Taxonomy of the Aerobic Pseudomonads: *Pseudomonas cepacia*, *P. marginata*, *P. alliicola* and *P. caryophylli*

By R. W. BALLARD, N. J. PALLERONI, M. DOUDOROFF
AND R. Y. STANIER

*Department of Bacteriology and Immunology, University of California, Berkeley,
California 94720, U.S.A.*

AND M. MANDEL

*Section of Molecular Biology, Department of Biology, The University of Texas,
M. D. Anderson Hospital and Tumor Institute, Houston,¹ Texas 77025, U.S.A.*

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SUMMARY

On the basis of phenotypic characterization and DNA–DNA homology studies of strains of phytopathogenic *Pseudomonas* species, it is concluded that *P. cepacia* is so similar to *P. multivorans* that the latter name should be regarded as a synonym. On similar grounds, *P. alliicola* appears to be a synonym of *P. marginata*. *P. caryophylli* is a readily distinguishable species. From the DNA–DNA hybridization studies all of these species seem to be related to each other and to the animal pathogens *P. pseudomallei* and *P. mallei*.

INTRODUCTION

As one outcome of their taxonomic study of aerobic pseudomonads, Stanier, Palleroni & Doudoroff (1966) proposed a new species, *Pseudomonas multivorans*, for a distinctive cluster of non-fluorescent strains. The first representatives of this species had been isolated from soil and water in Trinidad by Morris & Roberts (1959). *Pseudomonas multivorans* is characterized by its remarkable nutritional versatility. Most strains can use as sole sources of carbon and energy a substantially larger number of organic compounds than any of the fluorescent pseudomonads, previously considered the most omnivorous members of the genus *Pseudomonas*. The compounds utilizable by *P. multivorans* include a few—notably, D-fucose, cellobiose and L-threonine—that are not attacked by most other members of the genus. Many strains of *P. multivorans* produce pigments identified as phenazines (Morris & Roberts, 1959). Poly- β -hydroxybutyric acid (PHB) is accumulated as a reserve material, but cannot be utilized as an exogenous substrate. Redfearn, Palleroni & Stanier (1966) simultaneously characterized, by the same taxonomic methods, two *Pseudomonas* species pathogenic for animals: *P. pseudomallei* and *P. mallei*. These closely related organisms are also non-fluorescent pseudomonads that accumulate PHB as a reserve material and are nutritionally highly versatile. However, the substrates utilizable by the more versatile of the two species, *P. pseudomallei*, are fewer than those used by *P. multivorans*. Other characters which clearly distinguish *P. pseudomallei* and *P. mallei* from *P. multivorans* include the ability to denitrify, to utilize starch and maltose, and to hydrolyse and grow with exogenous PHB as carbon source. The DNAs of

P. pseudomallei and *P. mallei* also have a slightly higher G + C content than that of *P. multivorans* (Mandel, 1966).

Rogul, Brendle, Haapala & Alexander (1968) have recently demonstrated a close genetic relationship between *Pseudomonas pseudomallei* and *P. mallei* through experiments on DNA-DNA hybridization *in vitro*. Rogul *et al.* (1968) did not find evidence of genetic homology between the *P. pseudomallei*-*P. mallei* group and other species of aerobic pseudomonads with the exception of *P. multivorans*, which showed a relatively low but significant level of homology with both species. This work accordingly suggests that *P. pseudomallei*, *P. mallei* and *P. multivorans* constitute a species-cluster within the genus *Pseudomonas*, an interpretation that is consonant with the data of Redfearn *et al.* (1966) and Stanier *et al.* (1966) on the phenotypic characters of these three species.

There are many nomenspecies of phytopathogenic pseudomonads, most of which have been very inadequately characterized. They include aerobic pseudomonads which do not produce fluorescent pigments, and some known to accumulate PHB. In naming *P. multivorans*, Stanier *et al.* (1966) did not consider its possible synonymy with any of these species, primarily because there was no evidence to implicate *P. multivorans* as an agent of plant disease. However, Dr David Sands (personal communication), who has recently applied the methods of phenotypic characterization developed by Stanier *et al.* (1966) to a large number of phytopathogenic pseudomonads, found that a strain of the phytopathogen *Pseudomonas cepacia* Burkholder (1950) had a nutritional spectrum virtually identical with that of *P. multivorans*. Furthermore, an extensive study of the host ranges of bacteriophages for aerobic pseudomonads (Dr G. Cosens & Dr M. P. Starr, personal communication) has shown that the specific host range of certain bacteriophages encompasses both *P. multivorans* and a cluster of phytopathogenic nomenspecies: *P. marginata* (McCulloch) Stapp (1928) *P. alliicola* (Burkholder) Starr & Burkholder (1942), *P. cepacia* and *P. caryophylli* (Burkholder) Starr & Burkholder (1942). It appeared desirable to examine the possible relationships of *P. multivorans* to this group of phytopathogenic pseudomonads. We have therefore undertaken a complete phenotypic characterization of authentic strains of *P. marginata*, *P. alliicola*, *P. cepacia* and *P. caryophylli* by the methods used to characterize *P. multivorans* (Stanier *et al.* 1966). At the same time, we have extended the work of Rogul *et al.* (1968) on *in vitro* DNA-DNA hybridization. The strain labelled *P. marginata* (ATCC 17556), which was studied by Jessen (1965), Stanier *et al.* (1966) and Dr David Sands (personal communication), is misnamed. It is not a representative of this non-fluorescent species, and can be assigned to *P. fluorescens* biotype A (Stanier *et al.* 1966).

