A Taxonomic Study of some Gram-negative Facultatively Methylotrophic Bacteria

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One hundred and fifty pink-pigmented facultatively methylotrophic bacteria (PPFMs) of the '*Pseudomonas extorquens*' type, 28 other facultative methylotrophs and 16 non-methylotrophic marker strains of the genera *Pseudomonas*, *Alcaligenes*, *Mycoplana* and *Microcycylus* were compared in a numerical phenetic study using 140 unit characters. Data were analysed using the simple matching (S_{SM}) and Jaccard (S_J) coefficients and single, complete and average linkage algorithms. Cluster composition was largely the same with each similarity coefficient and linkage method. Four major and seven minor clusters, containing 187 of the 194 strains were defined above the 80% similarity level. The non-pink methylotrophs were recovered in two major, four minor and two single-membered clusters. One of the major clusters could be equated with *Microcycylus aquaticus* but strains in the other were not identified. Strains in two of the minor clusters were identified as *Pseudomonas* species but members of the other two minor and one of the single-strain clusters were not positively identified. They had pseudomonad properties but were not closely associated with any marker strains. The other single-membered methylotroph cluster was thought to be a cytophaga or flexibacter. The remaining minor and single-membered clusters contained only marker strains. All the PPFMs were recovered in the two remaining major clusters which were closely related to each other but not to the rest of the organisms studied. The generic assignment of the PPFMs is discussed and the suggestion is made that the genus *Methylobacterium* may be the most appropriate place for them, despite their apparent inability to utilize methane.

INTRODUCTION

Facultatively methylotrophic bacteria can utilize, as sole carbon source, C_1 compounds more reduced than CO_2 in addition to more complex organic compounds. Interest in the biochemistry of methylotrophy has led to the isolation of many such organisms in recent years, but their taxonomy has received relatively little attention. Although there have been some comparative studies (Stocks & McCleskey, 1964; Kouno & Ozaki, 1975), the need (Quayle, 1972) for more detailed taxonomic investigations of these organisms has remained.

The majority of facultative methylotrophs isolated have been Gram-negative or Gram-variable pink-pigmented organisms exemplified by '*Pseudomonas extorquens*'. They have been found in a wide range of habitats and have been assigned variously to the genera *Bacillus* (Bassalik, 1913), '*Protaminobacter*' (den Dooren de Jong, 1927), *Vibrio* (Bhat & Barker, 1948; von Graevenitz & Grehn, 1976), *Pseudomonas* (Krasil'nikov, 1959; Kaneda & Roxburgh, 1959; Peel & Quayle, 1961; Heumann, 1962; Anthony & Zatman, 1964; Kirikova, 1970; Mehta, 1973; Rock *et al.*, 1976; Austin & Goodfellow, 1979), *Mycoplana* (de Vries & Derx, 1953), *Flavobacterium* (Bassalik *et al.*, 1960), *Corynebacterium* (Gräf & Bauer, 1973), *Chromobacterium* (Dickinson *et al.*, 1975) and *Methylobacterium* (Patt *et al.*, 1976). This last genus is restricted to organisms able to utilize methane facultatively.

In the present study a large number of pink-pigmented strains, either already reported or shown in this laboratory to be facultatively methylotrophic, were examined along with some other Gram-negative facultative methylotrophs in an attempt to clarify their taxonomy.

METHODS

Maintenance of strains. Stock cultures of 178 facultatively methylotrophic bacteria and 16 marker strains (Table 1) were grown at 25 °C and maintained at 20 °C on the following media. Glycerol peptone (GP) medium [1.0% (w/v) Difco peptone, 1.0% (v/v), glycerol, 1.5% (w/v) Difco agar; pH 7.0] was used for the pink-pigmented facultative methylotrophs (PPFMs) including *Methylobacterium organophilum* NCIB 11278. Attempts to maintain this organism in a methane atmosphere on the modified (Patt *et al.*, 1974) NMS and AMS media of Whittenbury *et al.* (1970) failed. The MS medium of van Ert & Staley (1971) was used for the *Microcycus* strains, mineral medium (Colby & Zatman, 1973) with either 0.1% (w/v) trimethylamine hydrochloride or 0.1% (v/v) methanol was used for the other methylotrophs and nutrient agar (Oxoid CM55) for all other strains.

Tests. Unless stated otherwise, tests were done in the appropriate maintenance medium. Results were recorded after incubation at 25 °C for 10 d for the PPFMs and 3 d (plates) or 5 d (broths) for other strains.

Morphology and staining. No medium was suitable for all strains; therefore GP medium was used for the PPFMs, MS medium for the microcycli and nutrient agar or broth (Oxoid CM67) for all other strains. Bacteria from shaken and static broth cultures were examined by phase-contrast microscopy for motility and morphology at 6, 12, 24, 48 and 72 h. Static cultures were also examined for the presence of a pellicle or surface ring. Bacteria growing on agar plates were examined *in situ* by phase-contrast microscopy for cellular branching and pleomorphism at 1, 3, 7, 10 and 14 d. Gram stains were done on smears from 72 h plate cultures using Jensen's modification (Cruickshank, 1960). Smears from both 3 and 7 d cultures were prepared in duplicate; one set was stained with Sudan black and the other with modified Albert's stain (Cowan, 1974) to detect lipid and volutin inclusions, respectively.

Fine structure. Flagellation of motile non-pigmented methylotrophs and representative PPFMs, and the cell wall fine structure of the latter were kindly examined by Mr N. C. Houston, at Torrey Research Station, in the transmission electron microscope by the methods of Henderson & Hodgkiss (1973). Preparations of PPFMs were made from 4 d slope and 2 d shaken broth cultures and of other bacteria from 2 d slope cultures.

Biochemical tests. Strains were tested for lipase and lecithinase (Willis & Hobbs, 1958) and tyrosinase (Lelliot *et al.*, 1966) production, nitrate reduction (Spencer, 1969), OF reaction (Hugh & Leifson, 1953; up to 21 d incubation) and according to the methods of Cowan (1974) for methyl red (MR) reaction, KCN tolerance, production of acetylmethylcarbinol (VP), indole (method 3), urease (up to 21 d incubation), oxidase, catalase (all method 1), β -galactosidase (ONPG; up to 10 d incubation) and DNAase, and hydrolysis of starch, Tweens (both method 1) and gelatin (method 3). Milk agar plates [Oxoid Blood Agar Base + 1% (w/v) Difco skim milk] were examined for up to 3 weeks for clearing indicating casein hydrolysis. Cellulose digestion was tested by Dubos's filter paper strip method (Skerman, 1969) using both Dubos salts and the appropriate liquid maintenance medium (incubation up to 42 d). Hydrogen sulphide production was tested in liquid maintenance medium containing 0.1% (w/v) cysteine hydrochloride (up to 3 weeks incubation; detection with lead acetate paper).

