Taxonomy of the Aerobic *Pseudomonads*: the Properties of the *Pseudomonas stutzeri* Group

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SUMMARY

Strains of *Pseudomonas stutzeri* and related denitrifying bacteria were compared in their phenotypic properties and mean deoxyribonucleic acid (DNA) base composition. On the basis of this comparison and of *in vitro* DNA hybridization experiments, it was concluded that, using practical diagnostic tests, no more than two nomenspecies can be recognized within the group. One, *P. stutzeri*, was extremely variable in phenotypic characteristics and in DNA base composition; we included in it the strains previously assigned to *P. stanieri*. The other was a new species, *P. mendocina* Palleroni, which was more homogeneous in phenotypic characters, and in DNA base composition and homology. The comparative properties of known denitrifying pseudomonads are tabulated.

INTRODUCTION

Pseudomonas stutzeri is a non-fluorescent, denitrifying pseudomonad, widely distributed in soil and water. It was discovered and described as *Bacillus denitrificans* II by Burri & Stutzer in 1895, and renamed *Bacterium stutzeri* by Lehmann & Neumann in 1896. Thereafter, the taxonomic and nomenclatural status of this well-characterized species became confused, and was cleared up by van Niel & Allen only in 1952, when they retraced the history of the species, and redescribed it. *P. stutzeri* can be distinguished from other *Pseudomonas* spp. by many properties, of which the salient ones are: vigorous denitrifying ability; use of starch and maltose as carbon sources; and a characteristic and unusual colony structure; colonies of freshly isolated strains are wrinkled, tough and coherent, and have a light-brown colour which reflects the unusually high cytochrome *c* content of the cells.

Stanier, Palleroni & Doudoroff (1966) extended the description of *Pseudomonas stutzeri*, on the basis of a detailed examination of 17 strains isolated in diagnostic laboratories in Denmark and France from clinical specimens. A concurrent determination of the buoyant densities of DNA from these strains by Mandel (1966)

revealed a marked bimodal distribution: six strains had a guanine+cytosine (GC) content of about 62 moles %, and 11 of about 65 moles %; he assigned the former strains to a new species, *P. stanieri* with strain 224 as holotype, and with the reservation that strains 223, 320 and 228 were atypical and might not belong to the species. He limited *P. stutzeri* to the strains of the higher GC content. Stanier *et al.* (1966) found few consistent phenotypic differences between the two groups of strains and they did not accept Mandel's taxonomic proposal. The number of strains examined in this study was relatively small, and the principal source from which they had been isolated (clinical specimens) was certainly atypical, as there is no evidence that these denitrifying bacteria are associated with vertebrates as parasites, pathogens or commensals. It therefore seemed desirable to enlarge the study of these organisms.

Two of us (N. J. Palleroni & R. E. Solanes) isolated many additional strains of non-fluorescent denitrifying pseudomonads by enrichment procedures from soil and water samples collected in the province of Mendoza, Argentina; most of these strains had the typical distinguishing features of *Pseudomonas stutzeri* outlined above. However, some failed to attack starch and maltose, and formed smooth colonies on primary isolation. Further study showed that these strains were representatives of a related but readily distinguishable species, which we shall name *P. mendocina*. In this paper, we shall describe the phenotypic and genotypic properties of *P. mendocina* and of the *P. stutzeri*-*P. stanieri* complex.

MATERIALS AND METHODS

Origins of the strains. In addition to the strains described by Stanier *et al.* (1966) the following new isolates from denitrification enrichment cultures were examined. The source material and the conditions of the enrichment (carbon source and temperature) are indicated for each strain in parenthesis.

Strains assigned to the *P. mendocina stutzeri*-*P. stanieri* complex: CH 19 (soil, ethanol, 40°); CH 29 (soil, succinate, 30°); CH 39 (soil, L-tartrate, 40°); CH 44 (soil, L-tartrate, 30°); CH 48 (water, sebacate, 30°); CH 49 (soil, ethanol, 30°); CH 52 (water, ethanol, 30°); CH 53 (water, succinate, 30°); CH 54 (water, succinate, 30°); CH 58 (soil, L-tartrate, 30°); CH 60 (water, L-tartrate, 30°); CH 62 (soil, L-tartrate, 30°); CH 63 (soil, L-tartrate, 30°); CH 64 (soil, L-tartrate, 30°); CH 69 (water, ethanol, 40°); CH 70 (water, ethanol, 40°); CH 80 (soil, succinate, 30°); CH 82 (water, succinate, 30°); CH 85 (soil, succinate, 30°); CH 88 (water, L-tartrate, 30°); CH 90 (soil, ethanol, 30°); CH 118 (soil, sebacate, 40°).

Strains assigned to *Pseudomonas* sp. nov.: CH 20 (water, succinate, 40°); CH 34 (soil, succinate, 40°); CH 35 (soil, L-tartrate, 40°); CH 50 (soil, ethanol, 30°); CH 67 (water, L-tartrate, 40°); CH 74 (water, ethanol, 40°); CH 91 (soil, sebacate, 40°); CH 95 (water, ethanol, 30°); CH 110 (soil, ethanol, 40°); CH 113 (soil, ethanol, 40°); CH 120 (water, sebacate, 40°); CH 139 (soil, ethanol, 30°).

Phenotypic characterization. The phenotypic characterization of the strains was carried out by the methods described by Stanier *et al.* (1966).

DNA base compositions. DNA was isolated and the base composition determined as described by Mandel (1966).

