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Minireview

A Great Leap forward in Microbial Ecology

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Ribosomal RNA (rRNA) sequence-based molecular techniques emerged in the late 1980s, which completely changed our general view of microbial life. Coincidentally, the Japanese Society of Microbial Ecology (JSME) was founded, and its official journal "Microbes and Environments (M&E)" was launched, in 1985. Thus, the past 25 years have been an exciting and fruitful period for M&E readers and microbiologists as demonstrated by the numerous excellent papers published in M&E. In this minireview, recent progress made in microbial ecology and related fields is summarized, with a special emphasis on 8 landmark areas; the cultivation of uncultured microbes, *in situ* methods for the assessment of microorganisms and their activities, biofilms, plant microbiology, chemolithotrophic bacteria in early volcanic environments, symbionts of animals and their ecology, wastewater treatment microbiology, and the biodegradation of hazardous organic compounds.

Key words: Microbial community and function, 16S rRNA gene-based analysis, uncultured microbes, biofilms, symbiotic microorganisms

Introduction

This special minireview celebrates the twenty-fifth anniversary of both The Japanese Society of Microbial Ecology (JSME) and its official journal "Microbes and Environments (M&E)" and provides an excellent opportunity to look at the progress that has been made over the past 25 years. Small subunit of ribosomal RNA (rRNA) sequence-based molecular techniques for the description of microbial diversity provided the foundation for a significant step forward in microbial ecology in the mid-1980s. Using culture-independent molecular techniques, a vast number of new lineages in the domains *Bacteria* and *Archaea* have been retrieved from environments. It has been demonstrated that the microbial world is genetically and functionally more complex and diverse than previously predicted on the basis of culture-dependent studies. However, even though new microorganisms continue to be isolated, it is now widely recognized that only a small fraction of extant microorganisms have been grown in pure cultures and characterized. Consequently, the majority of relevant microorganisms have not been cultured and so their ecophysiological roles in natural and human-made ecosystems remain largely unknown. Therefore, concerted efforts must continue to expand our understanding of the microbial world and to develop the novel techniques required to elucidate the kinds of microorganisms out there and the roles they play.

This special minireview encompasses 8 landmark areas of microbial ecology that have been covered in M&E. It is our hope that this review by interdisciplinary groups of experts will significantly improve our understanding of the current research trends in microbial ecology and related areas.

Does the cultivation of uncultured organisms provide new insights into microbial ecology?

Research trends in any field of science change with time. However, the changes in microbial ecology over the last 4–5 years have been particularly remarkable. Metagenomic, metatranscriptomic and metaproteomic-based approaches have impacted the whole concept of microbial ecology, and overwhelming datasets are now being accumulated. Such "meta" approaches are often referred to as state-of-the-art culture-independent technologies and used to comprehensively capture genes, gene transcripts, and proteins in complex microbial communities.

These trends have raised questions: what is the significance and importance of isolating yet-to-be cultured organisms in the omics era of analyzing massive datasets produced using "meta" approaches? Together with metagenomics, if single cell genomics allows sequencing of the complete genome of an organism, is the isolation of target organism needed? In turn, if the "meta" approaches could decipher the

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substantial metabolism in complex communities, how could we take advantage of such information to develop new ways of isolating target organisms?

If scientists are interested in seeking particular molecules, for instance, an enzyme for industrial purposes, perhaps the isolation of organisms under selective pressure would be canonical, but convenient. As a matter of fact, those studies are still going on. However, if scientists are interested in the structure and functions of a complex microbial community in which a number of metabolic and physiological interactions between different taxa occur, "meta" approaches might be the most powerful way to better understand and overview the community.

Nonetheless, it should be noted that the isolation of organisms still needs to be done and is still a challenging process (5, 35, 68, 74, 149). If all scientists rush to "meta"-based research, phylogenetic trees based on rRNA or functional genes would be covered with an enormous number of sequences from uncultured organisms. This is really happening. However, if the numerous sequences from uncultured organisms are surrounded by or juxtaposed with those of cultured organisms, one could readily imagine, to a greater extent, the entities of those uncultured organisms by looking into the physiological and genetic traits of the neighboring isolates. Without isolates, one could never guess who they are, what they are doing, and what they could potentially do, even though culture-independent techniques such as stable isotope probing (SIP), fluorescence *in situ* hybridization (FISH), and omics data tell us something. It should be noted that comparing sequences with those of cultured organisms may lead to far-fetched conclusions, so that we should keep in mind that such a one-way approach has certain limitations.

