

## ***Pseudomonas fluorescens* Biovar V: Its Resolution into Distinct Component Groups and the Relationship of These Groups to Other *P. fluorescens* Biovars, to *P. putida*, and to Psychrotrophic *Pseudomonads* Associated with Food Spoilage**

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A numerical taxonomic analysis was performed to evaluate the appropriateness of a single biovar designation (biovar V) for all *Pseudomonas fluorescens* isolates negative for denitrification, levan production and phenazine pigmentation and to determine the relationship of biovar V strains to other taxa within the same *Pseudomonas* RNA homology group. Seventy-two strains assigned to *P. fluorescens* biovar V and four strains of *P. fragi* were characterized and the data subjected to a numerical taxonomic analysis along with comparable data for 17 previously characterized strains of this biovar and 89 *P. putida* strains. Seven distinct biovar V clusters containing three or more strains were revealed, and the carbon sources useful for their differentiation were identified. Cluster 1 (38 strains) closely resembled two atypical *P. fluorescens* I strains. It was also related to *P. fluorescens* biovar IV and to *P. fragi*. Cluster 2 (5 strains) was related to cluster 1. Cluster 3 (7 strains) was identical to a major group of meat spoilage psychrotrophic pseudomonads (*P. lundensis*). Cluster 4 (3 strains) was not related to any other group examined. Cluster 5 consisted of six isolates initially designated *P. putida* A along with four *P. fluorescens* biovar V strains all of which resembled *P. putida* more than they resembled the other *P. fluorescens* groups. Cluster 6 (16 strains) was distinct from the other biovar V clusters, but was closely related to *P. fluorescens* biovars I and II. Cluster 7 (3 strains) shared many characteristics with cluster 5. Separate *P. fluorescens* biovar designations are proposed for cluster 6 and for the combined clusters 1 and 2. A new *P. putida* biovar is proposed for the combined clusters 5 and 7.

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### INTRODUCTION

*Pseudomonas fluorescens* biotype G, now referred to as *P. fluorescens* biovar V (Palleroni, 1984), had the dubious distinction of being the only unsatisfactory fluorescent biotype delineated by Stanier *et al.* (1966) in their extensive taxonomic study which became the cornerstone of virtually all subsequent *Pseudomonas* taxonomy. Biotype G consisted of fluorescent strains which were negative for the three primary characteristics used by Stanier *et al.* (1966) for fluorescent biotype assignment, namely levan production, phenazine pigmentation and denitrification. The heterogeneity of the group was revealed in the nutritional studies which, in the case of the other biotypes, had strongly supported biotype assignment as based on the primary characteristics. Because the heterogeneity of the group has precluded the designation of typical strains, representatives of *P. fluorescens* biovar V have not been included

in most subsequent studies of the relationships between the several fluorescent biovars (Palleroni *et al.*, 1972, 1973; Champion *et al.*, 1980; Whitaker *et al.*, 1981*a, b*) or between *P. fluorescens* and other *Pseudomonas* species (Palleroni & Doudoroff, 1972; Palleroni *et al.*, 1973; Molin & Ternström, 1982, 1986; Shaw & Latty, 1982, 1984).

The exclusion of *P. fluorescens* biovar V representatives in such studies has not been perceived as a problem because it has generally been thought that this biovar consists primarily of strains which properly belong to other biotypes, but which have lost one or more of the properties considered to be of primary diagnostic importance (Stanier *et al.*, 1966; Palleroni, 1984). However, there is no experimental evidence that favours this hypothesis over the alternative possibility that biovar V consists of strains quite distinct from the other biovars. The latter possibility is suggested by the report that fluorescent strains which would be assigned to biovar V using the criteria of Stanier *et al.* (1966) constitute more than 75% of the fluorescent soil isolates in at least some areas of the world (Sands & Rovira, 1971). The primary goal of the work reported here was to address the question of the uniqueness of *P. fluorescens* biovar V.

The secondary goal was to examine the relationship of recently characterized pseudomonad food isolates to strains which comprise biovar V. The vast majority of oxidase-positive pseudomonads isolated from meat fall into two groups, both of which resemble *P. fluorescens* in certain respects (Molin & Ternström, 1982, 1986; Shaw & Latty, 1982, 1984). The first group contains the type strain of *P. fragi*. Although these strains are not fluorescent, their carbon source utilization matches the 'ideal fluorescent phenotype' outlined by Stanier *et al.* (1966). Furthermore, Whitaker *et al.* (1981*a, b*) have shown that the allostery of the *P. fragi* phenylalanine biosynthetic enzymes supports assignment to *Pseudomonas* RNA homology group I, which includes the fluorescent species (Palleroni *et al.*, 1973). The second major group of meat isolates contains fluorescent strains which would have been assigned to biovar V on the basis of the primary characteristics noted above (Molin & Ternström, 1982, 1986; Shaw & Latty, 1982, 1984). Molin *et al.* (1986) proposed separate species designation (*P. lundensis*) for this group. Numerical taxonomic analyses by both groups of investigators indicated a closer relationship between these two major groups of meat isolates than between either of them and any well-characterized fluorescent biovars. However, representatives of biovar V were not included in those studies.