METHODS

Origins of the strains examined. We shall refer to the strains by the numbers assigned to them in the collection of pseudomonads maintained in the Department of Bacteriology and Immunology of the University of California, Berkeley. The majority of the phytopathogenic strains examined were obtained from the International Collection of Phytopathological Bacteria, Department of Bacteriology, University of California, Davis, through the courtesy of its curator, Dr M. P. Starr. Additional strains of *Pseudomonas cepacia* and *P. alliicola* were received from Dr R. Dickey, Department of Plant Pathology, Cornell University, New York. The strains of *P. solanacearum*

Table 1. *Origins of the strains*

Strains received as <i>Pseudomonas cepacia</i>	
714 Starr PC 22	Received from W. H. Burkholder, New York, 1949
715 Starr PC 23	Received from W. H. Burkholder, New York, 1949
716 Starr PC 24	Received from W. H. Burkholder, New York, 1949
717 Starr PC 25	Received from W. H. Burkholder, New York, 1949
725 Dickey 63-53	Isolated from decayed onion, Orange Co., New York, 1963
726 Dickey 63-46	Received from Dr D. C. Hildebrand; isolated from muck soil, Prattsburg, New York, 1963
727 Dickey 64-20	Isolated from muck soil, Orange Co., New York, 1964
738 Dickey 64-22	Isolated from muck soil, Orange Co., New York, 1964
739 Dickey 64-32	Isolated from muck soil, Orange Co., New York, 1964
740 Dickey 63-46	Isolated from muck soil, Prattsburg, New York, 1963
741 Dickey 61-52	Isolated from decayed onion, Orange Co., New York, 1961
742 Dickey 63-56	Isolated from decayed onion, Orange Co., New York, 1963
743 Dickey 63-56b	Isolated from 742 by Mrs V. Vitanza as a colony variant
744 Dickey 63-71	Isolated from decayed onion, Orange Co., New York, 1963
745 Dickey 63-71b	Isolated from 744 by Mrs V. Vitanza as a colony shape variant
746 Dickey 63-75	Isolated from decayed onion, Orange Co., New York, 1963
747 Dickey 63-75b	Isolated from 746 by Mrs V. Vitanza as a colony shape variant
748 Dickey 63-87	Isolated from decayed onion, Orange Co., New York, 1963
Strains received as <i>P. marginata</i>	
701 Starr PM 2	(Clara G40) Isolated by W. H. Burkholder from gladiolus leaves, 1938
702 Starr PM 12	(NCPPB 1888) Isolated from gladiolus leaves in Ithaca, New York, 1945
703 Starr PM 13	(NCPPB 1889) Isolated in Ithaca, New York, from a greenhouse, 1945
704 Starr PM 107	(NRRL B-793) (ATCC 10248) (NCPPB 1891) Received from W. C. Haynes, NRRL, Peoria, Ill., 1948
705 Starr PM 109	(Dowson 116) Received from W. J. Dowson, 1950
706 Starr PM 175	(NCPPB 1051) (Hayward B670) Isolated from gladiolus sp. in S. Rhodesia, 1961;
722 Starr PC 144	(Robbs 349) Received from C. F. Robbs as unidentified isolate, Brazil, 1965, isolated from rotted onion
723 Starr PM 106	(NRRL B-792) (NCPPB 1890) (ATCC 10247) Received from W. C. Haynes, NRRL, Peoria, Ill., 1948
737 Starr PM 11	(NRRL B-851) (NCPPB 1887) Isolated from gladiolus leaves in Lima, New York, 1945
Strains received as <i>P. alliicola</i>	
707 Starr PA 6	(NRRL B-823) Isolated by W. H. Burkholder, 1939
708 Starr PA 7	(ATCC 19302) Isolated by W. H. Burkholder, 1939
709 Starr PA 8	Isolated by W. H. Burkholder, 1939
710 Starr PA 9	Isolated by W. H. Burkholder, 1939
711 Starr PA 10	Isolated by W. H. Burkholder, 1939
712 Starr PA 15	Isolated as onion pathogen, 1945
713 Starr PA 16	(NRRL B-828) Isolated as onion pathogen, 1945
724 Dickey 61-3	Isolated from decayed onion, Orange Co., New York, 1961
Strains received as <i>P. caryophylli</i>	
718 Starr PC 101	Isolated by K. F. Baker from carnations at Sato greenhouses, Hawthorne, California, 1950
719 Starr PC 102	Isolated by K. F. Baker from carnations at Sato greenhouses, Hawthorne, California, 1950
720 Starr PC 113	Received from N. A. MacLean, Plant Pathology, University of California, Berkeley; isolated from carnation, white Sims variety, 1951
721 Starr PC 115	(Hellmers 89) Received from E. Hellmers, Denmark; isolated from Danish carnation 'Harvest Moon', 1959
Strains received as <i>P. solanacearum</i>	
729 Strain 139b	Received from Mrs V. Vitanza; isolated by I. Buddenhagen from banana plant, Honduras
730 Strain P-28	Received from Mrs V. Vitanza; isolated by I. Buddenhagen from a tomato plant, Honduras

were received from Mrs V. Vitanza, Department of Plant Pathology, University of California, Berkeley. The designations and histories of the phytopathogenic strains examined are listed in Table 1. The histories of the other strains of aerobic pseudomonads examined are given by Stanier *et al.* (1966), with the exception of *P. saccharophila* (Doudoroff, 1940).

Methods of phenotypic characterization. These have been described by Stanier *et al.* (1966). A few compounds included in the nutritional screening by Stanier *et al.* were omitted in the present study; they were: oxalate, eicosanedioate, methanol, isophthalate, terephthalate, L-kynurenine, *m*-aminobenzoate, *p*-aminobenzoate, methylamine, *n*-dodecane and *n*-hexadecane.

