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Identification of a Functionally Important Population in Phenol-Digesting Activated Sludge with Antisera Raised against Isolated Bacterial Strains

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Antisera were raised against nine strains which had been isolated from phenol-acclimated oil refinery activated sludge. Although several antisera reacted significantly with the activated sludge during a period of adaptation to phenol, only an antiserum against one of the isolates, *Alcaligenes* sp. E2, reacted with the activated sludge after the adaptation period. A kinetic pattern of phenol-oxygenating activity of the activated sludge after the adaptation period was similar to that of strain E2. These results suggest that a functionally important population in the phenol-digesting activated sludge was serologically identified.

In activated-sludge processes, bacteria play dominant roles in digestion of organic and inorganic pollutants in wastewater (1, 3, 8), and it is generally thought that fluctuations of bacterial populations in an activated-sludge ecosystem exert serious effects on the overall process. For this reason, bacteria inhabiting activated sludge have been extensively studied. In recent years, attempts have been made to investigate bacterial community structures in activated sludge by direct-detection methods (non-culture-dependent methods) (15, 17). Hiraishi et al. characterized a bacterial population structure in an anaerobic-aerobic activated-sludge system on the basis of respiratory quinone profiles (10). Manz et al. analyzed microbial consortia in municipal activated sludge using in situ hybridization techniques (12). The results of these studies suggest that culture-dependent methods are inadequate for describing community structures of activated sludge. However, as suggested by Wagner et al. (19), a study using a pure culture of the isolated microorganism is a prerequisite for a detailed analysis of the organism's physiology and consequently its function. Thus, in order to better understand the microbial community, it would be very helpful to identify and characterize isolated microorganisms that relate to dominant populations in the community (detected by a direct-detection method).

The purpose of this study was to identify functionally important populations in a phenol-digesting activated-sludge microbial community. Because phenol and related phenolic derivatives are known to be common constituents in industrial aquatic wastes, a number of studies have focused on bacterial degradation of such compounds (5, 6, 9). However, a population within a microbial community which dominantly digests phenol has not yet been identified; this may be the main reason why little is known about the ecological nature of bacterial phenol degradation. In this study, antisera raised against nine bacteria which had been isolated from a target activated sludge were used in an immunological direct-detection method, i.e., enzyme-linked immunosorbent assay (ELISA). To identify functionally important populations, ELISA data were compared with results of kinetic analysis on phenol-oxygenating activity of the activated sludge. Phenol-oxygenating activity was employed because it is useful for characterizing phenol-degrading bacteria (21).

Bacterial strains against which antisera were raised had been isolated from activated sludge in the wastewater-treatment facility of Tonen Kawasaki Refinery (oil refinery activated sludge) as reported previously (22). These were *Alcaligenes* spp. R2, R5, and E2; *Flavobacterium* spp. R3, R4, E3, and E4; *Achromobacter* sp. E1, and *Pseudomonas* sp. E6. Apparent kinetic parameters in Haldane's formula (5), K_s, K_{SI}, and V_{max}, for the phenol-oxygenating activity of these strains, with the exception of strain E4, have been previously described (21). Apparent kinetic parameters of strain E4 were determined by the previously described method (21) to be as follows: K_s, 0.60 ± 0.11 μM; K_{SI}, 210 ± 34 μM; and V_{max}, 54 ± 7 U/g.

