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REVIEW

Isoprenoid Quinones as Biomarkers of Microbial Populations in the Environment

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Isoprenoid quinones are lipid molecules present in all species of respiratory and photosynthetic microorganisms and exhibit marked structural variations depending upon the microbial taxon. Taking advantage of this, quinones have been used not only as chemotaxonomic markers in microbial systematics but also as good measures of microbial populations in the environment in terms of quantity, quality, and activity. Basically, this biomarker approach, called the quinone profile method, is applicable to all environmental samples from which an absolute amount of microbial biomass $\geq 10^9$ cells can be collected. The quinone profile method allows good measurement of both fundamental and applied aspects of ecological and environmental microbiology. In particular, numerical cluster analyses of quinone profiles are useful for monitoring microbial population shifts in an ecosystem which is not amenable to conventional culture methods and molecular techniques. The combined use of molecular techniques and the quinone profile method in this research area should provide more accurate and reliable data regarding population dynamics and community structures.

[**Key words:** isoprenoid quinones, quinone profiles, biomarkers, microbial diversity]

Isoprenoid or terpenoid quinones (called quinones hereafter) are lipid-soluble substances found in almost all species of organisms. In prokaryotic cells, quinones are located exclusively in their cytoplasmic, intracytoplasmic, and thylakoid membranes. In eukaryotic cells, most of the quinones are associated with the inner membranes of mitochondria and with the thylakoid membranes of chloroplasts. The most important biological aspects of quinones are their functions as electron carriers in respiratory chains and photosynthetic electron transport systems coupled to proton translocation. For example, ubiquinones mediate the electron transfer between complex I (NADH dehydrogenase) and complex III (bc_1 complex) in the respiratory chain of aerobic proteobacteria and mitochondria. Plastoquinones and phylloquinone are involved in electron transfer in oxygenic photosynthesis. Some other functions of quinones have recently been reviewed by Soballe and Poole (1).

In addition to their biological importance, quinones have attracted attention in connection with their significance in microbial systematics. Early studies on the natural occurrence of quinones were conducted mainly in regard to their biochemical aspects but provided circumstantial evidence that inherent structural variations shown by microbial quinones might be of chemotaxonomic significance (2–4). The pioneering systematic surveys of bacterial quinones by Jeffries and co-workers (5, 6), Yamada and co-workers (7–12), and Collins and coworkers (13–17) in the late 1960's to 1970's established a satisfactory basis for the use of quinone structural types as taxonomic criteria. Since then, quinone profile data have been accumulated from numerous microbial species, and quinone analysis has been fully recognized to be one of the most important chemotaxonomic approaches to the classification and identification of both prokaryotic and eukaryotic taxa (18–22).

Advances in methodologies of microbial quinone analysis, *e.g.*, HPLC separation techniques and computer software for data analysis, have made it possible to easily separate and identify quinone mixtures extracted not only from pure microbial strains but also from complex microbial populations in the environment. In 1986, the first demonstration of the value of quinone analysis in ecological studies was made by Hedrick and White (23), who indicated the usefulness of menaquinone/ubiquinone ratios for the evaluation of the redox state of natural microbial communities. Following this report, the analysis of quinone profiles was more strongly suggested to have great promise as a tool for the characterization of different microbial communities in natural environments without the need for isolation and cultivation (24, 25). In recent years, this biomarker approach, called the quinone profile method (26, 27), has been successfully developed for the determination of microbial community structures in various environments, such as wastewater environments (24–26, 28–37), natural aquatic systems (38, 39), hot springs (40, 41), soil (42, 43), and compost (44).

Chemical biomarker approaches by profiling cell envelope constituents such as phospholipids and fatty acids are widely used for the *in situ* characterization of microbial communities in natural environments (45). Compared to these biomarker approaches, the quinone profile method has some advantages in characterizing these microbial communities. For example, since one homolog type of quinone generally predominates in a microbial species, the quinone composition of mixed microbial communities can be interpreted to be a direct reflection of the proportion of microbial taxa with different quinone types. Although the quinone composition of certain bacteria occasionally varies depending upon culture conditions (46-48), the dominant quinone type of a single species is generally unchanged. In addition, because of its intrinsic quality as a direct extraction process

for environmental lipids, the quinone profile method produces much less biased results than conventional culture methods and molecular techniques involving nucleic acid extraction and PCR amplification. This feature of the quinone profile method is convenient particularly for the analysis of microbial communities from which it is difficult to isolate microbes as cultivable strains or to extract nucleic acids quantitatively.

This article is concerned with the significance of quinones as biomarkers of microbial populations in terms of quantity, quality, and activity. The distribution patterns of quinones in microorganisms and in natural environments are also discussed in relation to microbial ecology and evolution.

STRUCTURAL TYPES OF MICROBIAL QUINONES

Microbial quinones are categorized into two major structural classes, the naphthoquinones and the benzoquinones, which are represented by menaquinones (formerly vitamin K_2) and ubiquinones (formerly coenzyme Q), respectively (Fig. 1).

Menaquinone was first isolated from bacterially putrefied fishmeal in 1939 (49), and its chemical structure was determined in 1958 (50). Menaquinone molecules are derivatives of 2-methyl-1,4-naphthoquinone in which the 3-position carries a polyisoprenyl chain of variable length. They are named according to the number (*n*) of isoprene units in the side chain; that is, menaquinone homologs with *n* isoprene units are called MK-*n*. If menaquinones have a partially hydrogenated side chain, they are expressed as MK-*n*(H_x), where *x* indicates the number of saturating hydrogen atoms. Thus far, menaquinone species with 5 to 14 isoprene units have been found as the primary components in bacteria. Phylloquinone, which was first isolated from alfalfa in 1939, represents another group of vitamin K that has a monounsaturated phytyl group at the 3-position (51). In addition to menaquinones and phylloquinone, several natural derivatives of naphthoquinones have been discovered.

