Base Composition, Size and Sequence Similarities of Genome Deoxyribonucleic Acids from Clinical Isolates of *Pseudomonas putrefaciens*

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The mean base compositions of DNA from 27 strains of *Pseudomonas putrefaciens*, *P. rubescens* and *P. piscicida* ranged from 43.4 to 53.2 mol % GC with genome sizes from 3.04×10^9 to 4.23×10^9 daltons. On the basis of *in vitro* DNA-DNA binding, estimated spectrophotometrically from initial renaturation rates, *P. putrefaciens* strains were heterogeneous in the extent to which they shared similar nucleotide sequences, and were divided into four DNA homology groups. The DNA characteristics of strains in these groups correlated with several biochemical characteristics that facilitated identification of clinical isolates of *P. putrefaciens*. The two species *P. putrefaciens* and *P. rubescens* appear to be synonymous and none of the four groups of *P. putrefaciens* was related in DNA sequences to *P. piscicida*. *Pseudomonas putrefaciens* should therefore be retained as a single species and characteristics for identifying the various groups within the species are listed.

INTRODUCTION

The Gram-negative, rod-shaped bacterium *Pseudomonas putrefaciens* (Derby & Hammer 1931) Long & Hammer 1941 was listed by Doudoroff & Palleroni (1974) as a *species incertae sedis* in the eighth edition of *Bergey's Manual of Determinative Bacteriology* because it did not conform to the generic description of *Pseudomonas*. This species was excluded from *Pseudomonas* mainly because strains had DNA base compositions in the range 43.5 to 59.0 mol % GC (Colwell, Citarella & Ryman, 1965; Herbert *et al.*, 1971; Levin, 1972; Lee, Gibson & Shewan, 1977), whereas the GC contents of typical *Pseudomonas* species range from 58 to 70% (Mandel, 1966).

Pseudomonas putrefaciens is widely distributed (Hugh, 1970); some strains have caused spoilage of fish and poultry (Shewan, 1971; Barnes & Melton, 1971), while others have been isolated from human clinical specimens (Minagawa, 1963; King, 1964; von Graevenitz & Simon, 1970; Gilardi, 1972; Riley, Tatum & Weaver, 1972; Fourquet *et al.*, 1975). Some clinically isolated strains were aetiologically linked to disease in man although the resulting infections were relatively benign (Debois *et al.*, 1975; Holmes, Lapage & Malnick, 1975; Rosenthal, Zuger & Apollo, 1975).

Two groups of strains within *P. putrefaciens* were reported (Riley *et al.*, 1972; Levin, 1972; Holmes *et al.*, 1975): one with GC contents ranging from 47.8 to 50.8 % and unable to grow in 6 % NaCl, the other with GC contents ranging from 55.9 to 59.0 % and salt tolerant. Bacteriocins (Williams & Levin, 1975) and autolysis (Levin & Van Sickle, 1976) were also used to separate these groups.

Pseudomonas rubescens (Pivnick, 1955) and *P. piscicida* (Hansen, Weeks & Colwell, 1965) resemble *P. putrefaciens* in having low GC contents of 44 to 48 % (Mandel, Weeks & Colwell, 1965; De Ley *et al.*, 1966; Levin, 1972) and for this reason were also listed by

Table 1. *Source of strains*

Species	Strain no.	Source
<i>Pseudomonas putrefaciens</i>	NCTC10735	Oil brine (Iizuka & Komagata, 1964)
	NCTC10736 (NCMB1733)	Haddock, strain P-19X of Levin (1968)
	NCTC10737	Cuttlefish
	NCTC10738 (NCIB11157)	Faeces, strain 136-2 of Minagawa (1963)
	NCTC10762	Pus, strain 734 of von Graevenitz & Simon (1970)
	NCTC10763	Bottled blood
	ATCC8071 (NCIB10471)*	Butter (Derby & Hammer, 1931)
	ATCC8072 (NCIB10472)	Butter (Derby & Hammer, 1931)
	ATCC8073 (NCIB10473)	Butter (Derby & Hammer, 1931)
	CL27/72	Ear swab, otitis media, strain 4 of Holmes <i>et al.</i> (1975)
	CL432/72	Pleural fluid, strain 5 of Holmes <i>et al.</i> (1975)
	CL256/73	Cerebrospinal fluid, strain 6 of Holmes <i>et al.</i> (1975)
	CL549/73	Tap swab, strain 7 of Holmes <i>et al.</i> (1975)
	CL595/73	Ear swab, otitis externa, strain 8 of Holmes <i>et al.</i> (1975)
	E631	Sputum, strain 1 of Holmes <i>et al.</i> (1975)
	E988	Nasal swab, strain 2 of Holmes <i>et al.</i> (1975)
	CL246/75	Oil, machine cooling system, from Dr W. Sheppard†
	CL389/75	Throat swab, from Drs P. Kohut & M. Rusinko‡
	CL104/76	Faeces
	CL105/76	Empyema
<i>Pseudomonas rubescens</i>	NCTC10695 (NCIB8768, ATCC12099)*	Urine, case 7 of Debois <i>et al.</i> (1975)
	ATCC19857	Sputum, case 10 of Debois <i>et al.</i> (1975)
<i>Pseudomonas rubescens</i>		Wound exudate, case 9 of Debois <i>et al.</i> (1975)
<i>Pseudomonas piscicida</i>	ATCC15251*	Urine, case 4 of Debois <i>et al.</i> (1975)
		Cutting oil (Pivnick, 1955)
		Not known
		Marine unspecified, strain 15 of Hansen <i>et al.</i> (1965)