#### METHODS

*Strains.* Strains included in the numerical analyses are listed in Table 1. Strains with the prefix RYS were described by Stanier *et al.* (1966). Strains characterized in this study (prefix PJ) were obtained from Professor Hans Lautrop of the Statens Serum Institut, Copenhagen, Denmark. Four strains of *P. fragi* were included in the numerical analyses: ATCC 4973, ATCC 27362 and ATCC 27363 (from the American Type Culture Collection) and FSBC 9 (from the Department of Food Science collection, originally isolated from cottage cheese). Additional *P. fragi* isolates were obtained by streaking out samples of spoiled cottage cheese or skim milk on nutrient agar (Difco) incubated at 30 °C. Purified isolates which were Gram-negative, polarly flagellated rods and which were oxidase-positive, strictly respiratory, non-pigmented, and capable of growth at 4 °C were considered presumptive *P. fragi*.

*Characterization methods* Bacterial strains were screened for 147 characteristics using the methodology of Stanier *et al.* (1966). The following characteristics were used by Stanier *et al.* (1966) but were not included in these studies: Tween 80 and egg yolk reactions, autotrophic growth with hydrogen, number of flagella, and the utilization of methanol, methylamine, oxalate, eicosanedioate, isophthalate, terephthalate, testosterone, *m*-aminobenzoate, *p*-aminobenzoate, *n*-dodecane and *n*-hexadecane. Similarity ( $S_j$ ) and matching ( $S_{SM}$ ) coefficients were calculated and the cluster analyses (group average method) performed using the methods of Sokal & Sneath (1963). DNA was isolated using the procedure of Marmur (1961) and G + C content determined using the method of Mandel (1966).

#### RESULTS AND DISCUSSION

##### *Delineation of clusters comprising P. fluorescens biovar V*

Seventy-two *P. fluorescens* strains assigned to biovar V on the basis of their lack of phenazine pigments and their inability to denitrify or produce levan were screened along with four *P. fragi*

Table 1. Strains included in the numerical analyses

Cluster*	Strains	Initial designation†
Pf V-1	PJ254, PJ259, PJ295, PJ310, PJ387, PJ391, PJ690, PJ700, PJ701, PJ704, PJ715, PJ760, PJ771, PJ855, PJ856, PJ861, PJ867, PJ835, PJ870, PJ877, PJ950, PJ952, PJ953, PJ954, PJ955, PJ956, PJ965, PJ977, PJ980, PJ981, PJ984, PJ985 RYS164, RYS169, RYS171, RYS172, RYS206, RYS269	<i>P. fluorescens</i> V <i>P. fluorescens</i> V‡
Pf V-2	PJ277, PJ389, PJ942, PJ958, PJ974	<i>P. fluorescens</i> V
Pf V-3	PJ63, PJ66, PJ720, PJ727, PJ728, PJ738, PJ899	<i>P. fluorescens</i> V
Pf V-4	RYS1, RYS99, RYS124	<i>P. fluorescens</i> V‡
Pf V-5	PJ783, PJ873, PJ878, PJ949 PJ91, PJ98, PJ275, PJ276, PJ892, PJ897	<i>P. fluorescens</i> V <i>P. putida</i> A
Pf V-6	PJ57, PJ68, PJ77, PJ270a, PJ270b, PJ282, PJ329, PJ694, PJ751, PJ836 RYS33, RYS34, RYS149, RYS166, RYS195, RYS271	<i>P. fluorescens</i> V <i>P. fluorescens</i> V‡
Pf V-7	PJ805, PJ808, PJ940	<i>P. fluorescens</i> V
Pp A	PJ6, PJ67, PJ80, PJ81, PJ82, PJ88, PJ89, PJ92, PJ93, PJ96, PJ229, PJ235, PJ242, PJ279, PJ280, PJ303, PJ305, PJ312, PJ324, PJ325, PJ392, PJ399, PJ498, PJ530, PJ534, PJ542, PJ677, PJ698, PJ726, PJ750, PJ768, PJ777, PJ780, PJ790, PJ891, PJ992, PJ997, PJ999 RYS5, RYS6, RYS7, RYS26, RYS42, RYS43, RYS44, RYS49, RYS51, RYS56, RYS76, RYS77, RYS82, RYS87, RYS89, RYS90, RYS100, RYS111, RYS115, RYS118, RYS119, RYS122, RYS128, RYS130, RYS144, RYS145, RYS154, RYS160, RYS266, RYS276	<i>P. putida</i> A <i>P. putida</i> A‡
Pp B	PJ707, PJ874, PJ948, PJ951, PJ961, PJ991 PJ994 PJ770 RYS53, RYS96, RYS107, RYS110, RYS153, RYS157, RYS158, RYS167	<i>P. putida</i> B§ <i>P. putida</i> A <i>P. fluorescens</i> V <i>P. putida</i> B‡
P fr	ATCC 4973, ATCC 27362, ATCC 27363, FSB 9	<i>P. fragi</i>
(None)	PJ724, PJ725, PJ758, PJ797, PJ893, PJ946, PJ962, PJ978, PJ982, PJ995 PJ90, PJ94, PJ95, PJ983, PJ993 RYS267, RYS272 RYS8 RYS98	<i>P. fluorescens</i> V <i>P. putida</i> A <i>P. fluorescens</i> V‡ <i>P. putida</i> A‡ <i>P. putida</i> B‡

\* Strains are arranged by cluster as shown in Fig. 1. Notations: Pf V, *P. fluorescens* biovar V; Pp A, *P. putida* biovar A; Pp B, *P. putida* biovar B; P fr, *P. fragi*.