In vitro DNA-DNA hybridization experiments. The bacteria were grown in the standard mineral base described by Stanier *et al.* (1966) with the addition of Difco yeast extract 5 g./l. and sodium lactate 2 g./l. Radioactive DNA was obtained by the addition of ¹⁴C-labelled adenine to a culture in a medium prepared by adding vitamin-free casaminoacids 2 g./l. and sodium lactate 2 g./l. to the standard mineral base. The adenine was added between 30 and 60 min. (about 0.5 generations) before harvesting the culture which was grown on a rotary shaker at 30°. Prior to DNA isolation, the bacteria were harvested by centrifugation, washed, and resuspended in saline-EDTA (saline-EDTA contains 0.15 M-NaCl and 0.1 M-EDTA, pH 8.0) to a concentration of 10% (wet weight/volume). Lysis was effected by the addition of sodium lauryl sulphate (15 g./l.) and heating the mixture in a water bath at 60° for 5 min. (Marmur, 1961). The mixture was cooled in ice, one-half volume of liquefied phenol was added, and the mixture shaken until homogeneous. The mixture was then centrifuged and the aqueous phase removed. The semisolid material at the aqueous DNA:phenol interphase was mixed well with 50 to 100 ml. of saline-EDTA and extracted with one-half volume of liquefied phenol. The pooled aqueous phases were precipitated with 2 vol. of 95% (v/v) ethanol. The DNA was redissolved in one-tenth strength SSC (SSC contains 0.15 M-NaCl and 0.015 M-Na citrate, pH 7.0) and dialysed against one-tenth strength SSC for 15 hr at 4°. The solution was then incubated with 50 µg./ml. ribonuclease (A-grade bovine pancreatic, Calbiochem, Los Angeles, Calif.) for 2 hr at 37°. Then 100 µg./ml. pronase (B-grade, Calbiochem, Los Angeles, Calif.) was added and the mixture incubated for an additional 3 hr. The salt concentration was increased to that of SSC and the DNA deproteinized by repeated extraction with chloroform+isoamyl alcohol, 24+1 (v/v) (Marmur, 1961). The DNA was then precipitated by adding 2 vol. ethanol, redissolved in one-tenth strength SSC, reprecipitated with isopropyl alcohol as described by Marmur (1961) and finally redissolved in one-tenth strength SSC. The DNA was stored until use at 4° in the presence of chloroform.

The DNA hybridization experiments were performed using the optimal conditions and techniques established by Johnson & Ordal (1968). Membrane filters (10.5 mm. diam.) contained from 25 to 35 µg. of immobilized denatured DNA. The filters were incubated in the mixture recommended by Denhardt (1966) made with double strength SSC for 2 hr at 65°. The filters were immersed singly in 0.25 ml. of solution which contained 1 µg. of denatured radioactive DNA, homologous to the DNA immobilized on the filter, and 150 µg. of denatured competitor DNA in double strength SSC. The DNA in solution was previously sheared to a mol. wt of about 500,000 by passage through a French pressure cell 15,000 lb./in.² (McCarthy &

Bolton, 1963) and denatured by heating at 102° for 8 min. in one-tenth strength SSC. Controls with homologous radioactive DNA and without competitor were included. The vials were incubated 12 hr at temperatures of 72° (25° below the T_m of the reference DNA) and 80°. The amount of radioactive homologous DNA bound to the filters in the absence of competitors was generally between 22 and 27 % of the initial input after incubation at 72° and between 12 and 16 % at 80°. By using radioactive high mol. wt DNA from *Pseudomonas aeruginosa* (67 % GC) and *P. stutzeri* (62 % GC) immobilized on filters identical with those used in the present experiments, it could be shown that about 53 to 61 % of the immobilized DNA was retained on the filter after 12 hr incubation at 70° and about 31 to 41 % at 80°. No significant differences in percentage retention were observed with DNA from the two species, or between experiments without competitor and those with 150 µg. of either homologous or heterologous sheared preparations of competitor DNA. After incubation, the filters were washed in double-strength SSC at the temperature used for incubation, dried and counted in a Nuclear-Chicago Mark I liquid scintillation counter. All experiments were done in duplicate and the counts, which generally agreed within 5 %, were averaged for the calculations. The results are expressed as 'percentage competition', i.e. the relative competition obtained in each case as compared to the competition with homologous DNA, which is arbitrarily assigned a value of 100 %:

$$\text{Percentage competition} = 100 \times X - Y / X - Z.$$

X = ¹⁴C retained without competitor.

Y = ¹⁴C retained with heterologous competitor.

Z = ¹⁴C retained with homologous competitor.

Preparations of DNA isolated from *Pseudomonas pseudomallei* and *P. mallei* were kindly furnished by Dr M. Rogul, Veterinary Immunology Section, Division of Veterinary Medicine, Walter Reed Army Medical Centre, Washington, D.C. 20012. These were isolated from *P. pseudomallei*, strains 4845 (ATCC 15682), NCTC 1691 and 295; and from *P. mallei* 4.

Determination of the base composition of DNA. The moles percentage G + C in the DNA of various strains was determined from buoyant density measurements in CsCl gradients (Mandel, 1966).

RESULTS

Phenotypic characterizations of phytopathogenic nomenclatures

Eighteen strains of *Pseudomonas cepacia* were examined. They are Gram-negative rods with polar multitrichous flagella as previously described by Burkholder (1950). None of the strains produces fluorescent pigments; however, all strains produce greenish yellow pigments, the chemical nature of which has not been ascertained. All strains accumulate poly-β-hydroxybutyric acid (PHB) as intracellular reserve material. They are oxidase-positive. They grow at 41° but not at 4°. Most strains produce slime in mineral media containing 2 or 4 % sucrose (negative: 714, 715, 716, 745), give a positive egg-yolk reaction (negative: 714, 715, 716, 743, 747) and hydrolyse Tween 80 (negative: 743, 747). Ten strains (715, 717, 738, 739, 740, 741, 742, 745, 746, 747) hydrolyse gelatin. None hydrolyses starch or PHB. None produces gas anaerobically in a complex medium containing nitrate. None synthesizes the enzymes of the arginine

dihydrolase system constitutively. The cleavage of protocatechuate by toluene-treated cells grown with *p*-hydroxybenzoate is of the *ortho* type. The strains of *P. cepacia* are nutritionally homogeneous. They use a wide variety of organic compounds as sole sources of carbon and energy (Tables 2, 3). Fourteen of the strains can use at least 102 of the 136 substrates tested in the screening. The list of compounds used by

