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Microbial respiratory quinones in the environment

I. A sensitive liquid chromatographic method

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Summary

Bacterial respiratory quinones in environmental samples are proposed as sensitive indicators of the aerobic versus anaerobic metabolism of bacterial populations. A liquid chromatographic method for the analysis of respiratory quinones is presented, and the proportions of ubiquinone, menaquinone and desmethylmenaquinone in aerobic and anaerobic enrichments and several environmental samples are reported. These preliminary data support the utility of the method.

Key words: *Desmethylmenaquinone – Environmental respiratory quinones – Menaquinone – Ubiquinone*

Introduction

The ability to measure the relative importance of aerobic and anaerobic metabolism in environmental samples would be useful in many areas of microbial ecology. For example, this measure could provide predictive power when an environmental pollutant undergoes different rates or pathways of biodegradation in aerobic versus anaerobic environments. However, the direct measurement of redox potential or dissolved oxygen is not possible for many samples, and enumeration of aerobic versus anaerobic microorganisms using plating or most probable number techniques is grossly inaccurate [1]. We propose that the bacterial respiratory quinones are useful, specific biomarkers for the type of energy metabolism found in microbial environments.

The common bacterial respiratory quinones are ubiquinones (UQ, 1-methyl-2-isoprenyl-3,4-dimethoxyparabenzoquinone), menaquinones (MK, 1-isoprenyl-2-methyl-naphthoquinone) and desmethylmenaquinones (DMK, 1-isoprenylnaphthoquinone). Each of these three types of quinones is a homologous

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series of compounds differing by the number of isoprene units in the sidechain. The type of quinone and length of the sidechain varies with the type of bacteria [2] and growth conditions [3, 4]. Ubiquinones are the respiratory quinones found in eukaryotes and are present in some gram-negative bacteria. Menaquinones are found in gram-negative and -positive bacteria and archaebacteria. Desmethylmenaquinones are not so widely distributed, having only been reported in some pathogenic enterobacteria and *Streptococcus faecalis* [2]. Quinones have been used as taxonomic markers for bacteria [2, 5].

There are three reasons to believe that the quinone content of a bacterial community would shift with changes in the availability of oxygen. The respiratory quinones act as mobile redox carriers between elements of the electron transport chain. The redox potentials of the quinones (+112 mV for UQ, +36 mV for DMK, and −74 mV for MK, measured at pH = 7 in isopropanol-water (9:1) with 0.1 M Tris-HCl) [6, 7] are appropriate for different roles. There are some energy-yielding reactions in aerobic environments that are less available to bacteria containing only MK, and some in anaerobic environments less available to those containing only UQ [7]. Bacteria with the appropriate quinone for the environment may have an energetic advantage and overgrow their competitors.

The second reason to expect a change in quinones with a change in the availability of oxygen is that aerobes tend to have UQs and anaerobes tend to have MKs. Almost every aerobic gram-negative bacteria examined has UQs, anaerobic gram-negative bacteria that have quinones have MKs, and some facultative gram-negative bacteria have DMKs as well as UQs and/or MKs [2]. It has also been shown that the quinone content of some bacteria is responsive to the redox state of the environment [8]. The ratio of UQ/MK in *Proteus rettgeri* changed from 10 to 0.12 when the electron acceptor was changed from oxygen to fumarate [9].

The third reason to expect a shift in quinones is that to maintain a respiratory chain for synthesis of ATP requires μmoles of respiratory quinones per gram bacterial protein, whereas substrate level phosphorylation does not [5, 10]. An increase in the proportion of ATP synthesized in a bacterial community by substrate level phosphorylation could be reflected in a decrease in the ratio of quinones to total biomass. Biomass is measured as lipid phosphate (LP) [11, 12]. Lipid phosphate has been shown to agree with other measures of biomass, e.g., enzyme activities, muramic acid levels, total adenosine triphosphate and respiratory activity [11].

