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Short Communication

Significance of Lipoquinones as Quantitative Biomarkers of Bacterial Populations in the Environment

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The direct total count of bacteria and the concentration of isoprenoid quinones were measured in 82 samples from different aquatic and soil environments. The geometric mean of the total count (TC)/total quinine (TQ) ratios obtained with different environments ranged from 1.1 to 2.2×10^3 cells $fmol^{-1}$. When the concentration of the total respiratory quinone (TRQ), i.e., ubiquinones and menaquinones, were taken into account, the geometric mean of the TC/TRQ ratios fell into a range of 2.2 to 4.1×10^3 cells $fmol^{-1}$. A high positive correlation was noted between TC and TQ (r^2=0.9864) or TRQ (r^2=0.9990). Based on the relationship between TC and TRQ, 1 nmol of the total quinone was estimated to be equivalent to 2.5×10^9 cells of bacteria on average. These results indicate that the concentration of the respiratory quinones can be used as a good measure of bacterial counts and biomass in the environment.

Key words: total bacterial counts, quinones, biomarkers

The quinone profiling method is not only widely used to provide an important chemotaxonomic criterion in microbial systematics[1)] but also recognized as one of the most promising lipid biomarker approaches to research on microbial ecology[5)]. Since one homolog type of quinone predominates in a single genus or species of microorganisms in general, the content of each quinone homolog in complex microbial communities may be interpreted as a direct reflection of the content of microbial taxa with different quinones. Taking this advantage, the quinone profiling method has been applied to characterize microbial community structure in various environments, such as sewage and activated sludge[4,8–10,13,17,18,20)], aquatic sediments[6,24)], hot springs[11)], soil[2,7,15,16)] and compost[12)]. Although the universality of quinone profiles as tools for estimating microbial community changes over time and space has been realized, the available information about the quantitative relationship between quinones and microbial biomass or cell counts is still limited. An early report in this research area showed that the ratio of total quinones to microbial biomass as measured by membrane lipid phosphate in sediment samples fluctuated under different environmental conditions[3)]. On the other hand, our previous studies indicated that there was a positive correlation between the total quinone content (TQ) and microbial biomass or the total count (TC) of bacteria in activated sludge and compost[5,10)]. Preliminary estimation based on these limited data showed that 1 nmol of total quinones corresponded to 0.83 mg of microbial biomass in activated sludge[5)]. Also, a high correlation between the quinone content and microbial biomass was found in soil environments[15,16)]. Thus, the purpose of this study was to more thoroughly evaluate the significance of quinones as quantitative biomarkers with special emphasis on their

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Table 1. Total bacterial counts and quinone concentrations in various environments

Sample	No. of samples tested	Geometric mean (and range) of total count (cells ml^{-1} or g^{-1} dry wt)	Geometric mean (and range) of total quinone content (fmol ml^{-1} or g^{-1} dry wt)	Mean TC/TQ ratio ($\times 10^3$ cells $fmol^{-1}$)
Aquatic environment				
Sea water	6	7.2 (3.2–23)$\times 10^5$	5.97 (2.67–10.1)$\times 10^2$	1.2 (4.1)[a]
Lake water	5	1.1 (0.67–1.7)$\times 10^6$	9.14 (2.28–23.0)$\times 10^2$	1.2 (3.7)
Lake sediment	6	3.8 (1.2–14)$\times 10^8$	3.06 (0.886–12.1)$\times 10^5$	1.3 (3.1)
River water	8	3.3 (0.71–26)$\times 10^6$	2.40 (0.710–8.60)$\times 10^3$	1.1 (2.7)
Wastewater environment				
Raw sewage	11	1.2 (6.0–51)$\times 10^7$	1.05 (0.238–72.0)$\times 10^4$	1.8 (2.2)
Activated sludge	12	5.5 (3.9–8.5)$\times 10^9$	2.55 (1.52–4.75)$\times 10^6$	2.2 (2.2)
Soil environment				
Forest soil	9	6.8 (1.6–38)$\times 10^8$	3.11 (0.880–19.8)$\times 10^5$	2.2 (2.2)
Farmland soil	9	5.8 (2.3–11)$\times 10^9$	2.73 (1.13–5.22)$\times 10^6$	2.1 (2.1)
Compost	16	2.6 (0.22–7.8)$\times 10^{11}$	1.26 (0.158–3.57)$\times 10^8$	2.1 (2.1)

[a] Figures in parentheses indicate the ratio of total count to total respiratory quinone content (ubiquinones plus menaquinones).

relationship to total bacterial counts. For this, we studied the TC/TQ ratios in a broad range of aquatic and soil environments as well as in artificial ecosystems.