Table 2. *Substrates used by all strains of P. cepacia, P. marginata, P. alliiicola and P. caryophylli*

D-Ribose, D-xylose, D-arabinose, L-arabinose, D-glucose, D-mannose, D-galactose, D-fructose, sucrose, cellobiose, gluconate, 2-keto-gluconate, saccharate, mucate, salicin
 Acetate, propionate, butyrate, isobutyrate
 Malonate, succinate, fumarate
 D-Malate, L-malate, *meso*-tartrate, DL- β -hydroxybutyrate, DL-lactate, glycerate, hydroxymethyl-glutarate
 Citrate, α -keto-glutarate, pyruvate, aconitate
 Mannitol, sorbitol, *meso*-inositol, glycerol
n-Propanol
p-Hydroxybenzoate, phenylacetate, quinate
 L-Alanine, β -alanine, L-serine, L-aspartate, L-glutamate, DL-arginine, γ -aminobutyrate, L-histidine, L-proline, L-tyrosine, L-phenylalanine, L-tryptophan
 Betaine, hippurate

Substrates not used by any strain of P. cepacia, P. marginata, P. alliiicola or P. caryophylli

Maltose, lactose, starch, inulin
 Maleate
 Poly- β -hydroxybutyrate
 Erythritol, ethyleneglycol
iso-Propanol, geraniol
 D-Mandelate, phthalate, phenylethanediol, phenol, naphthalene
 D-Tryptophan
 Creatine, pantothenate

Table 3. *Substrates used by some strains studied*

Substrate	Names under which strains were received			
	<i>P. cepacia</i> (18 strains)	<i>P. marginata</i> (9 strains)	<i>P. alliiicola</i> (8 strains)	<i>P. caryophylli</i> (3 strains)
D-Fucose	100*	89	100	100
L-Rhamnose	0	0	0	100
Trehalose	83	100	100	33
Valerate	100	100	87	100
Isovalerate	100	33	38	0
Heptanoate	100	89	100	0
Caprylate, caprate, caproate, pelargonate	100	100	100	0
Glutarate	100	44	38	0
Adipate, azelate, sebacate	100	100	100	0
Pimelate, suberate*	100	0	0	0
D-(−)-Tartrate	0	100	100	0
L-(+)-Tartrate	100	100	100	0
Glycollate	100	0	0	100
Levulinate	100	0	0	0
Citraconate	100	100	100	0
Itaconate	0	0	38	0
Mesaconate	0	100	100	0

* % strains positive.

Table 3 (cont.)

	Names under which strains were received			
	<i>P. cepacia</i> (18 strains)	<i>P. marginata</i> (9 strains)	<i>P. alliicola</i> (8 strains)	<i>P. caryophylli</i> (3 strains)
Adonitol*	100†	100	100	0
Propyleneglycol*	0	22	25	0
2,3-Butyleneglycol	100	0	0	100
Ethanol	72	100	100	0
<i>n</i> -Butanol	94	44	87	100
<i>iso</i> -Butanol	22	0	12	0
L-Mandelate, benzoylformate	94	0	0	33
Benzoate	94	100	100	0
<i>o</i> - & <i>m</i> -Hydroxybenzoate	100	0	0	0
Testosterone	94	0	0	0
Glycine	0	22	38	0
D-Alanine	100	100	100	0
L-Threonine*	100	100	100	33
L-Leucine	33	100	100	33
L-Isoleucine, L-lysine	100	100	100	0
DL-Norleucine	0	33	0	0
D-Valine	94	100	100	33
DL-Ornithine*	100	89	100	0
DL-Citrulline	100	78	75	0
DL- α -Aminobutyrate	0	67	87	0
DL- α -Aminovalerate	0	33	0	0
δ -Aminovalerate	94	0	0	0
Kynurenate	100	100	100	0
Anthranilate	100	89	87	0
Ethanolamine	100	89	100	0
Benzylamine	89	0	12	0
Putrescine	94	0	0	0
Spermine	17	0	0	0
Histamine	94	0	0	0
Tryptamine, α -amylamine	100	0	0	0
Butylamine	94	0	12	0
Sarcosine	89	56	87	0
Acetamide	100	0	0	0
Nicotinate	0	100	100	0
Trigonelline	0	100	100	0

Strain numbers of negative strains for each substrate used by some strains of each species are listed below.

P. cepacia: trehalose (714, 715, 716), butanol (740), isobutanol (all except 725, 726, 738, 743), L-mandelate, benzoylformate and benzoate (725), testosterone (726), L-leucine (all except 714, 715, 716, 717, 725, 727), L-valine (741), δ -aminovalerate (744), benzylamine (725, 738), putrescine (738), spermine (all except 715, 739, 745), histamine (725), butylamine (727), sarcosine (717, 727)

P. marginata: D-fucose (705), isovalerate (all except 701, 703, 737), heptanoate (705), glutarate (all except 701, 702, 704, 722), propyleneglycol (all except 722, 723), butanol (all except 704, 705, 722, 723), glycine (all except 702, 704), norleucine (all except 703, 706, 737), DL-ornithine (706), DL-citrulline (705, 706), α -aminobutyrate (705, 723, 737), α -aminovalerate (all except 703, 706, 737), anthranilate (723), ethanolamine (722), sarcosine (705, 706, 722, 723).

P. alliicola: valerate (711), isovalerate (all except 708, 709, 712), glutarate (all except 712, 713, 724), itaconate (all except 707, 710, 724), propyleneglycol (all except 710, 724), butanol (711), *iso*-butanol (all except 710), glycine (all except 708, 709, 712), DL-citrulline (709, 711), α -aminobutyrate (724), anthranilate (724), benzylamine (all except 724), butylamine (all except 724), sarcosine (724),

P. caryophylli: trehalose (719, 720), L-mandelate, benzoylformate (719, 720), L-threonine (719, 720), L-leucine (720, 721), L-valine (720, 721).

