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Numerical Taxonomy of Psychrotrophic Pseudomonads

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The taxonomy of 218 psychrotrophic pseudomonad strains (200 field strains from meat and 18 type and reference strains) was numerically studied by 174 biochemical and physiological tests. All strains were Gram-negative rods, oxidase positive and motile by means of one or more polar flagella. The strains clustered into 15 groups, of which 9 were regarded as major clusters. The major clusters were designated as *Pseudomonas fragi* (112 strains), *P. fluorescens* biotype III (7 strains), *P. fluorescens* biotype I (16 strains), *P. aureofaciens/chlororaphis* (3 strains), *P. fluorescens* biotype II (3 strains), *P. putida* biotype I (4 strains), *Alteromonas putrefaciens* (10 strains) and *Aeromonas hydrophila* biotype I (5 strains). One major cluster, containing 21 strains (cluster 2), was left unassigned. The phenotypic data indicate that this cluster might represent a new species. The *P. fluorescens/P. putida* complex matched closely the descriptions of Stanier *et al.* (1966), but the two largest clusters (1 and 2) were not in agreement with any species described in the eighth edition of *Bergey's Manual of Determinative Bacteriology*. Cluster 1 included the type strain (ATCC 4973) of the hitherto incompletely described *P. fragi*. A simplified scheme for the separation between *P. fragi, P. fluorescens, P. putida* and cluster 2 is presented.

INTRODUCTION

The taxonomy of *Pseudomonas* is, at present, based primarily on the work of Stanier *et al.* (1966). This study was later supplemented by Ballard *et al.* (1968, 1970), Misaghi & Grogan (1969), Davis *et al.* (1970), Sands *et al.* (1970), Ralston *et al.* (1973), Austin & Goodfellow (1979), Champion *et al.* (1980), Palleroni (1980) and Pickett & Greenwood (1980). Psychrotrophic *Pseudomonas* strains were only included in four of the above papers, and two of these considered mainly plant pathogenic strains.

In the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Doudoroff & Palleroni, 1974) and in the above-mentioned reports only three oxidase-positive psychrotrophic *Pseudomonas* species are described: *P. fluorescens, P. putida* and *P. cichorii* (phytopathogenic). According to the descriptions, these species would all be able to produce fluorescent pigment, and 90% or more of the strains have been found to do so (Stanier *et al.*, 1966 and Sands *et al.*, 1970, respectively). Thus, the incapacity to produce fluorescent pigments seems to be an unusual characteristic for described psychrotrophic pseudomonads. However, the microbial spoilage flora of refrigerated meat often consists to a high degree of non-fluorescent pseudomonads (Enfors *et al.*, 1979; Blickstad *et al.* 1981). These apparently 'atypical' psychrotrophic pseudomonads could be non-fluorescent varieties of *P. fluorescens* and/or *P. putida*. On the other hand, they could constitute one or several homogeneous groups of their own, with phenotypical differences from the fluorescent pseudomonads in addition to their inability to form fluorescent pigment. Davidson *et al.* (1973) studied 231 psychrotrophic pseudomonads and suggested that 'the majority of commonly occurring pseudomonads on meat and meat products do not conform with the species proposed by Stanier *et al.* (1966)'. Similar results have been reported by Shaw & Latty (1981) and, from fish, by Gray & Stewart (1980).

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The intention of the present study was to characterize psychrotrophic pseudomonads and to analyse their taxonomy in accordance with the principles suggested by Stanier *et al.* (1966).

METHODS

Strains. Strains used in the study are listed in Table 1.

Isolation of field strains. Psychrotrophic counts on tryptone glucose extract agar (Oxoid; incubated at 4 °C for 10 d in air) were made from meats of different origin. Colonies were randomly picked and screened for oxidase production (Kovacs, 1956), Gram-negative reaction (Bacto Gram stain set; Difco), motility [phase-contrast microscopy after growth in nutrient broth (NB; Oxoid) for 20 h at 25 °C] and polar flagellation (Mayfield & Inniss, 1977).

The isolates were picked from meat, representing a contamination level of 10^2–10^9 psychrotrophic colony-forming units cm^{-2}. Only one isolate was taken from each piece of meat.

All the isolations were made during a 2 month period. The isolates were grown in NB at 25 °C for 3 d (static cultures). The cultures were then stored at 2 °C and used as stock cultures. All isolates were tested within a 6 month period after the isolation and then, after testing, checked for purity and reaction on the first 10 biochemical tests performed. All the isolates were tested on a particular test at the same time. The type and reference strains were treated in the same way.

Properties examined.

Including the tests used in the screening procedure, a total of 192 characteristics were studied. Tests were done at 25 °C and reactions were read after 4 d unless otherwise stated.

Morphology. Cell shape, cell size (NB for 16–96 h) and colony appearance [nutrient agar (NA) and blood agar for 1–10 d] were studied.

Pigment production. Pink or yellow pigmentation was noted on nutrient agar after 2 and 7 d. Phenazine pigments were noted on King A medium and fluorescent pigments on King B medium after 1–5 d (King *et al.*, 1954).

Growth at different temperatures. Growth or no growth was recorded in NB incubated at 4 °C (10 d), 37 °C (2 d) and 42 °C (2 d).

Growth at different NaCl concentrations. Growth or no growth was recorded on NA plates supplemented with 3% and 6% (w/v) NaCl.

Oxidative and fermentative utilization of glucose. Test tubes with the medium of Hugh & Leifson (1953) supplemented with agar to a total strength of 1·5% were incubated aerobically and anaerobically (Gas Pak Anaerobic System, BBL). In the anaerobic incubation 8 ml 0·1% (w/v) bromocresol purple was used as indicator instead of bromothymol blue. The tubes were examined for acid and gas production, as indicated by a change of colour and disruption of the agar, respectively.

Acid production from carbohydrates. The medium of Hugh & Leifson (1·5% agar, plates) was supplemented with the following sterile filtered carbohydrates (final concentration 1%, w/v); adonitol, amygdalin, L-arabinose, cellobiose, dextrin, dulcitol, D-fructose, D-galactose, glycerol, glycogen (from oyster), *meso*-inositol, inulin, lactose, maltose, mannitol, D-mannose, melezitose, melibiose, raffinose, rhamnose, D-ribose, sucrose, salicin, sorbitol, L-sorbose, starch, trehalose, xylitol, and xylose.

