A Numerical Taxonomic Study of Some *Pseudomonas*-like Marine Bacteria

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SUMMARY

The abilities of 26 marine and 12 other *Pseudomonas*-like strains of bacteria to grow on single carbon sources, their antibiotic sensitivities and complement of some enzymes of intermediary metabolism were numerically analysed and gave five phenons. Two phenons contained strains with more than 55 mol % GC and were regarded as *Pseudomonas* spp., two were similar to the genus *Alteromonas*, and one was an intermediate phenon. Tests for identifying such organisms are proposed. *Pseudomonas putrefaciens* Derby & Hammer 1931 and *P. rubescens* Pivnick 1955 should be considered synonyms of *Alteromonas putrefaciens* Derby & Hammer comb. nov.

INTRODUCTION

The identification of Gram-negative rods from aquatic and food sources has proved difficult due to the unsatisfactory state of their taxonomy. The scheme of Shewan, Hobbs & Hodgkiss (1960) has proved as useful as any other to the applied bacteriologist: it includes the possession of pigmentation, cytochrome oxidase, catalase and the mode of metabolism of glucose in Hugh & Leifson's (1953) test. *Pseudomonas* species of their groups I and II correspond to those of the genus *Pseudomonas* as defined by Palleroni & Doudoroff (1972). The organisms in groups III and IV include such named strains as *P. atlantica*, *P. putrefaciens*, *P. rubescens* and *P. testosteroni*, all of which are of considerable economic importance. Indeed, Shewan (1974) regards *P. putrefaciens* as one of the most important organisms encountered in the spoilage of protein foods kept at chill temperatures (2 to 4 °C). Many isolates from the sea (Herbert *et al.*, 1971), from cutting oils (Pivnick, 1955) and from clinical material (Levin, 1972; Riley, Tatum & Weaver, 1972) are similar to it. The mol % GC of the DNA of many organisms of groups III and IV is much lower (40 to 55 %) than that of the genus *Pseudomonas* (58 to 70 %; Palleroni & Doudoroff, 1972). However, in routine bacteriological tests they are indistinguishable from other group III and IV strains whose mol % GC fit the genus. It seemed, therefore, appropriate that a more detailed study of the groups III and IV pseudomonads be undertaken especially to distinguish the low mol % GC strains from the true *Pseudomonas*.

While the work described here was in progress, Baumann *et al.* (1972) reported on the taxonomy of 218 strains of Gram-negative, non-fermentative, marine bacteria. All the polarly flagellated organisms required Na⁺ for growth and had 30.5 to 64.7 mol % GC. Those with mol % GC above 57.8 were placed in the genus *Pseudomonas*; those with mol % GC between 43.2 and 48 were placed in a new genus *Alteromonas*, which was distinct from the former solely on the grounds of DNA base composition, despite extensive characteriza-

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tion. The rest of the strains were unassigned. These workers emphasized the limitations of their results due to the very restricted ecology of their isolates. The genus *Alteromonas* as described includes organisms that would be placed in groups III and IV of Shewan *et al.* (1960). It is therefore necessary to compare any groups of low mol % GC described here with *Alteromonas* species.

Since the groups III and IV strains gave negative results in most routine bacteriological tests, they were subjected to a nutritional screening and some rapid biochemical tests (Kerstens, 1967). This paper describes a numerical taxonomic analysis of these organisms based on the results obtained. A preliminary report has been presented (Lee, Gibson & Shewan, 1973).

METHODS

Bacterial strains and their culture. Their designation and sources are shown in Table 1. Those requiring sea water for optimum growth were grown routinely in Leifson's (1970) medium M which contained (g per l distilled water): Bacto-casitone (Difco), 2; Bacto-yeast extract (Difco), 1; NaCl, 20; KCl, 1; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 4; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1; and Tris, 0.5. The Tris was added as a solution adjusted with HCl to pH 7.5 to 8.0 and the medium was solidified, if required, with agar (15 g l^{-1}). Medium L, containing 5 g NaCl l^{-1} but otherwise the same as medium M, was used for the cultivation of other strains. All cultures and growth tests were incubated at 20°C .

Buffers. Phosphate buffers were prepared from 0.2 M stock solutions of Na_2HPO_4 and KH_2PO_4 , and Tris/HCl buffers were prepared from 0.4 M stock solutions of Tris and HCl. For marine strains, phosphate plus marine salts buffer was used. The salts (NaCl, 17.5 g; KCl, 1.0 g; and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.6 g) were dissolved in 850 ml distilled water, the phosphate solutions were mixed (to give 0.01 M) and added to the salts solution and the volume was adjusted to 1 l. The buffers were not sterilized.

Requirement for Na^+ for growth. Strains were tested for their ability to grow in basal medium, basal medium plus $2.9 \text{ g NaCl l}^{-1}$ and basal medium plus $17.5 \text{ g NaCl l}^{-1}$. The basal medium contained (g per l deionized distilled water): KCl, 0.5; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.0; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5; Tris, 6.0; H_3PO_4 , 0.032; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.008; glucose, 1.0; and 0.25 g each of L-methionine, L-alanine, L-valine, L-asparagine, L-proline, and L-threonine. The Tris was added as a solution adjusted to pH 7.5 with HCl. Tubes ($150 \times 16 \text{ mm}$) containing 5 ml medium sterilized at 121°C for 15 min were inoculated in duplicate, using a straight wire, from 2-day-old plate cultures of medium M or L. The ability of a strain to show visible growth on one or both Na^+ -containing media but not on the basal medium within 10 days indicated a requirement for Na^+ for growth.

