REVIEW

Environmentally Relevant Microorganisms

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The development of molecular microbial ecology in the 1990s has allowed scientists to realize that microbial populations in the natural environment are much more diverse than microorganisms so far isolated in the laboratory. This finding has exerted a significant impact on environmental biotechnology, since knowledge in this field has been largely dependent on studies with pollutant-degrading bacteria isolated by conventional culture methods. Researchers have thus started to use molecular ecological methods to analyze microbial populations relevant to pollutant degradation in the environment (called environmentally relevant microorganisms, ERMs), although further effort is needed to gain practical benefits from these studies. This review highlights the utility and limitations of molecular ecological methods for understanding and advancing environmental biotechnology processes. The importance of the combined use of molecular ecological and physiological methods for identifying ERMs is stressed.

[Key words: environmental biotechnology, molecular microbial ecology, bioremediation, activated sludge]

INTRODUCTION: CONSTRAINTS IN ENVIRONMENTAL BIOTECHNOLOGY

Environmental biotechnology is a technology that applies biological systems to clean up environmental pollutants. This has been thought to be advantageous over physical and chemical treatments due to its relatively low cost and little disturbance to the environment (1, 2). In addition, organic pollutants are biodegraded to inorganic compounds (e.g., CO₂, H₂O, and Cl⁻), whereas physical and chemical processes, e.g., vaporization, adsorption and extraction, simply transfer the pollutants to different locations (1). The activated-sludge wastewater-treatment process has been a common practice for over 80 years (3), while bioremediation of polluted soil, groundwater and marine environments has only recently been initiated (1, 4, 5).

Environmental biotechnology relies on the pollutant-degrading capacities of naturally occurring microbial consortia (6), in which bacteria generally play central roles. Researchers are thus studying pollutant-degrading bacteria which inhabit polluted environments. These studies include the isolation of bacteria from the environment, their classification and physiological characterization, molecular analyses of their degradative enzymes and sometimes the construction of ‘superbugs’. Consequently, extensive knowledge of bacterial physiology and the molecular features of degradative enzymes has been obtained (7-10). However, discrepancies between the physiology of isolated pollutant-degrading bacteria and the nature of in situ pollutant biodegradation have been reported (11). For example, the kinetics of the phenol-degrading activity exhibited by total phenol-digesting activated sludge are significantly different from the kinetics of phenol-degrading bacteria isolated from activated sludge and intensively studied in the laboratory (Fig. 1). This finding suggests that we cannot directly extrapolate the data obtained using these laboratory-isolated phenol-degrading bacteria to assess the phenol removal performance of activated sludge processes.

A practical aspect of environmental biotechnology is the use of microbial consortia as ‘black boxes’ without analyzing the constituent microbial populations. An example is bioremediation of oil-contaminated beaches (4, 5, 12) in which inorganic nutrients, e.g., nitrogen and phosphorus, are supplemented to stimulate indigenous hydrocarbon-degrading bacteria. Although the biodegradation potentials expressed by total marine consortia have been well characterized, the microbial populations involved in the in situ hydrocarbon degradation have not been identified during the actual bioremediation trials (4).

There seems to exist two separate modes of study in environmental biotechnology: one biased toward laboratory science that focuses on isolated bacteria, and the other toward ‘pure practice’. Fusion of these modes of study would be a promising way of advancing environmental biotechnology, although there are hurdles that hamper this. The first hurdle is the insufficient knowledge obtained from laboratory studies to interpret what is happening in the real environment. The complexity of microbial consortia would be another hurdle that makes it difficult to analyze the structures of relevant microbial consortia. Microbial consortia involved in environmental biotechnology, e.g., activated sludge and soil consortia, are generally very complex, enabling them to act on a variety of pollutants.