† Initial designations of PJ strains based on gelatin liquefaction, pigmentation, levan production and ability to denitrify. Strains were characterized in this study unless otherwise indicated.

‡ Strains characterized by Stanier *et al.* (1966).

§ Strains characterized by Champion *et al.* (1980).

strains with respect to 147 characters (Stanier *et al.*, 1966). Preliminary results revealed that several strains resembled *P. putida* in their nutritional spectrum; like *P. fluorescens* V and *P. fragi*, *P. putida* consists of strains negative for phenazine pigments, denitrification and levan production. To enable recognition of *P. fluorescens* biovar V clusters which might be more correctly designated *P. putida*, it was thus necessary to include as many *P. putida* strains as possible in our numerical taxonomic analysis. A total of 49 *P. putida* strains were characterized. In the subsequent analyses, these results for all strains were pooled with the comparable data of Stanier *et al.* (1966) for 'biotype G' (17 strains), *P. putida* biovar A (32 strains), and *P. putida* biovar B (9 strains), plus the data of Champion *et al.* (1980) for an additional six *P. putida* B strains. Similarity coefficients were calculated for all pairs and a phenogram was constructed using the group average method (Fig. 1).

The biovar V strains formed seven distinct clusters of three or more strains at the 77%  $S_j$  level. *P. fragi*, *P. putida* A and *P. putida* B also formed distinct clusters at or above this level. Clusters V-1, V-2, V-3, and *P. fragi* were united at 71%  $S_j$  along with four other biovar V strains (PJ962, PJ978, PJ946, PJ995) and two strains initially assigned to *P. putida* A (PJ94 and PJ95). At 72%

