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Sensitive characterization of microbial ubiquinones from biofilms by electrospray/mass spectrometry

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Summary

Utilizing high-performance liquid chromatography/electrospray/tandem mass spectrometric analysis of the neutral lipid extract of microbial cells and biofilm communities, respiratory ubiquinone (UQ) (1-methyl-2-isoprenyl-3,4-dimethoxyparabenzoquinone) isoprenologues can be separated isocratically in minutes and assayed with a limit of quantification (LOQ) of 9 p.p.b. (11.1 fmol UQ_9 μL^{-1}). This corresponds to about 1.29×10^7 cells of *Pseudomonas putida*. Highest sensitivity is achieved using flow-injection analysis with multiple reaction monitoring wherein ammoniated molecular ions of specific isoprenologues pass through quadrupole one, are collisionally dissociated in quadrupole two and identified from the product ion fragment at *m/z* 197.1 in quadrupole three. This assay has a repeatability of between 6% and 10% over three orders of magnitude ($r^2 = 0.996$). Quinone profiling based on dominant isoprenologue patterns provides taxonomic insights. Detection of prominent UQ isoprenologues indicates presence of microeukaryotes and α *Proteobacteria* with UQ_{10}, obligatory aerobic Gram-negative bacteria with $UQ_{4\text{-}14}$, facultative Gram-negative (and some γ *Proteobacteria* growing in microniches with oxygen or to a much lesser extent nitrate as a terminal electron acceptor with UQ_8, and other γ *Proteobacteria* with UQ_9. High sensitivity is essential as the phospholipid fatty acid (PLFA) to UQ molar ratios are 130 or greater. Previous studies have established that recovery of sediment communities with high PLFA/UQ ratios corresponded to areas of aerobic metabolism, an important consideration in bioremediation or nuclide mobilization.

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Introduction

Respiratory quinone isoprenologue lipids in microbial plasma membranes of microorganisms have two major structures (Pennock, 1966): (i) ubiquinones (UQ) 1-methyl-2-isoprenyl-3,4-dimethoxyparabenzoquinone) with the 2-isoprenyl chains containing 1–14 isoprene units (Fig. 1), and (ii) the menaquinones (MK, 1-isoprenyl-2methylnaphthoquinone) and desmethylmenaquinones (DMK, 1-isoprenylnaphoquinone). UQ are found in aerobic *Proteobacteria* and in eukaryotes, but not in obligatory Gram-negative bacteria. MK and DMK are found in Gram-positive bacteria. Facultative anaerobic Gram-negative bacteria may contain various proportions of all three respiratory quinones depending on the electron donors and terminal electron acceptors. Homologous series differing in the number of isoprene units in the side-chain of each component are found in various species of bacteria and can be utilized taxonomically (Collins *et al*., 1979, 1980; Collins and Jones, 1979, 1981; Gilbart and Collins, 1984; Collins and Gilbart, 1985) The quinone isoprenologues are designated by numbers representing the number of isoprene units in the side-chain: UQ_{10} has 10 isoprene units and 50 carbon atoms in its side chain. MK are found in the Archae, anaerobically grown Gram-negative and many Gram-positive bacteria. DMK is found in some pathogenic bacteria and *Streptococcus fecalis* (Collins and Jones, 1981). Dominant homologues have been assigned to phylogenetic groups such as UQ_8 to the β-subclass of the *Proteobacteria* together with some members of the γ-subclass, UQ_9 to other members of the γ-subclass such as *Acinetobacter* and *Pseudomonas*; UQ_{10} to the α-subclass and to eukarya (Yokota *et al*., 1992). Quinone profiles have been utilized to define community composition along thermal gradients in geothermal hot springs (Hiraishi *et al*., 1999), in sewage sludge (Hiraishi *et al*., 1989) and in marine shallow and deep-sea sediments (Hedrick and White, 1986).