Effect of Na^+ concentration on growth. Twenty four Na^+ -requiring strains were grown in triplicate in Leifson's liquid medium M, modified to contain 0.005, 0.05, 0.10, 0.20, 0.30, 0.40, 0.50, 0.75 and 1.33 M-NaCl , dispensed in 5 ml amounts in $150 \times 16 \text{ mm}$ tubes. Tubes were inoculated with one drop of 24 h broth culture. After 24 h incubation, growth was stopped by the addition of 0.2 ml 36 % formaldehyde and determined by measuring $E_{580}^{1.0\text{cm}}$ in a Pye Unicam SP600 spectrophotometer.

Antibiotic sensitivities. The sensitivities of the strains were determined using Multodisks (Oxoid) applied to flood inoculated dried plates of medium M for the marine strains, or Oxoid CM3 for the others.

Deoxyribonuclease. DNAase was detected by the method of Jeffries, Holtman & Guse (1957) using Bacto-DNAase Test Agar (Difco) supplemented for marine strains with 2 % (w/v) NaCl.

Table 1. *Strains used and their sources*

Strain no.	Original designation	Source	Mol % GC*
<i>Pseudomonas</i> spp. of groups I/II†			
NCMB129	<i>P. fluorescens</i>	Cod	61·7
NCMB320	<i>P. fragi</i>	Cod	58·8
NCMB406	<i>P. putida</i>	Cod	61·7
NCMB1354		White fish (Castell & Greenough, 1959)	60·5
NCMB1792		Cod (Herbert <i>et al.</i> , 1971)	63·4
NCMB1793		Cod (Herbert <i>et al.</i> , 1971)	60·5
NCIB10649	<i>P. cepacia</i>	Urine (Jonsson, 1970)	67·3
<i>Pseudomonas</i> spp. of groups III/IV†			
NCIB8955	<i>P. testosteroni</i>	Soil (Marcus & Talalay, 1956)	62·9
NCIB9203	<i>P. maltophilia</i>	(Hugh & Ryschenkow, 1961)	67·1
NCIB9681	<i>P. acidovorans</i>	Soil (den Dooren de Jong, 1926)	68·8
NCIB11158		Faeces, strain 142-2 of Minigawa (1963)	55·1
<i>Pseudomonas putrefaciens</i> and related pseudomonads			
NCMB335		Cod	51·0
NCMB1733	<i>P. putrefaciens</i>	Haddock, strain P 19x of Levin (1968)	45·9
NCMB1735	<i>P. putrefaciens</i>	Haddock, strain Syl of Levin (1972)	48·1
NCIB8615	<i>P. putrefaciens</i>	Butter	47·3
NCIB8768	<i>P. rubescens</i>	Cutting oil (Pivnick, 1955)	46·9
NCIB10471	<i>P. putrefaciens</i>	Butter (Derby & Hammer, 1931)	44·7
NCIB10472	<i>P. putrefaciens</i>	Butter (Derby & Hammer, 1931)	47·3
NCIB10761	<i>P. putrefaciens</i>	Poultry, strain EB/E 44/148 of Barnes & Melton (1971)	54·1
SS65		Sea water	49·0
SS191	<i>P. putrefaciens</i>	Sea water	46·1
NCIB11156		Man, strain from group 1b of von Graevenitz & Simon (1970)	46·9
NCIB11157		Faeces, strain 136-2 of Minigawa (1963)	54·7
<i>Pseudomonas</i> spp.			
NCMB19		Clams, strain B16 of MacLeod, Onofrey & Norris (1954)	42·7
NCMB130		Cod	41·7
NCMB224		Dogfish	41·5
NCMB301	<i>P. atlantica</i>	Seaweed (Yaphe, 1957)	42·7
NCMB398		Sea water	58·0
NCMB399		Sea water	40·1
NCMB400		Sea water	41·7
NCMB1516		Cod	42·2
NCMB1520		Cod	44·7
NCMB1521		Cod	44·2
NCMB1731		Cod	42·5
NCMB1788		Cod (Herbert <i>et al.</i> , 1971)	46·4
NCMB1789		Cod (Herbert <i>et al.</i> , 1971)	42·2
NCMB1790		Cod (Herbert <i>et al.</i> , 1971)	41·5
<i>Vibrio</i> sp.			
NCMB6	<i>V. anguillarum</i>	Diseased cod (Bagge & Bagge, 1956)	44·4

NCMB, National Collection of Marine Bacteria; NCIB, National Collection of Industrial Bacteria, both of Torrey Research Station; SS, Statens Seruminstitut, Copenhagen, Denmark.

* Mol % GC determined by Gibson & MacLeod (unpublished results).

† Groups I/II and III/IV refer to the pseudomonad groups of Shewan *et al.* (1960).

Reduction of trimethylamine oxide. The ability of the strains to reduce trimethylamine oxide (TMAO) to trimethylamine was determined according to the method of Laycock & Regier (1971) except that 20 % KOH was used instead of K_2CO_3 (Murray & Gibson, 1972).

Hydrogen sulphide production. The ability to produce H_2S from L-cystine was tested on Bacto-Motility Sulphide Medium (Difco) supplemented for marine strains with 2 % NaCl.

