Deoxyribonucleic Acid Base Composition in the Genus *Pseudomonas*

By M. MANDEL

*Section of Molecular Biology, Department of Biology, The University of Texas,
M. D. Anderson Hospital and Tumor Institute, Houston, Texas*

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SUMMARY

The base compositions of deoxyribonucleic acids extracted from aerobic pseudomonads were determined by examination of the buoyant density of each sample in caesium chloride (CsCl) gradients. A collection of 165 strains, representing many of the principal biotypes among aerobic pseudomonads, was subjected to this examination. The similarities and differences in the base compositions of the genetic material have been compared with the taxonomic arrangements suggested by the comparison of phenotypic (largely nutritional and enzymic) features. The data on base composition of the deoxyribonucleic acids of the various cultures support, for the greater part, the conclusions of Stanier, Palleroni & Doudoroff (1966) and of Redfearn, Palleroni & Stanier (1966). Data are also presented on pseudomonads not included in the latter studies.

INTRODUCTION

Taxonomic groupings, if presented as representing natural groupings of organisms by virtue of common descent or heritage, should demonstrate some uniformity of genetic capacity. Evidence of this sort has proved quite difficult to obtain within the microbial world. A recent review of this evidence (Marmur, Falkow & Mandel, 1963*a*) indicates the limits which the paucity of known genetic mechanisms have placed upon the application of biological hybridization as a tool in bacterial taxonomy. Lee, Wahl & Barbu (1956) recognized that different bacteria have quite different proportions of the purine and pyrimidine bases present in their deoxyribonucleic acids (DNA). They also recognized that bacterial species and genera which were classified together were more often similar in base composition than were those of greater separation in current schemes of classification. Lanni (1960) proposed that a minimum requirement for genetic exchange was a similarity of base compositions of the DNA of sexual partners. Within the genus *Bacillus* it was then demonstrated that a necessary, but not sufficient, condition for transformation to occur was identity of base composition (Marmur, Seaman & Levine, 1963*b*).

Colwell & Mandel (1964*a*) showed that groups of Gram-negative bacteria of differing DNA base composition were well separated by the computer groupings of Adansonian analysis. A similar correlation for Gram-positive cocci has been provided by Silvestri & Hill (1965).

At present, there appears to be no reasonable evidence that the mean DNA base composition of an organism is subject to either induced or spontaneous change within the limits of our gross analytical techniques. Claims to the contrary can be

attributed to the failure to recognize contamination as such, or to failures in analytical techniques (De Ley, 1964*a*). We have, therefore, determined the DNA base composition of a large and representative sampling of the strains of *Pseudomonas* reported in the companion papers by Stanier, Palleroni & Doudoroff (1966) and of representatives of *Pseudomonas pseudomallei* and *P. mallei* examined by Redfearn, Palleroni & Stanier (1966). These cultures have been subjected to intensive biochemical characterization, as has been the case for *P. lemoignei* (Delafield *et al.* 1965).

The DNA base compositions reported in the present communication have been deduced from the buoyant densities determined at equilibrium in a caesium chloride (CsCl) gradient. The choice of the method was dictated by several considerations, of which the large number of samples to be examined was the most demanding. Schildkraut, Marmur & Doty (1962) pointed out that purified DNA samples are not required for use in this method: consequently, the preparation time of samples is considerably shortened and the nucleic acids are of high molecular weight because of the lessened risk of shear incidental to increased manipulation during purification. A further advantage of the technique is that only small amounts of material are required for analysis. The disadvantages are that the determinations lack some of the precision of comparisons obtainable by means of optical absorbance changes as a function of temperature. Offsetting this additional precision is the danger of recording spurious melting temperatures as a consequence of inequalities in ionic strength, presence of heavy metals or polyamines as contaminants of the preparations (Schildkraut & Lifson, 1965; Mandel, 1962). Although the presence of an unusual base replacing either thymine or cytosine and glycosylation of the constituent bases may lead to density determinations higher than those predicted by knowledge of the base compositions of the DNA (Schildkraut *et al.* 1962), thus far no such anomaly has been found in any DNA sample isolated from a bacterial source. Reports of discrepancies between base composition determined by melting temperature and buoyant density (Frontali, Hill & Silvestri, 1965) are probably attributable to the uncertainty of the linearity of the relationships of melting temperature to base composition at extreme ratios of guanine + cytosine to adenine + thymine (Marmur & Doty, 1962; Colwell & Mandel, 1964*a*). The determination of buoyant density of DNA samples can consequently be used with confidence to assay reproducibly a large number of samples for comparative estimates of base composition. Additionally, the technique has the bonus advantage of revealing non-Gaussian distributions of molecules in the gradient if this departure is evident (as will be shown for the DNA of two strains of *Pseudomonas stutzeri*). Forty-four replicate determinations of the buoyant density in CsCl of one sample of DNA extracted from a strain of *Caulobacter crescentus* averaged 1.7255 g./cm.³ with a standard deviation of 0.0005 g./cm.³ and a standard error of less than 0.0001 g./cm.³ permitting a convenient comparison with the determinations reported here.

METHODS

Organisms. The strains examined were received on nutrient agar slopes via airmail from the donors. Usually three slopes were shipped to Texas and a fourth retained at the University of California laboratories for diagnostic examination when

necessary. Cultures were often identified only as to number, thus eliminating operator bias.