Production of lipases. Lipase activity was tested on Tween 80 (Sierra, 1957) and on tributyrin (Collins & Lyne, 1970).

Production of proteases. Proteolytic activity on gelatin was recorded on the following medium (g l^{-1}): meat extract (Oxoid), 3·0; peptone (Oxoid), 5·0; gelatin (Oxoid), 120·0. Plates were examined after 1, 2, 7 and 30 d. Before reading, the plates were chilled for 2 h. Plates that remained fluid were scored positive.

Casein breakdown was investigated in a medium consisting of 30% (v/v) skim milk (Semper, Stockholm) and 1·5% (w/v) agar (sterilized at 110 °C for 20 min). After incubation the plates were flooded with a 10% (w/v) solution of $HgCl_2$. Positive reaction was indicated by clearing around the confluent growth.

Egg-yolk reaction. Plates were poured with NA supplemented with 10% (v/v) egg-yolk solution (Oxoid) and 1% (w/v) NaCl. Plates were examined for an opaque precipitation ('egg-yolk reaction') and for zones of clearing around the confluent growth due to lipolytic activity.

Hydrolysis of tyrosine. Plates were poured with NA supplemented with 5·0% (w/v) tyrosine. Clearing around the confluent growth was scored positive.

Hydrolysis of starch. Plates were poured with NA supplemented with 0·3% (w/v) soluble starch. Plates were flooded with Lugol's solution after incubation. Zones of clearing were scored positive.

Decomposition of hippurate. Plates were poured with the following medium (g l^{-1}): agar, 15·0; NaCl, 5·0; sodium hippurate, 3·0; $NH_4H_2PO_4$, 1·0; and phenol red, 0·001% (w/v). The pH was adjusted to 6·9 and the

medium was then sterilized at 110 °C for 20 min. Positive reaction was indicated by a red zone around the confluent growth.

Decomposition of arginine. This was tested as described by Thornley (1960).

Haemolysis on blood agar. Plates were poured with NA supplemented with 7% (v/v) ox blood. The plates were observed daily for up to 4 d and haemolysis was recorded.

Denitrification. Plates were poured with the following medium (g l^{-1}): agar, 15·0; yeast extract (Oxoid), 5·0; KNO_3, 5·0; K_2HPO_4, 1·0. The pH was adjusted to 6·8 and then the medium was sterilized at 121 °C for 15 min. The plates were incubated anaerobically; denitrification was indicated by growth.

Miscellaneous tests. Methods and media for methyl red and Voges–Proskauer reactions, growth on alginate agar and on Simmon's citrate agar, deamination of phenylalanine, and production of DNAase, H_2S and urease were all as described by Edwards & Ewing (1972). Production of RNAase was tested in the same base medium as DNAase, but supplemented with 0·2% or 2·0% (w/v) RNA (two tests).

Utilization of compounds as sole carbon source. A basal medium with the following composition (g l^{-1}): purified agar (Oxoid) 10·0; Na_2HPO_4, 3·0; KH_2PO_4, 2·3; NH_4Cl, 1·0; $MgSO_4$, 0·5; $CaCl_2$, 0·005; pH adjusted to 6·8 before autoclaving (121 °C for 15 min) was aseptically supplemented with 0·13% (w/v or v/v) of the substances listed in Table 2. All substances which could be filtered were sterilized by filtering through a membrane filter (pore size 0·45 μm). The others were autoclaved or, if heat labile, pasteurized at 80–90 °C.

Plates with the supplemented basal medium were inoculated with the different strains by drawing a patch with a cotton swab. The number of patches (isolates) on the same plate was limited to five to avoid substrate exhaustion and/or cross-feeding reactions. All patches showing growth greater than the control (without carbon source) were regarded as positive. In a few cases 'mutants' developed in an otherwise negative test. These were considered as 'no consequence tests' and were not included in the computation of data.

Analysis of data. A total of 174 quantitative characters were codified as 0, negative, 1, positive, and 2, of no consequence. None of the characteristics were the same for all the strains. Similarity coefficients using simple matching [$S_M = (m \times 100)/(n - nc)$, where m is the sum of positive and negative matches, n is the total number of tests and nc is the number of invalid tests] were obtained between every pair of strains.

The cluster analysis package 'Clustan' was used for the subsequent clustering (D. Wishart, Program Library Unit; version 1c, release 2; Edinburgh University, 1978). Four methods with all possible combinations of similarity coefficients with 'hierarchy' options are applicable: (1) nearest neighbour, (2) furthest neighbour, (3) group average and (4) McQuitty's similarity analysis. Method (3) (also called average linkage) was chosen as the basis for the analysis and plotting of the dendrogram. The other methods were used to check the stability of the results.

RESULTS

Clustering of the strains

Of the total of 218 test strains, 198 formed 15 clusters. Figure 1 shows the clusters and the clustering pattern obtained by applying the group average method. The clusters formed at the similarity level of 88% were stable when the cluster analysis was checked by the furthest neighbour and McQuitty's similarity analysis methods. Thus, the three methods yielded the same major clusters and these merged in the same order. The differences were marginal and consisted of occasional reversals of the order in which some straggler strains joined the major clusters. The nearest neighbour method gave roughly the same results, but in this case all the clusters merged one by one directly into cluster 1. This behaviour is inherent in the method, which tends to favour a cluster that is already large. The order of fusion remained the same for the major clusters but there were several reversals of order for the minor clusters and the stragglers.

The allocation of strains to clusters is shown in Table 1.

Significant clusters

Nine of the 15 clusters were regarded as significant. Thus, they were stable and either were large (>4 strains) or included type or reference strains. The distribution of the physiological and biochemical characters among the defined taxa is shown in Table 2.

