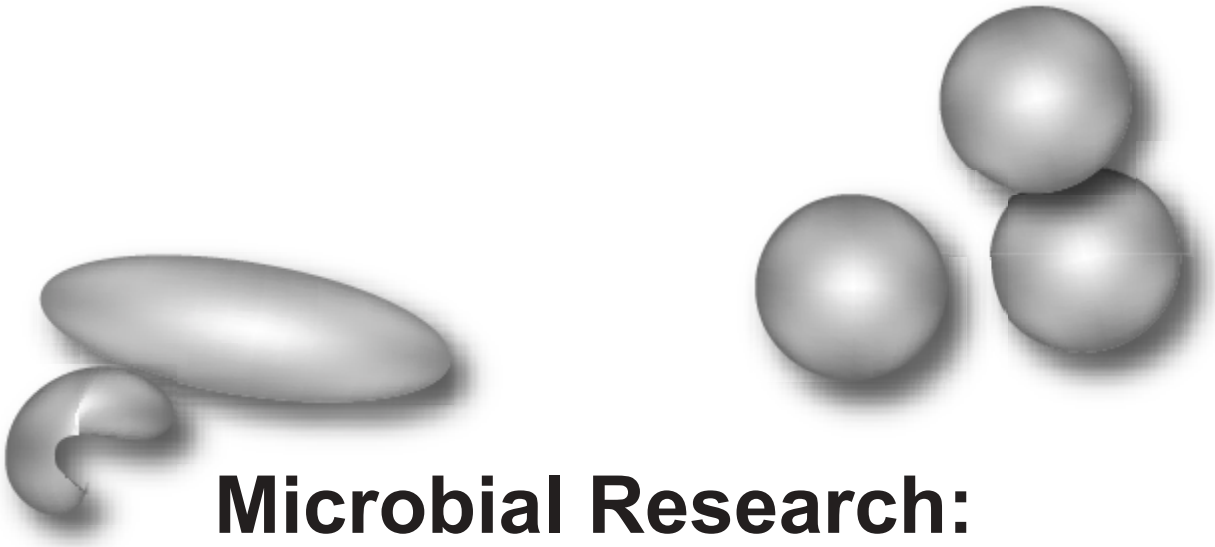
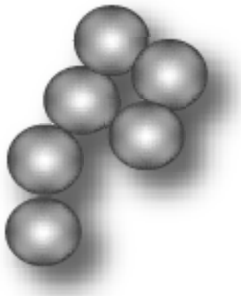


Microbial Research: Progress and Potential

NSF Microbial Observatory/Life in Extreme Environments
Principal Investigators' Workshop
Sept. 22-24, 2002, Arlington, VA



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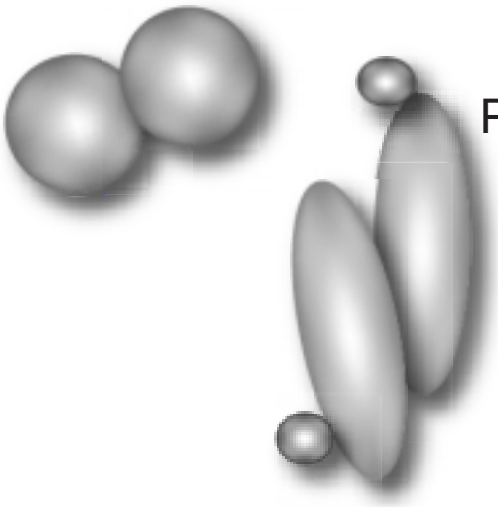

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Acknowledgements

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NSF Microbial Observatory/Life in Extreme Environments Principal Investigators' Workshop
Sept. 22-24, 2002, Arlington, VA

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Executive Summary

Introduction

The National Science Foundation (NSF) Microbial Observatory (MO) and Life in Extreme Environments (LExEn) programs have fostered significant advances in microbial ecosystems research in a wide variety of natural environments. The investigators funded by these programs have extended the frontiers of microbial diversity and microbial biogeochemistry research, discovering novel microbial lineages, describing the complexity of natural microbial communities, and linking microbial taxa to critical ecosystem functions. This workshop, the first of its kind, provided a platform for MO and LExEn researchers to discuss recent accomplishments and future directions in microbial ecosystem research.

