

Deoxyribonucleic Acid Similarities among *Pseudomonas* Species

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Deoxyribonucleic acid similarity experiments in which the S1 nuclease methodology was used were performed on strains of *Pseudomonas* species, including some that had not been previously subjected to similarity measurements. The results obtained with this technique confirmed and expanded previous observations on the low levels of deoxyribonucleic acid similarity among species of the genus as determined by membrane competition experiments. A general discussion of the results and nomenclatural status of some of the species is included.

The genus *Pseudomonas* has been subdivided into five groups on the basis of ribosomal ribonucleic acid (rRNA)-deoxyribonucleic acid (DNA) hybridization experiments (23, 24, 27). This classification has received ample confirmation from work done in various laboratories in which a number of different experimental approaches have been used. In the years since it was first proposed, no significant changes have been required, with the exception of assignment of organisms to some of the groups of some *Pseudomonas* species that were not included in the original studies. Thus, in the recent rRNA-DNA hybridization study of Byng et al. (3), *Pseudomonas panici*, *Pseudomonas synxantha*, *Pseudomonas angulata*, and *Pseudomonas fragi* were found to belong to group I; *Pseudomonas pyrocinia* and *Pseudomonas andropogonis* were found to belong to group II; and *Pseudomonas geniculata* was found to belong to group V. In addition, *Alcaligenes paradoxus* was found to be related to organisms belonging to group II, and *Alcaligenes eutrophus* was found to be related to group III organisms.

The homogeneity within each *Pseudomonas* rRNA similarity cluster is very high, with most species having nearly 100% rRNA similarity (3, 27), although the levels of DNA similarity between species within each rRNA cluster are low (25). In contrast, we have not always found this clear clustering among groups of anaerobic bacteria (16-18). In order to confirm this rather interesting observation on the *Pseudomonas* groups and to extend DNA similarity measurements between additional species of the genus, the experiments described here were performed. In addition, some of the species were not included on the 1980 Approved Lists of Bacterial Names (33), and others appear to be synonyms. Nomenclatural proposals concerning some of these organisms are included below.

MATERIALS AND METHODS

Bacterial strains. The organisms used in this study were the same as those used in a previous rRNA similarity study (3). All were obtained from the American Type Culture Collection, Rockville, Md.

DNA preparation. The organisms were grown in a medium containing 1% tryptone (BBL Microbiology Systems, Cockeysville, Md.), 0.5% yeast extract (Difco Laboratories, Detroit, Mich.), and 0.5% glucose. Erlenmeyer flasks (volume, 2 liters) containing 500 ml of medium were inoculated with 5-ml portions of broth cultures and shaken overnight at 250 rpm on a rotary shaker at 30°C. The harvested

cells were suspended in a 0.15 M NaCl—0.01 M ethylenediaminetetraacetic acid salt solution (pH 8.0), and DNA was isolated by the method of Marmur (22). The DNA preparations were dissolved in 0.1× SCC (1× SCC is 0.15 M NaCl plus 0.15 M sodium citrate, pH 7.0) and stored at -20°C. The DNA preparations were fragmented and denatured by two passages through a French pressure cell at 16,000 lb/in², followed by heating in a boiling water bath for 5 min. The concentrations were adjusted to 0.4 mg/ml, and the preparations were stored at -20°C.

Preparation of labeled nucleic acids. Samples of the fragmented denatured DNA were labeled with ¹²⁵I by using a variation of the thallium chloride method (32, 36).

DNA similarity methods. DNA similarity values were determined by using an S1 procedure, as described previously (15). The reassociation reaction mixtures contained 10 µl of denatured labeled DNA (0.01 to 0.03 µg), 50 µl of denatured unlabeled DNA (20 µg), 25 µl of a salt solution consisting of 5.28 M NaCl and 1 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] (pH 7.0), and 25 µl of deionized formamide. For measuring background reassociation and the S1-resistant material in the labeled DNA preparations, the denatured unlabeled DNA was replaced with 50 µl (20 µg) of sheared native salmon sperm DNA. The reassociation vials were incubated at 63°C (25°C below the thermal melting points of native reference DNAs in this buffer) for 20 h. The S1 nuclease-resistant fragments from each vial were coprecipitated with 60 µg of fragmented salmon sperm DNA, collected on type GF/F glass fiber filters (Whatman, Ltd., Kent, England), and counted with a gamma counter.