Cluster 1 (*Pseudomonas fragi*). This cluster included the type strain for *P. fragi* (ATCC 4973; strain no. 213). Typical strains utilized 55 out of 118 tested carbon sources. Of

Table 1. *Designation and source of strains*

Lab no.	Source/collection no.
	Strains assigned to cluster 1 (*Pseudomonas fragi*)
1	Pork
2	Pork
27	Pork
14	Pork
53	Pork
47	Pork
186	Pork
187	Pork
86	Beef
88	Beef
93	Beef
147	Turkey
84	Turkey
112	High-pH beef
64	Pork
13	Pork
16	Pork
18	Pork
33	Pork
15	Pork
163	Frozen hare meat, imported from Hungary
164	Frozen hare meat, imported from Hungary
49	Pork
52	Pork
192	Spoiled venison meat
79	Beef
165	Frozen hare meat, imported from Hungary
109	Spoiled beef
19	Bone marrow, pig
73	Beef
3	Pork
4	Pork
38	Prepacked pork
179	Pork
185	Pork
43	Pork
48	Pork
50	Pork
68	Pork
98	Spoiled beef
197	Pork
182	Pork
194	Spoiled elk meat
200	Frozen antelope meat, imported from USSR
199	Frozen antelope meat, imported from USSR
146	Turkey
89	Beef
195	Spoiled elk meat
71	Beef
188	Pork
46	Pork
51	Pork
72	Beef
90	Beef
55	Pork
116	Beef
77	Beef
66	Pork
181	Pork
184	Pork
75	Prepacked beef
76	Prepacked beef
80	Beef
87	Prepacked beef
92	Beef
141	Turkey
144	Turkey
101	Beef
183	Pork
70	Pork
63	Pork
121	Spoiled high-pH beef
125	High-pH beef
173	Chicken
175	Spoiled prepacked pork
20	Bone marrow, pig
26	Pork
31	Pork
34	Pork
24	Pork
37	Prepacked pork
39	Prepacked pork
83	Beef
56	Pork
160	Chicken
213	*P. fragi*, ATCC 4973; type strain
59	Pork
21	Pork
32	Pork
25	Pork
174	Spoiled prepacked pork
176	Spoiled prepacked pork
85	Spoiled beef
177	Spoiled prepacked pork
23	Pork
99	Spoiled beef
108	Spoiled prepacked beef
106	Beef
107	Beef
191	Spoiled venison meat
100	Beef
119	Beef
122	High-pH beef
124	Spoiled high-pH beef
41	Prepacked pork
115	Beef
120	High-pH beef
91	Beef
196	Spoiled elk meat
198	Frozen antelope meat, imported from USSR
126	High-pH beef
17	Pork
	Strains assigned to cluster 2 (*Pseudomonas* sp.)
8	Bone marrow, pig
45	Pork
82	Spoiled beef
127	Prepacked beef

Table 1 (*continued*)

Lab no.	Source/collection no.
128	Prepacked beef
133	Prepacked beef
139	Prepacked beef
131	Prepacked beef
132	Prepacked beef
136	Prepacked beef
138	Prepacked beef
135	Prepacked beef
130	Prepacked beef
140	Prepacked beef
134	Prepacked beef
178	Prepacked beef
137	Prepacked beef
65	Pork
129	Prepacked beef
102	Spoiled beef
97	Prepacked beef
	Strains assigned to cluster 3 (*Pseudomonas* sp.)
78	Beef
162	Frozen hare meat, imported from Hungary
	Strains assigned to cluster 4 (*Pseudomonas* sp.)
5	Pork
6	Pork
7	Pork
81	Prepacked beef
	Strains assigned to cluster 5 (*Pseudomonas* sp.)
36	Slimy bone marrow, pig
142	Turkey
	Strains assigned to cluster 6 (*Pseudomonas* sp.)
9	Bone marrow, pig
10	Bone marrow, pig
153	Chicken
	Strains assigned to cluster 7 (*P. fluorescens* biotype III)
104	Beef
105	Beef
117	Beef
212	*P. fluorescens* biotype III, ATCC 17400 (R. Y. Stainer 18)
143	Turkey
206	*P. fluorescens* biotype III, DSM 50124 (R. Y. Stainer 214)
154	Chicken
	Strains assigned to cluster 8 (*P. fluorescens* biotype I)
11	Pork
12	Pork
54	Pork
22	Pork
149	Chicken
151	Chicken
217	*P. putida* DSM 50906
168	Chicken
169	Chicken
150	Chicken
152	Chicken
158	Chicken
167	Chicken
155	Chicken
159	Chicken
210	*P. fluorescens* biotype I, ATCC 13525 (R. Y. Stanier193); type strain.
	Strains assigned to cluster 9 (*P. aureofaciens*/*P. chlororaphis*)
201	*P. aureofaciens*, DSM 50082
202	*P. aureofaciens*, DSM 50139 (R. Y. Stanier 36)
203	*P. chloraphis*, DSM 50083 = ATCC 9446 (R. Y. Stanier 30); type strain
	Strains assigned to cluster 10 (*Pseudomonas* sp.)
123	High-pH beef
171	Chicken
	Strains assigned to cluster 11 (*P. fluorescens* biotype II)
204	*P. fluorescens* biotype II, DSM 50106 (R. Y. Stanier 411)
205	*P. fluorescens* biotype II, DSM 50108 (R. Y. Stanier 108)
208	*P. fluorescens*, ATCC 949
	Strains assigned to cluster 12 (*P. putida* biotype I)
209	*P. fluorescens*, ATCC 11172
216	*P. putida* biotype I, DSM 50208 (R. Y. Stanier 111)
214	*P. putida* biotype I, DSM 50198 (R. Y. Stanier 77)
215	*P. putida* biotype I, DSM 50202 = ATCC 12633 (R. Y. Stanier 90); type strain.
	Strains assigned to cluster 13 (*Pseudomonas* sp.)
28	Pork
166	Chicken
114	High-pH beef
157	Chicken
	Strains assigned to cluster 14 (*Alteromonas putrefaciens*)
30	Slimy bone marrow, pig
69	Pork
58	Pork
145	Turkey
44	Pork
180	Pork
62	Pork
110	Prepacked high-pH beef
111	Prepacked high-pH beef
113	Prepacked high-pH beef

Table 1 (*continued*)

Lab no.	Source/collection no.
	Strains assigned to cluster 15 (*Aeromonas hydrophila*)
29	Slimy bone marrow, pig
40	Prepacked pork
60	Pork
61	Pork
148	Turkey
	Strains not assigned to clusters
94	Beef
193	Spoiled elk meat
42	Prepacked pork
67	Pork
190	Spoiled venison meat
170	Chicken
74	Beef
96	Beef
35	Slimy bone marrow, pig
211	*P. fluorescens*, ATCC 17397
57	Pork
207	*P. fluorescens* biotype IV, DSM 50415
172	Chicken
218	*P. putida* biotype II, DSM 50222 (R. Y. Stanier 110)
95	Beef
103	Spoiled beef
189	Spoiled venison meat
118	Prepacked beef
156	Chicken
161	Chicken

the 112 strains, 110 were unable to produce fluorescent pigments, and 105 of the 112 did not produce gelatinase. Some critical tests whereby *P. fragi* may be separated from related taxa are listed in Table 3.