One of the best examples of a how culture-dependent strategy in connection with culture-independent strategies has contributed to speculation on the morphotypes, functions and genome structures of yet-to-be cultured organisms is the study of a subgroup of the phylum *Chloroflexi* (165). Organisms belonging to this phylum are cosmopolitans found in almost all environments. Despite such ubiquity, only a small number of cultured microorganisms were known. In particular, subclass 1 did not have any cultured representatives at all until 2001, and it had long been believed that those organisms are difficult to cultivate. However, much effort has been made over the last decade, and, currently, six genera within subclass 1 of *Chloroflexi* have been isolated and characterized. The genomes of the isolates are now being studied. Isolates from five of the genera are slowly growing fermentative organisms that favor sugars and complex nutrients. In addition, the growth of some of them was found to rely on hydrogenotrophic methanogens that could remove H_2 produced from the fermentative isolates forming a syntrophic relationship. Very interestingly, all the novel isolates are filamentous bacteria together with known genera within the other subclasses of *Chloroflexi* except for the genus *Dehalococcoides*.

Undoubtedly, isolation and cultivation are still the most convincing way to know the entity of organisms. The most serious problem that culture-dependent approaches are facing is that the methods currently being employed are based on those created in the late 19th century, are very laborious, and are far slower in accumulating data than "meta" approaches. Such a fatal flaw cannot be readily solved. To fill the gap, creating massive and high throughput isolation techniques (177) and new isolation devices (5, 66) to overcome those problems will be indispensable.

In situ *identification and functional analysis of microorganisms tells us their true nature*

Microorganisms are highly diverse and play key roles in ecosystems. Various methods have been developed to clarify the *in situ* relationships between the physicochemical and biological characteristics and abundance, activities, diversity and functions of microbes in environments of interest.

Determination of the abundance of microorganisms is essential in environmental microbiology. Hobbie *et al.* (45) developed the total direct counting method in 1977, whereby microorganisms are fluorescently stained, collected onto a polycarbonate filter and counted under a fluorescence microscope. This method is rapid and simple, and subsequent studies demonstrated that many more microorganisms exist than expected and most (90–99%) are hard to culture under conventional conditions.

One of the next concerns of environmental microbiologists was phylogenetic information on these abundant microorganisms, and FISH was developed to answer these questions (2, 22). This method enables the *in situ* phylogenetic identification of targeted microorganisms; we can know where and how they exist from microscopic images. By combining FISH and PCR-based approaches such as quantitative PCR and clone analysis, details of the ecology of microorganisms, e.g. in symbiosis (59) and in wastewater treatment (53), have been clarified. However, bacteria in natural environments are often less active than cultured forms, and the amplification of fluorescence signals from targeted bacteria is required in such cases. Enzymatic reactions such as the HNPP/Fast Red TR reaction or tyramide signal amplification (TSA) were applied to improve the sensitivity of FISH (88, 129, 167).

One can obtain phylogenetic information on microorganisms of interest by FISH, but estimations of their activities are difficult using FISH alone. Various FISH-based methods have been developed to obtain phylogenetic information and also physiological and metabolic activities of microorganisms simultaneously in environments. The microcolony method (132) is useful for determining bacterial proliferative activity because most bacteria in natural environments do not form macroscopic colonies but form microcolonies under general culture conditions. FISH combined with this microcolony method (microcolony-FISH) (28, 143) is especially suitable when a sample contains small fluorescent particles which inhibit reliable detection of bacteria at a single-cell level; microcolonies are larger than these "noise" particles and can be easily differentiated under a fluorescence microscope. Direct viable counts (DVCs) (84) also enable one to detect bacteria with proliferative activity using a rather simple procedure. Nishimura *et al.* (118) and Wu *et al.* (164) used FISH combined with DVC (DVC-FISH) to count viable bacteria in natural seawater samples and in cow manure, respectively. Tada *et al.* (148) used the bacterial

uptake of bromodeoxyuridine (BrdU) as a marker of metabolic activity and combined this BrdU immunocytochemistry with FISH (BIC-FISH) to determine the phylotype-specific productivity of marine bacterial populations. A similar approach was established using FISH combined with microautoradiography (MAR-FISH) (90, 123) to detect the bacterial uptake of radioisotope-labeled substrates as an indicator of bacterial productivity. A rather simple approach is the combination of FISH with fluorescent vital staining. 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is widely used as bacterial respiratory indicator (31, 133). Yamaguchi *et al.* (166) detected starved fecal contamination indicator bacteria in potable water selectively by signal-amplified FISH following formazan formation (TSA-CTC-FISH).

