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Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the Proteobacteria

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A polyphasic approach to bacterial taxonomy attempts to integrate phylogenetic relationships with phenotypic marker analysis. This study describes the application of membrane fatty acids as a phenotypic marker for methylotrophs. Detailed phospholipid, ester-linked fatty acid (PLFA) profiles are reported for 17 methylotrophic eubacterial strains. These profiles included verification of double bond positions and geometries, both critical features for this analysis. Multivariate cluster analysis was used to indicate groupings of these strains along with literature values of both methylotrophs and non-methylotrophs based on the PLFA phenotype. Like many phenotypic characteristics, PLFA profiles were influenced by environmental conditions. The instabilities displayed, however, were predictable from physiological studies including increased *trans/cis* and cyclopropyl/*cis* ratios. Cluster analysis of PLFA profiles generated by separate investigators with different culture conditions indicated reproducibility by strain and species. The PLFA phenotype relationships compare favourably with phylogenetic associations based on 16S rRNA data for methylotrophs and will continue to be a valuable phenotypic marker for Proteobacteria taxonomy.

Introduction

Studies of bacterial evolution are utilizing nucleic acid sequence analyses to propose new phylogenetic relationships which were not previously recognized with traditional classification techniques (Woese, 1987). This is especially true within the diverse Gram-negative eubacterial species once referred to as the purple bacteria and now termed the Proteobacteria (Murray *et al.*, 1990). Woese (1987) reminds microbiologists and evolutionary biologists, however, that phylogenies derived from such sequence data must not be over-interpreted. He suggests that these relationships be viewed as hypotheses to be tested and either strengthened or rejected using independent methodology. A recent report has re-emphasized the importance of a polyphasic taxonomic approach to Proteobacteria taxonomy, that is, the integrated use of both phylogenetic and phenotypic characteristics to test for evolutionary relationships (Murray *et al.*, 1990).

There are many diverse approaches to microbial chemotaxonomy. Chemical profiling techniques using chromatography or electrophoresis have been a common approach, and many such techniques have been recently reviewed (Goodfellow & Minnikin, 1985; Brondz & Olsen, 1986). Bacterial fatty acid analyses are one of the more popular profiling methods in chemotaxonomy (Tornabene, 1985).

For over a decade, this laboratory has been involved in microbial community structure analysis using membrane fatty acid profiles as a reproducible and quantitative method of investigating *in situ* ecological relationships (e.g. White *et al.*, 1979*a*, *b*; Guckert *et al.*, 1985; Guckert & White, 1988; Ringelberg *et al.*, 1989). Owing to the complexity of these fatty acid profiles, including multiple positional and geometric isomers of unsaturated fatty acids (e.g. Guckert *et al.*, 1986; Ringelberg *et al.*, 1989),

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Abbreviations: ECL, equivalent chain length; FAME, fatty acid methyl esters; PLFA, phospholipid, ester-linked fatty acid; SV, similarity value.

we have also been very sensitive to the analytical quality of these profiles, adapting many analytical verification procedures to these studies (e.g. Nichols *et al.*, 1986*a*; Guckert *et al.*, 1987).

Previous research in our laboratory indicated that one group within the Proteobacteria, the methylotrophs, had very diverse membrane fatty acid profiles with several unusual monounsaturated isomers (Nichols *et al.*, 1985; Jahnke & Nichols, 1986; Ringelberg *et al.*, 1989). The systematics of the methylotrophs is currently based on nucleic acid sequencing assays (Tsuji *et al.*, 1990; Bulygina *et al.*, 1990). This present study reports membrane fatty acid profiles for 17 methylotrophic eubacterial strains of which many have been placed on 16S rRNA phylogenetic trees (Tsuji *et al.*, 1990). In addition, we report groupings of these strains based on fatty acid profiles as a phenotypic marker, and these groupings are discussed in relation to literature profiles for both methylotrophs and non-methylotrophs along with their reported phylogenetic relationships. Discussions of membrane fatty acids as reproducible and stable phenotypic markers, as well as the use of these in a polyphasic approach to Proteobacteria taxonomy, especially for the methylotrophs, are also given.

Methods

Micro-organisms. Methylotrophic cultures were obtained as freeze-dried pellets from B. J. Bratina and R. S. Hanson of the Gray Freshwater Biological Institute, University of Minnesota. Descriptions of these strains are given in Table 1. Details of the origins of these strains are available in Tsuji *et al.* (1990), Tsien *et al.* (1990) or from R. S. Hanson. 16S rRNA phylogenetic trees have been produced which include nine of these strains (Tsuji *et al.*, 1990). The remaining strains are currently being sequenced for inclusion (Tsien *et al.*, 1990; B. J. Bratina & R. S. Hanson, unpublished). *Agrobacterium tumefaciens* (ATCC 33970) was obtained as a Preceptrol culture.

Culture conditions. Methylotrophic bacteria were grown as described by Tsuji *et al.* (1990) and Tsien *et al.* (1990) in a minimal salts medium (Dalton & Whittenbury, 1976) with either methane or methanol as the sole carbon source. In most cases, only one culture was analysed for each strain. Two independent replicates of the strain *Methylocystis parvus* OBBP were prepared. These replicates are abbreviated OBBP1 and OBBP2 in this study. Replicate cultures of *Methylosinus trichosporium* OB3b were also grown in a 1 μM-copper-supplemented medium to test for copper-induced membrane fatty acid changes. These replicates are abbreviated OB3b1a and OB3b1b for the 1 μM-, and OB3b0a and OB3b0b for the 0 μM-copper treatments, respectively. *A. tumefaciens* was grown at the University of Tennessee in Trypticase Soy Broth (BBL 11768, ATCC medium 18). Incubations to obtain cell mass for lipid extractions were conducted with continuous shaking at 27 °C for both 16 h (three replicates, exponential growth phase) and 64–120 hours (four replicates, stationary growth phase). This species is abbreviated AGRO in this study.

