An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids

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Summary

A simple small-scale procedure for the sequential extraction of isoprenoid quinones and polar lipids from bacterial cells was developed. Extraction with a biphasic mixture of petroleum ether (b.p. 60-80\degree C) and methanolic saline gave an upper phase containing isoprenoid quinones. The lower phase, containing the partially extracted organisms, was processed according to the Bligh and Dyer extraction method to give a polar lipid extract. As examples of the procedure, the isoprenoid quinones and polar lipids of \textit{Bacillus subtilis}, \textit{Mycobacterium avium}, \textit{Pseudomonas diminuta} and \textit{Streptomyces griseus} were extracted and analyzed.

Key words: Glycolipids – Lipid extraction – Phospholipids – Polar lipids – Menaquinones – Ubiquinones

Introduction

The analysis of complex free lipids has been used increasingly in recent years in bacterial systematics. Two main classes of lipids have provided good chemotaxonomic characters, namely polar lipids [1, 2] and isoprenoid quinones [3]. In the past decade analyses of both these lipid classes have been developed in these laboratories, principally to clarify the classification and identification of actinomycetes and the so-called coryneform bacteria [4–10]. Throughout these studies the procedures used for preparing lipid extracts have been carefully chosen so that they combine rapidity with ease of application and economy of both bacte-
rial biomass and extraction solvents. Where possible, the introduction of alternative methods, providing no significant improvement over published procedures, has been avoided.

Mixtures containing chloroform and methanol have been shown to provide efficient extraction of polar lipids, and the established procedure of Bligh and Dyer [11] has been conveniently modified for the extraction of bacterial lipids [12, 13]. Initial chemosystematic studies in these laboratories used both Bligh and Dyer procedures [11, 14, 15] and simple extraction with chloroform-methanol (2:1, v/v [16, 17]. The Bligh and Dyer [11] system has the advantage that lipids are extracted with a monophasic chloroform-methanol-water system, followed by addition of extra chloroform and water to give a lipid-containing chloroform layer and an aqueous supernatant which contains most of the non-lipid contaminants. The integral wash in the Bligh and Dyer [11] method may, however, remove relatively hydrophilic lipids [13, 18], whereas simple chloroform-methanol extractions may contain non-lipid components that can interfere in chromatographic analyses [15]. Since it is possible that treating bacterial biomass with organic solvents at ambient temperature may not ensure complete extraction of tightly bound lipids, a brief heating step as recommended by Card [19] has been incorporated into the modified [14, 15] Bligh and Dyer procedure [11].

Isoprenoid quinones are readily extracted from bacteria by a wide range of organic solvents. Acetone has often been used [20, 21] and in our initial studies [22, 23] chloroform-methanol (2:1, v/v) was employed since it was convenient to use the same mixture employed for polar lipid extraction [16, 17]. An alternative extraction procedure, developed principally for biochemical studies of isoprenoid quinones [24, 25] uses a biphasic petroleum ether-methanol-acetone mixture and this method has been employed in chemosystematic studies by Mannheim and colleagues [26, 27].

An objective in the development of our chemotaxonomic procedures has been to integrate the various extraction and analytical methods as much as possible to make the most efficient use of small amounts of biomass. It would, therefore, be useful to extract both isoprenoid quinones and polar lipids from the same sample. An extraction solvent mixture such as chloroform-methanol (2:1, v/v) will provide an extract containing all free lipids and, after chromatographic isolation of isoprenoid quinones, the polar lipids might be re-extracted from the origins of the chromatograms. It is, however, more advantageous to first extract isoprenoid quinones with a relatively non-polar solvent system and then to release polar lipids by treating the residue with polar extraction solvents. This paper describes a small-scale integrated procedure which has been developed for the extraction of bacterial isoprenoid quinones and polar lipids from the same small sample of biomass.

**Materials and Methods**

**Organisms**

Freeze-dried heat-killed *Mycobacterium avium* D4 was supplied by H.B. Lee, Central Veterinary Laboratory, Weybridge (UK). Dry cells of *Pseudomonas di-
minuta NCTC 8545 and Bacillus subtilis NCIB 3610 were available from previous studies [28, 29]. Streptomyces griseus ISP 5236 was grown at 30°C on modified Sauton's medium [30] for 5 days, harvested by centrifugation, washed with water and freeze-dried.

Lipid extraction

Organisms were extracted according to the procedures detailed in Fig. 1, using ordinary grade solvents. The extractions were carried out in 8.5 ml Corning 99449-13 culture tubes fitted with Corning 9998-13 polytetrafluoroethylene (PTFE) lined screw caps. The use of 0.3% aqueous sodium chloride and the heating stage was according to Card [19].