Phenol digestion by the oil refinery activated sludge. An activated-sludge process was simulated in a laboratory unit consisting of an aeration tank (3 liters) and a settling tank (2 liters) and equipped with on-line dissolved oxygen (DO) and pH meters. Approximately 8 g (dry weight) of the oil refinery activated sludge was inoculated into the aeration tank and was supplied with an inorganic medium (MP medium) (21), which contained 200 ppm of phenol, at a flow rate of 6 liters per day. MLSS (mixed-liquor suspended solid) was maintained between 2,500 and 3,000 ppm by discarding excess sludge from the aeration tank. The mean sludge residence time was calculated to be approximately 15 days. Air was constantly supplied at a rate of 2 liters per min, and the temperature was maintained at approximately 25°C. MLSS was measured according to the Japan Industrial Standards method K0102. The total cell count of activated sludge was determined by the fluorescent-microscopy method described by Wagner et al. (19) with modifications; acridine orange was used for cell staining. Phenol concentration in the aeration tank was measured for a filtered sample (0.2-μm-pore-size membrane) by a modified colorimetric assay (13) with Phenol Test Wako (Wako Pure Chemical, Osaka, Japan). The minimum detection limit of the phenol assay is 0.5 ppm. The concentration of dissolved organic carbon (DOC) in the aeration tank was measured for the filtered sample by using a total organic carbon meter (TOC-5000; Shimadzu Co. Ltd., Kyoto, Japan). Data were determined in triplicate, and significant differences were evaluated by the *t*

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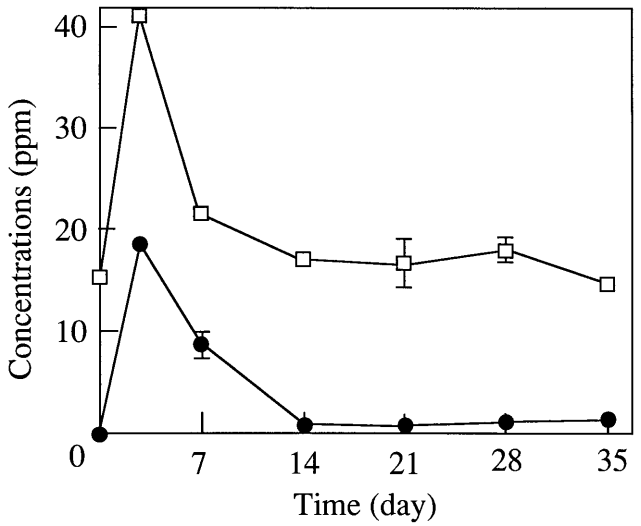

FIG. 1. Changes in the concentrations of phenol (●) and DOC (□). Error bars (1 standard deviation; $n = 3$) are shown when larger than the symbol.

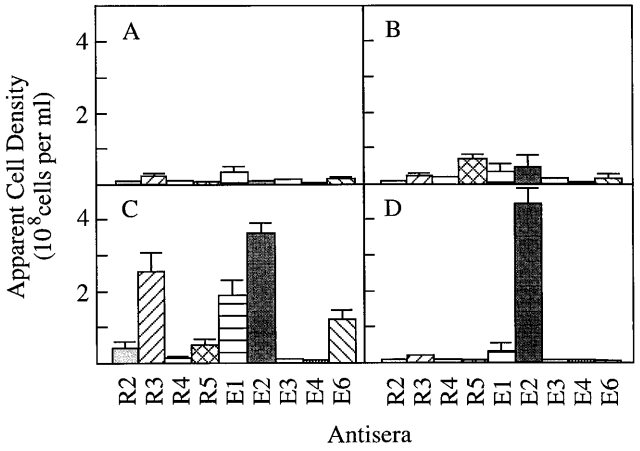

FIG. 2. Reactivities of the antisera against activated sludge. (A) Activated sludge from the municipal sewage plant. (B) Oil refinery activated sludge before commencing the phenol loading. (C) Phenol-digesting oil refinery activated sludge on day 7. (D) Phenol-digesting oil refinery activated sludge on day 21. Error bars indicate 1 standard deviation; $n = 3$.

test ($P < 0.05$). Reproducibility was confirmed in two independent experiments.