Extraction and preparation of membrane lipids. The equivalent of 20–25 mg dry weight of bacterial cells as extracted in a Bligh & Dyer (1959) single-phase solvent system modified to include phosphate buffer (White *et al.*, 1979*b*). During all procedures, care was exercised to minimize both contamination and artifactual changes in lipid structures using procedures recommended in Guckert & White (1988). Cells were extracted at room temperature in 142·5 ml chloroform/methanol/potassium phosphate buffer (1:2:0·8 by vol.; 50 mM, pH 7·4) for 3 h, at which time 37·5 ml each of chloroform and distilled water were added to separate the aqueous (upper) and organic (lower) phases overnight. The organic phase (containing the bacterial lipids) was collected and the solvent removed with a rotary evaporator at 37 °C. The total lipid extract was separated into lipid classes by silicic acid column chromatography as detailed in Guckert *et al.* (1985). The phospholipid-containing methanol fraction was further used in the evaluation of bacterial membrane lipid profiles. The phospholipid, ester-linked fatty acids (PLFAs) were prepared for gas chromatography (GC) analysis by a mild alkaline transesterification (Guckert *et al.*, 1985). The resultant fatty acid methyl esters (FAME) were separated, quantified and tentatively identified by capillary GC using a 50 m HP-1 (non-polar methyl silicone, Hewlett-Packard) column using the conditions described by Ringelberg *et al.* (1989). FAME structure was verified by GC/mass spectrometry (MS) as described in Ringelberg *et al.* (1989). Bacterial fatty acid double bond position and geometry was confirmed using GC/MS analysis of the dimethyl disulphide adducts of the monounsaturated FAME as described by Nichols *et al.* (1986*a*). Cyclopropyl FAME ring positions were determined by GC/MS analysis of hydrogenation products as reviewed by Guckert *et al.* (1985). Additional verification was done, as required, by equivalent chain length (ECL) analysis (Christie, 1989) with separations on several capillary GC column stationary phases

Table 1. *Descriptions of methylotrophic strains analysed in this study*

The pathway indicated is used to assimilate formaldehyde. Ribulose monophosphate pathway methylotrophs are historically referred to as 'type I' methylotrophs and serine pathway methylotrophs as 'type II' methylotrophs. Abbreviations given are used throughout this paper. Details of strains are available in Tsuji *et al.* (1990), Tsien *et al.* (1990) and references contained therein.

Strain	Pigments	Abbreviation
Ribulose monophosphate pathway methanotrophs		
Methylococcus capsulatus BATH		MCAP
Methylococcus luteus	Yellow	MLUT
Methylomonas methanica	Pink	MMET
Methylomonas gracilis	Brown	MGRA
Ribulose monophosphate pathway methylotrophs unable to use methane		
Methylobacillus glycogenes		MGLY
Methylomonas methanolica		MLIC
Methylomonas methylovora		MLOY
Methylophilus methylotrophus AS1		AS1
Methylotrophic sp. DM11		DM11
Serine pathway methanotrophs		
Methylosinus trichosporium OB3b		OB3b
Methylosinus methanica 81Z		81Z
Methylosinus sp. B		STR B
Methylocystis parvus OBBP		OBBP
Serine pathway methylotrophs unable to use methane		
Methylobacterium organophilum XX	Pink	XX
Methylobacterium extorquens AM1	Pink	AM1
Methylobacterium sp. DM4	Pink	DM4
Hyphomicrobium sp. DM2		DM2

including HP-1 (Hewlett-Packard), Rtx-1 (non-polar dimethyl polysiloxane, Restek) and Rtx-225 (polar 50% cyanopropylmethyl/50% phenylmethyl polysiloxane, Restek).

Fatty acid nomenclature. Fatty acids are designated as A:BωC, where A is the total number of carbon atoms, B is the number of double bonds, and C is the position of the double bond from the aliphatic (ω) end of the molecule. Geometry of this bond is indicated as 'c' for *cis* and 't' for *trans*. The prefixes 'i' and 'a' refer to *iso* and *anteiso* methyl-branching, respectively (Kates, 1986). Cyclopropyl fatty acids are designated as 'cy', with the ring position in parenthesis relative to the aliphatic (ω) end.

Literature sources of methylotrophic fatty acid profiles. Any successful phenotypic marker for chemotaxonomic studies must be directly comparable with literature values. Part of the data analysis presented in this study is a comparison of the PLFA profiles from the methylotrophs listed in Table 1 with similar and identical strain PLFA profiles available in the literature. Not all published methylotrophic PLFA profiles, however, are analytically precise enough to be included into a rigorous multivariate statistical analysis (see Wold *et al.*, 1984). For instance, the membrane fatty acid profile for *Methylosinus trichosporium* OB3b was first published in 1975 (Weaver *et al.*, 1975); however, no 16:1 or 18:1 isomers were separated. Nichols *et al.* (1985) reported that this same strain had several 18:1 isomers with 18:1ω8c, 18:1ω7c, and 18:1ω8t accounting for over 80% of the entire PLFA profile. The non-resolved data set of Weaver *et al.* (1975) is clearly not appropriate for broad chemotaxonomic studies.

Literature fatty acid profiles of sufficient quality were found for the following strains: *Methylococcus capsulatus* BATH, with several replicates grown under both high and low oxygen culture conditions (abbreviated MCAPhi, MCAPlo) (Jahnke & Nichols, 1986); *Methylomonas* sp. 761 (abbreviated 761); *Methylosinus trichosporium* OB3b (abbreviated OB3b); *Methylobacterium organophilum* XX (grown with either methane or methanol as sole carbon source and abbreviated as XXCH4 or XXMETH, respectively); and *Methylobacterium organophilum* RG (abbreviated RG) (Nichols *et al.*, 1985).

In addition to the inclusion of methylotrophic lipid profiles from the literature, several non-methylotrophic eubacteria were also included in this analysis. These had been previously assigned to specific divisions within the Proteobacteria using 16S rRNA analysis [after Woese (1987) and Devereux *et al.* (1989)] and had good-quality PLFA profiles published. These strains included: *Vitreoscilla stercoraria* and two other *Vitreoscilla* strains (Nichols *et al.*, 1986*b*); *Legionella* sp. (Pfiffner *et al.*, 1988); *Desulfovibrio* spp. (Edlund *et al.*, 1985); and *Desulfobacter* spp. (Dowling *et al.*, 1986).