Recently, the generation of non-'leaky' mutants in the genes of UQ synthesis has allowed demonstration of critical role UQ in *Escherichia coli* for gene regulation, protection from oxidative stress and in effective aerobic metabolism (Soballe and Poole, 1999, 2000). The UQ content is highly influenced by the degree of oxygen availability. The results of quinone function studies have indicated that UQ functions as a redox mediator in aerobic and to a much lesser extent in nitrate respiration whereas

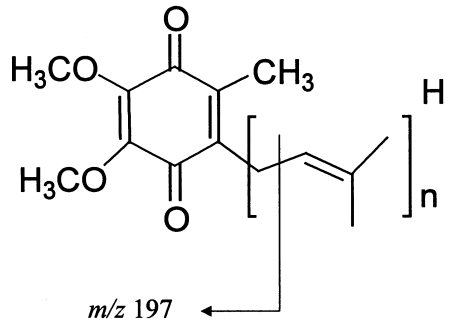

Fig. 1. Structure of ubiquinone (UQ).

MK functions under anaerobic conditions. This reciprocal relationship accounts for much of the plasticity of the electron transport system in responding to myriad environmental conditions. Mutants defective in UQ biosynthesis show pleiotrophic phenotypes with increased resistance to heat and antibiotics, but sensitivity to thiols, perchlorate, visible and gamma irradiation, and peroxide stress, together with loss of siderophore and flagella formation (Soballe and Poole, 1999).

Recently, we have shown that pollution impacts are most sensitively predicted by assessments of *in situ* microbial community composition determined by lipid biomarker analysis (LBA) of all physical and other biomarker measures (White *et al*., 1998). One of the great advantages of the LBA is that it not only reflects the genetic composition of the community but provides insight into the physiological status of the microbes (White, 1995). The microbial community LBA reflects the conditions in the microniches they occupy. One of the most important properties of the microniches in regulating the rates and pathways of microbial metabolic processes is the redox level and concentration of the terminal electron acceptors for the microbial electron transport systems. The provision of high potential electron acceptors is often the rate-limiting step in the subsurface bioremediation of pollutants. Gram-negative facultative anaerobic bacteria shift the composition and proportions of their respiratory quinones between aerobic high potential (UQ) and anaerobic microniche conditions (MK) (Hedrick and White, 1986; Gennis and Stewart, 1999).

Clearly, UQ isoprenologue identification would be an excellent addition to the LBA. The problem has not been in the separation of the isoprenologues but in the detection that would be sufficiently sensitive to detect them in environmental samples. Separation of respiratory quinone isoprenologues by high-performance lipid chromatography (HPLC) is readily accomplished. The problem has been in the sensitivity of detection. UV adsorption can be used but is relatively insensitive, but may be applied to $>10^9$ cells (Hiraishi, 1999) ($\sim$8 umol sample^{-1} or 5×10^{15} cells the size of *E. coli*, based on the UQ_8 content shown in Table 1). Electrochemical detection proved sensitive ($\sim$3 pmol sample^{-1}, $\sim 2 \times 10^9$ bacteria the size of *E. coli* grown aerobically) (Hedrick and White, 1986). The electrochemical detection was cumbersome requiring routine interruption to repassivate the electrodes with nitric acid followed by extensive flushing and proved so difficult it has not been used routinely for environmental samples. Electrochemical detection in thoroughly degassed methanol/ethanol solvents the absence of traces of water proved inherently unstable.

In this paper, we describe a method based on high-performance liquid chromatography/electrospray/tandem mass spectrometry (HPLC/ES/MS/MS) with rapid isocratic UQ isoprenologue separation, requiring no derivatization, and with a sensitivity 4.3×10^6 cells μL^{-1} of *Pseudomonas putida*, containing 3 p.p.b. (3.7 fmol μL^{-1}) of UQ_9. The assay has a repeatability of between 6% and 10%. Unfortunately, neither MK nor DMK isoprenologues give definitive base peaks so could not be analysed using this method.

Results

HPLC separation and electrospray mass spectrometry of ubiquinones

Microbial ubiquinones were readily separated on a C18 column using an isocratic mobile phase consisting of methanol–isopropyl alcohol (MeOH/IPA) (80:20 v/v) containing 10 mM ammonium acetate. The quinones elute as a homologous series differing in the number of isoprene groups. Addition of 10 mM ammonium acetate produced

Table 1. Ubiquinones detected in 2.1×10^9 cells of *Pseudomonas aeruginosa,* 4.3×10^9 cells of *P. putida*, 1.8×10^9 cells of *Escherichia coli* and 4.3×10^9 cells of *Acidovorax delafieldii* 670a