In vitro DNA hybridizations. Extraction and purification of DNA was by the procedure of Marmur (1961). Radioactive DNA was obtained by addition of ¹⁴C adenine

to a culture in a medium prepared with the standard mineral base described by Stanier *et al.* (1966), with the addition of 0.2% (w/v) vitamin-free Casamino acids, and 0.2% (w/v) of sodium lactate. Adenine was added 30 to 60 min. (about 1.5 generations) before harvesting the culture, which was grown on a rotary shaker at 30°.

DNA hybridizations *in vitro* were performed by the technique of Johnson & Ordal (1968), fully described by Ballard *et al.* (1970). By this technique, competition with the homologous DNA, rather than direct binding, was determined. The results were expressed as 'per cent competition', i.e. the relative competition obtained in each case compared with the competition with homologous DNA, arbitrarily assigned a value of 100%.

RESULTS

Isolation of Pseudomonas mendocina from enrichment cultures. The strains of *P. mendocina* described in this paper were isolated from water and soil by enrichment cultures with different carbon sources, either at 30 or 40°. Nine of the twelve isolates were from enrichments at 40°; in seven the same inoculum yielded *P. stutzeri* when incubated at 30°, with the same carbon source.

Table 1. *Pseudomonas stutzeri*–*P. stanieri* complex. Substrates used by 90% or more of the strains

Carbohydrates and sugar derivatives	Polyalcohols and glycols
Glucose*	Glycerol*
Maltose*	Ethylene glycol
Starch*	Propylene glycol*
Fatty acids	Alcohols
Acetate	Ethanol*
Butyrate*	Non-nitrogenous aromatic and other
Caprylate*	cyclic compounds
Caprate*	(None)
Dicarboxylic acids	Aliphatic amino acids
Succinate	L- α -Alanine*
Fumarate*	D- α -Alanine*
Azelate*	L-Leucine*
Sebacate*	L-Glutamate*
Hydroxyacids	Amino acids and related compounds
L-Malate*	containing a ring structure
DL- β -Hydroxybutyrate*	L-Proline*
DL-Lactate	Amines
Glycollate*	(None)
Miscellaneous organic acids	Miscellaneous nitrogenous compounds
Citrate*	(None)
α -Ketoglutarate*	Paraffin hydrocarbons
Pyruvate	(None)
Itaconate*	
Mesaconate*	

* Substrates not used by all strains. Negative strains are listed in Table 2.

Common properties of the stutzeri group. It may be useful to summarize the properties shared by the *Pseudomonas stutzeri*–*P. stanieri* complex and *P. mendocina* n.sp., and indicate those characters that set them apart from other major species groups among the aerobic pseudomonads.

The members of this group were similar in structure. The rod-shaped bacteria were

Table 2. *Pseudomonas stutzeri*–*P. stanieri* complex. Substrates used by a fraction of the strains

Substrate	Total of positive strains	Negative strains
Xylose	1	All except CH 85
L-Arabinose	1	All except CH 85
Glucose	38	CH 62
Mannose	2	All except 275, CH 85
Fructose	25	223, 225, 275, 316, 319, 419, CH 19, CH 48, CH 52, CH 53, CH 54, CH 62, CH 80, and CH 82
Maltose	36	229, 316, CH 62
Starch	35	275, 229, 316, CH 62
Gluconate	28	228, 319, 320, 321, 419, CH 29, CH 53, CH 54, CH 62, CH 80 and CH 82
Saccharate	25	220, 221, 224, 225, 226, 227, 275, 316, 319, 419, CH 19, CH 53, CH 54 and CH 118
Mucate	18	All except 222, 223, 228, 229, 318, 230, 321, CH 39, CH 52, CH 58, CH 62, CH 63, CH 64, CH 69, CH 70, CH 85, CH 88 and CH 90
Propionate	24	220, 223, 224, 225, 228, 275, 319, 320, 321, 419, CH 49, CH 52, CH 58, CH 88 and CH 90
Butyrate	35	275, CH 29, CH 58, CH 60
Isobutyrate	3	All except CH 53, CH 70 and CH 80
Valerate	28	220, 229, 275, 318, 321, 419, CH 29, CH 49, CH 52, CH 58 and CH 63
Isovalerate	29	275, 320, 321, CH 29, CH 49, CH 52, CH 60, CH 62, CH 90 and CH 118
Caproate	30	275, 320, 321, CH 29, CH 49, CH 52, CH 58, CH 62 and CH 90
Heptanoate	34	223, CH 29, CH 49, CH 58 and CH 60
Caprylate	36	CH 29, CH 60 and CH 62
Pelargonate	33	223, CH 29, CH 49, CH 52, CH 60 and CH 90
Caprate	35	CH 60, CH 64, CH 70, CH 85
Malonate	33	228, 229, 320, 321, 419 and CH 58
Fumarate	37	CH 49 and CH 60
Glutarate	32	228, CH 29, CH 44, CH 49, CH 52, CH 58 and CH 60
Adipate	4	All except 223, CH 44, CH 58 and CH 85
Pimelate	1	All except CH 58
Suberate	2	All except 223 and CH 85
Azelate	35	228, 229, 275 and 419
Sebacate	37	275 and 419
D-Malate	4	All except 228, 316, 320 and 321
L-Malate	37	CH 19 and CH 48
L(+)-Tartrate	2	All except 419 and CH 85
M-Tartrate	1	All except CH 85
DL- β -Hydroxybutyrate	35	CH 29, CH 49, CH 53 and CH 62
Glycollate	35	CH 29, CH 44, CH 58 and CH 60
Glycerate	16	All except 223, 275, 319, 320, 321, 419, CH 19, CH 48, CH 52, CH 58, CH 63, CH 69, CH 70, CH 80, CH 82 and CH 85
Hydroxymethylglutarate	4	All except 222, 226, 227 and CH 48
Citrate	38	318
α -Ketoglutarate	37	228 and CH 29
Aconitate	18	All except 220, 221, 223, 224, 225, 228, 275, 316, 319, 320, 321, 419, CH 48, CH 69, CH 70, CH 80, CH 82 and CH 118
Levulinate	2	All except CH 48 and CH 60
Itaconate	37	223 and CH 62
Mesaconate	37	223 and CH 62

Table 2 (cont.)

