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Characterization of the Bacterial Population Structure in an Anaerobic-Aerobic Activated Sludge System on the Basis of Respiratory Quinone Profiles

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Bacterial respiratory quinones were used as biomarkers for studying the bacterial population structure, especially the content of *Acinetobacter* species, in a laboratory-scale anaerobic-aerobic activated sludge system and in the standard aerobic system. All tested sludges contained both ubiquinone and menaquinone, with a molar ratio of about 1:0.5. High-performance liquid chromatography showed that ubiquinone with eight isoprene units (Q-8) was present as the predominant ubiquinone, Q-10 was the second most common type, and Q-9 and other homologs were minor components in the anaerobic-aerobic sludge and the standard aerobic sludge. Bacteriological examination indicated that, in both sludge systems, Q-8-containing bacteria constituted a large proportion of the aerobic heterotrophic bacterial flora, but only a few strains with Q-9 were found. These findings demonstrate that the population of *Acinetobacter* species, which contain Q-9 as the major quinone, is negligible in those environments. The present results suggest that the introduction of anaerobic conditions into the aerobic batch process has little influence on the bacterial community structure.

Anaerobic-aerobic or anoxic-oxic activated sludge systems are characterized by their capacity for efficient phosphate removal from wastewater (3). Since the enhanced phosphate removal is possibly attributable to the presence of certain bacteria capable of rapid uptake and accumulation of phosphate, the study of the bacterial population structure in the anaerobic-aerobic system is a subject of great concern. The available information on this matter, however, is still fragmentary and unclear. Numerous investigators have reported that *Acinetobacter* species are involved in phosphate removal processes and/or predominate in the bacterial population in the anaerobic-aerobic system (5, 6, 8–10, 15–17). On the other hand, some species of bacteria other than *Acinetobacter* species were suggested to have important roles in biological phosphate removal (4, 16, 22).

The limited and confusing information regarding the sludge bacteria results partly from methodological difficulties in quantitative isolation and identification of the bacteria by conventional procedures. To conquer this problem, a new approach to research in this area is required. Recently, bacterial respiratory quinones have been successfully used as biomarkers for identifying different bacterial populations in activated sludge (12) and for monitoring changes in the respiratory state of microbial communities in the environment (11). The quinone composition is now recognized as one of the most important traits in bacterial chemotaxonomy (7).

In the present study, the bacterial community structure of activated sludge in an anaerobic-aerobic system was characterized on the basis of bacterial quinone profiles in comparison with that in the standard aerobic system. In particular, we surveyed the distribution of *Acinetobacter* species in those environments, taking advantage of the fact that these bacteria contain ubiquinone with nine isoprene units as the major quinone component (7, 18). The main objectives of the research were to determine (i) the effect of anaerobic stress on the bacterial flora and (ii) the relationship between enhanced phosphate removal and the presence of *Acinetobacter* in anaerobic-aerobic systems.

MATERIALS AND METHODS

Sludge samples. Activated sludge was taken from the Tsudanuma sewage treatment plant, Chiba, Japan, and cultivated in our laboratory with synthetic sewage (12) containing mineral salts, acetate, peptone, and meat extract. The laboratory system consisted of two jar fermentors (2-liter capacity each) equipped with a dissolved oxygen controller unit (Iwashiya ADM, Ltd., Tokyo, Japan). One of the reactors was operated on a fill-and-draw basis with a 24-h batch cycle consisting of an 8-h anaerobic period, a 15-h aerobic period, and a 1-h settling stage (anaerobic-aerobic system). The other was also a 24-h batch process, consisting of a 23-h aerobic period and 1 h for settling (standard aerobic system). Anaerobic conditions were achieved by stopping aeration, and all aerobic stages were kept at a dissolved oxygen tension between 3 and 5 mg/liter. The initial sludge concentration in a batch cycle was adjusted to 2,000 to 2,500 mg of dry sludge per ml, and the waste-loading rate was 0.35 kg of biological oxygen demand per kg of dry sludge per day. After complete acclimation, sludge was sampled from the reactors at the end of the anaerobic or aerobic phase of operation and then prepared suitably for quinone analysis and bacteriological examination as described previously (12).