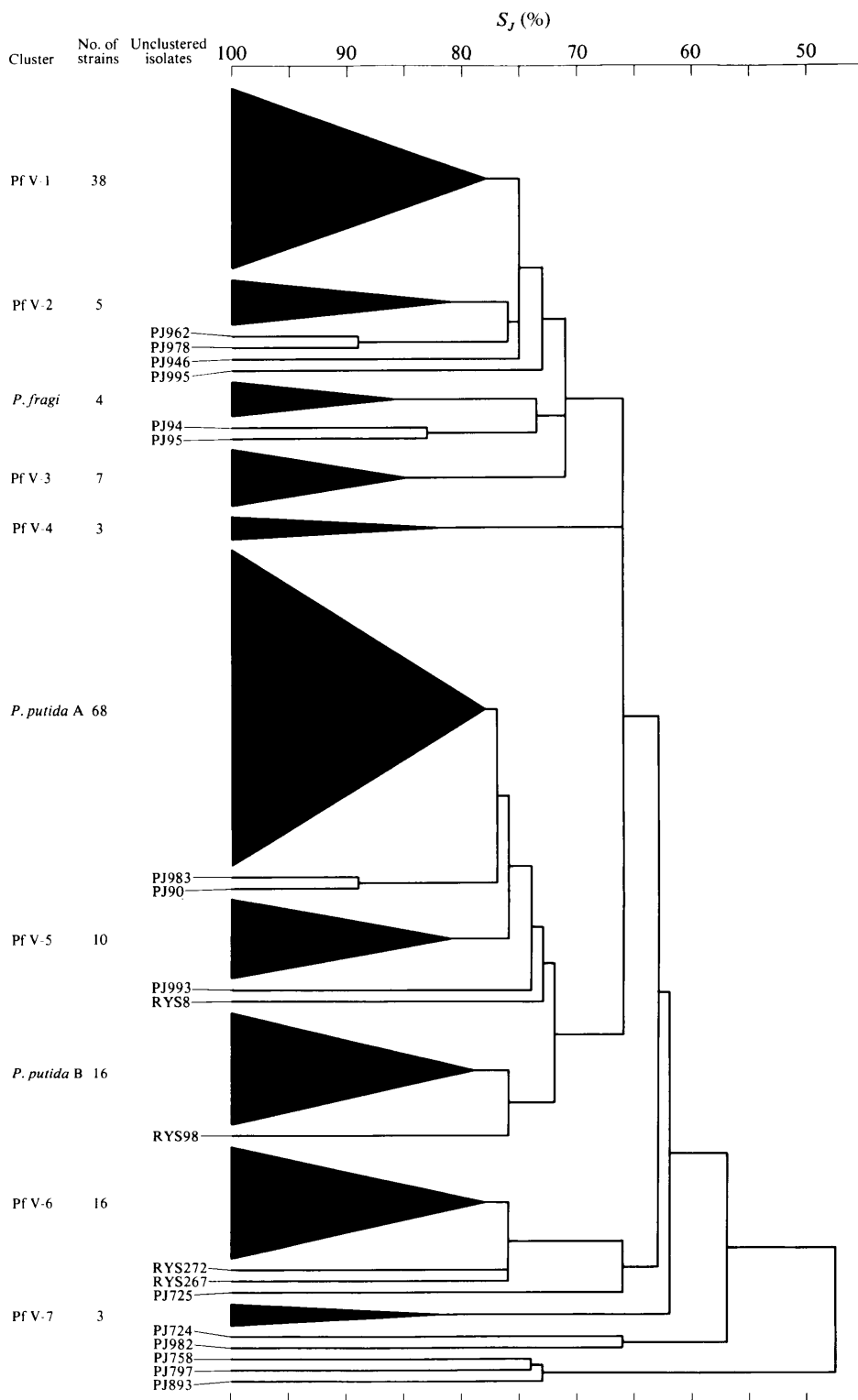


Fig. 1. Simplified phenogram based on the group average method analysis of a similarity matrix derived from the  $S_j$  coefficient. *P. fluorescens* biovar V clusters are designated as Pf V.

$S_J$ , cluster V-5 was united with *P. putida* A, *P. putida* B and five single *P. putida* strains (PJ90, PJ983, PJ993, RYS8, RYS98). The two large taxa joined each other and cluster V-4 at 66%  $S_J$ . Clusters V-6 and V-7 appeared to be only distantly related to the above groups; cluster V-6 was joined by three single biovar V strains (RYS267, RYS272 and PJ725) before joining the rest at 63%  $S_J$  and cluster V-7 joined at 62%  $S_J$ . Five *P. fluorescens* biovar V strains joined all the rest at  $S_J$  levels below 60% (PJ724, PJ982, PJ758, PJ797, PJ893).

Most of the PJ strains included in our analysis were previously examined by Jessen (1965), who subdivided a large collection of fluorescent pseudomonads into 82 biotypes using primarily acid production from various carbohydrates. Our cluster analysis shows significant agreement with Jessen's biotype assignments for the same strains. For example, Jessen's biotypes 18, 21 and 25-28 contained the PJ cluster V-1 strains, and his biotypes 10, 46 and 47 consisted of the cluster V-5, V-7 and V-3 strains, respectively.

The carbon sources useful for the differentiation of the various clusters are shown in Table 2. Previously characterized fluorescent pseudomonad groups are also included in this table for comparison. They are divided into the clusters revealed by Champion *et al.* (1980), who used the same phenotypic characters as were used in this study. The remaining carbon sources tested which do not appear in this table apparently contributed little to the clustering based on all characteristics as they fell into one of three classes: (a) used by no strains or used by very few strains, none of which constituted more than 20% of any cluster (D-fucose, L-rhamnose, cellobiose, lactose, maltose, starch, inulin, salicin, pimelate, poly- $\beta$ -hydroxybutyrate, maleate, D(-)tartrate, glycollate, ethylene glycol, isopropanol, geraniol, D-mandelate, L-mandelate, *o*-hydroxybenzoate, *m*-hydroxybenzoate, phenylethanediol, phthalate, naphthalene, phenol, L-threonine, L-norleucine, DL- $\alpha$ -aminobutyrate, DL- $\alpha$ -aminovalerate, D-tryptophan, acetamide, pantothenate, *n*-dodecane and *n*-hexadecane); (b) used by most strains of all clusters (D-glucose, D-fructose, D-ribose, gluconate, 2-ketogluconate, acetate, propionate, caproate, heptanoate, caprylate, pelargonate, caprate, succinate, glutarate, fumarate, L-malate, DL-lactate, DL- $\beta$ -hydroxybutyrate, DL-glycerate, citrate, 2-oxoglutarate, pyruvate, aconitate, glycerol, L- $\alpha$ -alanine, D- $\alpha$ -alanine,  $\beta$ -alanine, L-leucine, L-isoleucine, L-valine, L-aspartate, L-glutamate, L-arginine, L-ornithine, L-histidine, L-proline, L-tyrosine,  $\gamma$ -aminobutyrate,  $\delta$ -aminovalerate, putrescine, spermine, betaine and sarcosine); or (c) used by many strains in one or more clusters, but of little diagnostic value (D-mannose, sucrose, butyrate, isobutyrate, valerate, isovalerate, azelate, suberate, D-malate, L(+)tartrate, *meso*-tartrate, citraconate, laevulinate, butanol, isobutanol, butylene glycol, benzoate, L-serine, L-lysine, L-citrulline, L-phenylalanine and ethanolamine). Utilization patterns for the latter group of carbon sources are shown in Table 3.

All strains were positive for motility and oxidase reaction and were negative for denitrification, levan production, phenazine pigmentation, growth at 41 °C and poly- $\beta$ -hydroxybutyrate accumulation. Strains of all clusters formed fluorescent pigment with the exception of one strain in cluster V-5 and three *P. putida* strains. Results for gelatin liquefaction and ability to grow at 4 °C are shown in Table 3.