By using FISH techniques, we can obtain phylogenetic information on targeted microorganisms in the environment, and can estimate their activities by the new FISH techniques described above. The next important issue is the investigation of functions of individual cells in complex microbial communities. FISH usually targets highly abundant rRNA in cells, and conventional FISH is unable to detect low copy numbers of targeted nucleic acid molecules. However, various new FISH techniques are being developed to detect single copy genes inside cells combined with *in situ* gene amplification techniques. Tani *et al.* (150) determined the dynamics of phenol-degrading bacteria in groundwater during bioaugmentation by *in situ* PCR-FISH. Kenzaka *et al.* (73) and Maruyama *et al.* (102) developed cycling primed *in situ* amplification-fluorescence *in situ* hybridization (CPRINS-FISH) and *in situ* rolling circle amplification (*in situ* RCA) to examine the possible range and frequency of gene transfer among bacterial cells. Hoshino and Schramm (48) improved *in situ* RCA to detect nitrite and nitrous oxide reductase genes (*nirS* and *nosZ*) in *Pseudomonas stutzeri*.

In situ targeted gene amplification improves the sensitivity and specificity of FISH, while the design of probes and/or primers for reactions is sometimes complicated. Kawakami *et al.* (71) improved the signal intensity of TSA-FISH by their Two-Pass TSA-FISH, which repeats TSA twice, in order to detect single copy genes without *in situ* gene amplification. On the other hand, Zwirglmaier *et al.* (178) developed FISH with polynucleotide probes (RING-FISH) for detection of the nitrite reductase gene (*nirK*) in denitrifiers (130) while oligonucleotide probes are usually used in FISH. In RING-FISH, a multiply labeled transcript polynucleotide probe is used to intensify signals through multiple labeling and detect a single gene on the bacterial chromosome during FISH.

Another way to detect a single copy gene inside targeted cells is to improve the signal detection system. Nanometer-resolution secondary ion mass spectrometry (NanoSIMS) is a powerful tool in environmental microbiology. This system has unique elemental and isotopic sensitivity and accuracy, and Behrens *et al.* (10) visualized the fate of substrates labeled with ^{13}C and ^{15}N in a microbial consortium consisting of filamentous cyanobacteria and *Alphaproteobacteria*, while individual cells were identified simultaneously with a covalently halogen-Cy3-labeled probe.

One can obtain large data sets of rRNA and functional gene sequences of microorganisms from databases and these databases are rapidly growing. New FISH techniques will reveal the nature of microorganisms in various environments with cultivation-independent approaches.

Life is not as simple as it seems

Bacteria have long been considered to live independently of neighboring cells. However, recent research has changed this view revealing an ability to form multicellular communities and to communicate with each other through signaling chemicals.

In *Pseudomonas aeruginosa*, three chemically distinct molecules that mediate cell-to-cell communication have been well characterized (163). Two signaling systems utilizing distinct *N*-acylhomoserine lactones (AHLs) which are common among Gram-negative bacteria (146), are the LasR-LasI (*las*) and RhlR-RhlI (*rhl*) systems. Another type of signaling molecule is 2-heptyl-3-hydroxy-4-quinolone which is referred to as the *Pseudomonas* quinolone signal (PQS). These cell-to-cell communication systems were first extensively studied in relation to the virulence of the bacteria. Now, there is evidence that cell-to-cell communication has more to do than merely the regulation of virulence factors.