A total of 82 samples to be analyzed for TC and TQ were collected from different areas in Japan (see Table 1). Surface water and sediment samples were taken from Toyogawa River (Aichi Prefecture), Umeda River (Aichi Prefecture), Lake Hamana (Shizuoka Prefecture), Lake Suwa (Nagano Prefecture), Lake Teganuma (Chiba Prefecture), Atsumi Bay (Aichi Prefecture) and Sagami Bay (Kanagawa Prefecture). Sewage and activated sludge were collected from sewage treatment plants in Nagoya, Osaka and Toyohashi. Soil and compost samples were collected as described previously[12,14]. Direct total counts of bacteria were measured by epifluorescence microscopy with 4,6-diamino-2-phenylindole (DAPI)[22] or ethidium bromide (EtBr)[23] staining. Also, another nucleic acid-specific fluorochrome, SYBR Green II (Molecular Probes, Inc., Eugene, OR, USA), was used to stain and count cells in sewage and activated sludge as reported previously[17]. A preliminary study showed that there was no significant difference in cell counts among the DAPI, EtBr and SYBR Green staining methods. Stained samples were observed under an Olympus BX-50 epifluorescence microscope equipped with a Flovel FD-120M digital CCD camera (Flovel Co., Tokyo, Japan). The number of stained cells was counted using the image analysis program WINROOF (Flovel), where 12–16 fields per sample and a total of 1,600–2,000 cells per sample were taken to count. For quinone analysis, microbial biomass and suspended solids from 3 to 10 L of water samples were harvested by centrifugation at 12,600×*g* for 15 min. The resultant supernatant was further filtered through membrane filters with a pore size of 0.2 μm to collect the remaining and carry-over biomass. Sediment, sewage, activated sludge, soil and compost samples were prepared for quinones analysis as previously reported[9,14]. Quinones were extracted with acetone and a chloroform-methanol mixture, fractionated into the menaquinone and ubiquinone fractions by column chromatography using Sep-Pak Vac silica gel cartridges (Waters, Milford, MA, USA) and then separated for identification and quantification by reverse-phase HPLC and photodiode array detection with external quinone standards[9,14]. Mass spectrometry with atomic pressure chemical ionization was also used for identification of quinone components of the menaquinone fraction[12].

Table 1 summarizes the data on TC and TQ obtained with all test samples. The molar fractions of different quinone classes, ubiquinones, plastoquinones, phylloquinone, unsaturated menaquinones and partially saturated menaquinones, detected in different environments are also shown in Fig. 1. The calculated geometric means of the TC/TQ ratios differed with different environments but fell into a relatively narrow range of 1.1–2.2$\times 10^3$ cells $fmol^{-1}$. All of the samples from the natural aquatic environments contained significant amounts of the photosynthetic quinones, plastoquinone and phylloquinone (Fig. 1), most of which were possibly derived from eukarytotic phytoplanktons. Thus, when only the total respiratory quinone (TRQ) content (i.e., ubiquinones

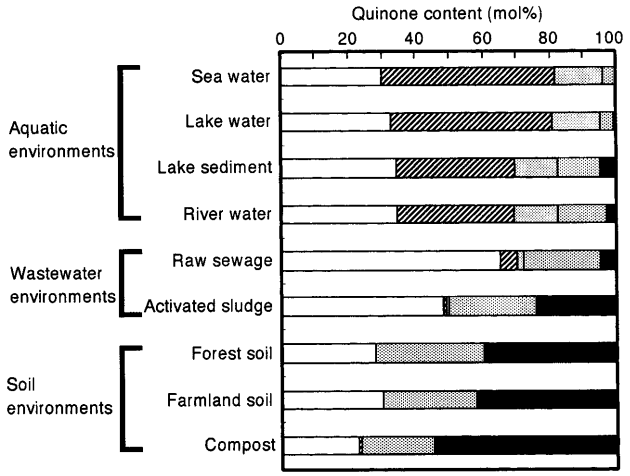

Fig. 1. Quinone composition of microbial communities in different environments. Data show average mol% of ubiquinones (open area), plastoquinones (striped area), phylloquinone (dotted area), unsaturated menaquinones (gray area) and partially saturated menaquinones (closed area). The number of samples from each environment is given in Table 1.

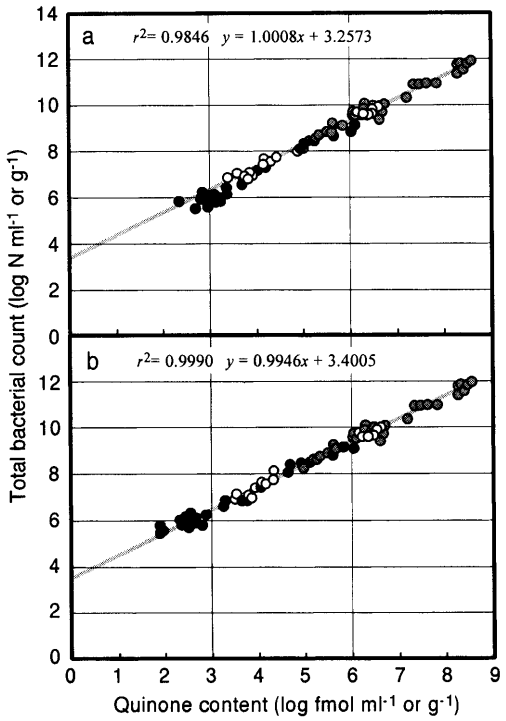

Fig. 2. Correlation between the total count and the total quinone (a) or the total respiratory quinone (b) in the environment. All data obtained with 82 samples from aquatic (closed circles), wastewater (open circles) and soil (gray circles) environments were plotted.