Antibiotic sensitivity. Sensitivity to 25 antibiotics (those listed in Table 2, plus penicillin and bacitracin) was tested using impregnated paper discs (Mast Laboratories Ltd, Liverpool, U.K.) placed on surface seeded plates. Results were recorded after 7 d (PPFMs) or 2 d (other strains).

Temperature and pH tolerance. Strains were tested for the ability to grow at 1, 5 and 10 °C (28 d), 15, 20 and 25 °C (14 d) and 30, 37 and 45 °C (5 d) and at initial pH 4, 5, 9 and 10 (adjusted aseptically, after sterilization, with 1 M-HCl or 1 M-NaOH).

Growth factor requirements. Calcium pantothenate (0.5 mg l⁻¹), nicotinic acid (0.5 mg l⁻¹), biotin (10.0 µg l⁻¹), riboflavin (0.5 mg l⁻¹), vitamin B₁₂ (2.5 µg l⁻¹), *p*-aminobenzoic acid (10.0 µg l⁻¹), folic acid (10.0 µg l⁻¹) and pyridoxal. HCl (2.0 mg l⁻¹) were tested individually as growth requirements. The basal medium was liquid mineral medium (Colby & Zatman, 1973) without vitamins and glassware was cleaned by the method of Owens & Keddie (1968). Results were recorded after three successive subcultures.

Carbon source utilization tests. The basal medium was that of Palleroni & Doudoroff (1972) supplemented with biotin (10 µg l⁻¹), thiamin. HCl (0.5 mg l⁻¹), cyanocobalamin (2.5 µg l⁻¹) and methionine (10 µg l⁻¹), and solidified with 1.5% (w/v) Oxoid purified agar (L28). The medium was autoclaved at 121 °C for 15 min and test compounds (see Table 2 and Results) were added afterwards as autoclaved or filter-sterilized solutions to a final concentration of 0.1% (w/v), except for phenol (0.025%, w/v), geraniol (present in the vapour phase) and methane (50%, v/v, mixture with air in an anaerobic jar). Media were dispensed in plastic Replidishes (Sterilin, Teddington, U.K.; Sneath & Stevens, 1967) and inoculated with thrice-washed cells using the replication method of Stewart & Widanapratirana (1972). Tests were read after 7 and 14 d incubation. Results with the following

Table 1. Designation, source and cluster assignment of strains

Name	Collection/Strain no.	Reference
Strains assigned to cluster 1		
<i>Pseudomonas</i> sp.	NCIB 11590 (strain MA)	Shaw <i>et al.</i> (1966)
<i>Pseudomonas</i> sp.	NCIB 11591 (strain MS)	Wagner (1964)
<i>Pseudomonas</i> sp.	1A3 ^a	Colby & Zatman (1973)
Unnamed strains	27 ^b , 32, 36, 44	
	NCIB 11347 (strain 5H2)	Hampton & Zatman (1973)
Strains assigned to cluster 2		
<i>Mycoplana dimorpha</i>	*NCIB 9439	Gray & Thornton (1928)
<i>Mycoplana bullata</i>	*NCIB 9440	Gray & Thornton (1928)
Strains assigned to cluster 3		
<i>Microcycylus aquaticus</i>	*NCMB 1801	
<i>Microcycylus aquaticus</i>	ATCC 27068 (strain M)	van Ert & Staley (1971)
<i>Microcycylus aquaticus</i>	ATCC 27069 (strain B)	van Ert & Staley (1971)
' <i>Microcycylus polymorphum</i> '	NCIB 10516	
' <i>Microcycylus eburneus</i> '	ATCC 21373	Kouno & Ozaki (1975)
<i>Microcycylus</i> sp.	H ^c , W	Kanopka <i>et al.</i> (1976)
Unnamed strain	414 ^d	
Strains assigned to cluster 4		
Unnamed strains	29 ^b , 23A1, 33WH	
	NCIB 11346 (strain 5B1)	Colby & Zatman (1973)
Strains assigned to cluster 5		
Unnamed strains	M1 ^b , M5	
Strains assigned to cluster 6		
<i>Pseudomonas aeruginosa</i>	*NCIB 8295	
<i>Pseudomonas putida</i>	*NCIB 9494	
<i>Pseudomonas</i> spp.	42(2) ^e , 42(3)	
Strains assigned to cluster 7		
' <i>Pseudomonas cepacia</i> '	NCIB 9089	
<i>Pseudomonas fluorescens</i>	*NCIB 9046	
Strains assigned to cluster 8		
<i>Pseudomonas mendocina</i>	*NCIB 10541	
<i>Pseudomonas pseudoalcaligenes</i>	*NCIB 9946	
Strains assigned to cluster 9		
' <i>Pseudomonas oxalaticus</i> '	NCIB 8642	
<i>Pseudomonas testosteroni</i>	*NCIB 8955	
<i>Pseudomonas acidovorans</i>	*NCIB 9681	
Unnamed strains	5A1 ^b	
	1B1S ^b	
Strains assigned to cluster 10		
' <i>Corynebacterium rubrum</i> '	^f	Gräff & Bauer (1973)
<i>Methylobacterium organophilum</i>	*NCIB 11278 (strain XX)	Patt <i>et al.</i> (1976)
' <i>Mycoplana rubra</i> '	NCIB 10409	
' <i>Protaminobacter ruber</i> '	NCIB 2879	den Dooren de Jong (1927)
' <i>Protaminobacter ruber</i> subsp. <i>machidanus</i> '	ATCC 21611–21614	
' <i>Protaminobacter ruber</i> '	^g	Sato <i>et al.</i> (1977)
' <i>Pseudomonas extorquens</i> '	NCIB 9399	Janota-Bassalik & Pedyk (1961)
' <i>Pseudomonas extorquens</i> '	3	Unknown
' <i>Pseudomonas methylica</i> '	2 ^h	Kirikova (1970)
<i>Pseudomonas rhodos</i>	NCIB 9421	Heumann (1962)
<i>Pseudomonas</i> sp.	NCIB 9133 (strain AM1)	Peel & Quayle (1961)
<i>Pseudomonas</i> sp.	NCIB 9686 (strain M27)	Anthony & Zatman (1964)
<i>Pseudomonas</i> spp.	1 ⁱ , 135	Rock <i>et al.</i> (1976)
<i>Pseudomonas</i> spp.	NCIB 9141, 9144, 9145	Hayward (1960)

Table 1. (*Continued*)