Exponential growth phase *Escherichia coli* data from a recent review of microbial lipids (Ratledge & Wilkinson, 1988) were also included. This review did not specify double bond positions; however, this laboratory has verified the double bond and cyclopropyl ring positions for *E. coli* (unpublished data). The abbreviations used for these strains are given in the legend of Fig. 2.

Statistical analysis. All averaged data which include an indication of variability about the mean are shown ± sample SD. Data used in the calculations of these standard deviations are detailed in Results and Discussion. The bacterial membrane lipid profiles, or more specifically, their PLFA profiles were treated as multivariate data and analysed using cluster analysis. Dendrograms were constructed using a complete-linkage, farthest neighbour method with the PC-based software package Ein•Sight (Infometrix). The dendrograms presented are essentially identical to others produced with other clustering algorithms available both through Ein•Sight and the mainframe software package SPSSX (Version 3.0). In addition, the dendrograms were stable to the exclusion of minor (<0·1 mol%), unidentified PLFA and highly correlated PLFA. We feel, therefore, that the relationships presented are robust to the method of clustering utilized. The similarity values (1·00 = identical) given were determined by the Ein•Sight program using modified Euclidean distances and were stable to the inclusion and exclusion of different strains of eubacteria. A conservative minimum similarity value (SV) of 0·65 was used to define a clustered 'group'. Average PLFA profiles were calculated for members of groups or separate clusters within groups.

Results and Discussion

PLFA profiles are given for the 17 methylotrophic strains in Table 2. There are four unidentified (UNK 1, 2, 3, 4) fatty acids listed in Table 2. Characteristics of these fatty acids, including ECL values on two GC columns and GC/MS ion information, are given in Table 3. The GC/MS spectra and ECL values on the non-polar Rtx-1 column suggest that these are diunsaturated 18-carbon FAME. The ECL values on the more polar Rtx-225 are not consistent with this identification, with co-elution of these unknowns occurring with mono-unsaturated 18-carbon FAME. These unknown fatty acids were not shown to be an important discriminating parameter in the multivariate cluster analysis used to determine taxonomy relationships.

PLFA values are given in Table 2 as the percentage of total moles of PLFA for each strain (mol%). The total molar amount of PLFA recovered per gram dry weight of culture material is also given. The average PLFA content for all strains was 114 ± 87 μmol (g dry wt)$^{-1}$, which compares favourably to the reported value of 100 μmol (g dry wt)$^{-1}$ for 'typical' eubacteria (White *et al.*, 1979*a*). The range of this value is large [12·08–344·45 μmol (g dry wt)$^{-1}$], but this is due to media salts included in cultures during freeze-drying and will not affect the analysis.

Reproducibility of PLFA phenotypes

All strains in Table 2 are represented by one analysis except for *Methylocystis parvus* OBBP and *Methylosinus trichosporium* OB3b for which mean values for the replicates discussed above are given. The PLFA profiles for the methylotrophic strains in Table 2 (excluding the OBBP and OB3b mean values), the two replicates of OBBP (OBBP1 and OBBP2), the four samples of OB3b under different conditions of copper supplementation (OB3b0a, OB3b0b, OB3b1a, OB3b1b) and the literature methylotroph PLFA profiles were analysed by cluster analysis (Fig. 1; literature values are marked with the symbol '■' on the dendrogram). No effort was made to exactly mimic culture conditions for strains which have been previously analysed and published. In spite of this, replicate strains from different sources were closely clustered, as described below. The dendrogram (Fig. 1) has five groups (SV > 0·65), each marked with a star.

Table 2. *PLFA profiles of 17 methylotrophic eubacterial strains*

Results are expressed as a percentage of total PLFA recovered. Fatty acids are listed in their order of elution off a non-polar methyl silicone stationary phase (e.g. HP-1 or Rtx-1). Strain abbreviations are defined in Table 1.

Fatty acid	XX	AM1	DM4	81Z	DM2	SIN B	OBBP	OB3b	MCAP	MLUT	MGRA	MGLY	DM11	MLOV	MLIC	ASI	MME
14:0	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·01	2·35	3·45	0·00	0·33	1·12	0·54	0·79	1·28	20·76
15:0	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	1·22	0·00	2·31	0·20	0·00	0·30	0·17	0·00	0·3(
16:1ω8c	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	40·9(
16:1ω7c	0·00	5·34	6·41	9·90	0·00	15·19	0·67	10·71	18·66	47·93	45·20	55·68	43·84	37·21	37·60	35·47	10·8(
16:1ω6c	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	10·26	6·26	0·00	0·00	0·00	0·00	0·00	0·00	3·3(
16:1ω7t	0·00	0·00	0·00	0·00	0·00	0·64	0·00	0·37	8·54	0·00	0·00	0·00	0·00	2·08	0·00	0·72	1·9(
16:1ω6t	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·57
16:1ω5c	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	12·36	1·39	0·00	0·00	0·00	0·00	0·00	0·00	0·8(
16:1ω5t	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·38	17·79	0·00	0·00	0·00	0·00	0·00	0·00	15·5
16:0	6·36	4·94	3·05	1·52	3·04	1·02	0·26	1·17	32·53	15·19	52·49	36·15	39·83	42·60	41·32	43·32	4·2(
i17:0	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·06	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
a17:0	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·10	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
cy17:0(ω7,8)	0·00	0·00	0·00	0·26	0·00	0·00	0·00	0·00	7·37	0·00	0·00	1·96	14·52	11·30	12·79	18·47	0·0(
cy17:0b	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	5·17	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
17:0	0·40	0·22	0·22	0·00	0·21	0·00	0·00	0·03	0·00	0·00	0·00	0·25	0·00	0·35	0·25	0·00	0·0(
UNK 1	0·00	0·00	0·00	11·87	0·00	0·00	10·44	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
UNK 2	0·00	0·00	0·00	0·00	0·00	0·00	2·63	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
UNK 3	0·00	0·00	0·00	0·00	2·23	0·00	4·62	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
UNK 4	0·00	0·00	0·00	0·23	0·00	0·00	1·12	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
18:1ω9c	0·00	0·00	0·00	0·00	3·40	32·34	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
18:1ω8c	0·00	0·00	0·00	0·00	0·00	32·28	64·68	62·02	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
18:1ω7c	84·63	81·06	82·76	75·12	70·41	10·48	12·53	17·35	0·81	8·00	0·00	5·03	0·42	3·48	6·55	0·46	0·2(
18:1ω8t	0·00	0·00	0·00	0·00	0·00	7·57	1·71	7·50	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
18:1ω7t	0·00	0·00	0·68	0·00	0·46	0·00	0·00	0·00	0·14	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
18:1ω5c	0·00	0·00	0·00	0·00	0·36	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
18:0	8·61	8·44	6·88	1·01	12·20	0·47	0·36	0·66	0·21	0·00	0·00	0·40	0·27	0·62	0·52	0·29	0·0(
cy19:0(ω7,8)	0·00	0·00	0·00	0·08	7·35	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	1·52	0·00	0·00	0·0(
20:0	0·00	0·00	0·00	0·00	0·34	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·0(
Σμmol (g dry wt)$^{-1}$	48·5	67·24	42·51	92·52	25·03	160·02	135·04	105·35	260·96	12·08	52·74	93·64	161·07	44·88	144·91	156·81	344·4