#### *Relationship of P. fluorescens V clusters to other fluorescent biovars*

By definition, *P. fluorescens* V consists of *P. fluorescens* strains which are negative for denitrification, levan production and phenazine pigmentation while all other *P. fluorescens* biovars consist of strains positive for one or more of those characteristics (Stanier *et al.*, 1966; Palleroni, 1984). It was suggested previously (Stanier *et al.*, 1966) that *P. fluorescens* biovar V may consist of strains which, on the basis of overall phenotype, more closely resemble strains of other biovars than each other, but which have lost one or more of the aforementioned primary traits. If this were the case, then the respective nutritional spectra of the various biovar V clusters should each parallel that of another fluorescent group which is positive for one or more of the primary traits. This hypothesis can be tested by comparing the carbon source utilization data for the biovar V clusters with the patterns for other fluorescent groups. To permit quantification of the comparison, an additional numerical analysis was performed: matching coefficients ( $S_{SM}$ ) were calculated for all pairs of strains with respect to the carbon sources listed in Table 2. Two additional carbon sources important in differentiation among the other

Table 2. Carbon sources which differentiate the *Pseudomonas* clusters from each other and from previously described biovars

Species . . .	Strains examined in this study (percentage positive)												Typical patterns for other biovars*†											
	fluorescens						putida						fragi						fluorescens				chl.	aur.
Biovar . . .	V	V	V	V	V	V	V	V	V	V	V	V	A	B	A	B	I	II	III	IV	K	L	chl.	aur.
Cluster . . .	1	2	3	4	5	6	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
No. of strains . . .	38	5	7	3	10	16	3	—	—	—	—	—	68	17	4	4	—	—	—	—	—	—	—	—
D-Arabinose	100	100	0	0	0	6	33	7	12	100	100	7	12	100	—	—	—	—	—	—	—	—	—	—
L-Arabinose	100	80	100	0	0	100	0	22	94	100	100	0	22	94	100	—	—	—	—	—	—	—	—	
D-Xylose	76	0	0	0	0	94	100	9	82	100	100	9	82	100	50	50	—	—	—	—	—	—	—	—
D-Galactose	97	80	14	0	20	100	100	1	88	50	75	1	88	50	75	75	—	—	—	—	—	—	—	—
Trehalose	84	0	86	100	0	100	100	0	6	100	100	0	6	75	—	—	—	—	—	—	—	—	—	
Saccharate	100	80	0	0	20	100	0	100	94	100	100	100	94	100	—	—	—	—	—	—	—	—	—	
Mucate	100	100	0	0	0	100	0	100	100	100	100	100	100	100	—	—	—	—	—	—	—	—	—	
Adonitol	0	0	0	0	0	94	0	1	0	0	0	0	1	0	—	—	—	—	—	—	—	—	—	
Erythritol	0	0	0	0	0	88	0	0	0	0	0	0	0	0	—	—	—	—	—	—	—	—	—	
Mannitol	97	100	0	33	0	100	0	9	76	25	25	9	76	25	—	—	—	—	—	—	—	—	—	
Sorbitol	0	0	0	0	0	100	0	1	18	0	0	1	18	0	—	—	—	—	—	—	—	—	—	
myo-Inositol	0	0	100	100	0	100	0	0	6	25	25	0	6	25	—	—	—	—	—	—	—	—	—	
Propylene glycol	0	0	0	100	100	62	100	84	24	0	0	84	24	0	—	—	—	—	—	—	—	—	—	
Malonate	95	80	0	100	30	100	0	72	100	0	0	72	100	0	—	—	—	—	—	—	—	—	—	
Adipate	0	0	0	100	0	0	0	3	6	0	0	3	6	0	—	—	—	—	—	—	—	—	—	
Sebacate	0	0	0	100	0	0	0	0	0	0	0	0	0	0	—	—	—	—	—	—	—	—	—	
Itaconate	0	0	0	67	0	100	0	15	0	50	50	15	0	50	—	—	—	—	—	—	—	—	—	
Mesaconate	0	0	0	33	0	100	0	12	0	0	0	12	0	0	—	—	—	—	—	—	—	—	—	
Hydroxymethylglutarate	0	0	0	0	0	94	0	0	0	0	0	0	0	0	—	—	—	—	—	—	—	—	—	
Ethanol	0	0	14	100	100	69	67	91	76	0	0	91	76	0	—	—	—	—	—	—	—	—	—	
Propanol	0	0	14	100	100	69	100	97	76	100	100	97	76	100	—	—	—	—	—	—	—	—	—	

<i>p</i> -Hydroxybenzoate	92	100	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	+	+	+	+	+	+	+	+	+	
Quinate	97	100	0	100	100	100	100	100	100	97	100	100	100	100	100	100	100	100	100	100	100	+	+	+	+	+	+	+	+	+
Benzoyl formate	0	0	0	100	0	0	0	0	0	34	6	0	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Phenylacetate	0	80	0	67	100	12	67	91	94	0	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Glycine	32	100	0	0	0	0	0	87	88	50	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
L-Tryptophan	0	0	0	100	0	100	0	0	82	0	0	82	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Kyurenine	0	0	0	100	0	100	0	1	88	0	1	88	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Kyrenate	0	20	0	67	0	0	0	3	82	0	3	82	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Antranilate	0	0	0	100	0	100	0	3	100	0	3	100	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Benzylamine	5	100	14	67	10	0	0	97	100	0	97	100	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Histamine	11	100	0	100	10	12	0	99	82	0	99	82	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Tryptamine	0	60	0	0	0	6	0	78	82	0	78	82	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Butylamine	3	80	0	100	80	0	67	88	94	0	67	88	94	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
$\alpha$ -Amylamine	3	80	0	100	80	6	67	88	94	0	67	88	94	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Creatine	0	0	86	0	0	0	100	94	47	100	94	47	100	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Hippurate	0	40	0	0	0	0	0	75	71	100	75	71	100	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Nicotinate	0	0	0	33	0	88	0	75	0	0	75	0	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
Trigonelline	97	100	0	33	0	94	0	100	82	0	100	82	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-