	P. aeruginosa 2.1×10^9 cells	*P. putida* 4.3×10^9 cells	*E. coli* 1.8×10^9 cells	*A. delafieldii* 670a 4.3×10^9 cells
UQ_6	ND	ND	0.04 pmol μL^{-1}	
UQ_7	ND	ND	0.26 pmol μL^{-1}	
UQ_8	0.08 pmol μL^{-1}	0.12 pmol μL^{-1}	2.63 pmol μL^{-1}	0.064 pmol μL^{-1}
UQ_9	1.24 pmol μL^{-1}	4.28 pmol μL^{-1}	0.01 pmol μL^{-1}	1.940 pmol μL^{-1}
UQ_{10}	0.01 pmol μL^{-1}	0.05 pmol μL^{-1}	ND	0.037 pmol μL^{-1}

ND, not detectable $< 3.7 \times 10^{-15}$ mol uL^{-1}.

ammoniated adducts of the neutral ubiquinones. Under increased orifice voltages, the ubiquinones produced a major benzylium ion at $m/z = 197$. Because all ubiquinones contain the same 'base' structure (Fig. 1) they all have this common fragment. Analysis with a single quadrupole mass spectrometer in the selected ion monitoring mode (SIM) at *m/z* 197 showed the ion currents (IC) of ammoniated adducts of UQ_6, UQ_7 and UQ_{10} eluted by HPLC (Fig. 2A). To be characterized as a ubiquinone, peaks thus had to contain the appropriate ammoniated molecular ion and the common fragment at *m/z* 197. The peaks at 2.54 min and approximately 3.3 min are coenzyme UQ_8 and UQ_9. These were determined to be ubiquinones found in the coenzyme UQ_7 standard that was reported to be 85% pure by its certificate of analysis. Figure 2B shows the IC for *E. coli*. Coenzyme UQ_8 is the only ubiquinone detected from *E. coli*. Lyophilized cells from *Legionella pneumophila* were also analysed by ES/MS. Figure 2C shows the ion current obtained from the analysis of *Legionella pneumophila*. Ubiquinones detected consist of coenzyme UQ_{11}, UQ_{12} and UQ_{13}. These higher chain isoprene units for ubiquinones have been reported in *Legionella* (Gilbart and Collins 1984).

MS/MS analysis of ubiquinones

When tandem mass spectrometry (MS/MS) was used for analysis, orifice/ring voltages were optimized to allow maximum transmission of the ammoniated molecular ion through quadrupole one. These ions were collisionally dissociated in quadrupole two and the products were scanned in quadrupole three. Figure 3A shows the ES mass spectrum for UQ_6. The ammoniated molecular ion region is magnified for ease of viewing. The ion at *m/z* 591.6 corresponds to the protonated molecular ion of UQ_6. The ion at *m/z* 608.6 corresponds to the ammonium adduct of the molecular ion (M+18) of UQ_6, whereas the ion at *m/z* 629.6 corresponds to the potassium adduct. Figure 3B shows the product ion spectrum for *m/z* 608.6. The major ion detected was *m/z* 197.1. *Pseudomonas aeruginosa* was previously reported to contain coenzyme UQ_9 (Collins and Jones, 1981). ES/MS/MS established *P. aeruginosa* does contain coenzyme UQ_9. Figure 4 shows the precursor ion mass spectrum for *m/z* 197.1 in *P. aeruginosa*. The only ion detected corresponds to the ammonium adduct of the molecular ion for coenzyme UQ_9, *m/z* 812.9, thus confirming the presence of UQ_9. Because all ubiquinones give a common product ion at