RESULTS AND DISCUSSION

DNA similarities. The DNA similarity results for the *Pseudomonas* species representing each rRNA similarity cluster are shown in Tables 1 through 4. There are examples where there are high levels of DNA similarity between species, which are discussed below, but most of the species within each rRNA cluster have interspecies similarity values in the 5 to 20% range. Although the similarity values are low, reciprocal values (Table 1) are very close, so that we believe that most of the values that differ by 5% are significant. In addition, there appears to be a good correlation between these small DNA similarity differences and the rRNA similarity results (3). The average DNA similarity values between species belonging to different rRNA similarity clusters are very low (not more than 1 to 2%) (Table 5).

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TABLE 1. Levels of DNA similarity among rRNA similarity group I species

Source of unlabeled DNA	% Similarity with labeled DNA from strain:										
	ATCC 15692	ATCC 14909 ^{Ta}	ATCC 25411 ^T	ATCC 17591 ^T	ATCC 25941 ^T	ATCC 9890 ^T	ATCC 13525 ^T	ATCC 9447	ATCC 12633 ^T	ATCC 13453	ATCC 10857 ^T
<i>P. aeruginosa</i> ATCC 15692 ^b	(100)		19		15			18	6	9	
<i>P. alcaligenes</i> ATCC 14909 ^T	15	(100)	23	14	12	10	9	14	14	8	8
<i>P. mendocina</i> ATCC 25411 ^T	13	23	(100)	14	10	9	8	12	11	6	8
<i>P. stutzeri</i> ATCC 17591 ^T	10	15	13	(100)	7	6	6	8	9	4	6
<i>P. agarici</i> ATCC 25941 ^T	9	10	10	6	(100)	16	16	20	13	10	12
<i>P. agarici</i> ATCC 25943	9	10	10	7	100	16	16	20	14	10	11
<i>P. synxantha</i> ATCC 9890 ^T	10	11	12	7	21	(100)	43	25	17	12	13
<i>P. fluorescens</i> ATCC 13525 ^T	10	11	11	7	21	44	(100)	27	16	12	14
<i>P. chlororaphis</i> ATCC 9447 ^c	16	16	16	11	27	29	28	(100)	22	15	18
<i>P. aureofaciens</i> ATCC 13986	17	16	15	10	27	28	26	81	21	15	18
<i>P. putida</i> ATCC 12633 ^T	11	14	13	9	15	15	15	19	(100)	11	13
<i>P. fragi</i> ATCC 4973 ^T	8	9	8	8	15	17	16	17	13	9	11
<i>P. angulata</i> ATCC 13453	7	9	10	8	14	14	13	15	12	(100)	25
<i>P. syringae</i> ATCC 19304 ^d	6	8	10	7	15	13	18	18	12	76	23
<i>P. syringae</i> ATCC 19875 ^c	8	9	9	7	21	14	13	16	12	53	24
<i>P. cichorii</i> ATCC 10857 ^T	8	9	10	7	15	14	13	15	13	21	(100)

^a T = type strain.^b Strain ATCC 10145^T exhibits 78% similarity with strain ATCC 15692.^c Strain ATCC 9446^T exhibits 100% similarity with strain ATCC 9447.^d *P. syringae* pv. *phaseolicola*.^e *P. syringae* pv. *panici*.

Our S1 nuclease similarity results differ from the previous membrane competition results (1, 25, 29) by being about 15 to 20% lower. This is due to the digestion by S1 nuclease of short noncomplementary regions that were included by the membrane competition measurements (1).

The dendrogram shown in Fig. 1 summarizes the results of our DNA similarity experiments on members of rRNA similarity group I. By taking into consideration DNA similarity values above 50%, single branches are drawn for the three clusters constituted by (i) the two strains of the mushroom pathogen *Pseudomonas agarici*, (ii) the strains of *Pseudomonas chlororaphis* and *Pseudomonas aureofaciens*, and (iii) the strains of the nomenclatures *Pseudomonas syringae*, *P. angulata*, and *P. panici*.

In our interspecies comparisons, the highest similarity values for group I organisms were obtained between the strains of *P. chlororaphis* and *P. aureofaciens*. This finding is in agreement with previous results (25) and casts new doubts on the convenience of maintaining these two taxa under different species names. Consequently, a formal recommendation for assignment of all strains of the two groups to a single species is included below. Our use of strain ATCC 9447 of *P. chlororaphis* instead of the type strain, strain ATCC 9446, seems justified, since these two strains share 100% DNA sequence similarity (Table 1).