Table 3. *Characteristics of unknown FAME listed in Table 2 including equivalent chain lengths (ECL) and GC/MS diagnostic ions (positive electron impact)*

Molecular ions (M$^+$) were also verified by positive chemical ionization GC/MS.

Unknown FAME	ECL		GC/MS Ions		
	Rtx-1	Rtx-225	M$^+$	Base	Others
UNK 1	17·48	18·10	294	55	262, 245, 220, 209
UNK 2	17·53	18·15	294	55	262, 245, 229, 209
UNK 3	17·56	18·21	294	67	262, 245, 220, 209
UNK 4	17·60	18·19	-	67	245, 220, 209

In group 1, the two literature strains of *Methylobacterium organophilum* XX (from Nichols *et al.*, 1985) and the sample obtained for this study group very closely along with *M. organophilum* RG (from Nichols *et al.*, 1985), *M. extorquens* AM1, and *Methylobacterium* sp. DM4 (SV = 0·90, Fig. 1). All of these strains are pink-pigmented methylotrophs which use the serine pathway to assimilate formaldehyde (Table 1). This group wa previously referred to as the 'type II' methylotrophs.

The other grouping of the serine methylotrophs was i group 2 and included the two replicates of *Methylocystis parvus* OBBP clustered at SV = 0·91, and five cultures o *Methylosinus trichosporium* OB3b [including one litera ture value from Nichols *et al.* (1985)] grouped a SV = 0·82.

The high- and low-oxygen incubation effects o membrane lipids of *Methylococcus capsulatus* BATI have been discussed by Jahnke & Nichols (1986). Th high-oxygen cultures formed one cluster (SV = 0·9 within group 4), whereas the low-oxygen culture was i group 3 with the strain grown by the University o Minnesota researchers (SV = 0·82). The cultures pro duced for this study were grown for maximum biomas production, principally for 16S rRNA sequence analysis Although they were incubated with constant agitatio (see Tsuji *et al.*, 1990 for details), it is likely that thi culture was also 'low-oxygen'. The other methylotrophi literature profile included in this analysis was that o *Methylomonas* sp. 761 (Nichols *et al.*, 1985), which wa most closely matched in group 5 to *Methylomona*

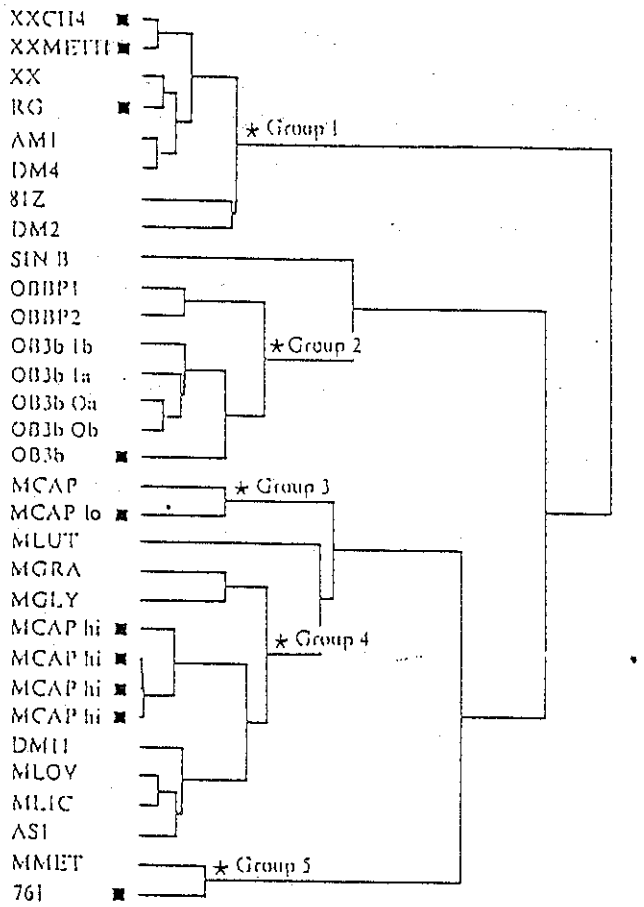

Fig. 1. Dendrogram of methylotrophic eubacterial PLFA profiles for strains detailed in the text. Values obtained from the literature are marked with a '■' symbol. Strain abbreviations are as shown in Table 1 and described in the text. Groups defined by an SV > 0·65 are marked with a star and labelled for discussion purposes.

methanica (SV = 0·77), a pink-pigmented methylotroph which uses the ribulose monophosphate pathway to assimilate formaldehyde. This group was previously referred to as the 'type I' methylotrophs.