\* Biovar subdivisions of *P. fluorescens* based on the numerical analyses of Champion *et al.* (1980), which employed the same characteristics as were used in this study: biovar I-1, major cluster of 'biotype A' strains; biovar I-2, biotype A strains RYS184 and RYS185; biovar II-1, major cluster of 'biotype B' strains; biovar II-2, biotype B strains RYS400 and RYS402; biovar II-3, biotype B strains RYS2 and RYS93; biovar III-1, major cluster of 'biotype C' strains (groups C-I, C-II, C-III, and C-V of Champion *et al.*, 1980; biovar III-2, biotype C strains RYS204, RYS210, RYS212 and PJ832 (group C-IV of Champion *et al.*, 1980); biovar IV-1, the two biotype F strains of Stanier *et al.* (1966); biovar IV-2, strains PJ890, PJ976 RYS407 and RYS413 (the latter two formerly assigned to biotype B by Stanier *et al.*, 1966). Biovars 'K' and 'L' were created by Champion *et al.* to accommodate strains originally designated biotype C which did not resemble any of the *P. fluorescens* biovars included in that study. *P. chlororaphis* (*chl.*) and *P. aureofaciens* (*aur.*) correspond to biotypes D and E, respectively, of Stanier *et al.* (1966). Carbon source utilization data for the above groups were published by Stanier *et al.* (1966) excepting data for clusters IV-2, biotype 'K', and biotype 'L', which have not been previously published.

† Notation for previously described biovars: +, used by 75% or more strains; -, used by 25% or fewer strains; v, used by 26-74% of the strains.

Table 3. *Additional characteristics for which strains of P. fluorescens biovar V differ*

Species . . .	Strains examined in this study (percentage positive)									
	<i>fluorescens</i>							<i>putida</i>		<i>fragi</i>
Biovar . . .	V	V	V	V	V	V	V	A	B	—
Cluster . . .	1	2	3	4	5	6	7	—	—	—
No. of strains . . .	38	5	7	3	10	16	3	68	17	4
Mannose	92	100	43	33	10	94	100	26	82	100
Sucrose	45	0	0	33	0	6	0	4	7	0
Butylene glycol	11	0	0	67	90	56	100	71	47	0
Butyrate	45	100	100	100	100	38	67	97	100	100
Isobutyrate	74	80	100	33	100	50	67	94	87	100
Valerate	89	100	100	100	100	56	67	100	100	100
Isovalerate	87	100	100	100	100	38	67	100	100	75
Suberate	0	0	0	33	0	0	0	0	0	0
Azelate	0	0	0	67	0	0	0	0	0	0
D-Malate	60	60	86	0	100	31	100	66	53	NT
L(+)-Tartrate	0	0	0	0	0	6	0	49	12	NT
meso-Tartrate	50	20	0	0	80	94	67	57	0	NT
Citraconate	29	80	0	0	0	62	0	6	65	0
Laevulinat	45	40	0	33	40	19	0	35	67	0
Butanol	24	0	29	100	100	50	100	94	82	100
Isobutanol	8	0	29	67	100	56	0	97	71	75
Benzoate	29	100	71	100	20	81	0	100	100	50
L-Serine	100	80	71	33	70	100	33	81	80	0
L-Lysine	82	60	86	100	100	81	100	100	82	75
Citrulline	26	40	0	100	100	69	0	91	35	100
L-Phenylalanine	97	100	57	33	100	69	100	97	94	100
Ethanolamine	74	40	71	100	100	62	100	74	24	25
Gelatin liquefaction	92	100	71	100	40	81	0	0	18	0
Growth at 4 °C	100	100	100	100	80	100	100	13	100	100

NT, Characteristics not tested.

fluorescent biovars were also included in this analysis: propionate (uniquely negative for *P. fluorescens* biovar IV), and sucrose (negative for biovar III but positive for all other *P. fluorescens* biovars). For each pair of clusters to be compared, the  $S_{SM}$  values which related, respectively, each member of the one cluster to each member of the other cluster were averaged. The internal homogeneity of each group was also evaluated by calculation of the average  $S_{SM}$  between all pairs within it. The results are compiled in Fig. 2.

The analysis revealed significant similarity of *P. fluorescens* V-1 to *P. fluorescens* I-2 (average  $S_{SM} = 89\%$ ). In contrast, the average  $S_{SM}$  relating the I-2 strains to the major group within their own biovar (*P. fluorescens* I-1) was 67%. Apparently on the basis of carbon source utilization, biovar I-2 strains would be more correctly assigned to biovar V. However, the two biovar I-2 strains do produce levan from sucrose (Stanier *et al.*, 1966), which is the primary characteristic used to differentiate biovar I strains from biovar V strains. Close similarity was also revealed between cluster V-1 and the two *P. fluorescens* IV-1 strains (average  $S_{SM} = 85\%$ ). The biovar IV-1 strains were both originally designated *P. lemonnieri* and were described as producing a blue insoluble phenazine pigment, although the pigment production has proved to be an unstable characteristic (Stanier *et al.*, 1966). Both denitrify and produce levan from sucrose, and thus differ from biovar V strains on the basis of all three primary characteristics used for biovar assignment. Molin & Ternström (1986) described a cluster of *P. fluorescens* strains isolated from soil which were readily assigned to biovar IV on the basis of carbon source utilization, but many of which did not denitrify, produce levan, or form a blue pigment. Perhaps their group is synonymous with our cluster V-1.