The efficiency of isoprenoid quinone extraction using the procedure shown in Fig. 1 was assessed by comparing the amounts of menaquinone in three extracts of M. avium. Dry organisms (50 mg) were extracted in the first experiment with chloroform-methanol (2:1 v/v) (20 ml) by stirring overnight in a conical flask protected from light by aluminium foil [22, 23]. Cells were removed by filtration and solvent removed under reduced pressure at < 37°C. The lipid residue was then dissolved in petroleum ether (0.5 ml) and transferred to a small vial, and the solvent removed under nitrogen at < 37°C. The second extract was prepared as in Fig. 1 but with the heating stage carried out after adding methanol-water (2 ml) in stage 1 but before adding petroleum ether (2 ml); the final extract was prepared as in Fig. 1. Each extract was dissolved in spectroscopic grade hexane (100 μl), and 50 μl of this solution was added to spectroscopic grade ethanol (3 ml) in a cuvette. Ultraviolet spectra were recorded for each solution using ethanol in the reference cell and a Unicam SP-800 spectrophotometer.

Lipid analysis

Extracts considered to contain isoprenoid quinones (Fig. 1) are dissolved in petroleum ether (b.p. 60–80°C) and applied as 4 cm bands to 10 × 10 cm pieces of Merck 5735 plastic-backed silica gel sheets. After development with petroleum ether-acetone (95:5, v/v) separated components were revealed using short wave (254 nm) ultraviolet light. Bands were marked with pencil, cut from the plates and extracted in 8.5 ml tubes by shaking twice for 15 min with a 1 ml diethyl ether; the solvent was removed by evaporation under a stream of nitrogen at < 37°C. Standards of vitamin K1 and ubiquinone-50 (coenzyme Q10) (Sigma) were used to locate bands corresponding to menaquinones (RF 0.8–0.9) and ubiquinones (RF 0.3–0.4), respectively. The identity of the isoprenoid quinones extracted from test organisms was confirmed by mass spectroscopy [22, 23].

Polar lipid extracts were dissolved in chloroform-methanol (2:1, v/v) (60 μl) and 10 μl samples were applied, using a 50 μl syringe fitted with a Hamilton PB 600 repeating dispenser, to the corners of four 6.6 × 6.6 cm pieces of Merck 5554 aluminium-backed silica gel sheets. Chromatography was carried out using chloroform-methanol-water (65:25:4, by vol.) in the first direction, followed by chloroform-acetic acid-methanol-water (40:7.5:6.2, by vol.) in the second. Spraying one plate with 5% ethanolic molybdo phosphoric acid, followed by charring at
1. To dry biomass (ca. 50 mg) in polytetrafluoroethene capped tube, add methanol-water (100:10) (2 ml) and petroleum ether (b.p. 60-80°C) (2 ml)
   Mix on tube rotator (15 min), transfer upper layer to small vial
2. Add petroleum ether (1 ml)
   Mix again, remove upper layer, evaporate combined upper layers with N₂ (< 37°C) 
   Isoprenoid quinone extract
   Heat lower layer in boiling water bath (5 min), cool in water bath at 37°C (5 min)
3. Add chloroform-methanol-water (90:100:30) (2.3 ml)
   Mix (60 min), centrifuge, transfer supernatant to 8.5 ml tube
4. Add chloroform-methanol-water (50:100:40) (0.75 ml)
   Mix (30 min), centrifuge, supernatant combined with above
5. Add chloroform-methanol-water (50:100:40) (0.75 ml)
   Mix (30 min), treat as step 4
6. Add chloroform (1.3 ml) and water (1.3 ml) to combined supernatants (from steps 3-5)
   Mix thoroughly, centrifuge, remove upper layer, evaporate lower layer with N₂ (< 37°C)

Polar lipid extract

\textsuperscript{a} 0.3% aqueous sodium chloride [19].
\textsuperscript{b} Centrifuge if necessary to ensure separation of layers.

Fig. 1. Combined extraction of isoprenoid quinones and polar lipids.

180°C for 15 min revealed the presence of all lipids. Another plate was sprayed with ninhydrin (0.2% in water-saturated butan-1-ol), heated at 110°C for 15 min, pink spots marked lightly with pencil, and the cold plate sprayed with the lipid phosphate reagent of Dittmer and Lester [31] to reveal the presence of phospholipids as blue spots, that were stable for a few hours. The third plate was sprayed with \(\alpha\)-naphthol-sulphuric acid [32] revealing the presence of glycolipids as brown spots after heating at 110°C for 15 min. The periodate-Schiff spray reagent [33] was applied to the final plate for the detection of \(\alpha\)-glycols.

Results and Discussion

The lipid extraction procedure given in Fig. 1 has been developed during extended chemosystematic studies on bacterial lipids. The aim has been to produce a small scale but comprehensive method giving rapid isolation of potentially unstable isoprenoid quinones followed by efficient extraction of the polar lipids from the same sample of biomass. The integrated procedure has been designed for the easy extraction of organisms during a working day, the number of samples processed depending on the capacity of instruments such as laboratory centrifuges. The tube rotators used in the extractions (Fig. 1) were constructed in this laboratory but commercial instruments are available. The speed of rotation during extraction should be kept low to avoid packing organisms at the bottom of tubes.