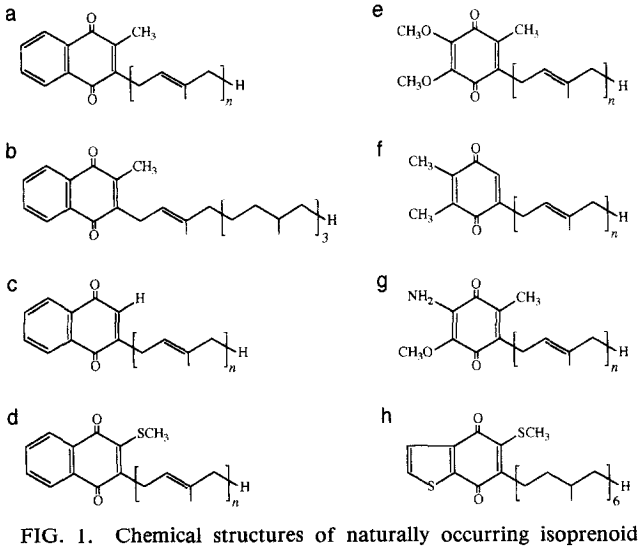

FIG. 1. Chemical structures of naturally occurring isoprenoid quinones. (a) Menaquinone (MK, MK-*n*); (b) phylloquinone (K_1); (c) demethylmenaquinone (DMK, DMK-*n*); (d) methionaquinone (MTK, MTK-*n*); (e) ubiquinone (UQ, Q-*n*); (f) plastoquinone (PQ, PQ-*n*); (g) rhodoquinone (RQ, RQ-*n*); and (h) caldariellaquinone (CQ).

These include chlorobiumquinones (52, 53), demethylmenaquinones (DMK-*n*) (54, 55), dimethylmenaquinones (56), methylmenaquinones (thermoplasmaquinones) (57, 58), and methionaquinones (MTK-*n*) (59).

Ubiquinones were discovered somewhat later than the naphthoquinones, and fully characterized in 1958 (60). They are derivatives of 2,3-dimethoxy-5-methyl-1,4-benzoquinone which has a polyisoprenyl side chain at the 6-position. Ubiquinones as well as menaquinones are named according to the number (*n*) of isoprene units and the degree of hydrogenation in the side chain, and are designated as Q-*n* and Q-*n*(H_x), respectively. The dominant species of ubiquinones found in microorganisms are Q-6 to Q-14. Another major class of benzoquinones is plastoquinone, which was first isolated from alfalfa in 1946 (61). Plastoquinones are derivatives of 2,3-dimethyl-1,4-benzoquinone having a polyisoprenyl side chain (62–64). Examples of naturally occurring derivatives of ubiquinones are epoxyubiquinones (65), methylubiquinones (66, 67) and rhodoquinones (RQ-*n*) (68, 69).

In addition to the naphthoquinones and benzoquinones noted above, there is a novel group of benzothiophenquinones, such as caldariellaquinones and sulfolobusquinones (70–73). Benzothiophenquinones as well as methionaquinones represent the major groups of natural sulfur-containing quinones so far discovered.

TAXONOMIC AND PHYLOGENETIC IMPLICATIONS

As described above, there is a wide variety of native structural types of quinones in the microbial world. All types of structural variations exhibited by these quinones may be useful as taxonomic criteria. Primary variations are found in the quinone ring structure and the number of isoprene units and the degree of saturation in the side chain. There is general agreement that quinone isoprenolog types can be used as criteria for the classification of microorganisms at the generic or higher taxonomic levels, apart from the biological significance of the length of the quinone side chain. It has recently been shown that polyprenyl diphosphate synthase essentially defines the length of the side chain of ubiquinones in their biosynthetic process (74). Although different ubiquinone isoprenologs are biologically functional in cells or reconstituted membranes, microbial species seem to prefer their own type of quinones for activities requiring maximum electron transport (75, 76).

The ring structure of quinones has more important implications than the structure of their side chains in relation to physiology, biochemistry, and phylogeny. One of the reasons for this is that the ring structure of quinones is directly related to their redox properties. For example, the menaquinone/menaquinol couple has a low redox potential (−74 mV), whereas the ubiquinone/ubiquinol couple has a higher one (+110 mV). Therefore, ubiquinones are thermodynamically unsuitable as electron mediators for anaerobic respiration that involves the use of a low-potential terminal electron acceptor such as sulfate or fumarate (77). In view of these redox properties of quinones, it is suggested that microbes with menaquinones and those with ubiquinones differ significantly from each other in their physiological and ecological aspects. In fact, strictly anaerobic respiratory bacteria contain menaquinones and/or their analogues as the sole

quinone components. On the other hand, ubiquinones occur mainly in strictly aerobic microbes, including proteobacteria, yeast, and fungi. Another reason for the phylogenetic importance of the quinone ring structure is the marked differences in the biosynthetic pathway between naphthoquinones and benzoquinones (46, 78). The biosynthesis of menaquinones and ubiquinones involves several respective steps of enzymatic activities (79–81), suggesting an evolutionary distinct status of microbial species containing naphthoquinones and benzoquinones.

