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A direct comparison between fatty acid analysis and intact phospholipid profiling for microbial identification

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Abstract

Two chemical methods for characterization of micro organisms were compared: phospholipid ester-linked fatty acid (PLFA) analysis by gas chromatography/mass spectrometry, and intact phospholipid profiling (IPP) using liquid chromatography/electrospray ionization/mass spectrometry. Both methods were tested on five reference pseudomonad strains: *Pseudomonas putida* mt-2, *Pseudomonas putida* F1, *Burkholderia cepacia* G4, *Burkholderia pickettii* PKO1, and *Pseudomonas mendocina* KR1. PLFA detected eight major fatty acids in these pseudomonads, ranging in chain length from C_{14} to C_{19}. IPP detected 16 phospholipids in three different classes: phosphatidylglycerol, phosphatidylethanolamine and phosphatidyl-dimethylethanolamine. Factor analysis of the data showed that IPP is superior to the PLFA technique in microbial differentiation and identification.

Keywords: Fatty acids; Intact phospholipid profile; *Pseudomonas*; *Burkholderia*; GC/MS; LC/ESI/MS

1. Introduction

Traditional isolation and culture techniques are inadequate for microbial characterization in environmental studies because the techniques (1) are selective and not quantitative (Vestal and White, 1989; White et al., 1997), (2) provide little insight into microbial consortium interactions (White et al., 1997), and (3) may introduce disturbance artifacts because these techniques involve subsampling and separation of micro organisms from the environmental matrix (Findlay et al., 1990). Furthermore, most micro organisms in the environment are viable but not cultivable (Xu et al., 1982; McCarthy and Murray, 1996). Viable counts of bacteria in environmental samples determined with classical methods represent only a small fraction (0.1%–10%) of the active microbial community (White et al., 1997).

Catabolic gene and 16S rRNA probes have been used successfully to assess the presence of specific micro organisms in environmental samples (e.g. Sayler et al., 1985). Such methods, however, are labor intensive and experience limitations when measuring community functionality under stress or competition (Findlay, 1996). Moreover, since the sequence of the universal primers is based on cultured organisms, the applicability of this technique for community analysis in environmental samples remains questionable (Pace, 1996). Such limitations have motivated the development of chemical characterization techniques to determine microbial biomass and community structure without prior isolation and cultivation of micro organisms.

Current approaches used for chemical characterization of microbial populations in natural environments include two techniques that analyze the cell membrane phospholipids. These are (1) phospholipid ester-linked fatty acid (PLFA) analysis by gas chromatography/mass spectrometry (White et al., 1979), and (2) intact phospholipid profiling (IPP) using liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/

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MS) analysis of bacterial membrane phospholipids (Fang and Barcelona, 1998). Both techniques rely on the fact that phospholipids are found in the membranes of all living cells, but not in storage lipids, and are rapidly turned over in dead cells. Thus, their quantification provides an estimation of viable biomass (Balkwill et al., 1988).

Identification of microorganisms by either PLFA or IPP is subject to potential confounding effects of overlapping phospholipid profiles and potential changes in phospholipid composition due to differences in growth conditions (Haack et al., 1993; White et al., 1997). Nevertheless, both techniques can give valuable insight into microbial community structure, based on the premise that there are a great number of dissimilar fatty acids in bacterial phospholipids and some bacteria contain unique fatty acids.

The aim of this study was to characterize the PLFA and IPP of five reference pseudomonad strains and compare the efficacy of these two methods for bacterial differentiation and identification.

2. Materials and methods

2.1. Bacterial cultures

Pseudomonas putida F1, *P. putida* mt-2, *Burkholderia cepacia* G4, *B. pickettii* PKO1, and *Pseudomonas mendocina* KR1 were selected for the application of microbial phospholipid profiling. These reference strains represent the archetypes of organisms using the five known aerobic degradation pathways of toluene (Zylstra, 1994). All cells were grown at about 25°C on mineral salts base medium (Stanier et al., 1966) amended with 10 mM succinate. Cells were harvested in late exponential growth phase, washed, and resuspended in mineral medium to an OD_{600} of about 0.5.