Decarboxylases. Lysine and ornithine decarboxylases were detected by the method of Møller (1955).

Arginine dihydrolase. Arginine dihydrolase was detected by the method of Thornley (1960).

Nutritional screening. The strains were tested in triplicate for their ability to grow on 72 compounds at 0·1 % (w/v) final concentration as the sole source of carbon, with ammonia and nitrate as nitrogen source. The basal medium for the marine strains contained the following salts (g): NaCl, 20; KCl, 1; $MgCl_2 \cdot 6H_2O$, 4; $CaCl_2 \cdot 2H_2O$, 1; $(NH_4)_2SO_4$, 1; NH_4NO_3 , 1; and $FeCl_3 \cdot 6H_2O$, 0·008. Distilled water (250 ml) and 0·2 M-Tris buffer (pH 6·5; 250 ml) containing 330 μ mol H_3PO_4 were added and the mixture was autoclaved at 121 °C

for 20 min before adding sterile distilled water or sterile 2 % (w/v) purified agar (Oxoid) (500 ml). The basal medium for the other strains was the liquid minimal medium or minimal agar of Clowes & Hayes (1968). If sufficiently soluble, the carbon sources were prepared as concentrated solutions which were sterilized by membrane filtration; otherwise they were dissolved in the minimal medium and autoclaved at 115 °C for 10 min.

The media were dispensed in 25 compartment Replidishes (Sterilin, Richmond, Surrey) to avoid the possibility of the strains cross-feeding, and inoculation was by means of a simple replicating device (Lee, 1973). Positive growth was recorded for any strain which, after 14 days incubation, showed more growth on the particular substrate than on the control medium.

Pseudomonas maltophilia NCIB9203 requires methionine for growth; it was examined separately in methionine (0.1 %) supplemented media. *Pseudomonas atlantica* NCMB301 can grow on agar as the sole source of carbon and was therefore tested in liquid media.

Cell-free extracts. Strains were grown in either medium M or L supplemented with 0.1 % (w/v) sodium gluconate and 0.1 % (w/v) glucose in four 2 l conical flasks each containing 500 ml medium. Each flask was inoculated with 1 ml of an overnight starter broth culture and incubated at 20 °C in a cooled orbital incubator (model IH-465, Gallenkamp) at 250 rev. min⁻¹. Bacteria were harvested in the late-exponential phase of growth (E_{580}^{lum} approximately 1.5) by centrifuging at 8000 g for 10 min at 20 °C and washed twice in 0.01 M-phosphate buffer, pH 7.0. About 10 g packed wet wt cells were suspended in 15 ml 0.01 M-phosphate buffer, pH 7.0, and added to a 50 ml MSK bottle (Braun, Melsungen, Germany) containing 50 g glass beads (0.10 to 0.11 mm diam.) all at 4 °C. The cells were broken by shaking for 3 min at 4000 rev. min⁻¹ in a cooled Braun Disintegrator. The mixture was fractionated into a soluble enzyme and a particulate fraction according to the scheme of Kersters (1967).

No extracts could be prepared from NCMB335 because it was not possible to grow sufficient organisms.

Enzyme assays. The method of Kersters & De Ley (1968) was used to assay 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase, and the method of Sols & Salas (1966) for phosphofructokinase. The NAD- and NADP-linked dehydrogenase activities were determined on the soluble cell fractions and the 2,6-dichlorophenolindophenol-linked dehydrogenases on the particulate fractions by the method of Kersters (1967). The substrates used are listed in Tables 4 and 5. Any change compared with a control without substrate after 1 h incubation at 20 °C was regarded as positive.

Numerical analysis of results. The results from all the above tests except the effect of Na⁺ on growth, H₂S production, decarboxylases and arginine dihydrolase were used in a computer-assisted numerical taxonomic analysis. All results were regarded as two-state characters. Soluble NAD- and NADP-linked and particle-linked dehydrogenases were counted as three separate characters. Since several strains had many negative characters, the coefficient S_j of Jaccard was used to calculate similarities (Sokal & Sneath, 1963). Analyses were performed on the enzyme assay data alone (88 characters/strain), on the combined physiological and nutritional data (77 characters/strain) and on all the data combined (165 characters/strain). Dendrograms were constructed by complete linkage analysis (Sørensen, 1948) from the similarity matrices which were scanned at decrements of 0.01 S_j .

RESULTS AND DISCUSSION

The results from enzyme screening assays on subcellular fractions have not previously been used in numerical taxonomic analyses. We therefore computed these data separately

Table 2. Comparison of phenons derived from enzyme, physiological and nutritional, and combined data using the similarity coefficient S_s and clustering by complete linkage

Phenon	Strains always present	Level of clustering (%) and additional strains present		
		1*	2*	3*
A	NCIB10649 NCMB1792 NCMB1793 NCMB406 NCMB1354 NCMB129 NCMB320	52 %	56 %	54 %
B	NCIB11158 NCIB9681 NCIB8955	31 %	46 % NCMB398	37 % SS65† NCIB9203 NCMB398
C	NCMB301 NCMB1516 NCMB1789 NCMB224 NCMB1788 NCMB19 NCMB399 NCMB1731	46 % NCIB8768 NCMB130	43 %	49 %
D	NCMB1520 NCMB400	47 %	58 % NCMB1521	50 % NCMB1521
E	NCIB11156 NCMB1735 NCIB11157 SS191 NCIB8615 NCMB1733 NCIB10471	50 % NCIB10472 NCMB1521	41 % SS65 NCIB10761 NCIB8768 NCMB335	50 % NCIB10761 NCIB8768 NCIB10472

* 1, Data on enzyme analysis of subcellular fractions; 2, nutritional and physiological data; 3, all data combined. The phenons from analysis 3 were those finally accepted.