* Type strain.

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Doudoroff & Palleroni (1974) as *species incertae sedis*. *Pseudomonas rubescens* and *P. putrefaciens* were reported to be similar to each other phenotypically (Hugh, 1970; Holmes *et al.*, 1975) and Lee *et al.* (1977), on the basis of a numerical taxonomic analysis, proposed that they be renamed *Aleromonas putrefaciens*. Members of the genus *Aleromonas* have base compositions of 43 to 48 % GC and are separated from species of *Pseudomonas* solely on this characteristic (Baumann *et al.*, 1972).

This study investigated the taxonomic relationships based on DNA characteristics of strains classified as *P. putrefaciens*. The base compositions, genome sizes and base sequence similarities of DNA from various clinical isolates of *P. putrefaciens* are described and compared with those of *P. putrefaciens* strains from other sources and of type strains of *P. rubescens* and *P. piscicida*. Correlations between phenotypic and DNA characteristics are examined for tests that might improve the identification of potentially pathogenic clinical isolates of *P. putrefaciens*.

METHODS

Strains used and conditions for growth. Twenty-seven strains of *Pseudomonas* were used (Table 1). *Escherichia coli* (strain B) NCTC10537 and *Serratia marcescens* NCTC1377 were used as DNA reference strains for the determination of base compositions and genome molecular weights. Bacteria were grown overnight at 30 °C in 200 ml Nutrient Broth no. 2 (Oxoid) with aeration in an orbital incubator (Gallenkamp), except for *P. piscicida* ATCC15251 which was grown at 30 °C for 48 h in the above medium supplemented with 2.5 % (w/v) NaCl.

DNA extraction and purification. Bacteria were harvested, washed and resuspended in 0.15 M-NaCl and 0.1 M-EDTA buffer pH 8.0 (5 g wet wt in 50 ml buffer) and lysed at 60 °C with sodium dodecyl sulphate at a final concentration of 2 % (w/v). DNA was extracted and purified by a combination of the methods of Marmur (1961) and Brenner *et al.* (1969), which included treatment twice with 50 µg ribonuclease ml⁻¹ (bovine pancreas; BDH), treatment with 50 µg pronase ml⁻¹ (Koch-Light) and deproteinization with aqueous phenol. The DNA samples were precipitated with 95 % (v/v) ethanol and finally with either propan-2-ol or 2-ethoxyethanol. Samples were redissolved in 0.1 × SSC buffer (0.15 M-NaCl buffered with 0.015 M-trisodium citrate at pH 7.0) and stored at 4 °C over chloroform. Samples stored for a month or more were reprecipitated before use. DNA purity was assessed from the 280:260:230 nm extinction ratios (Marmur, 1961).

Estimation of base composition. The mean base composition of the DNA was estimated from the thermal denaturation temperature (T_m) by the method of Marmur & Doty (1962). Thermal denaturation was carried out in a Pye Unicam SP1800 spectrophotometer equipped with an SP 876 series 2 temperature programme controller and heated cell block; ΔE_{260} was recorded automatically. The temperature of the DNA solution in the cuvette was measured with a thermistor thermometer as described by Hill (1968). The DNA mean base composition, expressed as percentage guanine and cytosine to total base content (mol % GC), was calculated from the T_m determined in $0.1 \times$ SSC buffer, and expressed relative to the chemically determined value of 50.9 % for *Escherichia coli* strain B (De Ley, 1970) by the equation:

$$\% \text{ GC} = 50.9 + 2.08 (T_{mx} - T_{mE})$$

where T_{mx} is the melting temperature of the unknown DNA in $0.1 \times$ SSC and T_{mE} is the melting temperature of DNA from *E. coli* strain B in $0.1 \times$ SSC buffer.

Fragmentation of double-stranded DNA. The DNA concentration was adjusted to approximately $70 \mu\text{g ml}^{-1}$ in $5 \text{ ml } 0.1 \times$ SSC buffer. Solutions were sonicated for three periods of 1 min at 4°C at a frequency of 20 to 25 kHz and an amplitude of 2 to $5 \mu\text{m}$ in a 100 W ultrasonic disintegrator (MSE). This yielded fragments of average molecular weight 2×10^5 to 3×10^5 (Owen & Snell, 1976).