Substrate	Total of positive strains	Negative strains
Mannitol	24	226, 227, 316, 318, 319, 320, 321, 419, CH 19, CH 48, CH 53, CH 54, CH 80, CH 82 and CH 118
Glycerol	38	275
Propylene glycol	35	221, CH 53, CH 54 and CH 60
2,3-Butylene glycol	34	226, 227, 229, 275 and CH 48
Ethanol	36	CH 53, CH 54 and CH 60
Propanol	34	221, CH 53, CH 54, CH 60 and CH 64
Butanol	31	275, CH 49, CH 53, CH 54, CH 58, CH 60, CH 62, and CH 64
Isobutanol	31	226, 227, 229, 275, 320, 321, CH 58 and CH 60
D-Mandelate	1	All except CH 88
L-Mandelate	1	All except CH 88
Benzoyl formate	1	All except CH 88
Benzoate	29	220, 221, 223, 224, 225, 275, 318, 320, 321 and CH 85
<i>p</i> -Hydroxybenzoate	6	All except 226, 227, 318, 419, CH 69 and CH 85
Quinate	1	All except CH 48
Glycine	34	CH 29, CH 49, CH 58, CH 60 and CH 90
L- α -Alanine	37	CH 49 and CH 60
D- α -Alanine	35	CH 29, CH 44, CH 60 and CH 62
β -Alanine	3	All except CH 48, CH 69 and CH 70
L-Serine	4	All except CH 48, CH 69, CH 80 and CH 82
L-Leucine	38	CH 60
L-Isoleucine	34	275, 319, 419, CH 60 and CH 62
L-Valine	33	228, 229, 275, CH 39, CH 49 and CH 60
L-Aspartate	27	221, 229, 275, 419, CH 19, CH 39, CH 53, CH 54, CH 62, CH 69, CH 88 and CH 90
L-Glutamate	37	CH 49 and CH 60
α -Amino-butyrate	25	220, 221, 222, 224, 225, 228, 275, 316, 318, 320, 321, CH 19, CH 29 and CH 62
δ -Amino-valerate	21	221, 222, 223, 224, 225, 228, 229, 275, 316, 320, 321, CH 19, CH 29, CH 44, CH 52, CH 60, CH 88 and CH 118
L-Histidine	1	All except CH 85
L-Proline	37	419 and CH 62
L-Tyrosine	34	223, 229, 318, CH 29 and CH 60
L-Phenylalanine	9	All except 223, 275, 319, CH 19, CH 48, CH 70, CH 80, CH 82 and CH 85
Kynurenate	2	All except 316 and 319
Ethanolamine	8	All except 220, 221, 224, 225, 316, 319, 320 and 321
Putrescine	29	229, 316, 419, CH 44, CH 49, CH 53, CH 58, CH 60, CH 90 and CH 118
Spermine	8	All except 319, 320, 321, CH 48, CH 69, CH 70, CH 80 and CH 82
Betaine	3	All except 223, CH 48 and CH 85
Sarcosine	1	All except CH 48
Nicotinate	1	All except 319
Trigonelline	1	All except 319

0.75 to 0.85 μm . wide and 1.4 to 2.8 μm . long in exponentially growing cultures. All bore a single polar or subpolar flagellum with a wavelength of 1.75 to 2.0 μm . (Pl. 1). In several strains of *Pseudomonas mendocina* (notably CH 39, 110 and 120), lateral flagella of shorter wavelength (about 1 μm .) (Pl. 2) were also produced, particularly in young cultures on complex solid media. Two of ten strains of *P. stutzeri* (227 and

319) also had lateral flagella of short wavelength, although they occurred on fewer bacteria than in *P. mendocina*. In this group, the flagella of shorter wavelength were easily shed during the manipulations of flagella staining.

No member of the stutzeri group accumulated poly- β -hydroxybutyrate as a reserve material. Neither fluorescent nor phenazine pigments were produced. Growth factors were not required. A fairly wide, though variable, range of simple organic compounds could serve as sole sources of carbon and energy; the most distinctive substrates, which supported growth of nearly all strains of the group, but were rarely used by other *Pseudomonas* species, were ethylene glycol and glycolic acid. All strains grew well under anaerobic conditions, with abundant gas production, in media that contained sufficient nitrate. It was noted that the capacity for vigorous denitrification might be lost after prolonged cultivation of strains on nitrate-free media, and a period of adaptation might be required to restore this activity to a high level (Stanier *et al.* 1966). The temperature range was relatively large; all strains grew well at 40°, and none grew at 4°.

Phenotypic properties of newly isolated strains assigned to the Pseudomonas stutzeri-P. stanieri complex. Twenty-two strains of *P. stutzeri* from soil and water in Argentina isolated by enrichment techniques for denitrifying bacteria were characterized, and some of the nutritional properties of the strains previously examined (Stanier *et al.* 1966) were rechecked. The nutritional spectrum of all 39 strains is shown in Tables 1 and 2. The nutritional variability of the complex, evident from earlier work, became even clearer. Although 29 compounds among those tested could be utilized by 90% or more of the strains, only acetate, succinate, lactate, pyruvate and ethylene glycol were universal substrates (Table 1). Glucose, caprylate, caprate, fumarate, L-malate, glycollate, ethanol, L- and D-alanine and glutamate, universally used by the 17 strains studied by Stanier *et al.* (1966), were not attacked by some of the strains isolated in Argentina.