Cluster 2. This cluster could be described as intermediate between *P. fragi* and *P. fluorescens*. The resemblance to *P. fluorescens* is indicated by the fact that 10 of the 21 strains produced fluorescent pigments and 20 produced gelatinase. However, the strains clustered next to *P. fragi* and utilized significantly fewer carbon sources (45) than *P. fluorescens* (65).

We would predict that cluster 2 represents a new species, but it requires at least a guanine plus cytosine analysis before this can be verified.

Cluster 7 (*P. fluorescens biotype III*). This cluster included the two biotype III reference strains DSM 50124 (no. 206) and ATCC 17400 (no. 212), both described by Stanier *et al.* (1966), who used the term biotype C. All the clustered strains met the description of biotype III (Doudoroff & Palleroni, 1974) with the exception of the utilization of L-arabinose. Six of the strains in our study were able to utilize this compound.

Cluster 8 (*P. fluorescens biotype I*). This cluster included the type strain for *P. fluorescens*, ATCC 13525 (no. 210). Ten of the 14 field strains were isolated from chicken. All the strains met the description of biotype I (Doudoroff & Palleroni, 1974).

Cluster 9 (*P. aureofaciens/P. chlororaphis*). This cluster included only type and reference strains, which all met the descriptions of Doudoroff & Palleroni (1974): *P. chlororaphis* DSM 50083 (no. 203 = ATCC 9446) is the type strain and *P. aureofaciens* strain DSM 50139 (no. 202) was described by Stanier *et al.* (1966) as *P. fluorescens* biotype E.

Cluster 11 (*P. fluorescens biotype II*). This cluster consisted only of reference strains, which all met the description of Doudoroff & Palleroni (1974). The strains DSM 50106 and DSM 50108 (nos 204 and 205, respectively) were described by Stanier *et al.* (1966) as biotype B.

Cluster 12 (*P. putida biotype I*). The cluster consisted only of type and reference strains, all with high resemblance to the description of Doudoroff & Palleroni (1974). Three of the strains were described by Stanier *et al.* (1966), including the type strain DSM 50202 (no. 215 = ATCC 12633). The fourth strain, ATCC 11172 (no. 209), was labelled as a *P. fluorescens* (ATCC Catalogue of Strains I, 14th edn, 1980). It should be pointed out that after prolonged incubation this strain was able to denitrify, a character not previously recorded in *P. putida*.

Cluster 14 (*Alteromonas putrefaciens*). This cluster, which consisted only of field strains, differs distinctly from the other pseudomonad strains examined. Thus, the strains utilized only 10 carbon sources for growth and produced only small amounts or no acid at all from the tested carbohydrates. The cluster could easily be separated from the other psychrotrophic

pseudomonads by the following: ability to produce H_2S; pink pigmentation; inability to produce arginine dihydrolase; and ability to produce DNAase (10 out of 14).

Cluster 15 (*Aeromonas hydrophila*). This cluster is not related to the *Pseudomonas* strains. The test results (Table 2) match the description for *A. hydrophila* biotype I (subsp. *hydrophila*; Popoff & Veron 1976).

Insignificant clusters

Clusters 3–6, 10 and 13 may be regarded as insignificant. They consisted of 2–4 isolates and are mostly intermediates between named species or within the *P. fluorescens* complex.

Cluster 4 (4 strains) was unstable as it changed position when the different methods of cluster analysis were applied.

Cluster 6 (3 strains) joined the *P. fluorescens* biotype III cluster (cluster 7) at the 85% level and might be regarded as a subgroup of biotype III. Similar organisms were included in biotype C of Stanier *et al.* (1966).

Cluster 13 (4 strains) comprised strains grouped together due to their almost completely negative response to the utilization tests. If negative matches were excluded, the cluster disintegrated.

Three reference strains—*P. fluorescens* ATCC 17397 (no. 211), *P. fluorescens* biotype IV, DSM 50415 (no. 207) and *P. putida* biotype II, DSM 50222 (no. 218)—were stragglers. The *P. fluorescens* biotype IV strain joined the *P. fluorescens* complex as the last strain at the 81% level.

DISCUSSION

The utilization tests of the present study followed closely the procedure of Stanier *et al.* (1966). However, the basal medium was that described by Palleroni & Doudoroff (1972) (citrate eliminated) and the incubation temperature was 25 °C instead of 30 °C. The ability to produce acid from 30 different compounds was tested. Most of these were also used in the utilization tests and it could be argued that the same features were tested twice. However, there were often discrepancies between the ability of an organism to utilize a particular compound and to produce acid from it. Thus, an organism may be positive in the utilization test but negative in the acid production test or vice versa. This has also been pointed out by Palleroni & Doudoroff (1972).

Cluster 1 was designated as *P. fragi*. The name *fragi* (*Bacterium fragi*) was originally used by Eichholz (1902) on a psychrotrophic milk spoiling organism which gave off a strawberry odour. The organism was considered by Hussong *et al.* (1937) to be identical to an organism called *Pseudomonas fragariae* by Grüber (1902). *Pseudomonas fragi* was isolated from various sources (milk, cream, cheese, butter, sheep fat and water) and reported to be a non-fluorescent, proteolytic and 'May apple'-smelling organism (Hussong *et al.*, 1937). The organism has been further described by Breed *et al.* (1948) and Pereira & Morgan (1957), and a neotype strain for *P. fragi* (ATCC 4973) was proposed by Lysenko (1961); this has since become the type strain (Skerman *et al.*, 1980). This particular strain has since then been used as test organism in a number of studies, and has not only been connected with the spoilage of dairy products but also that of meat (Tarrant *et al.*, 1973; Enfors & Molin, 1981) and fish (Florin, 1972). In spite of this, taxonomic information on *P. fragi* is sparse and too limited to provide a base for differentiation from other psychrotrophic *Pseudomonas* species. Consequently, the organism is not described in the eighth edition of *Bergey's Manual of Determinative Bacteriology*. Nevertheless, cluster 1 seems to have much in common with the descriptions of *P. fragi*. The exceptions are the proteolytic activity on gelatin and the acid production on maltose. However, earlier methodological descriptions are vague, making it

Table 2. *Percentage frequencies of positive characters found in the major clusters and clusters containing type and reference strains*

All strains had the following properties: Gram-negative; oxidase positive; motile rod morphology; growth at 3% but not at 6% NaCl; no growth at 42 °C; negative for methyl red reaction; no growth on alginate; negative for deamination of phenylalanine; negative for acid production from adonitol, amygdalin, dulcitol, *meso*-inositol and inulin; negative for utilization of D-fucose, butyrate, maleate, pimelate, glycollate, *cis*-aconitate, ethylene glycol, L-mandelate, testosterone, DL-norvaline, α-amylamine, 2-aminoethanol and DL-α-aminobutyrate.