Preparation of DNA samples. Lysates of bacteria were prepared by washing the growth from the surface of the agar slopes with a small amount of saline EDTA (0.15 M-NaCl + 0.1 M-ethylenediaminetetra-acetic acid, EDTA; pH 8.0) followed by the addition of sodium lauryl sulphate to 2 % (w/v). The lysate was deproteinized by shaking with neutralized phenol saturated with saline EDTA, then with chloroform + isoamyl alcohol (24 + 1, by vol.). The nucleic acids were precipitated with 2 vol. of ethanol, collected by spooling on a glass rod, washed twice with 70 % (v/v) ethanol in water and then dissolved in 0.15 M-NaCl containing 0.015 M-Na₃ citrate (pH 7.0).

Cultures received as lyophilized samples were cultivated in nutrient broth (Difco) at 30° on a rotary shaker. The cultures were streaked on nutrient agar (Difco) and examined microscopically with phase-contrast optics to assure cultural purity. DNA was isolated and purified by the method of Marmur (1961), following lysis and one deproteinization with phenol as described above. Lysates of *Pseudomonas pseudomallei* and *P. mallei* were prepared within plastic isolation hoods and removed to the room through a phenol solution only after the addition of phenol to the lysate.

Buoyant density. The buoyant density in CsCl solutions and the guanine + cytosine (GC) content were determined as described by Schildkraut *et al.* (1962). Centrifugation was performed in a Spinco Model E analytical ultracentrifuge at 42,040 rev./min. and 25.0° for 22–23 hr. Each sample was also analysed by centrifugation in another instrument at 44,770 rev./min. and 25.0° for 19–21 hr. Photographs of the DNA bands at equilibrium in the gradient were obtained on Kodak Ortho Contrast Film by using monochromatic light at 265 m μ . Each centrifuge cell received approximately 1–2 μ g. of the DNA of unknown density and 0.5 μ g. of a reference DNA sample from SP8 bacteriophage (*Bacillus subtilis* host) of a density of 1.742 g./cm.³ as calculated by comparison with DNA of *Escherichia coli* taken to be the primary reference of 1.710 g./cm.³. Microdensitometer tracings of the ultraviolet (u.v.) photographs were made with a Joyce-Loebl double-beam recording microdensitometer. The GC content was calculated from the peak of each u.v.-absorbing band by using the equation of Schildkraut *et al.* (1962).

Values of standard deviations and statistical significance were determined with the aid of an IBM 1620 computer and standard program.

RESULTS

The fluorescent pseudomonads

Of the 175 strains studied by Stanier *et al.* (1966), 66 were analysed for DNA base composition. The mean buoyant density calculated from a minimum of duplicate determinations of the density of the DNA of each strain is listed in Table 1. Each species or biotype, in the terminology of Stanier *et al.* (1966), appears to be composed of individuals of homogeneous mean DNA base composition. The organisms comprising the species *Pseudomonas putida* can be divided into two types on the basis of their GC content; biotype A consists of organisms whose mean DNA base composition is 2 % higher in GC content than organisms of biotype B and this difference is significant at the 99 % confidence level (Table 2). Stanier *et al.* (1966)

Table 1. *Buoyant density and GC content of DNA of representatives of the fluorescent pseudomonads*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Species	Biotype	Strain	Mean density (g./cm. ³)	Mean value and standard deviation calculated for each group	
				Density (g./cm. ³)	GC (moles %)
<i>Pseudomonas aeruginosa</i>	—	45	1.726	1.7259 ± 0.0011	67.2 ± 1.1
		52	1.726		
		55	1.7255		
		58	1.726		
		181	1.727		
		182	1.726		
		278	1.725		
		280	1.725		
		282	1.726		
		286	1.7265		
		291	1.7255		
<i>P. fluorescens</i>	A	12	1.719	1.7193 ± 0.0011	60.5 ± 1.1
		126	1.719		
		182	1.7195		
		392	1.7195		
	B	2	1.719	1.7201 ± 0.0011	61.3 ± 1.1
		400	1.721		
		401	1.7195		
		403	1.720		
		404	1.721		
		411	1.720		
	C	18	1.7195	1.7194 ± 0.0008	60.6 ± 0.8
		50	1.719		
		181	1.720		
		191	1.719		
		213	1.7195		
		217	1.7195		
	D	30	1.7215	1.7222 ± 0.0009	63.5 ± 0.9
		31	1.722		
		32	1.722		
		35	1.7225		
		388	1.7215		
		389	1.7225		
		390	1.722		
		391	1.7225		
		393	1.7225		
		394	1.722		
	E	36	1.722	1.7223 ± 0.0008	63.6 ± 0.8
		37	1.7215		
		38	1.7225		
		39	1.723		
		41	1.722		
		86	1.7225		
	F	83	1.718	1.7182 ± 0.0008	59.4 ± 0.8
		143	1.7185		
	G	33	1.720	1.7193 ± 0.0010	60.5 ± 1.0
		34	1.7195		
		267	1.7195		
		269	1.7185		
		271	1.719		
		272	1.719		

Table 1 (cont.)