plus menaquinones) was taken into account, the average TC/TRQ ratios ranged from 2.1 to 4.1×10^3 cells fmol^{-1}. In this case, the TC/TRQ ratios became higher in the natural aquatic environments than in the wastewater and soil environments. This suggests that the quinone content of individual bacterial cells was lower in the natural aquatic environments. However, if cyanobacteria, containing plastoquinone and phylloquinone as the major quinones, were incorporated into the TCs measured, it was less significant to compare TC/TRQ ratios in different environments. Apart from this question, the results shown in Table 1 indicate that the size of the quinone pool of whole microbial communities is relatively constant irrespective of environment.

When all TC data were plotted against the quinone concentrations, a high positive correlation was noted between TC and TQ (r^2=0.9846) or TRQ (r^2=0.9990) (Fig. 2). Based on a regression equation deduced for the relationship between TC and TRQ, 1 nmol of the total quinone is estimated to correspond to 2.5×10^9 cells of bacteria on average. Previous studies showed that the TC/TRQ ratios were 1.3×10^9 cells nmol^{-1} in activated sludge[10] and 3.0×10^9 cells nmol^{-1} when combined data on activated sludge and compost were used[5]. The differences in TC/TRQ values among the studies are possibly due to differences in accuracy of cell counting and/or quinone content and cell size of bacteria in samples under investigation.

It has been shown that the quinone content of bacterial cultures differs from species to species and varies depending upon environmental conditions[19,21,25]. In this respect, we also examined the relationship between the quinone concentration and the cell density in pure cultures of 8 representative species of gram-negative and gram-positive bacteria (Table 2). The quinone content and the cell count per g of biomass were found to differ significantly from species to species. However, the geometric mean of the TC/TQ ratios for all test bacteria (2.6×10^9 cells nmol^{-1}) was similar to those for the environmental samples noted above.

The results of this study demonstrate that there is a strong positive correlation between TC and TQ or TRQ in a broad range of environments. Probably, the quinone content of individual cells or species of bacteria present in complex communities is much different and varies in response to environmental stress. However, since the quinone profiling method detects the bulk of quinones from whole microbial communities, the variations in the quinone content among the bacteria present may be leveled off. This is a possible reason why the size of the quinone pool of whole microbial communities in different environments falls into a relatively narrow range. In conclusion, the analysis of quinones can provide quantitative information as well as qualitative data

Table 2. Quinone content and cell count per g (dry wt) of biomass of different bacterial strains.

Test organism[a]	Growth medium used[b]	Total quinone content (μmol g^{-1} dry wt)	Major quinone type(s)	Cell count ($\times 10^{12}$ g^{-1} dry wt)
Acinetobacter calcoaceticus IAM 12087	PBY	2.66	Q-9	5.8
Bacillus subtilis IAM 12118	PBY	0.981	MK-7	1.2
Brachymonas denitrificans JCM 9216	PBY	3.06	Q-8+RQ-8	6.7
Brevundimonas diminuta IAM 12691	PBY	2.55	Q-10	11
Escherichia coli IAM 12119	PBY	0.610	Q-8+MK-8+DMK-8	3.6
Micrococcus luteus IAM 1056	PBY	1.98	MK-8+MK-8 (H_2)	5.5
Porphyrobacter sanguineus IAM 12620	PBY+1% NaCl	2.69	Q-10	6.7
Rhodopseudomonas palustris DSM 123	MYS	1.14	Q-10	3.2
Geometric mean		1.71		4.4

[a] The strains with DSM, IAM and JCM numbers were obtained from the DSMZ (Deutsche Summlung von Mikroorganismen und Zellkulturen GmbH), Braunschweig, Germany, IAM Culture Collection, The University of Tokyo, Tokyo, Japan, and the Japan Collection of Microorganisms, Wako, Japan, respectively. All strains tested are the type strains of the respective species.

[b] PBY medium contained 0.5% peptone, 0.3% beef extract and 0.1% extract (pH 7.0); MYS medium contained 0.36% sodium malate, 0.1% ammonium sulfate, 0.05% yeast extract and a mineral base. Cell were grown aerobically with reciprocal shaking and harvested by centrifugation at the late exponential phase of growth for testing.

on bacterial populations or biomass in complex environments. Since quinone profiling is a direct chemical method for the analysis of lipid molecules, it can be easily applied for samples in which cell counting or biomass estimation is difficult to do. For example, this method has been successfully used for the characterization and biomass estimation of endosymbiotic bacteria associated with invertebrates dwelling in chemosynthetic communities of hydrothermal vents and cold seep fields[26].

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