Name	Collection/Strain no.	Reference
<i>Pseudomonas</i> spp.	NCIB 10597–10604, 10606–10612	
' <i>Vibrio extorquens</i> '	190B ^j , 381B, 385B, AM1 var. 471	Chandra & Shethna (1975)
' <i>Vibrio extorquens</i> '	^k	von Graevenitz & Grehn (1976)
Unnamed strains	GB ^c , CS51, 82A B46 ^l 109 ^d , 111, 212, 216, 218, 270, 339, 390, 393, 463, 466, 471 B014 ^m , B015, B016, B017, B018, B019, B020, B021, B022, B023, B024, B025, B026, B040, B041 2 ⁿ , 9, 20, 39, 82, 83, 205, 285, 449, 453, 456, 465, 509, 530, 539, 691, 706, 711, 712, 724, 789, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, N-11, N-12, M159-1	Kouno & Ozaki (1975)
Strains assigned to cluster 11		
' <i>Mycoplana rubra</i> '	R14 ^o	
' <i>Mycoplana rubra</i> '	NCIB 11272 ^p	
<i>Pseudomonas mesophilica</i>	*NCIB 11561 (strain A47)	Austin & Goodfellow (1979)
<i>Pseudomonas rhodos</i>	9–6 ^q	Schmitt <i>et al.</i> (1974)
<i>Pseudomonas</i> spp.	NCIB 9142, 9143	Hayward (1960)
' <i>Vibrio extorquens</i> '	602 ^l	Chandra & Shethna (1975)
Unnamed strains	D123 ^l B044 ^m EP1 ^c , PB, 2St.A, 2CZ.A, 2CR, 2Gel.A, 2TBA 044 ^d , 115, 269 35 ⁿ , 37, 45, 58, 317, 420, 434, 437, 470, 488, 532, 617, 670, 679, 696, 767, 781, 787, 788, 790, 823, O-6, O-7, O-20, O-24, O-31, O-46, N-2	Kouno & Ozaki (1975)
Strains assigned to single-membered clusters		
<i>Pseudomonas</i> sp.	MOX	Chandra & Shethna (1975)
<i>Pseudomonas maltophilia</i>	*NCIB 9203	
<i>Pseudomonas diminuta</i>	*NCIB 9393	
<i>Alcaligenes faecalis</i>	*NCIB 8156	
<i>Pseudomonas alcaligenes</i>	*NCIB 9945	
<i>Pseudomonas stutzeri</i>	*NCIB 11358	
<i>Cytophaga/Flexibacter</i> sp.	6A1 ^b	

* Type strain.

† Binomials which are not included in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980) are shown in quotation marks.

Sources of strains: a, Dr J. Colby, University of Warwick, U.K.; b, Dr A. J. Smith, University College of Wales, Aberystwyth, U.K.; c, Dr A. E. Konopka, Purdue University, U.S.A.; d, ICI Ltd, Billingham, Cleveland, U.K.; e, Dr M. E. Rhodes-Roberts, University College of Wales, Aberystwyth, U.K.; f, Dr W. Gräf, University of Erlangen-Nürnberg, W. Germany; g, Dr K. Sato, Nagoya University, Japan; h, Dr N. N. Kirikova, Moscow State University, U.S.S.R.; i, Dr R. I. Mateles, Hebrew University, Jerusalem, Israel; j, Dr T. S. Chandra, Indian Institute of Science, Bangalore, India; k, Professor A. von Graevenitz, Yale University, U.S.A.; l, Dr B. Austin, University of Newcastle-upon-Tyne, U.K.; m, Dr T. G. Mitchell, British-American Tobacco Co., Southampton, U.K.; n, Dr K. Kouno, Sanraku-Ocean Co. Ltd, Fujisawa, Japan; o, Dr R. M. Lafferty, Technische Universität, Grätz, Austria; p, The Boots Co. Ltd, Nottingham, U.K.; q, Dr F. Mayer, University of Erlangen-Nürnberg, W. Germany. Strains bearing NCIB or NCMB numbers were obtained from the National Collection of Industrial Bacteria and National Collection of Marine Bacteria, Aberdeen, U.K. Unless otherwise stated strains bearing ATCC numbers were obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A.

compounds were difficult to interpret and therefore were not recorded: oxalate, glyoxylate, glycollate, *cis*-aconitate, asparagine, serine, tryptophan, pantothenate, 2-oxogluconate, formamide and xanthine.

Computer analysis. Test results were recorded as two-state unit characters. The 13 characters common to all strains were omitted from the data matrix, leaving 140 characters for 194 strains. Data were analysed on an IBM 1130 computer using programs written by H. R. Sanders and G. L. Smith of the Torrey Research Station. Similarities between strains were calculated using both the simple matching (S_{SM} ; Sokal & Michener, 1958) and the Jaccard (S_J ; Sneath, 1957) coefficients which include and exclude negative matches, respectively. Clustering was achieved using single, complete and unweighted average (UPGMA) linkage algorithms (Sneath & Sokal, 1974).

DNA base composition. DNA from representative strains was prepared according to the method of Gibson & Ogden (1979). The mol % guanine + cytosine (% GC) was determined by the thermal denaturation method (Marmur & Doty, 1962) using the equation of De Ley (1970).

Citrate synthase properties. The rapid techniques of Harford *et al.* (1976) were used to determine whether the molecular size and the effect of NADH and ATP on the activity of the citrate synthases of strains D123, B46, '*Corynebacterium rubrum*' and *Pseudomonas* sp. NCIB 9133 were typical of Gram-negative bacteria (Weitzman & Jones, 1968). Shaken GP broth cultures (4 d, 25 °C) were used for the molecular size determinations and GP plate cultures (7 d, 25 °C) were used for the enzyme inhibition tests. The same strains were also kindly tested by Professor P. D. J. Weitzman who found identical results.

RESULTS

Clustering of strains using the S_{SM} coefficient and the UPGMA algorithm

In the S_{SM} /UPGMA analysis, 187 of the 194 strains were recovered in four major (8 to 103 strains) and seven minor (2 to 5 strains) clusters (Fig. 1; Table 1). The other analyses yielded clustering patterns essentially the same except that clusters were formed at lower (about 10%) similarity levels in the S_J analyses, minor clusters 6 and 8 merged in both single linkage runs, and two outlying strains (B46 and *Pseudomonas rhodos* NCIB 9421) of cluster 10 moved to cluster 11 in the S_J /UPGMA analysis.