Quinone analysis. Quinones were extracted from fresh wet sludge with a mixture of chloroform-methanol (2:1, [vol/vol]), purified by thin-layer chromatography, and then analyzed spectrophotometrically for verification of purity and determination of concentrations. Quinone components were separated by reverse-phase high-performance liquid chromatography and then identified by comparing their retention

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times with those of standard quinones. These analytical procedures have been described previously (12, 13). In this paper, ubiquinone and menaquinone with n isoprene units are abbreviated Q-n and MK-n, respectively, and MK-$n(H_x)$ represents menaquinone having n isoprene units of the side chain saturated with x hydrogen atoms.

Bacteriological examination. Aerobic heterotrophic bacteria were isolated quantitatively from activated sludge by the plate count method as reported previously (12). The plating technique with the acetate medium of Deinema et al. (9) was also used for selective isolation of *Acinetobacter* species. Colonies recovered on countable plates used for the enumeration were picked randomly for standard purification and then subjected to quinone analysis and several phenotypic tests for identification as previously described (12). Bacterial identification was performed by comparing the results of characterization tests with the descriptions in a previous report (12) and in *Bergey's Manual of Systematic Bacteriology*, vol. 1 and 2 (14, 21). As reference organisms, the following bacterial strains were used: *Acinetobacter calcoaceticus* IAM 12087 (=ATCC 23055, type strain), *A. calcoaceticus* IFO 12552, and *Acinetobacter lwoffii* GIFU 1951 (=ATCC 15039, type strain), which were obtained from the Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan, the Institute for Fermentation, Osaka, Japan, and the Department of Microbiology, Gifu University School of Medicine, Gifu, Japan, respectively.

Phosphate determination. P_i concentration in wastewater was determined colorimetrically by the ascorbic acid method (2).

RESULTS

P_i release and removal by anaerobic-aerobic sludge. After several weeks of operation in the laboratory, the sludge in the intermittently anaerobic treatment unit came to show features characteristic of anaerobic-aerobic sludge, i.e., P_i release under anaerobic conditions and P_i uptake during the aerobic phase. Typical data obtained in a batch test are shown in Fig. 1. In this case, the extracellular soluble phosphate increased from 0.42 to 1.20 mmol/liter, with an initial release rate of 0.15 mmol of P_i per h per g (dry weight) of sludge during the anaerobic phase, and subsequent aerobic treatment resulted in removal of 85% of the soluble phosphate. The P_i release rate in the laboratory sludge was similar to those of other anaerobic-aerobic systems reported elsewhere (17, 20) and was considerably higher than those recorded for pure *Acinetobacter* cultures (1, 10, 19) and other bacteria (23).

Quinone profiles of sludge. The anaerobic-aerobic sludge in the steady state noted above was examined for isoprenoid quinone composition in comparison with the plant sludge used as the seed sludge and the laboratory sludge in the standard aerobic system. All tested sludges contained both ubiquinone and menaquinone, at concentrations of 0.36 to 0.44 and 0.18 to 0.23 μmol/g (dry weight) of sludge, respectively. The menaquinone/ubiquinone ratios for these sludges varied slightly between 0.47 and 0.52, with an average value of 0.50, suggesting no marked difference in the respiratory states (11) among the sludges. Although activated sludge was reported to produce demethylmenaquinone and/or rhodoquinone in some cases (12), such quinones were not detected in any tested sludge.

The homologous distribution of quinones extracted from the three types of sludge was analyzed by high-performance liquid chromatography (Fig. 2). The sludges were quite similar in ubiquinone profiles. In all cases, Q-8 was present as the predominant ubiquinone, Q-10 was the second most common type, and Q-9 and other homologs occurred as minor components. Quantitative data analysis with five different determinations for the anaerobic-aerobic sludge indicated that the relative amounts of Q-8, Q-9, and Q-10 were 66 to 73, 3 to 6, and 17 to 24%, respectively. Similar results were obtained with standard activated sludge studied here (data not shown) and elsewhere (12). On the other hand, the three sludges differed in menaquinone composition (Fig. 2). The major types in the seed sludge were MK-7, MK-10, and MK-11, while both laboratory sludges produced MK-7, but not the long-chain homologs, as the major menaquinones. Significant proportions of MK-8 and MK-9 were also noted in the laboratory sludges.