	P. fragi	*Pseudomonas* sp.	*P. fluorescens* biotype III	*P. fluorescens* biotype I	*P. aureofaciens/ chlororaphis*	*P. fluorescens* biotype II	*p. putida* biotype I	*Alteromonas putrefaciens*	*Aeromonas hydrophila*
Cluster no.	1	2	7	8	9	11	12	14	15
No. of strains	112	21	7	16	3	3	4	10	5
Colonial morphology:									
Fluorescent pigments	2	48	86	75	100	100	100	0	0
Phenazine pigments	0	0	0	0	100	0	0	0	0
Pink pigments	0	0	0	0	0	0	0	100	0
Yellow pigments	2	0	0	0	0	0	0	0	0
β-Haemolysis	0	0	0	0	0	0	0	10	100
Growth at:									
4 °C	100	100	100	100	100	100	100	80	100
37 °C	0	0	0	6	33	0	50	0	100
Biochemical tests:									
Arginine hydrolysis	99	100	100	94	100	100	100	0	100
Citrate, Simmon's	99	100	100	94	100	100	100	0	80
Hippurate hydrolysis	90	62	71	88	67	100	100	50	60
H_2S	0	0	0	0	0	0	0	90	0
Indole	3	0	0	0	0	0	0	0	100
$NO_3 \rightarrow N_2$	4	0	100	6	0	100	25	40	0
Urease	71	67	14	94	100	100	0	40	0
Voges–Proskauer	0	0	0	0	0	0	0	0	100
Degradation tests:									
Casein	1	0	100	56	100	0	0	10	100
DNA	0	0	0	0	0	0	0	70	60
Egg-yolk reaction	6	19	100	94	100	33	0	20	40
Egg-yolk clearing	14	76	86	56	100	67	0	100	100
Gelatin	6	95	100	88	100	33	0	100	100
RNA	0	0	0	0	0	0	0	40	80
Starch	0	0	0	6	0	0	0	0	80
Tributyrin	75	75	100	100	100	100	100	80	80
Tween 80	2	0	100	50	100	0	0	80	100
Tyrosine	98	95	86	100	100	67	100	100	20
Acid from:									
L-Arabinose	100	100	100	100	67	100	100	20	100
Cellobiose	99	100	14	13	0	0	0	0	100
Dextrin	0	0	0	0	0	0	0	0	100
D-Fructose	0	0	0	0	0	0	0	0	100
D-Galactose	100	100	100	100	100	100	100	10	100
D-Glucose (aerobic)	100	100	100	100	100	100	100	20	100
D-Glucose (anaerobic)	0	0	0	0	0	0	0	0	100
Glycerol	7	0	0	13	0	0	0	0	100
Glycogen	0	0	0	0	0	0	0	0	100
Lactose	16	5	0	0	0	0	0	0	60
Maltose	100	95	0	6	0	0	0	10	100
Mannitol	4	0	0	19	0	0	0	0	100
D-Mannose	100	95	100	100	100	100	100	10	100

Table 2 (*continued*)

Cluster no.	1	2	7	8	9	11	12	14	15
Melezitose	0	5	0	0	0	0	0	0	0
Melibiose	100	95	100	100	100	33	50	0	0
Raffinose	0	0	0	0	67	0	0	0	0
Rhamnose	8	10	57	0	0	0	0	0	0
D-Ribose	99	95	100	100	100	100	0	0	100
Sucrose	0	0	0	69	100	67	0	0	100
Salicin	0	0	0	0	0	0	0	0	100
Sorbitol	1	0	0	63	0	100	0	0	40
L-Sorbose	0	0	0	6	0	0	0	0	0
Starch	0	0	0	0	0	0	0	0	100
Trehalose	0	0	0	0	0	0	0	0	100
Xylitol	1	5	0	6	0	0	0	0	0
Xylose	99	100	100	100	67	100	100	0	0
Growth on sole carbon source:									
Carbohydrates:									
D-Arabinose	94	52	29	13	0	0	50	0	0
L-Arabinose	66	91	86	100	67	100	75	20	100
Cellobiose	1	10	67	0	0	100	75	0	100
D-Fructose	99	100	100	100	100	100	100	0	100
D-Galactose	26	0	86	100	67	100	25	0	100
Gluconate	100	100	100	100	100	100	100	70	100
D-Glucose	100	100	100	100	100	100	100	100	100
Lactose	0	0	0	0	0	0	0	0	40
Maltose	26	14	29	0	0	100	75	100	100
D-Mannose	68	0	100	100	100	100	0	0	100
Mucate	62	0	86	100	100	100	100	0	0
L-Rhamnose	1	5	86	0	0	100	50	0	0
D-Ribose	100	19	100	100	100	100	25	0	100
Saccharate	93	0	0	73	100	100	100	0	20
Salicin	1	0	14	0	0	0	0	0	100
Starch	1	0	14	0	33	0	50	70	100
Sucrose	12	0	14	56	100	67	0	30	100
Trehalose	90	10	100	93	100	100	0	30	100
D-Xylose	100	5	0	88	0	67	25	0	0
Fatty acids:									
Acetate	98	100	100	94	100	100	100	10	100
iso-Butyrate	0	0	0	0	0	0	33	0	0
Caprate	90	95	100	100	100	67	100	10	40
Caproate	13	15	100	69	100	0	100	0	0
Caprylate	96	95	100	100	100	100	100	0	0
Heptanoate	98	100	100	100	100	100	100	0	100
Pelargonate	95	100	100	100	100	67	100	0	0
Propionate	94	100	100	100	100	100	100	0	0
Valerate	28	0	0	13	0	0	0	0	0
iso-Valerate	0	0	0	6	0	0	100	0	0
Dicarboxylic acids:									
Adipate	1	5	50	0	0	0	0	0	0
Glutarate	100	100	100	100	100	100	100	0	20
Malonate	1	0	14	31	0	0	50	0	0
Sebacate	0	0	57	0	0	0	0	0	0
Suberate	1	0	14	0	0	0	0	0	20
Succinate	100	100	100	100	100	100	100	90	100
Hydroxyacids:									
DL-Glycerate	100	91	100	100	100	100	100	70	80
DL-β-Hydroxybutyrate	83	5	100	100	100	100	100	0	0
Hydroxymethylglutarate	0	0	0	81	0	100	0	0	0
DL-Lactate	100	100	100	100	100	100	100	50	0
D-Malate	36	0	0	0	0	0	100	0	0
L-Malate	67	91	86	50	100	100	100	0	20