Cluster 1 contained three strains received as *Pseudomonas* spp. and five unnamed strains; 2 contained the two marker strains of *Mycoplana*, *M. dimorpha* NCIB 9439 and *M. bullata* NCIB 9440; 3 contained all seven *Microcycilus* strains and one unnamed ring-forming organism; 4 contained four unnamed strains; 5 contained two unnamed strains; 6 contained two strains received as *Pseudomonas* spp. and the marker strains *Ps. putida* NCIB 9494 and *Ps. aeruginosa* NCIB 8295; 7 contained the marker strains '*Ps. cepacia*' NCIB 9089 and *Ps. fluorescens* NCIB 9046; 8 contained the marker strains *Ps. mendocina* NCIB 10541 and *Ps. pseudoalcaligenes* NCIB 9946; 9 contained two unnamed strains and '*Ps. oxalaticus*' NCIB 8642, *Ps. testosteroni* NCIB 8955 and *Ps. acidovorans* NCIB 9681. Clusters 10 and 11 contained all 150 PPFMs. The larger cluster (10) contained 103 strains and included most of the named PPFMs except the type strain of *Ps. mesophilica* (NCIB 11561) and six others. These last seven strains were among the 47 strains of cluster 11.

The seven strains that formed single member clusters were the marker strains of *Ps. diminuta* (NCIB 9393), *Ps. maltophilia* (NCIB 9203), *Ps. alcaligenes* (NCIB 9945) and *Ps. stutzeri* (NCIB 11358), *A. faecalis* (NCIB 8156) and two non-pigmented facultative methylotrophs (*Pseudomonas* strain MOX and unnamed strain 6A1).

Characterization of strains

The 13 characters common to all strains were excluded from the data matrix since they had no discriminating value. Thus, all strains were catalase-positive, MR- and VP-negative, non-cellulolytic, resistant to penicillin and bacitracin, did not produce indole or H_2S and did not utilize thiourea, tetramethylurea, methane, hexane or benzene as sole carbon sources. The distribution of the remaining characters to the 11 clusters is shown in Table 2. The main features of clusters are described below and characters which differentiate between them are given in Table 3.

Fig. 1. Simplified dendrogram showing clusters obtained with the S_{SM} coefficient and average linkage algorithm, and the position of certain named strains.

Table 2. Percentage of strains in major and minor clusters positive for unit characters

Character	No. of strains . . .	Major clusters				Minor clusters						
		1 8	3 8	10 103	11 47	2 2	4 4	5 2	6 4	7 2	8 2	9 5
<i>Morphology</i>												
Rods 1–3 µm		100	100	100	100	100	100	100	100	100	100	100
Rods >3–8 µm		0	0	100	100	100	0	0	0	0	0	0
Coccobacilli		100	0	0	0	0	100	100	100	100	100	100
Branching		100	0	100	100	100	0	0	0	0	0	20
Ring-shaped cells		0	100	0	0	0	0	0	0	0	0	0
L-shaped cells		0	0	100	100	0	0	0	0	0	0	0
Pleomorphic cells		0	0	100	100	100	0	0	0	0	0	0
Sudanophilic inclusions		33	0	100	100	100	0	100	0	0	0	100
Gram variability		0	0	100	100	0	0	0	0	0	0	0
Colonies red/pink		0	0	100	100	0	0	0	0	0	0	0
Colonies white/off-white		100	100	0	0	100	100	100	100	100	100	100
Colonies yellow/orange		0	0	0	0	0	0	0	0	0	0	0
Colony diameter ≤ 1 mm		100	100	78	15	100	25	100	0	0	100	60
Colony diameter > 1–3 mm		13	0	23	85	0	100	0	100	100	50	80
Colony diameter > 3 mm		0	0	0	0	0	0	0	50	0	0	0
Broth pellicle and/or ring		100	100	100	100	100	100	0	100	100	100	100
Broth uniform turbidity		100	100	0	0	100	100	100	100	100	50	100
Motility		100	0	100	100	100	75	100	100	100	100	100
<i>Biochemical and physiological tests</i>												
Oxidase		100	100	100	100	100	100	100	100	100	100	100
Nitrate → Nitrite		25	25	17	15	0	75	0	25	0	50	100
Gas from nitrate		25	0	0	0	0	50	0	25	0	50	20
Gelatinase		0	0	1	2	50	0	0	25	100	0	0
Starch hydrolysis		0	0	2	2	0	0	0	0	0	0	0
Casein hydrolysis		0	0	1	0	0	0	0	25	100	0	0
β-Galactosidase		0	100	0	0	0	0	100	0	100	0	0
Lipase		0	0	9	0	0	0	0	0	50	0	0
Lecithinase		0	0	0	0	0	0	0	0	100	0	0
DNAase		0	0	2	0	0	0	0	0	0	0	0
Urease		100	84	100	100	0	50	100	100	100	100	100
Tween 20 hydrolysis		13	13	16	7	0	0	100	50	100	100	80
Tween 80 hydrolysis		0	0	0	2	0	0	100	25	100	100	60
Growth in presence of KCN		100	86	0	0	50	100	100	100	100	100	100
OF: alkaline		0	0	27	53	0	0	0	0	0	100	50
OF: oxidative		0	0	0	0	0	0	0	100	100	0	0
Biotin requirement		0	100	0	0	50	0	0	0	0	0	0
Thiamin requirement		0	0	0	0	50	33	0	0	0	0	0
Other growth factor requirement		0	0	0	0	0	0	0	0	0	0	0
Growth at:												
5 °C		100	100	9	2	0	0	0	75	50	100	75
10 °C		100	100	20	47	0	0	0	100	100	100	100
15 °C		100	100	99	100	100	100	50	100	100	100	100
37 °C		0	100	94	96	100	100	0	100	100	100	100
pH 4		0	0	2	63	0	0	0	0	50	0	0
pH 5		100	0	99	100	0	67	100	100	100	50	100
pH 9		100	100	99	87	100	100	0	100	100	100	100
pH 10		100	88	61	2	100	100	0	100	50	100	100
Sensitivity to:												
Nitrofurantoin		43	100	1	0	0	67	50	0	50	100	67
Kanamycin		29	100	100	100	100	100	100	100	50	100	100
Oxytetracycline		100	100	100	100	100	100	100	50	100	100	100
Novobiocin		100	71	92	83	100	100	0	0	50	0	75
Methenamine m.		100	100	11	9	100	100	100	100	100	100	100
Polymyxin B		29	17	1	0	0	100	100	100	100	100	80
Colistin sulphate		25	100	1	0	50	100	100	100	50	100	80
Paromomycin		13	100	47	34	100	100	100	100	50	100	20

Table 2 (*continued*)