Based on the reproducibility of these phenotypic markers for replicates from different sources (Fig. 1), the PLFA profiles for the three XX strains, the two OBBP strains, the five OB3b strains, the two low-oxygen MCAP strains, and the four high-oxygen MCAP strains were separately averaged for further chemotaxonomic analyses.

PLFA profiles and phenotype stability

To date only rRNA sequences have been shown to provide a reliable bacterial phylogeny because of their universality, genetic stability, and conservation of structure (Murray *et al.*, 1990). This phylogenetic analysis will not alone, however, provide conclusive taxonomic definitions for the eubacteria, and the importance of coupling these analyses to phenotypic descriptions is well established (Murray *et al.*, 1990). Phenotypic characteristics will only be stable under environmental conditions which do not modify genotypic expression. PLFA profiles are no different. For instance, oxygen concentration has been shown to influence lipid profiles of *Methylococcus capsulatus* BATH (Jahnke & Nichols, 1988; see Fig. 1). Not all changes in culture conditions will affect PLFA profiles, however, and PLFA changes that do occur will only occur within the range of biochemical modifications available to a particular genotype. Two examples are given below.

When *Methylosinus trichosporium* OB3b was grown in 0 and 1 μM-copper-supplemented medium, there were minimal PLFA profile changes (Table 4) although the addition of copper does promote significant physiological changes in this strain (R. S. Hanson, unpublished). Although not statistically significant, there was an increase in the *trans/cis* ratio on the addition of copper (Table 4). Since this is a well-documented indicator of physiological stress in eubacteria (see Guckert *et al.*, 1986, 1987, and discussion below), further work is being conducted to attempt to correlate the observed physiological changes with membrane lipid stress markers (D. B. Ringelberg, D. C. White & R. S. Hanson, unpublished).

Other eubacteria, like *Agrobacterium tumefaciens*, undergo what appear to be drastic PLFA changes during their normal growth phase. As shown in Table 4, when *A. tumefaciens* is in stationary growth phase, both the *trans/cis* and cyclopropyl/*cis* ratios for the 16:1ω7 and 18:1ω7 isomers significantly increase. Although these changes are significant, and would no doubt affect the classification of this species, they can also be predicted on the basis of known stress-induced biochemical modifications of the principal end products of eubacterial anaerobic desaturase fatty acid synthesis, 16:1ω7c and 18:1ω7c (see Guckert *et al.*, 1986 and references within for review). These modifications include a methyl donation across the ω7c double bond from *S*-adenosyl-L-methionine catalysed by cyclopropyl synthetase to form the corresponding cyclopropyl ring, e.g. 16:1ω7c → cy17:0(ω7,8) (see Guckert *et al.*, 1986 for discussion). Although this reaction is well characterized, it is not clear what benefit this metabolically-expensive stationary phase modification has to eubacterial survival. Even less is known about eubacterial *trans* PLFA synthesis, although the possibility of a constitutive isomerase along with preferential loss (degradation) of the *cis* isomer has been suggested as a mechanism for stress-induced increases in *trans/cis* ratios (Guckert *et al.*, 1986, 1987).

Table 4. *PLFA profile changes with culture conditions*

Results are expressed as mol% of total recovered, with known eubacterial lipid stress ratios included. Details of culture conditions are given in the text.

	Methylosinus trichosporium strain OB3b		*Agrobacterium tumefaciens* (ATCC 33970)	
Fatty acid	Low copper growth (0 μM-Cu)	High copper growth (1 μM-Cu)	Exponential phase (< 16 h)	Stationary phase (> 16 h)
14:0	0·02 ± 0·03	0·00 ± 0·00	0·15 ± 0·13	2·22 ± 0·15†
i15:0	0·05 ± 0·07	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00
15:0	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·73 ± 0·15†
16:1ω7c	10·87 ± 2·64	10·56 ± 0·75	4·99 ± 0·80	0·48 ± 0·19†
16:1ω7t	0·10 ± 0·07	0·63 ± 0·19	0·02 ± 0·03	0·06 ± 0·02
16:0	0·63 ± 0·59	1·72 ± 0·40	9·94 ± 3·01	19·31 ± 2·03†
i17:0	0·06 ± 0·06	0·05 ± 0·07	0·00 ± 0·00	0·00 ± 0·00
a17:0	0·10 ± 0·08	0·10 ± 0·14	0·00 ± 0·00	0·00 ± 0·00
cy17:0(ω7,8)	0·00 ± 0·00	0·00 ± 0·00	2·00 ± 1·74	22·15 ± 0·76†
17:0	0·06 ± 0·01	0·00 ± 0·00*	0·00 ± 0·00	0·06 ± 0·04
18:1ω8c	64·87 ± 2·42	59·16 ± 0·16	0·00 ± 0·00	0·00 ± 0·00
18:1ω7c	16·65 ± 0·71	18·06 ± 4·78	71·95 ± 11·81	4·68 ± 1·76†
18:1ω8t	6·06 ± 0·07	8·93 ± 5·54	0·00 ± 0·00	0·00 ± 0·00
18:1ω7t	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·28 ± 0·05†
18:0	0·53 ± 0·16	0·79 ± 0·17	0·60 ± 0·08	0·25 ± 0·02†
cy19:0(ω7,8)	0·00 ± 0·00	0·00 ± 0·00	10·35 ± 6·22	49·77 ± 3·22†
Trans/cis ratios				
16:1ω7	0·010 ± 0·009	0·061 ± 0·022	0·004 ± 0·006	0·128 ± 0·016†
18:1ω7	0 ± 0	0 ± 0	0 ± 0	0·065 ± 0·016†
18:1ω8	0·094 ± 0·004	0·151 ± 0·093	0 ± 0	0 ± 0
Cyclopropyl/*cis* ratios				
16:1ω7	0 ± 0	0 ± 0	0·37 ± 0·32	53·00 ± 24·37*
18:1ω7	0 ± 0	0 ± 0	0·16 ± 0·10	12·18 ± 5·57*

* Change in PLFA content significant ($0·01 < P < 0·05$) for changes in culture conditions.
† Change in PLFA content significant ($P < 0·01$) for changes in culture conditions.