Phenotypic properties of Pseudomonas mendocina Palleroni sp. nov. Colonies were flat, smooth, butyrous, of brownish yellow colour. The wrinkled appearance of freshly isolated colonies of *P. stutzeri* was not observed. The yellow colour was due to the presence of carotenoid pigments in the cells.

Starch, gelatin and poly- β -hydroxybutyrate were not hydrolysed and the egg-yolk reactions were negative. All strains were arginine dihydrolase positive. Strains that grew with benzoate or *p*-hydroxybenzoate attacked the respective diphenolic intermediates (catechol and protocatechuate) by *ortho* cleavage, and therefore degraded aromatic substrates through the β -ketoadipate pathway. The temperature optimum was about 37°; all strains could grow at 41°, and none at 4°.

The nutritional spectrum of *Pseudomonas mendocina* is described in Tables 3 and 4. In nutrition, the species was far more homogeneous than the *P. stutzeri*-*P. stanieri* complex. Of the 67 compounds that can be used by the species as a whole, 46 were used by all twelve strains and 55 by at least eleven strains. The species differed from *P. stutzeri* in many nutritional respects; it failed to use starch, maltose, azelate and sebacate, used by most strains of *P. stutzeri*; it grew with spermine, betaine, sarcosine and geraniol, rarely used by strains of *P. stutzeri*. In nutrition, and in the possession of arginine dihydrolase, *P. mendocina* resembled species of the fluorescent group, particularly *P. aeruginosa*. Properties shared by these two species included: polar monotrichous flagella, denitrifying ability, capacity for growth at 41°, and ability to

use geraniol, an alcohol not attacked by other *Pseudomonas* spp. *Pseudomonas mendocina* and *P. aeruginosa* could, however, be readily distinguished from one another by differences in pigmentation, in attack on mannitol, azelate and suberate, and in gelatinase production.

Table 3. *Pseudomonas mendocina*. Substrates utilized by 90% or more of the strains

Carbohydrates and sugar derivatives	Alcohols
D-Glucose	Ethanol
Gluconate	Propanol
Saccharate	n-Butanol
Mucate*	Isobutanol
Fatty acids	Geraniol
Acetate	Non-nitrogenous aromatic and other cyclic compounds
Propionate	Quinate*
Butyrate	Aliphatic aminoacids
Valerate	L- α -Alanine
Isovalerate*	D- α -Alanine
Caproate*	β -Alanine*
Heptanoate	L-Serine
Caprylate	L-Leucine
Pelargonate	L-Isoleucine
Caprate	L-Valine
Dicarboxylic acids	L-Aspartate
Malonate	L-Glutamate
Succinate	DL-Arginine
Fumarate	α -Aminobutyrate
Glutarate	α -Aminovalerate
Hydroxyacids	Aminoacids and related compounds having a ring structure
DL- β -Hydroxybutyrate	L-Histidine
DL-Lactate	L-Proline
Glycollate	L-Tyrosine
DL-Glycerate*	Amines
Miscellaneous organic acids	Putrescine
Citrate	Spermine
α -Ketoglutarate	Miscellaneous nitrogenous compounds
Aconitate	Betaine
Levulinate*	Sarcosine
Itaconate*	Paraffinic hydrocarbons
Mesaconate	(None)
Polyalcohols and glycols	
Ethylene glycol*	
Propylene glycol	

* Substrates not used by all strains. Negative strains are listed in Table 4.

Nutritional characters common to the two species of the *stutzeri* group, but rarely if ever encountered in species of the fluorescent group, included the ability to use ethylene glycol and glycollate, and the failure to use 2-ketogluconate.

DNA base composition of the stutzeri group. DNA base compositions were determined from buoyant density measurements for many of the strains isolated in Argentina (Table 5). The mean base composition of the DNA of *Pseudomonas mendocina* showed little variation from strain to strain; all values clustered closely around 63.2 moles % GC. However, values for the remaining 22 newly isolated strains ranged from 62.2 to 66.3 mole % GC; the distribution of values for the individual strains was about Gaussian, with a peak of 64.3 % GC (Fig. 1). When these data were combined with the

Table 4. *Pseudomonas mendocina*. Substrates utilized by a fraction of the strains

Substrate	Total of positive strains	Negative strains
D-Fructose	9	CH 20, CH 34, CH 110
Mucate	11	CH 95
Isobutyrate	7	CH 20, CH 34, CH 35, CH 50 and CH 67
Isovalerate	11	CH 95
Caproate	11	CH 95
D-Malate	5	All except CH 20, CH 34, CH 35, CH 50 and CH 67
L-(+)-Tartrate	5	All except CH 35, CH 67, CH 95, CH 110 and CH 139
DL-Glycerate	11	CH 95
Hydroxymethylglutarate	9	CH 34, CH 50 and CH 95
Pyruvate	10	CH 91 and CH 95
Levulinate	11	CH 91
Citraconate	10	CH 20 and CH 120
Itaconate	11	CH 95
Glycerol	9	CH 91, CH 95 and CH 120
Ethylene glycol	11	CH 95
Benzoate	10	CH 74 and CH 110
Quinate	11	CH 67
Glycine	10	CH 91 and CH 95
β -Alanine	11	CH 91
L-Phenylalanine	5	All except CH 20, CH 34, CH 35, CH 50 and CH 67
Tryptamine	9	CH 74, CH 110 and CH 113

