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Evaluation of a hexane/isopropanol lipid solvent system for analysis of bacterial phospholipids and application to chloroform-soluble Nuclepore (polycarbonate) membranes with retained bacteria

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Summary

The recovery of phospholipid, ester-linked fatty acids and phospholipid phosphate from the eubacterium *Pseudomonas atlantica* was shown to be equivalent for the modified Bligh and Dyer (chloroform/methanol/water) procedure and a hexane/isopropanol (HIP) lipid extraction. In addition to its previously suggested lower toxicity, the HIP solvent system allows lipid analyses to be routinely done on chloroform-soluble materials such as Nuclepore polycarbonate membranes, useful for well-defined size fractionation studies of bacterioplankton communities.

Key words: Bacterial lipid; Lipid analysis; Nuclepore filter

Introduction

The analysis of membrane phospholipids has become an important tool for the quantitative description of the viable microbial biomass, community structure, and nutritional status in complex, natural environments [1]. There are a diversity of procedures utilized, however, for the extraction, isolation and purification of these lipids. These procedures may not all be equivalent in recovery of the lipid classes of interest for microbial ecology research. New or modified methods need to be evaluated in terms of quality and quantity of lipid recovered with respect to standard analyses.

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The most widely used solvent system is chloroform:methanol (CM, 2:1, v/v) which is reported to extract lipid more completely from animals, plants or bacteria than most other 'simple' solvent systems [2]. This extraction system was improved by E. G. Bligh and W. J. Dyer [3]. The 'Bligh and Dyer' (B/D) extraction uses a monophasic solution of chloroform:methanol:water (1:2:0.8, v/v/v) for the primary extraction. Water helps the extraction of the polar lipids as it causes cellular swelling which facilitates the permeability of the cell walls for the solvents [4]. After the primary extraction, chloroform and water are added to a final ratio of 2:2:1.8 (chloroform:methanol:water, v/v/v) to form two phases. These ratios result in a lower phase close to 100% chloroform (lipid-rich) and the upper phase largely of methanol/water containing the non-lipids. Recovery is reported to be 95% of the total lipid with a single extraction [3]. White et al. [5] substituted a 50 mM phosphate buffer (8.7 g K_2HPO_4 per liter, adjusted with 1 N HCl to pH 7.4) for water in the original proportions for environmental samples to minimize pH shifts which could result in transesterification artifacts occurring during the lipid extraction procedure [2]. The modified B/D (mB/D) was used in this research.

The mB/D extraction does have limitations. A principal concern for lipid analysis in microbial ecology is the chloroform-solubility of many common materials which could contribute interference to the analysis. This includes certain plastics that may be of interest as substrata in biofouling research [6], and Nuclepore filters for quantitative size fractionation studies of aquatic microbiota [7].

An extraction system of hexane/isopropanol/water (HIP) was suggested as a low-toxicity substitute for the B/D procedure [8]. The HIP would permit lipid analysis of material retained on Nuclepore filters since polycarbonate is stable in these solvents [Nuclepore Catalog, 1984]. In addition, isopropanol is an inhibitor of phospholipase D and has been used in previous work to keep plant lipids in their native state [2]. Other advantages of HIP reported have been that it extracts less pigment and non-lipids (especially proteolipids) than CM [8].

A comparison of HIP vs. mB/D was carried out for recovery of phospholipid, ester-linked fatty acids (PLFA) of a eubacterial culture. PLFA, the principal lipids of eubacteria [9], are used as biochemical markers of the viable microbiota for environmental samples [1] and are the specific lipids of interest for much of current microbial ecology lipid research [10]. One purpose of this comparison was to validate a lipid extraction system compatible with Nuclepore filters to allow lipid analyses to be done on bacterioplankton communities defined by specific size-fractionation. The recovery of bacterial lipid from cells retained on Nuclepore filters was also included in the comparison. Analysis of recovered lipid was by molar quantities of phospholipid phosphate analysed colorimetrically and total PLFA per g dry weight of cell culture quantified by gas chromatography. The profiles of PLFA recovered were evaluated for possible selectivity of the solvent systems.

Materials and Methods

Bacterial culture conditions

Pseudomonas atlantica, Humm (ATCC 19262 NCMB 301) was grown for 18 h in 1 liter of medium containing: 10 g galactose, 5 g casamino acids, and sufficient

artificial sea salts (approximately 25 g Forty Fathoms Sea Salts) to give a final solution salinity of 32 parts per thousand.