Species	Biotype	Strain	Mean density (g./cm. ³)	Mean value and standard deviation calculated for each group	
				Density (g./cm. ³)	GC (moles %)
<i>P. putida</i>	A	5	1.7205	1.7213 ± 0.0009	62.5 ± 0.9
		6	1.722		
		7	1.721		
		26	1.7215		
		42	1.721		
		90	1.722		
	B	53	1.719	1.7195 ± 0.0011	60.7 ± 1.1
		96	1.7185		
		98	1.720		
		110	1.7195		
		153	1.7205		
		157	1.7195		
		158	1.719		
		167	1.719		
Unclassified		95	1.7185	1.7185	59.7

Table 2. Significant differences of mean GC content of DNAs of fluorescent pseudomonads

<i>P. fluorescens</i> biotypes	A	14.537 (28)*							
	B	14.406 (32)*	1.651 (18)‡						
	C	17.690 (32)*	0.460 (18)	1.672 (22)‡					
	D	11.593 (40)*	7.070 (26)*	5.568 (30)*	8.369 (30)*				
	E	9.916 (32)*	7.200 (18)*	5.432 (22)*	8.700 (22)*	0.306 (30)			
	F	15.824 (26)*	2.095 (12)‡	3.719 (16)*	3.162 (16)†	9.375 (24)*	10.330 (16)*		
	G	16.912 (32)*	0.000 (18)	1.878 (22)†	0.439 (22)	8.078 (30)*	7.907 (22)*	2.238 (16)†	
<i>P. putida</i> biotypes	A	12.247 (32)*	4.841 (18)*	3.062 (22)*	5.686 (22)*	2.450 (30)*	2.719 (22)†	7.638 (16)*	5.352 (22)*
	B	17.519 (36)*	0.531 (22)	1.377 (26)‡	0.223 (26)*	7.787 (34)*	7.344 (26)*	2.718 (20)†	0.609 (26)
			A	B	C	D	E	F	G
<i>P. aeruginosa</i>		<i>P. fluorescens</i> biotypes							<i>P. putida</i> biotype

Number of degrees of freedom are given in parentheses for values of *t* calculated by the method of Fisher (1925). Pairs significantly different at the 99 % confidence level are marked *, at the 95 % level †, at the 80 % confidence level ‡. Pairs not marked are not significantly different.

have pointed out that biotype B is more *fluorescens*-like than is biotype A. Indeed this resemblance extends to the GC contents and biotype B of *P. putida* is indistinguishable from *P. fluorescens* biotypes A, B and G on this basis. Biotype A of *P. putida*, on the other hand, has DNA more nearly like *P. fluorescens* biotypes E and

D, but significantly different from both at the 95% confidence level. *P. putida* biotype B thus probably warrants designation as a species. In concert with my Californian colleagues, I also would refrain from such designation until comparisons can be made with the various named plant pathogens or authentic surviving holo-types of the named species which have been reduced to synonymy with *P. putida*.

Table 2 is a display of the statistical tests for significant differences in the mean GC content representative of each set of fluorescent pseudomonads. The data concerning the GC content of the representatives of *Pseudomonas fluorescens* biotypes A, B, C and G clearly demonstrate no highly significant differences in the overall composition of the genetic material. While biotype F appears to be the lowest in GC content of all the fluorescent pseudomonads, too few determinations have been made to separate this biotype from biotype A with any high degree of confidence. Biotypes D and E, on the other hand, are distinctly different in GC content from the remainder of the *P. fluorescens* group. On the basis of this difference and the phenotypic differences set forth by Stanier *et al.* (1966) a specific epithet for *P. chlororaphis* and *P. aureofaciens* appears to be justifiable and one must reject the proposal that these species be reduced to synonymy with *P. fluorescens*. The difference in GC content of as much as 3% between *P. fluorescens* and these phenazine-

Fig. 1. The GC content of DNAs of the aerobic pseudomonads. Values plotted are means $\pm$ one standard deviation. Species and biotypes represent those studied by Stanier *et al.* (1966), the pseudomallei group of Redfearn *et al.* (1966) and the monotypic *Pseudomonas lemoignei* of Delafield *et al.* (1965).

producing organisms sets them apart, but does not preclude the existence of substantial genetic information shared in common. Again, the high degree of phenotypic similarity and of GC content of *P. chlororaphis* and *P. aureofaciens* indicates the advisability of reducing the latter to synonymy with the former. Quite striking is the difference observed when all these fluorescent pseudomonads are contrasted with *P. aeruginosa*. The DNA of the latter species has GC 67.2 %, distinctly separate from the GC content of all other fluorescent pseudomonads. Obviously, the conclusion of Lysenko (1961) that *P. chlororaphis* and *P. aureofaciens* are closely allied to *P. aeruginosa* is not substantiated. This solitary position of *P. aeruginosa* is indeed unfortunate, for this is the sole pseudomonad in which a system for genetic recombination is known (Holloway, 1955; Holloway & Monk, 1959), and if experience with the Enterobacteriaceae, Bacillaceae and *Neisseria* are any guide, interspecific hybridizations of *P. aeruginosa* with other fluorescent pseudomonads are of slight likelihood (see Marmur *et al.* 1963*a*).

Fig. 1 provides a summary of the mean GC contents of the representatives of the fluorescent pseudomonads in comparison with the aerobic pseudomonads described below.

The acidovorans group

The 26 strains placed in the acidovorans group by Stanier *et al.* (1966) have all been examined for GC content of their DNA with results as listed in Table 3. Fifteen of the strains are readily separable into a set possessing 66.8 moles % GC and another 9 strains can be assigned to a set possessing 61.8 % GC. This division of the cultures into the two species *Pseudomonas acidovorans* and *P. testosteroni* is further correlated with the nutritional differences established by Stanier *et al.* (1966). Two strains (60, 298) which bear phenotypic resemblances to the acidovorans group differ sufficiently from each other and from each of the named species in GC content and in nutritional properties to preclude their identification with either of the two species of the acidovorans group. It is possible that these represent additional species which have not otherwise been encountered in the study of Stanier *et al.* (1966).