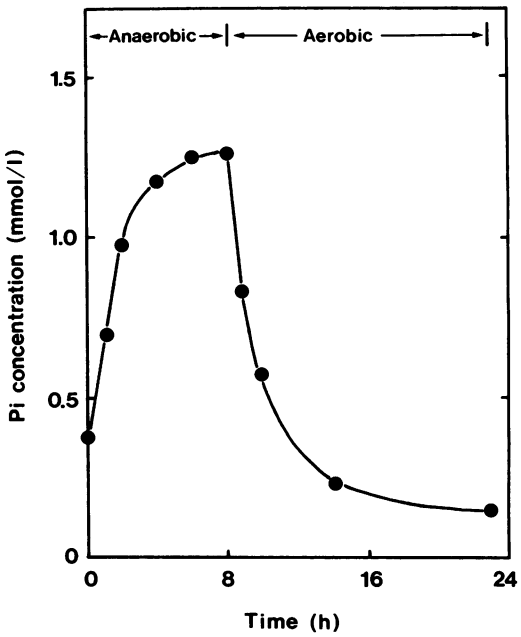

FIG. 1. P_i release and uptake by laboratory anaerobic-aerobic sludge in a batch cycle.

The results shown in Fig. 2 provide evidence that a shift in menaquinone composition took place from the seed sludge to the anaerobic-aerobic sludge. However, this may not have been due to the introduction of anaerobic conditions but rather to other factors, such as the chemical composition of the wastewater, since there was more similarity in menaquinone profiles between the two laboratory sludges than between the anaerobic-aerobic sludge and the plant sludge.

Comparative studies of sludges at the anaerobic and aerobic stages showed that there was no difference in lipoquinone profiles between the two sludges (data not shown). For the anaerobic-aerobic system, therefore, only data obtained with sludge at the aerobic stage is presented below.

Quinone composition of *Acinetobacter* species. The fact that *Acinetobacter* species contain ubiquinone as the sole quinone, with Q-9 predominating (7, 18), was verified in this study. The concentrations and homologous distribution of ubiquinone in *A. calcoaceticus* and *A. lwoffii* are shown in Table 1. The ubiquinone content of these species ranged from 2.50 to 3.28 μmol/g (dry weight) of cells. These values are much higher than those obtained for the sludge samples. All tested strains produced Q-9 as the major type, with small amounts of the next lower and higher homologs.

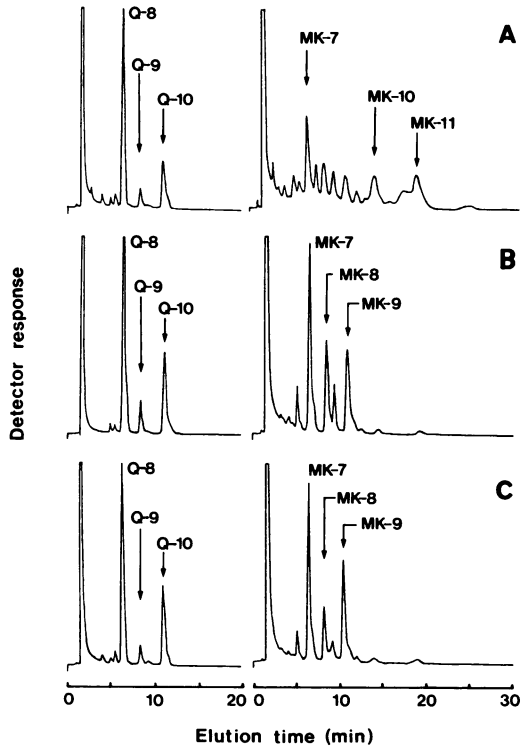

FIG. 2. High-performance liquid chromatography of ubiquinones (left) and menaquinones (right) from three types of activated sludge. (A) Plant aerobic sludge used as the seed; (B) laboratory sludge in the standard aerobic system; (C) laboratory sludge in the anaerobic-aerobic system (aerobic stage). Analytical conditions: pump, Shimadzu LC-6A; column, Cosmosil $5C_{18}$ (4.6 [inside diameter] by 150 mm); mobile phase, methanol-isopropyl ether (10:3 [vol/vol] for ubiquinone and 3:1 [vol/vol] for menaquinone); flow rate, 1 ml/min; column temperature, 22°C; ubiquinone and menaquinone samples were monitored at 275 and 270 nm, respectively.

Bacterial flora of sludge. Bacteriological examination of the anaerobic-aerobic sludge showed that aerobic heterotrophic bacteria occurred at 3.9×10^8 CFU/mg (dry weight) of sludge, while the presumptive *Acinetobacter* count estimated with the acetate medium was 3.8×10^6 CFU/mg of dry sludge. Thus, even if the colonies recovered on the acetate agar plates were all *Acinetobacter* species, their numbers accounted for only 1% of the total viable population. Similar bacterial counts were observed in the standard aerobic sludge (data not shown).