A search for possible solutions to these constraints in environmental biotechnology began in the early 1990s, when good molecular biological techniques were developed to study microbial ecology (called molecular microbial ecology). With this new discipline, we can understand natural microbial consortia in a more realistic manner. This review describes how researchers have applied this new discipline to environmental biotechnology and also discusses how practical benefits can be drawn from such studies. A possible scheme to achieve this goal is then proposed, which include the structure analy-
digesting activated sludge originating from a municipal wastewater strain BH (3.20 μM), the municipal sludge (0.70 μM) and oil-refinery activity. The K_s values were estimated for strain CF600 (2.99 μM), follow Haldane's equation, \( v = \frac{V_{max}}{[s]} + \frac{K_s + [s]}{V_{max}} \), where [s] is the substrate concentration, K_s is the half-saturation constant, K_inh is the inhibition constant, and V_max is the theoretical maximum activity. The K_s values were estimated for strain CF600 (2.99 μM), strain BH (3.20 μM), the municipal sludge (0.70 μM) and oil-refinery sludge (0.26 μM).

sis of microbial consortia by molecular methods, functional analysis of in situ populations, isolation and detailed physiological analyses of functionally important populations (i.e., environmentally relevant organisms, ERMs), and the control of ERMs in the microbial consortia.

**APPLICATION OF MOLECULAR MICROBIAL ECOLOGY TO ENVIRONMENTAL BIOTECHNOLOGY**

Molecular microbial ecology The advent of new methods redefines our scope. This has been the case for microbial ecology in the last two decades, when microbiologists started to use molecular ecological methods, particularly that known as the 16S rRNA framework (13-15), for analyzing natural bacterial populations. Previously, microscopic observation and cultivation were the methods widely used for identifying bacteria in the natural environment, even though these methods are thought to be insufficient for these purposes. Problems associated with microscopic observation are that (i) the morphology of bacterial cells is generally too simple to serve as a basis for sound identification and to allow reliable classification and (ii) microorganisms may adopt different morphologies under different physiological conditions (16). Cultivation methods, e.g., viable plate count and most-probable-number (MPN) techniques, have been used for quantification of active cells in environmental samples. However, because the medium used in these methods always selects for certain organisms, the results are always biased toward these organisms (called cultivation bias). In addition, some bacterial cells may be viable but not able to replicate under stress conditions (16, 17). These problems have been realized by the observation that direct microscopic counts of bacteria in aquatic and soil habitats exceed viable plate counts by several orders of magnitude (18-20). In most cases, conventional cultivation methods can detect (and hence recover) only a small fraction of the microbial consortia.

In contrast to traditional microbial ecology, molecular microbial ecology describes the consortium structure based on DNA sequences recovered from the consortium without cultivation. The DNA is extracted directly from a microbial consortium, so that the cultivation bias is eliminated. Certain gene fragments of different organisms are then cloned or amplified by PCR from the extracted DNA in order to determine their sequences. Gene coding for the small subunit of ribosomal RNA (16S rDNA for bacteria and archaea, and 18S rDNA for eukaryotes) is most commonly used for this purpose (13-15). The advantages of the use of this gene are: (i) all organisms harbor this gene, and their evolutionary relationships can be deduced (21), (ii) a large number of sequences of different organisms are stored in databases (22), (iii) universal PCR primers can be designed using sequences in several highly conserved regions, and (iv) bacterial cells can be detected by in situ hybridization targeting abundant ribosomes in cells. Using the 16S rDNA sequences, bacteria are classified into the phylogenetic groups proposed by Woese (21), and the identification of natural populations follows this phylogenetic classification.

A typical scheme for analyzing bacterial populations in the environment is illustrated in Fig. 2 (refer to the legend of this figure for the scheme). Among the methods, denaturing/temperature gradient gel electrophoresis (D/TGGE) is widely used in recent years for profiling microbial consortia (23, 24). The separation of DNA fragments by D/TGGE is based on the decreased electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants (DGGE) or a temperature gradient (TGGE). Molecules with different electrophoretic mobility migrate to different positions in a gel. This method is particularly useful when temporal and spatial dynamics of the population structure are analyzed, since it avoids the laborious aspects of cloning and sequencing. In situ hybridization with a fluorescence-labeled specific probe is applicable for the quantitative analysis of a microbial population (15). This method directly counts labeled bacterial cells under microscopic observation and compares the count with the total cell count determined with a DNA-binding fluorescent dye, such as 4',6-diamidino-2-phenylindole (DAPI) or acridine orange (AO).