† Considered to be a member of phenon E as it has an overall mean similarity S_T of 60 % with strains of phenon E and only 45 % with phenon B strains.

Strains NCMB1790, 130 and 6, together with NCMB335 for which there was no enzyme data (see Methods), were not finally allocated to any phenon.

from the combined physiological and nutritional data, as well as combined with them. The three analyses yielded very similar phenons (Table 2), showing that the enzyme screening assays are suitable for taxonomic analysis and suggesting that the phenons are distinct and formed on the basis of a wide range of characters. The phenons derived from the analysis of the combined data (Table 2, Fig. 1) are those referred to in the following discussion.

Table 3 summarizes the results of the nutritional and physiological tests useful in separating the phenons. There is a marked correlation between the mol % GC of the strains and their clustering in the numerical analysis. Thus phenons A and B contain only strains of more than 55 mol % GC and include all the named *Pseudomonas* species of Palleroni & Doudoroff (1972) used in this study. Phenons C, D and E contain only strains with less than 55 mol % GC.

Many strains required Na^+ for growth (Table 3) but the response of the strains in the different phenons to increasing concentrations of Na^+ varied (Fig. 2). No strain grew on methionine, butan-1-ol, *o*-hydroxybenzoate, formate, malate or on any of the amines. *Pseudomonas putrefaciens* NCIB10472 did not grow on any compound as the sole source of carbon but did not have a vitamin requirement as it grew on vitamin-free Casamino acids. The other strains which used few carbon sources were those not clearly belonging to any phenon.

The dehydrogenase activities that correlate with the phenons are shown in Tables 4 and 5. Activities on sugar phosphates were generally present and not helpful in separating the

Fig. 1. Dendrogram constructed from a complete linkage analysis of the similarities (S_j) calculated from all the data.

phenons. L-Serine was the only substrate tested that did not stimulate dehydrogenase activity in any strain.

Most strains possessed the enzymes characteristic of the Entner-Doudoroff pathway, 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase. Strains NCMB400 and NCMB1790 had only the latter enzyme, perhaps indicative of the modified pathway described by De Ley *et al.* (1970). Neither enzyme could be detected in extracts from NCBI11158 nor in *Vibrio anguillarum*, NCMB6, the only strain with phosphofructokinase activity. Extracts from most strains could dehydrogenate 6-phosphogluconate and glucose 6-phosphate, perhaps indicative of the pentose phosphate pathway. Most strains could grow on glucose as the sole source of carbon (Table 3). Thus, although many strains did not produce visible signs (acid or gas) of the metabolism of glucose in Hugh & Leifson's (1953) test, they could metabolize glucose.

Table 3. Some differences between phenons in physiological and nutritional attributes

	Phenon				
	A	B	C	D	E
No. of strains in phenon	7	5	8	3	11
Mean mol % GC	62.0	62.4	42.5	43.5	48.3
Range	58.8-67.3	55.1-68.8	40.1-46.4	41.7-44.7	44.7-54.7
Mean no. of single carbon sources utilized	44	24	25	13	19
Requirement for Na ⁺	—	1	+	+	2
DNAase	—	1	+	+	+
Reduction of TMAO	—	—	—	+	+
Production of H ₂ S	1	2	—	+	+
Inhibition by:					
Tetracycline (10 µg)	6	4	—	—	+
Erythromycin (50 µg)	3	+	+	+	+
Oleandomycin (10 µg)	—	2	+	+	10
Chloramphenicol (10 µg)	—	+	+	+	+
Furazolidone (50 µg)	—	3	7	+	+
Neomycin (10 µg)	6	4	2	+	+
Nitrofurantoin (50 µg)	—	3	3	+	+
Novobiocin (30 µg)	1	4	1	1	1
Penicillin (1.5 i.u.)	—	1	—	+	—
Polymyxin B (300 i.u.)	6	+	+	+	+
Streptomycin (25 µg)	6	4	7	2	+
Growth on:					
Glycine	2	—	7	—	—
Isoleucine	6	3	1	—	9
Threonine	1	1	3	—	8
Aspartate	+	4	5	—	—
Arginine	+	1	7	2	—
Proline	+	+	+	2	—
Succinate	+	+	7	—	+
Malate	+	+	1	+	7
Glycerol	+	1	—	—	—
Glucose	+	2	+	+	8
Ribose	+	1	—	+	2
Galactose	6	1	+	—	1
N-Acetylglucosamine	3	1	2	1	7
Glucosamine	+	1	—	—	8
Benzoate	3	2	—	—	—
<i>p</i> -Hydroxybenzoate	6	2	—	—	—
<i>m</i> -Hydroxybenzoate	3	2	—	—	—
Malonate	4	2	—	—	—
Itaconate	4	1	—	—	—
Protocatechuate	7	1	—	—	—

+, Property present in all strains; numbers (*n*) indicate the property was present in *n* strains of the phenon; —, property absent in all strains.