As part of an ongoing program of methods development for the analysis of bacterial marker lipids in microbial ecology, we present a sensitive liquid chromatographic method for the analysis of bacterial respiratory quinones in environmental samples and data on their usefulness in distinguishing aerobic from anaerobic environments. This method [13] has been adapted to environmental samples in such a way that several other classes of lipids may also be examined to obtain additional information on the biomass and community structure of the microbiota [11, 12, 14, 15]. The benefits of a similar integrated procedure have been discussed [16].

Materials and Methods

Isolation of quinones

Special precautions

Respiratory quinones break down under the influence of light, oxygen, alkali or heat [2]. Alkali is not a problem in this procedure and the normal precautions against oxygen and heat used in lipid analysis are sufficient [17]. Samples were handled under dim light and never exposed to direct fluorescent light. To avoid contamination the sample did not come in contact with any plastic after the beginning of the extraction.

Extraction and silicic acid fractionation

The modified Bligh-Dyer extraction, silicic acid fractionation and lipid phosphate determination procedures were done as previously reported [14, 18] except for protecting the quinones from light. Quinones were found in the neutral (chloroform) fraction after silicic acid fractionation of the lipid extract. Lipid phosphate analysis was performed on the phospholipid (methanol) fraction. Chloroform and methanol used were Baker resi-analyzed grade (Baker, Phillipsburg, NJ).

Thin layer chromatography

The silicic acid thin layer plates (Whatman, Clifton, NJ) were prewashed with 85:15 (v:v) petroleum ether (boiling range, 35–60 °C)/ethyl ether. Approximately 1 nmol each of UQ and MK were spotted as retention standards and the sample was streaked on the plate. The pre-eluted plate with standards and samples was then developed in the dark with the same solvent system.

The positions of the TLC retention standards were determined by the sequential use of two spray reagents prepared fresh daily [19]: 1% (w:v) sodium borohydride (Aldrich Chem. Co., Milwaukee, WI) in 50% (v:v) aqueous ethanol (200 proof, Florida Distillers Co., Lake Alfred, FL) and 0.2% (w:v) aqueous neotetrazoleum bromide (NTZ) (Sigma Chemical Co., St. Louis, MO). The retention standards were sprayed with the sodium borohydride solution. Samples were protected with cardboard during spraying to prevent decomposition of the quinones. The plate was allowed to dry completely before spraying the NTZ solution. Quinones appeared as blue spots. The UQ spot was made darker by placing the standard track flat on a 100 °C heating block, taking care not to heat the sample quinones.

A band ±1 cm from the center of each retention standard was scraped from the TLC plate and poured into a 5 ml luer-lock glass syringe fitted with a 0.45 μm nylon filter (Micron Separations, Inc., Honeoye Falls, NY). Quinones were eluted from the silica with four 2 ml aliquots of chloroform and the solvent then removed under a stream of nitrogen at 37 °C. Samples were dissolved in 1 ml of ethanol prior to chromatography.

High performance liquid chromatography

Apparatus

The HPLC apparatus is illustrated in Fig. 1. All components contacting the mobile phase were stainless steel or glass. The degasser was a magnetically stirred thick walled one liter vacuum flask with two 1/8 inch stainless steel tubes passing through the removable rubber stopper. A steel clamp held the stopper under pressure. With the rubber stopper removed, a 47 mm wide vacuum filter holder (Millipore Filter Corp., Bedford, MA) with a 0.45 μm pore size teflon filter (Gelman Sci. Inc., Ann Arbor, MI) could be put in its place to filter the mobile phase before degassing. The 375 ml capacity ISCO model 314 syringe pump (Instrument Specialties Co., Lincoln, NE) could be switched from accepting mobile phase from the degasser, expelling it to waste, or metered delivery to the chromatographic system. The parallel glassy carbon working electrodes of the Bioanalytical Systems LC4-B dual detector (Bioanalytical Systems, West Lafayette, IN) were set at -60 mV and -300 mV versus the standard calomel electrode.