Another useful method for consortium analysis (not included in Fig. 2) is terminal restriction fragment length polymorphism (T-RFLP) analysis of PCR-amplified 16S rDNA fragments (25, 26). This method identifies 16S rDNA fragments based on the restriction endonuclease digestion patterns rather than the sequences. It appears to be advantageous over D/TGGE due to its resolution and simplicity of identification.

Since these molecular methods are capable of detecting microbial populations that are hardly detected by conventional culture-dependent methods (27-29), researchers have started to apply them for analyzing microbial consortia in environmental biotechnological processes. These applications are summarized below, and their utility and limitations are discussed.

**Activated sludge** The activated sludge process is currently the most popular biological system for the
FIG. 2. A typical scheme for analyzing a microbial consortium using molecular ecological methods. DNA is first extracted from a microbial consortium in the environment and used as a template for PCR to amplify 16S rDNA fragments with a set of universal primers. Thereafter, the PCR products (of the same length but with different sequences) are either separated by D/TGGE (see text for the separation mechanism) or each product is cloned into E. coli. 16S rDNA fragments are then sequenced, and the determined sequences are compared with the sequences stored in nucleotide databases to phylogenetically identify the detected populations. Moreover, the sequence information can be used to design an oligonucleotide probe for the detection and quantification of a specific bacterial population by fluorescence in situ hybridization (FISH). (a) Cells stained with DAPI showing the total population in a groundwater sample. (b) DGGE profiles showing the diversity and relative abundance of PCR-amplified 16S rDNA fragments; lanes 1 to 6 represent consortia in petroleum-contaminated groundwater, while lanes 7 to 10 represent those in control groundwater. (c) A phylogenetic tree showing the position of the band-1 sequence; band 1 was affiliated with the epsilon subclass of the class Proteobacteria and most closely related to the genus Thiomicrospira. (d) Cells labeled with a DNA probe specific for the band-1 population, an example of FISH.
different from the structure determined by in situ hybridization with group-specific probes. Problems frequently associated with the activated-sludge operation are bulking and foaming, which result in poor sludge settling (56). Microscopic observations have indicated that overgrowth of filamentous bacteria is involved in these problems (37, 38). Although many filamentous organisms have been isolated from activated sludge (39-42), identification of organisms relevant to these problems by conventional microbiological methods is difficult due to their similar morphology. In situ hybridization with group-specific probes (43) and probes designed from isolated bacteria (44, 45) and cloned 16S rDNA fragments (45, 46) has thus been applied to detect and identify these organisms. de los Reyes et al. have demonstrated that a vast majority of foaming-related *Mycobacterium* in activated sludge comprised *Gordona* populations, although *Gordona amarae*, the most intensively studied *Gordona* bacterium in the laboratory, made up only a small percentage of the *Gordona* populations (43).

**Removal of inorganic compounds** Removal of nitrogen (47) and phosphorus (48, 49) is also an important role of the activated sludge process. Scientists have attempted to identify bacterial populations responsible for nitrogen (50-52) and phosphorus (53-55) removal by molecular methods. Juretschko et al. have suggested *Nitrosooccus* and *Nitrospira*-like populations to be the dominant ammonia- and nitrite-oxidizers, respectively (52), although *Nitrosomonas* and *Nitrobacter* are the most frequently isolated counterparts. Bond and coworkers have analyzed the phylogenetic structures of efficient and nonefficient phosphate-removing activated-sludge consortia (54, 55), and found that populations affiliated with the *Rhodococcus* group in the beta subclass of *Proteobacteria* were abundantly detected only in the efficient sludge, suggesting that these populations are involved in phosphate removal.