There have been other situations in which the ability to denitrify was found not to correlate





The *P. fluorescens* V-5 cluster, which consists of strains initially designated *P. putida* A as well as *P. fluorescens* V strains, was found to resemble *P. putida* A more than any biovar of *P. fluorescens*. None of the V-5 strains used trehalose; Stanier *et al.* (1966) noted that trehalose was used by virtually all strains of *P. fluorescens*, but no strains of *P. putida*. Cluster V-5 also closely resembled the 'biotype L' strains of Champion *et al.* (1980). A separate biotype status was proposed for these strains in the latter study because they differed significantly from all the other *P. fluorescens* strains examined. However, *P. putida* strains were not included in that study.

The results compiled in Fig. 2 also revealed that the three strains constituting *P. fluorescens* biovar V-7 were very similar to the biovar V-5 and 'biotype L' strains, although they differed from the V-5 strains with respect to the utilization of trehalose. The relationship between V-5 and V-7 was not revealed in the phenogram (Fig. 1). A review of the  $S_J$  values used to construct that phenogram indicated that the apparent discrepancy was a result of the method of phenogram construction; the average  $S_J$  relating the V-7 strains to cluster V-5 by itself was 72%. They were not joined at this  $S_J$  level because cluster V-5 was more related to *P. putida* A ( $S_J = 75\%$ ). However, the average  $S_J$  relating V-7 to *P. putida* A was very low (61%), so that when it was joined to the combined V-5/*P. putida* A cluster (68 *P. putida* A strains and 10 cluster V-5 strains), the average  $S_J$  was only 62%, and the V-5/V-7 relationship was thus obscured. On the other hand, a two-dimensional array such as that shown in Fig. 2 does not obscure relationships between two groups when only one of them is more closely related to a third group.

The *P. fluorescens* biovar V cluster V-6 was found to be closely related to *P. fluorescens* biovars I and II (84%  $S_{SM}$  with biovar I-1, and 84%, 83% and 77% with II-1, II-2 and II-3, respectively). Biovar I and II strains all form levan from sucrose; strains of biovar II, but not I, also denitrify. There are very few carbon sources that distinguish biovar I strains from biovar II strains. Similarly, we found very few that distinguished most biovar V-6 strains from either I-1 or II strains. In fact, two V-6 strains (PJ77 and PJ270a) were related to each of three *P. fluorescens* biovar II strains (RYS406, RYS411 and RYS414) by an  $S_{SM}$  of 95%.

Cluster V-4 was not found to be closely related to any other group examined, its closest relative being *P. chlororaphis* with an average  $S_{SM}$  of 76%. However, the relationship of the cluster V-4 strains to the other groups did not appear to parallel the relationship of *P. chlororaphis* to the same groups.

#### *Relationships between biovar V clusters and other 'unassigned' fluorescent strains*

In the first numerical analysis, based on  $S_J$  values, five *P. fluorescens* biovar V strains were less than 60% related to any cluster (PJ724, PJ982, PJ758, PJ797 and PJ893). To see if any of them resembled any of the other groups with respect to the 41 carbon sources used for the second numerical analysis,  $S_{SM}$  values relating these strains to the members of all the groups included in Fig. 2 were calculated. Two (PJ724 and PJ982) were found to resemble cluster V-1; each had an average  $S_{SM}$  relating them to the V-1 strains greater than 80%. The other three (PJ758, PJ797 and PJ893) were even more closely related to cluster V-3; for each the average  $S_{SM}$  relating it to the seven V-3 strains was greater than 88%. The apparent discrepancy between these results and those represented in Fig. 1 is a reflection of the different methods used for the respective cluster analyses. In the initial study,  $S_J$  values were used; thus only positive results were taken into account. When a cluster is characterized by a limited range of possible carbon sources, as is cluster V-3, the differences between component strains are magnified as compared to differences among members of a group of versatile strains. Matching coefficients were not used in that analysis because the strains shared many negative characteristics, a feature which increases the percentage similarity among all strains, thereby compressing the range of significant differences between clusters. Furthermore, the second analysis involved only those carbon sources which were especially useful for cluster definition. While this selection of carbon sources might be looked upon as deliberate bias, it is important to remember that the clusters on which we were attempting to focus were exactly those clusters revealed in the analysis involving all 147 characteristics.