Measurement of DNA reassociation. Reassociation was determined spectrophotometrically. Changes in E_{260} due to the reassociation of the denatured DNA fragments were measured on a recorder with a full scale deflection of 0.10 extinction units. The buffer solutions were filtered through a nitrocellulose membrane ($0.2 \mu\text{m}$ pore size; Sartorius Membranfilter GmbH, Göttingen, West Germany), and the final DNA concentration was adjusted to $60 \mu\text{g ml}^{-1}$. The sheared DNA in $1.8 \text{ ml } 0.1 \times$ SSC buffer was denatured at 95°C for 10 min, and the Na^+ concentration was adjusted to 0.51 M with 0.2 ml of 5 M-NaCl . To measure the binding between DNA from different strains, an equimolar mixture of the two DNAs in $0.1 \times$ SSC was also prepared. The DNA solutions were then quickly transferred to preheated cuvettes in the spectrophotometer cell compartment at the optimum temperature of renaturation (TOR). This temperature was approximately 25°C below the DNA melting temperature in 0.51 M -sodium buffer, and was calculated from the equation: $\text{TOR} = 51.9 + (0.47 \times \% \text{ GC})$.

The renaturation of the DNA fragments was monitored by the decrease in extinction during the initial 40 min of the reaction. The apparent renaturation rate constant (k') expressed in arbitrary units was calculated from the equation of Gillis, De Ley & De Cleene (1970): $k' = v/C^2$, where v is the initial renaturation rate expressed as the decrease in extinction at 260 nm min^{-1} , and C is the DNA concentration expressed in mm nucleotide pairs. The DNA concentrations were based on estimates determined chemically.

Estimation of genome size. The genome size (molecular weight) of a prokaryotic organism is the amount of double-stranded chromosomal DNA it contains expressed in numbers of base pairs or more commonly as its molecular weight. The renaturation rate constant of a DNA containing non-repetitive sequences was shown to be inversely proportional to its molecular complexity (Britten & Kohne, 1966). Genome sizes were therefore calculated relative to a reference DNA (*Escherichia coli* strain B) with a mean molecular weight of 2.71×10^9 (Gillis & De Ley, 1975) from the formula:

$$\text{Genome size} = \frac{k'(E. coli) \times 2.71 \times 10^9}{k'(\text{unknown})}$$

Estimation of DNA binding. The experiments to determine the degree of binding between DNA from different strains were done in 0.51 M -sodium buffer and at the TOR as described above. If the two DNAs in the mixture differed in base composition by 5 % GC or less, the mean of their respective TOR values was used. When GC differences exceeded 5 %, the TOR of the reference DNA was used. All determinations were repeated in duplicate or triplicate. The degree of binding in a mixture of two DNAs at approximately the same molar nucleotide pair concentration was calculated using the equation given by De Ley, Cattoir & Reynaerts (1970):

$$D = 100 [4V_M - (V_A + V_B)]/2V_A$$

where V_A , V_B and V_M are the renaturation rates (change in extinction at 260 nm min^{-1}) of DNA samples (A, B) and the mixture (M) respectively.

RESULTS

Mean DNA base compositions

The base compositions of the 27 strains examined (Table 2) were broadly distributed in the range 43.4 to 53.2 % GC within which were three narrower ranges. The 43.4 to 44.4 % GC range comprised six strains: *P. piscicida* ATCC15251, *P. rubescens* ATCC19857 and