Examination by ultraviolet spectroscopy of the \textit{M. avium} extract containing menaquinones prepared as in Fig. 1, with one made by stirring overnight with chloroform-methanol (2:1, v/v), gave practically superimposable spectral traces. However, if the heating stage was carried out before the addition of petroleum
ether, the absorption spectrum of the crude menaquinones was reduced by approximately 30%. The recommended procedure given in Fig. 1, therefore, effectively extracts menaquinones from *M. avium* more rapidly than the chloroform-methanol (2:1, v/v) used in early studies in these laboratories [22, 23]. Semi-quantitative data for a wide range of bacteria, involving inspection of the intensity of isoprenoid quinone bands on TLC, has also indicated that the procedure in Fig. 1 extracts menaquinones and ubiquinones just as efficiently as stirring with chloroform-methanol. It is interesting to note that in a pioneering study [34] extraction of dry cells with octane was recommended. The present procedure was developed from the methods of Kröger and colleagues [24, 25] as used by Mannheim and collaborators in taxonomic studies [26, 27], the main modification being to omit acetone which would extract polar glycolipids.

Examination of the isoprenoid quinone extracts from the four test strains showed that a ubiquinone was present in *P. diminuta* NCTC 8545, and that the remaining three organisms contained menaquinones. Mass spectrometry showed that ubiquinone-10 (Q-10) was the major component (M+ m/z 862) from *P. diminuta*, this apparently [3] being the first time that the isoprenoid quinones of this species have been studied. The major menaquinones of *B. subtilis* NCIB 3610, *M. avium* D4 and *S. griseus* ISP 5236 were found by mass spectrometry to be MK-7, MK-9 (H2) and MK-9 (H4, H6, H8) in accordance with previous studies [3] on representatives of these genera. Isoprenoid quinones may also be characterized by reverse-phase [20, 35] and argentation [20] thin-layer chromatography, and reverse-phase [36–39] and argentation [38] high-performance liquid chromatography. Mass spectrometry alone is not reliable for mixtures of isoprenologues differing widely in their mass ranges and quinol-quinine interconversions in the heated inlet system of a mass spectrometer may produce erroneous M+2 peaks [40].

The extraction of polar lipids in the latter part of the scheme outlined in Fig. 1 is essentially as previously described [12–19]. The convenience of the procedure has been improved, in comparison with our previous studies [14, 15], by reducing the scale of operations so that all manipulations can be carried out using inexpensive Corning culture tubes. Comparative studies have shown that the efficiency of polar lipid extraction has not suffered by reducing the quantities of the extraction mixtures.

Typical patterns of polar lipids are shown in Fig. 2. The amounts of sample applied to the chromatograms have been found to give satisfactory patterns for a large number of organisms. The use of high-performance (HPTLC) plates, such as Merck 5548 aluminium-backed silica gel sheets, did not give significantly superior polar lipid patterns though the increased sensitivity of such sheets would be of value in the analysis of very small samples. The polar lipid patterns recorded for the test organisms (Fig. 2) are in general accord with previous studies [2, 17, 28, 29] on such bacteria.

The extraction procedures described are based on well-tried methods and have been evaluated by application to large numbers of different bacteria; several of these studies have recently been published [41–43]. Other non-polar lipids such as free fatty acids, acylglycerols and carotenoid pigments [13, 18] would also probably
be present in the petroleum ether 'isoprenoid quinone' extract (Fig. 1). The possible presence of such lipids did not interfere with the extraction and purification of isoprenoid quinones. Polar glycolipids and phospholipids were not detected in the isoprenoid quinone extract (Fig. 1). Defatted organisms remaining after extraction of free lipids using the present system may be analyzed for bound lipids such as mycolic acids [7] and for wall components such as peptidoglycan amino acids [44].

Fig. 2. Two-dimensional thin-layer chromatography of polar lipids of (a) *B. subtilis* NCIB 3610, (b) *P. diminuta* NCTC 8545, (c) *S. griseus* ISP 5236 and (d) *M. avium* D4. Chloroform-methanol-water (65:25:4) was used in the first direction, followed by chloroform-acetic acid-methanol-water (40:7.5:6:2) in the second direction. Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; LPG, lysophosphatidylglycerol; G, diglucosyl diacylglycerol; G1, glucosyl diacylglycerol; G2, unknown glycolipid; G3, glucuronosyl diacylglycerol; G4, glucosylglucuronosyl diacylglycerol; P, uncharacterized phospholipids; PI, phosphatidylinositol; PIDM-M, monacylated phosphatidylinositol dimannoside; PIDM-D, diacylated phosphatidylinositol dimannoside.
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References