The following substrates were also used but were of lesser value in separating the phenons: alanine, asparagine, cysteine, cystine, glutamate, glutamine, histidine, leucine, lysine, methionine, phenylalanine, serine, tryptophan, tyrosine, valine (all amino acids were in the L form), propan-1-ol, butan-1-ol, acetate, caproate, citrate, formate, fumarate, glycollate, glyoxylate, *o*-hydroxybenzoate, DL-β-hydroxybutyrate, DL-lactate, maleate, oxalate, propionate, pyruvate, DL-tartrate, D-arabinose, L-arabinose, D-fructose, D-glucuronate, D-glucuronic acid, DL-glyceraldehyde, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, D-xylose, methylamine.HCl, dimethylamine.HCl, trimethylamine.HCl, trimethylamine oxide, ethylamine.HCl, diethylamine.HCl and triethylamine.HCl.

Table 4. *Presence of soluble NAD- and NADP-linked dehydrogenases in cell extracts*

Substrate	Phenon									
	A (7)*		B (5)		C (8)		D (3)		E (11)	
	NAD	NADP	NAD	NADP	NAD	NADP	NAD	NADP	NAD	NADP
Alanine	—	—	—	—	3	3	1	1	—	—
Valine	—	—	1	—	+	+	+	+	10	10
Threonine	1	1	—	—	+	+	+	+	9	7
Glutamate	—	5	1	3	—	—	2	1	7	5
Acetate	—	—	—	—	5	3	1	1	2	1
Succinate	—	—	1	—	1	—	2	—	1	—
Malate	6	6	3	3	2	+	+	+	+	+
Citrate	2	1	3	+	7	7	+	2	7	6
Isocitrate	+	+	+	+	+	+	+	+	10	+
Glycerol	—	—	1	—	—	—	1	—	6	1

+, Present in all strains; *n*, present in *n* strains; —, absent in all strains.

* Numbers in parentheses indicate number of strains in phenon.

The following substrates were also examined but were of lesser value in separating the phenons: glycine, serine, lysine, arginine, proline, glycollate, D-lactate, L-lactate, glyoxylate, pyruvate, D-arabinose, L-arabinose, ribose, xylose, D-fucose, L-fucose, rhamnose, fructose, galactonate, galactose, gluconate, glucose, mannitol, mannose, sorbitol, sorbose, maltose, sucrose, fructose 6-phosphate, glucose 6-phosphate, 6-phosphogluconate, galactose 6-phosphate, and ribose 5-phosphate.

Table 5. *Particle-bound 2,6-dichlorophenolindophenol-linked dehydrogenases*

Substrate	Phenon				
	A (7)*	B (5)	C (8)	D (3)	E (11)
Glutamate	4	4	6	1	1
Glycollate	—	2	—	2	1
D-Lactate	5	4	—	2	6
L-Lactate	—	4	—	—	2
Glyoxylate	2	1	—	1	1
Succinate	1	3	+	+	10
L-Malate	6	3	—	—	1
Citrate	—	—	—	2	1
Isocitrate	—	—	—	2	—
L-Arabinose	+	—	—	—	—
Xylose	+	—	—	—	—
D-Fucose	6	—	—	—	—
Galactonate	6	—	—	—	—
Galactose	+	—	—	—	—
Gluconate	+	—	—	—	—
Glucose	+	—	—	—	—
Mannose	+	—	—	—	—
Maltose	+	—	—	—	—

+, Present in all strains; *n*, present in *n* strains; —, absent in all strains.

* Numbers in parentheses indicate number of strains in phenon.

The following substrates were also used but were of lesser value in separating the phenons: alanine, glycine, valine, serine, threonine, lysine, arginine, proline, acetate, pyruvate, glycerol, D-arabinose, ribose, L-fucose, rhamnose, fructose, mannitol, sorbitol, sorbose, sucrose, fructose 6-phosphate, glucose 6-phosphate and 6-phosphogluconate.

Taxonomic considerations

Phenon A. This phenon of fairly closely related strains corresponds to the genus *Pseudomonas*, in particular to groups I and II of Shewan *et al.* (1960), and includes the species *P. fluorescens*, *P. fragi*, *P. putida*, and *P. cepacia* (NCMB129, 320, 406 and NCIB10649 respectively). As would be expected from the results of Stanier, Palleroni & Doudoroff (1966), they were nutritionally the most versatile and *P. cepacia* NCIB10649 could grow on more compounds as sole sources of carbon than any other strain.

Phenon B (excluding ss65). This is the phenon of lowest internal similarity and is analogous to the *Pseudomonas* groups III and IV of Shewan *et al.* (1960). It includes three type strains, *P. testosteroni*, *P. maltophilia*, and *P. acidovorans* (NCIB8955, NCIB9203 and NCIB9681 respectively).

Strain NCMB398 was the only strain in the phenon requiring Na^+ for growth. It has 58 mol % GC, within the range reported for the genus *Pseudomonas* (Palleroni & Doudoroff, 1972), and had similar characteristics to the three Na^+ -requiring species *P. doudoroffii*, *P. marina* and *P. nautica* described by Baumann *et al.* (1972). It is most similar to *P. nautica* but differs from it in its ability to grow on valine, glucose, fructose, gluconate and glycerol. It seems inadvisable therefore to assign NCMB398 to an existing species but the strain is considered to be a member of the genus *Pseudomonas*.