2.2. Lipid extraction

Total lipids were extracted with a modified Bligh and Dyer extraction method (White et al., 1979; Fang and Findlay, 1996). Approximately 5 ml of liquid bacterial culture were added to a test tube filled with 20 ml of methanol, dichloromethane (DCM), and phosphate buffer (2:1:0.8) extraction solution. The extraction mixture was allowed to stand overnight in darkness at 4°C. The lipids were then partitioned by adding DCM and water such that the final ratio of DCM-methanol-water was 1:1:0.9. The upper aqueous phase was discarded and the lower organic phase was decanted through a cellulose No. 4 filter into a test tube. The solid residue retained on the filter was washed with 3×1 ml DCM. The total lipid extract was dried under a gentle stream of nitrogen and then dissolved in methanol.

2.3. Column chromatography

Total lipids were separated into different lipid classes using miniature champagne columns (Supelco Inc., Bellefonte, PA). Neutral lipids, glycolipids, and phospholipids were obtained by eluting with 4 ml of chloroform, acetone, and methanol, respectively. The phospholipid fraction was split into two parts, one for intact phospholipid profiling using liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS), and the other for determinations of fatty acid methyl esters using gas chromatography/mass spectrometry (GC/MS).

2.4. Analysis of fatty acid methyl esters (FAMEs) by gas chromatography/mass spectrometry (GC/MS)

Ester-linked phospholipid fatty acids were subjected to a mild alkaline trans-methylation procedure to produce fatty acid methyl esters (Fang and Findlay, 1996). Double-bond position and geometry of monounsaturated fatty acids were determined by using methods described by Dunkelblum et al. (1985).

FAMEs were analyzed on a Hewlett-Packard 5890 II GC interfaced with an HP 5972 Mass Selective Detector. Analytical separation of the compounds was accomplished using a 30-m×0.25-mm i.d. (0.25 μm film thickness) DB-5 MS fused-silica capillary column (J&W Scientific, Folsom, CA). The column temperature was programmed from 50 to 120°C at 10°C/min, then to 280°C at 3°C/min. Response factors were obtained for each compound. The concentrations of individual compounds were calculated based on the chromatographic response of the internal standard $C_{18:0}$ ethyl ester after correction for recovery efficiency using $C_{22:0}$ ethyl ester as a surrogate standard.

Method blanks were extracted with each set of samples and were assumed to be free of contamination if chromatograms of the blanks contained no peaks. A standard containing known concentrations of eleven fatty acids was analyzed daily on the GC/MS instrument to check analytical accuracy (>90%). Duplicate analyses of samples were done to ensure reproducibility (variation ≤20%).

2.5. Liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS)

The LC/ESI/MS analysis was performed on a HP 1090 liquid chromatography/HP 5989B single quadrupole mass spectrometer with an electrospray interface (Fang and Barcelona, 1998). The LC was equipped with a 250 μl sample loop. A HP reverse phase HPLC column (Zorbax 150 × 4.6 mm, 5 μm) was used for the chromatographic separation of phospholipids. A gradient solvent system composed of solvent A (10 mM

ammonium acetate), solvent B (methanol) and C (acetonitrile) was used with a flow rate of 0.5 ml/min. At the beginning of the gradient, the mobile phase was 25% of A and 75% of B for 2 min. Solvent B was increased to 90% and solvent C increased to 10% at 30 min. The mobile phase was then held isocratically for 5 min.

The mass spectrometer was operated in the negative ionization mode and was scanned from 70 to 1000 at approximately 0.4 scans/s. The concentrations of phospholipids were calculated based on chromatographic area response of individual phospholipids relative to that of an internal standard (18:1-lyso-phosphatidylglycerol) and are reported as μg/ml of liquid culture.