Passivation

Ions leached from the metal tubing and pump are reduced at the working electrode leading to unacceptable background currents. This was prevented by passivating the system before use or once every 2 weeks (Jacobs, W., personal communication). The column was disconnected from the injector, and the purging flask, pump, injector and all connecting tubing were filled with 6 N nitric acid (Baker, Phillipsburg, NJ) for 2 h. This was replaced for 2 h with an aqueous solution: 2 M in potassium

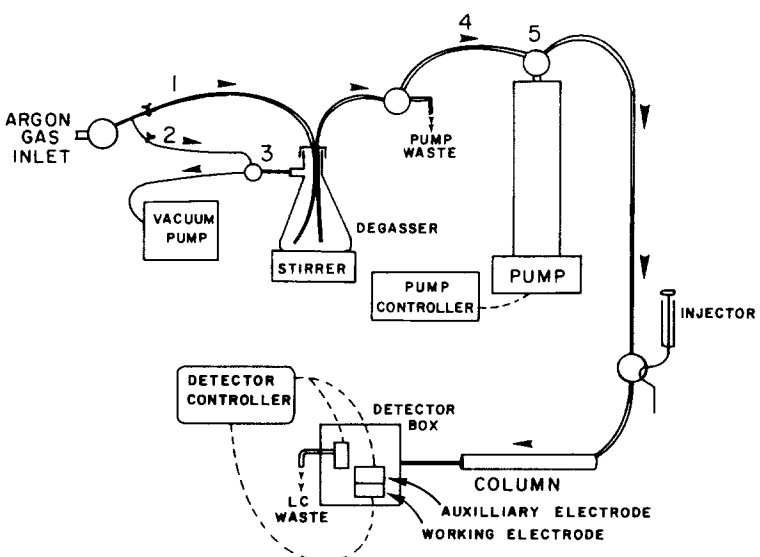

Fig. 1. High performance liquid chromatography apparatus built for the separation of microbial quinones from environmental samples. Operation is described in the text.

phosphate dibasic (Fisher Scientific Co., Fair Lawn, NJ) and 0.2 M in ethylenediaminetetraacetic acid disodium salt (Sigma, St. Louis, MO), adjusted to pH 8. The system was flushed thoroughly with deionized water, then methanol.

Mobile phase preparation and oxygen removal

Seven grams anhydrous sodium perchlorate (Fisher Scientific Co., Fair Lawn, NJ), and 1.0 ml of 60% (v/v) aqueous perchloric acid (Mallinckrodt, St. Louis, MO) were dissolved in one liter of 3:2 ethanol/methanol (v/v), and filtered into the degassing flask. The mobile phase was purged with argon for 5 min, put under 138 kPa vacuum for 1 min, and then pressurized under 69 kPa of argon for 1 min. This 1 min evacuation/1 min pressurization cycle was repeated seven times and followed by 5 min of 138 kPa vacuum [20]. The syringe pump was flushed thoroughly with mobile phase and filled. The column and detector were also thoroughly flushed with mobile phase (10 min) before turning on the detector.

Standards and samples

Retention time and concentration standards

Individual homologs were named as follows: the abbreviation of the type of quinone (UQ, MK or DMK), a dash, and the number of isoprene units in the sidechain. For example, UQ-10 stands for the homolog of ubiquinone with 10 isoprene units (50 carbons) in its sidechain. Quinone standards for UQ-8 and MK-8 (with small amounts of the shorter homologs) were extracted from lyophilized *Escherichia coli* (ATCC 11303), MK-7 from *Bacillus subtilis* (ATCC 6633), MK-7, -8 and -9 from *Staphylococcus aureus* (Newman D_2c strain) [2]. Lyophilized bacteria and UQ-10 were purchased from Sigma Chemical Co., St. Louis, MO. A culture of *Haemophilus parainfluenzae,* generously supplied by Dr. Johan H. Stuy of the Department of Biological Science, Florida State University, was the source of DMK-5, -6 and -7. The retention times for quinones for which no standards were available were obtained from linear plots of the natural logarithm of the homolog's adjusted retention time against the number of isoprene units in that homolog [21].