**Anaerobic granules** Anaerobic reactors are used to treat various organic wastes that are ultimately converted to methane. The upflow anaerobic sludge blanket (UASB) is a popular anaerobic reactor that utilizes microorganisms to form a granular structure up to several millimeters in diameter (56). This type of reactor is thus able to maintain a high concentration of biomass and to treat an extremely high volumetric loading rates of organic pollutants. Microscopic and immunological studies have revealed the ultrastructure of layered organisms in a granule (57, 58), in which aceticlastic methanogenic archaea are surrounded by fermentative bacteria. This structure is considered important for the transformation of organic pollutants first to acetate and then to methane. This ultrastructure has been confirmed by in situ hybridization with group-specific probes (59 61). Cloning and sequencing of granule 16S rDNA fragments have revealed the existence of novel bacterial populations (62), and specific cloned sequences have been used successfully for in situ hybridization of sectioned granules to investigate their spatial distribution (63).

**Groundwater bioremediation** Contamination of groundwater and underground soil with halogenated organic compounds (64, 65) and petroleum hydrocarbons (66, 67) is a frequent occurrence. Organic compounds persist in these environments due to the low oxygen concentration, so that great effort is required for the remediation of contaminated sites. Extensive studies have been performed on the bioremediation of trichloroethylene (TCE) and related chlorinated compounds, and so far these compounds are known to be cometabolically transformed by bacteria that contain nonspecific oxygenases, e.g., methane-oxidizing bacteria (68-70), aromatic hydrocarbon-degrading bacteria (71-73) and ammonia-oxidizing bacteria (74). Based on this knowledge, workers have attempted in situ bioremediation by injecting methane (75), phenol (76) and toluene (77), although information on bacterial populations relevant to in situ transformation of TCE has been quite limited (75). Bowman et al. examined the distribution of methanotrophs in TCE-contaminated groundwater by culture-dependent techniques and reported that most of the isolated methanotrophs were affiliated with type II methanotrophs (75). Since the type II bacteria are known to possess soluble methane monooxygenases (sMMO) capable of efficiently transforming TCE, they have suggested that TCE can be effectively removed by stimulating these methanotrophs with methane (73).

We have analyzed bacterial populations in TCE-contaminated soil cores before and during methane injection for in situ biostimulation by using DGGE analysis of consortium 16S rDNA fragments (unpublished data). It was found that the population structure significantly changed due to the methane injection and bacterial populations closely related to some type I methanotrophs abundantly occurred after the methane injection. In that study, we also analyzed methanotrophs by a MPN-culture method and found that a population affiliated with a type II methanotroph was detected most abundantly regardless of the methane injection. The discrepancy is considered to be ascribable to a bias associated with the MPN method in that only type II methanotrophs would have grown well under the culture conditions employed. The result thus suggests the inadequacy of conventional culture-dependent methods for counting and identifying methanotrophic populations in the environment.

Two groups of scientists have analyzed bacterial populations in underground aquifers contaminated with JP-4 jet fuel by 16S rRNA methods (78, 79). The aims of such population analyses are to obtain a measure of the bioremediation effectiveness and data to determine the endpoint of the treatment (80). The population analyses also provide information concerning the biodegradation pathways of pollutants. Dojka et al. cloned 16S rDNA sequences closely related to *Syntrophus* spp. and *Methanoseta* spp. from a fuel-contaminated underground aquifer, and have hypothesized that acetoclastic methanogenesis is the terminal step of hydrocarbon biodegradation (78).

**Marine bioremediation** Petroleum spills have had a serious impact on marine environments (4, 5). A famous case is the *Exxon Valdez* oil spill in Alaska; in March 1989, approximately 41 million liters of Alaskan North Slope crude oil was spilled from a tanker, the T/V *Exxon Valdez*, resulting in oil contamination of over 2000 km of rocky intertidal shorelines. Another case is the *Nakhodka* oil spill in Japan. In January 1997, more than 5000 tons of heavy fuel oil was spilled from the Russian tanker *Nakhodka*, which ran aground and sank in the sea of Japan close to Oki island; the oil contaminated more than 500 km of the Japan sea coastline. In these cases, the low oxygen environment accelerated the natural degradation of residual oil. The effectiveness of bioremediation has been extensively evaluated,
which includes chemical and microbiological studies (4, 5, 12, 81), although researchers have not employed molecular ecological methods.