Developing Molecular Technologies

LExEn and MO projects have greatly benefited from the use of new molecular technologies to identify organisms and their activities in natural environments. Molecular methods have provided microbial ecologists with invaluable information about the enormous reservoir of uncultured microbes; 16S rRNA work alone has identified more than 13,000 new prokaryotes. These technologies have also allowed access to functional genes, providing a mechanism to assess both capability for and expression of ecologically important microbial processes in natural environments. The challenges now facing microbial-life researchers are to move beyond the limitations of 16S rRNA-based approaches and characterized functions to access the unknown organisms, functions, and activities of uncultured members of microbial communities.

Metagenomics

Genomic analysis, now used primarily to observe the structures of genomes of individual organisms, can also be used to study the genetic reservoir of entire communities, i.e., the metagenome (Vergin et al., 1998; Rondon et al., 2000; Béjà et al., 2000b). With this approach, genetic material from a natural community can be analyzed without first culturing all the organisms. Several MO projects are making use of this new technology to describe and discover microbes and microbial products from environments as diverse as soils, the ocean, and the guts of insects. Before metagenomics can take a leading position in microbial-life research, however, technological capabilities must be improved for obtaining sufficient DNA from natural environments; for cloning, sequencing, and storing metagenomic DNA; and for capturing the dynamics of microbial communities over time and space through gene sequencing approaches.

Environmental Microarrays

Use of microarray technology represents another potentially valuable, yet challenging, prospect for microbial-life research. A reasonably complete set of functional genes collected from an environment or collection of environments provides a window into the composition and activity of complex microbial communities. Sensitivity

and specificity are chief among the challenges of using environmental microarrays when working with small amounts of highly complex DNA or RNA.

Other Molecular Approaches and Issues

Proteomics and protein arrays will play important future roles in understanding microbial activities, as will faster and more affordable genetic fingerprinting methods. The expansion of molecular ecology tool kits to include eukaryotic microbes is also needed. Long-term storage of environmental samples and culture collections, including mechanisms for distribution to other researchers, is costly but necessary for enhancing the value of microbial life data.

Recommendations for Future Research

- The continued development of environmental genomics with particular attention to capturing ecological heterogeneity;
- The continued development of environmental microarray technology with particular emphasis on understanding sensitivity and complexity;
- The development of environmental proteomics;
- The expansion of molecular environmental methods to include eukaryotic organisms;
- The encouragement of bioinformatics and modeling as research tools for solving critical problems (e.g., reconstructing genomic information from mixed samples, understanding the ecological significance of genomic complexity and evolution); and
- The development of cost-effective mechanisms for environmental sample and culture collection archiving and for dissemination to microbial-life researchers.

Novel Approaches for Isolating and Culturing Microorganisms

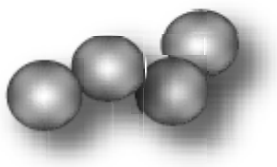
Interestingly, the recent emphasis on molecular characterization of microbial communities is leading to a renewed interest in cultivating representatives of microbes known only through nucleic acid-based studies. It is generally argued that less than one percent of all microorganisms is known and culturable. But are any microorganisms truly "unculturable," or have our attempts simply failed to provide the environmental conditions essential for growth? The challenges now lie in overcoming the limitations of traditional culturing techniques—those of selectivity—and improving culturing techniques to isolate novel organisms known only from 16S rRNA sequences.

Recommendations for Future Research

- The continued development of culture-based technologies that take advantage of recent advances in materials, microfluidics, and other micro- and nanotechnologies;
- Development of improved microsensor techniques to identify and quantitate important organic and inorganic metabolites in situ, as well as to follow reactant sources and products in real time; and
- Building of new data schema for rapidly identifying novel microbes that can be coupled with advances in molecular genotyping and phenotyping methods.

Environmental Sequence Databases

Existing public sequence databases are insufficient for ecological purposes. The development of sequence databases with an environmental slant will build knowledge of where, when, and under what conditions microbial sequences were retrieved. Such databases can provide mechanisms for data exchange within the research community, enhance the value of sequences obtained in single laboratories, and provide a data



catalog that can be mined at different times and with different questions by microbial diversity and microbial biogeography researchers.