The commercially available Microbial Identification System (Microbial ID Inc., Newark, DE, USA), which is based on the analysis of whole-cell, ester-linked fatty acid profiles, recognizes the importance of phenotypic stability to library searching for identification of unknown strains. This procedure requires the unknown strains to be grown on defined media under specific conditions to provide the biomass for lipid analysis (Microbial Identification System Operating Manual, Version 3.0).

Since the Microbial Identification System is directed at the identification of clinical isolates, the incubation medium and conditions are generally not too restrictive. The reproducibility of the PLFA profiles described above (and in Fig. 1) suggests that these extreme precautions might not be required for methylotrophs; however, one does need to be cautious of culture conditions, especially the growth phase. It is suggested that exponential growth phase biomass be used whenever possible and that PLFA profiles are monitored for known modifications occurring during cell stress, such as cyclopropyl formation and high (> 0·1) *trans/cis* ratios (Guckert *et al.*, 1986, 1987). Several methylotrophs h been shown to increase cyclopropyl/*cis* PLFA ratio stationary phase cultures (Urakami & Komagata, 19

Integrating PLFA phenotype and phylogenetic relationships

Taxonomic relationships for methylotrophic eubacte have been reported utilizing 16S rRNA (Tsuji *et* 1990), 5S rRNA (Ando *et al.* 1989; Bulygina *et al.*, 19 DNA base composition with DNA–DNA homolo (Urakami *et al.*, 1985; Hood *et al.*, 1987), as wel multivariate analyses of characters based on m phology, physiology and biochemistry (Green Bousfield, 1982; Jenkins & Jones, 1987). Most of th studies have not used the same strains discussed in work; hence, integration of relationships is difficult. have chosen, therefore, to interpret the PLFA phenot relationships by reference to the 16S rRNA phylogen work of Tsuji *et al.* (1990), from whom the test str used in the present study were obtained.

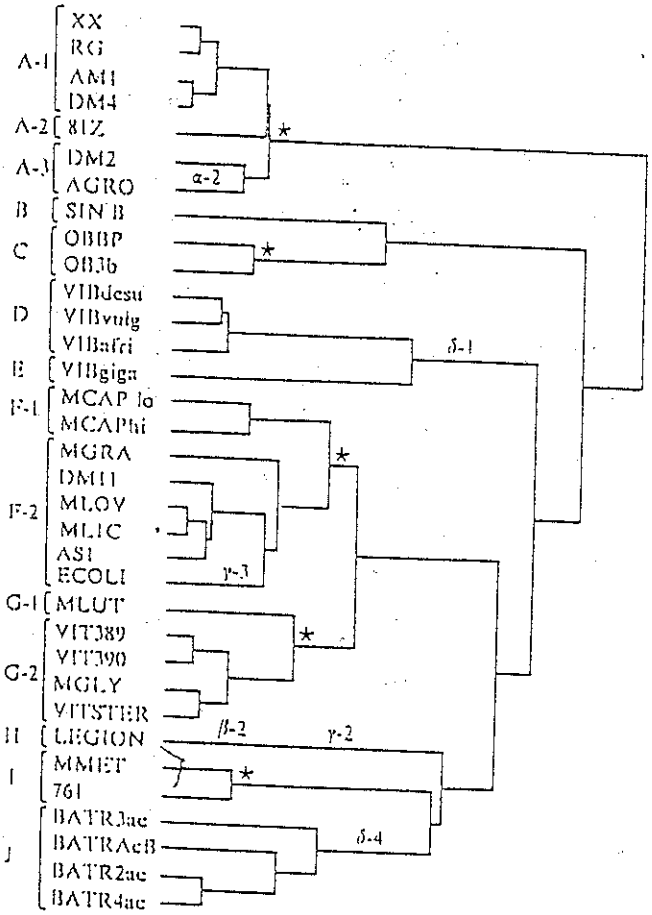

Fig. 2. Dendrogram of average methylotrophic PLFA profiles compared to non-methylotrophic species as described in the text. Groups (SV > 0·65) are marked with a star. Clusters, as presented in Table 5, are also marked for discussion purposes. Strain abbreviations are as shown in Table 1 and described in the text. Other abbreviations are as follows: *Desulfovibrio desulfuricans*, VIBdesu; *Desulfovibrio vulgaris*, VIBvulg; *Desulfovibrio africanus*, VIBafri; *Desulfovibrio gigas*, VIBgiga; *Escherichia coli*, ECOLI; *Vitreoscilla* sp. 389, VIT389; *Vitreoscilla* sp. 390, VIT390; *Vitreoscilla stercoraria*, VITSTER; *Legionella* sp., LEGION; *Desulfobacter* sp. 3ac10, BATR3ae; *Desulfobacter* sp. AcBa, BATRAeB; *Desulfobacter postgatei* 2ac9, BATR2ae; *Desulfobacter* sp. 4ac11, BATR4ae.

In addition to having 16S rRNA and PLFA results from the same methylotroph strains, we included PLFA profiles from several other eubacteria with well-established phylogenetic relationships. Fig. 2 shows the relationships for these Proteobacteria. Groups (SV > 0·65) are marked with a star. Separate clusters are also marked and detailed in Table 5 with the strains, SV and a rank order listing of the mean (±SD) for the characteristic PLFA from each cluster. In the left-hand column of Table 2, the 16S rRNA division is listed for the non-methylotrophic strains as reported in Woese (1987) or Devereux *et al.* (1989).