* These substrates were tested with only seven strains of *P. cepacia* (714, 715, 716, 717, 725, 726, 727) and with eight strains of *P. marginata* (all except 737). The percentages are calculated on the basis of the strains tested.

† % strains positive.

P. cepacia is identical with that reported by Stanier *et al.* (1966) to be utilized by *P. multivorans*. These two species cannot be distinguished by any of the phenotypic characters that we have examined.

Nine strains of *Pseudomonas marginata* and eight strains of *P. alliicola* were examined. These two species are Gram-negative rods, motile by polar multitrichous flagella, as reported by McCulloch (1921) and Burkholder (1942). None of the strains produces fluorescent pigments. All strains accumulate PHB as reserve material. They are oxidase-positive, although some strains give a very weak reaction. They grow at 41° but not at 4°. They produce slime in mineral media containing 2 or 4 % sucrose, give a positive egg-yolk reaction and hydrolyse Tween 80 and gelatin. None hydrolyses starch or PHB. None of the strains produces gas anaerobically in a complex medium containing nitrate. None synthesizes the enzymes of the arginine dihydrolase system constitutively. The cleavage of protocatechuate by toluene treated cells grown with *p*-hydroxybenzoate is of the *ortho* type. The nutritional spectra of the strains of these two species are essentially identical (Tables 2, 3). They are versatile; all strains use at least 78 of the 136 substrates tested.

Four strains of *Pseudomonas caryophylli* were examined. They are Gram-negative rods motile by polar multitrichous flagella as described by Burkholder (1942). None of the strains produces fluorescent pigments. All strains accumulate PHB as reserve material. They are oxidase-positive. They grow at 41° but not at 4°. None of the strains produces slime in mineral media containing 2 or 4 % sucrose. One strain (720) produces a positive egg-yolk reaction and two strains (720 and 721) hydrolyse Tween 80. None hydrolyses gelatin, starch or PHB. They produce gas anaerobically in a complex medium containing nitrate and synthesize the enzymes of the arginine dihydrolase system constitutively. The cleavage of protocatechuate by toluene-treated cells grown with *p*-hydroxybenzoate is of the *ortho* type. One of the strains of *P. caryophylli* (722) requires unidentified growth factors, and hence cannot grow in the defined mineral medium used for nutritional screening. The nutritional patterns were thus determined only for the other three strains (Tables 2, 3). These three strains of *P. caryophylli* utilize from 61 to 65 of the compounds tested as sole sources of carbon and energy.

On the basis of these phenotypic characterizations, it appears that *Pseudomonas cepacia* and *P. multivorans* are indistinguishable species, as are *P. marginata* and *P. alliicola*. However, the *P. cepacia*-*P. multivorans* complex, the *P. marginata*-*P. alliicola* complex and *P. caryophylli* represent three phenotypically distinct clusters of strains, even though they do share many common properties.

Mean DNA base compositions

Table 4 summarizes data obtained by buoyant density measurements for the mean base composition (moles percentage G+C) of DNA from representative strains of four nomenclatures of non-fluorescent phytopathogenic pseudomonads. Also included are data for two additional strains of *Pseudomonas multivorans*; data for strains belonging to this species have been published previously by Mandel (1966). The mean value and standard deviation for the DNA base composition of *P. multivorans* shown in Table 4 have been calculated on the basis of data for all strains of this species so far examined. Data for *P. pseudomallei* and *P. mallei*, from Mandel's (1966) earlier work, are included.

It is evident that the mean DNA base compositions of *Pseudomonas cepacia*, *P. multivorans*, *P. marginata* and *P. alliicola* did not differ significantly. The values for *P. pseudomallei* and *P. mallei*, not significantly different from one another, were slightly higher, while the value for *P. caryophylli* was slightly lower.

Table 4. Buoyant density and GC content of the DNA of *Pseudomonas multivorans*, *P. pseudomallei*, *P. mallei* and some phytopathogenic nomenspecies*

Species	Strain	Mean density (g./cm. ³)	Mean value and standard deviation calculated for each group	
			Density (g./cm. ³)	GC (moles %)
<i>P. cepacia</i>	714	1·726	1·7256 ± 0·0009	66·9 ± 0·9
	715	1·7265		
	717	1·7265		
	725	1·725		
	726	1·725		
	727	1·725		
<i>P. multivorans</i>	85	1·7265	1·7263 ± 0·0006	67·6 ± 0·6
	104	1·727		
	249	1·7265		
	382	1·7255		
	383	1·7255		
	384	1·726		
	385	1·726		
	386	1·7265		
	387	1·7265		
	396	1·727		
	397	1·727		
	398	1·726		
<i>P. marginata</i>	399	1·7265	1·7271 ± 0·0008	68·5 ± 0·8
	424	1·726		
	702	1·727		
	704	1·7275		
<i>P. alliicola</i>	706	1·727	1·7271 ± 0·0010	68·5 ± 1·0
	707	1·7275		
	709	1·727		
<i>P. caryophylli</i>	724	1·727	1·7240 ± 0·0006	65·3 ± 0·6
	719	1·724		
	720	1·7235		
	721	1·7245		
<i>P. pseudomallei</i>	(6 Strains)	—	1·7281 ± 0·0007	69·5 ± 0·7
<i>P. mallei</i>	(7 Strains)	—	1·7276 ± 0·0010	69·0 ± 1·0

* Values given for mean density are raw averages of two determinations of CsCl buoyant density and include those previously reported by Mandel (1966).