Figure 2 illustrates a 16S rRNA-based phylogenetic tree of modern species of prokaryotes with different ring structural classes of major quinones. The sulfur-containing quinones, methionaquinones, have been found in members of the *Aquifex-Hydrogenobacter* group ("Aquificales") (41, 59, 82–84), which is the earliest branching lineage of (hyper-)thermophilic, chemolithotrophic, oxygen-reducing bacteria (85, 86). It has recently been shown that the hyperthermophilic aerobic archaeon *Aeropyrum pernix* contains DMK-6(H_{12}) as the major quinone and MTK-6(H_{12}) as the second component depending upon culture conditions (87). Another sulfur quinone group, the assembly of benzothiophenquinones, occurs in the hyperthermophilic acidophilic archaea, *Sulfolobus* species (70–73). These may suggest that the sulfur-containing quinones are essential in aerobic (or microaerophilic) respiratory chains of early branching prokaryotes. Members of the next branching thermophilic lines of *Bacteria*, such as the chemotrophic aerobe *Thermus* and the anoxygenic phototroph *Chloroflexus*, contain menaquinones as the sole quinones. Menaquinones and/or their analogs are the sole components in all quinone-producing members of the domain *Archaea* and in all other phylogenetic groups of the domain *Bacteria*, except cyanobacteria and the α, β, and γ subclasses of the class *Proteobacteria*. The oxygenic phototrophs, cyanobacteria, branch from this large group of menaquinone-producing bacteria, and characteristically contain plastoquinones and phylloquinone. Ubiquinones are distributed only in α-, β-, and γ-proteobacteria among prokaryotes. In eukaryotic microorganisms, ubiquinones and plastoquinones (plus phylloquinone) are present in mitochondria and chloroplasts, respectively. Menaquinones are absent in eukaryotes.

Quinone structural types that predominate in different phylogenetic groups of *Bacteria* are also summarized in Table 1 on the basis of information from the literature (18, 20–22, 88–90). In addition to the cases noted above, some phylogenetic groups contain group-specific structural types of quinones. For example, partially hydrogenated menaquinones are uniquely present in many species of the class *Actinobacteria* and in limited members of the sulfate-reducing proteobacteria. In *Proteobacteria*, Q-10 is found mostly in the α subclass, Q-8 in the β subclass, and Q-9 in the γ subclass. Interestingly, some species of these proteobacteria contain rhodoquinones as the second component (91–93). Although rhodoquinones are derivatives of benzoquinones produced from ubiquinones as precursors in their biosynthetic process (94, 95), they are similar to menaquinones rather than to

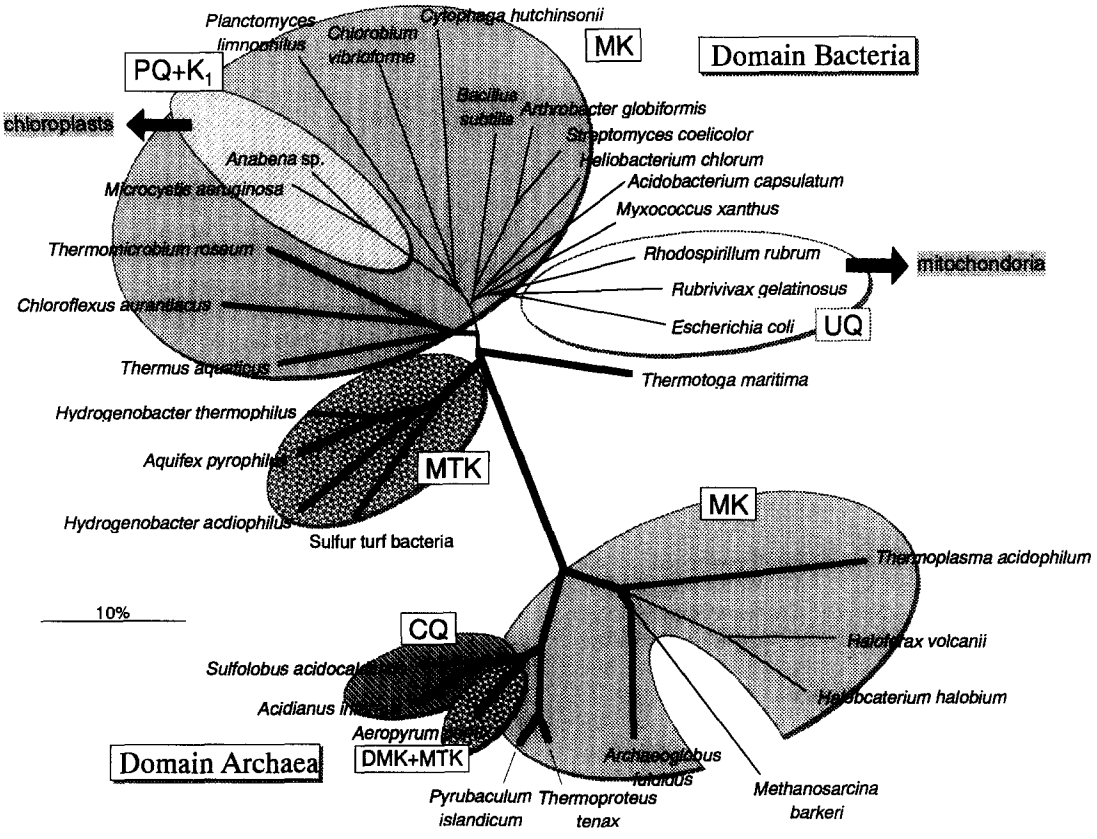

FIG. 2. Neighbor-joining phylogenetic tree of 16S rRNAs of prokaryotic species with different ring structural classes of major quinones. The 16S rRNA sequence information was obtained from the DDBJ/EMBL/GenBank databases and analyzed with the CLUSTAL W program (116) for tree construction. Each phylogenetic group with a specific quinone ring class is surrounded by an oval. The thickest and the medium-thick lines indicate the lineages of hyperthermophiles and thermophiles, respectively.