Tsuji *et al.* (1990) reported that the methylotrophs are not an evolutionarily coherent group, but are scattered throughout the α-, β- and γ-subdivisions of the Proteobacteria as defined by Woese (1987). The PLFA phenotypic analysis agrees with this conclusion. When non-methylotroph PLFA profiles from all subdivisions of the Proteobacteria (α-, β-, γ-, δ-) were included in the cluster analysis, methylotrophic strains grouped (SV > 0·65) with all eubacteria except members of the δ-subdivision, the sulphate-reducing bacteria (SRB). PLFA profiles differentiated the SRB from other Proteobacteria as well as the *Desulfovibrio* spp. group (δ-1) from the *Desulfobacter* spp. group (δ-4). The representative PLFA profiles from the literature for these groups are listed in Table 5; however, these groups will not be discussed further since they are both phenotypically and phylogenetically distinct from the methylotrophs.

Phylogenetic analysis has indicated that all methylotrophs using the serine pathway ('type II') to assimilate formaldehyde fall into two groups within the α-subdivision (Tsuji *et al.*, 1990). One group included the pink-pigmented 'type II' methylotrophs and the other most of the non-pigmented 'type II' methylotrophs. PLFA analysis provided the same results. The 'type II' pigmented strains were tightly clustered (cluster A-1, Fig. 2) due to characteristically high proportions of 18:1ω7c (Table 5). Non-pigmented serine pathway methylotrophs were divided between clusters A-2 and A-3, which could be grouped (SV > 0·65) with A-1, and clusters B and C (Fig. 2).

Agrobacterium tumefaciens is a common reference strain for phylogenetic discussions and has been established within the α-2 subdivision (Woese, 1987). Its PLFA profile is included in the group of clusters A-1, A-2 and A-3 (Fig. 2), suggesting agreement between the PLFA and 16S rRNA taxonomic placement of the serine pathway methylotrophs in the α subdivision.

A potential discriminating feature of these pink-pigmented methylotrophs (cluster A-1) and the two most similar non-pigmented serine pathway methylotrophs (81Z and DM2) is the level of cy19:0(ω7,8) (Table 5). As discussed above, this value may be influenced by culture conditions, so a definitive analysis of phenotypic relationships for this group would require strict attention to culture conditions. It is curious, though, that with over 80% of their PLFA as 18:1ω7c, there was no cy19:0(ω7,8) detected for any of the pink-pigmented methylotrophs using the serine pathway to assimilate formaldehyde (Table 2). Urakami & Komagata (1979) also noted that not all methylotrophic species in their study increased their cyclopropyl content during stationary phase. The lack of cyclopropyl synthetase may, therefore, provide another key characteristic for differentiating this group.

Both the 16S rRNA and PLFA analyses divided the serine pathway methylotrophs into two groups. The

Table 5. *PLFA characteristics of clustered strains*

16S rRNA division*	Strains grouped	SV	Mol% PLFA (mean ± SD)		Clusters from Fig. 1
	Methylobacterium organophilum XX		18:1ω7c	(83·6 ± 2·4)	
	M. organophilum RG	0·92	18:0	(8·2 ± 1·6)	
	M. extorquens AM1		16:0	(3·4 ± 1·1)	A-1
	Methylobacterium sp. DM4		16:1ω7c	(2·9 ± 3·4)	
			18:1ω7t	(1·2 ± 1·0)	
	Methylosinus methanica 81Z		(see Table 2 for profile)		A-2
	Hyphomicrobium sp. DM2		18:1ω7c	(71·2 ± 1·1)	
α-2	*Agrobacterium tumefaciens*	0·86	cy19:0(ω7,8)	(8·8 ± 2·1)	
			16:0	(6·5 ± 4·9)	A-3
			18:0	(6·4 ± 8·2)	
			16:1ω7c	(2·5 ± 3·5)	
	Methylosinus sp. B		(see Table 2 for profile)		B
	Methylocystis parvus OBBP	0·83	18:1ω8c	(62·1 ± 3·7)	
	Methylosinus trichosporium OB3b		18:1ω7c	(15·7 ± 4·5)	
			16:1ω7c	(5·5 ± 6·9)	C
			UNK 1	(5·2 ± 7·4)	
			18:1ω7t	(4·8 ± 4·3)	
δ-1	*Desulfovibrio desulfuricans*		i17:1ω7c	(28·7 ± 3·9)	
δ-1	*D. vulgaris*	0·88	16:0	(18·0 ± 3·8)	
δ-1	*D. africanus*		i15:0	(16·3 ± 4·1)	
			16:1ω7c	(8·5 ± 2·5)	
			i17:0	(8·2 ± 0·2)	D
			a15:0	(3·5 ± 1·6)	
			18:0	(3·1 ± 2·2)	
			18:1ω7c	(2·9 ± 2·2)	
			a17:0	(2·5 ± 1·2)	
			a17:1ω7c	(2·3 ± 2·0)	
δ-1	*Desulfovibrio gigas*		(see Edlund *et al.*, 1985 for profile)		E
	Methylococcus capsulatus BATH		16:0	(39·5 ± 3·6)	
	grown under low and high O_2	0·84	16:1ω7c	(27·1 ± 6·7)	
			16:1ω6c	(10·5 ± 3·4)	
			16:1ω5c	(7·9 ± 2·7)	F-1
			14:0	(4·5 ± 2·2)	
			cy17:0(ω7,8)	(3·8 ± 3·9)	
			cy17:0(ω6,7)	(3·4 ± 5·3)	
	Methylomonas gracilis		16:0	(43·3 ± 4·7)	
	Methylotrophic sp. DM11	0·77	16:1ω7c	(38·5 ± 5·0)	
	Methylomonas methylovora		cy17:0(ω7,8)	(10·4 ± 6·7)	F-2
	Methylomonas methanolica		18:1ω7c	(4·5 ± 6·2)	
	Methylophilus methylotrophus AS1				
γ-3	*E. coli*				
	Methylococcus luteus		(see Table 2 for profile)		G-1
	Methylobacillus glycogenes	0·87	16:1ω7c	(52·9 ± 2·9)	
β-2	*Vitreoscilla stercoraria*		16:0	(30·8 ± 4·1)	
	Vitreoscilla spp. strains 389, 390		18:1ω7c	(7·5 ± 1·8)	G-2
			14:0	(5·3 ± 4·3)	
γ-2	*Legionella* sp.		i16:0	(34·6 ± 3·0)†	
			a15:0	(19·1 ± 3·3)	
			a17:0	(15·0 ± 2·4)	
			16:1ω7c	(11·5 ± 1·0)	H
			i16:1	(7·3 ± 0·4)	
			a17:1	(2·8 ± 0·5)	
			16:0	(1·4 ± 0·4)	
			18:0	(1·0 ± 0·2)	