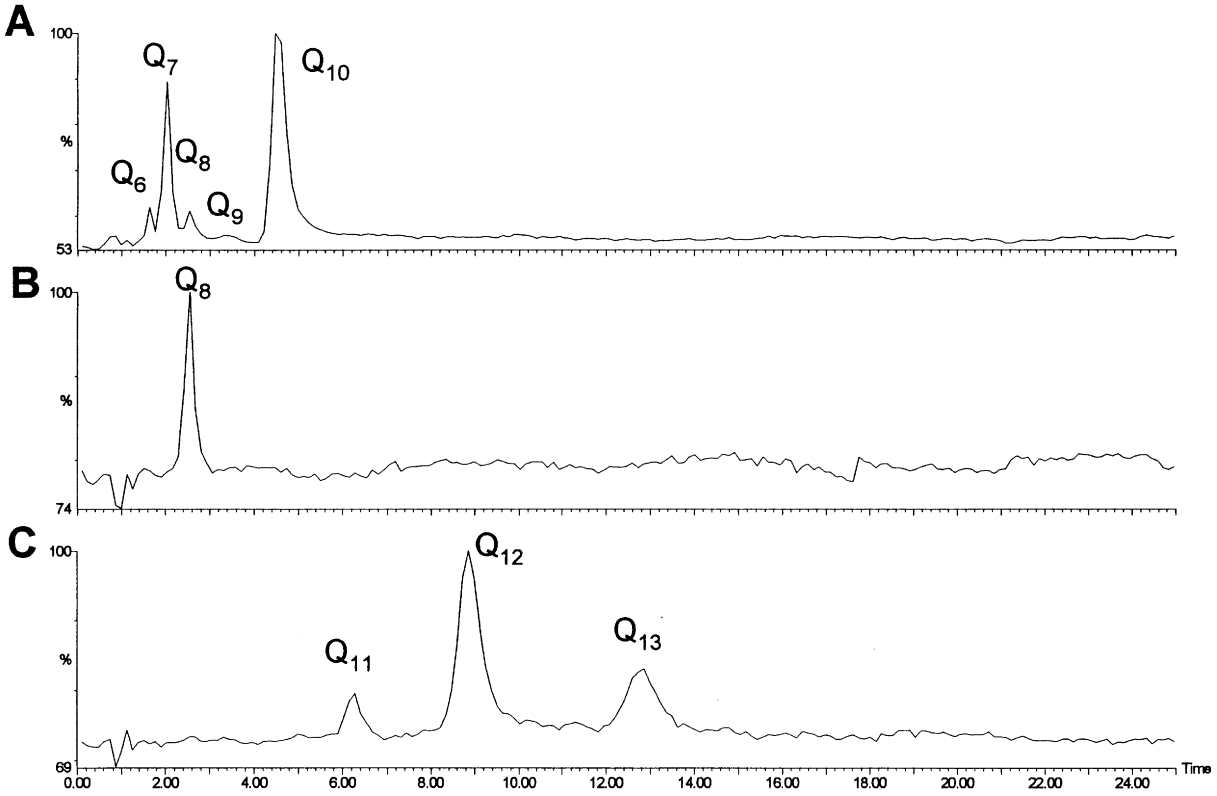

Fig. 2. A. ES/MS Ion current for authentic UQ_6–UQ_{10} eluted isocratically from C18 column.
B. ES/MS ion current for UQ_8 from *Escherichia coli*.
C. ES/MS ion current for UQ_{11}, UQ_{12} and UQ_{13} from *Legionella pneumophila*.