TABLE 1. Distribution of quinone structural types in different phylogenetic groups of the domain Bacteria*

Phylogenetic group	MTK	MK-*n*		MK-*n*(H_X)	$PQ+K_1$	Q-*n*			RQ
		$n\leq 8$	$n\geq 9$			$n=8$	$n=9$	$n=10$	
"Aquificales"	++	−	−	−	−	−	−	−	−
Green nonsulfur bacteria	−	+	+	−	−	−	−	−	−
Thermus-Deinococcus group	−	++	−	−	−	−	−	−	−
Cytophaga-Flavobacterium	−	++	*	−	−	−	−	−	−
Green sulfur bacteria	−	++	−	−	−	−	−	−	−
Planctomycetales	−	++	−	−	−	−	−	−	−
Acidobacterium group	−	++	−	−	−	−	−	−	−
Gram positive bacteria (low G+C)	−	+	*	−	−	−	−	−	−
Actinobacteria	−	*	+	+	−	−	−	−	−
Cyanobacteria	−	−	−	−	+	−	−	−	−
Proteobacteria									
α subclass	−	−	*	−	−	*	*	++	*
β subclass	−	*	−	−	−	++	−	−	*
γ subclass		*	−		−	+	+	−	−
δ/ε subclasses	−	++	−	*	−	−	−	−	−

* Abbreviations and symbols: MTK, methionaquinone; MK-*n*, menaquinone with *n* isoprene units; MK-*n*(H_X), menaquinone with the side chain saturated with *x* hydrogen atoms; PQ, plastoquinone; K_1, phylloquinone; Q-*n*, ubiquinone with *n* isoprene units; RQ, rhodoquinone; ++, present in all or most species (>90%); +, present in 10–90% of species; *, present in few species (<10%); −, absent. Information from references 40, 51, and 81–83 for "Aquificales", 18 and 90 for green nonsulfur bacteria, 89 for planctomycetales, 88 for *Acidobacterium* group, and 18 and 22 for all others.

ubiquinones in terms of the redox potential (96).

In yeast and fungi, various ubiquinone species (Q-6 to Q-11) are present as well. The occurrence of partially hydrogenated ubiquinones has so far been limited to certain fungal species (20).

ANALYTICAL TECHNIQUES

Extraction and purification Quinones are easily extracted from microbial cells with an appropriate organic solvent mixture. The extraction method commonly used in chemotaxonomic studies is the direct extraction of lyophilized cells with a mixture of chloroform-methanol (2 : 1, v/v) or of wet cells with acetone (19). For environmental samples, the direct extraction from wet biomass with acetone and/or with chloroform-methanol is desirable because lyophilization or drying treatment may promote the degradation of quinones in some cases. Detailed information on the extraction procedure for environmental samples has been given by Hiraishi and coworkers. (33, 38–41). The sample volume to be subjected to the analysis differs from sample to sample, *e.g.*, 0.5–1 g (wet wt) for activated sludge and compost, 10–50 g for soil and sediment, and 1–10 *l* for surface water of aquatic environments. The extract is evaporated under vacuum and treated with *n*-hexane-water (1 : 1, v/v). Then, the quinone extract in the hexane layer is saved, concentrated, and dissolved in a small amount of *n*-hexane or other organic solvents.

The lipid extract is fractionated into the menaquinone and ubiquinone fractions by an appropriate technique prior to HPLC separation. In chemotaxonomic studies, silica-gel TLC is the most commonly used technique for this purpose (19). During the course of quinone profiling of activated sludge, however, the recovery efficiency by TLC proved to be low (33, 97); therefore quinone concentration was underestimated. To overcome this problem, column chromatography with Waters Sep-Pak cartridges has been used for quinone profiling of environmental microbial populations (32, 33, 38, 97). The menaquinone fraction thus obtained may contain plastoquinones in addition to the naphthoquinones, whereas the ubiquinone fraction may contain rhodoquinones. The total concentration of menaquinones and ubiquinones in the respective fractions can be determined spectrophotometrically by monitoring reduced-minus-oxidized difference spectra (98). However, the application of this technique is difficult when each fraction contains different ring structural types of quinones. For the determination of quinone concentration, it is easier to use HPLC as described below.

A simplified scheme for the analysis of quinones in environmental samples is shown in Fig. 3.

HPLC analysis Quinone components can be separated by reverse-phase partition HPLC with a mixture of methanol-isopropyl ether, methanol-isopropanol, or methanol-chlorobutane as the mobile phase (99–101). The elution is monitored with a UV spectrophotometric detector at 270 nm for menaquinones and at 275 nm for ubiquinones. Quinone species are identified by comparing HPLC elution time of samples with standard quinones. Authentic phylloquinone and ubiquinones can be purchased from manufactures. Unfortunately, most menaquinone and plastoquinone species and other quinone derivatives found in nature are not commercially available. For these quinone types, therefore, it is necessary to use known species of bacteria and sewage activated sludge as the source of standard quinones. When the logarithmically transformed HPLC retention times of quinone isoprenologs are plotted against the number of isoprene units, a linear relationship is found for each quinone series. Based on this relationship, the "equivalent number of isoprene units" (ENIU) values (100) can be calculated for unknown quinone components. This is true for a series of partially saturated quinones. However, differences in the location of hydrogenation of the side chain may affect HPLC patterns.

The use of a photodiode array detector facilitates the identification of quinone species by recording their absorption spectra (33). In a methanol-isopropyl ether mixture (9 : 2, v/v), menaquinones and phylloquinone show absorption maxima at 243, 248 (major peak), 262, and 269 nm, ubiquinones at 275 nm, plastoquinones at 255 (major peak) and 262 nm, and rhodoquinones at 283

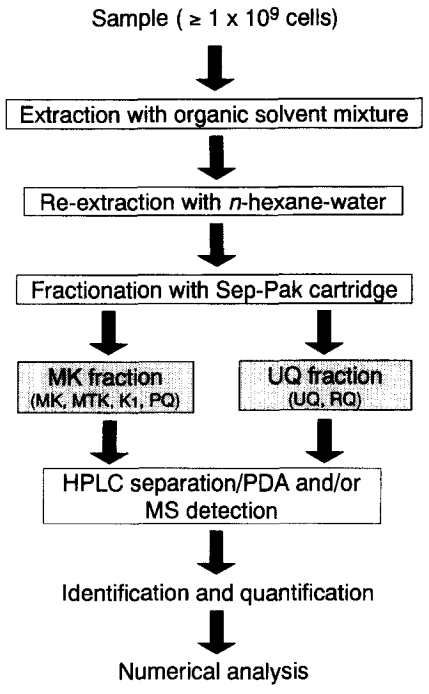

FIG. 3. Simplified flow chart for the quinone analysis of environmental samples.