TABLE 2. Levels of DNA similarity among rRNA similarity group II species

Source of unlabeled DNA	% Similarity with labeled DNA from strain:			
	ATCC 25416 ^T	ATCC 10248 ^T	ATCC 23060	ATCC 17697 ^T
<i>P. cepacia</i> ATCC 25416 ^T	(100)	7	3	8
<i>P. pyrocinia</i> ATCC 15958 ^T	15	12	13	6
<i>P. gladioli</i> ATCC 10248 ^T		(100)	4	8
<i>P. gladioli</i> ATCC 24517	2	98	4	8
<i>P. andropogonis</i> ATCC 23060	3	1	(100)	2
<i>P. solanacearum</i> ATCC 10692	6	0	1	11
<i>A. eutrophus</i> ATCC 17697 ^T	7	5	2	(100)

Aside from the *P. chlororaphis*-*P. aureofaciens* relationship, the highest level of similarity in Fig. 1 is represented by the species *P. synxantha* and *Pseudomonas fluorescens*. These two species are phenotypically similar, as shown by Jessen (14), who found that strains of both species are positive for gelatin liquefaction, denitrification, growth at low temperature (4 to 5°C), utilization of galactose for growth, and acid formation from trehalose. All of these characteristics are typically positive for *P. fluorescens* biotypes (35). *P. synxantha* and *P. fluorescens* join the *P. chlororaphis*-*P. aureofaciens* cluster in Fig. 1 at a substantial level of DNA similarity, which is not surprising because these two taxa were previously included among the biotypes of *P. fluorescens* (35).

The high level of similarity between the *P. angulata* strain and *P. syringae* (Table 1) supports the lumping of these two taxa under a single species name, *P. syringae* (see below). These two taxa are integrated here into the *P. syringae* branch of the dendrogram (Fig. 1). Jessen (14) examined strains NCPPB 79, NCPPB 214, and NCPPB 263 of *P. angulata* and assigned them to three different biotypes (biotypes 63, 69, and 70) in his system of classification. One strain under the same label was studied by Sands et al. (30) and was placed in a group with *P. syringae*; however, later, Pecknold and Grogan (28) placed their four strains (named *Pseudomonas tabaci*, a synonym of *P. angulata*) in their "morsprunorum" group, which was separated from the "syringae" group of these authors.

Another phytopathogenic species, *Pseudomonas cichorii*, joins the *P. syringae* branch in Fig. 1 at a 23% similarity level, which is consistent with the fact that this species is separated from the rest of the fluorescent plant pathogens by virtue of a positive oxidase reaction (24). Low similarity values between *P. cichorii* and other *Pseudomonas* species have been reported previously (25, 28).

Finally, the dendrogram in Fig. 1 shows the rather isolated position of *Pseudomonas aeruginosa*. As mentioned elsewhere (21, 35), this species is very sharply defined on phenotypic grounds and by DNA base composition. Its isolation is also emphasized in tridimensional representa-

TABLE 3. Levels of DNA similarity among rRNA similarity group II species

Source of unlabeled DNA	% Similarity with labeled DNA from strain:						
	ATCC 15946 ^T	ATCC 17724 ^T	ATCC 17506	ATCC 19860 ^T	ATCC 15668 ^T	ATCC 17713 ^T	ATCC 11996 ^T
<i>P. saccharophila</i> ATCC 15946 ^T	(100)	9	6	6	6	7	3
<i>P. palleronii</i> ATCC 17724 ^T	6	(100)	11	10	7	10	6
<i>P. delafieldii</i> ATCC 17506	6	12	(100)	15	13	10	4
<i>P. delafieldii</i> ATCC 17505 ^T	6	12	83	15	11	10	4
<i>P. facilis</i> ATCC 11228 ^T	10	12	49	15	10	10	8
<i>P. avenae</i> ATCC 19860 ^T	7	12	16	(100)	13	12	12
<i>P. acidovorans</i> ATCC 15668 ^T	6	13	15	16	(100)	10	12
<i>P. acidovorans</i> ATCC 17476	6	15	15	17	80	10	9
<i>A. paradoxus</i> ATCC 17713 ^T	7	15	13	16	10	(100)	6
<i>P. testosteroni</i> ATCC 11996 ^T							(100)
<i>P. testosteroni</i> ATCC 17510	5	9	10	10	16	6	77

tions of similarity data (D. C. Hildebrand and N. J. Palleroni, unpublished data). For our experiments we used strain ATCC 15692, and not the type strain (strain ATCC 10145). However, these two strains have a very high DNA similarity level (Table 1).