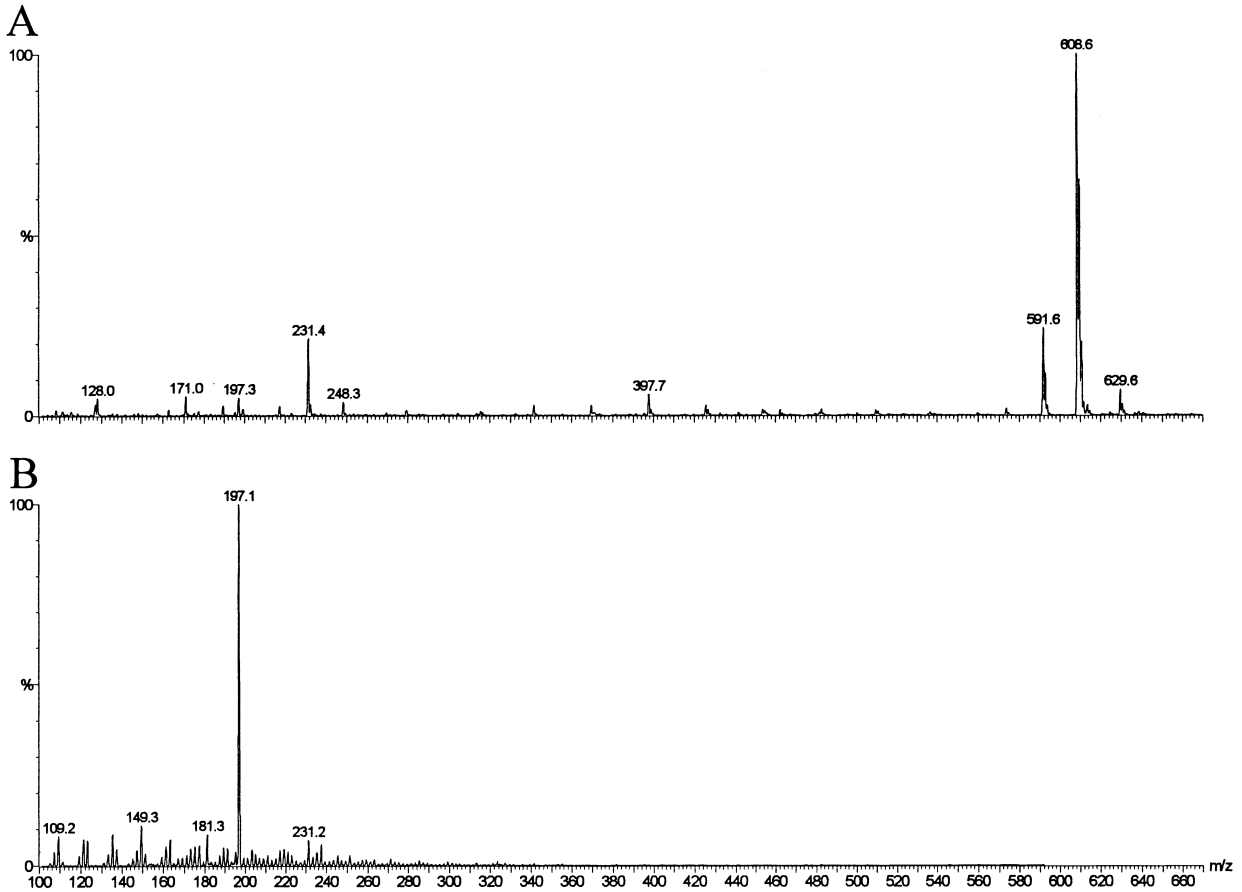

Fig. 3. A. ES mass spectrum of coenzyme UQ6 showing ammoniated molecular ion at *m/z* 608.6.
B. Product ion spectrum of *m/z* 608.6 showing fragment at *m/z* 197.1.

m/z 197, a precursor ion scan can be used to detect all ubiquinones in the sample, provided they are present at detectable concentrations.

Escherichia coli, *P. aeruginosa*, *P. putida* and *Acetovorax delafieldii* 670a were also analysed by MS/MS. Transitions corresponding to the ammoniated molecular ion of UQ_6 through UQ_{10} and the fragment at *m/z* 197 were monitored for the presence of ubiquinones in these samples. Table 1 shows the concentrations of ubiquinone iosprenologues detected. Authentic UQ_6 was used as the internal standard for the quantification of ubiquinones in both *Pseudomonas* species. An external calibration curve for UQ_6 was generated to quantify the ubiquinones in *E. coli* as *E. coli* contains traces of UQ_6.

Ratio of ubiquinone to phospholipid fatty acid

The ratio of phospholipid fatty acid (PLFA) to ubiquinones from 4.3×10^{10} cells of *P. putida* grown aerobically to late stationary phase was 120:1 (2926 pmol PLFA μL^{-1}, 50 pmol UQ_9 μL^{-1}). The ratio pf PLFA to ubiquinones for 1.0×10^{10} cells of *E. coli* was about 150:1 (3550 pmol PLFA μL^{-1}, 24.5 pmol UQ_8 μL^{-1}).

Sensitivity of ubiquinone assay

Pseudomonas putida was grown as previously described and cell counts performed. Dilutions were then made to give cell counts ranging from 4.3×10^6 to 4.3×10^{10} cells. Coenzyme UQ_6 was added as the internal standard at a concentration of 1 ppm. These cells were extracted and concentrated. Samples were analysed by ES/MS/MS in the multiple reaction monitoring (MRM) mode. The transition from *m/z* 608.8 → 197.0 was monitored for the internal standard, whereas *m/z* 812.8 (197.0 was monitored for coenzyme UQ_9. Because both parent ions of interest produce a product ion having identical mass, a 200 ms pause time was included between transitions to eliminate cross-talk. The limit of detection (LOD), as defined by a signal-to-noise ratio (s/n) ≥ 3 was experimentally determined to be 4.3×10^6 cells of *P. putida*, which contained 3 p.p.b. (3.7 fmol μL^{-1}) of UQ_9. The limit of quantification (LOQ) was experimentally determined to