2.6. Nomenclature

Fatty acids are designated according to convention by the total number of carbon atoms:number of double bonds (i.e. a 16 carbon alkanoic acid is $C_{16:0}$). The position of the double bond is indicated with an ω number closest to the aliphatic methyl end of the fatty acid molecule. Phospholipids are designated as follows: $C_{1:d1}/C_{2:d2}$-PL (e.g., $C_{16:0}/C_{18:1}$-PG), where C_1 and C_2 are the numbers of carbon atoms in the fatty acyl chains on the *sn*-1 and *sn*-2 positions, respectively; d1 and d2 are the numbers of double bonds of the *sn*-1 and *sn*-2 fatty acyl chains, respectively; PL is the abbreviation for phospholipids (Table 1). Positions of fatty acids on the *sn* positions were determined based on the ratio of intensity of fragment ions representing each fatty acid (Fang and Barcelona, 1998).

2.7. Factor analysis

Factor analysis (STATISTICA, Tulsa, OK) was conducted to compare the microbial differentiation and identification capabilities of the IPP and PLFA techniques. Phospholipid and fatty acids concentrations were analyzed without any sort of data normalization, and there were no null values in the data set. In the factor

Table 1
Concentrations (μg/ml) of phospholipid ester-linked fatty acids and intact phospholipids detected in five pseudomonad strains

Peak[a]	Compound	F1	mt-2	G4	KR1	PKO1
Phospholipid ester-linked fatty acids						
1	14:0	0.01	0.01	0.01	0.10	0.02
2	16:1 ω9	0.43	0.19	0.19	1.48	0.72
3	16:1 ω7	0.24	0.03	< 0.01	0.02	< 0.01
4	16:0	1.78	0.46	0.40	2.46	1.75
5	17:1	0.25	0.01	0.02	0.06	0.12
6	18:1 ω9	2.49	1.48	1.32	7.87	4.34
7	18:0	0.02	0.02	0.01	0.01	0.04
8	19:1	0.01	0.01	0.01	0.01	0.01
Phospholipids[b]						
1	14:0/16:1-PG	< 0.1	< 0.1	< 0.1	< 0.1	9.8
2	16:0/16:1-PG[c]	< 0.1	< 0.1	< 0.1	< 0.1	11.6
3	16:1/16:1-PG	< 0.1	< 0.1	< 0.1	2.7	< 0.1
4	18:1/16:1-PG[c]	< 0.1	1.8	< 0.1	< 0.1	10.6
5	16:1/16:1-PDME	< 0.1	5.1	< 0.1	< 0.1	< 0.1
6	16:0/16:1-PG[c]	4.4	12.1	32.4	38.9	18.7
7	18:1/16:1-PG[c]	26.6	1.1	5.7	30.2	22.3
8	16:0/17:1-PG	30.3	< 0.1	< 0.1	< 0.1	13.9
9	16:0/16:0-PG[d]	< 0.1	< 0.1	< 0.1	19.0	< 0.1
10	16:0/17:1-PG	< 0.1	< 0.1	0.6	< 0.1	< 0.1
11	17:1/18:1-PG	4.6	< 0.1	< 0.1	8.5	5.4
12	18:1/18:1-PG	< 0.1	< 0.1	< 0.1	1.1	< 0.1
13	16:1/16:1-PE	< 0.1	2.0	< 0.1	3.5	< 0.1
14	16:0/16:1-PE	32.1	1.9	0.3	7.4	6.3
15	18:1/16:1-PE	25.3	9.8	2.3	46.3	16.0
16	17:0/17:1-PE	17.3	< 0.1	< 0.1	3.0	1.7

[a] Peak numbers are depicted in Fig. 1.

[b] Phospholipid head group designation: PG = phosphatidylglycerol, PE = phosphatidylethanolamine, and PDME = phosphatidyldimethylethanolamine.

[c] Phospholipids 2 and 6, and 4 and 7 have the same designation because they contain fatty-acid isomers that differ only in the position of their double bonds.

[d] 16:0/16:0-PG co-eluted with 16:0/17:1-PG and 18:1/16:1-PG.