The carbon sources listed in Table 2 were also used as a basis for examining the 11 'unassigned' fluorescent strains in the original taxonomic work of Stanier *et al.* (1966) for

possible overlap with the biovar V clusters. Four of them, RYS17, RYS47, RYS59 and RYS91, were found to be closely related to the V-5 cluster; the average  $S_{SM}$  relating each to all the V-5 strains was greater than 85%, and one of them (RYS91) was related to a V-5 strain (PJ783) by an  $S_{SM}$  of 100%. Two of Stanier's unassigned strains (RYS10 and RYS127) were related to several V-1 strains by  $S_{SM}$  values greater than 80%. The remaining five strains did not appear related to any of the groups examined in this study.

#### *Relationship between P. fragi and P. fluorescens biovar V*

In the early descriptions of *P. fragi*, the chief identifying characteristics were the production of 'fruity' spoilage odour (Hussong & Long, 1937; Morrison & Hammer, 1941) and the formation of an 'acid ring' in litmus milk (Hussong & Long, 1937). Although neither characteristic lends itself to accurate strain identification, it has been shown that the type strain deposited many years ago in the American Type Culture Collection is representative of a large and unique class of meat spoilage pseudomonads (Molin & Ternström, 1982, 1986; Shaw & Latty, 1982). Like biovar V strains, *P. fragi* neither denitrifies nor produces levan from sucrose. Arginine dihydrolase was positive for all four strains as it is for all the fluorescent pseudomonads tested by Stanier *et al.* (1966). The G + C content of the three ATCC strains was found to be 58.3–58.5 mol %. This value is at the lower limit for *P. fluorescens* (Mandel, 1966). The fluorescent biovars at the lowest end are, in fact, the ones closest to *P. fragi* in our phenotypic analysis, namely biovar IV and strains of biovar V which fell into cluster 1 (Mandel, 1966).

The results of our carbon source utilization tests agreed closely with the comparable results reported by Molin & Ternström (1982, 1986) and Shaw & Latty (1982) for their major cluster of meat spoilage pseudomonads. About 130 carbon sources included in this study were also used by Molin & Ternström (1982, 1986); our results differed only with respect to the utilization of butyrate, isobutyrate, propanol, butanol and citrulline, all of which we scored as positive, but which were reported as negative by the other investigators. We also examined 20 Gram-negative motile rods isolated from separate spoiled dairy products and found seven which closely resembled the named strains of *P. fragi* in carbon source utilization pattern. This indicates that the same species is common in dairy products as well as in meat. All the *P. fragi* strains failed to grow at 37 °C, which may be a useful distinguishing characteristic.

The analyses revealed close relationships between *P. fragi* and *P. fluorescens* clusters V-1 and V-3. We had found the latter group to be synonymous with the second major group of meat spoilage pseudomonads, now designated *P. lundensis* (Molin *et al.*, 1986). Molin & Ternström (1982, 1986) found *P. fragi* to be more closely related to *P. lundensis* than any *P. fluorescens* biovars, but biovar V was not included in those studies.

#### *Taxonomic conclusions*

The foregoing results have shown that the currently defined *P. fluorescens* biovar V consists of several distinct groups, most of which are as different from each other on the basis of overall phenotype as are the recognized *P. fluorescens* biovars. The results suggest the need to redefine biovar V in terms of just one of the clusters revealed in this study and to designate at least two new fluorescent biovars for the other clusters. We propose that cluster V-6 alone should retain the designation biovar V because it contains the greatest number of the original 'biotype G' strains of Stanier *et al.* (1966). This newly defined biovar V is part of a group of closely related biovars that includes I and II.

The largest biovar V cluster, V-1, was quite distinct from cluster V-6 and should be designated as a new biovar (biovar VI) which also includes the *P. fluorescens* I-2 strains (RYS184 and RYS185). Because the small number of V-2 strains available for this study does not warrant separate biovar designation at this time, and because this cluster was closely related to V-1, the V-2 strains could also be included in biovar VI for the time being.

An additional new fluorescent biovar is also suggested for cluster V-5. Because it was more related to *P. putida* A than to any *P. fluorescens* group, it should be designated as a *P. putida* biovar, presumably biovar C. The fact that many fluorescent strains which, in previous studies, could not be assigned to one of the established biovars, were shown here to have the V-5

phenotype supports the recognition of this group as a unique biovar. Cluster V-7 was related to V-5 in the second numerical analysis (Fig. 2) and thus might be considered a subgroup of *P. putida* C until more strains are available for further study.

Cluster V-3 was shown to be identical to the group of meat isolates now designated *P. lundensis* (Molin *et al.*, 1986). The latter investigators have described the new species in sufficient detail to permit its differentiation from the other fluorescent groups.

Cluster V-4 did not closely resemble any of the other groups examined, but its small size argues against designation of a new biovar at this time. Studies of additional similar strains are needed to clarify the taxonomic position of this cluster.

The results concerning *P. fragi*, like those of previous investigators (Molin & Ternström, 1982, 1986; Shaw & Latty, 1982, 1984), support the designation of this group as a unique (and now well-defined) *Pseudomonas* species. The similarity of *P. fragi* to *P. fluorescens* V-1 supports the suggestion that it is part of RNA homology group I (Whitaker *et al.*, 1981*a, b*). Perhaps its phenotype is indicative of the lack of the need for fluorescent pigmentation for survival in the rich environment of meats and other foods.

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We wish to dedicate this paper to Dr Luis F. Leloir on the occasion of his 80th birthday.

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