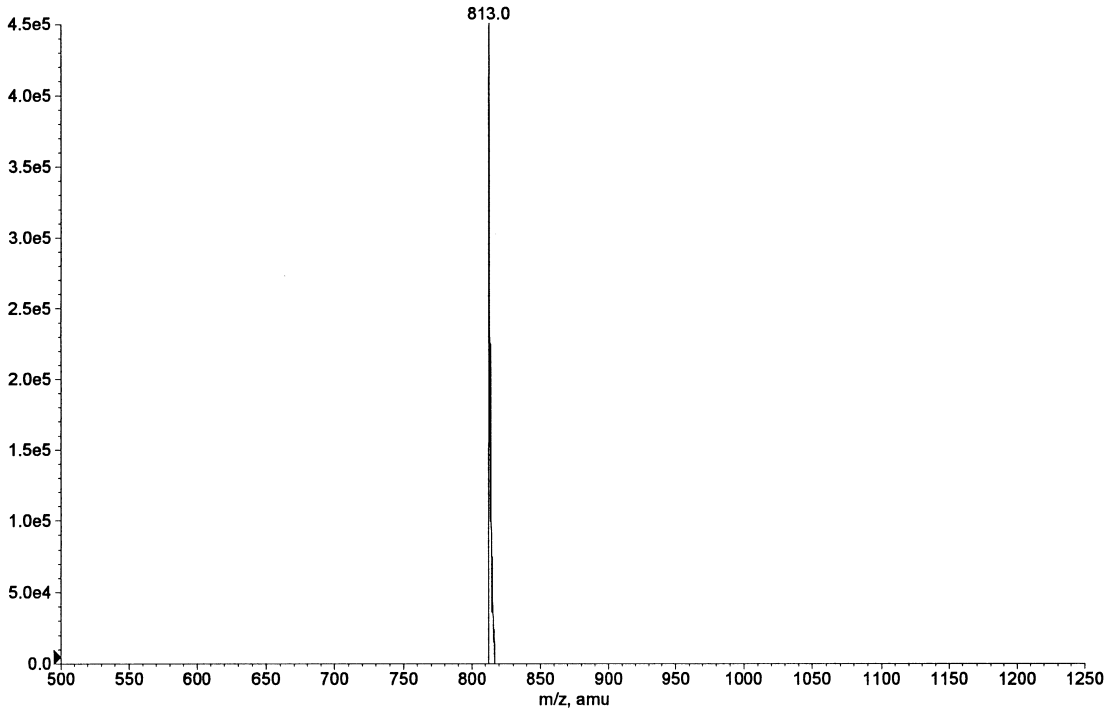

Fig. 4. Precursor ion scan for *m/z* 197.1 from *P. aeruginosa,* (Scan Q-1 Fix Q3), showing an ammoniated molecular ion at *m/z* 812.8, which is UQ_9.

be 1.29×10^7 cells of *P. putida*, which corresponds to 9 p.p.b. (11.1 fmol μL^{-1}).

Reproducibility

Flow-injection analysis (FIA) was utilized to generate a calibration curve using the dilution series of *P. aeruginosa*, in triplicate, ranging from 1×10^6 to 1×10^{10} cells. UQ analysis was performed utilizing MRM for maximum sensitivity. Relative standard deviation (%RSD) was determined to be 25% at 1×10^7 cells, 7% at 1×10^8 cells, 10% at 1×10^9 cells and 6% at 1×10^{10} cells. The correlation coefficient, r^2, was experimentally determined to be 0.996.

Detection of ubiquinones in a triculture biofilm

Acidovorax delafieldii 670a, *Bacillus* sp. 3h47e and *P. putida* sp. were grown as previously described (White *et al*., 1999) and pumped through the biofilm accumulation chamber (BAC). After 3 days the coupon was removed, extracted and the ubiquinone fraction collected, concentrated and analysed. Upon analysis by FIA utilizing MRM, 36 fmol μL^{-1} of UQ_9 was detected, which corresponds to about 4×10^7 cells cm^{-2}. The concentration of UQ_8 and UQ_{10} were below the detection limit.