Culture harvesting

The cultures were harvested by centrifugation (9000 × *g*, 20 min). In addition, three 10 ml aliquots of the *P. atlantica* cell suspension were filtered through separate Nuclepore polycarbonate filters (47 mm diameter, 0.2 μm pore, Nuclepore Corp.). All filters used for the cell harvesting were cleaned overnight in hexane:isopropanol (3:2) and then air dried prior to dry weight tare and filtration. Both the cells on filters and cell pellets from centrifugation were quickly frozen in liquid nitrogen and lyophilized immediately.

Sample handling

Dry weights of the filtered cell material were calculated by weight difference with the previously tared filters. The three replicates averaged 8.1 (± 1.3) mg. Six aliquots of the homogeneous sample of dry cells collected by centrifugation were set up with dry weights averaging 10.6 (± 0.9) mg. These nine samples were randomly coded and the six centrifuged cell samples randomly assigned to one of the lipid extraction methods. The samples were processed in order of code number and were treated identically after the initial lipid extraction.

Lipid extractions

The chloroform/methanol method used was the modified Bligh and Dyer (mB/D) [5]. The dried cells were placed into a separatory funnel and a single phase solvent system of chloroform:methanol:phosphate buffer (18.75 ml:37.5 ml:15 ml) was added. The cells were shaken in the solvent system and extracted overnight before chloroform:distilled water (18.75 ml:18.75 ml) was added to form two phases. The phases were allowed to separate overnight before the lower organic phase was removed and filtered through a Whatman 2V filter paper into a round-bottom flask to dry under vacuum (< 37 °C).

The hexane/isopropanol (HIP) method used was the single phase system described by Hara and Radin [8]. The dried, centrifuged or filtered cells (still on filters) were extracted in separatory funnels with *n*-hexane:isopropanol:distilled water (40 ml:26.7 ml:1.7 ml) overnight. The single phase was then removed and filtered through a Whatman 2V filter into a round bottom flask, and the separatory funnel rinsed twice with additional 10 ml washes of hexane which were also filtered into the round bottom flask. This solvent mixture easily dried in vacuo (< 37 °C) using a Buchi 011 Rotavapor which has little dead volume between the sample flask and the solvent collection area with coldfinger. This configuration allows the azeotropic mixture consisting of hexane and isopropanol to form and condense on the coldfinger without fractionation, allowing the sample flask to dry quickly.

Lipid analysis

The phospholipid, ester-linked fatty acids were analysed as described in Guckert et al. [11]. Briefly, the total lipids were separated by silicic acid column chromatography into neutral, glyco-, and phospholipid fractions. The phospholipid

fraction was subjected to a mild alkaline methanolic transesterification which resulted in fatty acid methyl esters (FAME) and aqueous-soluble glycerol phosphate. The FAME were quantified by capillary gas chromatography (GC) without any further purification. The GC conditions are as described [11] except that the temperature program was 80 °C – 210 °C at 3°/min followed by a 10 min isothermal program. Peak areas were quantified using the Nelson Analytical 3500 Chromatography Software package. The internal standard option within the Target Compound software program was employed relative to 50 pmol of 19:0 added as the internal injection standard just prior to GC analysis. Identification of the *P. atlantica* FAME was based on identical retention indices with previously analysed *P. atlantica* lipid which was structurally verified using GC/mass spectrometry (GC/MS) [11], a dimethyl disulfide (DMDS) derivatization with GC/MS [12] to determine double bond position and geometry, and Fourier transform infrared spectroscopy to verify the unusual *trans*-geometry of some of *P. atlantica*'s PLFA [13].

Phospholipid phosphate analysis

All lipid fractions from the silicic acid chromatography were analysed with perchloric acid digestions and colorimetric for phospholipid phosphate ($PLPO_4$) determination as described in White et al. [5].

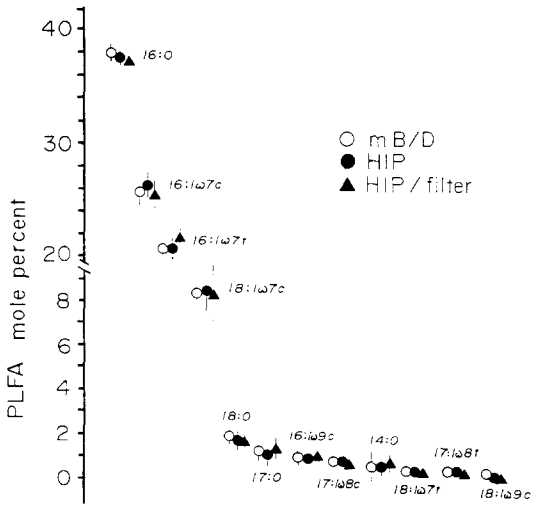

Fig. 1. Comparison of the phospholipid fatty acid (PLFA) profiles recovered by modified Bligh and Dyer (mB/D) extraction, hexane/isopropanol (HIP) and HIP of cells retained on Nuclepore polycarbonate membrane filters (HIP/filter). The symbols indicate the average ($n = 3$) percent of the total molar quantity recovered for each fatty acid. The error bars indicate ±1 SD. When error bars are not visible, the SD was equal to or less than the width of the symbol.