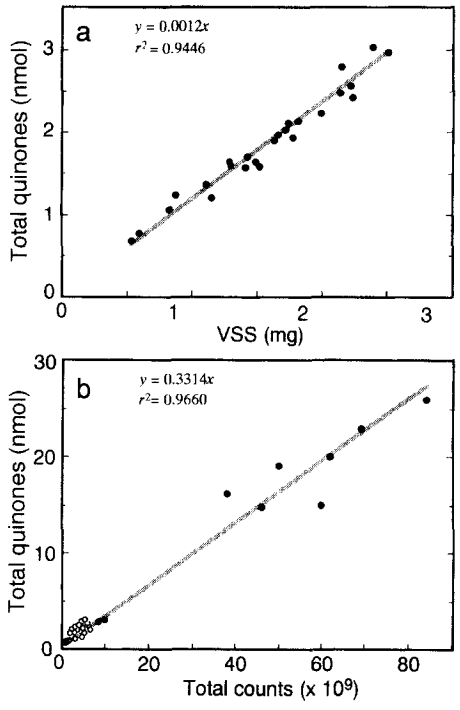

FIG. 4. Relationship between the concentration of total quinones and microbial biomass. (a) Quinones vs. volatile suspended solids (VSS) in activated sludge; (b) quinones vs. total bacterial count in activated sludge and compost. Data from references 37, 44, and A. Hiraishi (unpublished) are plotted.

nm. Since lipid components other than quinones from environmental samples are frequently detected by HPLC, it is essential to use a photodiode array detector for the accurate determination of quinone composition.

Mass spectrometry and other techniques Mass spectrometry is the most powerful technique for the structural determination of quinones. This technique provides not only accurate molecular weights of unidentified quinones but also structural information on the quinone ring and side chain. Mass spectrometric detection in addition to photodiode array measurement adds a large dimension to the HPLC system enabling the accurate identification of components in complex quinone mixtures (102).

Some different quinone types have very similar HPLC retention times. In particular, this is the case in PQ-9 vs. MK-8(H_2), MK-9 vs. MK-8(H_4), MK-10 vs. MK-9(H_4), and MK-9(H_8) vs. MK-10(H_4). In early studies of environmental quinone profiles (24, 25, 31), MK-8(H_4) and MK-9(H_4) might have been misidentified as MK-9 and MK-10, respectively. Simple methods for the identification of partially saturated menaquinones are two-dimensional TLC and silver ion-modified TLC (103), the latter of which allows the separation of quinone species according to the degree of hydrogenation of the side chain.

QUINONES IN DIFFERENT ENVIRONMENTS

Quinones as measures of biomass Whether or not the total concentration of quinones can be used as a measure of microbial biomass in the environment is a subject of major concern. Quinone contents and profiles of microbial communities may vary not only depending upon the quantity and quality of microbial biomass present but also in response to different environmental conditions. It has been shown that the quinone content of a single bacterial strain changes depending upon the growth phase and culture conditions (104–106). Hedrick and White (23) indicated that the ratio of total quinones to biomass (as measured by membrane lipid phosphate) in sediment samples was different under different environmental conditions. On the other hand, a previous study on activated sludge quinones showed that the total concentration of quinones was highly correlated with microbial biomass (as volatile suspended solids [VSS]) and the total bacterial count (37).

The relationships between the total quinone concentration and microbial biomass are shown in Fig. 4, where all data obtained thus far from activated sludge and composting systems (refs. 37, 44, and A. Hiraishi [unpublished data]) are plotted. There is a highly positive correlation between the total quinone concentration and microbial organic matter (as VSS) or total bacterial count in these environments. Based on the data shown in Fig. 4, it can be estimated that 1 nmol of total quinones corresponds to 0.83 mg of VSS (equivalent to 1.11 mg of dry sludge) and a total bacterial count of 3.0×10^9. These values are somewhat higher than those recorded previously for activated sludge (37). In a compost system, the amount of microbial biomass deduced from the quinone concentration was found to coincide with that of the biomass determined by the chloroform fumigation-extraction method (44). The relationship between quinone content and microbial biomass in other environments awaits further study.

Wastewater environments Activated sludge and other wastewater environments have received intensive study on quinone profiles as measures of microbial populations (24–26, 28–38). Microbial communities of activated sludge in municipal sewage treatment plants exhibit similar quinone profiles regardless of the scale of reac-

tors and geographical locations. The molar ratios of menaquinones to ubiquinones which have been recorded for sewage activated sludge operated at a normal biochemical oxygen demand (BOD)-loading rate (0.2–0.4 kg·dry sludge·kg^{-1}·d^{-1}) are between 0.6 and 1.0, mostly ranging from 0.9 to 1.0 (30, 33, 37). However, the MK/UQ ratios increase to 1.2 to 4.0 in sludge operated at a lower BOD-loading rate or with long sludge retention time (A. Hiraishi, unpublished observations). In the ubiquinone fraction from activated sludge, Q-8 is predominant, Q-10 is the second most common type, and Q-9 and other homologs are minor components. In addition, Q-8 is the most abundant type among the total quinone contents. In the MK fraction, either MK-7, MK-8, or MK-8(H_4), is the predominant type; in some cases, MK-6 or MK-9(H_8) is most abundant. These quinone profiles indicate that members of the β subclass of *Proteobacteria* are predominant in sewage activated sludge operated under normal conditions. α-Proteobacteria, actinobacteria, and other phylogenetic groups containing MK-6 to MK-8 (*e.g.*, *Cytophaga-Flavobacterium* group) constitute lower but significant proportions of the activated sludge microbiota. Similar results have been obtained by molecular techniques such as PCR-aided 16S rDNA cloning and sequencing and rRNA-targeted fluorescent *in situ* hybridization (FISH) (36, 107–110).