Stanier *et al.* (1966) point out the discrepancy between the growth of strains 61 and 62, which they received as *Comamonas terrigena* but have identified as typical representatives of *Pseudomonas acidovorans*, and of ATCC 8461, the type culture of *C. terrigena*. The DNA of the latter culture and of its peritrichate mutant have been examined by Leifson & Mandel (1966). By using identical techniques and reference standards, they find the type culture to contain DNA of 64 % GC, or 2–3 % poorer in GC than the DNA of strains 61 and 62. Regardless of what taxonomic lodging *Comamonas terrigena* may eventually find, the epithet *terrigena* cannot be used as an earlier synonym for *P. acidovorans*.

The alcaligenes group

The DNAs of all 7 strains included in this group by Stanier *et al.* (1966) were analysed; Table 4 gives these data. There was no significant difference between the density determinations of the DNAs of 6 strains assigned to *Pseudomonas pseudoalcaligenes*, but the representative of *P. alcaligenes* was significantly richer in GC content by 3.5 %; this difference is significant at the 99.9 % probability level.

When the GC contents of the representatives of the alcaligenes group are contrasted with those of the acidovorans group, *Pseudomonas alcaligenes* DNA does not differ significantly from that of *P. acidovorans*. The 1% difference in GC content

Table 3. *Buoyant density and GC content of DNAs of representatives of the acidovorans group*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Species	Strain	Mean density (g./cm. ³)	Mean value and standard deviation calculated for each group	
			Density (g./cm. ³)	GC (moles %)
<i>P. acidovorans</i>	14	1.7255	1.7255 ± 0.0010	66.8 ± 1.0
	24	1.7255		
	29	1.7255		
	61	1.726		
	62	1.725		
	80	1.725		
	102	1.726		
	103	1.726		
	105	1.725		
	106	1.726		
	114	1.727		
	125	1.725		
	129	1.7255		
	146	1.7245		
	148	1.7255		
<i>P. testosteroni</i>	15	1.7205	1.7206 ± 0.0011	61.8 ± 1.1
	16	1.721		
	25	1.720		
	27	1.7205		
	28	1.721		
	78	1.720		
	79	1.720		
	138	1.721		
	139	1.7215		
Unclassified	60	1.7175	1.7175	58.7
	298	1.723	1.723	64.3

Table 4. *Buoyant density and GC content of DNAs of representatives of the alcaligenes group*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Species	Strain	Mean density (g./cm. ³)	Mean value and standard deviation calculated for each group	
			Density (g./cm. ³)	GC (moles %)
<i>P. alcaligenes</i>	142	1.725	1.7250 ± 0.0007	66.3 ± 0.7
<i>P. pseudoalcaligenes</i>	63	1.721	1.7216 ± 0.0009	62.8 ± 0.9
	65	1.721		
	66	1.722		
	297	1.7225		
	299	1.721		
	417	1.722		

between *P. pseudoalcaligenes* and *P. testosteroni* is significant at the 95 % confidence level. Hence the GC content can aid in distinguishing these species, even when used only in conjunction with conventional bacteriological diagnostic tests. Neither unclassified member of the acidovorans group has GC contents which match either species of the alcaligenes group.

Pseudomonas multivorans

Of the 19 strains studied by Stanier *et al.* (1966), 12 were analysed for GC content. The data in Table 5 show that the groups of strains examined are quite alike in GC content and the defective strains (385, 396, 397, 399) did not display any difference in this regard.

Table 5. *Buoyant density and GC content of DNAs of representative strains of Pseudomonas multivorans*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Strain	Mean density (g./cm. ³)	Mean value and standard deviation calculated for each group	
		Density (g./cm. ³)	GC (moles %)
85	1.7265	1.7263 ± 0.0008	67.6 ± 0.8
104	1.727		
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		
399	1.7265		

There seems little chance that *Pseudomonas multivorans* could be mistaken for *P. aeruginosa*, *P. maltophilia*, *P. acidovorans*, *P. mallei* or *P. pseudomallei* on cultural and physiological comparison. These high GC representatives all differ in base composition from *P. multivorans* by relatively small amounts, the smallest difference being found with *P. aeruginosa*. The *t*-test indicates a significant difference at 85 % probability; the other species cited above have differences calculated to be of significance at the 95 % probability level or better.

Pseudomonas stutzeri

The 17 strains studied by Stanier *et al.* (1966) were analysed for GC content, with the results shown in Table 6. Two clearly differentiated groups are revealed by this analysis. Group B contains the neotypes proposed by van Niel & Allen (1952) and by Stanier *et al.* (1966); hence, *P. stutzeri* is characterized by DNA of 65 % GC content. Group A differs in having DNA of 62.1 % GC and the disparity is significant at the 99.9 % confidence level. If these bacteria have genomes containing as few as 10⁷ nucleotide pairs in their DNA, then this difference represents approxi-

mately 2.9×10^4 GC substitutions for adenine + thymine (AT) pairs in the DNA of organisms of group B as contrasted with group A organisms. If these base-pair substitutions are spread at random, we should predict little or no genetic material in common. Stanier *et al.* (1966) were reluctant to propose the designation of a new