Fig. 1. Distribution of guanine + cytosine values (mole %) among the strains of *Pseudomonas stutzeri* isolated in Argentina.

data of Mandel (1966) on the 17 strains of *P. stutzeri* studied by Stanier *et al.* (1966), the bimodal distribution of GC values which then led Mandel to propose a separation into two species remained, but was less clear-cut (Fig. 2). The majority of values clustered between 64.3 and 65.3 mole % GC; there was still a minor peak at 62.2, but it was now connected by intervening values to the major peak. There is little probability that these values can be fitted to a single Gaussian distribution on the assumption that the standard deviation of the values is about 1 % GC, that is, no greater than that

Fig. 2. Distribution of guanine + cytosine values (mole %) among all the strains of *Pseudomonas stutzeri* described in this paper.

found for other *Pseudomonas* species (Mandel, 1966). The extreme limits of base composition were represented by one strain with a value of 60.6 mole % GC, and two with 66.3. Mandel's (1966) data for other *Pseudomonas* species in which the DNA base composition of many strains had been determined, showed that well-characterized species generally had a remarkably constant mean base composition. The wide variation in the DNA base composition of the *P. stutzeri*-*P. stanieri* complex was exceptional among aerobic pseudomonads, although a comparable situation occurred in the *Acinetobacter* group (Baumann, Doudoroff & Stanier, 1968). The variation in GC content may be related to the very high degree of nutritional heterogeneity in the strains of this complex (see Table 2).

In vitro DNA hybridization in the *stutzeri* group. To obtain a further insight into the genotypic structure of the *stutzeri* group, we performed experiments on *in vitro* DNA hybridization, using several reference strains of the *Pseudomonas stutzeri*-*P. stanieri* complex, with different DNA base compositions, and one reference strain of *P. mendocina*. In most competition experiments, annealing temperatures of 70 to 72° (about 25° below the *T_m* of the DNA samples being compared) were used. A few competition

Table 5. *CsCl* buoyant densities* and calculated GC (mole %) in the DNA of strains of the *stutzeri* group isolated in Argentina

<i>P. stutzeri</i> - <i>P. stanieri</i> complex			<i>P. mendocina</i>		
Strain	ρ_{CsCl}	GC	Strain	ρ_{CsCl}	GC
CH 19	1.725	66.3	CH 20	1.723	64.3
CH 29	1.723	64.3	CH 35	1.7215	62.8
CH 39	1.7235	63.8	CH 47	1.7215	62.8
CH 44	1.7225	63.8	CH 50	1.723	64.3
CH 48	1.7225	63.8†	CH 67	1.723	64.3
CH 49	1.7225	63.8	CH 74	1.7215	62.8
CH 52	1.7235	64.8	CH 91	1.722	63.3
CH 53	1.724	65.3	CH 95	1.7215	62.8
CH 54	1.725	66.3	CH 110	1.721	62.2
CH 58	1.723	64.3	CH 120‡	1.7215	62.8
CH 60	1.723	64.3	CH 139	1.7215	62.8
CH 62	1.723	64.3	$\bar{X} = 1.7219 \pm 0.0010$		
CH 63	1.722	63.3	$\bar{GC} = 63.2 \pm 1.0$ mole % (22 determinations of ρ)		
CH 64	1.723	64.3			
CH 69	1.724	65.3			
CH 70	1.723	64.3			
CH 80	1.724	65.3			
CH 82	1.7235	64.8			
CH 85	1.721	62.2			
CH 88	1.723	64.3			
CH 90	1.722	63.3			
CH 118	1.7235	64.8			
$\bar{X} = 1.7230 \pm 0.00093$					
$\bar{GC} = 64.3 \pm 0.9$ mole % (46 determinations of ρ)					

* Mean of two determinations for each, except CH 48.

† CH 48: four values.

‡ Satellite at 1.7095, even in re-cloned population.

experiments were also made at a higher annealing temperature (80°), where conditions for DNA-DNA hybridization were much more stringent; the occurrence of detectable competition at this annealing temperature probably indicated some virtually complete base sequence homologies between the two organisms.

As one of our goals was to assess the genetic significance of the variable DNA base composition of the *Pseudomonas stutzeri*-*P. stanieri* complex, reference DNA was prepared from five strains: three (220, 224 and 320) had DNA containing about 62 mole % GC, and two (221 and 222) had DNA of about 65 mole % GC. In preliminary experiments, the homology of one reference strain from each group was determined with strains of *P. mendocina*, *P. aeruginosa*, *P. fluorescens* (denitrifying biotypes), *P. putida*, *P. acidovorans* and *P. testosteroni*. These experiments (Table 6) revealed that the *P. stutzeri*-*P. stanieri* complex was genetically well isolated from other species of the genus *Pseudomonas*, with the exception of *P. mendocina*. At an annealing

temperature of 70 to 72°, competition by DNAs from all species except *P. mendocina* was less than 20%, whereas competition by DNA of *P. mendocina* ranged from 20 to 40%.