One example is the regulation of respiratory activity. *P. aeruginosa* is capable of utilizing N-oxide as an electron acceptor in the absence of oxygen. Denitrification is generally regulated by physicochemical conditions such as the presence of electron acceptors through certain regulatory proteins (106, 125). In addition, it has been demonstrated that denitrification is regulated by AHLs and PQS in *P. aeruginosa* (157, 158). While the AHL signals regulate the transcription of the denitrifying genes, PQS is considered to act directly on the denitrifying enzymes.

The direct effect of PQS on enzymatic activity indicates a new function of this signaling molecule suggesting a broad impact on other bacterial species. Indeed, PQS affects the growth of a broad spectrum of bacteria from Gram-negative to Gram-positive bacteria (159). While PQS represses the consumption of oxygen in some bacteria, the mechanism by which it represses growth is still unknown. Several mechanisms may be involved in this growth repression since PQS has been reported to chelate iron and produce oxidative stress (12, 26, 52) besides affecting respiratory enzymatic activity. Nevertheless, the concentration of iron is a key factor in determining the effect of PQS on respiratory activity and growth indicating that the surrounding conditions control the interspecies interaction (157, 159). By tuning signal production in response to the environment, bacteria could be more flexible in coping with neighboring bacteria according to the change in the environment.

Another characteristic of the PQS molecule is that it could induce the production of outer membrane vesicles that contain active proteins and perform diverse biological processes (103, 153). Several mechanisms have been proposed for the biogenesis of the outer membrane vesicles (87, 152). In *P. aeruginosa*, PQS is able to induce outer membrane vesicle production and these vesicles facilitate cell-to-cell communication by carrying PQS (103). Interestingly, PQS also induces the production of outer membrane vesicles in other species (154), including Gram-negative and Gram-

positive bacteria.

These multifunctional effects of PQS on other bacteria suggest an important role in interspecies interaction. Concerning the interspecies interaction via PQS, it is important to state that PQS production is repressed by indole compounds that are produced extracellulary in many bacteria (89, 151). Moreover, *Bukholderia* and *Alteromonas* species produce PQS-related compounds that could possibly enhance the production of PQS in *P. aeruginosa* (25).

As growing evidence indicates that bacteria interact among inter and intra species (169), technical demands to handle complex communities arise. One example of such communities is biofilms in which bacteria are attached to a surface and embedded in extracellular matrices forming heterogeneous three-dimensional structures (44, 120). It has been estimated that the majority of bacteria exist in biofilms in nature rather in a planktonic free-living state. In order to reveal the behavior of bacteria in their natural habitat it is necessary to develop a nondestructive method that can monitor the bacteria without fixation. Confocal laser scanning microscopy (CLSM) combined with fluorescent protein-tagged bacteria is one of the preferred techniques in this category, however, its use is limited to bacteria that can express fluorescent proteins, which makes it difficult to handle natural samples and conditions where fluorescent proteins are not expressed. By applying a confocal reflection microscopy (CRM) technique to biofilms, this problem can be solved. Natural samples such as activated sludge were observed with this technique as were model organisms, resulting in a high resolution that is comparable to the utilization of fluorescent proteins and dyes (170, 171). By combining this technique with other techniques, it is possible to measure gas metabolites under anaerobic conditions and observe the correlation of the biofilm structure and material diffusion in biofilms (170, 172).

Microbiology has classically depended on pure cultures of single species, yet little is known of how bacteria behave in a complex community. How bacteria interact with each other in a complex community and what changes as a result, would be one of the most exciting things to know about in the near future.

Microbial diversity and functions in plant-soil ecosystems: how do plants select specific microbes from soil microbial communities?

Rhizobia are symbiotic nitrogen-fixing bacteria that form nodules on legume roots, and include diverse phylogenetic groups mainly within *Alphaproteobacteria*. Their symbiotic interactions and survival are still crucial for plant microbiology and global environmental conservation. Fujihara (30) and Lim (94) summarized recent developments in biogenetic amines in rhizobia and TonB-dependent receptors based on rhizobial genomes in excellent minireviews, respectively. Host symbiosis genes (98) and malic enzymes (21) are involved in rhizobial infection and nitrogenase function, respectively. Masuda *et al.* (104) verified that the *cbbL* gene encoding ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is required for chemoautotrophic growth of *Bradyrhizobium japonicum*. Inaba *et al.* (58) observed a correlation between N_2O emissions from soybean rhizospheres and microbial community changes including rhizobia. These results show survival strategies and ecological functions of free-living rhizobia in soil environments.