Chromatographic peaks were quantitated either by peak area integration with a Hewlett-Packard 3350 laboratory automation system or by manual measurement of peak height. Linear calibration curves for UQ-8 and MK-8 were used to calculate the amounts of other homologs in samples, expressed as equivalent response to UQ-8 and MK-8. The Student's *t*-test [22] was used to determine the significance level of differences between the logarithmically transformed data from aerobic and anaerobic enrichments. When no quinones were detected, for statistical calculations they were entered at the limit of detection.

Samples

Estuarine sediments from the top 1 cm of a subtidal mud flat approximately 25 m offshore from the Florida State University Marine Laboratory, Turkey Point, Florida were either extracted to serve as field controls or used as inocula to aerobic and anaerobic enrichments, as described by Guckert et al. [14]. Aliquots of the broth from a primary aerobic culture were used to inoculate secondary aerobic/aerobic

(AER/AER) and aerobic/anaerobic (AER/AN) enrichments. The primary anaerobic culture was likewise used to inoculate secondary anaerobic/aerobic (AN/AER) and anaerobic/anaerobic (AN/AN) enrichments. The aerobic and anaerobic media contained D-glucose (Mallinckrodt, St. Louis, MO), lipid-extracted yeast extract (Difco Laboratories, Detroit, MI) and sufficient artificial seawater salts (Forty Fathoms Marine Mix, Marine Enterprises, Baltimore, MD) to yield 30 parts per thousand. Aerobic conditions were maintained with sterile air bubbling into the media. Anaerobic cultures additionally contained resazurin as redox indicator and cysteine as a reductant. Purging with oxygen-free nitrogen:carbon dioxide (80:20, v/v) established anaerobic conditions, as evidenced by the resazurin remaining clear. The cell mass of all cultures was harvested by centrifugation.

North Atlantic deep-sea sediment samples (depth, 4820 m) from the High Energy Benthic Boundary Layer Experiment (Hebble) [15, 18] were provided by Bruce Baird after collection and storage as described [15]. This unusual area is characterized by relatively cold water and high current velocities. Frequent abyssal storms occur during which current velocities exceed 60 cm/s. The bottom sediment consists of silt and clay, and is typically oxidized to a depth of 10 cm or more (determined visually) [15, 23]. Sediment samples were stored at $-70\,^{\circ}C$ until extraction [15, 18]. They were divided into shallow (0–9 cm) and deep (= 11 cm) portions while still frozen and extracted immediately.

Results and Discussion

Chromatography

The chromatographic method described here gave baseline resolution of the major bacterial respiratory quinones (see Fig. 2). Bacterial cultures used as sources of retention and concentration standards gave the peaks expected based upon reported quinone contents for those species [2]. Further, the retention times for UQ-7 and -8 from *E. coli* and UQ-10 purchased from Sigma consistently gave linear plots of log adjusted retention time versus homolog number, as did MK-7 from *B. subtilis*, MK-8 from *E. coli* and MK-7, -8 and -9 from *S. aureus*. Also, all homologs gave the elution order expected for reverse phase chromatography, i.e., UQ-X, DMK-X and then MK-X. The relative response of the two working electrodes to UQ versus MK was also used to validate the identity of the peaks. The −60 mV working electrode had approximately equal molar response to UQ and MK. The −300 mV electrode had about four times the response to MK as to UQ.

The desmethylmenaquinones were found in the MK band from TLC plating and were identified by the following criteria: they followed the structurally very similar MKs through the extraction and chromatographic steps, were part of a homologous series that paralleled the MKs, had a relative response at the two electrodes that matched DMK standards, and coeluted with DMK standards. DMKs were expressed as equivalent response to MK-8.