Table 2. DNA base compositions of bacterial strains

DNA source	Strain	Mean $T_m \pm$ S.D. in 0.1 $\times$ SSC buffer*	Mean GC†	Published values (mol % GC)
<i>P. piscicida</i>	ATCC15251	71.7 $\pm$ 0.10	43.4	44.5 (Mandel <i>et al.</i> , 1965)
<i>P. rubescens</i>	NCTC10695	71.8 $\pm$ 0.05	43.6	46.9 (Lee <i>et al.</i> , 1977)
<i>P. putrefaciens</i>	ATCC8071	71.9 $\pm$ 0.00	43.8	43.4 and 45.4 (Colwell <i>et al.</i> , 1965), 48.4 (Levin, 1972), 44.7 (Lee <i>et al.</i> , 1977)
<i>P. putrefaciens</i>	CL246/75	72.0 $\pm$ 0.10	44.0	
<i>P. rubescens</i>	ATCC19857	72.1 $\pm$ 0.00	44.2	46.1 (De Ley <i>et al.</i> , 1966), 48.1 (Levin, 1972)
	NCTC10736	72.2 $\pm$ 0.15	44.4	45.9 (Lee <i>et al.</i> , 1977), 49.3 (Levin, 1972)
	ATCC8073	73.0 $\pm$ 0.00	46.1	50.1 (Levin, 1972)
	CL106/76	73.0 $\pm$ 0.00	46.1	
	NCTC10735	73.1 $\pm$ 0.05	46.3	
	E631	73.1 $\pm$ 0.05	46.3	
	CL549/73	73.1 $\pm$ 0.05	46.3	
	CL107/76	73.1 $\pm$ 0.00	46.3	
	NCTC10737	73.2 $\pm$ 0.05	46.5	
	CL389/75	73.2 $\pm$ 0.10	46.5	
	E988	73.2 $\pm$ 0.10	46.5	
<i>P. putrefaciens</i>	ATCC8072	73.5 $\pm$ 0.00	47.2	47.3 (Lee <i>et al.</i> , 1977), 50.1 (Levin, 1972)
	CL256/73	74.1 $\pm$ 0.10	48.4	
	CL432/72	74.2 $\pm$ 0.00	48.6	
	NCTC10738	75.8 $\pm$ 0.10	51.9	54.7 (Lee <i>et al.</i> , 1977)
	CL105/76	75.8 $\pm$ 0.00	51.9	
	NCTC10762	76.1 $\pm$ 0.05	52.6	
	CL27/72	76.1 $\pm$ 0.05	52.6	
	CL595/73	76.1 $\pm$ 0.30	52.6	
	CL108/76	76.2 $\pm$ 0.00	52.8	
	NCTC10763	76.2 $\pm$ 0.05	52.8	
	CL109/76	76.3 $\pm$ 0.10	53.0	
	CL104/76	76.4 $\pm$ 0.00	53.2	
<i>E. coli</i>	NCTC10537	75.3 $\pm$ 0.05	50.9	

* Values given for mean T_m are averages of two or three separate determinations and the standard deviation.

† Expressed relative to a value of 50.9 % GC (chemically determined; De Ley, 1970) for *E. coli* strain B.

NCTC10695, and three strains of *P. putrefaciens* including the type strain ATCC8071. The 46.1 to 48.6 % GC range comprised 12 *P. putrefaciens* strains of which 10 had base compositions of 46.1 to 47.2 % GC and two (CL256/73 and CL432/72) had slightly higher base compositions of 48.4 and 48.6 % GC. The 51.9 to 53.2 % GC range comprised the remaining nine strains of *P. putrefaciens*.

Our base composition results (Table 2) agreed with the previously published data on *P. putrefaciens* also given in Table 2 (Colwell *et al.*, 1965; Mandel *et al.*, 1965; De Ley *et al.*, 1966; Lee *et al.*, 1977) but showed a consistent discrepancy of about 4 % GC from the values of Levin (1972). This difference was attributed to our results being expressed relative to a value of 50.9 % GC (De Ley, 1970) for the DNA of *E. coli* strain B, whereas Levin (1972) reported a higher value of 54.0 % for DNA of this strain.

DNA renaturation rate constants

The apparent renaturation rate constant (k') values of DNA from the strains of *P. putrefaciens*, *P. rubescens* and *P. piscicida* were determined in 0.51 M-sodium buffer. Four selected DNAs (*E. coli* NCTC10537, *S. marcescens* NCTC1377, *P. putrefaciens* NCTC10735 and *P. rubescens* NCTC10695) were also tested in 0.39 M-sodium buffer ($2 \times$ SSC). This buffer was not used for the other DNAs as the renaturation rates were too slow for accurate measurement of gradients. The DNA of *E. coli* under our experimental conditions had an