Apart from rhizobia, diverse bacteria reside in the phytosphere as endophytes and epiphytes in nature (57). However, their ecology is largely unknown, because they do not form a special organ in plants like the root nodules formed by rhizobia. M&E have published many articles of bacterial endophytes in agricultural settings by using culture-dependent and -independent procedures. Mano and Morisaki (100) have published an excellent minireview of bacterial endophytes in rice plants based on culture-dependent analyses (67, 99). Okubo *et al.* (126) also showed that communities of bacterial endophytes are dependent on soybean nodulation genotypes by culture-dependent analysis. These articles indicate the significance of classical culture-dependent analyses for bacterial endophytes irrespective of culture biases. Interestingly, this situation is similar to endosymbiotic bacteria in insects (74).

Meanwhile, Saito *et al.* (136) emphasized the significance of culture-independent methodologies to investigate the diversity of plant-associated microbes (54, 55, 135). Generally, culture-independent methods offer advantages when observing microbial diversity because of the existence of unculturable microbes in the phytosphere (57). In this regard, it is important how microbial DNA is isolated from plant tissues. Microbial DNA extraction directly from leaf tissues (147) and bacterial cell enrichment by centrifugation (56) were developed for this purpose.

As for functional aspects of plant-associated microbes, nitrogen-fixing endophytes has been extensively reported in M&E: *nifH* expression in field grown sweet potato (155), the behavior of *gfp*-tagged *Herbaspirillum* sp. in sugarcane (175, 176), the effects of organic fertilizers (70), a community analysis of the *Melastoma* rhizosphere (139), ^{15}N dilution analysis of sugarcane (110), and the effects of *Azospirillum* sp. in paddy rice fields (60).

Rhizosphere bacteria reside at the interfaces between plants and soils, and often show crucial functions in plant-soil ecosystems, because plants excrete photosynthate from their roots. Phytase-producing bacteria in the rhizosphere of plants help phosphorous uptake by plants (64). One fundamental question of microbial ecology in plant-soil systems is how plants select specific microbes from soil microbial communities. What are the functions of plant-associated microbes? Minamisawa (107) have pointed out the significance of metagenomic analyses of plant-associated microbes to address this issue. Strong support from bioinformatics and rational genome databases (17) are needed to attain this goal.

Significance of chemolithotrophic bacteria in early volcanic environments

One major challenge in general soil microbiology is to identify the pioneer microbes colonizing new soil substrates such as lava, tephra, and volcanic ash and to know their ecological roles and functions in the formation of new soils and ecosystems. Such volcanic substrates are characterized partly by large amounts of reduced minerals (e.g., sulfides)

and always by little or no organic carbon and nitrogen (13). Much of the research showed that such substrate systems result in a rich variety of microbes independent of organic carbon as the energy source, *i.e.*, chemolithotrophic microbes. According to King's notion (80), the habitats which support the growth of sulfur- and iron-oxidizing chemolithotrophs can be categorized as reductant-rich volcanic systems. In reductant-poor volcanic systems, chemolithotrophs may also play significant roles in the early ecosystem's development through the ability to use atmospheric H_2 and CO for chemolithotrophic growth (80). *In situ* and *ex situ* assays of H_2 and CO uptake by recent Kilauea volcanic deposits in Hawaii suggested that H_2 accounts for 20–25% of respiratory reducing equivalent flow, while CO accounts for 2–10% (78). Similarly, the consumption of H_2 and CO was detected at sites of a 23-year-old scoria deposit on Miyakejima, a volcanic island in the Izu chain southeast of Tokyo, Japan (81). Methane in the atmosphere is another probable substrate for bacterial growth. King and Nanba (82) measured the atmospheric CH_4 oxidation of Hawaiian volcanic deposits and soils. Their results indicated that methanotrophs colonized volcanic substrates slowly and likely depended on interactions with plants and other microbial communities.