Strain NCIB11158 has 55 mol % GC which is just below the range suggested for the genus *Pseudomonas*. Unlike the pseudomonads of phenons A and B, this strain lacked the enzymes of the Entner–Doudoroff pathway and had such a high background fluorescence with NAD that it was impossible to detect NAD-linked dehydrogenase activities. Strains NCIB10761 and NCIB11157 had similar mol % GC but were placed in phenon E and a third, NCMB335, did not clearly belong to any phenon but was most closely related to strains of phenon E. Strain NCIB11158 had relatively high similarity to these three strains and to some other strains of phenon E. Thus, although nearer to phenon B than E, NCIB11158 is an intermediate and may represent those strains of intermediate mol % GC that Baumann *et al.* (1972) did not assign to a genus.

Phenon C. This phenon contains quite closely related strains, requiring Na^+ for growth with an optimum at about 0.3 M (Fig. 2c). They were nutritionally the most versatile of the low mol % GC organisms. Except for *P. atlantica* (NCMB301) the nutritional spectra and mol % GC of strains in phenon C were very similar to those described for *Alteromonas marinopraesens* Baumann *et al.* 1972, which has been renamed *A. haloplanktis* Reichelt & Baumann 1973. This species includes strain NCMB19 (MacLeod's B16). We would therefore identify all strains of phenon C, except NCMB301, as *A. haloplanktis*. *Pseudomonas atlantica* NCMB301 just qualifies for phenon C. In its nutritional spectrum it resembled *A. macleodii* but it could also liquefy agar, a property not possessed by any alteromonad described by Baumann *et al.* (1972). As we did not have the strains of *A. macleodii* for comparison we identify this strain just as an alteromonad.

Phenon D. This small phenon contains strains intermediate to those in phenons C and E. All strains required Na^+ for growth but at lower levels than the strains in phenon C with an optimum at 0.2 to 0.3 M- Na^+ (Fig. 2b). They do not correspond to any previously described species but are probably alteromonads. There are too few strains to justify designation of a new species.

Phenon E (including ss65). This phenon contains the strains previously identified as *P. putrefaciens* including NCIB10471 and 10472, two of the original strains of Derby & Hammer (1931) and *P. rubescens* NCIB8768 (Pivnick, 1955) isolated from cutting oils. Only NCIB11157

Fig. 2. Effect of Na^+ on growth. Twenty four strains were tested as described in Methods. (a) Response of NCMB1354 (phenon A). Similar results were obtained with NCMB129, 320 and 406, of phenon A, NCMB1733 and 1735, NCIB8768 and 11157, SS65 and 191 of phenon E, and NCMB335. (b) Response of NCMB1521 (phenon D). Similar results were obtained with the other two strains of phenon D. (c) Response of NCMB1789 (phenon C). Similar results were obtained with NCMB19, 224, 399, 1516, 1731 and 1788 of phenon C, NCMB398 of phenon B, and NCMB130 and 1790.

and NCMB1733 required Na^+ for growth but both could grow optimally in the presence of 0.005 M- Na^+ (Fig. 2a). All except NCIB11156 and NCIB11157 possessed ornithine decarboxylase, a property not found in any other strain studied. This test has been suggested as being useful for the identification of *P. putrefaciens* (Hugh, 1970).

All the low mol % GC strains examined appear to be inter-related. Phenon D and NCMB130, 335 and 1790 are intermediate to the two major phenons C and E. The clusters differ too little in properties to warrant being placed in separate genera. The strains of phenon C (excluding NCMB301) are identifiable as *A. haloplanktis*. Recently, single strains of the species *A. communis*, *A. vaga*, *A. macleodii* and *A. haloplanktis* when grown on glucose have been shown to have the characteristic enzymes of the Entner-Doudoroff pathway and no detectable phosphofructokinase (Baumann & Baumann, 1973). These data are similar to the results reported here except that two, NCMB400 and 1790, had only 2-keto-3-deoxy-6-phosphogluconate aldolase.

According to Baumann *et al.* (1972) the genera *Pseudomonas* and *Alteromonas* are differentiated solely on the basis of mol % GC. They stated that strains of the genus *Alteromonas* had a mol % GC of 43 to 48 but they included organisms of 41.8 to 49.0. All the low mol % GC strains studied here fell within the latter range except NCMB335 (51.0 %), NCIB10761 (54.1 %) and NCIB11157 (54.7 %). Strains NCIB10761 and 11157 fell in phenon E and NCMB335 was also closely related to phenon E strains. Accordingly, these three strains are sufficiently related to the other low mol % GC strains to be included in the same genus. Therefore, all the non-fermentative strains with mol % GC of 55 or less are placed in the genus *Alteromonas* of Baumann *et al.* (1972).

Levin (1972) has suggested that *P. putrefaciens* strains could be divided into two groups on the basis of mol % GC and the ability to grow in the presence of 6 % NaCl. Although phenon E includes two subclusters which differ slightly in their mol % GC, there are no distinctive differences between these two groups. All the strains of phenon E have been examined for their ability to grow in the presence of 6 % NaCl and they could not be divided as described by Levin. Therefore, although the phenon has a wider range (44.7 to 54.7) of mol % GC than would generally be accepted for a single species, there is insufficient evidence at present to divide it into separate species or biotypes. It does not correspond to any species of alteromonad described by Baumann *et al.* (1972) but includes strains of *P. putrefaciens* and *P. rubescens* which are considered synonymous by Hugh (1970) as is corroborated by

Table 6. *Proposed identification scheme for Gram-negative, polarly flagellated, catalase and oxidase positive, non-fermentative bacteria*