Table 5—*continued*

16S rRNA division*	Strains grouped	SV	Mol% PLFA (mean ± sd)		Clusters from Fig. 1
	Methylomonas methanica	0·86	16:1ω8c	(35·4 ± 7·7)	
	Methylomonas sp. 761		14:0	(19·3 ± 2·0)	
			16:1ω7c	(14·3 ± 4·9)	
			16:1ω5c	(8·6 ± 10·9)	I
			16:1ω5t	(7·9 ± 10·9)	
			16:0	(5·6 ± 1·9)	
			16:1ω7t	(3·8 ± 2·6)	
			16:1ω6c	(1·7 ± 2·4)	
δ-4	*Desulfobacter* sp. 3ac10	0·67	16:0	(27·0 ± 6·3)	
δ-4	*Desulfobacter* sp. AcBa		cy17:0(ω7,8)	(22·2 ± 10·4)	
δ-4	*Desulfobacter postgatei* 2ac9		10Me16:0	(15·4 ± 6·9)	
δ-4	*Desulfobacter* sp. 4ac11		14:0	(15·2 ± 10·8)	J
			16:1ω7c	(4·8 ± 2·6)	
			i15:0	(3·4 ± 3·6)	
			16:1ω5c	(1·8 ± 0·2)	
			cy19:0(ω7,8)	(1·3 ± 1·5)	

* After Woese (1987) and Devereux *et al.* (1989).
† Variability for group as reported by Pfiffner *et al.* (1988).

second PLFA group was characterized by PLFA which include 18:1ω8c. These strains were *Methylosinus* sp. B (in cluster B), *Methylocystis parvus* OBBP and *Methylosinus trichosporium* OB3b (both in cluster C). The membrane lipids of OB3b have been the subject of several studies, in fact the results of Nichols *et al.* (1985) are included in this analysis (Fig. 1). We have emphasized the importance of adequate analytical verification of PLFA structure for this type of analysis by pointing out that in an initial description of the membrane fatty acids of OB3b (Weaver *et al.*, 1975) the 18:1 isomers were not separated. Note that if all 18:1 isomers were not resolved for this analysis, the cluster of OBBP and OB3b (cluster C) would contain approximately 78% 18:1 and would not be separable from the pink-pigmented 'type II' methylotrophs (Table 5).

The 16S rRNA phylogenetic analysis for the 'type I' (ribulose monophosphate pathway) methylotrophs suggested a split into the β- and γ-subdivisions of the Proteobacteria (Tsuji *et al.*, 1990). 'Type I' methylotrophs are generally characterized by a dominance of 16:1 isomers with few PLFA of any longer chain lengths (Nichols *et al.*, 1985). The PLFA analysis indicated two principal groups either clustered with *E. coli* (γ-3, cluster F-2) or *Vitreoscilla stercoraria* (β-2, cluster G-2) (Fig. 2) with some outliers.

One of these outliers was *Methylococcus capsulatus* BATH (MCAP, cluster F-1). The physiology of this organism is apparently not completely resolved and it has been described as a 'type X' methylotroph (Whittenbury & Dalton, 1980; Tsuji *et al.*, 1990). The PLFA phenotype of this strain is unusual due to the presence of the 16:1ω6c isomer (Table 5), regardless of the oxygen content of the culture medium (Jahnke & Nichols, 1986). Another strain which contained 16:1ω6c was *Methylomonas methanica* (MMET, Table 2), a pink-pigmented ribulose monophosphate methylotroph (Table 1). The 16S rRNA phylogenetic analysis suggested that both of these strains fall into the γ subdivision, although sequence differences between the two were over 20% (Tsuji *et al.*, 1990). Although they both have the unusual 16:1ω6c, there is a clear separation by PLFA analysis. MCAP is most similar to the group of methylotrophs grouped with *E. coli*, a γ-3 organism. MMET is most closely related to another *Methylomonas* strain, 761 (see Nichols *et al.*, 1985) due to its diversity of 16:1 isomers: 16:1ω8c, 16:1ω7c, 16:1ω7t, 16:1ω6c, 16:1ω6t, 16:1ω5c and 16:1ω5t (Table 5).

The other ribulose monophosphate obligate methylotrophs included in the 16S rRNA tree of Tsuji *et al.* (1990) were *Methylophilus methylotrophus* AS1 and methylotrophic DM11. These were found to be most closely phylogenetically related to eubacteria in the β-3 subdivision. The PLFA phenotypes for these strains are also very similar (Table 5) and group with the other ribulose monophosphate methylotrophs (cluster I, Fig. 2); *Methylomonas gracilis*, *Methylomonas methylovora* and *Methylomonas methanolica*. This group of methylotrophs, however, had a profile similar to that of the γ-3 *E. coli*, rather than a β-subdivision eubacterium like *Vitreoscilla*. Woese (1987) notes that the β- and γ-subdivisions are very closely related and may, in fact, be branches of the same, larger subdivision. Additional PLFA profiles from these subdivisions of the Proteo-

bacteria are needed to help determine these relationships.

In conclusion, we have reported PLFA profiles for 17 methylotrophs with sufficient analytical detail for use in future taxonomic evaluations. The ability to include such literature data for multivariate analyses was demonstrated. Cluster analysis of PLFA profiles generated by separate investigators with different culture conditions indicated reproducibility by strain and species. The methylotroph data have shown that these profiles are not 'inadequate for varieties of micro-organisms with similar metabolic traits' (Tsuji *et al.*, 1990). The phenotypic relationships described here compare favourably with phylogenetic associations based on 16S rRNA data for these same strains (Tsuji *et al.*, 1990), and will continue to be a valuable phenotypic marker for Proteobacteria taxonomy.

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