The majority of chemolithotrophs in the Hawaiian volcanic deposits were identified as facultative not obligate chemolithotrophs by clone library analyses for the gene coding for the large subunit of the form I RubisCO (*rbcL*) (116). This result coincides with those of several culture-based studies on volcanic deposits. A bacterium that resembled *Bacillus schlegelii*, capable of heterotrophic growth and autotrophic growth in the presence of hydrogen and carbon dioxide, was isolated from geothermal soil collected from Mount Erebus, Ross Island, Antarctica (49). Hydrogen-oxidizing, facultatively chemolithotrophic bacteria, *Cupriavidus pinatubonensis* and *Cupriavidus laharis*, were reported to be abundant in 2- to 3-year-old volcanic mudflow deposits from Mt. Pinatubo (The Philippines) (140, 141). A heterotrophic bacterium capable of chemolithotrophic growth by oxidizing thiosulfate was isolated from 23-year-old Miyakejima volcanic deposits (95). Recently, this thiosulfate-oxidizing bacterium was characterized as a novel species, *Limnobacter litoralis* (96). These observations collectively suggest that facultative chemolithotrophs represent early colonists on organic-poor volcanic deposits. Furthermore, many of the known CO- or H_2-oxidizing isolates have been reported to fix N_2 or to harbor nitrogenase genes (79, 104), and thus they may also contribute to nitrogen dynamics and the evolution of nitrogen cycling in volcanic deposits.

An iron-oxidizing chemolithotrophic bacterium, *Leptospirillum ferrooxidans*, is known to dominate acid mine drainage biofilms (11, 27, 161), bioleaching systems (19, 131), and metal-rich and extremely acidic river environments (36). Very recently, it was reported that *L. ferrooxidans* was abundant in 8-year-old, acidic, volcanic ash deposits near the crater (Mt. Oyama) of Miyakejima and further, the isolates possessed nitrogenase activity (142). The site of isolation was frequently exposed to volcanic gases (mainly, SO_2) ejected from the crater (72) and remained essentially unvegetated. In extreme environments, iron-oxidizing chemolithoautotrophs may represent pioneer colonists and play a significant role in the accumulation of carbon and nitrogen and the initiation of new soils and ecosystems. The development of a suitable SIP with $^{15}N_2$ gas will give proof of the *L. ferrooxidans in situ* N_2-fixing activity (142).

Soil bacteria have diverse ecological functions. Nitrous oxide (N_2O) and methane (CH_4) are greenhouse gases, which are emitted from soils during microbial processes and likely increase global warming. N_2O is emitted during the microbial transformation of inorganic nitrogen during nitrification and denitrification processes. Several articles in M&E have dealt with microbial diversity and functional genes of nitrification (4, 65, 115) and denitrification (69, 137) in soil environments in Asia.

Living together or separately?: ecology of animal symbionts

Due to technological advances in molecular microbial ecology, researchers can now investigate individual microorganisms or microbial communities that are associated with animals. In the early stages of the 25-year history of M&E, microbial associations or symbioses were major topics but often merely descriptive. Nowadays, advances have changed our views to a great extent, showing that symbioses are widespread, and offer many fascinating novelties. For instance, until recently, human intestinal bacteria had been considered mere commensal residents having little effect on the host. We now have recognized that they have a profound effect on the digestive physiology of the host (91, 93). This kind of awareness has led to interdisciplinary research between microbiologists and others such as animal physiologists in health and environmental science.

One of the most widespread symbioses in animals involves obligate and facultative intracellular bacteria (endosymbionts) that live in special tissues of insects. Although molecular ecological and genomic studies have revealed their diversity, function, and adaptative evolution, some model systems with culturable symbionts that can be engineered genetically are emphasized for understanding the molecular mechanisms for interactions with host insects (74, 134). Another exciting example of symbioses in insects involves termites and their microbial symbionts which play an essential role in the digestion of recalcitrant lignocellulose. There are a number of reports in recent volumes of M&E that investigate not only the microbial symbionts of bacteria (24, 156), archaea (23, 109), protists (83) (these three inhabit their gut), and fungi in the nest of termites (114), but also associations of bacteria or archaea with the protists (37, 59, 119). Consequently, the gut microbial community of termites, though complex and highly structured, has become an attractive model system to study microbial diversity, structure and function of the community, and coevolution. Indeed, genome analyses of the symbionts have unveiled their roles in the associations (46, 121 for reviews).