Table 2 (*continued*)

Cluster no.	1	2	7	8	9	11	12	14	15
D-Tartrate	2	0	0	0	0	33	0	0	20
L-Tartrate	0	0	0	0	33	0	0	0	0
meso-Tartrate	82	0	0	19	0	0	100	0	0
Miscellaneous organic acids:									
Citraconate	0	0	29	6	0	33	0	0	0
Citrate	100	100	100	100	100	100	100	0	40
Itaconate	63	0	43	81	100	100	0	0	0
Laevulinate	0	0	0	6	67	0	0	0	0
2-Oxoglutarate	100	100	100	100	100	100	100	90	20
Polyalcohols and glycols:									
Adonitol	1	0	86	94	0	0	0	0	0
Butane-2,3-diol	1	0	86	13	100	100	100	0	0
Glycerol	100	100	100	100	100	100	100	0	100
meso-Inositol	17	95	100	100	100	100	0	0	0
Mannitol	66	0	100	100	100	100	0	0	100
Propane-1,2-diol	3	0	86	0	0	100	100	0	0
Sorbitol	3	0	0	94	0	100	0	0	60
Alcohols:									
iso-Butanol	0	0	71	0	0	100	100	0	0
N-Butanol	20	91	57	31	100	100	100	0	20
Ethanol	2	5	71	0	0	100	75	0	0
Geraniol	0	0	14	0	0	0	100	0	0
N-Propanol	4	5	14	0	0	67	25	0	0
Non-nitrogenous aromatic and other cyclic compounds:									
Benzoate	85	67	100	44	100	0	100	0	0
Benzoylformate	0	0	0	0	100	0	75	0	0
m-Hydroxybenzoate	0	0	14	0	33	67	75	0	0
o-Hydroxybenzoate	4	0	0	0	0	0	0	0	0
p-Hydroxybenzoate	96	5	43	100	100	100	100	0	0
Phenol	0	0	0	0	0	0	50	0	0
Phenylacetate	2	0	0	0	67	0	75	0	0
D-Quinate	97	5	100	100	100	100	100	10	20
Aliphatic amino acids:									
D-α-Alanine	100	100	100	100	100	100	100	0	100
L-α-Alanine	100	100	100	100	100	100	100	0	100
β-Alanine	98	100	100	94	100	100	100	0	20
γ-Aminobutyrate	98	100	100	100	100	100	100	0	0
DL-α-Aminovalerate	0	0	0	6	0	0	100	0	0
δ-Aminovalerate	51	100	100	25	100	100	100	0	0
DL-Arginine	99	100	100	100	100	100	100	0	100
L-Aspartate	100	100	100	100	100	100	100	50	100
DL-Citrulline	1	0	100	88	0	33	75	0	0
L-Glutamate	100	100	100	100	100	100	100	30	100
Glycine	75	0	0	0	0	0	100	0	20
L-Isoleucine	81	76	100	100	100	100	100	30	0
DL-Norleucine	1	0	0	0	0	33	0	0	0
L-Leucine	99	100	100	100	100	100	100	80	40
L-Lysine	7	0	29	0	0	0	100	0	0
DL-Ornithine	96	95	100	94	33	67	100	0	20
L-Serine	4	0	100	100	100	33	100	0	100
L-Threonine	0	0	0	0	0	0	0	0	60
L-Valine	85	100	100	100	100	100	100	0	0
Amino acids and related compounds:									
Anthranilate	2	0	100	100	100	100	0	0	0
L-Histidine	100	100	100	100	100	67	100	0	100
Kynurenate	8	0	100	75	33	0	50	0	0
L-Phenylalanine	97	91	100	38	67	67	100	0	0

Table 2 (*continued*)

Cluster no.	1	2	7	8	9	11	12	14	15
L-Proline	100	100	100	100	100	100	100	0	20
D-Tryptophan	0	0	0	6	0	0	0	0	0
L-Tryptophan	0	0	100	100	100	100	0	0	0
L-Tyrosine	100	95	100	100	100	100	100	10	80
Amines:									
Benzylamine	0	0	0	0	0	0	100	10	0
Butylamine	0	0	0	0	0	0	100	0	0
Ethanolamine	96	95	86	94	100	67	100	30	80
Histamine	4	0	0	40	100	0	100	0	0
Putrescine	100	100	100	100	100	100	100	40	100
Spermine	100	100	86	100	100	100	100	10	20
Tryptamine	0	0	29	0	0	100	0	0	0
Miscellaneous nitrogenous compounds:									
Acetamide	0	0	0	0	0	0	25	0	0
Betaine	100	86	100	100	100	100	100	10	20
Hippurate	86	5	14	13	0	0	100	10	20
Nicothinate	16	0	0	19	0	0	100	0	0
Sarcosine	76	81	60	88	100	100	100	0	20
Trigonelline	1	0	0	0	0	33	100	0	0

Table 3. *Simplified scheme for the differentiation of P. fragi, cluster 2, P. fluorescens and P. putida*

Key: –, 10% or less of strains positive; +, 90% or more of strains positive; v, variable reaction (percentage of positive strains shown in parentheses); d, differs between biotypes.