Test	Phenon	...	Pseudomonads		Alteromonads		
			A	B	C	D	E
Glucose metabolism (Hugh & Leifson, 1953)			O	N/A	N/A	N/A	N/A
TMAO reduction			—	—	—	+	+
Requirement for Na ⁺			—	—	+	+	—
DNAase			—	—	+	+	+
Arginine dihydrolase			+	—	—	—	—
Ornithine decarboxylase			—	—	—	—	+
Sensitivity to penicillin (1.5 i.u.)			—	—	—	+	—

O, Oxidative; N/A, no change or alkaline; —, property absent in all strains; +, property present in all strains.

our results. We therefore propose that *P. putrefaciens* Derby & Hammer 1931 and *P. rubescens* Pivnick 1955 be considered synonyms of *Alteromonas putrefaciens* Derby & Hammer comb. nov. (phenon E). The strain NCIB10471 is designated as the type strain of this species.

The results of the work described here and the inclusion of *A. putrefaciens* in the genus would require that the description of the genus *Alteromonas* include the following modified statements. The mol % GC content of the DNA ranges from 43 to 55. They are strict aerobes except for those species which can use denitrification as a means of anaerobic respiration. Nearly all strains are capable of growth on a minimal medium containing artificial sea water with D-glucose as the sole source of carbon and energy, and ammonia as the sole source of nitrogen. Sodium ions but no organic growth factors may be required for growth. Some species may produce an extracellular DNAase. Some strains can hydrolyse agar.

The tests used in the analysis and some of the more commonly used bacteriological tests most useful in separating the phenons are shown in Table 6. The latter were easier to perform than some of the tests used to establish the groups. It is suggested that the tests (Table 6) could be useful in identifying *A. putrefaciens* and for dividing new isolates into the other phenons described here. Our results suggest that the strains of phenon C and Na⁺-requiring pseudomonads may prove difficult to distinguish. The only high mol % GC, Na⁺-requiring strain studied was NCMB398 which differed from phenon C strains solely in the absence of DNAase. Unfortunately, no other high mol % GC Na⁺-requiring strains were available for study. Obviously a much larger number of strains including more Na⁺-requiring pseudomonads needs to be studied to evaluate the usefulness of the proposed identification scheme. However, the Gram-negative, non-fermentative, oxidase positive and polarly flagellated organisms corresponding to *Pseudomonas* groups III and IV of Shewan *et al.* (1960) and of different mol % GC content can now be clearly subdivided on characters other than mol % GC alone.

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REFERENCES

- BAGGE, J. & BAGGE, O. (1956). *Vibrio anguillarum* as the cause of ulcerous disease in codfish. *Nordisk Veterinaermedicin* **8**, 481-492.
- BARNES, E. M. & MELTON, W. (1971). Extracellular enzymic activity of poultry spoilage bacteria. *Journal of Applied Bacteriology* **34**, 599-609.
- BAUMANN, L. & BAUMANN, P. (1973). Enzymes of glucose catabolism in cell-free extracts of non-fermentative marine bacteria. *Canadian Journal of Microbiology* **19**, 302-304.
- BAUMANN, L., BAUMANN, P., MANDELL, M. & ALLEN, R. D. (1972). Taxonomy of aerobic marine eubacteria. *Journal of Bacteriology* **110**, 402-409.
- CASTELL, C. H. & GREENOUGH, M. F. (1959). The action of *Pseudomonas* on fish muscle: 4. Relation between substrate composition and the development of odours by *Pseudomonas fragi*. *Journal of the Fisheries Research Board of Canada* **16**, 21-31.
- CLOWES, R. C. & HAYES, W. (1968). *Experiments in Microbial Genetics*. Oxford and Edinburgh: Blackwell Scientific Publications.
- DE LEY, J., KERSTERS, K., KHAN-MATSUBARA, J. & SHEWAN, J. M. (1970). Comparative D-gluconate metabolism and DNA base composition in *Achromobacter* and *Alcaligenes*. *Antonie van Leeuwenhoek* **36**, 193-207.
- DEN DOOREN DE JONG, L. E. (1926). Bijdrage tot de kennis van het mineralisatieproces. Rotterdam: Nijgh and Van Ditmar.
- DERBY, H. A. & HAMMER, B. W. (1931). Bacteriology of butter. IV. Bacteriological studies on surface taint butter. *Iowa Agricultural Experimental Station Research Bulletin* **145**, 389-416.
- GRAEVENITZ, A. VON & SIMON, G. (1970). Potentially pathogenic, non-fermentative, H₂S-producing Gram-negative rod (1b). *Applied Microbiology* **19**, 176.
- HERBERT, R. A., HENDRIE, M. S., GIBSON, D. M. & SHEWAN, J. M. (1971). Bacteria active in the spoilage of certain seafoods. *Journal of Applied Bacteriology* **34**, 41-50.
- HUGH, R. (1970). A practical approach to the identification of certain non-fermentative Gram-negative rods encountered in clinical specimens. *Public Health Laboratory* **28**, 168-187.
- HUGH, R. & LEIFSON, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *Journal of Bacteriology* **66**, 24-26.
- HUGH, R. & RYSCHENKOW, E. (1961). *Pseudomonas maltophilia*, an *Alcaligenes*-like species. *Journal of General Microbiology* **26**, 123-132.
- JEFFRIES, C. D., HOLTMAN, D. F. & GUSE, D. G. (1957). Rapid method for determining the activity of micro-organisms on nucleic acids. *Journal of Bacteriology* **73**, 590-591.
- JONSSON, V. (1970). Proposal of a new species *Pseudomonas kingii*. *International Journal of Systematic Bacteriology* **20**, 255-257.
- KERSTERS, K. (1967). Rapid screening assays for soluble and particulate bacterial dehydrogenases. *Antonie van Leeuwenhoek* **33**, 63-72.
- KERSTERS, K. & DE LEY, J. (1968). An easy screening assay for the enzymes of the Entner-Doudoroff pathway. *Antonie van Leeuwenhoek* **34**, 388-392.
- LAYCOCK, R. H. & REGIER, L. W. (1971). Trimethylamine-producing bacteria on haddock (*Melanogrammus aeglefinus*) fillets during refrigerated storage. *Journal of the Fisheries Research Board of Canada* **28**, 305-309.
- LEE, J. V. (1973). *Some comparative biochemical and physiological studies on selected Gram-negative bacteria*. Ph.D. thesis, University of Aberdeen.
- LEE, J. V., GIBSON, D. M. & SHEWAN, J. M. (1973). Comparative biochemical analysis of some pseudomonads. *Journal of General Microbiology* **77**, xii.
- LEIFSON, E. (1970). Motile marine bacteria. IV. Ionic relationships of marine and terrestrial bacteria. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung II)* **125**, 170-206.
- LEVIN, R. E. (1968). Detection and incidence of specific species of spoilage bacteria on fish. 1. Methodology. *Applied Microbiology* **16**, 1734-1737.
- LEVIN, R. E. (1972). Correlation of DNA base composition and metabolism of *Pseudomonas putrefaciens* isolates from food, human clinical specimens, and other sources. *Antonie van Leeuwenhoek* **38**, 121-127.
- MACLEOD, R. A., ONOFREY, E. & NORRIS, M. E. (1954). Nutrition and metabolism of marine bacteria: I. Survey of nutritional requirements. *Journal of Bacteriology* **68**, 680-686.
- MARCUS, P. I. & TALALAY, P. (1956). Induction and purification of α - and β -hydroxy-steroid dehydrogenases. *Journal of Biological Chemistry* **218**, 661-674.
- MINIGAWA, M. (1963). Studies on the strains closely related to *Vibrio parahaemolyticus* and reddish brown pigment-producing *Pseudomonas* isolated from the stools of patients of acute enteritis. *Annual Report of the Institute of Food Microbiology, Chiba* **16**, 9-23.
- MØLLER, V. (1955). Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. *Acta pathologica et microbiologica scandinavica* **36**, 158-172.