As expected, the nonfluorescent members of the group (*Pseudomonas alcaligenes*, *Pseudomonas mendocina*, *Pseudomonas stutzeri*, and *P. fragi*) are only marginally related to the fluorescent species.

The data obtained in DNA hybridization experiments performed with members of *Pseudomonas* rRNA similarity group II are shown in Table 2. The strain of *P. andropogonis* (synonym, *Pseudomonas stizolobii*), a plant-pathogenic species, has a very low level of similarity with other species of the group. The strain used in our experiments (strain ATCC 23060) is not the type strain (strain ATCC 23061), but these two strains are very similar phenotypically and were assigned to the same pathovar (*P. andropogonis* pv. *andropogonis*), since both are pathogenic for species of the Gramineae. *P. andropogonis* was tentatively classified in group III by Byng et al. (4). This organism was later clearly shown to belong to rRNA similarity group II, but groups II and III are the most closely related of the rRNA clusters (3). The organism is peculiar in a number of properties (A. C. Hayward, personal communication), including the fact that the cells have a sheathed flagellum. Perhaps *P. andropogonis* should be placed in an intermediate position between groups II and III.

A substantial level of DNA similarity has been demonstrated between some *Pseudomonas cepacia* strains and *P. pyrrrocinia* (data not shown), but there is only a modest relationship between the latter species and the type strain of *P. cepacia* (Table 2). As shown by Elander et al. (8), pyrrolnitrin, the antibiotic characteristic of *P. pyrrrocinia*,

can also be produced by strains of *P. cepacia*. On the other hand, the strains of *P. cepacia* show a marked heterogeneity in DNA sequence similarity (1). Therefore, it would be worthwhile to subject *P. pyrrrocinia* strains in the future to a detailed phenotypic analysis to define other points of similarity between the two species.

A. eutrophus and the other group II species exhibit high levels of similarity in their rRNA sequences (3) and are also linked by low levels of DNA similarity (Table 2). Phenotypically, *A. eutrophus* resembles the members of group III, particularly *Pseudomonas acidovorans* (D. H. Davis, Ph.D. thesis, University of California, Berkeley, 1967), again reflecting the higher levels of rRNA relatedness between group II and III organisms.

Only one strain of *Pseudomonas solanacearum*, which was not the type strain, was examined in our study. This species is quite complex, and DNA hybridization experiments have shown various degrees of sequence similarity (26); these findings indicate the limited value of our results for this species and the need to include more strains in future studies.

One small cluster can be recognized in Table 3, represented by the hydrogen pseudomonad *Pseudomonas facilis* and its obligate heterotrophic counterpart, *Pseudomonas delafieldii*. These two species are almost identical in phenotype. The differences between them are in the capacity for utilization of a few substrates, in gelatin liquefaction (*P. facilis* has a stronger activity than *P. delafieldii*), and in the capacity for living autotrophically in contact with an atmosphere containing hydrogen, oxygen, and carbon dioxide. In a previous study (29) the two species were found to be indistinguishable by DNA reannealing experiments, but in our study, this total DNA identity was not evident, although the level of similarity was relatively high (49%).

TABLE 4. Levels of DNA similarity among rRNA similarity group V species

Source of unlabeled DNA	% Similarity with labeled DNA from strain:					
	ATCC 17806	ATCC 13637 ^T	ATCC 19867	ATCC 33913 ^T	ATCC 19865	ATCC 8721
<i>X. maltophilia</i> ATCC 17806	(100)	48	54	16	3	13
<i>X. maltophilia</i> ATCC 13637 ^T	52	(100)	71	2	1	11
<i>X. maltophilia</i> ATCC 19867	50	62	(100)	13	5	13
<i>P. geniculata</i> (<i>X. maltophilia</i>) ATCC 19374	53	64	71	14	4	13
<i>X. campestris</i> ATCC 33913 ^T				(100)	22	
<i>X. campestris</i> ATCC 19865	15	13	16	33	(100)	77
<i>X. campestris</i> pv. <i>pelargonii</i> ATCC 8721	16	13	15	14	64	(100)
<i>X. campestris</i> pv. <i>phaseoli</i> ATCC 9563	16	13	17	25	22	45
<i>X. campestris</i> pv. <i>malvacearum</i> ATCC 9924	14	13	16	16	20	43