Table 6. *Buoyant density and GC content of DNAs of Pseudomonas stutzeri and P. stanieri sp.nov.*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Group	Strain	Mean density (g./cm. ³)	Mean value and standard deviation calculated for each group	
			Density (g./cm. ³)	GC (moles %)
A (<i>P. stanieri</i> sp.nov.)	220	1.721	1.7209 $\pm$ 0.0010	62.1 $\pm$ 1.0
	223	1.7195		
	224	1.721		
	225	1.7215		
	228	1.7215		
	320	1.721		
B (<i>P. stutzeri</i>)	221	1.724	1.7237 $\pm$ 0.0010	65.0 $\pm$ 1.0
	222	1.7235		
	226	1.7235		
	227	1.7243		
	229	1.724		
	275	1.723		
	316	1.7245		
	318	1.724		
	319	1.724		
	321	1.724		
	419	1.724		

species for organisms of group A. Such a separate designation is felt to be necessary by the present author, despite the lack of as clear a set of phenotypic traits correlated with this different base composition of the DNA as was observed in the acidovorans group. The variety of phenotypic traits displayed in the group of cultures studied as *P. stutzeri* would seem to make it quite difficult to demonstrate such a correlation using the data at hand. Sebald & Véron (1963) have already established a precedent for altering nomenclatural designations based upon determinations of DNA base composition, and Campbell & Postgate (1965) have provided descriptions of the species of *Desulfotomaculum* in which the GC content of the described species are definitive, along with one or two other distinguishing phenotypic traits. For these reasons I propose that the organisms of group A be separated from the species *P. stutzeri* and given a specific designation.

A review of the nomenclatural status of *Bacillus denitrificans* II Burri and Stutzer by van Niel & Allan (1952) elucidated the unwarranted confusion attending the classification of this organism and the proper recognition of *Pseudomonas stutzeri* (Lehmann and Newmann) Kluyver as the combined epithets. Further confusion would result if one of the specific synonyms for *P. stutzeri* were now used to describe the species of low GC content, particularly when neither holotypes nor neotypes of the cultures which have borne the synonyms appear to exist for which the base composition can be determined. It is therefore proposed that the new species be

named after Professor R. Y. Stanier in recognition of his contributions to the knowledge of the physiology and taxonomy of the genus *Pseudomonas*. A description follows, based in the main on the data of Stanier *et al.* (1966).

Pseudomonas stanieri sp.nov., description as for *P. stutzeri* conforming in all respects to the ideal phenotype of Stanier *et al.* (1966). DNA contains 62.1 moles % GC. Differentiated from most strains of *P. stutzeri* by the ability to utilize ethanolamine and mannitol and the inability to utilize propionate. No growth at 43°. Strains 224 and 225 conform strictly to this description and strain 224 (strain AB 212 of Dr H. Lautrop, Statens Seruminstitut, Copenhagen; ATCC 17591) is proposed as the holotype.

Strains 223, 320 and 228 are atypical in many nutritional characters. It is possible that these strains may not be *P. stanieri*, but it would be premature to consider the creation of still another species.

Two strains (226, 227) of *Pseudomonas stutzeri* have yielded DNA specimens which display a departure from the Gaussian distribution of DNA molecules in the CsCl gradient found with all other specimens reported in this study (as well as for over 1200 other samples of DNAs of bacterial origin examined in the author's laboratory in the past years). Figure 2 shows the appearance of a microdensitometer tracing of the DNA of strain 227. A DNA satellite is evident on the less dense side of the main DNA band. There was no difference in the position and amount of the satellite band in DNA extracted from 226. To ensure that the satellite was indeed composed of DNA, samples were diluted with water to 1% the salt concentration, boiled for 10 min. and chilled in ice. This denaturation was accomplished at low concentrations of DNA (5–10 µg./ml.) to guard against aggregation phenomena (Marmur, Rownd & Schildkraut, 1963). Upon examination of the CsCl gradient, with DNA of *Escherichia coli* as a reference standard, both satellite and main DNA bands were found to be increased in density by 0.014–0.015 g./cm.³ and the bands were increased in distribution width as would be expected if both represented double-stranded DNA. Subcultures of both strains were re-examined by Dr N. J. Palleroni and not found to be contaminated nor to be different from the previous description. The cultures were streaked on nutrient agar and single colonies again streaked. Six clones of each strain were then cultivated in nutrient broth, and the DNAs isolated and purified by the method of Marmur (1961). Again each specimen contained the same satellite and in the same proportion as in the original observations, irrespective of the fact that one sample of each strain was cultivated at 37° rather than 30° as had been the remainder. The satellite DNA represents 10–15% of the total DNA, as estimated from the area under the curve of the ultraviolet-absorbing material. If the satellite DNA corresponded to the presence of a bacterial contaminant in each culture, the contaminant would have to make up approximately that proportion of the population. Since microscopic examination of the suspended cultures did not reveal any tendency for the organisms to aggregate in groups, it is safe to dismiss contamination as the source of the satellite DNA. The satellite DNA is remarkably like that reported to be present in species of *Halobacterium* (Joshi, Guild & Handler, 1963). The latter authors concluded that an episomal element was probably present. Preliminary experiments by Mandel & Goldschmidt (unpublished) would indicate that it is likely that some form of cytoplasmic DNA (plasmid or episome) is present. Growth of strains 226 and 227 in nutrient broth containing neutral acriflavine 25 µg./ml.

Fig. 2. Microdensitometer tracing of the ultraviolet absorbing bands of purified DNA of *Pseudomonas stutzeri* strain 227. Film exposed after 22 hr at 44,770 rev./min. in 5.7 M-CsCl at 25.0°. The band at the left is that of the reference DNA (0.5 μ g.) of SP 8 bacteriophage.