Phenol loading was commenced on day 0 at a phenol-loading rate of 0.4 g/liter/day, and the loading rate was kept constant thereafter. Phenol was detected on day 3 and day 7 at concentrations of 18.5 ppm and 8.4 ppm, respectively, and was not detected at concentrations higher than 1 ppm after day 7 (Fig. 1). This indicates that the oil refinery activated sludge started to digest phenol before day 3, although complete digestion was not observed until day 14. The period from day 0 to day 7 was, thus, considered as an adaptation period of the activated sludge to phenol. Throughout the experiment, DOC exceeded 15 ppm (Fig. 1), and the total cell count ranged from 3.4×10^9 to 4.1×10^9/ml. The high level of DOC on day 3 was due to phenol remaining in the aeration tank. It is assumed that the DOC supported heterotrophic populations, other than phenol-digesting populations, in the activated-sludge microbial community.

Immunoreactive populations in the phenol-digesting activated sludge. Rabbit antisera were raised against the nine strains according to the method described by Harlow and Lane (7). Cross-reactions between these antisera and the nine antigen strains were not detected by ELISA, in which pure cultures of the nine strains in nutrient broth containing 4×10^8 to 6×10^8 cells per ml were used (data not shown). Reactivities of these antisera with activated sludge were determined in triplicate by ELISA as described previously (23). Significant differences were evaluated by the t test ($P < 0.05$). In this study, reactivity of an antiserum with activated sludge is expressed as an apparent cell density determined from a standard curve which was produced with dilutions of pure culture of the respective antigen strain (23).

Reactivities of these antisera with activated sludge of a municipal sewage plant (Takinoshita, Kawagoe, Saitama, Japan) were below 5×10^7 cells per ml (which was considered to be background and estimated to be less than 3% of the total cell count) (Fig. 2A). This suggests that ELISA can be used with these antisera for the detection of major immunoreactive populations in activated sludge. Earlier reports described serological detection of populations in activated sludge (11, 16, 18). Saraswat et al. described the detection of *Nitrosomonas europaea* in activated sludge by using an enzyme immunoassay (16). They used a prepurified polyclonal antibody and reported that the apparent lower detection limit was 5×10^6 cells per ml. It is probable that this type of prepurified antiserum or monoclonal antibodies are useful for reducing the background level.

Reactivities of the antisera with the oil refinery activated sludge before the phenol loading are shown in Fig. 2B. Most of the antisera, with the exception of R5 antiserum, reacted with the oil refinery activated sludge at levels equivalent to the background. In contrast, R5 antiserum reacted with a 1/10 diluted activated-sludge suspension at a significant level; the apparent cell density was estimated to be 7.2×10^7 cells per ml. After commencing the phenol loading, reactivities of these antisera with the activated sludge were investigated at 7-day intervals. On day 7, several antisera reacted with the activated sludge at significant levels (Fig. 2C); apparent cell densities were 2.6×10^8 (R3 antiserum), 2.0×10^8 (E1 antiserum), 3.7×10^8 (E2 antiserum), and 1.3×10^8 (E6 antiserum) cells per ml. In contrast, from day 14 onward, only E2 antiserum reacted with the activated sludge at significant levels; apparent cell densities determined with E2 antiserum on days 14, 21, 28, and 35 were 4.4×10^8, 4.5×10^8, 5.2×10^8, and 3.3×10^8 cells per ml, respectively. Figure 2D shows a result on day 21; at this time, the apparent cell density determined with E2 antiserum was approximately 15% of the total cell count.

Phenol-oxygenating activity of the activated sludge. Phenol-oxygenating activity (phenol-oxygenation-dependent oxygen consumption rate) of the activated sludge was measured by the previously described method used to measure the activities of phenol-degrading bacteria (21). In the previous study (21), the method was used to determine the activity at phenol concentrations greater than 0.3 μM. For an identical sample, the activity typically varied ±10% or less. The activity of the activated sludge was measured at phenol concentrations from 0.5 μM to 20 mM. One unit of the activity was arbitrarily defined as 1 μmol of oxygen consumed per min. The specific activity was defined as the activity per g of suspended solids. Kinetic analysis of the activity (v) was conducted as described previously (21), according to Haldane's formula (5); i.e., $v = V_{max} \cdot [S]/([S]+K_s+[S]^2/K_{SI})$, where [S] is a substrate concentration, K_s is the apparent half-saturation constant, K_{SI} is the apparent inhibition constant, and V_{max} is the apparent maxi-