Recently, molecular methods in combination with fatty-acid analysis have been applied for evaluating bioremediation of an experimental oil spill at a sandy beach (82). In that study, DGGE analysis indicated that the spilled oil promoted the growth of bacteria within the alpha-Proteobacteria and Flexibacter-Cytophaga-Bacteroides phyla. A comparison of bacterial populations by DGGE between oil-contaminated and uncontaminated plots revealed significant differences even when the biodegradation had ceased. The data clearly suggest the persistent influence of spilled oil on marine biota.

Utility and limitations of molecular ecological methods As described above, molecular methods have enabled detection of numerous uncultured bacteria in environmental biotechnology processes, some of which constitute major populations. It is natural to consider that major populations play some important roles in these processes. The molecular methods can also provide insight into the functions of microbial populations, although only indirectly. If population dynamics correspond to fluctuations of a performance expressed by the total consortium, the detected population may be relevant to that performance. An example is phosphate-removing activated sludge (54, 55). In addition, spatial population dynamics may also provide useful information concerning the functions of the detected populations; this principle has been applied to analyzing anaerobic granules (59-61, 63).

Another purpose of molecular methods is to provide measures to assess the influences of pollution and enforced remediation practices on the natural biota (80). Since a food web starts from bacterial mineralization of organic matter, analyses of the transition of bacterial populations are quite important for such assessment.

The above examples also suggest limitations of the 16s rRNA approaches. First, we should consider biases associated with PCR amplification and DNA extraction (83-87), which can affect PCR-mediated quantification of a microbial population. We should also bear in mind that some populations are still undetected. The abundance of a target population (a target sequence, if accurately described) can be more reliably estimated by using specifically designed quantitative PCR (88-92) and/or in situ hybridization (15), although each method would include its own biases. Table 1 presents comparison of ratios of a bacterial population to the total population determined by three different methods (unpublished data). The ratios obtained by PCR-mediated methods, i.e. cloning and DGGE, are larger than the ratios obtained by fluorescence in situ hybridization (FISH). It has been realized that PCR-mediated methods tend to overestimate the abundance of a detected population, since these methods fail to detect some populations whose DNA fragments are difficult to amplify. In contrast, FISH analysis sometimes underestimates the abundance of a microbial population, particularly of a slowly growing population, since metabolically inactive cells (not growing rapidly) contain a small amount of ribosomes and thus are hardly stained with a fluorescent probe targeting 16s rRNA. Cross checking of the results of several methods is thus recommended for quantitative population analyses (83, 93). In addition, understanding inherent biases of each method is also important for the quantitative evaluation of the results of molecular approaches.

The second limitation is related to the level of diversity of the 16s rRNA sequences. Relatively conserved 16s rRNA sequences are very suitable for presenting the phylogenetic relationships of a wide range of bacteria, although the sequences often fail to discriminate between closely related bacterial populations (92-94). For example, two phenol-degrading populations in activated sludge harboring an identical 16s rDNA sequence (but their gyrB sequences were different) showed different behaviors regarding floc formation, i.e., one is a floc-forming bacteria, and the other is nonflocculating bacteria (93). Quantitative PCR with specific gyrB-targeting primers showed different population dynamics of these two bacteria (93); gyrB was selected due to its higher molecular evolution rate (95).

Third, the 16s rRNA approaches reveal only the phylogenetic trait of existing populations; the detected populations are thus called phylogenetic strains. We hence assume the function of a phylogenetic strain from the physiological traits of closely related isolates, although the phylogenetic feature is not necessarily correlated with important physiological traits (93).

In summary, we are now able to analyze the structures of microbial consortia in environmental biotechnology processes by molecular ecological methods. This knowledge should be related to the functional understanding of microbial populations in order to gain practical insight, and the molecular methods themselves are being improved in order to move toward this goal.