(Hirota & Iijima, 1957) has yielded populations where the satellite DNA is either absent or so decreased in amount as not to be discernible on the ultraviolet photographs of the extracted DNA at equilibrium in the CsCl gradient. Single colonies of strain 227 lacking the satellite are now being studied in the laboratory of Professor Stanier for alteration of phenotypic traits and in this laboratory for the ability to re-acquire the trait by transmission from the wild-type strains. It is of interest that in the data of Stanier *et al.* (1966) strains 226 and 227 deviate from the remainder of their *P. stutzeri* strains in eight common features, five of which are positive characters.

Pseudomonas maltophilia

This species is represented by 4 strains analysed of the 23 studied by Stanier *et al.* (1966). The indicated GC content was 66.9 moles % and all 4 strains were quite uniform in this respect (Table 7). The sample is too small to guarantee that there is no other GC type present, but the uniformity of phenotypic characters present in the 23 strains does not raise any such suspicion.

Table 7. *Buoyant density and GC content of DNA of representative strains of Pseudomonas maltophilia*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Strain	Mean density (g./cm. ³)	Mean value and standard deviation calculated for the group	
		Density (g./cm. ³)	GC (moles %)
67	1.726	1.7256 ± 0.0008	66.9 ± 0.8
72	1.7255		
301	1.7255		
303	1.7255		

The pseudomallei-mallei group

Table 8 lists the buoyant densities of the DNA of 6 of the 26 strains of *Pseudomonas pseudomallei* and 7 of the 15 strains of *P. mallei* studied by Redfearn *et al.* (1966). These bacteria had DNA of the highest GC content found in the pseudomonads. The difference in GC content for the two groups is not of high significance (80 % probability).

Table 8. *Buoyant density and GC content of DNAs of representatives of pseudomallei group*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Redfearn *et al.* (1966).

Species	Strain	Mean density (g./cm. ³)	Mean value and standard deviation calculated for each group	
			Density (g./cm. ³)	GC (moles %)
<i>P. pseudomallei</i>	NBL 111	1.728	1.7281 ± 0.0007	69.5 ± 0.7
	NBL 113	1.728		
	NBL 114	1.728		
	NBL 117	1.728		
	NBL 121	1.7285		
	NBL 123	1.728		
<i>P. mallei</i>	NBL 1	1.728	1.7276 ± 0.0010	69.0 ± 1.0
	NBL 2	1.728		
	NBL 4	1.727		
	NBL 7	1.7265		
	NBL 16	1.7275		
	NBL 17	1.7278		
	NBL 19	1.7275		

Pseudomonas lemoignei sp. nov.

Delafield *et al.* (1965) have described a new species of aerobic pseudomonad which has been distinguished from the bulk of the pseudomonads by the criteria used by R. Y. Stanier and his collaborators. The holotype and sole strain of this poly-β-hydroxybutrate-digesting strain has DNA of 58.2 % GC (Table 9). It therefore represents the lowest GC content found among the aerobic pseudomonads reported above.

Table 9. *Buoyant density and mean GC content of the DNA of additional cultures received as species of Pseudomonas*

Organism	Strain designation and source	Density (g./cm. ³)	GC (moles %)
<i>P. lemoignei</i> sp.nov.	Strain 443 of Delafield <i>et al.</i> (1965); received as harvest of organisms from Dr N. J. Palleroni, University of California, Berkeley	1·717	58·2
<i>P. pseudoalcaligenes</i>	Strain 63AD of Dr M. Véron, Institut Pasteur; received from Prof. R. Y. Stanier as 440	1·721	62·2
	Strain 63AS of Dr M. Véron; received from Prof. R. Y. Stanier as 441	1·722	63·2
<i>P. aeruginosa</i>	ATCC 8707	1·726	67·3
	ATCC 8689	1·726	67·3
	ICPPB 2020; received from Dr M. P. Starr, University of California, Davis	1·7257	67·0
	NRRL B 23; studied as strain 54 by Stanier <i>et al.</i> (1966); received from Dr J. Marmur, Albert Einstein Medical College, New York	1·727	68·4
<i>P. polycolor</i>	PP2; received from Dr M. P. Starr, University of California, Davis	1·726	67·3
<i>P. aminovorans</i>	Strain 26 of den Dooren de Jong (1926); received from Dr E. R. Leadbetter, Amherst College, Amherst	1·722	63·2
<i>P. schuylkilliensis</i>	NRRL B 1104; from Dr W. C. Haynes, U.S. Department of Agricul- ture, Peoria, Ill.	1·7195	60·7
	NRRL B 1105; from Dr W. C. Haynes	1·7197	60·9
<i>P. geniculata</i>	NRRL B 1603; from Dr W. C. Haynes	1·719	60·2
	NRRL B 2080; from Dr W. C. Haynes	1·725	66·3
	NRRL B 2337; from Dr W. C. Haynes	1·726	67·3
<i>P. atlantica</i>	NCMB 301 Received from Dr J. M. Shewan, Torry Research Station, Aberdeen	1·7025	43·5
<i>P. mallei</i>	NCTC 3709; received from Mr Paul Baumann, University of California, Berkeley	1·728	69·4
<i>P. tabaci</i>	PT 1; received from Dr M. P. Starr, pathogenic for tobacco	1·717	58·2
<i>P. phaseolicola</i>	PM 142; received from Dr M. P. Starr, pathogenic for beans	1·7177	58·9
<i>P. methanica</i>	Harvested organisms received from Dr J. W. Foster, The University of Texas, Austin	1·711	52·1
<i>P. xanthe</i>	F 3-0; received from Dr O. B. Weeks, New Mexico State University, University Park	1·7285	69·9

Other aerobic pseudomonads

Table 9 lists a number of additional cultures which were analysed for the GC content of their DNAs. Two other strains of *Pseudomonas pseudoalcaligenes* have been identified as such by Drs Stanier, Palleroni and Doudoroff; their GC contents fall within the range established for the cultures previously described.