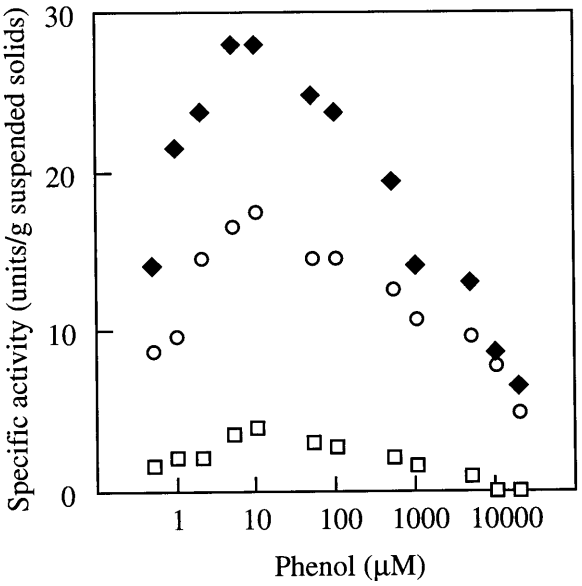

FIG. 3. Phenol-oxygenating activity of the activated sludge on days 0 (□), 7 (◆), and 21 (○).

mum activity. The kinetic data were statistically analyzed by the *t* test ($P = 0.05$).

Phenol-oxygenating activity of the activated sludge was measured on days 0, 7, 14, 21, 28, and 35. Figure 3 shows specific activities of the activated sludge on days 0, 7, and 21. The activities on days 14, 28, and 35 were nearly the same as that on day 21. Before the phenol loading was commenced, the activated sludge expressed phenol-oxygenating activity at a low level (day 0). This may be consistent with the observation that phenol in the feed was partly digested by the activated sludge before day 3, probably indicating that this level of activity was not sufficient for complete digestion of phenol in the feed. Maximum specific activity was observed on day 7, especially at low phenol concentrations. In contrast, the activity at high phenol concentrations remained at a similar level throughout the experiment. On day 7, the activated sludge was capable of digesting not only phenol supplied from the feed but also that remaining in the aeration tank, resulting in a decrease in phenol concentration in the aeration tank (Fig. 1). As shown in Fig. 2, several antisera reacted significantly with the activated sludge on day 7. In addition, MLSS and the total cell count remained stable throughout the experiment. These data suggest that phenol remaining in the aeration tank aided in specific development of several phenol-digesting populations in the activated sludge, thus resulting in the high specific phenol-oxygenating activity. On the other hand, the activity after the adaptation period was stable. It is probable that this level of activity was sufficient for the activated sludge to completely digest phenol in the feed.

Apparent kinetic parameters in Haldane's equation for phenol-oxygenating activity of the activated sludge were estimated (Table 1), and these were compared with the parameters of the isolated strains (21). It was shown in our previous study (21) that phenol-degrading bacteria isolated from the oil refinery activated sludge by means of continuous enrichment cultivation expressed phenol-oxygenating activities with low K_s (below 1 μM), and that these were different from the activities of phenol-degrading bacteria isolated from batch enrichments. The K_s values presented in Table 1 were below 1 μM, suggesting that phenol-degrading bacteria expressing low K_s played dominant roles in the phenol-digesting activated sludge. Our previous study (21) also showed that strain E2 had a conspicuous activity with an extremely high K_{SI} value (7,360 ± 500 μM). The result of the kinetic analysis in the present study shows that the activated sludge after the adaptation period also expressed high K_{SI} values which were comparable to the K_{SI} value of strain E2. In addition, the K_s values of the activated sludge were identical to that of strain E2 (0.36 ± 0.06 μM, within a range of one standard error). These data indicate that the activated sludge after the adaptation period predominantly expressed E2-type phenol-oxygenating activity.