TABLE 5. Levels of intergroup DNA similarity among rRNA similarity groups

rRNA similarity group	% Similarity with rRNA similarity group:			
	I	V	II	III
I		2.1	2.0	2.0
V	1.0		0.9	2.1
II	2.0	1.8		3.2
III	1.5	2.0	2.1	
IV	1.4		1.0	2.7

The data in Table 3 clearly show that, despite the fact that rRNA similarity group III includes all of the hydrogen pseudomonads, the three species which we studied (*Pseudomonas saccharophila*, *Pseudomonas facilis*, and *Pseudomonas palleronii*) exhibit relatively low levels of DNA similarity, supporting the concept of the limited taxonomic value of hydrogen utilization at the species level (6; Davis, Ph.D. thesis).

In the same group III (Table 3), *P. acidovorans* and *Pseudomonas testosteroni* appear to be related at only a low level as determined by DNA similarity experiments, as expected. Our values are in agreement with the low values reported previously (25). The two species have marked phenotypic similarities (35), but their DNA base compositions differ by 5 mol% (21).

Table 4 summarizes the hybridization data for members of rRNA group V. The group V strains are divided into two subgroups; one subgroup contains *Xanthomonas campestris* strains, and the other contains *Xanthomonas maltophilia* and *P. geniculata*. There is substantial heterogeneity among the strains in each subgroup. Heterogeneity among *X. maltophilia* strains has also been observed by Ikemoto and collaborators (13), who delineated five clusters, all having intercluster similarity values similar to those shown in Table 4.

Strains of different *Pseudomonas* species within each of the rRNA groups share substantial degrees of rRNA similarity (3, 27), while most of the DNA sequences seem to have undergone considerable divergence, as judged by the low levels of DNA similarity obtained in hybridization experiments (25; this paper). In contrast, strains of different

species of other genera may show in their DNAs a degree of conservatism approaching that of the ribosomal genes (18). It is tempting to attribute these peculiar properties of the pseudomonads to the ubiquitous occurrence of these organisms and the concomitant need for adjustment to the selective pressures of highly variable natural habitats (17).

Nomenclatural considerations. (i) **Comments on some of the strains used in the nucleic acid hybridization experiments.** The names of some of the strains used in this and previous work (3) deserve comments, since they are either synonymous with other names or have been changed in current taxonomic treatments of the genus.

One of the two *P. synxantha* strains studied by Jessen (14) was included in this study. The second strain was examined for its phenotypic properties by us and was found to belong to biotype G (biovar V) of *P. fluorescens* (data not shown).

The species name *P. angulata* is a synonym of *P. tabaci*, which has priority over *P. angulata*, but at present it designates a pathovar of *P. syringae*. Consequently, the accepted name is *P. syringae* pv. *tabaci* (7, 24).

There is considerable confusion in the literature and in collection catalogs concerning the specific epithets *panici* and *panacis*. Recently, a recommendation has been made for the rejection of the epithet *panici*, which was not meant to be applied to fluorescent organisms (11); however, the strain included in our studies, as well as in the studies of other workers (3, 7, 28, 30), is definitely a pathovar of *P. syringae*, although its origin is unclear.

P. geniculata (*P. maltophilia* ATCC 19374) was included in our study for the first time in DNA hybridization experiments, and until its recent assignment to group V (3), its taxonomic position appeared to be uncertain (24). That the uncertainty still continues will perhaps be clear after the discussion below. *P. geniculata* was described by Haynes and Burkholder in *Bergey's Manual of Determinative Bacteriology*, 7th ed. (10), among the fluorescent pseudomonads. According to the original description (5), the strains of this organism produce a green color in some media, but fluorescence is not specifically mentioned. The colonies are yellowish, and under certain conditions a diffusible brown pigment may be produced. The type strain of *P. geniculata* has not survived, and it is clear from a search of the literature that the description of the colors has been interpreted differently by later authors. Thus, three strains of the species were examined by Stanier et al. (35), who found them to be typical fluorescent pseudomonads and to correspond to *P. fluorescens* biotype G. On the other hand, one strain studied by Lysenko (20) produced no fluorescent pigment but had yellowish colonies and produced a diffusible brown pigment. Recently, this strain was examined by Byng et al. (3) for its rRNA composition and was assigned to rRNA group V. In all of the papers by this group of workers this organism is included under the name *P. geniculata*. This strain is listed in the American Type Culture Collection Catalog, 15th ed. (American Type Culture Collection, Rockville, Md.), among the strains of *P. maltophilia*, a species with which the strain shares the same colony color and brown diffusible pigment (24). Our DNA hybridization data confirm this diagnosis, but whether the original *P. geniculata* type was a green fluorescent pseudomonad or a representative of one of the *P. maltophilia* similarity clusters remains an unanswered question.