Competition at an annealing temperature of 70 or 72° among DNA preparations from strains studied by Stanier *et al.* (1966) and Mandel (1966) is shown in Table 7. Strains could be subdivided into four groups in terms of GC content and their relative

Table 6. DNA competition among strains of the *Pseudomonas stutzeri* group and other species of aerobic pseudomonads*

DNA competition within the <i>P. stutzeri</i> group	Range of % DNA competition at	
	70–72°	80°
<i>P. stanieri</i> v. <i>P. stanieri</i>	81–100	76–92
<i>P. stutzeri</i> v. <i>P. stutzeri</i>	81–92	71–83
<i>P. stanieri</i> v. <i>P. stutzeri</i>	52–70	30–56
<i>P. mendocina</i> v. <i>P. mendocina</i>	98–100	85–95
<i>P. mendocina</i> v. <i>P. stanieri</i>	19–38	7–9
<i>P. mendocina</i> v. <i>P. stutzeri</i>	28–40	13
DNA competition between the <i>P. stutzeri</i> group and other <i>Pseudomonas</i> spp.		
<i>P. stanieri</i> (220, 224) v. <i>P. aeruginosa</i> (52)	0–18	
<i>P. stanieri</i> (220, 224) v. <i>P. putida</i> (90)	0–4	
<i>P. stanieri</i> (224) v. <i>P. fluorescens</i> (2, 50)	0–7	
<i>P. stanieri</i> (224) v. <i>P. acidovorans</i> (105)	0	
<i>P. stanieri</i> (224) v. <i>P. testosteroni</i> (78)	0	
<i>P. stutzeri</i> (221, 222) v. <i>P. aeruginosa</i> (52)	0–5	
<i>P. stutzeri</i> (221, 222) v. <i>P. putida</i> (90)	0–8	
<i>P. stutzeri</i> (221, 222) v. <i>P. fluorescens</i> (2, 50)	0–3	
<i>P. stutzeri</i> (221) v. <i>P. acidovorans</i> (105)	0	
<i>P. stutzeri</i> (221) v. <i>P. testosteroni</i> (78)	0	
<i>P. mendocina</i> (CH 20) v. <i>P. aeruginosa</i> (52)	9	
<i>P. mendocina</i> (CH 20) v. <i>P. fluorescens</i> (2, 50)	18–19	
<i>P. mendocina</i> (CH 20) v. <i>P. fluorescens</i> (31)	21	
<i>P. mendocina</i> (CH 20) v. <i>P. fluorescens</i> (83)	21	
<i>P. mendocina</i> (CH 20) v. <i>P. acidovorans</i> (14)	0	
<i>P. mendocina</i> (CH 20) v. <i>P. testosteroni</i> (78)	0	

* Values for homologous DNA (100%) are not included. The data for competition within the *P. stutzeri* group are collected from Tables 7, 8 and 10. For all other DNA competition experiments, the strain numbers are shown in parentheses, with the reference underlined.

and absolute competitions with high and low GC reference strains. Five low GC strains (group I) showed consistently high homology (80 to 100% competition) with the low GC reference strains, and a lower homology (58 to 78% competition) with the high GC reference strains. Six high GC strains (group II) showed the inverse pattern of homology. Repetition of some experiments at an annealing temperature of 80° gave essentially similar results (Table 8). The strains of groups I and II appear to constitute two intraspecific clusters, each characterized by comparatively high internal genetic homology. However, the correlations between GC content and genetic homology were by no means perfect. Five strains, one of low GC content (group III) and four of high GC content (group IV) showed relatively low competition values (20 to 65%)

Table 7. *Percentage DNA competition among strains of the Pseudomonas stutzeri-P. stanieri complex (annealing temperature: 70 or 72°)*

Group	Mean DNA base composition mole % GC	Competitor DNA strain number	Reference DNA strain number				
			220	224	320	221	222
I	62.2	220	—	100	81	68	58
	62.2	224	100	—	81	60	61
	62.7	225	90	90	82	68	59
	62.7	228	83	86	84	70	63
	62.2	320	81	83	—	70	64
II	65.2	221	60	64	60	—	81
	64.7	222	64	57	60	86	—
	64.7	226	67	57	60	88	83
	65.5	227	64	60	65	80	83
	65.2	318	52	59	62	89	87
	65.2	321	61	60	68	92	86
III	60.7	223	33	35	32	33	37
IV	64.2	275	20	38	47	29	36
	65.7	316	31	49	34	65	47
	65.2	319	46	35	33	49	49
	65.2	419	33	31	44	56	50

Table 8. *DNA competition among strains of the Pseudomonas stutzeri-P. stanieri complex (annealing temperature: 80°)*

Group (mole % GC)	Competitor DNA strain number	Reference DNA strain number				
		220	224	320	221	222
		DNA competition* (%)				
I (62 % GC)	220	(100)*	—	84	—	47
	224	—	(100)	80	55	48
	225	92	—	88	—	47
	228	—	—	84	—	40
	320	76	82	(100)	48	48
II (65 % GC)	221	33	30	54	(100)	71
	222	37	47	56	83	(100)
	226	—	—	52	—	—
	227	—	—	48	—	78

* Figures in parentheses are control values for homologous DNA.

Table 9. *DNA competition between strains of the Pseudomonas stutzeri-P. stanieri complex of the CH series, and strains of P. stanieri and P. stutzeri*

Strain	% GC	DNA competition with reference strain (%)	
		224 (62.2 % GC)	222 (64.7 % GC)
CH 63	63.3	57	77
CH 39	63.9	64	75
CH 48	64.3	60	74
CH 54	66.3	45	62

with all the reference strains; and in most of these cases, the values obtained with reference strains of similar GC content were not significantly or consistently greater than the values obtained with reference strains of markedly different GC content. It should be noted that these five strains (223, 275, 316, 319 and 419) are also aberrant in nutritional respects (Table 2). This analysis accordingly showed that the strains of high GC and of low GC content studied by Stanier *et al.* (1966) were genetically heterogeneous.