TABLE 1. Apparent kinetic parameters for phenol-oxygenating activity of phenol-digesting activated sludge

Day	Apparent kinetic parameters for specific phenol-oxygenating activity[a]		
	K_s (μM)	K_{SI} (μM)	V_{max} (U/g of suspended solids)
0[b]	0.73 ± 0.27	810 ± 310	3.5 ± 0.28
7	0.29 ± 0.15	3,500 ± 1,300	26 ± 1.7
14	0.41 ± 0.14	7,800 ± 2,200	18 ± 0.86
21	0.36 ± 0.15	8,500 ± 2,800	15 ± 0.89
28	0.37 ± 0.19	6,800 ± 2,700	19 ± 1.3
35	0.32 ± 0.16	8,200 ± 3,000	18 ± 1.2

[a] Data are estimated values ± standard deviations; $n = 12$.
[b] Before commencing the phenol loading.

The K_s values of the activated sludge obtained in this study were low compared with those reported in previous studies for phenol-degrading activities of pure cultures (5, 14) and of a mixed consortium (4). Two explanations for this difference are possible. The first is the difference in the organisms examined. As described previously (21), it is likely that low-K_s-type phenol-degrading bacteria were enriched in the activated-sludge system, which is considered a continuous-culture system. The second concerns the methods applied to the measurements of the activity. Folsom et al. (5) used a phenol-disappearance assay to measure phenol-degrading activity. They stated, however, that the apparent K_s value obtained was not so accurate because of limitations in the sensitivity of the assay for rate determination at low phenol concentrations. In this study, phenol-oxygenating activity was measured at phenol concentrations from 0.5 μM, which is much lower than the limit of the phenol-disappearance assay.

ELISA results clearly showed that only the antiserum against strain E2 reacted significantly with the activated sludge after the adaptation period. The population reacting with E2 antiserum was estimated to be 10 to 15% of the total cell count. At the same time, i.e., after the adaptation period, the activated sludge had V_{max} values that were approximately 10 to 12% of the V_{max} value of strain E2 (155 ± 7 U/g of dry cells) (21), as determined by a weight-based estimation. From this estimation, it is conjectured that ratios of E2 populations to total populations would be approximately 10 to 12%, which would be in agreement with the ratios estimated from the ELISA data. It is, therefore, suggested that a functionally important population in the phenol-digesting activated sludge in this time period was identified by means of ELISA with E2 antiserum.

As described above, of the antisera that reacted with the activated sludge during the adaptation period, only E2 antiserum reacted significantly with the activated sludge after that period. This indicates that shifts in microbial populations occurred in the course of the adaptation of the activated sludge

to phenol. Atlas and Bartha reported that species diversity probably peaks during the early or middle stages of succession and may decline inordinately in the stable climax community (2). The adaptation process observed in this study is considered to demonstrate this ecological theory. In an early stage of the adaptation period, phenol may have been supplied to many phenol-digesting populations, and thus, they probably had opportunities to enhance their densities. However, the occurrence of many phenol-digesting populations probably caused competition among them, thereby resulting in the survival of one population with selective growth advantages over other phenol-digesting populations in the activated sludge, i.e., the E2 population. Among the nine strains, E2 was one of the strains expressing high specific phenol-oxygenating activities (though not the highest) (21). Furthermore, it was the only strain capable of forming flocs in its pure culture (20, 23). It is thus assumed that this floc-forming ability, in addition to the high phenol-oxygenating activity, was important for the survival of the E2 population.

In conclusion, this study shows a methodology for identifying a functionally important population in a microbial community. This methodology includes direct detection of major populations in a microbial community in combination with kinetic analyses of the microbial community and of isolated strains related to the major populations. The successful application of these procedures to the phenol-digesting population in the oil refinery activated sludge is presented. Further studies on the physiological characteristics of the isolated strain related to the functionally important population will contribute to a better understanding of the phenol-digesting activated-sludge microbial community.

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