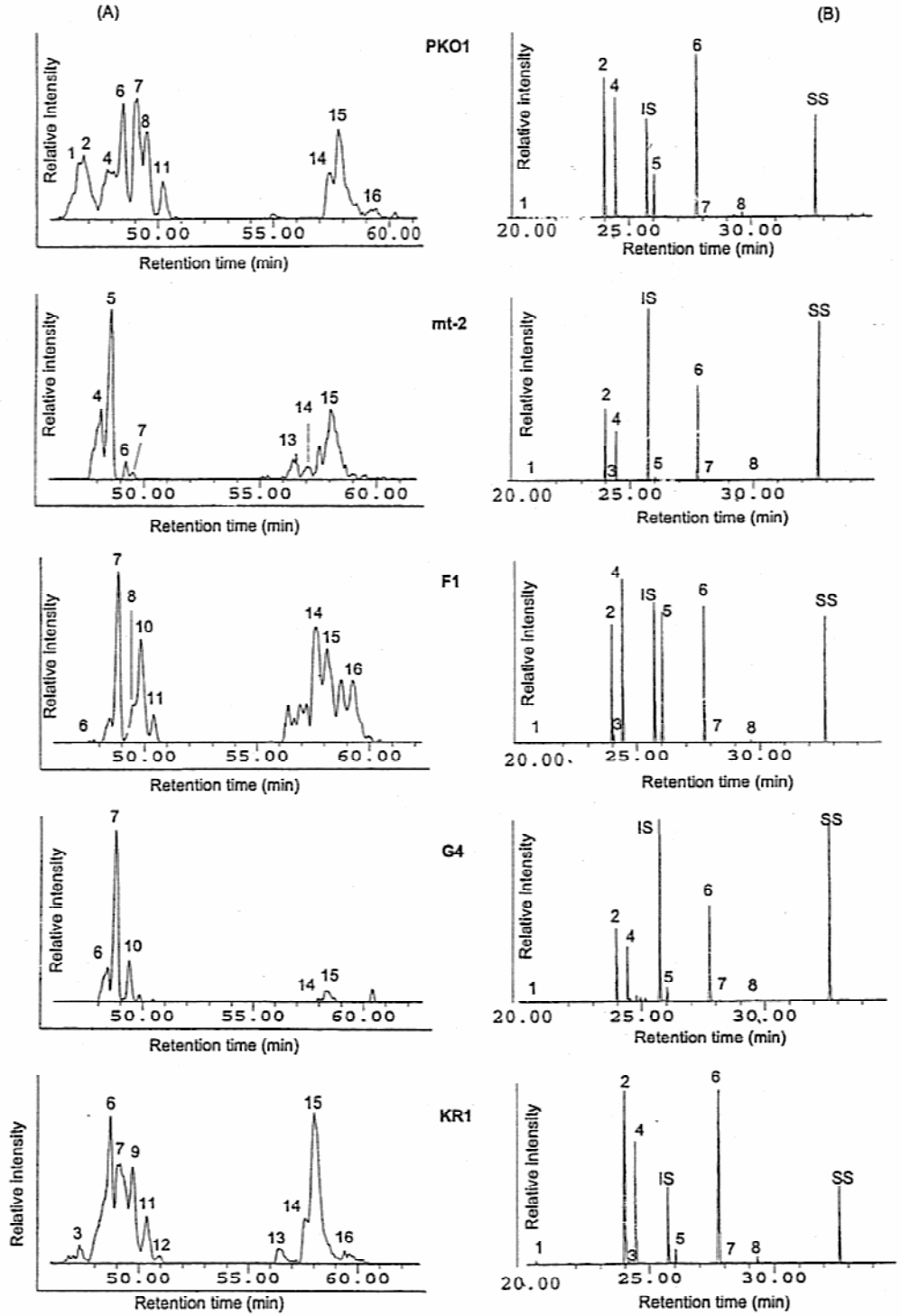

Fig. 1. LC/MS total ion chromatograms of intact phospholipids (A) and GC/MS total ion chromatograms of phospholipid ester-linked fatty acids (B) from five pseudomonad strains grown on succinate. PKO1 = *Burkholderia pickettii* PKO1; mt-2 = *Pseudomonas putida* mt-2; F1 = *P. putida* F1; G4 = *B. cepacia* G4; KR1 = *P. mendocina* KR1. IS = internal standard ($C_{18:1}$-lyso-phosphatidylglycerol); SS = surrogate standard ($C_{22:0}$ ethyl ester). For compound identification (peak numbers), see Table 1.