Character	No. of strains . . .	Major clusters				Minor clusters						
		1 8	3 8	10 103	11 47	2 2	4 4	5 2	6 4	7 2	8 2	9 5
Sulphamethoxazole		88	0	0	0	100	0	100	0	100	100	100
Cephalexin		67	100	56	33	100	100	100	0	0	0	60
Neomycin		100	100	56	36	100	100	100	100	100	100	60
Erythromycin		100	63	4	64	100	100	100	0	0	0	25
Gentamycin		100	100	96	100	100	100	100	100	100	100	50
Cycloserine		14	40	78	76	50	100	100	0	0	100	40
Carbenicillin		100	75	1	0	100	100	100	0	0	0	20
Albamycin T		100	100	98	100	100	100	100	33	100	100	100
Nalidixic acid		33	50	2	0	0	100	100	75	100	100	100
Streptomycin		14	100	100	98	100	100	100	100	100	50	40
Cephalothin		88	0	1	4	D	100	100	0	0	0	0
Oleandomycin		0	100	64	72	100	50	0	0	0	0	20
Framycetin		100	100	99	98	100	100	100	100	100	100	100
Spiramycin		50	100	1	28	100	100	100	0	0	0	0
Cephaloridine		88	100	86	58	100	100	D	0	0	100	0
<i>Sole carbon sources</i>												
L-Arabinose		71	100	0	94	100	75	100	0	100	0	0
D-Xylose		100	100	0	98	100	25	100	0	50	0	0
L-Rhamnose		100	0	0	2	0	0	100	0	0	0	0
D-Fucose		75	14	0	93	D	0	100	0	0	0	0
L-Sorbose		33	0	0	0	0	25	0	25	0	0	0
D-Glucose		100	100	2	96	100	100	100	100	100	50	0
D-Fructose		100	100	74	38	100	100	100	100	100	100	80
D-Mannose		100	13	0	0	100	100	100	50	100	0	0
D-Galactose		100	100	1	93	100	75	100	0	100	0	25
Sucrose		100	0	0	0	0	75	0	0	D	0	0
Trehalose		100	0	0	0	0	25	0	0	100	0	0
Maltose		100	0	0	0	0	100	0	0	50	0	0
Cellobiose		100	0	0	0	0	25	D	0	0	0	0
Starch		0	0	0	0	0	0	0	0	0	0	0
Inositol		100	0	0	0	50	100	100	0	100	0	0
Glycerol		100	100	100	100	100	100	100	100	100	100	100
Sorbitol		100	100	0	0	100	100	100	0	100	0	0
Mannitol		100	100	0	0	100	100	100	25	100	0	40
Erythritol		0	0	0	0	0	100	0	0	50	0	40
Adonitol		0	100	0	2	50	100	100	0	0	0	0
Malonate (Na)		0	33	100	100	0	100	100	50	100	50	75
Succinate (Na)		0	88	99	100	0	100	100	100	100	100	100
Fumarate (Na)		0	50	99	100	0	100	100	100	100	100	100
L-Aspartate (Na)		0	0	10	91	0	33	100	25	100	50	80
L-Glutamate (Na)		71	100	15	96	100	100	100	100	100	100	80
Adipic acid		0	0	0	87	0	0	100	25	50	0	100
Sebacic acid		0	0	0	86	0	0	100	50	100	50	100
2-Oxoglutarate		0	100	100	100	0	75	100	100	100	100	100
DL-Lactate (Na)		100	100	100	100	100	100	100	100	100	100	100
DL-Malate (Na)		0	71	99	100	50	100	100	100	100	100	100
D-Tartrate (Na)		0	0	50	24	0	0	100	25	50	0	60
Acetate (Na)		100	100	99	100	100	100	100	100	100	100	100
Propionate (Na)		100	88	0	0	0	75	100	100	100	100	100
D-Laevulinate (Na)		0	0	1	3	0	0	50	100	100	100	100
Pelargonate (Na)		0	0	0	0	0	0	100	100	100	100	25
Pyruvate (Na)		100	100	100	100	100	100	100	100	100	100	100
Citrate (Na)		0	50	4	100	50	33	100	100	100	100	100
Citraconate (Na)		60	38	0	98	0	25	100	0	100	100	100
Saccharic acid		0	0	1	96	0	0	100	75	100	50	100
DL-Arginine		100	20	0	0	100	100	100	100	100	100	20
L-Alanine		100	100	1	13	100	100	100	100	100	100	75
L-Valine		100	0	0	0	0	50	100	100	100	50	75

Table 2 (continued)

Character	No. of strains . . .	Major clusters				Minor clusters						
		1 8	3 8	10 103	11 47	2 2	4 4	5 2	6 4	7 2	8 2	9 5
L-Leucine		86	0	0	0	0	25	100	50	0	50	100
Glycine		100	0	0	0	0	33	100	100	D	100	75
L-Histidine		83	0	0	4	100	100	100	100	100	100	80
DL-Phenylalanine		0	0	0	2	100	33	100	100	100	100	67
L-Proline		100	100	2	0	100	100	100	100	100	100	100
Ethanol		0	100	99	98	0	100	0	100	0	100	100
Propylene glycol		0	100	99	98	0	100	0	100	0	50	40
2,3-Butylene glycol		0	100	1	2	50	100	50	100	50	0	80
Geraniol		0	D	0	0	0	0	0	25	0	100	25
Methanol		0	100	100	98	0	25	0	50	0	0	0
Methylamine		100	D	99	32	0	100	0	0	0	0	0
Trimethylamine		100	0	34	5	0	100	100	0	0	0	25
Trimethylamine N-oxide		100	0	4	0	0	100	100	0	0	0	0
Ethylamine		60	100	100	94	0	100	0	100	0	100	75
Glucosamine		100	0	0	2	100	0	100	25	100	0	0
Ethanolamine		75	100	82	71	0	67	100	100	100	100	20
Butylamine		25	0	11	2	0	75	50	75	50	0	20
Tryptamine		0	0	0	0	0	0	100	0	50	50	0
Amylamine		0	0	9	0	0	100	100	100	50	0	40
Histamine		0	0	0	0	0	25	0	100	50	0	0
Putrescine		0	0	0	0	100	25	0	100	100	100	0
Formate (Na)		0	100	100	98	0	100	D	50	0	0	50
Tetramethylammonium Cl		0	0	0	2	0	25	0	0	0	0	0
Dimethylglycine		100	100	44	17	50	100	100	100	100	100	0
p-Hydroxybenzoate		50	0	0	2	0	33	100	100	100	0	100
m-Hydroxybenzoate		0	0	0	0	0	0	100	50	50	0	100
Betaine		100	100	76	17	50	100	100	100	100	100	0
Testosterone		0	0	0	0	0	0	0	0	0	0	100
Sarcosine		0	0	0	0	0	0	50	0	0	0	100
Phenol		0	0	0	0	0	0	0	0	0	0	25

D, Doubtful results.