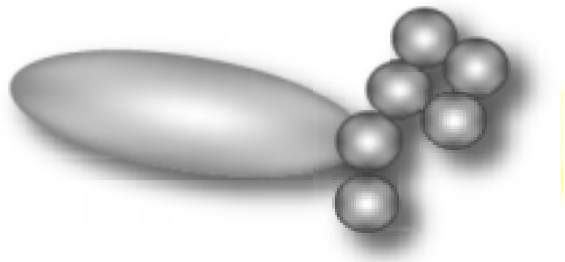
Recommendations for Future Research

- Determination of the feasibility and desirability of building a centralized, ecological sequence database that will serve as a community-wide resource; and
- Consideration of the relationship of an environmental database to existing gene archives, including the Ribosomal Database Project (RDP; rdp.cme.msu.edu/html) and NCBI/GenBank (www.ncbi.nlm.nih.gov).

Recommendations for Microbial Life Research Funding at NSF

Workshop participants judged the MO program to be overwhelmingly successful in addressing a critical research need in site-based microbial discovery and activity. Yet, despite the success of this and other NSF environmental microbiology funding opportunities, significant funding gaps were identified. Critical areas that currently fall outside existing programs and special competitions include:

- Microbial discovery that is not site-based;
- Microbe-microbe interactions;
- Microbial community interactions (physiological, biochemical, genetic);
- Natural patterns of microbial distribution;
- Environmental proteomics and functional genomics;
- Exploring extreme environments for biochemical and phylogenetic diversity;
- Specific programs to support eukaryotic microbial studies and soil microbial studies at a high level;
- Bioremediation; and
- Discovering natural products from microorganisms.



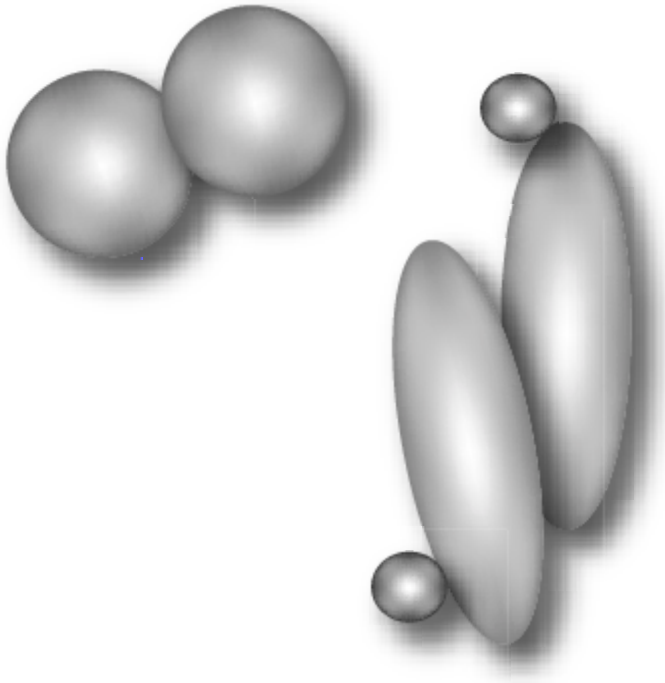
Final Recommendations

1. The MO Program has played a major role in advancing research on microbial life and should be continued in its current form, perhaps broadening its scope to include extreme environments and cover habitat types, rather than single sites.
2. Nonetheless, significant and critical gaps exist in funding research on microbial life that can only be filled with an increased investment by NSF.
3. Consideration should be given to establishing long-term, renewable MO projects, perhaps analogous to the Long-Term Ecological Research (LTER) projects that focus on diversity and function, and are not necessarily restricted to a single locale.
4. Consideration should be given to establishing a core funding program for ecological microbiology.
5. Special short-term programs are a particularly valuable approach for funding research on microbial life, since they allow flexibility in responding to a rapidly changing field.
6. Continued support of multidisciplinary research that welcomes collaborations between microbiologists, geochemists, and molecular biologists should be encouraged.
7. New mechanisms for funding needs unique to research on microbial life should be considered. These needs include: maintenance of culture collections, sample archiving, establishment of environmental sequence databases, updating instrumentation, and increasing accessibility of molecular and in situ technologies to environmental microbiologists.