analysis, the original variables (phospholipids and fatty acids) were orthogonally transformed and new uncorrelated (or orthogonal) variables called factors were extracted consecutively. The factors are independent of each other. The first factor accounts for most of the original variability; the second factor contains the second largest variability, etc., as indicated by the eigenvalues. The number of factors retained was determined by the Kaiser criterion (eigenvalues $=1$) (Kaiser, 1960). The relationships between bacteria can be evaluated based on factor loadings plots (e.g. Fig. 2). Bacteria that cluster together (i.e. that have similar values on factors) would have similar phospholipid or PLFA compositions.

3. Results and discussion

The phospholipid ester-linked fatty acids (PLFA) and intact phospholipid profiles (IPP) of the five archetypes of pseudomonad strains are shown in Fig. 1 and Table 1. A total of eight different fatty acids were detected in the pseudomonad strains (Table 1). All of the strains except G4 and PKO1 contained two hexadecenoic acid isomers. $C_{18:1}$ ω9 was the dominant fatty acid in all strains, followed by $C_{16:1}$ ω9 and $C_{16:0}$. The PLFA patterns of the pseudomonad strains were quite similar, based on visual observation of the chromatograms (Fig. 1) and the relatively high Pearson's correlation coefficients for $\alpha = 0.01$ (highly correlated) (Table 2).