Bacterial populations in anaerobic-aerobic activated sludge showing enhanced phosphate removal were also characterized by quinone profiling (28, 29, 36, 37). The results of these analyses indicate that the bacterial community structure in activated sludge processes may be affected more by the nature of influent wastewater than by the introduction of an anaerobic stage into the process.

Microbial community structures in influent sewage as indicated by quinone profiling are different from those of activated sludge (33). The MK/UQ ratios are much lower in sewage than in activated sludge. The photosynthetic quinones, PQ-9 and K_1, occur in significant proportions, and MK-6 is the major component of the menaquinone fraction in untreated sewage.

Natural aquatic systems There has been much less information about quinone profiles in natural aquatic systems such as rivers, ponds, lakes, and marine environments. Unlike microbial populations in wastewater environments, microbial communities in rivers and lakes are characterized by a high production of plastoquinones as well as ubiquinones (38, 39). Microbial quinone compositions of lake sediment in Japan were studied by Hiraishi and Kato (39). The sediment produced PQ-9 or Q-8 as the most abundant quinone type regardless of geographic location and depth. These results indicate that oxygenic phototrophic microorganisms and Q-8-containing bacteria (*i.e.*, mostly β-proteobacteria) constitute major parts of microbial populations in lake sediment. In surface water from the same sampling sites, plastoquinones and phylloquinone were present in much higher amounts.

Thermal environments Respiratory and photosynthetic quinones of microbial mats in sulfide-containing neutral hot spring streams in Japan were recently analyzed (40, 41). One of the most interesting observations is that there are changes in quinone patterns and the physiological type of microbial mats in response to *in situ* temperature (40). All microbial mats present at temperatures above 68°C were so-called sulfur-turf bacterial mats and produced methionaquinones (MTK-7) as the major quinones. This observation is consistent with molecular data which reveal that a novel 16S rDNA phylotype belonging to "Aquificales" is abundant in the sulfur-turf mats from similar environments (111). *Chloroflexus*-mixed mats were found at 61 to 65°C and contained MK-10 as the major component together with a significant amount of either MTK-7 or PQ-9. The sunlight-exposed biomats from 45 to 56°C were all cyanobacterial mats, in which the photosynthetic quinones (PQ-9+K_1) predominated and MK-10 was the next highest component in most cases. Ubiquinones were not found or were detected only in small amounts in the biomats from 50°C and above, whereas the majority of the quinone content of a purple photosynthetic mat at 34°C was represented by Q-8. Thus, in hot spring microbial communities, the major quinones varied with decreasing temperature in the following order: methionaquinones (MTK-7)→menaquinones (MK-10)→plastoquinones (PQ-9)+K_1→ubiquinones (Q-8). This change in quinone patterns demonstrates the succession of microbes with different energy-yielding systems in response to the temperature of the environment. Namely, the mode of energy-yielding systems of microbes changes with the thermal gradient in the following order: MTK-mediated respiration by Aquificales→MK-involved anoxygenic photosynthesis by *Chloroflexus*→PQ-involved photosynthesis by cyanobacteria→UQ-involved anoxygenic photosynthesis by purple bacteria.

The feature of the phylogenetic tree of modern organisms provides circumstantial evidence that life on Earth originated from hyperthermophilic ancestry (112, 113), and that the four phylogenetic groups noted above branch more deeply in the following order: Aquificales←*Chloroflexus*←cyanobacteria←purple bacteria. Therefore, the succession of the different quinone-containing bacterial populations found in extant hot spring environments with a thermal gradient is considered to be a reflection of the evolutionary history of prokaryotes and their energy-yielding systems, which occurred in the Precambrian age on Earth.

The distribution of quinones in another type of thermal environment, marine hydrothermal vents, has not yet been investigated but is of great interest from ecological and evolutionary viewpoints.

Soil and compost There are some reports on the quinone profiles of microbial populations in soil and compost (38, 42–44). Microbial communities in these environments are characterized by the exhibition of high MK/UQ ratios and the production of no or trace amounts of plastoquinones and phylloquinone. The major portion of the menaquinone fraction consists of partially hydrogenated types and long-chain isoprenologs. These profiles indicate the predominance of actinobacterial species in soil and compost. Partially hydrogenated ubiquinones are also frequently detected in soil and compost, suggesting the presence of fungal species in considerable amounts. The finding that the concentrations of the photosynthetic quinones in soil environments are very low, if any, indicates the occurrence of free-living oxygenic phototrophs as minor constituents of the soil microbiota. In view of the quinone profile data, microbial communities in soil contrast with those in natural aquatic environments (also see Fig. 6).

NUMERICAL ANALYSES

Parameters Changes or differences in microbial quinone patterns among environmental samples over time and space can be evaluated simply by visual interpretation of HPLC profiles. To enhance the objectivity of the information, however, the processing of data by appropriate numerical and statistical methods is required. One of the most interesting issues in this respect is how to estimate differences in quinone profiles quantitatively. Numerical cluster analysis of quinone profiles was first performed by using the dissimilarity (D) index (30). This is given by:

$$D(i, j) = 1/2 \sum_{k=1}^{n} |x_{ik} - x_{jk}| \quad \text{(a)}$$

where $x_{ik}, x_{jk} \geq 0.01$, $\Sigma x_{ik} = \Sigma x_{jk} = 100$, and x_{ik} and x_{jk} indicate the mol% of quinone homolog k in samples i and j, respectively. As seen in formula (a), the D index is a modification of "city block distance". The D values can be interpreted to reveal the extent of differences in microbial community structures among samples.