TABLE 1. Ubiquinone composition of strains of *Acinetobacter* species[a]

Species and strain	Total ubiquinone content (μmol/g [dry wt])	Homolog (%)			
		Q-7	Q-8	Q-9	Q-10
A. calcoaceticus					
IAM 12087	2.56	tr[b]	6	88	5
IFO 12252	2.50	tr	7	86	6
A. lwoffii GIFU 1951	3.28	1	11	86	2

[a] Results obtained with cells grown aerobically in PPY broth (0.3% proteose peptone, 0.3% Polypepton, 0.3% yeast extract [pH 7.0]) (12) for 2 to 3 days.
[b] tr, Trace (less than 1% of total peak area).

TABLE 2. Distribution of aerobic heterotrophic bacteria by quinone category and taxonomic group in anaerobic-aerobic activated sludge and standard aerobic sludge

Quinone	Genus or group	% of total isolates	
		Anaerobic-aerobic sludge (n = 65[a])	Standard aerobic sludge (n = 52)
Q-8	*Comamonas-Pseudomonas*	46.2	50.0
	Alcaligenes	7.7	5.8
Q-9	*Pseudomonas* (fluorescent group)	3.1	1.9
Q-10	*Methylobacterium*	1.5	
	Paracoccus	12.3	11.5
	Unidentified (gram-negative rods)		1.9
Q-8 + MK-8	*Aeromonas*	1.5	1.9
MK-7	*Flavobacterium-Cytophaga*	16.9	13.5
	Bacillus		1.9
	Staphylococcus	1.5	
MK-8	*Micrococcus*		1.9
MK-8(H_2)	Coryneform	3.1	5.8
	Rhodococcus	1.5	
MK-9(H_2)	*Arthrobacter*		1.9
MK-10	Unidentified (gram-positive cocci)	1.5	
MK-11 + MK-12	*Aureobacterium-Microbacterium*	3.1	1.9

[a] Number of strains isolated and tested.

A total of 117 strains of aerobic heterotrophic bacteria were isolated from both laboratory sludges and identified on the basis of their phenotypic properties and quinone systems. The distribution of the isolates in various taxonomic groups and quinone categories is shown in Table 2. No marked difference was observed in the bacterial composition between the anaerobic-aerobic and standard aerobic sludges. The majority of the isolates were Q-8-type bacteria, most of which were identified as *Comamonas* species or certain *Pseudomonas* species. Bacteria with Q-10 and MK-7, principally belonging to the genus *Paracoccus* and the *Flavobacterium-Cytophaga* group, respectively, also constituted a significant proportion of the bacterial flora. Such menaquinone-producing bacteria as the coryneform and nocardioform groups occurred in small percentages. No *Acinetobacter* strains were found among the isolates, and only a few strains with Q-9, probably belonging to a typical *Pseudomonas* species (fluorescent group), were isolated. These bacteriological and chemotaxonomic data are consistent with the bacterial quinone profiles obtained with the sludge samples themselves.

Viability and survival of *Acinetobacter* species in anaerobic-aerobic systems. The findings presented above concerning the sludge quinone profiles and bacterial flora contradict the idea that *Acinetobacter* species are predominant bacteria in the anaerobic-aerobic sludge system. This raises the question whether exogenous *Acinetobacter* strains can really grow and survive in this system. To answer this question, we investigated the population dynamics of *Acinetobacter* species in an anaerobic-aerobic reactor seeded with pure cultures of these bacteria, using Q-9 as the indicator.

The changes in ubiquinone profiles and P_i release and uptake rates in the *Acinetobacter*-incorporated sludge system during 1 month of cultivation are shown in Table 3. As expected, a marked increase in the Q-9 content relative to the total ubiquinones in the reactor was observed just after the addition of *Acinetobacter* cells. With time, however, the Q-9 content declined gradually and decreased finally to the

TABLE 3. Changes in ubiquinone profiles and P_i release and uptake rates of anaerobic-aerobic sludge after addition of *Acinetobacter* cultures[a]

Operation period (days)	Ubiquinone[b]				Soluble P_i in wastewater (mmol/liter)	
	Total content (μmol/g [dry wt])	Homolog (%)			Released during anaerobic phase	Removed during aerobic phase
		Q-8	Q-9	Q-10		
0 (before addition)	0.42	70	4	24	0.67	0.75
0 (after addition)	0.97	48	38	11	0.95	1.03
2	ND[c]	63	17	17	0.80	0.88
7	0.46	71	9	18	0.74	0.83
14	ND	74	5	19	0.69	0.72
30	0.44	68	5	26	0.68	0.75