Experiments on DNA–DNA hybridization *in vitro*

Genetic relationships among the species studied were examined by the technique of DNA–DNA hybridization *in vitro*, using as reference standards strain 382 of *Pseudomonas multivorans* (Table 5), strain 704 of *P. marginata* (Table 6) and strain 721 of *P. caryophylli* (Table 7). Competition experiments were conducted at two annealing temperatures, 72 and 80°. The former temperature, about 25° below the melting point of the reference DNA, is the annealing temperature commonly used in such

experiments. Competition at the higher temperature (80°) is a more stringent test of homology. Hence, similar competition values at both 72 and 80° indicate a close genetic relationship between the test strain and the reference strain, whereas a sharp decline in the competition value at 80° relative to that at 72° indicates a much looser genetic homology between the test strain and the reference strain.

Table 5. *DNA competition experiments using DNA of Pseudomonas multivorans 382 as reference standard*

DNA of	Strain no.	Per cent competition at	
		72°	80°
<i>P. multivorans</i>	85	59	58
<i>P. multivorans</i>	104	79	57
<i>P. multivorans</i>	249	52	20
<i>P. multivorans</i>	383	66	59
<i>P. multivorans</i>	385	54	40
<i>P. multivorans</i>	387	54	42
<i>P. multivorans</i>	396	46	31
<i>P. multivorans</i>	424	75	65
<i>P. cepacia</i>	727	76	79
<i>P. cepacia</i>	725	66	70
<i>P. marginata</i>	704	35	6
<i>P. alliicola</i>	709	30	18
<i>P. alliicola</i>	724	28	0
<i>P. caryophylli</i>	721	24	14
<i>P. pseudomallei</i>	295	19	14
<i>P. pseudomallei</i>	1691	43	4
<i>P. pseudomallei</i>	4845	24	10
<i>P. mallei</i>	4	23	16
<i>P. solanacearum</i>	729	0	—
<i>P. solanacearum</i>	730	0	—
<i>P. saccharophila</i>	—	0	—
<i>P. aeruginosa</i>	131	0	—
<i>P. putida</i>	90	0	—
<i>P. fluorescens</i>	31	0	—
<i>P. fluorescens</i>	38	0	—
<i>P. stutzeri</i>	222	0	—
<i>P. stutzeri</i>	224	0	—
<i>P. acidovorans</i>	105	0	—

The most extensive series of experiments was conducted using DNA of *Pseudomonas multivorans* 382 as the reference standard (Table 5). The competition values with DNA from eight other strains of this species ranged from 46 to 79% at 72°. In most cases, the values did not decline significantly at 80°; the largest decline occurred with DNA from strain 249, for which the competition value fell from 52% at 72° to 20% at 80°. The DNA of two strains of *P. cepacia* have competition values in the same range (66 and 76%) at 72°, and the values did not decline significantly at 80°. Lower but significant competition values (19 to 43%) were obtained at 72° with DNA from strains of *P. marginata*, *P. alliicola*, *P. caryophylli*, *P. pseudomallei* and *P. mallei*; in every case the values fell substantially at 80° (0 to 18%). No competition was detected at 72° with DNA from strains of *P. solanacearum*, *P. saccharophila*, *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. stutzeri* or *P. acidovorans*. These results provide independent confirmation of the specific identity of *P. cepacia* and *P. multivorans*. They also show that there is a significant degree of genetic relationship between these

two nomenspecies and *P. marginata*, *P. alliicola*, *P. caryophylli*, *P. pseudomallei* and *P. mallei*.

Table 6 presents the results of competition experiments using DNA of *Pseudomonas marginata* 704 as the reference standard. DNA from one other strain of this species and from two strains of *P. alliicola* showed high levels of competition (79 to 100 %) at 72°, not significantly depressed (63 to 81 %) at 80°. Lower levels of competition at 72° (17 to 46 %) were shown by DNA from strains of *P. cepacia*, *P. multivorans*, *P. caryophylli*, *P. pseudomallei* and *P. mallei*. In nearly every case, the competition fell to a much lower level (0 to 17 %) at 80°; however DNA of *P. cepacia* strain 727, which had given the highest value (46 %) at 72°, also gave a high value (36 %) at 80°. No competition was detected at 72° with DNA from strains of *P. solanacearum*, *P. saccharophila*, *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. stutzeri* or *P. acidovorans*. These results provide independent confirmation of the specific identity of *P. marginata* and *P. alliicola*.

Table 6. DNA competition experiments using DNA of *Pseudomonas marginata* 704 as reference standard

DNA of	Strain no.	Per cent competition at	
		72°	80°
<i>P. marginata</i>	702	100	81
<i>P. alliicola</i>	709	79	79
<i>P. alliicola</i>	724	82	63
<i>P. cepacia</i>	727	46	36
<i>P. cepacia</i>	725	17	11
<i>P. multivorans</i>	382	35	17
<i>P. multivorans</i>	249	23	0
<i>P. caryophylli</i>	721	28	3
<i>P. pseudomallei</i>	4845	27	13
<i>P. mallei</i>	4	30	5
<i>P. solanacearum</i>	729	0	—
<i>P. solanacearum</i>	730	0	—
<i>P. saccharophila</i>	—	0	—
<i>P. aeruginosa</i>	131	0	—
<i>P. putida</i>	90	0	—
<i>P. fluorescens</i>	38	0	—
<i>P. stutzeri</i>	222	0	—
<i>P. stutzeri</i>	224	0	—
<i>P. acidovorans</i>	105	0	—
<i>P. testosteroni</i>	78	0	—

Table 7 presents the results of competition experiments using DNA of *Pseudomonas caryophylli* 721 as the reference standard. The DNA of two other strains of this species gave extremely high competition values (99 and 100 % at 72°, 99 and 94 % at 80°). The DNA of two strains of *P. pseudomallei* showed much lower competition at 72° (24 and 29 %), but the competition values remained at similar levels at 80°. Significant competition (8 to 29 %) was also obtained at 72° with DNA from strains of *P. mallei*, *P. cepacia*, *P. multivorans*, *P. marginata* and *P. alliicola*, but the values fell to much lower levels (0 to 13 %) at 80°. No competition was detected at 72° with DNA from strains of *P. solanacearum*, *P. saccharophila*, *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. stutzeri* or *P. acidovorans*. These results confirm the phenotypic data, which suggest that *P. caryophylli* is a distinctive species.