Four additional cultures of *Pseudomonas aeruginosa* were analysed. The first two listed under this name in Table 9 had originally been erroneously described as *P. fluorescens*, as noted in the catalogue of the American Type Culture Collection (1964). All four strains have DNA of GC content characteristic for *P. aeruginosa*. The representative of *P. polycolor* had DNA of a GC content like that of *P. aeruginosa*, adding weight to the proposal of Elrod & Braun (1942) that this plant pathogen is identical with *P. aeruginosa*, despite some differences in cultural characteristics. Dr T. Feary (unpublished results) has examined the sensitivity of this culture to *aeruginosa* phages and found it to be attacked by more virulent and temperate phages than any other strain of *P. aeruginosa*.

The holotype of *Pseudomonas aminovorans* (den Dooren de Jong, 1926) has 63.2 moles % GC. It cannot be placed with any certainty into any of the species delineated by Stanier *et al.* (1966).

Two additional strains of *Pseudomonas schuylkilliensis* have GC contents not significantly different from the *P. fluorescens* biotype G, in which group Stanier *et al.* (1966) placed strain 267 (NRRL B 9). The latter culture was also identified as *P. fluorescens* by Dr W. C. Haynes, who provided these cultures. The epithet should be reduced to synonymy with *P. fluorescens*.

Strains of *Pseudomonas geniculata* pose another problem. The description of this species is that of a fluorescent pseudomonad isolated from the Schuylkill River in Pennsylvania. Strains 269 (NRRL B 1606) and 271 (NRRL B 1612) were studied by Stanier *et al.* (1966) and placed in *P. fluorescens* biotype B; each has DNA of 60 % GC content. There are an additional 3 strains received as *P. geniculata* in Table 9. One of these (NRRL B 1608) has DNA of 60 % GC composition while the other two have 66 or 67 % GC. De Ley & Van Muylem (1963) reported the GC content of *P. geniculata* 338 to be 67.7 %. Their culture was obtained from Lysenko, who proposed it as the neotype for the species (Lysenko, 1961). His description of the organism is that of a non-fluorescent pseudomonad of little enzymic capacity.

Strains NRRL B 2080 and B 2337, with 66–68 % GC have recently been identified by Dr Palleroni as *P. multophila*. The same is probably true for the organisms studied by De Ley & van Muylem (1963).

The strain of *Pseudomonas atlantica* analysed is shown in Table 9 to have DNA of 43.5 moles % GC. The source of this culture is ostensibly the same as that organism analysed by De Ley & Friedman (1965) and found by them to have DNA of 66.4 % GC. The culture examined in this laboratory was obtained directly from Dr J. M. Shewan (National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland), while De Ley & Friedman received their specimen from Dr O. Lysenko, who reported having received it from Dr Shewan. The report of Lysenko (1961) noted that his culture differed in some characters from the original description. Another specimen of an agar-utilizing and alginate-utilizing Gram-negative rod labelled *P. atlantica* was found to have DNA of 55 % GC content

(Colwell & Mandel, 1964*b*). In view of this confusion it is impossible to state what the GC content of *P. atlantica* is and whether it shows any affinity with the genus *Pseudomonas* as defined by Stanier *et al.* (1966).

Table 9 also lists one further specimen of *Pseudomonas mallei*. The GC content of the DNA matches the composition found for the strains provided by Redfearn *et al.* (1966; Table 8).

Data for two phytopathogenic fluorescent pseudomonads, *Pseudomonas tabaci* and *P. phaseolicola*, are given in Table 9. Both have DNA of 58 to 59 moles % GC content, at the low GC extreme of the fluorescent group. Whether these, and other similar fluorescent phytopathogenic pseudomonads, can be accommodated within one of the established biotypes of *P. fluorescens* must await full examination of the nutritional and enzymic properties of this economically important group of bacteria.

Dworkin & Foster (1956) recommended that *Methanomonas methanica* be transferred to the genus *Pseudomonas*. The GC content of the authentic culture provided by Dr J. W. Foster is 52 moles % (Table 9) and would thereby be remote in base composition from those organisms which Stanier *et al.* (1966) and the present author would include in the genus *Pseudomonas*. The representatives of *Hydrogenomonas* (which are to be reported on elsewhere by Doudoroff and collaborators and by myself) have DNA base compositions in the range reported for *Pseudomonas*. The proper generic classification of the methane-oxidizing pseudomonad is obscure.

The last culture listed in Table 9, *Pseudomonas xanthe*, exemplifies another problem in the classification of bacteria. The culture is apparently Gram-negative on routine examination but does not lyse when treated with high concentrations of sodium lauryl sulphate (up to 10 %, w/v). In the author's experience, all Gram-negative bacteria (and a few Gram-positive organisms) lyse promptly upon addition of this detergent. Dr O. B. Weeks has advised me that this strain, as well as others bearing the same name, gives an equivocal Gram reaction by the Hucker method but is definitely Gram positive when stained by Kopeloff's procedure. Zettnow's original culture apparently is not available for comparison.