Table 3. DNA renaturation rate constants and bacterial genome sizes

DNA source	Strain	Sodium molarity	Temp- erature of renatur- ation* (TOR)	No. of determin- ations	Apparent renaturation rate constant ($10^2 \times k' \pm \text{S.D.}$)	$10^{-9} \times$ genome size (daltons)	Size relative to <i>E. coli</i> B
<i>P. rubescens</i>	NCTC10695	0.51	72.4	22	19.72 ± 3.00	3.038	1.12
		0.39 (2 × SSC)	69.2	2	12.81 ± 0.19		
<i>P. putrefaciens</i>	NCTC10762	0.51	76.6	3	19.32 ± 0.44	3.101	1.14
	CL246/75	0.51	72.6	2	19.14 ± 0.63	3.131	1.16
	E631	0.51	73.7	5	18.92 ± 1.36	3.167	1.17
	CL27/72	0.51	76.7	4	18.01 ± 0.59	3.327	1.23
	CL256/73	0.51	74.6	30	17.88 ± 1.26	3.351	1.24
<i>P. rubescens</i>	ATCC19857	0.51	72.7	2	17.64 ± 0.51	3.397	1.25
	NCTC10738	0.51	76.3	14	17.47 ± 1.53	3.430	1.27
	ATCC8071	0.51	72.5	4	17.38 ± 1.18	3.448	1.27
	CL432/72	0.51	74.7	4	17.31 ± 1.20	3.462	1.28
	NCTC10763	0.51	76.7	4	17.14 ± 0.78	3.496	1.29
	CL389/75	0.51	73.8	4	17.07 ± 0.72	3.510	1.30
	CL595/73	0.51	76.6	4	17.04 ± 2.15	3.516	1.30
	NCTC10735	0.51	73.7	27	17.03 ± 1.38	3.523	1.31
	CL549/73	0.39 (2 × SSC)	70.6	2	9.85 ± 0.44		
		0.51	73.7	4	16.81 ± 0.89	3.564	1.32
<i>P. putrefaciens</i>	E988	0.51	73.8	5	16.59 ± 0.92	3.612	1.33
	NCTC10737	0.51	73.8	7	16.01 ± 1.84	3.743	1.38
	CL108/76	0.51	76.7	2	15.92 ± 1.22	3.764	1.39
	NCTC10736	0.51	72.8	5	15.86 ± 0.99	3.778	1.39
	CL106/76	0.51	73.6	4	14.42 ± 1.43	4.155	1.53
	ATCC8072	0.51	74.1	4	14.20 ± 1.19	4.220	1.56
	ATCC8073	0.51	73.6	5	14.19 ± 0.89	4.223	1.56
<i>P. piscicida</i>	ATCC15251	0.51	72.3	6	15.22 ± 1.80	3.937	1.45
<i>E. coli</i>	NCTC10537	0.51	75.8	4	22.11 ± 0.95	2.710†	1.00
		0.39 (2 × SSC)	73.0	2	14.71 ± 0.97		
<i>S. marcescens</i>	NCTC1377	0.51	80.6	4	15.10 ± 1.01	3.968	1.46
		0.39 (2 × SSC)	78.2	2	10.42 ± 0.42		

* Calculated from TOR = $51.9 + 0.47\%$ GC for the 0.51 M-sodium buffer, and from TOR = $47.0 + 0.51\%$ GC for the 0.39 M-sodium buffer (Gillis *et al.*, 1970).

† From Gillis & De Ley (1975).

apparent rate constant of 22.11×10^{-2} , which was similar to the two previously determined values for this DNA of 19.1×10^{-2} (Gillis *et al.*, 1970) and 22.2×10^{-2} (Swings & De Ley, 1975). The latter rates were determined using slightly different experimental conditions (approximately $80 \mu\text{g}$ DNA ml^{-1} in $2 \times \text{SSC}$ buffer) from those used here.

Genome sizes

The molecular weights of the genome DNAs from strains of *P. putrefaciens* and *P. rubescens* ranged from 3.04×10^9 to 4.23×10^9 (Table 3) which represented an overall size difference of 36 %. Differences in size of 0.5×10^9 or less were within the limits of experimental reproducibility and were not considered to be taxonomically significant. The genome size of *P. piscicida* ATCC15251 (3.94×10^9 daltons) was also within the *P. putrefaciens* range. All strains had genome sizes larger than that of *E. coli* strain B, in some cases by as much as 56 %, but they were within the range of 1×10^9 to 7×10^9 reported for the molecular weights of various other bacterial species (Bak, Christiansen & Stenderup, 1970; Gillis & De Ley, 1975). The DNA of *Serratia marcescens*, a micro-organism taxonomically unrelated to *P. putrefaciens*, was included as a reference standard for the estimation of genome

Table 4. Reassociation of DNA samples from *P. putrefaciens*, *P. rubescens* and *P. piscicida*, with DNA from reference strains *P. rubescens* NCTC10695 and *P. putrefaciens* NCTC10735

The extent of binding between DNA from the test strains and DNA from the reference strains is expressed as a percentage relative to the reference DNA using the formula: $D = 100[4V_M - (V_A + V_B)]/2V_A$ where V_M is the renaturation rate of the mixture, V_A is the renaturation rate of the reference DNA, and V_B is the renaturation rate of the test strain. ΔGC is the difference in the mean % GC contents of the DNAs in the reassociation mixture. Genome sizes are expressed relative to that of the respective reference DNA.