**ENVIRONMENTALLY RELEVANT MICROORGANISMS**

Functional analyses of microbial populations in the environment One important current issue in microbial ecology is to analyze the functional (physiological features) of microbial populations that are detected by molecular ecological methods. Several culture-independent methods for analyzing the in situ functions and physiology of microbial populations have been developed.

Metabolically active members of microbial consortia can be identified by quantifying rRNA molecules of different species, since the ribosome content of bacterial cells is linearly related to their growth rate (96-98). Nogales et al. have identified presumptive metabolically active populations in moorland soil highly polluted with polychlorinated biphenyls by sequencing of cloned
reverse transcription-PCR (RT-PCR) products of 16S rRNA generated from total RNA extracts (99). Consortium 16S rDNA fragments have been amplified by PCR from extracted DNA and by RT-PCR from extracted RNA, and their D/TGGE profiles have been compared in order to detect metabolically active populations (100-102). In this way, Felske et al. demonstrated that uncultured Actinobacteria were active in soil (100). The fluorescence intensity of individual cells labeled by FISH is correlated with the ribosome content of a bacterial cell (15, 98), which is thus another possible indicator of the in situ activity (the growth rate).

Microautoradiography of individual cells after incubation with a radioactive substrate has been used in combination with fluorescence in situ hybridization to identify metabolically active cells (103). Urbach et al. demonstrated another usage of substrate uptake to identify metabolically active cells without cultivation (104); DNA labeled with exogenously supplied bromodeoxyuridine (BrdU) was immunologically purified from total DNA extracted from a lakewater consortium and subjected to length heterogeneity PCR (85) to identify metabolically active ribotypes.

It is very helpful for analyzing functions of localized populations if one can know the chemical gradients coupled with spatial population dynamics, and microsensors have been applied for this purpose (105). This technique has been applied in combination with molecular ecological methods to analyze sulfidogenic biofilms (106), nitrifying biofilms (107, 108) and activated sludge (109). These studies have shown that some of the 16S rRNA probe labeled cells are metabolically active.

Catabolic gene fragments are amplified by PCR from environmental DNA samples and sequenced to analyze the composition and diversity of catabolic populations. Genes used for this purpose include ammonia monoxygenase (110), nitrilase monoxygenase (111-113), catechol dioxygenase (114, 115), phenol hydroxylase (116) and hydrogenase of Desulfovibrio spp. (117, 118). Detection of the 16S rRNA gene, is thus necessary for comprehensive analysis of catabolic populations (110, 116).

**Isolation of microorganisms**

Although the above-mentioned techniques are quite useful for studying functional and physiological traits of microbial populations in the environment, pure culture experiments are indispensable for detailed analyses of functions of each population (27, 93), particularly for manifesting concealed physiological traits likely to be important for the establishment of the consortium. To date, many pollutant-degrading bacteria have been isolated from natural mixed populations after batch-culture enrichment in media containing relatively high concentrations of the pollutant. However, this batch-culture enrichment is not considered suitable for ecological studies, because such methods isolate a very limited number of bacteria that always grow most rapidly in laboratory media (119). Dunber and coworkers have thus proposed an alternative method, namely autoradiographic direct plating, for isolating diverse microbial species with unique catabolic traits (119, 120). It has also been demonstrated that continuous culture enrichment is useful for isolating diverse phenol-degrading bacteria with high affinities for the substrate (121, 122). Marine oligotrophic bacteria have been successfully isolated by the dilution culture technique proposed by Button et al. (123). This technique simply dilutes raw seawater with sterile seawater (low concentrations of organic nutrients may be added), and pure cultures may be obtained by the MPN manner. These alternative isolation techniques have facilitated the isolation of diverse bacteria, most of which exhibit genotypic and phenotypic traits different from those of the batch-enrichment isolates.