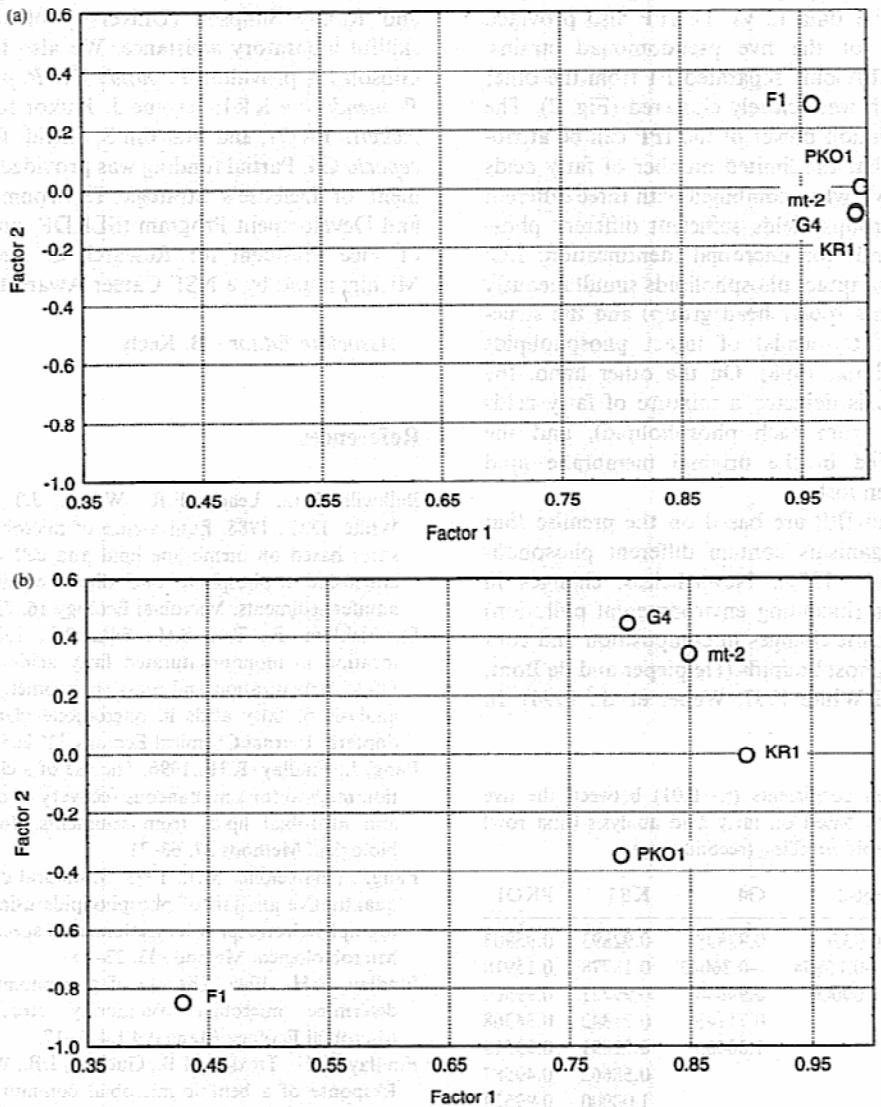

Fig. 2. Factor analysis of phospholipid ester-linked fatty acids (a) and intact phospholipid profiling (b) of the five pseudomonad strains.

Sixteen major intact phospholipids were detected in the five archetypes of pseudomonad strains (Table 1). All of the fatty acids identified in the PLFA analyses were detected in the intact phospholipid profiles except $C_{19:1}$, probably because of its low concentration. The fatty acids were distributed in three different classes of phospholipids: phosphatidylglycerol, phosphatidylethanolamine and phosphatidyldimethylethanolamine. The phospholipid profiles of the five pseudomonad strains were statistically distinguishable, based on the relatively low Pearson's correlation coefficients for $\alpha = 0.01$ (Table 2).

Factor analysis was applied to the PLFA and IPP data to compare the efficacy of these methods for microbial identification (Fig. 2). The number of variables with high loadings ($>|1.0|$) was greater in IPP data than in PLFA data (2 vs. 1). IPP also provided better separation of the five pseudomonad strains, whereas using PLFA only separated F1 from the other four strains, which were closely clustered (Fig. 2). The superior differentiation power of the IPP can be attributed to the fact that the limited number of fatty acids (eight in this study), when combined with three different classes of head groups yields sufficient different phospholipid compounds for microbial identification. LC/ESI/MS analysis of intact phospholipids simultaneously determines the class (polar head group) and the structure (individual fatty acids) of intact phospholipids (Fang and Barcelona, 1998). On the other hand, the total PLFA analysis detected a mixture of fatty acids (two fatty acids from each phospholipid), and the information carried in the original membrane lipid molecules had been lost.

Both PLFA and IPP are based on the premise that different microorganisms contain different phospholipids (White et al., 1979). Nevertheless, changes in growth conditions (including environmental pollution) can induce significant changes in composition and content of microbial phospholipids (Heipieper and de Bont, 1994; Pinkart and White 1997; Weber et al., 1994). In addition, lipid-pattern overlap could confound the taxonomic identification of individual strains when mixed cultures are analyzed. Therefore, caution should be exercised in using PLFA or IPP for taxonomic identification of samples from contaminated environments. The true power and limitations of the IPP method will become evident as more well-characterized microbial cultures and functional groups are examined in detail and future improvements in methodology allow the fatty-acid double bond positions to be determined in intact phospholipids.

Table 2
Pearson's correlation coefficients ($\alpha = 0.01$) between the five pseudomonad strains based on fatty acid analysis (first row) and intact phospholipid profiling (second row)

	F1	mt-2	G4	KR1	PKO1
F1	1.00000	0.93267	0.92898	0.92893	0.95903
		−0.15954	−0.36043	0.18778	0.15918
mt-2		1.00000	0.99948	0.99721	0.99506
			0.71143	0.71342	0.34368
G4			1.00000	0.99891	0.99519
				0.58662	0.49587
KR1				1.00000	0.99524
					0.58232
PKO1					1.00000

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