Reference DNAs from strains 222 (high GC) and 224 (low GC) were tested at an annealing temperature of 70 or 72° with DNA preparations from five strains of the *Pseudomonas stutzeri*–*P. stanieri* complex isolated in Argentina, selected for differences in GC content. The competition data are summarized in Table 9; the test strains are arranged in order of increasing GC content, but the clusters of strains that constitute groups I and II do not stand out sharply. *In vitro* hybridization data accordingly suggest that there may be considerable genetic homology among all strains of the complex, overlain by a substantial amount of genetic variation. The mean DNA base composition of a given strain is therefore not necessarily indicative of its genetic relatedness to other strains of the complex.

Table 10. DNA competition between strains of the *Pseudomonas stutzeri* group and *P. mendocina* CH 20 (annealing temperature: 70°)

Competitor DNA	% GC	DNA competition (%)
<i>P. stutzeri</i> 220	62.2	33
<i>P. stutzeri</i> 223	60.7	35
<i>P. stutzeri</i> 224	62.2	20
<i>P. stutzeri</i> 225	62.7	38
<i>P. stutzeri</i> 228	62.7	32
<i>P. stutzeri</i> 320	62.2	19
<i>P. stutzeri</i> 221	65.2	40
<i>P. stutzeri</i> 222	64.7	34
<i>P. stutzeri</i> 226	64.7	40
<i>P. stutzeri</i> 227	65.5	28
<i>P. stutzeri</i> 319	65.2	29
<i>P. mendocina</i> CH 50	64.3	98
<i>P. mendocina</i> CH 120	62.8	99
<i>P. mendocina</i> CH 139	62.8	100

With *Pseudomonas mendocina* strain CH 20 as the source of reference DNA, virtually total competition was obtained with DNA from three other strains of the same species, indicating a high degree of intraspecific genetic homology (Table 10). DNAs from high and low GC strains of the *P. stutzeri*–*P. stanieri* complex showed considerably lower, but still substantial, competition at an annealing temperature of 70°. Even at an annealing temperature of 80°, some competition by these DNAs was evident (Table 6).

Experiments with DNA from other *Pseudomonas* species (Table 6) at an annealing temperature of 70° showed significant competition by biotypes B, C, D and F of *P. fluorescens*; in all these cases, however, competition fell to zero at an annealing temperature of 80°.

The hybridization studies accordingly suggested that *Pseudomonas mendocina* was a genetically uniform species, with some genetic relationship to the *P. stutzeri*–*P. stanieri* complex, and a lesser genetic relationship to some of the fluorescent pseudomonads.

DISCUSSION

The problem of nomenspecies in the Pseudomonas stutzeri group. Within the stutzeri group, the twelve strains assigned to *P. mendocina* constituted a uniform and readily differentiable species in terms of all the criteria that we applied: DNA base composition, *in vitro* DNA hybridization and phenotypic properties. Judged by the same criteria, the remaining 39 strains of the group constituting the *P. stutzeri*–*P. stanieri* complex, emerged as a heterogeneous array which presented a difficult problem in terms of species recognition. The GC contents of the DNA of these strains ranged from 60.7 to 66.3 mole %, and it was primarily because this range appeared too wide to characterize a single species, that Mandel (1966) proposed to limit the circumscription of *P. stutzeri* to strains with DNA of high GC content (about 65 mole %) and to create a second species for strains with DNA of low GC content (about 62 mole %). Such a specific separation could not be substantiated in terms of phenotypic differences among the 17 strains of the complex originally examined (Stanier *et al.* 1966), and the study of an additional 22 strains has not improved the situation. The apparently clear-cut bimodal distribution of DNA base compositions observed by Mandel (1966) in the 17 strains was not evident in the 22 newly examined strains, which showed an approximately Gaussian distribution of DNA base composition. When both sets of data were combined (see Fig. 2), the distribution of DNA base composition appeared to be polymodal, although the number of samples was still too small to permit any firm conclusion concerning the frequency distribution.

The variability of the nutritional characters of this group, evident from our original work (Stanier *et al.* 1966) became even more marked by the inclusion of data for an additional 22 strains (Tables 1, 2). A detailed analysis of nutritional data (not presented here) showed that strains of widely different GC content might be virtually indistinguishable in phenotypic respects; for example, strains 320 (62 mole % GC) and 321 (65 mole % GC) differed by only one of 80 nutritional characters variable within the group. We were unable to detect among our strain any clusters of differing GC content that showed sufficiently clear-cut phenotypic differences to justify their recognition as separate species.

The data on DNA hybridization with selected strains representative of the span of DNA base composition found in the complex indicated (as might be expected) that strains of similar GC content tend to show a higher level of homology with one another than with strains of different GC content. However, there were exceptions. Furthermore, all strains of the complex showed a considerable degree of genetic relatedness.

In short, we do not consider that a useful specific subdivision of this complex can be justified at present and we propose to include all strains of the *P. stutzeri*–*P. stanieri* complex in the single nomenspecies, *P. stutzeri*. This taxonomic treatment does not, of course, preclude the possibility of an eventual subdivision of the complex into two (or more) species, but work on a larger collection of strains, using an expanded phenotypic characterization, will undoubtedly be required to provide the necessary grounds for such a subdivision.