DISCUSSION

Stanier *et al.* (1966) have proposed a rigorous definition for the genus *Pseudomonas* based upon morphological and physiological properties. The inclusion of a range of DNA base compositions was deferred. The arguments for setting such a range as part of the definition of *Pseudomonas* follow. If *Pseudomonas* was described as containing organisms having DNA with not less than 57 nor more than 70 moles % guanine + cytosine (GC), all the aerobic pseudomonads encountered within this study would be included. Aerobic pseudomonads which are at present classified in other genera, but which might be assigned to the genus *Pseudomonas*, have DNA base compositions which fall within these suggested limits. Analyses have been published for representatives of *Xanthomonas* (Colwell & Mandel, 1964*a*; De Ley & Van Muylem, 1963), for the polarly flagellate *Acetomonas* (*Gluconobacter*), by De Ley & Schell (1963), for *Comamonas* (Leifson & Mandel, 1966; Sebald & Véron, 1963), for *Vibrio sensu lato* (Sebald & Véron, 1963; Colwell & Mandel, 1964*a*), and all have GC contents well within the suggested range. The hydrogenomonads likewise have DNA base compositions within this range (data to be published). No information

is available for representatives of *Alginomonas*, *Cellulomonas* and *Cellvibrio*. The use of the above suggested range of GC contents as diagnostic for *Pseudomonas* would exclude *Methanomonas* if the value for the single culture examined in this study should prove to be representative of the genus.

The morphological and physiological definition does not exclude the newly proposed genera *Campylobacter* (Sebald & Véron, 1963) and *Bdellovibrio* (Stolp & Starr, 1963). The 30–34 moles % GC content of the DNA of *Campylobacter* was one of the chief criteria used by Sebald & Véron (1963) in assigning *Vibrio fetus* and *V. bubulus* to the new genus. Drs Mandel, Starr & Baigent will report elsewhere that representatives of *Bdellovibrio* have DNAs of low GC content and thus may be excluded from *Pseudomonas*.

The marine bacterium *Flavobacterium piscicida* Bein has been found to be polarly flagellate (Hansen, Ingebritsen & Weeks, 1963) and hence reassigned to the genus *Pseudomonas* by Buck, Meyers & Leifson (1963). Mandel, Weeks & Colwell (1965) have shown that 20 strains of this species have DNA of 44.5 moles % GC. The above proposed limit of GC contents would exclude this species from the genus *Pseudomonas*. The computer taxonomic study of Hansen, Weeks & Colwell (1965) showed this phenon to bear a 67–70 % similarity to certain *Pseudomonas* species. The species of *Pseudomonas* bearing the greatest similarity to *P. piscicida* were the strains of Lysenko (1961) labelled *P. geniculata* and *P. atlantica*; my doubts about the identity of these cultures are expressed above. No disservice to the taxonomy of *Pseudomonas* will be done by further chequering the career of the chromogenic ichthyotoxigenic marine bacterium by its exclusion from the genus. This bacterium, as well as the low-GC organisms *P. cruciviae* and *P. atlantica*, should be compared with the marine vibrios of similar base compositions (Colwell & Mandel, 1964*b*).

If the genus *Pseudomonas* is then defined, in part, as having species whose GC contents range from 57 to 70 moles %, how does this compare with other taxonomic units? The compilation of DNA base compositions of Marmur *et al.* (1963*a*) lists members of the genus *Bacillus* as having a still broader range (35–51 % GC), and members of *Corynebacterium* range from 53 to 59 % GC. Mandel, Bergendahl & Pfennig (1965) established that the green sulphur bacteria of the genus *Chlorobium* have at least 51–58 % GC contents of their DNAs. *Caulobacter* species range from 62 to 67 % GC, and the related *Asticcacaulis* has DNA of 55 % GC (Poindexter, 1964). *Lactobacillus* species are reported as having DNAs of 35–53 % GC, by Suzuki & Kitahara (1964).

The family Enterobacteriaceae has representatives whose DNA GC contents are as low as 39 moles % (*Proteus*) and as high as 59 % (*Serratia*). The actinomycetes appear to be more uniform and, though usually accorded higher taxonomic rank, GC contents apparently range from 65 to 75 % GC. In summary, the suggested range of GC contents for definition of the genus *Pseudomonas* cannot *a priori* be accounted as either too narrow or too broad. The decision on the propriety of using DNA base composition as part of a generic definition is then based upon the uniformity provided to the assemblage contained within the taxon.

One major test of the genetic and perhaps phylogenetic relatedness of the aerobic pseudomonads has been initiated by De Ley & Friedman (1965). They found substantial hybrid formation of DNA molecules extracted from various species of

Xanthomonas, *Pseudomonas* and acetic acid bacteria, with the DNA of *X. pelargoni*. Indeed, the degree of relatedness expressed by these estimates of hybrid formation are surprisingly high.

Marmur *et al.* (1963*a*) suggested that the limits of episomal transfer, based upon experiences with the Enterobacteriaceae, could define a family. The apparent episome found in *Pseudomonas stutzeri* may provide a similar test for the pseudomonads. Mandel & Rownd (1964) showed that *Aeromonas liquefaciens* and *Serratia marcescens* contain satellite DNA matching *Escherichia coli* DNA in base composition but present in only one-tenth the proportion of satellite to main DNA found in *P. stutzeri*. The physiological definition excludes *Aeromonas* and *Serratia* from *Pseudomonas* but the notion persists that *Aeromonas* is related to *Pseudomonas* and may serve as an evolutionary link between the enteric bacteria and pseudomonads (De Ley, 1964*b*; Lysenko, 1961). Needless to say, neither molecular hybridizations nor genetic tests have been performed which would lend support to this view; the task is a formidable one.

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