	*P. fragi**	Cluster 2*	*P. fluorescens*†	*P. putida*†
Fluorescent pigments	–	v (48)	+	+
Gelatinase	–	+	+	–
Acid from cellobiose and maltose	+	+	–	–
Utilization of:				
Saccharate	+	–	d	+
Trehalose	+	–	+	–
meso-Inositol	v (17)	+	+	–
Benzylamine	–	–	–	+

* Based on the present study.
† Based on the present study and data from Stanier *et al.* (1966).

difficult to compare the results with the present ones. It may be pointed out that the gelatinase test applied in the present study gives a positive reaction only at a comparatively high activity. *Pseudomonas fragi* seems to be widely distributed in nature and is apparently the most important part of the microflora of refrigerated meat.

Cluster 2 strains differed significantly from *P. fragi* as well as the *P. fluorescens* complex (Fig. 1). This taxon was after *P. fragi* the most commonly occurring among the field isolates.

All biotypes of *P. fluorescens* described in the eighth edition of *Bergey's Manual of Determinative Bacteriology* are represented in the present study. Stanier *et al.* (1966) divided *P. fluorescens* into six well-defined biotypes (A to F) and one provisional biotype (G). Biotypes A, B, C and F are in *Bergey's Manual of Determinative Bacteriology* (8th edition) represented by biotypes I, II, III and IV, respectively, and biotypes D and E are listed as *P.*

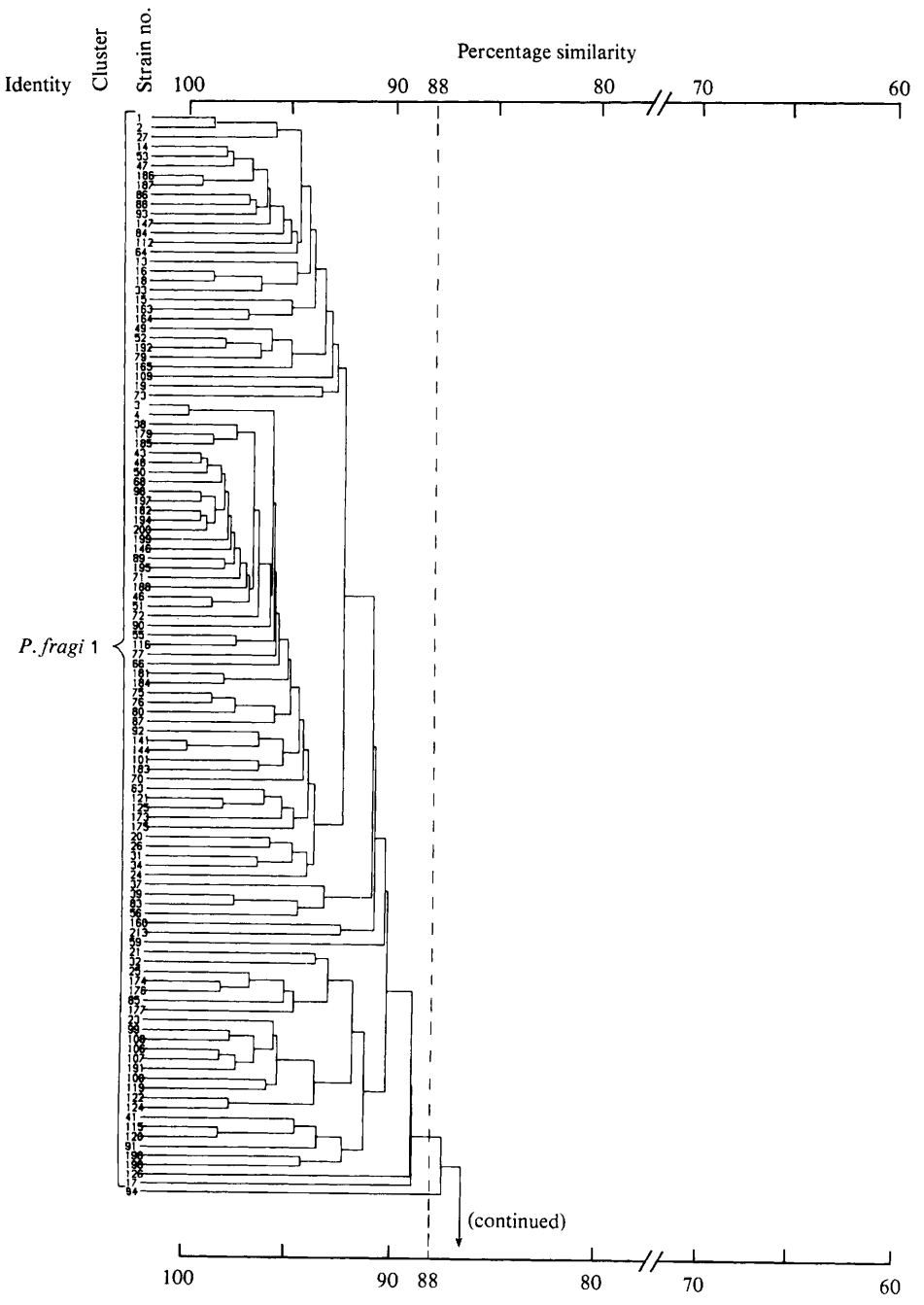

Fig. 1. Dendrogram of 218 pseudomonad strains (oxidase-positive; Gram-negative; motile by polar flagellation) showing the relationship between clusters based on the S_M coefficient and unweighted group average linkage clustering.