Molecular ecological methods are also useful for the monitoring of enrichment and isolation processes. Teske et al. used DGGE phylogenetic data from sulfate-reducing enrichment to select methods (e.g., culture media) for isolating the component microorganisms from the enrichment (124). Heuer et al. have reported a method for searching for bacterial isolates that correspond to populations detected by a molecular ecological method (125). They generated digoxygenin-labeled polynucleotide probes from major bands in TGGE community fingerprints, and the probes were used to screen bacterial isolates by dot blot hybridization (125). In situ hybridization has successfully been applied to check the purity of a bacterial phytype in an enrichment culture and in purification steps by density gradient centrifugation (126). In this way, Strous et al. obtained an almost pure cell suspension of planctomycetes (99.6%) that could not be cultivated by conventional microbiological techniques and showed anaerobic ammonia oxidation (126).

**Environmentally relevant microorganisms (ERMs)**

A microorganism that plays a major role in the environment (or in an environmental biotechnology process) is termed an environmentally relevant microorganism (ERM), while, in this review, this term is specifically used for an isolated bacterium having such ecological characteristics (hence, its physiology and genetics should be characterized in the laboratory). To date, only a few microorganisms can be considered as ERMs, and these few examples are presented below in order to depict how they were isolated and how they are different from typical laboratory strains.

The first example is high-affinity methanotrophs (127). It has been recognized that the apparent half saturation constant $K_{\text{m(app)}}$ for methane oxidation in soil is 1 to 3 orders of magnitude lower than those observed with methanotrophs previously isolated in the laboratory. Dunfield et al. applied low gaseous mixing ratios (<275 parts per million of volume) for the enrichment of methanotrophs from soil, and a stable mixed culture was obtained which exhibited a $K_{\text{m(app)}}$ compatible with that of the organisms in soil. DGGE with the methane monoxygenase gene and 16S rDNA fragments showed that a population affiliated with the type II methanotroph dominated the enrichment. This population was then isolated by direct plating under a low gaseous mixing ratio of methane. However, when the enrichment was transferred to a batch-culture enrichment (over 1%), the $K_{\text{m(app)}}$ value increased without a change in the DGGE pattern, suggesting that the $K_{\text{m(app)}}$ value is...
dependent on the growth condition (the physiological state) rather than different $K_{app}$ values being expressed by different methanotrophs. This paper emphasizes the importance of choosing appropriate conditions for laboratory experiments in ecological studies.

It has been well known that the majority of marine bacteria resist cultivation in standard laboratory media and the culturability seldom exceeds 0.01% (123). The uncultured bacteria are believed to be oligotrophs, because the total organic concentration in seawater is generally very low. Oligotrophs only are able to utilize diluted organic compounds and exist in the form of very small cells. They cannot attain high cell concentrations. An isotope incorporation experiment has suggested that oligotrophs predominately incorporate ambient organic compounds in seawater (128). This is also likely to be the case for marine oil spills, which has been suggested by a toluene turnover study (129). A marine oligotrophic bacterium capable of growing on toluene, xylene, and some polynuclear aromatics was isolated and identified as *Cycloclastics us oligotrophus* (130). It was found that this bacterium harbors genes for aromatic hydrocarbon degradation, similar to those of soil Pseudomonads (130), and that the bacterium exhibits an affinity constant for toluene incorporation (e.g., 1.3 µg per liter) similar to that measured in seawater (131). These data suggest that this organism is involved in toluene degradation in the marine environment, although molecular ecological methods have not yet been applied to its detection in the marine environment.