Hu (Hu, H.-I., Ph. D. thesis, Yokohama Univ., Yokohama, 1993) proposed another parameter, DQ, which showed the extent of divergence of quinone types detected. This parameter was renamed the microbial divergence index (MD_q) by Iwasaki and Hiraishi (38). The microbial divergence index is given by:

$$MD_q = \left(\sum_{k=1}^{n} \sqrt{x_k} \right)^2 \quad \text{(b)}$$

where $x_k \geq 0.001$ and x_k indicates the molar ratio of quinone homolog k to the total quinone content as 1. If all quinone types detected constitute equal molar proportions, the MD_q value becomes equivalent to the number of quinone types detected. Since MD_q values represent the divergence of quinone structural types detected, they can be used as indicators of microbial diversity.

As shown by formula (b), all quinone homologs are equally taken into consideration in the calculation of MD_q values. Therefore, for this parameter, only the number and proportion of quinone homologs detected are significant, whereas those of the quinone ring classes detected are independent of what. For more appropriate interpretation of microbial diversity based on quinone profiles, the divergence of quinone nucleus type should also be taken into consideration, because this may reflect both the divergence of different phylogenetic groups and that of microbes with different respiration or energy-yielding modes. Namely, ubiquinones are the sole quinones of many species of strict aerobes having a respiratory chain with oxygen as a terminal electron acceptor (*i.e.*, most proteobacteria and mitochondria). Plastoquinones are present together with phylloquinone in microorganisms which perform oxygenic photosynthesis (*i.e.*, cyanobacteria and chloroplasts). Menaquinones are the sole quinones of strictly anaerobic bacteria and of aerobic bacteria other than the aerobic proteobacteria. Based on this concept, the bioenergetic divergence index, BD_q, was proposed (38). This is given by:

$$BD_q = (\sqrt{UQ} + \sqrt{PQ+K_1} + \sqrt{MK})^2 \quad \text{(c)}$$

where UQ, $(PQ+K_1)$, $MK \geq 0.001$ and UQ, PQ, K_1, and MK indicate the molar fraction of ubiquinones, plastoquinones, phylloquinone, and menaquinones (plus their derivatives), respectively, to the total quinone content. Thus, BD_q is regarded as an indicator of the divergence of bioenergetic modes of microbes, *i.e.*, the balance of ubiquinone-mediated aerobic respiration, oxygenic photosynthesis, and menaquinone-mediated anaerobic and aerobic respiration.

A personal computer program was developed for the integrated numerical analysis of quinone profiles (38). This program, named "BioCLUST", was written for use

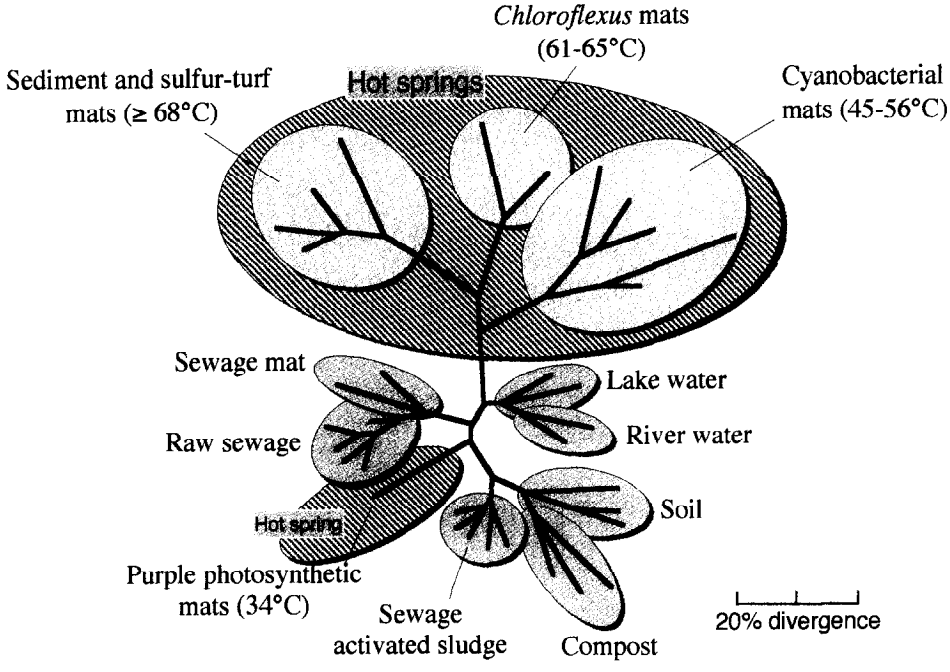

FIG. 5. Neighbor-joining dendrogram showing relationships among microbial community structures in different environments based on D matrix data. Ecologically and physicochemically similar environments are surrounded by ovals. Quinone profile data from references 37–40 and 44 were used for analysis with the BioCLUST program (38).

with IBM and compatible personal computers. Quinone profile data expressed as mol% ($k \geq 0.01\%$) are entered into the program for calculation of D, MD_q, and BD_q values, tabulation of D value matrix data, and construction of neighbor-joining dendrograms for cluster analysis. Neighbor-joining is an algorithm originally developed for constructing phylogenetic trees based on DNA and protein sequences (114). This method gives more accurate results in describing the topology of trees than the unweighted pair group method using arithmetic averages, which is commonly used for cluster analysis in ecological studies.