Table 7. *DNA competition experiments using DNA of Pseudomonas caryophylli 721 as reference standard*

DNA from	Strain no.	Per cent competition at	
		72°	80°
<i>P. caryophylli</i>	719	99	99
<i>P. caryophylli</i>	720	100	94
<i>P. pseudomallei</i>	1691	29	24
<i>P. pseudomallei</i>	4845	24	24
<i>P. mallei</i>	4	24	13
<i>P. cepacia</i>	725	13	0
<i>P. cepacia</i>	727	20	8
<i>P. multivorans</i>	382	29	6
<i>P. multivorans</i>	249	16	0
<i>P. marginata</i>	702	9	0
<i>P. marginata</i>	704	19	0
<i>P. alliicola</i>	709	9	0
<i>P. alliicola</i>	724	8	0
<i>P. solanacearum</i>	729	0	—
<i>P. solanacearum</i>	730	0	—
<i>P. saccharophila</i>	—	0	—
<i>P. aeruginosa</i>	131	0	—
<i>P. putida</i>	90	0	—
<i>P. fluorescens</i>	31	0	—
<i>P. fluorescens</i>	38	0	—
<i>P. stutzeri</i>	222	0	—
<i>P. stutzeri</i>	224	0	—
<i>P. acidovorans</i>	105	0	—
<i>P. testosteroni</i>	78	0	—

DISCUSSION

The pseudomallei group: nomenclature and internal relationships

The 18 strains of the phytopathogen *Pseudomonas cepacia* that we examined cannot be distinguished phenotypically from the strains, isolated from soil, water and clinical material, which Stanier *et al.* (1966) placed in the new species *P. multivorans*. Furthermore, our studies on DNA-DNA hybridization *in vitro*, using DNA from the type strain of *P. multivorans* as the reference standard, reveal that two strains of *P. cepacia* share a level of genetic homology with the type strain of *P. multivorans* at least as high as that of any of the eight other strains of *P. multivorans* examined. The mean DNA base composition of six strains of *P. cepacia* fall in the range of *P. multivorans*. The sole reported difference between the two species is the ability of *P. cepacia* to cause a plant disease, a root rot of onion bulbs. Inoculation experiments with onion bulb slices, performed by Mrs Vilma Vitanza and Dr D. C. Hildebrand (personal communication) showed that some strains of *P. multivorans* studied by Stanier *et al.* (1966) were capable of producing such a rot. In view of this fact, the two species are without question synonymous; and the correct specific designation is *Pseudomonas cepacia* Burkholder (1950) emend. The specific description should be amended to exclude phytopathogenic ability as a specific character, since not all strains of *P. multivorans* produce a clear-cut rot of onion bulb slices, and to include the many distinctive phenotypic characters established by Stanier *et al.* (1966) and by the present study. Since a type strain was not chosen by Burkholder (1950), we propose his isolate *P. cepacia* 717 (ATCC 25416) as the lectotype strain.

The nine strains of *Pseudomonas marginata* and the eight strains of *P. alliicola* that we studied cannot be distinguished phenotypically. Studies on DNA-DNA hybridization *in vitro*, using DNA from a strain of *P. marginata* as a reference standard, show that two strains of *P. alliicola* share with it a level of genetic homology almost as high as that of the only other strain of *P. marginata* tested. The mean DNA base compositions of three strains belonging to each species cannot be distinguished. The sole reported difference between these two species concerns their specific phytopathogenic behaviour; *P. alliicola* was described as the causative agent of a rot of onion bulbs (Burkholder, 1942), whereas *P. marginata* causes a rot of *Gladiolus* (McCulloch, 1921). However, Mrs Vitanza and Dr Hildebrand (personal communication) have shown that some of the strains of *P. marginata* we studied can rot onion bulb slices; the alleged difference between the two species is, therefore, probably not significant, and simply reflects the particular plant sources from which strains were isolated. These two species should be considered as synonymous; the correct specific designation, on grounds of priority, is *P. marginata* (McCulloch) Stapp (1928). The description should be amended to exclude the particular phytopathogenic properties originally attributed to *P. marginata* as characters of the species, and to include the many distinctive phenotypic characters established in the present study. Since a type strain was not originally chosen (McCulloch, 1921, 1924) we propose *P. marginata* 704 (ATCC 10248) as the lectotype strain.

Although we have studied only four strains of *Pseudomonas caryophylli*, this species appears to be valid. It is readily distinguishable from both *P. cepacia* and *P. marginata* in phenotypic respects; *in vitro* DNA hybridizations show that the three strains examined share a high level of genetic homology, but share very low homology with either *P. cepacia* or *P. marginata*. Although *P. caryophylli* was originally described as the agent of a necrosis (wilt) of carnations, Dr V. Vitanza and Dr D. C. Hildebrand (personal communication) showed that the strains studied by us can cause a rot of onion bulb slices, like *P. cepacia* and *P. marginata*. The specific description should therefore be amended to exclude the particular phytopathogenic properties originally attributed to *P. caryophylli*, an omission which will not in any way impair recognition of this species, in view of its distinctive phenotypic characters, revealed by the present study. Since a type strain was not originally chosen (Burkholder, 1942) we propose *P. caryophylli* 720 (ATCC 25418) as the neotype strain.