Major clusters

Cluster 1. All strains were Gram-negative, occasionally branched rods, 1–3 μ m long, having one or more polar flagella; some also had single sub-polar or lateral flagella. Colonies were off-white, mostly ≤ 1 mm in diameter. Strains grew at 5 °C and 30 °C and at initial pH 10 and 5 but not at 37 °C or at initial pH 4. There was growth but no indicator change in the OF medium. All strains grew in the presence of KCN, were oxidase-positive and hydrolysed urea but were unreactive in other hydrolysis tests. All strains were methylotrophic and utilized as sole carbon source methylamine, trimethylamine and trimethylamine N-oxide but not formate or methanol. Various mono- and disaccharides and sugar alcohols, arginine, alanine, valine, glycine, proline, lactate, acetate, propionate, pyruvate, glucosamine, dimethylglycine and betaine were also utilized. Growth factors were not required. The DNA base composition of strains was in the range 60.8–64.7% GC.

Cluster 3. All strains were Gram-negative, non-motile rods, 1–3 μ m long, often curved or horseshoe-shaped and sometimes forming complete rings. Colonies were white or off-white, ≤ 1 mm in diameter. Strains grew at 5 °C, 37 °C and with one exception at initial pH 10, but not at 1 °C, 45 °C or at initial pH 5. There was growth but no indicator change in the OF medium. All strains were oxidase- and β -galactosidase-positive and all but one grew in the presence of KCN and hydrolysed urea. All were unreactive in other hydrolysis tests. All strains were methylotrophic and grew with methanol and formate (weakly) as sole carbon

Table 3. *Differential characters for and C₁ compounds utilized by clusters*

Character	Major clusters				Minor clusters							
	1	3	10	11	2	4	5	6	7	8	9	
Branching	+	—	+	+	+	—	—	—	—	—	—	
Curves, horseshoes	—	+	—	—	—	—	—	—	—	—	—	
L-shapes	—	—	+	+	—	—	—	—	—	—	—	
Sudanophilic inclusions	v	—	+	+	+	—	+	—	—	—	+	
Gram variable	—	—	+	+	—	—	—	—	—	—	—	
Red colonies	—	—	+	+	—	—	—	—	—	—	—	
Motility	+	—	+	+	+	v	+	+	+	+	+	
Gelatinase	—	—	—	—	v	—	—	v	+	—	—	
Caseinase	—	—	—	—	—	—	—	v	+	—	—	
β -Galactosidase	—	+	—	—	—	—	+	—	+	—	—	
Lecithinase	—	—	—	—	—	—	—	—	+	—	—	
Growth in presence of KCN	+	v	—	—	v	+	+	+	+	+	+	
OF: oxidative	—	—	—	—	—	—	—	+	+	—	—	
Biotin requirement	—	+	—	—	v	—	—	—	—	—	—	
<i>Sole carbon sources</i>												
L-Arabinose	v	+	—	+	+	v	+	—	+	—	—	
D-Xylose	+	+	—	+	+	v	+	—	v	—	—	
L-Rhamnose	+	—	—	—	—	—	+	—	—	—	—	
D-Fucose	v	v	—	+	D	—	+	—	—	—	—	
D-Glucose	+	+	—	+	+	+	+	+	+	v	—	
D-Mannose	+	v	—	—	+	+	+	v	+	—	—	
D-Galactose	+	+	—	+	+	v	+	—	+	—	v	
Sucrose	+	—	—	—	—	v	—	—	D	—	—	
Trehalose	+	—	—	—	—	v	—	—	+	—	—	
Maltose	+	—	—	—	—	+	—	—	v	—	—	
Cellobiose	+	—	—	—	—	v	D	—	—	—	—	
Inositol	+	—	—	—	v	+	+	—	+	—	—	
Sorbitol	+	+	—	—	+	+	+	—	+	—	—	
Mannitol	+	+	—	—	+	+	+	v	+	—	v	
Adonitol	—	+	—	—	v	+	+	—	—	—	—	
Malonate (Na)	—	v	+	+	—	+	+	v	+	v	v	
Succinate (Na)	—	v	+	+	—	+	+	+	+	+	+	
Fumarate (Na)	—	v	+	+	—	+	+	+	+	+	+	
L-Aspartate (Na)	—	—	—	+	—	v	+	v	+	v	v	
2-Oxoglutarate (Na)	—	+	+	+	—	v	+	+	+	+	+	
DL-Malate (Na)	—	v	+	+	v	+	+	+	+	+	+	
Propionate (Na)	+	v	—	—	—	v	+	+	+	+	+	
D-Laevulinate (Na)	—	—	—	—	—	—	v	+	+	+	+	
Pelargonate (Na)	—	—	—	—	—	—	+	+	+	+	v	
Citrate (Na)	—	v	—	+	v	v	+	+	+	+	+	
Citraconate (Na)	v	v	—	+	—	v	+	—	+	+	+	
Saccharic acid	—	—	—	+	—	—	+	v	+	v	+	
DL-Arginine	+	v	—	—	+	+	+	+	+	+	v	
L-Valine	+	—	—	—	—	v	+	+	+	v	v	
Glycine	+	—	—	—	—	v	+	+	D	+	v	
L-Histidine	v	—	—	—	+	+	+	+	+	+	v	
DL-Phenylalanine	—	—	—	—	+	v	+	+	+	+	v	
L-Proline	+	+	—	—	+	+	+	+	+	+	+	
Ethanol	—	+	+	+	—	+	—	+	—	+	+	
Propylene glycol	—	+	+	+	—	+	—	+	—	v	v	
2,3-Butylene glycol	—	+	—	—	v	+	v	+	v	—	v	
p-Hydroxybenzoate	v	—	—	—	—	v	+	+	+	—	+	
Ethylamine	v	+	+	+	—	+	—	+	—	+	v	
Glucosamine	+	—	—	—	+	—	+	v	+	—	—	
<i>C₁ substrates:</i>												
Methanol	—	+	+	+	—	v	—	v	—	—	—	
Methylamine	+	D	+	v	—	+	—	—	—	—	—	
*Dimethylamine	v	—	v	v	—	v	—	—	—	—	—	

Table 3 (continued)

Character	Major clusters				Minor clusters						
	1	3	10	11	2	4	5	6	7	8	9
Trimethylamine	+	—	v	—	—	+	+	—	—	—	v
Trimethylamine <i>N</i> -oxide	+	—	—	—	—	+	+	—	—	—	—
Formate (Na)	—	+	+	+	—	+	D	v	—	—	v
*Formamide	—	D	v	v	—	v	D	—	—	—	v

+, ≥90% of strains positive; —, ≤10% of strains positive; v, 10–90% of strains positive; D, doubtful results.

* These results were excluded from the numerical analyses.