Discussion

LBA provides a powerful, relatively rapid, quantitative and comprehensive means to define the viable and total microbial biomass, the *in situ* community composition and the nutritional/physiological status in a single analytical procedure (White, 1994; White *et al*., 1996). Recently, the procedure has been made more comprehensive with the substitution of HPLC/ES/MS/MS for gas chromatography/mass spectrometry (GC/MS) in the analysis of lipid components (White *et al*. 2000). HPLC allows a much higher range of molecular weights and in many cases does not require derivatization to make volatile analytes but at the cost of less chromatographic resolution. ES ionization is in many cases more efficient resulting in nearly quantitative ion formation compared wiht the small proportion of the analytes that are ionized with the electron impact ionization used with GC/MS. This increases the sensitivity. The power of tandem mass spectral analysis greatly increases the specificity of detection and decreases the chemical noise, increasing the sensitivity (Lytle *et al*., 2000).

To make the LBA even more effective as a comprehensive, quantitative analytical system for environmental microbial communities, the time and complexity of analytical manipulations needs to considerably decreased. To this end the 8 h room temperature/pressure solvent extraction followed by lipid fractionation on silicic acid columns, followed by derivatization of components is being compressed with a sequential high/pressure temperature

extractions with supercritical CO_2 to recover neutral lipids, lyse cells and make intracellular DNA more recoverable. This is followed by a high pressure/temperature solvent extraction to recover polar lipids from the once-extracted sample. This twice-extracted sample is then subjected to mild acid hydrolysis and re-extraction to recover the lipid A of the lipopolysaccharide of Gram-negative bacteria. Each fraction is then analysed by FIA HPLC/ES/MS/MS (White *et al*., 2000).

The research reported herein provides sensitive, rapid and quantitative UQ isoprenologue analysis to both supplement community composition data from the polar lipids as well as provide the deep insight into the genetic, physiological, oxidative stress responses reflected in the levels of UQ. At the simplest level, the presence of high ratios of UQ to PLFA announce the metabolic activity engendered by high potential electron acceptors and aerobic metabolism at specific microsites in the environmental samples. Besides high UQ/PLFA ratios, high proportions of *Actinomycetes* with mid-chain branched saturated PLFA, a ratio of iso15:0/anti-iso15:0 PLFA < 0.1 characteristic of Gram-positive *Micrococci* type of aerobic bacteria, and unbranched sphinganines from *Sphingomonas* strongly suggest aerobic microsites. Vinyl–ether plasmalogen polar lipids and possibly the isoprenoid alkyl ethers of the methanogenic *Archae* characteristic of anaerobes would not be found (White, 1995).

Unfortunately, the reciprocal indicators between high UQ for aerated sites and aerobic metabolism and high MK (and DMK) for anaerobic sites are not amenable to this analysis as neither MK nor DMK yield an identifiable diagnostic product ion from the molecular ion in FIA-HPLC/ES/MS/MS. Currently, atmospheric pressure chemical ionization (APCI) techniques for MK and DMK are being actively explored.

Experimental procedures

Materials

All solvents were of LC grade and were obtained from Baxter Scientific Products. All glassware was washed in a 10% (v/v) micro cleaner solution (Baxter Diagnostics), rinsed five times in tap water and then five times in deionized water. The glassware was then heated overnight in a muffle furnace at 450°C so as to remove any carbon contamination. Authentic ubiquinones, coenzyme UQ_6, UQ_7 and UQ_{10} were purchased from Sigma Chemical.

Bacterial growth

Pseudomonas aerugionasa JB from IAM culture collection was grown in 100 ml of tryptic soy broth (Difco, Fisher Scientific) as a batch culture. The culture was grown at room temperature, overnight, in a 1 litre flask with continuous shaking. The absorbance at 600 nm (A_{600}) was measured and the cells were centrifuged at 2500 r.p.m. for 15 min. The supernatant was removed and the cell pellet was stored at −80°C or on ice until further analysis. *Acidovorax delafieldii* 670a, *Bacillus* sp. 3h47e and *Pseudomonas putida*, also from the IAM culture collection, were grown as individual batch cultures in TSB overnight at room temperature. The A_{600} was measured and adjusted to an initial cell count of 5×10^7 cells ml^{-1} (A_{600} of 0.1) for each species. Lyophilized cells of *E. coli* were purchased from Sigma Chemical. *Legionella pneumophilia* was generously supplied as lyophilized cells by Dr William R. Mayberry, Department of Microbiology, East Tennessee State College of Medicine, Johnson City, TN.