Fatty acid nomenclature

Fatty acids are designated as total number of carbon atoms:number of double bonds with the position of the double bond closest to the aliphatic (omega, ω) end of the molecule indicated with the geometry 'c' for *cis* and 't' for *trans* (e.g. 16:1ω7t, *trans*-9-hexadecenoate).

Statistical analysis

All analyses were done on the Florida State University Cyber 730 mainframe computer utilizing programs available in the statistical software package SPSS. Logarithmic transformations of data were done to meet the homogeneity of error variance assumptions of the analysis of variance model used [14].

Results

Comparison of mB/D and HIP lipid extraction

A comparison of the PLFA profiles recovered by mB/D, HIP and HIP from filters is shown in Fig. 1. The relative fatty acid levels are expressed as percent of the total molar quantity recovered (mol%). The symbols indicate the mean of three replicates $\pm$ 1 standard deviation (SD). There were no statistically significant differences between these extraction methods for any PLFA (ANOVA $\alpha = 0.05$).

The total molar quantity of PLFA per g dry wt. cell material and the phospholipid phosphate ($PLPO_4$) per g dry wt. for the three lipid treatments are shown in Table 1. No significant differences ($\alpha = 0.05$) were found for either biochemical estimate of bacterial biomass for the lipid extraction methods. The ratios of PLFA/$PLPO_4$ for the three treatments are also shown in Table 1. There were no significant differences ($\alpha = 0.05$) found for these data, either.

Discussion

The HIP procedure is an equivalent substitute for the mB/D lipid extraction for both the quantity of total PLFA and $PLPO_4$ (Table 1) as well as the PLFA profiles (Fig. 1) recovered from a bacterial culture, whether harvested by centrifugation or Nuclepore filtration.

TABLE 1

COMPARISON OF PHOSPHOLIPID FATTY ACID (PLFA) AND PHOSPHOLIPID PHOSPHATE ($PLPO_4$) RECOVERIES FROM HEXANE:ISOPROPANOL (HIP) FOR CELLS HARVESTED BY CENTRIFUGATION OR NUCLEPORE MEMBRANE FILTRATION, COMPARED WITH THE CORRESPONDING MODIFIED BLIGH AND DYER (mB/D) EXTRACTION FOR *P. ATLANTICA* CELLS

	HIP	HIP/filter (μmol/g dry weight cells)	mB/D
PLFA	65.5 ± 12.4	70.5 ± 9.4	58.0 ± 15.5
$PLPO_4$	26.5 ± 1.4	22.6 ± 1.5	26.1 ± 2.1
PLFA/$PLPO_4$	2.5 ± 0.6	3.2 ± 0.6	2.2 ± 0.8

Previous evaluations of the HIP lipid extraction have compared HIP vs. B/D extractions of beef lipid [15] and HIP vs. CM extractions of rat brain lipid [8]. In both cases, the HIP was found to recover lower amounts of polar lipids, when evaluated by weight of lipid recovered. It has been suggested that proteolipids extracted with CM, but not HIP, accounted for this difference [8]. When the solvent systems were evaluated analytically by gas chromatography in the present study, no difference in the recovery of bacterial diacyl phospholipids was found, however, a companion study comparing the recovery of lipid classes from a unicellular green alga indicates that HIP does not recover the glyco- and polar lipids as well as the mB/D procedure for this eukaryotic membrane system [16]. Two of the previous HIP evaluations [8, 15] as well as the present study (Fig. 1) have shown that the profile of fatty acids recovered by the HIP is equivalent to the CM extraction procedures.

The mB/D efficiently removes non-lipids when the phases are separated [2]. Procedures have been suggested for an aqueous wash to remove non-lipids in the HIP extraction [8]. Non-lipids which might be recovered only interfere with non-specific analyses, such as gravimetric determinations of lipid recovery. The procedures used in the present study are specific for PLFA and $PLPO_4$ and are, therefore, unaffected by non-lipid contamination. When Na_2SO_4 was used to separate the primary HIP extraction into two phases for non-lipid removal as originally described [8], the total PLFA recovery by GC analysis decreased to 56% of the mB/D (unpublished data). These results suggested that no phase partitioning be used in the HIP procedure when specific analytical estimates of lipids minimize the interferences of non-lipid contaminants.