[a] Cell suspensions of *A. calcoaceticus* IAM 12087 and *A. lwoffii* GIFU 1951 were added to the mixed liquor to give counts of 2.2×10^8 and 1.0×10^8 CFU/ml, respectively. The *Acinetobacter*-incorporated system was then operated on a fill-and-draw basis with a 24-h batch cycle (see Materials and Methods) in which half of the effluent was exchanged with fresh sewage everyday.

[b] Results obtained with sludge at the aerobic stage.

[c] ND, Not determined.

same level as the initial content before the seeding. This observation indicates that the exogenous *Acinetobacter* strains were unable to grow and survive in the anaerobic-aerobic system. Although the addition of *Acinetobacter* cultures to the mixed liquor enhanced P_i removal to some degree, this effect was of short duration.

DISCUSSION

Recent research has demonstrated the usefulness of bacterial respiratory quinones not only as chemotaxonomic markers but as tools for studying the microbial population structure in the environment (11, 12). The results reported here indicate that there was no marked difference in ubiquinone profiles between the anaerobic-aerobic sludge and the standard aerobic sludge: both sludges produced Q-8 as the predominant homolog, Q-10 as the second most common type, and Q-9 as a minor component. These findings suggest that bacteria with Q-8 rather than those with Q-9 predominate in the bacterial community of anaerobic-aerobic sludge, as well as in standard aerobic sludge. If ubiquinone contents are not much different among the sludge bacteria, it can be estimated on the basis of our data that the content of Q-9-type bacteria relative to the total population of ubiquinone-containing bacteria in the sludge was 3 to 6%. It is therefore evident that *Acinetobacter* populations in the anaerobic-aerobic system were negligible, if they were present at all. The above-noted percentage estimated for the potential presence of *Acinetobacter* bacteria may not be underestimated, since the ubiquinone content of pure cultures of these bacteria was significantly greater than that of the sludge, as described above.

Bacteriological experiments demonstrated that the aerobic heterotrophic bacteria in the anaerobic-aerobic sludge, as well as in the standard aerobic sludge, were mainly such Q-8-type bacteria as *Comamonas* or *Pseudomonas* strains. We could not find *Acinetobacter* strains among the sludge-derived isolates. Moreover, our attempts to demonstrate the dominance of *Acinetobacter* strains in the sludge by selective isolation with the acetate medium have given negative results. These observations are in accord with the quinone profiles of the sludges.

The present results suggest that the introduction of anaerobic conditions into the batch aerobic treatment system has little influence on its bacterial community structure. Also, the results obtained with the laboratory sludge seeded with *Acinetobacter* cultures suggest that the anaerobic stress does not provide conditions favorable for their growth and survival. Our views may be supported by the fact that several researchers (4, 22) could find no evidence for the influence of anaerobic conditions on the bacterial flora of an activated sludge plant. In contrast, many investigators (5, 6, 8, 15–17) have reported the presence of significant numbers of *Acinetobacter* species in anaerobic-aerobic systems. This discrepancy may be explained partly by different experimental conditions due to the chemical composition of wastewater, the scale of reactors, and the mode of operation. Since our results were obtained merely with laboratory-scale reactors, further work on the quinone and bacterial composition of activated sludge in full-scale anaerobic-aerobic plants is necessary.

The anaerobic-aerobic sludge system studied here clearly showed rapid P_i release during the anaerobic phase and P_i uptake at the aerobic stage, as expected. In view of this, together with the findings as to the quinone profiles and bacterial flora of the sludge, it is reasonable to conclude that enhanced phosphate removal by the anaerobic-aerobic process does not always depend on the presence of *Acinetobacter* species. Thus, we prefer to claim that not only *Acinetobacter* species but many other bacteria which are already present in standard activated sludge systems are involved in biological phosphate removal or that a symbiotic interrelationship among these bacteria has important implications in this process. Some investigators (23) suggested symbiotic effects of sludge bacteria on phosphate removal in anaerobic-aerobic activated sludge systems.

In summary, it is likely that the introduction of anaerobic conditions into the batch aerobic system does not affect the bacterial population structure but does influence the phosphate metabolism in bacteria already present, whereby enhanced phosphate removal may take place.

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