The extraction and cleanup procedure presented here gave an overall recovery for a spiked solvent blank of 87% for MK-8 and 82% for UQ-8. The sensitivity varied from day to day with the condition of the working electrodes but was approximately

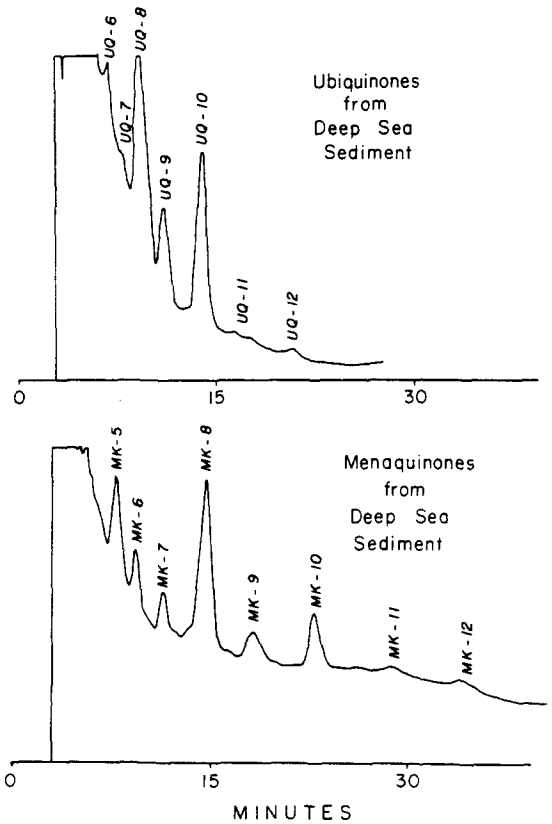

Fig. 2. Ubiquinone and menaquinone chromatograms of a HEBBLE deep sea sediment sample.

·3 pmol of each quinone homolog per sample. This corresponds to approximately 3 μg of bacterial protein (based upon 1 μmol respiratory quinones per g bacterial protein [10]) or 10×10^6 bacteria the size of *E. coli* (2 pg/cell, 15% protein by wet weight [16]). The reproducibility of the culturing and analysis was measured as the root mean squared relative standard deviation of UQ and MK measured for the field control and primary aerobic samples. The value of 12% indicated sufficient reproducibility for comparison.

Analysis of estuarine sediment enrichment cultures

The availability of oxygen affects the pattern of quinones produced

The data from the analysis of the estuarine mud and the cultures grown from it may be treated as three experiments, each time taking a population of bacteria and enriching it under aerobic and anaerobic conditions (see Fig. 3). The primary aerobic cultures were dominated by respiring bacteria with an almost identical pattern of quinones to the field control, while the primary anaerobic culture had much greater