It has been recommended (12) that the name *Pseudomonas marginata* should be changed to *Pseudomonas gladioli*.

Likewise, *Pseudomonas albobiprecipitans* has been invalidated in favor of *Pseudomonas avenae* (31).

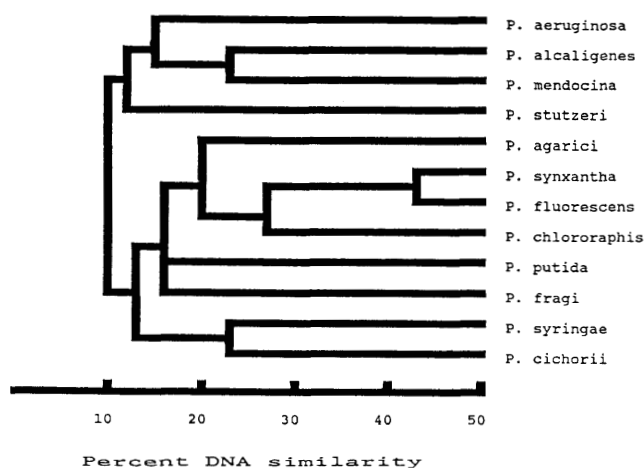


FIG. 1. Dendrogram of DNA similarity values for rRNA similarity group I species. The cluster analysis of the average DNA similarity values was calculated from Table 1 by using the unweighted pair group method.

(ii) **Proposal of synonymy of *P. chlororaphis* (Guignard and Sauvageau 1894) Bergey, Harrison, Breed, Hammer and Huntoon 1930, 166, and *P. aureofaciens* Kluver 1956, 406.** Strains of *P. chlororaphis* (Guignard and Sauvageau 1894) Bergey, Harrison, Breed, Hammer, and Huntoon 1930 (2) and *P. aureofaciens* Kluver 1956 (19) were examined by Stanier et al. (35) and were assigned to biotypes D and E of *P. fluorescens*, a species which they resemble in the production of fluorescent pigment and in gelatin liquefaction. *P. chlororaphis* and *P. aureofaciens* are identical to one another in many nutritional properties. Thus, the only absolute differences (i.e., properties found in all of the strains of one species and in none of the strains of the other) are the use of L-arabinose, D-alanine, and α -amylamine (24). However, a recent repetition of the nutritional tests has given discrepant results for D-alanine and α -amylamine; both species are positive for utilization of D-alanine, and the use of α -amylamine is not universal among *P. chlororaphis* strains.

Strains of both species produce levan from sucrose. Strains named *P. chlororaphis* produce the green phenazine pigment chlororaphin, while *P. aureofaciens* cultures produce the orange pigment phenazine monocarboxylate, which is closely related to chlororaphin (34). Only one of the species (*P. chlororaphis*) is capable of denitrification.

The strong similarities between the two species were further supported by their identical DNA base compositions (ca. 63.5 mol% guanine plus cytosine) (21) and a high level of DNA similarity (25; this paper). At the time that the last treatment of the genus *Pseudomonas* was written (24), the facts described above suggested the convenience of restoring the two biotypes to their former species status.

It is our opinion that the level of DNA similarity shared by the strains is sufficiently high to justify lumping the two species into one taxon, for which the name *P. chlororaphis* should be used based on priority.

Properties of interest for the differentiation of *P. chlororaphis* from most *P. fluorescens* biotypes are the capacity for formation of certain phenazine pigments (phenazine monocarboxylate or its amide, chlororaphin) (34), the utilization of benzoyl formate, and the inability to grow with D-xylose, L-rhamnose, citraconate, and sorbitol. Many additional phenotypic properties are listed in *Bergey's Manual of Systematic Bacteriology*, vol. 1 (24).

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