of similarity of each pair of strains was calculated from the number of enzymes which appear to be the same type of the total number of enzymes available for comparison for that pair. Data are from Table 2.*

monads and extend the examination of the internal structure of these species.

All cultures that we have examined contain DNA whose base composition does not depart significantly from 67 moles % G + C.

Eight of the nine strains of *P. cepacia* which we have studied form a very homogeneous group with respect to zymogram analysis. The ninth strain (249) bears close phenotypic resemblance to this group of cultures (1) but is distinctly different in the constitution of at least 7 of 11 enzyme proteins detected in common among these strains. These differences are paralleled by the low degree of DNA-DNA association of this strain as compared to the reference samples previously employed (1).

The lack of any significant sequence homology between the *P. cepacia* representative and the two *P. solanacearum* representatives is consistent with our failure to find any enzymes which might be identical in structure and conformation held in common between these two groups of organisms.

The general observation of Palleroni and Doudoroff (6) that *P. solanacearum* is a moderately homogeneous group is supported by our observations. We do not see any strong correlation of enzyme types reflecting the division by Hayward into four biotypes (4). A general division into two groups or biotypes can be supported by the overall biochemical and nutritional similarities within the two biotypes, their high order of DNA sequence homologies, and by the lack of differences in the patterns of electrophoretic migrations of the set of enzymes examined in this study.

The strains which we have examined in common with Palleroni and Doudoroff fall into two recognizably different, yet similar, groups. If we designate one as biotype I, we would include within it strains 735, 769, 772, 773, and 782. It includes both representatives of Hayward's biotype 2 and representatives of his biotype 1, but excludes strains 768 and 771. These latter strains we would include in biotype II together with the other representatives of Hayward's biotypes 3 and 4 (730, 776, 777, 780, and 781). These assortments are consistent with DNA homologies previously ascertained for these strains and serve to provide more homogeneous groupings of many of the phenotypic characters (6). For example, all members of biotype I utilize hippurate whereas none of biotype II do so; none of biotype I then utilizes *p*-hydroxybenzoate and only one culture of biotype II fails to do so; all strains of biotype II utilize DL-lactate and none of biotype I does so.

We conclude that *P. solanacearum* is a distinct species, readily differentiated from *P. cepacia*, and although divisible into two biotypes constitutes a reasonably homogeneous collection of strains displaying a somewhat wider range of variations than do other species of the genus.

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LITERATURE CITED

1. Ballard, R. W., N. J. Palleroni, M. Doudoroff, R. Y. Stanier, and M. Mandel. 1970. Taxonomy of the aerobic pseudomonads: *Pseudomonas cepacia*, *P. marginata*, *P. alliicola* and *P. caryophylli*. J. Gen. Microbiol. **60**:199–214.
2. Baptist, J. N., C. R. Shaw, and M. Mandel. 1969. Zone electrophoresis of enzymes in bacterial taxonomy. J. Bacteriol. **99**:180–188.
3. Gasser, F., and C. Gasser. 1971. Immunological relationships among lactic dehydrogenases in the genera *Lactobacillus* and *Leuconostoc*. J. Bacteriol. **106**:113–125.
4. Hayward, A. C. 1964. Characteristics of *Pseudomonas solanacearum*. J. Appl. Bacteriol. **27**:265–277.
5. Mandel, M. 1966. Deoxyribonucleic acid base composition in the genus *Pseudomonas*. J. Gen. Microbiol. **43**:273–292.
6. Palleroni, N. J., and M. Doudoroff. 1971. Phenotypic characterization and deoxyribonucleic acid homologies of *Pseudomonas solanacearum*. J. Bacteriol. **107**:690–696.
7. Palleroni, N. J., M. Doudoroff, R. Y. Stanier, R. E. Solanes, and M. Mandel. 1970. Taxonomy of the aerobic pseudomonads: the properties of the *Pseudomonas stutzeri* group. J. Gen. Microbiol. **60**:215–231.
8. Shaw, C. R., and R. Prasad. 1970. Starch gel electrophoresis of enzymes—a compilation of recipes. Biochem. Genet. **4**:297–320.
9. Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**:159–271.