Pseudomonas cepacia, *P. marginata* and *P. caryophylli* share many phenotypic properties with *P. pseudomallei* and *P. mallei*. Furthermore, the data on DNA-DNA hybridization *in vitro* of Rogul *et al.* (1968), which have been confirmed and extended in the present study, show varying degrees of genetic homology among all five species. Using DNA from *P. cepacia*, *P. marginata* and *P. caryophylli* as reference standards, we were unable to detect genetic homology with any of the other *Pseudomonas* species examined: *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. stutzeri*, *P. solanacearum*, *P. acidovorans* and *P. saccharophila*. For these reasons, it seems appropriate to regard *P. pseudomallei*, *P. mallei*, *P. cepacia*, *P. marginata* and *P. caryophylli* as a major and isolated species-cluster among the aerobic pseudomonads, which possibly deserves recognition at the generic or subgeneric level. Provisionally, we shall term this cluster the 'pseudomallei group'. The principal characters that serve to distinguish the five component species are summarized in Table 8.

The taxonomic significance of DNA homology groups

The aerobic pseudomonads constitute a large and varied sub-group among the eubacteria. Past attempts to create generic subdivisions among these organisms, as exemplified by such genera as *Hydrogenomonas*, *Comamonas* and *Xanthomonas*, have

Table 8. *Characters distinguishing the species of the pseudomallei group*

Character	<i>P. cepacia</i> *	<i>P. marginata</i>	<i>P. caryo- phylli</i>	<i>P. pseudo- mallei</i> †	<i>P. mallei</i> †
Moles % G+C in DNA	67.4±1.4	68.5±1.0	65.3±0.6	69.5±0.7	69.0±1.0
Dinitrification	—	—	+	+	v
Arginine dihydrolase	—	—	+	+	+
Extracellular Gelatin	v	+	—	+	+
hydrolases PHB	—	—	—	+	+
for Starch	—	—	—	+	+
No. of flagella	> 1	> 1	> 1	> 1	0
<i>Utilization of:</i>					
L-Rhamnose	v	—	+	—	—
Maltose, starch	(—)	—	—	+	v
Saccharate, mucate	+	+	+	—	—
Malonate	+	+	+	—	+
Glutarate	+	—	—	v	(—)
Adipate	+	+	—	+	+
Azelate, sebacate	+	+	—	+	v
L(+), D(—) & meso Tartrate	—	+	—	—	—
Glycollate	v	—	+	—	—
Hydroxymethylglutarate	+	+	+	+	—
Aconitate	+	+	+	+	—
Levulinate	+	—	—	+	—
Citraconate	+	+	—	—	—
Mesaconate	—	+	—	—	—
Erythritol	—	—	—	+	—
2,3-Butyleneglycol	+	—	+	—	—
Benzoate	+	+	—	+	+
D-Alanine	+	+	—	+	+
L-Valine	v	+	—	+	v
DL- α -Aminobutyrate	—	+	—	—	+
δ -Aminovalerate	+	—	—	+	v
Kynurenate, anthranilate	+	+	—	+	v
Ethanolamine, butylamine	(+)	+	—	+	—
Benzylamine	+	—	v	—	—
Putrescine	+	—	—	+	v
Spermine	+	—	—	v	—
Acetamide	+	—	—	—	—
Nicotinate	—	+	—	—	—

* Data in part from Stanier *et al.* (1966) and Mandel (1966).

† Data from Redfearn *et al.* (1966) and Mandel (1966).

() Character possessed by 90% or more of the strains.

been of questionable value: each of these genera is defined in terms of a limited number of arbitrarily selected phenotypic characters. For this reason, Stanier *et al.* (1966) favoured a very broad definition of the genus *Pseudomonas*.

Recent work suggests that the genetic homology group, as established through experiments on nucleic acid hybridization *in vitro*, may eventually provide the best

basis for the circumscription of the bacterial genus. The pseudomallei group, as defined through the hybridization experiments described in the present paper, provides one example among the aerobic pseudomonads of such a homology group, embracing a considerable number of species. A second large homology group, clustered around the type species of the genus *Pseudomonas*, *P. aeruginosa*, is also evident from recent work; it includes at least three other species of fluorescent pseudomonads: *P. putida*, *P. fluorescens* and *P. syringae* (under which designation we would place most of the nomenspecies of fluorescent phytopathogenic pseudomonads). We have established (unpublished data) that these four species all share a substantial level of genetic homology. Furthermore, work performed at Berkeley has shown that non-fluorescent denitrifying pseudomonads of the *P. stutzeri* species-group, closely related to one another in terms of genetic homology, also show a more distant relationship in these terms to some members of the fluorescent group. None of the members of the fluorescent-stutzeri group shows detectable DNA homologies with any of the species of the pseudomallei group.

The bacterial genus, defined as a cluster of species which belong to a single genetic homology group, would be a taxonomic entity of considerable biological significance; in effect, its component species could be construed as offshoots of a single evolutionary branch among the bacteria. The practical feasibility of assigning generic status to each homology group within a large bacterial assemblage such as the aerobic pseudomonads is still uncertain. Since nucleic acid hybridization *in vitro* is unlikely to become a routine taxonomic tool in the near future, genera defined in these terms would be of little value unless they could also be distinguished from one another by easily determinable phenotypic traits. To ascertain whether the two large homology groups now recognizable among the aerobic pseudomonads can be distinguished from one another at the phenotypic level, we compared the known characters that are common to all species of each homology group. From this it was evident that many phenotypic characters of great value in the differentiation of species among the aerobic pseudomonads—for example, denitrification, the oxidase reaction, the utilization of starch and poly- β -hydroxybutyric acid, the ability to grow at 41°—are not distinctive of either homology group, since they are variable in the component species. Only five known characters sharply differentiate these two large homology groups: the formation of PHB as a reserve material, and the ability to utilize D-arabinose, D-fucose, cellobiose and L-threonine. All five characters are positive in the pseudomallei group, and negative in the fluorescent-stutzeri group. It does not seem prudent to propose a generic differentiation on such slim phenotypic grounds.

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