Fig. 2. (a) Phase-contrast micrograph of '*Ps. extorquens*' (NCIB 9399) exhibiting branching. (b) Photomicrograph of Gram-stained preparation of '*Ps. extorquens*' (NCIB 9399) showing sudanophilic inclusions. The bar markers represent 10 μ m.

source. Various monosaccharides and sugar alcohols (but not disaccharides), alanine, proline, glutamate, 2-oxoglutarate, pyruvate, acetate, lactate, ethanol, propylene glycol, 2,3-butyleneglycol, ethylamine, ethanolamine, dimethylglycine and betaine were also utilized. All strains required biotin for growth. The DNA base composition of representative strains is in the range 67.1–68.8% GC (Kanopka *et al.*, 1976).

Cluster 10. All strains were rods, 1–8 μ m long, occasionally branched and pleomorphic (Fig. 2a), often containing much sudanophilic material (Fig. 2b) and sometimes volutin granules. The Gram reaction here and in members of cluster 11 was uncertain but the multilayered cell wall structure and the type of citrate synthase present (Weitzman & Jones, 1968) in representative strains of both clusters were typical of Gram-negative bacteria. Cells were motile (sometimes feebly), usually by polar or sub-polar flagella, but single lateral flagella were occasionally seen. Growth on nutrient agar was slow and colonies on GP agar were pale pink to bright orange-red, <1–3 mm in diameter. Almost all strains grew at 15 °C and 37 °C and at initial pH 5 and 9. There was growth but no acid production (some strains gave an alkaline reaction) in the OF medium, and no growth in the presence of KCN. Strains

were oxidase-positive and hydrolysed urea, but almost all were unreactive in other hydrolysis tests. All strains were methylotrophic and utilized methanol, formate and, with a single exception, methylamine as sole carbon sources. Malonate, succinate, fumarate, 2-oxoglutarate, lactate, malate, acetate, pyruvate, ethanol, propylene glycol and ethylamine were also utilized by almost all strains. Carbohydrates and amino acids were generally not utilized, except for glycerol (utilized by all strains) and fructose (utilized by 74% of strains). Growth factors were not required. The DNA base composition of representative strains was in the range 60.5–67.3% GC.

Cluster 11. Strains were morphologically and, in many respects, physiologically and biochemically very like those of cluster 10. However, they were nutritionally more versatile and, in addition to the substrates utilized by members of cluster 10, most could utilize several monosaccharides (but not disaccharides), aspartate, glutamate, citrate, citraconate and saccharic acid. Work done since the numerical analysis was completed (unpublished results) has shown that the organisms in this cluster, but not those in cluster 10, could also utilize suberate, pimelate, azelate and adipate as sole carbon sources. Growth factors were not required and the DNA base composition of representative strains was in the range 64.2–70.5% GC.

Minor clusters

Little weight can be given to the distribution of characters to clusters containing very few strains. However, since clusters 4, 5, 6 and 9 included facultatively methylotrophic strains, they are considered further. (Clusters 2, 7 and 8 contained only non-methylotrophic marker strains.)

All strains in clusters 4, 5, 6 and 9 were Gram-negative, unbranched, motile (with one exception) rods, 1–3 µm long. Flagellation was single or multiple polar (clusters 4, 5 and 6) or peritrichous (two strains in cluster 9). Strains in clusters 5 and 9 had sudanophilic inclusions. Colonies were white or off-white, up to 3 mm in diameter, and strains in cluster 6 (which included *Ps. aeruginosa* NCIB 8295 and *Ps. putida* NCIB 9494) produced green fluorescent diffusible pigments. Members of cluster 6 were also oxidative in the OF test whereas those of other clusters gave either an alkaline or no reaction. All strains were oxidase-positive and most hydrolysed urea. Strains in cluster 5 produced β-galactosidase and hydrolysed Tweens and *Ps. aeruginosa* (cluster 6) was proteolytic, but otherwise strains were unreactive in degradation tests. Strains in all clusters were nutritionally versatile utilizing a wide range of compounds as sole carbon sources, although only the strains of clusters 4 and 5 utilized a range of carbohydrates. All strains in clusters 4 and 5 were methylotrophic, utilizing (cluster 4) formate, methylamine, trimethylamine *N*-oxide, trimethylamine and methanol (one strain), and (cluster 5) trimethylamine and trimethylamine *N*-oxide. Cluster 6 included two methylotrophic strains utilizing methanol and formate, and cluster 9 contained two utilizing formate and one trimethylamine.

DISCUSSION

The dendrogram in Fig. 1 is divided into two distinct parts; one comprises all PPFMs and the other contains the rest of the organisms included in this study. Therefore the pink and non-pink methylotrophs will be discussed separately.

Non-pink-pigmented facultative methylotrophs

Several of the strains examined here have been assigned previously to the genus *Pseudomonas* but it seems likely that only some of them (all recovered in minor clusters) belong there. The two fluorescent strains in cluster 6 [42(2) and 42(3)] almost certainly are strains of *Ps. putida*, and strain 5A1 in cluster 9 can be identified as *Ps. acidovorans*. However, cluster 9 also contains '*Pseudomonas oxalaticus*' (Khambata & Bhat, 1953) and strain 1B1S which are peritrichously flagellate (and thus, by definition, excluded from the

genus *Pseudomonas*) suggesting that they belong to the genus *Alcaligenes* (Hendrie *et al.*, 1974), although they did not cluster with the type strain of *Alcaligenes faecalis* (NCIB 8156).

The two strains in cluster 5 (M1 and M5) and three in cluster 4 [5B1 (Colby & Zatman, 1973), 23A1 and 33WH] appear to be pseudomonads, but they are not associated with any of the marker strains and do not fit any described species of *Pseudomonas*. The remaining strain in cluster 4 (29) is a non-motile *Moraxella*-like organism (Lautrop, 1974).

The single strain *Pseudomonas* MOX (Chandra & Shethna, 1975) again has pseudomonad properties but cannot be identified with any particular species. The remaining single methylotrophic strain (6A1), unlike all the other organisms studied here, shows gliding motility and probably belongs to the *Cytophaga/Flexibacter* group.

The strains in the first of the major clusters (cluster 1) are difficult to classify. Although some have been called *Pseudomonas* species (Wagner, 1964; Shaw *et al.*, 1966; Colby & Zatman, 1973), morphologically they are much more like the two *Mycoplana* marker strains of cluster 2 in that cultures commonly contain branching cells. However, the two clusters are not closely associated and the identity of the cluster 1 strains remains uncertain.