- MURRAY, C. K. & GIBSON, D. M. (1972). An investigation of the method of determining trimethylamine in fish muscle extracts by the formation of its picrate salt: part 1. *Journal of Food Technology* **7**, 35–46.
- PALLERONI, N. J. & DOUDOROFF, M. (1972). Some properties and subdivisions of the genus *Pseudomonas*. *Annual Review of Phytopathology* **10**, 73–100.
- PIVNICK, H. (1955). *Pseudomonas rubescens*, a new species from soluble oil emulsions. *Journal of Bacteriology* **70**, 1–6.
- REICHEL, J. L. & BAUMANN, P. (1973). Change of the name *Alteromonas marinopraesens* (ZoBell and Upham) Baumann *et al.* to *Alteromonas haloplanktis* (ZoBell and Upham) comb. nov. and assignment of strain ATCC23821 (*Pseudomonas enalia*) and strain c-A1 of De Voe and Oginsky to this species. *International Journal of Systematic Bacteriology* **23**, 438–441.
- RILEY, P. S., TATUM, H. W. & WEAVER, R. E. (1972). *Pseudomonas putrefaciens* isolates from clinical specimens. *Applied Microbiology* **24**, 798–800.
- SHEWAN, J. M. (1974). The biodeterioration of certain proteinaceous foodstuffs at chill temperatures. In *Industrial Aspects of Biochemistry*. Edited by B. Spencer. London: North-Holland Publishing Company.
- SHEWAN, J. M., HOBBS, G. & HODGKISS, W. (1960). A determinative scheme for the identification of certain genera of Gram-negative bacteria with special reference to the Pseudomonadaceae. *Journal of Applied Bacteriology* **23**, 379–390.
- SOKAL, R. R. & SNEATH, P. H. A. (1963). *Principles of Numerical Taxonomy*. San Francisco and London: W. H. Freeman.
- SOLS, A. & SALAS, M. L. (1966). Phosphofructokinase. *Methods in Enzymology*, **9**, 436–442.
- SØRENSEN, T. (1948). A method of establishing groups of equal amplitude in plant sociology based on similarity of species content and its application to analyses of the vegetation of Danish commons. *Biologiske Skrifter* **5**, 1–34.
- STANIER, R. Y., PALLERONI, N. J. & DOUDOROFF, M. (1966). The aerobic pseudomonads: a taxonomic study. *Journal of General Microbiology* **43**, 159–271.
- THORNLEY, M. J. (1960). The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. *Journal of Applied Bacteriology* **23**, 37–52.
- YAPHE, W. (1957). The use of agarase from *Pseudomonas atlantica* in the identification of agar in marine algae (Rhodophyceae). *Canadian Journal of Microbiology* **3**, 987–993.