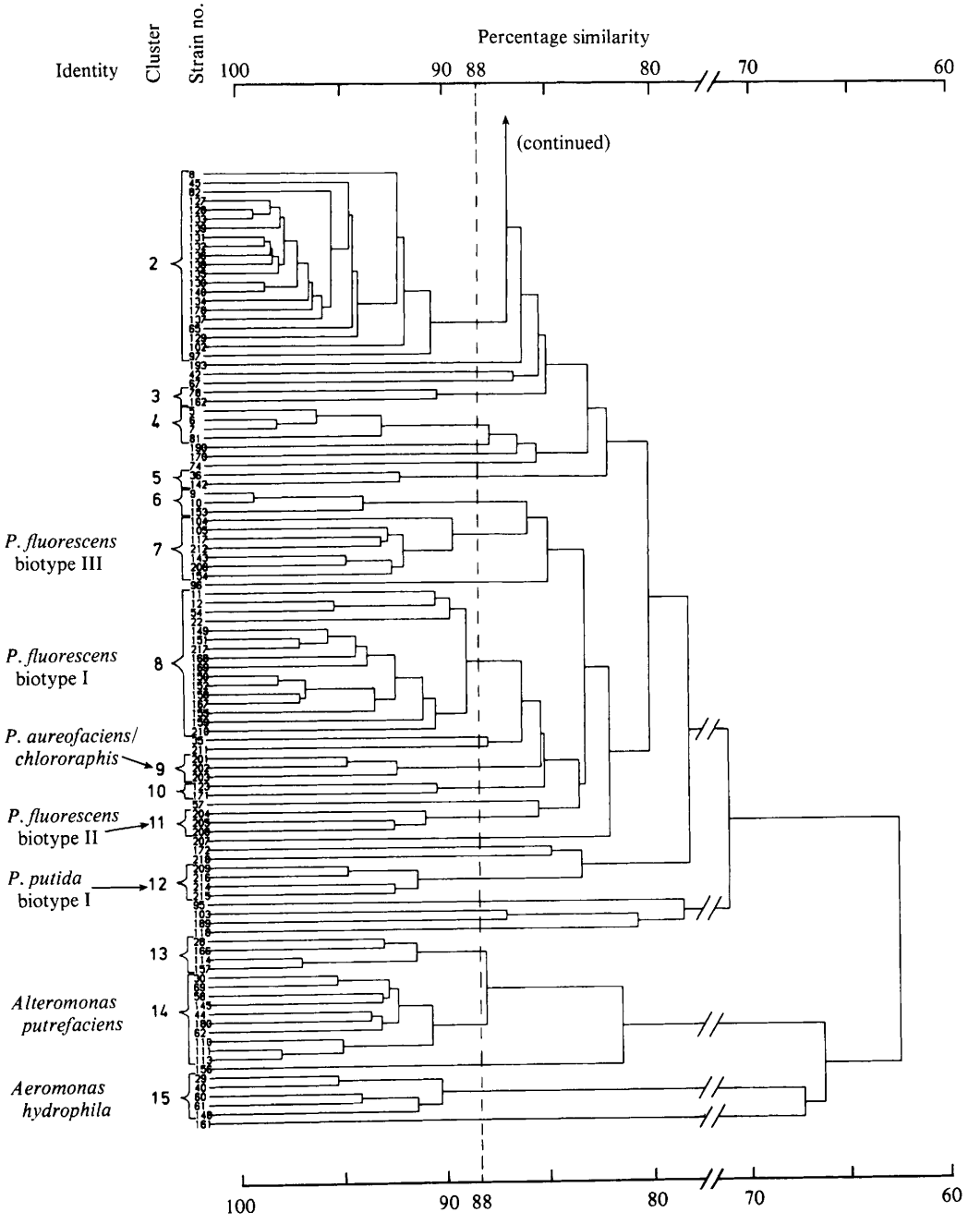

Fig. 1 (*continued*)

chlororaphis and *P. aureofaciens*, respectively. The differentiation of the last two from *P. fluorescens* is based mainly on their production of phenazine pigments. However, the recognition of these organisms as separate species is not supported by the nutritional data of the present study, where *P. aureofaciens* and *P. chlororaphis* join the *P. fluorescens* complex on the same similarity level as the biotypes. In consequence, *P. aureofaciens* and *P. chlororaphis*, in accordance with Stanier *et al.* (1966), should be regarded as biotypes of *P. fluorescens*. Furthermore, no evidence was found in the present study for separating *P. aureofaciens* and *P. chlororaphis*. Our type and reference strains (two *P. aureofaciens* and one *P. chlororaphis*) joined together at a similarity level of 95%. DNA homology studies by Palleroni & Doudoroff (1972) also support the suggestion that *aureofaciens* and *chlororaphis* are two names for the same organism. The numerical analysis (Fig. 1) showed that *P. fluorescens* biotype I was the clustering centre for the 38 strains of the *P. fluorescens* complex. Thus, biotype I can here be regarded as the most 'typical' *P. fluorescens* group, which is in agreement with Stanier *et al.* (1966).

Cluster 14, *Alteromonas putrefaciens*, consisted of 10 field strains. Here it should be pointed out that the clustering is somewhat uncertain as it is based mainly on negative matches. *Alteromonas putrefaciens* was originally found in tainted butter by Derby & Hammer (1931) and given the name *Achromobacter putrefaciens*, but was later transferred to the genus *Pseudomonas* (Long & Hammer, 1941). On the basis of the guanine plus cytosine content of some examined strains (44–48%), this species has been transferred to *Alteromonas* (Lee *et al.*, 1977). However, three of the pseudomonads isolated were able to produce H_2S and had at the same time a guanine plus cytosine content of more than 55%. Furthermore, studies on the lipid composition of *A. putrefaciens* do not support the proposal that the organism should be an *Alteromonas* (Wilkinson & Caudwell, 1980). Since *A. putrefaciens* is the only acknowledged name (Anon., 1981) this name has been used in the present study.

In conclusion, it can be said that the principle of Stanier *et al.* (1966) to differentiate *Pseudomonas* species by testing their ability to utilize different compounds as sole carbon source seems functional. The capacity to utilize different sources for growth decreases in the order *P. putida* < *P. fluorescens* < *P. fragi* < cluster 2 < *A. putrefaciens*. However, the diagnostic principles of Stanier *et al.* (1966) call for a rather large number of utilization tests, which may be inconvenient for studies not of strictly taxonomic nature. This is probably the reason why, for example, in food microbiology, the system of Shewan *et al.* (1960) is widely applied. This system is based on the ability to produce fluorescent pigments and on how the organism reacts on the medium of Hugh & Leifson (1953). If this system were applied to the present material, all strains except eight *A. putrefaciens* strains would be split between Shewan's groups I and II in accordance with the ability to produce fluorescent pigments. Thus, *P. fluorescens, P. putida* and about half the strains of cluster 2 would be placed in group I and *P. fragi*, while the other half of cluster 2 and two *P. putrefaciens* strains would be placed in group II. All the single strains and the strains of the insignificant clusters would also be divided between Groups I and II. A more accurate division of the present material could be accomplished by using the simplified identification scheme in Table 3.

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