In addition to insects, various associations with animal hosts have been dealt with in M&E. Probable chemoautotrophic endosymbionts are reported to associate with a marine beard worm, and because of the occurrence of the

related free-living species in the surrounding environment, the hosts are suggested to acquire the symbionts horizontally every generation (1, 86). Meanwhile, some *Vibrio* species are important pathogens of marine fishes and invertebrates or involved in the poisoning of marine foods. Contamination of drinking water and food with fecal bacteria of animals is of great significance to public health. Therefore, the detection and enumeration of the species responsible or indicator species like *Escherichia coli* are anticipated. A series of reports have contributed to the rapid and accurate detection of target species in environments (14, 32, 33, 61, 62, 128, 144, 164, 166) particularly by developing new methods, as well as to the detailed taxonomy of related species (62, 145). These studies have had a considerable impact on the ecology of animal-associated microorganisms by demonstrating that they likely share other ecological niches in natural environments, and represent the exciting recent research activities found in M&E.

Biological wastewater treatment systems: excellent teachers of microbial ecology

Biological wastewater treatment is undoubtedly one of the most important and largest of the biotechnological processes, which have been used for over a century to treat municipal and industrial wastewaters. It is now recognized that wastewater treatment processes harbor a vast variety of microorganisms, most of which are still yet-to-be cultured, and hence uncharacterized (3, 39, 168, 173). However, a number of new exciting insights into microbial structure and function in wastewater treatment processes have been recently gained by applying culture-independent molecular approaches, which has significantly expanded our understanding of process design, operation and control.

Various molecular techniques [ex. quinone profiling, FISH, denaturing gel gradient electrophoresis (DGGE), and DNA microarray (92, 162)] have been developed and applied to nitrification-denitrification (47, 105, 122), enhanced biological phosphorus removal (EBPR) (34, 160), anaerobic digestion (6, 117), scum-forming (42), microbial fuel cells (18, 38), reverse-osmosis water purification (8), membrane bioreactors (97, 108) and compost (31).

Molecular techniques have revealed that microbial communities are composed of a great variety of microorganisms. Narihiro *et al.* (117) collected granular sludge samples from twelve full-scale UASB plants and examined bacterial and archaeal populations based on a 16S rRNA gene cloning analysis. Their community structures were composed of 41–65 bacterial and 6–12 archaeal phylotypes, and the microbial composition differed among the twelve UASB plants. Iguchi *et al.* (53) further examined the distribution of uncultured members of the phylum *Nitrospirae* in those UASB plants by quantitative PCR. The members of the *Nitrospirae* group were commonly found and accounted for up to around 10.9% of all 16S rRNA genes, though their ecological function in granular sludge is unclear.

In addition, molecular techniques revealed that most of the model organisms suggested based on the outcome of culture-dependent methods are of minor relevance. One example is polyphosphate-accumulating organisms (PAOs). Until to the 1990s, the members of *Acinetobacter* sp. are thought to be a dominant PAO because they were frequently isolated from the activated sludge of EBPR processes and accumulated intracellular polyphosphate granules (29, 63). However, FISH analysis combined with chemical staining for intracellular polyphosphate granules revealed that the dominant PAOs were *Candidatus* 'Accumulibacter phosphatis' and *Actinobacteria* (20, 34, 85). Furthermore, Okunuki *et al.* (127) determined the abundance of *Candidatus* 'Accumulibacter phosphatis' and actinobacterial PAOs by quantitative PCR and found that the copy number of 16S rRNA genes of *Candidatus* 'Accumulibacter phosphatis' correlated with the phosphorus removal performance. Nowadays, the contribution of *Candidatus* 'Accumulibacter phosphatis' and actinobacterial PAOs to biological phosphorus removal is widely accepted.

Furthermore, MAR-FISH (123) allows us to observe ecophysiological interactions among community members. Kindaichi *et al.* have clearly indicated that heterotrophs in the nitrifying biofilm utilized soluble microbial products secreted from nitrifiers, using MAR-FISH with $^{14}CO_2$ as a tracer to nitrifying biofilms (77, 124).

A more comprehensive understanding of microbial structure and function is required to design microbial consortia having high treatment stability and efficiency. For this purpose, 'omics' technologies such as metagenomics (75) have recently been applied (101). The metagenomic approach represents a snapshot of microbial community structures, their potential function and interactions. Furthermore, highly sensitive FISH technology will be a powerful tool to study the phylogeny of particular genes found in metagenomic libraries.