Cluster analysis The cluster analysis of microbial communities in various environments has been successfully performed by quinone profiling and neighbor-joining tree construction (36–38, 40). Based on the available D data, the author reconstructed a neighbor-joining dendrogram for grouping of microbiota in various environments, including hot springs, rivers, lakes, sewage, activated sludge, and soil (Fig. 5). It is clear that the microbial communities in a physically and chemically similar environment form a single tight cluster. Thus, neighbor-joining cluster analysis based on D matrix data allows quantitative estimation of the differences in entire community structures among various environments. This performance is a characteristic feature of the quinone profile method.

The plotting of MD_q vs. BD_q values provides more quantitative information on the differences in microbial communities with respect to chemotaxonomic and bioenergetic diversity. As shown in Fig. 6, microbial communities from ecologically similar environments are grouped in a single narrow area on the MD_q vs. BD_q coordinate system. Of particular interest is the finding that the microbial diversity of hot spring environments becomes simpler as the temperature of the environment increases. Thus, temperature may be one of the most important factors defining the vector of the construction and evolution of microbial diversity. The combined use of D-based neighbor-joining dendrograms and MD_q vs. BD_q plot profiles is very attractive for determining microbial population shifts quantitatively.

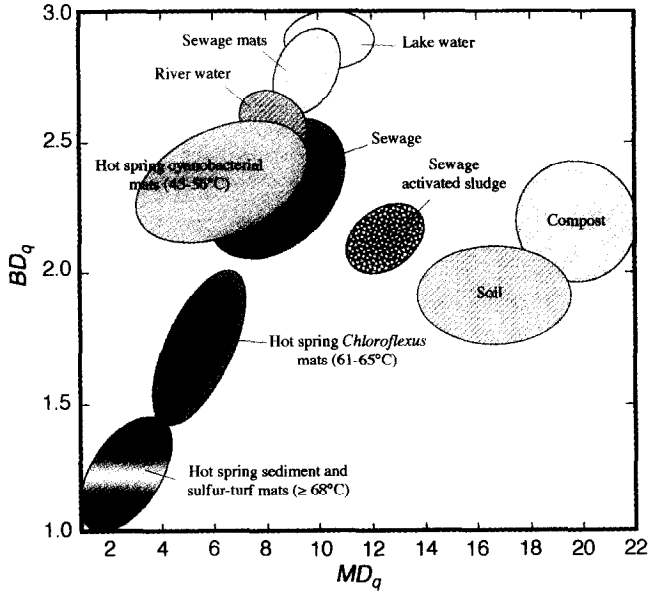

FIG. 6. MD_q-BD_q plot profile showing the extent of microbial diversity in different environments. The quinone profile data shown in Fig. 5 were used for calculating MD_q and BD_q values.

COMPARISON TO MOLECULAR APPROACHES

Genetic molecular methods by using rRNA sequence information, the so-called rRNA approaches (115), are now widely used for the characterization and identification of different phylogenetic taxa in microbial communities. The principles of the quinone profile method and the rRNA methods are completely different. Therefore, it is of great significance to compare the former method with the latter in characterizing microbial communities in environmental samples. The phylogenetic and taxonomic structures of bacterial populations in an anaerobic-aerobic activated sludge system were studied by quinone profiling, 16S rDNA cloning and sequencing, and FISH (36). Although the results of these approaches were similar as a whole, some differences in the detection of specific phylogenetic groups were found between the chemotaxonomic and molecular methods. The quinone profile data indicated the occurrence of a relatively high proportion of *Actinobacteria* (>23%), whereas both molecular methods detected small populations (3.1–6.9%) of this phylogenetic group. It is evident that the quinone method is inferior to the rRNA methods in terms of taxonomic and phylogenetic resolution. However, the limitations and potential biases of the molecular methods were suggested only when the quinone profile method was simultaneously used.

Recently, the bacterial community structure of activated sludge was characterized on the basis of both quinone profiles and terminal restriction patterns of PCR-amplified 16S rDNA (T-RFLP). Numerical cluster analyses demonstrated that there was a high correlation between the quantitative data of population shifts deduced from quinone and T-RFLP patterns (A. Hiraishi, unpublished data).

FUTURE PERSPECTIVES

Quinone profiling is one of the most useful culture-independent biomarker approaches to study microbial populations in the environment in terms of quantity, quality, and activity. Basically, this biomarker approach is applicable to all environmental samples from which an absolute amount of microbial biomass of more than 10^9 cells can be collected. The available information regarding quinone profiles is obtained mainly from man-made and natural environments that contain relatively high populations of microorganisms, such as activated sludge, sediment, and soil. The application of the quinone profile method to other environments has just begun. In this context, quinone analysis of microbial habitats with low biomass, particularly marine environments, is of great interest.

The quinone profile method not only allows good measurement of fundamental aspects of microbial ecology but also provides information of value for applied microbiology and biotechnology. The numerical cluster analysis of quinone profiles has enormous potential for application to environmental biotechnology, such as biological waste treatment and bioremediation. In particular, this approach may be useful for the quantitative analysis of microbial population shifts in bioremediation processes which are not amenable to conventional cul-

ture methods and molecular techniques.

A new aspect of the quinone profile method concerns the analysis of specific microbial activity. In general, measurements of microbial activity involve the incorporation or metabolism of a precursor compound with an appropriate label, and this procedure is applicable to quinone analysis with mass spectrometric detection. Recent studies in our laboratory have shown that, when sewage activated sludge is fed with ^{13}C-labeled acetate or phenol, the label is incorporated mainly into Q-8 among all the quinone species detected (unpublished data). This suggests that the bacteria containing Q-8, *i.e.*, β-proteobacteria, play a primary role in removing acetate or phenol as a BOD source in activated sludge processing.

Although culture-independent chemical biomarker and molecular methods are innovative in ecological and environmental microbiology, each of the methods still has limitations. To compensate for these limitations and rectify potential biases, a polyphasic approach using different methods is required. The combined use of molecular techniques and the quinone profile method in this area of research should provide more accurate and reliable data regarding population dynamics and community structures.

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