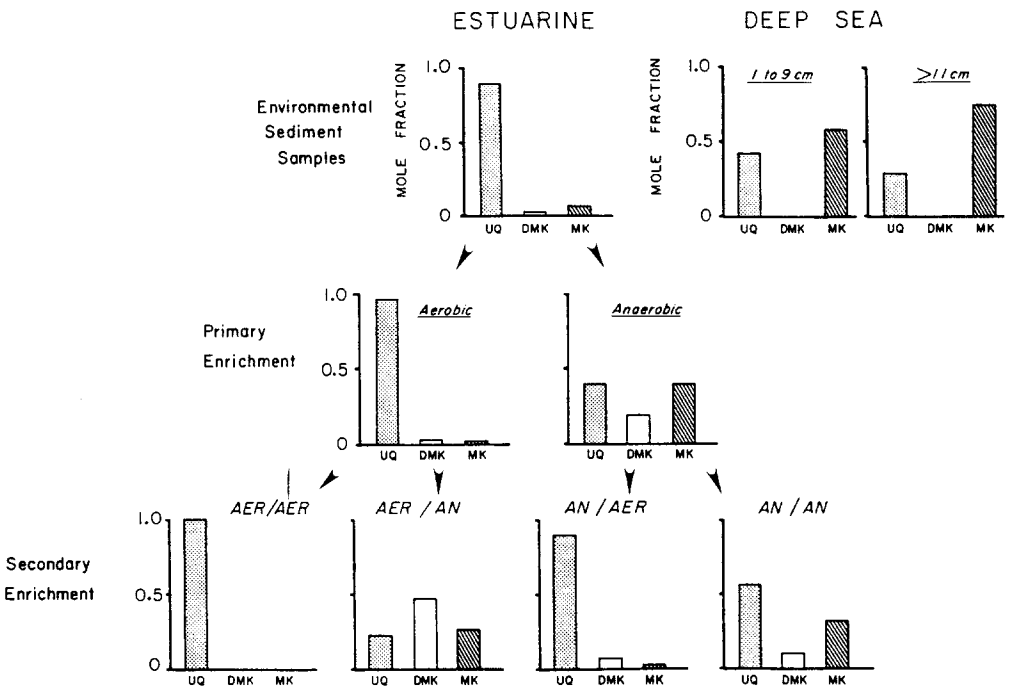

Fig. 3. Histograms of the proportions of total respiratory quinones in the estuarine sediment and the enrichment cultures. Estuarine sediment was the inoculum for the primary enrichment cultures. The aerobic primary enrichment culture was the inoculum for the secondary AER/AER and AER/AN enrichments and the anaerobic primary culture was the inoculum for the secondary AN/AER and AN/AN cultures. UQ, ubiquinone; DMK, desmethylmenaquinone; MK, menaquinone; AER, aerobic; and AN, anaerobic.

proportions of DMKs and MKs (see Fig. 3). Subculturing the primary aerobic enrichment aerobically and anaerobically produced clearly different patterns of respiratory quinones. The secondary AER/AER enrichment had no detectable MKs or DMKs, indicating the dominance of aerobic respiration after two subculturings under forced aeration. The secondary AER/AN culture produced a strikingly different pattern of respiratory quinones from the field control, the primary aerobic or the secondary AER/AER enrichments (Fig. 3) indicating the difference in the availability of oxygen. The major respiratory quinones were the DMKs, followed by the MKs and UQs. The secondary AN/AER and AN/AN cultures again were different from each other. The pattern found in the secondary AN/AER culture was very similar to those of the field control and the other two aerobically grown cultures, except for a slight increase in the DMKs. The secondary AN/AN culture was very similar to the primary anaerobic culture except for a greater proportion of UQs. Each of the aerobic cultures had much more UQ than MK or DMK. The anaerobic cultures all had greater proportions of MK than any of the aerobic cultures. This clearly demonstrates the usefulness of the quinones for detecting changes in the community structure or respiratory state of bacterial communities due to changes in the

availability of oxygen, and is in agreement with Guckert's analysis of the phospholipid fatty acid profiles of these same cultures [14].

Oxygen increases the ratio of ubiquinones to menaquinones

The ratio of UQ to MK + DMK in the field controls was shifted an order of magnitude lower in the primary anaerobic cultures and an order of magnitude higher in the primary aerobic cultures (see Fig. 4). Subculturing the primary aerobic culture aerobically gave UQ/MK + DMK values greater than the field controls, while subculturing the primary aerobic culture anaerobically gave a lower ratio than the anaerobic primary culture. The UQ/MK + DMK ratio increased significantly when the anaerobic primary culture was subcultured aerobically. The Student's *t*-test was used to determine the significance level of the difference between the UQ/MK + DMK ratios of aerobic and anaerobic cultures. The interpolated *p*-value obtained was 0.007. This indicates that the ratio UQ/MK DMK reliably distinguishes the aerobic from anaerobic enrichments.