DNA source		Reference DNA from strain:					
		NCTC10695			NCTC10735		
		Relative binding (%)	Δ GC	Relative genome size	Relative binding (%)	Δ GC	Relative genome size
DNA homology group I							
<i>P. rubescens</i> NCTC10695	100	0	1.00	47	2.7	0.86	
<i>P. putrefaciens</i> CL246/75	100	0.4	1.03				
<i>P. rubescens</i> ATCC19857	100	0.6	1.12	41	2.1	0.98	
<i>P. putrefaciens</i> ATCC8071	83	0.2	1.14	26	2.5	0.98	
DNA homology group II							
<i>P. putrefaciens</i>	NCTC10735	39	2.7	1.16	100	0	1.00
	NCTC10737	30	2.9	1.23	83	0.2	1.06
	ATCC8072				58	0.9	1.20
	NCTC10736	32	0.8	1.24	57	1.9	1.07
DNA homology group III							
<i>P. putrefaciens</i>	CL256/73	29	4.8	1.10	32	2.1	0.95
	CL432/72				31	2.3	0.98
	CL549/73				23	0	1.01
	E631				26	0	0.90
	E988	34	2.9	1.19	18	0.2	1.03
	ATCC8073				38	0.2	1.20
	CL389/75				14	0.2	1.00
	CL106/76				31	0.2	1.18
DNA homology group IV							
<i>P. putrefaciens</i>	NCTC10738	25	8.3	1.13	13	5.6	0.97
	NCTC10762	24	9.0	1.02	9	6.3	0.88
	CL595/73	6	9.0	1.16	24	6.3	1.00
	CL27/72	25	9.0	1.10	18	6.3	0.94
	NCTC10763	11	9.2	1.15	27	6.5	0.99
<i>P. piscicida</i> ATCC15251	20	0.2	1.30	19	2.9	1.12	

size, and our molecular weight value of 3.97×10^9 agreed with the 3.99×10^9 value previously reported for this species by Gillis & De Ley (1975). However, both of these values for *S. marcescens* were less than the estimate of 5.56×10^9 reported by Bak *et al.* (1970).

Estimates of interstrain DNA-DNA binding

Accuracy of binding estimates. From a total of 100 interstrain reassociations (including repeat determinations), the overall reproducibility of the spectrophotometric method for the estimation of DNA binding with our experimental conditions was $\pm 6\%$ (pooled standard deviation). This compared well with the overall standard deviation for this method of $\pm 4.7\%$ reported by De Ley *et al.* (1970), and $\pm 4.1\%$ reported by Gibbins & Gregory (1972). Binding values of $< 20\%$ were considered approximate estimates because of possible errors in measurements involving slow rates of reassociation.

DNA homology group I. Two strains of *P. rubescens* (NCTC10695 and ATCC19857) and two strains of *P. putrefaciens* (CL246/75 and ATCC8071) represented a DNA homology group with high levels of binding ($> 83\%$) relative to *P. rubescens* NCTC10695, the selected

Table 5. Reassociation of DNA samples from *P. putrefaciens*, *P. rubescens* and *P. piscicida*, with DNA from reference strains *P. putrefaciens* CL256/73 and NCTC10738

See Table 4 legend for details.

Reference DNA from strain:						
DNA source	CL256/73			NCTC10738		
	Relative binding (%)	Δ GC	Relative genome size	Relative binding (%)	Δ GC	Relative genome size
DNA homology group I						
<i>P. rubescens</i> NCTC10695	34	4.8	0.91	27	8.3	0.89
<i>P. putrefaciens</i> ATCC8071	32	4.6	1.03			
DNA homology group II						
<i>P. putrefaciens</i>	NCTC10735	28	2.1	1.05	13	5.6
	NCTC10737	31	1.9	1.12		
	ATCC8072	21	1.2	1.26		
	NCTC10736	26	4.0	1.13		
DNA homology group III						
<i>P. putrefaciens</i>	CL256/73	100	0	1.00	40	3.5
	CL432/72	72	0.2	1.03		
	CL549/73	61	2.1	1.06		
	E631	60	2.1	0.95		
	E988	48	1.9	1.08		
	ATCC8073	42	2.3	1.26		
	CL389/75	45	1.9	1.05		
	CL106/76	41	2.3	1.24		
DNA homology group IV						
<i>P. putrefaciens</i>	NCTC10738	44	3.5	1.02	100	0
	NCTC10762			96	0.7	0.90
	CL595/73			97	0.7	1.03
	CL27/72			87	0.7	0.97
	NCTC10763			87	0.9	1.02
	CL108/76			82	0.9	1.10
	CL104/76			90	1.3	0.88
	CL109/76			100	1.1	0.88
<i>P. piscicida</i> ATCC15251	15	5.0	1.18			

DNA reference strain (Table 4). This DNA had lower levels of binding, ranging from 6 to 39 %, with DNA from 10 other strains of *P. putrefaciens*. The four strains in this DNA homology group also had similar base compositions (mean 43.9 ± 0.3 % GC) and genome sizes (mean $3.25 \pm 0.20 \times 10^9$ daltons).

DNA homology group II. Table 4 also lists the degrees of binding between reference DNA from *P. putrefaciens* NCTC10735 and DNA from 20 other strains. Three *P. putrefaciens* strains (NCTC10735, NCTC10737 and ATCC8072) formed a DNA homology group with binding values of 57 to 83 % relative to NCTC10735. The strains had similar base compositions (mean 46.7 ± 0.5 % GC) and genome sizes except for the DNA of ATCC8072 which was approximately 20 % larger than the reference DNA. *Pseudomonas putrefaciens* NCTC10736 was 57 % related to NCTC10735 and was included in this homology group, although its GC composition of 44.4 % was lower than that of the other strains. There was 41 to 47 % binding between NCTC10735 and the two *P. rubescens* strains (NCTC10695 and ATCC19857) of group I, but the degrees of binding between NCTC10735 and the remaining 14 *P. putrefaciens* strains were relatively low (< 38 %).