Triculture biofilm apparatus

Acidovorax delafieldii 670a, *Bacillus* sp. 3h47e and *P. putida* sp. were isolated from a drinking water biofilm and grown as a reproducible triculture biofilm (White *et al*., 1999). The BAC used to grow the triculture biofilm was designed using 70×12 mm stainless-steel tubing. Each end was sealed with Swagelock (Ridge Valve and Fitting Co) reducing fittings. The 70×12 mm chamber contained two $10 \times 20 \times 0.2$ mm stainless-steel coupons or 4.5 g of 5-mm-diameter solid glass beads (Fisher Scientific). One coupon or a few glass beads were used to stain and view under the confocal laser microscope, whereas the other was used for lipid analysis. The BAC was connected to a 2 litre flask, that contained a 10-fold dilution of TSB. The flow was generated with a multichannel cartridge pump (Watson-Marlow) using Masterflex (L/S 14 IS 1.6 mm inside diameter tubing (Cole-Palmer). The flow rate was set at 30 r.p.m. (1.75 ml min^{-1}) and the diluted TSB was pumped through the chamber for approximately 20 min to fill the chamber and remove bubbles. The tube from the reservoir 2 litre flask was clamped, and 2 ml of the diluted triculture was injected into the chamber and incubated for 30 min, to allow the cells to attach to the coupons or beads. The clamp was removed and the pumping was initiated at 15 r.p.m. (0.88 ml min^{-1}). The effluent was pumped to waste distal to the BAC. The chamber was opened after 3 days at room temperature (23–24°C). One randomly chosen coupon was placed inside a 2 ml Eppendorf tube (Fisher Scientific) containing Live/Dead® BacLight™ Bacterial stain (Molecular Probes). The coupon was removed from the stain after an approximately 2 min incubation at room temperature, and placed on a slide cover for confocal microscopy. The other coupon was used for lipid analysis.

Isolation of ubiquinones

Lyophilized cells and glass beads were extracted with the modified Bligh/Dyer extraction and fractionated into general lipid classes (neutral lipid, glycolipid, and polar lipid) by silicic acid column chromatography as previously described (White and Ringelberg, 1998). Samples were kept under nitrogen and away from fluorescent light as carefully as possible. The neutral lipid containing chloroform fraction was recovered and passed through a 0.2 μm filter prior to HPLC analysis. The neutral lipid fraction was dried under a stream of nitrogen,

then reconstituted in a mixture of methanol–chloroform (80/20, v/v) containing 10 mM $NH_4C_2H_3O_2$.

Isolation and analysis of PLFA

After fractionation by silicic acid column chromatography, the polar lipid fraction was eluted in methanol followed by methanol–acetate buffer. The polar lipids was transesterified by mild alkaline methanolysis and the phospholipid ester-linked fatty acid methyl esters (PLFA) analysed by GC/MS (White and Ringelberg, 1998).

High-performance liquid chromatography

HPLC was performed on an Hewlett Packard model 1100 gradient instrument with an autosampler (Agilent Technologies), using a reversed phase HAISIL 300, 30 mm $\times$ 1 mm $\times$ 5 μ column (Higgins Analytical) at a flow rate of 40 μL min^{-1}. The mobile phase consisted of 80:20 methanol–isopropanol containing 10 mM $NH_4C_2H_3O_2$.

Mass spectrometry

Initial analysis of the ubiquinones was performed on a VG Platform II, single quadrupole mass spectrometer. Next, 1 μL of the neutral lipid fraction was injected onto the HPLC for FIA or injected directly with the Harvard Apparatus model 33-glass syringe pump. Electrospray was used to efficiently transfer the ions present in solution to the gas phase. The electrospray capillary was operated at -2.78 kV. The counter electrode was operated at 0.41 kV. The cone was set to -80 V, whereas the skimmer lens offset was set to 5 V. The source was operated at 100°C. For optimum sensitivity and structural confirmation, samples were analysed on a PE Sciex API 365 tandem quadrupole mass spectrometer utilizing TurboIon spray. The TurboIon spray was operated at 5500 V. The declustering potential, also known as the orifice was optimized at 11 V. The focusing potential, also known as the ring was optimized at 210 V. Samples were analysed by FIA utilizing MRM for maximum sensitivity. The mobile phase for FIA consisted of methanol–chloroform (80/20, v/v) containing 10 mM $NH_4C_2H_3O_2$.

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