Oxygen increases the ratio of total quinones to lipid phosphate

That the ratio of total quinones to membrane lipid phosphate Q/LP follows the redox state of the medium is shown in Fig. 4. Since non-respiring bacteria contain 0.1% of the quinones that respiring bacteria do [5, 10], an increase in the proportion

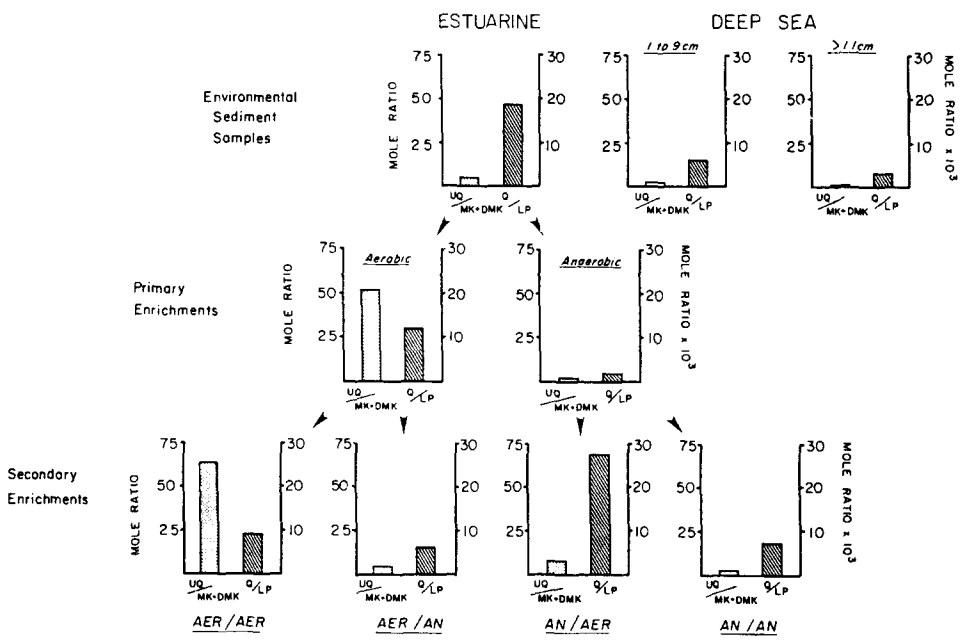

Fig. 4. Histograms of the ratio of ubiquinones to desmethylmenaquinones and menaquinones (UQ/MK + DMK) and the ratio of total respiratory quinones to lipid phosphate (Q/LP). The units are nmol/nmol and nmol/mmol, respectively. All other abbreviations are as in Fig. 3.

of bacteria gaining their energy by substrate level phosphorylation should be reflected in a decrease in the ratio of total quinones to lipid phosphate. This ratio decreased significantly when the field control was cultured anaerobically, and then increased again when the primary anaerobic culture was subcultured aerobically. In each of these subculturings, the ratio of Q/LP was greater in the aerobic than in the anaerobic culture. The Student's *t*-test was used to determine the significance level of the difference in Q/LP for each of the three inocula grown under aerobic and anaerobic conditions. The interpolated *P*-value was 0.063. The data presented in Fig. 4 shows that the secondary AER/AER enrichment is the one culture in which the aerobic enrichment did not have a ratio of Q/LP much greater than the corresponding anaerobic enrichment. This is also the culture that did not have a measurable amount of MKs or DMKs. Comparison of UQ/MK + DMK and Q/LP for AER/AER and AN/AER shows that AER/AER has a much lower ratio of Q/LP and a much higher ratio of UQ/MK + DMK than AN/AER. This shows that less UQ than MK or DMK was required to support a given amount of biomass measured as LP.