DNA homology group III. Reference DNA from *P. putrefaciens* CL256/73 had degrees of binding ranging from 41 to 72 % with eight *P. putrefaciens* strains (Table 5). Seven of these strains had GC compositions in the range 46.1 to 48.6 % (mean 46.9 ± 1.0 %) and

Table 6. Summary of DNA binding within and between the four *P. putrefaciens* DNA homology groups, and *P. piscicida*

DNA source	Relative binding (%) with DNA from:				<i>P. piscicida</i> ATCC15251
	<i>P. putrefaciens</i> DNA group				
	I	II	III	IV	
<i>P. putrefaciens</i> DNA group:					
I	83-100 (4)*				
II	26-47 (6)	57-83 (4)			
III	29-34 (4)	14-38 (11)	41-72 (8)		
IV	6-25 (6)	9-27 (5)	40-44	82-100 (8)	
<i>P. piscicida</i> ATCC15251	20	19	15	NT	100

NT, Not tested (see text).

* Numbers in parentheses show the numbers of strains tested (if more than 2).

were included in the same homology group. They were more heterogeneous in degrees of DNA binding than members of the other homology groups. *Pseudomonas putrefaciens* CL256/73 and NCTC10738 (homology group IV, see below) were 44 % related but binding with DNA from seven strains belonging to other homology groups was low (< 34 %).

DNA homology group IV. This group comprised eight strains (Table 5) with high levels of binding (> 82 %) relative to reference DNA from *P. putrefaciens* NCTC10738. These strains and an additional strain (CL105/76) had similar GC compositions (mean 52.6 ± 0.4 %) and genome sizes (mean $3.38 \pm 0.24 \times 10^9$ daltons).

Estimates of binding between *P. putrefaciens* homology groups and *P. piscicida*

Table 6 summarizes the degrees of binding within each of the *P. putrefaciens* DNA homology groups, as well as the ranges of binding observed between DNA from strains in the different homology groups, and from *P. piscicida* ATCC15251. The highest degrees of intergroup binding were between strains of groups I and II (47 %), groups III and IV (44 %), and groups II and III (38 %). *Pseudomonas piscicida* ATCC15251 was not similar in nucleotide sequences to any of the *P. putrefaciens* reference strains, although it was similar in base composition to strains of DNA homology group I. Reassociations between NCTC10738 (*P. putrefaciens* DNA homology group IV) and *P. piscicida* were not carried out. The two strains had a difference of 8.5 % GC in their mean DNA base compositions and the maximum fraction of common GC regions shared by these two DNAs was estimated as only 26 % (De Ley, 1969). The taxonomic relationships of *P. piscicida* will not be discussed further here. For each DNA reassociation reaction the difference between the mean base compositions of the reassociated DNAs was calculated (see Tables 4 and 5). High levels of binding (72 % and above) occurred only when the mean base compositions of the reassociated DNAs were similar ($\Delta GC < 1.3$ %). The genome sizes of several of the reassociated DNAs differed and this was taken into account in the calculation of degrees of binding.

DISCUSSION

Strains of *P. putrefaciens* (Derby & Hammer) Long & Hammer 1941 occur in a variety of clinical specimens and recently were associated with infections in man (Debois *et al.*, 1975; Holmes *et al.*, 1975; Rosenthal *et al.*, 1975). The identification of this species by conventional bacteriological tests is not difficult, but strain variation in a number of biochemical characteristics has been recorded and it was suggested that the species might comprise several groups or possibly species (Levin, 1972; Riley *et al.*, 1972; Holmes *et al.*, 1975). Most of the strains studied here, which were isolated from a variety of

Table 7. Characteristics for the practical differentiation of *P. putrefaciens* strains

Characteristics	Group I	Group II	Group III	Group IV
Total no. strains examined*	4	4	9	9
No. strains of clinical origin	0	0	7	9
Growth, 6% NaCl†	—	—	—	+ (1)
Growth, 42 °C	—	—	— (3)	+ (1)
Growth, 37 °C	+	—	+	+
Growth, 5 °C	+ (1)	+ (1)§	— (3)	—
Acid from: Arabinose‡	+ (1)	+ (1)	+	—
Glucose	—	+ (1)	+	—
Maltose	—	+ (1)	+	—
Sucrose	—	+ (1)	+ (1)	—
Growth, <i>Salmonella</i> / <i>Shigella</i> agar	+ (1)	—	— (1)	+
Alkali production, Christensen's citrate	—	+ (1)	— (1)	—
Mean GC±s.d. (mol %)	43.9±0.3	46.1±1.2	46.9±1.0	52.6±0.4

* Designation of strains. Group I: NCTC10695, ATCC8071, ATCC19857 and CL246/75; group II: NCTC10735, NCTC10736, NCTC10737 and ATCC8072; group III: ATCC8073, CL432/72, CL256/73, CL549/73, CL389/75, CL106/76, E631, E988 and CL107/76 (this strain was included in the group but its DNA was not reassocated); group IV: NCTC10762, NCTC10763, NCTC10738, CL27/72, CL595/73, CL104/76, CL108/76, CL109/76 and CL105/76 (this strain was included in the group but its DNA was not reassocated).