However, recent research has indicated the importance of bacteriophages and protozoa to the performance or stability of wastewater treatment processes. Barr *et al.* (9) indicated that a bacteriophage infection of *Candidatus* 'Accumulibacter phosphatis' caused a deterioration of phosphorus removal. Moreno *et al.* (111) examined a predator-prey relationship between autotrophic bacteria and protozoa by RNA stable isotope probing method and showed the importance of protozoa to study microbial population dynamics. This clearly indicated that the ecophysiology of not only bacteria or archaea but also of bacteriophages and protozoa must be studied to expand our understanding of process design, operation and control.

Biodegradation of hazardous chemicals: one of the fastest growing applications of environmental biotechnology

The biodegradation of hazardous or persistent organic compounds in the environment by soil microorganisms and enriched microbial consortia has been well documented in connection with natural attenuation and bioremediation. Huong *et al.* (50) isolated and characterized bacteria capable of degrading 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophoxyacetic acid (2,4,5-T) from Vietnamese solids historically exposed to Agent Orange. They also characterized a 2,4-D- and 2,4,5-T-degrading enrichment culture by 16S rRNA and *benA* gene-targeted PCR-DGGE, and suggested the major role of *Burkholderia* species in

degrading these chemicals (51). Sakai *et al.* (138) reported that the *Burkholderia cepacia* RASC type 2,4-D-degrading genes harbored on large plasmids spread out among 2,4-D-degrading bacteria isolated from soil in Japan. A soil bacterial community capable of degrading 3-chlorobenzoate was characterized by 16S rRNA and *benA* gene-targeted PCR-DGGE (112, 113). The bacterial community diversity of anaerobic fluidized bed bioreactors treating some aromatic compounds, such as phenol (15) and 2,4-dinitroaniaole (7), was studied. Kimura *et al.* (76) applied a metagenomic approach to the characterization of a 4-nitrotoluene-oxidizing enzyme from an activated sludge.

Bioremediation technology using specific microorganisms and microbial consortia has gained momentum as a cost-effective and ecologically sound approach to the remediation of environments contaminated with hazardous chemicals. Large numbers of structurally diverse haloorganic compounds, such as chloroethenes, polychlorinated dibenzo-*p*-dioxins/furans (PCDD/Fs), and polychlorinated biphenyls (PCBs), are common contaminants in soil, sediment, and groundwater. Anaerobic microbial redox processes can work effectively in the engineered bioremediation of these haloorganic contaminants (40, 41). Anaerobic microbial consortia with "*Dehalococcoides*" species as potent dechlorinators were enriched from a PCDD/F-contaminated microcosm (43). One of these dehalogenating cultures, designated TUT2264, dechlorinated tetrachloroethene to form dichloroethenes with the transcription of multiple reductive dehalogenase genes (35). Yoshida *et al.* (174) reported that an enrichment culture containing *Dehalobacter* species was able to reductively dechlorinate PCDDs and PCBs. In addition, Chiba *et al.* (16) reported that a *Bacillus* strain isolated from a marine sediment core had a haloacid dehalogenase that catalyzed the dehalogenation of monobromoacetic acid, monochloroacetic acid, and 2-chloropropionic acid.

Conclusions

The past quarter century was undoubtedly a golden era of culture-independent small subunit of rRNA gene-based analyses in all areas of microbial ecology. The advent of this technical breakthrough provided new insights into the composition and structure of microbial communities and revealed a remarkably vast microbial diversity including many hitherto-recognized and yet uncultured species in various microbial habitats. However, some challenges remain, for example, the significance of this microbial diversity and its relation to function is not fully understood. Fortunately, new powerful tools including metagenomic, metatranscriptomic and metaproteomic-based approaches are now available to confront such challenges. Since we are facing serious environmental issues that threaten our lives, a better understanding of the ecophysiology of environmentally relevant microorganisms is essential to solve problems such as global warming and environmental pollution, by converting anthropogenic waste into clean renewable energy. Therefore, the future of molecular-based microbial ecology seems to be bright and promising, but requires continuous efforts and works in collaboration with multiple disciplines.

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