In conclusion, the results from this examination of *P. atlantica* lipids indicates that the HIP extraction of Hara and Radin [8] is equivalent to the mB/D for bacterial biomass determinations of either $PLPO_4$ or total PLFA. The PLFA profiles for bacterial community structure determinations were also found to be equivalent for the two procedures indicating no preferential recovery of bacterial phospholipids. A companion study to this present research indicates that this analysis will only be valid in environments with low microeukaryotic biomass due to the lower recoveries of glyco- and polar lipids from these membrane systems by HIP [16]. The use of the HIP extraction in microbial ecology lipid analysis will permit the extraction of bacterial samples on chloroform-soluble components such as plastics used as artificial substrata in biofouling research as well as Nuclepore polycarbonate filters for size fractionation studies of aquatic environments. When these environments are primarily bacterial (e.g. groundwater, subsurface wells [17] and initial microfouling communities [18]), the utilization of lipid analyses for bacterial biomass, community structure and nutritional status [1] does not have to be restricted to chloroform-stable substrata or filters with the use of the HIP lipid extraction procedure.

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References

1 White, D.C. (1985) Quantitative physical-chemical characterization of bacterial habitats. In: Bacteria in Nature (Poindexter, J. and Leadbetter, E., eds), pp. 177–203, Plenum Press, NY.
2 Christie, W.W. (1973) Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids. Pergamon Press, Oxford, 338 pp.
3 Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Phys. 37(8), 911–917.
4 Zhukov, A.V. and Vereshchagin, A.G. (1981) Current techniques of extraction, purification and preliminary fractionation of polar lipids of natural origin. Adv. Lipid Res. 18, 247–282.
5 White, D.C., Davis, W.M., Nickels, J.S., King, J.D. and Bobbie, R.J. (1979) Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia (Berl.) 40, 51–62.
6 Marshall, K.C. (1976) Interfaces in Microbial Ecology. Harvard Univ. Press, Cambridge, MA, 156 pp.
7 Sieburth, J.McN., Smetacek, V. and Lenz, J. (1978) Pelagic ecosystem structure: heterotrophic compartments of the plankton and their relationship to plankton size fractions. Limnol. Oceanogr. 23, 1256–1263.
8 Hara, A. and Radin, N.S. (1978) Lipid extraction of tissues with a low-toxicity solvent. Anal. Biochem. 90, 420–426.
9 Lennarz, W.J. (1966) Lipid metabolism in the bacteria. Adv. Lipid Res. 4, 175–225.
10 Guckert, J.B. (1988) Phospholipid, ester-linked fatty acid analysis in microbial ecology. Proc. 4th Int. Symp. Microb. Ecol., in press.
11 Guckert, J.B., Antworth, C.P., Nichols, P.D. and White, D.C. (1985) Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiol. Ecol. 31, 147–158.
12 Nichols, P.D., Guckert, J.B. and White, D.C. (1986) Determination of monounsaturated fatty acid double bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts. J. Microbiol. Methods 5, 49–55.
13 Guckert, J.B., Ringelberg, D.B. and White, D.C. (1987) Biosynthesis of *trans* fatty acids from acetate in the bacterium, *Pseudomonas atlantica*. Can. J. Microbiol. 33, 748–754.
14 Winer, B.J. (1971) Statistical Principles in Experimental Design, 2nd edn. McGraw-Hill, NY, 907 pp.
15 Sahasrabudhe, M.R. and Smallbone, B.W. (1983) Comparative evaluation of solvent extraction methods for the determination of neutral and polar lipids in beef. J. Am. Oil Chem. Soc. 60(4), 801–805.
16 Guckert, J.B., Cooksey, K.E. and Jackson, L.L. (1988) Lipid solvent systems are not equivalent for analysis of lipid classes in the microeukaryotic green alga, *Chlorella*. J. Microbiol. Methods 8, 139–149.
17 Smith, G.S., Nickels, J.S., Kerger, B.D., Davis, J.D., Collins, S.P., Wilson, J.T., McNabb, J.F. and White, D.C. (1986) Quantitative characterization of microbial biomass and community structure in subsurface material: a prokaryotic consortium responsive to organic contamination. Can. J. Microbiol. 32, 104–111.
18 Odham, G., Tunlid, A., Westerdahl, G., Larsson, L., Guckert, J.B. and White, D.C. (1985) Determination of microbial fatty acid profiles at femtomolar levels in human urine and the initial marine microfouling community by capillary gas chromatography-chemical ionization mass spectrometry with negative ion detection. J. Microbiol. Methods 3, 331–344.