Problems associated with the use of flagellar insertion as a generic character. Only the polar insertion of flagella distinguishes the members of the genus *Pseudomonas* from the many other Gram-negative, rod-shaped eubacteria with DNA of relatively

high GC content. However, the use of this particular structural character as a generic one raises many difficulties, both practical and theoretical. One permanently immotile organism, *P. mallei*, was placed in *Pseudomonas* on account of its close phenotypic resemblance to the polarly flagellated *P. pseudomallei* (Redfearn, Palleroni & Stanier, 1966), and this assignment was confirmed by the demonstration of a substantial degree of nucleotide sequence homology between the two species (Rogul, Brendle, Haapala & Alexander, 1968). A different facet of the same problem is our observation on the modes of flagellar insertion characteristic of *P. stutzeri* and *P. mendocina*. Both species may possess lateral, as well as polar, flagella. Their characterization as polarly flagellated bacteria can be maintained only because the polar are distinguishable by their wavelength from the lateral flagella and predominate under certain conditions of cultivation. Comparable situations were described in *Aeromonas* and *Chromobacterium* (Rhodes, 1965). Such bacteria presumably possess two sets of genetic determinants that govern, respectively, polar and lateral flagellation, a supposition supported by Sneath's (1956) finding that the polar and lateral flagella of *Chromobacterium* are antigenically different, and also by the correlation between flagellar wavelength and insertion site. A bacterium in which polar and lateral flagella were of identical wavelength would, however, be characterized phenotypically as 'peritrichous' or 'degenerately peritrichous' even if it possessed two different sets of flagella subject to separate genetic control. The possibility that some apparently peritrichous Gram-negative bacteria may be related to one of the subgroups of *Pseudomonas* should be kept in mind by taxonomists.

Identification of denitrifying Pseudomonas spp. The early bacteriological literature on denitrification contains specific descriptions of many denitrifying organisms that are assignable to the genus *Pseudomonas*. No authentic strains of these species have survived, and the descriptions are so fragmentary that with the exceptions of *P. stutzeri*, *P. aeruginosa* and *P. fluorescens* it would be difficult to recognize a newly isolated strain as a representative of one of the named species. For this reason, we believe that most of the older names proposed for denitrifying pseudomonads should be treated as *nomina dubia*, and in this category we would place *Bacillus denitrificans* α and β Gayon & Dupetit (1886); *Bacillus denitrificans* Giltay & Aberson (1892); *Bacillus denitrificans* I Burri & Stutzer (1895); *Bacillus denitrificans agilis* Ampola & Garino (1896).

The present work, coupled with that of Stanier *et al.* (1966) and Redfearn *et al.* (1966), shows that the capacity for denitrification is characteristic of five well-defined *Pseudomonas* species that occur in soil and water. As an aid to their identification, we have prepared a tabular key of the characters that appear to have the greatest differential value (Table II).

A sixth species (*Pseudomonas caryophylli*) which to our knowledge has not been reported to be a soil inhabitant, but which is pathogenic for plants, is also included in the table. This vigorously denitrifying and readily recognizable organism has been characterized by Mr R. W. Ballard in our laboratory and the information shown is abstracted from his data. Some strains of another plant pathogen, *P. solanacearum*, also cause denitrification (Hayward, 1964). We have confirmed Hayward's report, but have not yet studied this *Pseudomonas* species in sufficient detail to include data for it in Table II. For each phenotypic character, we list the percentage of positive strains in each species; the values for *P. fluorescens* are based on the pooled phenotypic

data for the three denitrifying biotypes (B, C and D), and do not include data for non-denitrifying biotypes (A, E, F and G). The denitrifying biotypes of *P. fluorescens* can, in turn, be differentiated from one another in terms of additional characters (Stanier *et al.* 1966).

Table 11. *Characters of highest differential value for the identification of denitrifying aerobic pseudomonads*

Figures represent the percentages of strains which are positive for each of the characters

	<i>P. stutzeri</i> – <i>P. stanieri</i> complex (39 strains)	<i>P. mendo-</i> <i>cina</i> (12 strains)	<i>P. aeruginosa</i> (29 strains)	<i>P. fluorescens</i> (biot. B, C and D (44 strains)	<i>P. pseudo-</i> <i>mallei</i> (26 strains)	<i>P. caryophylli</i> (3 strains)
DNA (GC, mole %)	60.7–66.3	62.8–64.3	66.3–68.3	60.3–63.3	69.4–69.9	66
Structural characters						
Monotrichous flagellation	100	100	100	0	0	0
poly- β -Hydroxy- butyrate accumulation	0	0	0	0	100	100
Temperature relationships						
Growth at 40°	100	100	100	0	100	100
Growth at 4°	0	0	0	100	0	0
Pigmentation						
Fluorescent pigment	0	0	72	84	0	0
Pyocyanine	0	0	86	0	0	0
Carotenoids	0	100	0	0	0	0
Biochemical characters						
Arginine dihydrolase	0	100	100	100	100	100
Starch hydrolysis	98	0	0	0	100	0
poly- β -Hydroxy- butyrate hydrolysis	0	0	0	0	100	0
Nutritional characters						
Growth on:						
Xylose	3	0	0	34	0	100
Maltose	92	0	0	0	100	0
Saccharate	64	100	0	63	0	100
Mannitol	62	0	100	100	100	100
Ethylene glycol	100	92	0	2	8	0
2,3-Butylene glycol	87	0	93	73	0	100
Geraniol	0	100	100	0	0	0
Azelate	90	0	97	20	88	0
Levulinate	5	92	100	20	92	0
Glycollate	90	100	0	2	0	100
Serine	10	100	14	80	96	100
Arginine	0	100	100	100	100	100
Histidine	0	100	100	91	100	100
Betaine	5	100	100	100	100	100
Sarcosine	3	100	90	84	88	0

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EXPLANATION OF PLATES

PLATE I

Flagella stain (Leifson's method) of *Pseudomonas stutzeri*, strain 227(a), and of *P. mendocina*, strain CH 110(b). $\times 2000$.

PLATE 2

Flagella stain (Leifson's method) showing cells with lateral flagella of short wavelength: *Pseudomonas mendocina*, strain CH 110 (a) and (b); *P. stutzeri*, strain 319 (c), and *P. stutzeri*, strain 227(d).