† Phenotypic results from Holmes *et al.* (1975) and Holmes & Owen (unpublished observations). The number of strains which deviated from the recorded result is given in parentheses.

‡ Acid from carbohydrates in ammonium salt medium.

§ NCTC10736 was atypical of this group.

clinical and other sources, were not markedly different from each other in their overall genome sizes, yet the DNA base compositions and base sequence homologies showed that the species comprised genetically heterogeneous strains. As reported previously by Levin (1972), *P. putrefaciens* strains can have widely differing GC compositions and the range in values of 43 to 53 % found here was large compared with observed ranges of most other bacterial species (Normore, 1973).

Our DNA-DNA reassociation results provided evidence of at least four DNA homology groups within *P. putrefaciens*. Two of these groups (I and IV) had high levels (> 82 % DNA binding) of internal DNA relatedness whereas the other two groups (II and III) were characterized by comparatively greater heterogeneity in internal relatedness. It is difficult formally to relate DNA sequence relatedness and levels in the taxonomic hierarchy. Data on various members of the family Enterobacteriaceae (Brenner, 1973) indicated that strains classified in homogeneous phenetic species usually shared 70 % or more of their DNA sequences. This may not necessarily be true for other groups of bacteria; species of *Pseudomonas* for example, show considerable heterogeneity in DNA relatedness (Palleroni *et al.*, 1972). However, on this criterion *P. putrefaciens* groups I and IV could constitute distinct species with only a small amount of DNA in common with each other. The division of strains into these DNA homology groups correlated well with the results on DNA base compositions and with several phenotypic characteristics obtained previously (Debois *et al.*, 1975; Holmes *et al.*, 1975).

To facilitate the practical identification of clinical isolates, the principal characteristics distinguishing the DNA groups of *P. putrefaciens* are summarized in Table 7. All clinical isolates of *P. putrefaciens* belonged either to group III or to group IV. However, there were no consistent differences in biochemical characteristics between the clinical isolates and those from marine and other sources, except that the clinical isolates were unable to grow at 5 °C. *Pseudomonas putrefaciens* group IV was the most homogeneous group in DNA and phenotypic characteristics. Several characteristics (6 % NaCl tolerance, growth on SS agar, lack of saccharolytic activity and GC contents of 53 %), suggested these strains were closely related to certain other clinical isolates of *P. putrefaciens* described as group 2

(Riley *et al.*, 1972), and the high GC content (57.6 %) strains of Levin (1972). None of these strains were proved to be human pathogens.

Doudoroff & Palleroni (1974) considered that *P. putrefaciens* was not a valid species of *Pseudomonas*, and recently Lee *et al.* (1977) proposed that strains should be transferred, with the same specific epithet, to the genus *Alteromonas* (Baumann *et al.*, 1972). The taxonomic problems regarding the generic status of these bacteria are outside the scope of the present study, but our results on *P. putrefaciens* DNA homology group I provided evidence of close similarities between the type strains of *P. putrefaciens* and *P. rubescens* (ATCC8071 and NCTC10695 respectively). As concluded by Lee *et al.* (1977), these two species appear synonymous, and in accordance with the *International Code of Nomenclature of Bacteria, 1976 Revision* (Lapage *et al.*, 1975) the name *P. putrefaciens* (Derby & Hammer) Long & Hammer 1941 should be retained in preference to the junior synonym *P. rubescens* (Pivnick, 1955).

The estimation of degrees of DNA-DNA binding from the spectrophotometric measurement of renaturation rates has been used in relatively few taxonomic studies even though the method compares favourably in its accuracy and reproducibility with other reassociation techniques (Gibbins & Gregory, 1972; Bradley, 1973; Martini & Phaff, 1973; Crombach, 1974a; Swings & De Ley, 1975). We found the method convenient because it did not need isotopically labelled DNA, any combination of cross-reactions between DNAs from different strains could be tested (reducing the likelihood of selecting an aberrant reference strain), and genome sizes could be estimated. In our view, the method was not suitable for use as a batch procedure or for estimating base mismatching from thermal denaturation of the duplexes (see also Crombach, 1974b).

It is concluded that the strains at present classified as *P. putrefaciens* should be retained in this species, but DNA relatedness studies should be extended to determine relationships between the various groups of *P. putrefaciens* and species of *Alteromonas*.

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