A functionally dominant phenol-degrading population in phenol-digesting activated sludge has been identified by the combined use of molecular ecological detection and physiological comparison (116). In that study, TGGE analysis of PCR products of 16S rDNA and of the gene encoding phenol hydroxylase (LmPH) revealed a few dominant populations in activated sludge after 20 d of incubation with phenol. Bacteria closely related to *Comamonas* spp. were isolated by direct plating and continuous enrichment methods and were found to possess 16S rDNA and LmPH sequencese identical to those predominantly found by the TGGE analyses. Although these bacteria are capable of growing on phenol as the sole carbon source, their growth on phenol at concentrations of over 100 mg per liter was very poor. A kinetic analysis of the phenol-oxygenating activity indicated that the *Comamonas* bacteria exhibit the affinity and inhibition constants in Haldane’s equation almost identical to those of phenol-digesting activated sludge (Fig. 3). Since the three approaches (i.e., TGGE using 16S rDNA, that using LmPH and kinetic analysis) were consistent, the *Comamonas* bacteria have been identified to be the dominant phenol-degrading population in activated sludge. This study has thus demonstrated an ecological theory (132) that although naturally occurring microbial consortia harbor diverse bacteria possessing a specific function, only limited species dominate the niche under stable environmental conditions.

Concentrations of organic compounds in the natural environment are generally much lower than those in laboratory media. Hence, ERMs listed above show high affinities for organic substrates, suggesting the importance of kinetic studies for physiological identification of ERMs. If a microorganism expresses pollutant-degradation kinetics compatible with those observed in the environment and its population dynamics as analyzed by molecular ecological methods (hopefully by several methods) correspond to changes in a specific function, one is allowed to declare that an ERM has been identified. One important aspect is that all these ERMs have been enriched and isolated under low substrate concentrations.

**Control of ERMs in microbial consortia**

An example is presented in which the growth of ERMs was controlled to improve an environmental biotechnology process. When the phenol-loading rate of an activated sludge process was increased stepwise from 0.5 to 1.0, and then to 1.5 g l$^{-1}d^{-1}$, the process broke down within one week after the loading rate was increased to 1.5 g l$^{-1}d^{-1}$ (93). Transitions of major bacterial populations were analyzed using molecular tools (93), and we found that phenol-degrading populations changed from flocc-forming bacteria to nonflocculating bacteria at the loading rate of 1.5 g l$^{-1}d^{-1}$; this population change was observed concomitantly with sludge washout, suggesting that it was probably responsible for the process breakdown. To circumvent this problem, a preferential growth substrate for the flocc-forming phenol-degrading population was additionally supplied at high phenol-loading rates; this measure was found to be effective for enhancing the flocc-forming population and suppressing the nonflocculating populations in activated sludge (unpublished data). Consequently, the activated-sludge process was capable of degrading phenol at phenol-loading rate of 1.5 g l$^{-1}d^{-1}$. Although the supply of galactose is impractically expensive, this study has demonstrated that control of ERMs enables the improvement of environmental biotechnology processes.

Figure 4 illustrates factors governing the size of bacterial populations in a natural microbial consortium. The four factors indicated by arrows in the figure can be
FIG. 4. Factors governing the sizes of bacterial populations in a natural habitat. Arrows pointing toward bacteria work positively for bacterial populations, while those pointing away from bacteria work negatively.

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Bacterial populations, while those pointing away from bacteria work negatively.

used to control bacterial populations. Biostimulation supplements nutrients to activate pollutant-degrading microorganisms (shown by arrow 3). In the above example, we selectively stimulated the growth of the ERM population, which is hence termed selective biostimulation. The other two factors (indicated by arrows 2 and 4) are also known to exert large effects on the bacterial populations in the environment (133), so that these factors may also be applicable in the field of environmental biotechnology in order to improve microbial consortia.

CONCLUSION

Manipulation of ERMS is a possible way to microbiologically improve environmental biotechnology processes. To achieve this, we should understand the physiological traits of ERMS in detail and compare these with the traits of other major members of the microbial consortium. However, identification of an ERM is still laborious, even if we can effectively use molecular ecological tools. Furthermore, compared with typical laboratory strains, ERMS often grow very slowly in laboratory media, and the cell concentrations achieved in their cultures are generally very low, which hampers their physiological characterization. We therefore propose that an important subject that should be tackled in the next decade is the development of physiological means that facilitate the study of ERMS in the laboratory.

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VOL. 89, 2000
ENVIRONMENTALLY RELEVANT MICROORGANISMS

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