Agreement with phospholipid fatty acid data

Guckert et al. [14] reported the phospholipid fatty acid (PLFA) profiles for these estuarine sediment field controls and laboratory enrichments. The fatty acid methods and nomenclature used were described in Guckert et al. [14]. The amounts of cyclopropane fatty acids and 10Me16:0 were greater in the secondary AN/AN enrichments than in the secondary AER/AER enrichments. The amounts of 16:1w7c and 18:1w7c were less, and the amounts of the saturated straight chain from 13:0 to 18:0 were greater in the anaerobic enrichments, as were all the branched chain iso and anteiso PLFA detected (a15:0, i16:0, i17:0 and a17:0). Guckert concluded that the PLFA may be used to distinguish cultures enriched aerobically from those enriched anaerobically. In his analysis the secondary AER/AN and AN/AER cultures were grouped under the heading 'facultative'. Their PLFA profiles were found to be more similar to each other than to either the AER/AER or AN/AN enrichments. While these 'facultative' bacteria had very similar fatty acid profiles, their quinone profiles were very different. The quinone profiles clearly showed the difference in the metabolism of the bacterial communities in the two environments. These data could be interpreted as the survival of a similar group of bacteria through aerobic and then anaerobic enrichment to those surviving anaerobic and then aerobic enrichment.

Detection of desmethylmenaquinones in environmental samples

Desmethylmenaquinones were a minor component of the estuarine mud field control and were not detected in the deep sea sediment. A recent comprehensive review of bacterial quinones [2] only reported DMKs being found in some gram-negative facultatively anaerobic rods (species of *Erwinia, Escherichia, Klebsiella, Proteus, Aeromonas, Actinobacillus, Haemophilus* and *Pasteurella*) and in the gram-positive coccus *Streptococcus faecalis*. Each of these is a recognized pathogen [25]. It is not surprising, however, that this unusual lipid would only be reported from medically interesting bacteria since the lipids of so many more pathogens have been

analyzed than non-pathogenic bacteria. The top 1 cm of estuarine mud is predominantly aerobic. The major shifts in the amount of oxygen available to the inocula occurred when the field control and the primary aerobic culture were subcultured anaerobically. In both cases the DMKs greatly increased. Thus, the DMKs may be useful as environmental biomarkers for blooms of opportunistic bacteria due to drastic changes in the redox state of the environment, such as a sudden increase in the amount of degradable carbon compounds.

Analysis of deep sea sediments

The ability of this method to measure a difference in the respiratory state of environmental samples was tested on deep sea sediment from the HEBBLE experimental area. Two shallow sediment samples (1–9 cm) and one from greater than 11 cm were analyzed. The data in Fig. 3 shows that the quinone profiles for the deep sea sediments sampled was very different from the estuarine sediments analyzed, with a much smaller proportion of UQs. The profile is most similar to the primary anaerobic enrichment, except for the lack of DMKs. The values for UQ/MK + DMK and Q/LP plotted in Fig. 4 again show the deep sea sediment is more similar to the anaerobic enrichment of the estuarine sediment than to the field control. The differences in these parameters in the 1–9 cm compared to the >11 cm horizons are what would be expected if the bacterial community became more anaerobic with depth. Baird et al.'s analysis of the PLFA profiles [15] of the 0–1 cm and 9–10 cm horizons of samples from the same group of samples agrees with this conclusion. They found that the mole fraction of 16:1w7c and 18:1w7c decreased and 16:0, 18:0, 10Me16:0 and cyclopropane 17:0 and 19:0 increased with depth.

Respiratory quinones have for the first time been isolated, separated, and quantified from environmental samples. These preliminary data strongly support their utility as environmental biomarkers for the proportion of aerobic versus anaerobic metabolism in bacterial environments. More samples from various environments and laboratory enrichments under different conditions need to be analyzed to determine the effects of different electron donors and acceptors on the relative amounts of respiratory quinones. The detection of DMKs was unexpected as they have heretofore only been found in pathogenic bacteria, and more work needs to be done to determine their significance.

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