In contrast, there is little doubt that all the members of cluster 3 can be considered to be strains of *Microcycylus aquaticus*. Our results strongly support the opinions of van Ert & Staley (1971) and Kanopka *et al.* (1976) that strains H, W, ATCC 27068, ATCC 27069 and the type strain of *Mi. aquaticus* (NCMB 1801, ATCC 25396) are sufficiently alike to be assigned to the same species. By the same token, there seems little justification for regarding the industrial organisms '*Mi. eburneus*' (Kouno & Ozaki, 1975) and '*Mi. polymorphum*' (neither name is validly published) as species separate from *Mi. aquaticus*.

Pink-pigmented facultative methylotrophs

Major clusters 10 and 11 comprise all the PPFMs included in this study. The close association of the two clusters (about 77% similarity) supports previously expressed views that the PPFMs are a group of phenotypically similar organisms belonging to the same genus (Peel & Quayle, 1961; Quayle, 1961, 1972; Stocks & McCleskey, 1964; Anthony & Zatman, 1964; Kouno & Ozaki, 1975). The phenotypic data also indicate that clusters 10 and 11 might represent two species but the spread of GC values (6–7%) in each suggests that to regard them as such would be premature. In addition, recent DNA–DNA hybridization studies (Hanson, 1980) have shown a fairly low level of homology between *Methylobacterium organophilum* and *Pseudomonas* AM1, both of which are in our cluster 10. Therefore, at present, we simply note that cluster 10 includes several of the strains considered by Stocks & McCleskey (1964) to belong to the same species and that cluster 11 largely conforms to the description of *Ps. mesophilica* (Austin & Goodfellow, 1979) and contains the type strain of that species.

The generic placement of the PPFMs is beset with problems, as was recognized by Kouno & Ozaki (1975). The strains in clusters 10 and 11 bear a confusing variety of generic labels (Table 1) but at least two are clearly not applicable. Stocks & McCleskey (1964) and Quayle (1972) expressed reservations about assigning the PPFMs to the genus *Vibrio*, the present definition of which (Shewan & Véron, 1974) excludes such strictly aerobic organisms. Similarly, the properties (apart from cellular pleomorphism) of strains allocated to *Corynebacterium* (Gräf & Bauer, 1973; see also below) are not consistent with current ideas about the circumscription of this genus (see, for example, Keddie & Bousfield, 1980).

Many PPFMs appear to have some characteristics of the genus *Pseudomonas* and are often assigned there (see Introduction for references). In the present study, however, the lack of association of clusters 10 and 11 with any of the pseudomonads *sensu* Doudoroff & Palleroni (1974) included suggests that such a classification may not be appropriate. Furthermore, close examination shows that the PPFMs have properties at variance with the present definition of *Pseudomonas* (Doudoroff & Palleroni, 1974). Thus, the Gram reaction is often equivocal, flagellation is not always polar and the cellular size, pleomorphism and

branching shown by most strains are not typical of the genus (Fig. 2*a*). In fact, these features led Gräf & Bauer (1973) to propose a new species of *Corynebacterium*, '*C. rubrum*', to accommodate red organisms they had isolated from tapwater. A representative strain of '*C. rubrum*' included here was a typical PPFM, recovered in cluster 10. [Gräf & Bauer used the name '*C. rubrum*' illegitimately, since it had already been applied legitimately (Crowle, 1962) but incorrectly (Goodfellow & Alderson, 1977) to another organism.]

Morphologically the PPFMs resemble members of the genus *Mycoplana* and some of the strains included in cluster 10 have been identified previously as '*My. rubra*' (M. E. Rhodes-Roberts, personal communication; P. N. Green, unpublished results). This species name was a new combination proposed by de Vries & Derx (1953) to accommodate '*Protaminobacter rubrum*' (den Dooren de Jong, 1927) and other pink, motile, branching organisms thought to be identical with it. The new combination was considered necessary because the strain of '*Pr. rubrum*' examined by de Vries & Derx appeared to fit the description of the genus *Mycoplana* (Gray & Thornton, 1928) but did not conform to that of '*Protaminobacter*' (den Dooren de Jong, 1927). Certainly the strains of '*Pr. ruber*' (spelling corrected by Breed *et al.*, 1957) examined in the present study are quite unlike the Gram-positive, white or yellow-pigmented, non-motile strains of '*Pr. alboflavus*', the type species of '*Protaminobacter*' (Leadbetter, 1974; NCIB unpublished records). In addition, the taxonomic soundness of the genus '*Protaminobacter*' is questionable (Leadbetter, 1974). However, our results suggest that despite morphological similarities, the PPFMs are not closely related to the type species of *Mycoplana*, *My. dimorpha* (the type strain, NCIB 9439, was recovered in cluster 2), and that therefore their allocation to this genus also would be inappropriate.

The first Gram-negative facultative methylotrophs shown to utilize methane were isolated by Patt *et al.* (1974), and a new genus, *Methylobacterium*, was subsequently proposed to accommodate them (Patt *et al.*, 1976). The genus contained one species, *Me. organophilum*, and both the generic and specific descriptions were based on the properties of the single pink-pigmented strain (NCIB 11278) recovered here in cluster 10. Similarities between this strain and other PPFMs were mentioned, but no direct comparison was reported. [However, more recent work has indicated that this strain is more closely related to the PPFMs than to other methylotrophs (Hanson, 1980).] The ability to utilize methane was regarded as sufficient to warrant the allocation of the strain to a new genus, the definition of which excluded methane non-utilizing (and thus almost all) PPFMs. Nevertheless, our results suggest there is little to distinguish *Me. organophilum* from many other PPFMs, apart from the ability to utilize methane. Even this property may be plasmid-borne in *Me. organophilum* and is easily lost (R. S. Hanson, personal communication; Hanson, 1980); for instance, our cultures of *Me. organophilum* no longer appear able to grow on methane. Thus, the use of facultative methanotrophy *per se* as a primary taxonomic criterion and especially as a major generic determinant should be viewed with caution. In our opinion this, together with the high level of similarity (>95% in some cases) of *Me. organophilum* NCIB 11278 to the other strains in cluster 10 makes the argument that this one strain belongs to a different genus difficult to uphold.

While *Me. organophilum* appears to be very similar to the other PPFMs, the relationship between the latter and two more recently described monotypic species, '*Me. ethanolicum*' and '*Me. hypolimneticum*' (Lynch *et al.*, 1980), is not clear. These two species were placed in *Methylobacterium* on the basis of their facultative methanotrophy but were considered sufficiently different from *Me. organophilum* and from each other to merit individual species status. We have not yet had the opportunity to examine cultures of these non-pink organisms, but a comparison between them, the PPFMs and other methylotrophs undoubtedly would be taxonomically useful. There is a need to establish if facultative methanotrophy in Gram-negative bacteria is indeed restricted to closely related organisms or if, like facultative methylotrophy, it can be found in taxonomically diverse groups.

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