Conclusion

Despite the great successes of the LExEn and MO programs, there still is a need for expanded funding for research on microbial life: from identifying organisms in all environments (soil, ocean, air, and extreme environments), to determining the role of the organisms in the ecosystem, to sequencing the organisms' genetic material for phylogenetic and evolutionary studies.

The meeting of LExEn and MO grantees lauded the programs' successes, but also pointed out some areas that remain unfunded and others that require additional funding. The grantees listed areas of critical concern, and requested that programs developed to address them be continued and expanded. NSF is one of the few sources of research funds for microbial biology. For such research to continue and expand, additional NSF programs remain essential.



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Mary Ann Moran and Sherry L. Cady, editors

MICROBIAL RESEARCH: PROGRESS AND POTENTIAL

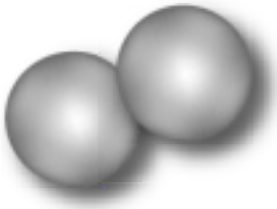
Introduction

The National Science Foundation (NSF) Microbial Observatory (MO) and Life in Extreme Environments (LExEn) programs have fostered significant advances in microbial ecosystems research in a wide variety of natural environments. The investigators funded by these programs have extended the frontiers of microbial diversity and microbial biogeochemistry research, discovering novel microbial lineages, describing the complexity of natural microbial communities, and linking microbial taxa to critical ecosystem functions. This workshop, the first of its kind, provided a platform for MO and LExEn researchers to discuss recent accomplishments and future directions in microbial ecosystem research. Principal investigators included molecular and ecological microbiologists, geomicrobiologists and geochemists, biochemists, chemical engineers, and computer modelers. This report highlights a number of insightful contributions generated by MO and LExEn projects, and underscores areas for which future funding is needed and can have a significant impact.

An important theme for the MO program—one that emerged from the LExEn program—is the need for comprehensive multidisciplinary characterization of microbial systems. The LExEn program, funded for five years (1996-2001), focused on microorganisms in extreme environments. Since many of these ecosystems were only recently discovered, comprehensive characterization of the microbiology, geochemistry, and physical constraints of these ecosystems were research priorities. This work provides an environmental context for advanced studies of these systems, such as those of the ongoing MO program that focus on the discovery and characterization of undescribed microorganisms.

Development of new technologies and instruments for studying microbial ecosystems—another theme initiated through the LExEN program—represents a timely and significant aspect of future microbial research. A combination of technological advances applied to ecosystem characterization studies promises to accelerate our understanding of the interactions between organisms and the physical and chemical constraints of their environment.

The inherent cross-disciplinary nature of MO and LExEn projects produced a new generation of cross-disciplinary scientists, an added value of microbial ecosystem studies fostered by investigators from a wide range of disciplines. Many of these scientists, including postdoctoral associates and students of the principal investigators, along with postdoctoral associates funded through the NSF Microbial Biology Postdoctoral program, participated in the workshop.

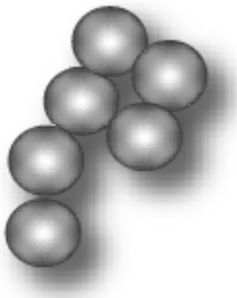


"The investigators funded by these programs have extended the frontiers of microbial diversity and microbial biogeochemistry research".

Developing Molecular Technologies

LExEn and MO projects have greatly benefited from the use of new molecular technologies to identify organisms and their activities in natural environments. Molecular methods have provided microbial ecologists with invaluable information on the enormous reservoir of microbes that have not been cultured; 16S rRNA work alone has identified the presence of more than 13,000 new prokaryotes. These technologies have also allowed access to functional genes, providing a mechanism to assess both capability for and expression of ecologically important microbial processes in natural environments.

However, these successes have only scratched the surface of the diversity and activity of the microbial world. Now the challenges are to: 1) move beyond the limitations of 16S rRNA-based approaches to link members of microbial communities with function and 2) move beyond the limitations of characterized functions into unknown and unexpected microbial activities. These approaches can provide insights into known microbial processes, glimpses of unsuspected microbial processes, and access to novel microbial products, such as naturally produced antibiotics, immunosuppressants, antitumor agents, and other potential pharmaceuticals manufactured within microbes.



Metagenomics

Genomic analysis, now used primarily to observe the structures of genomes of individual organisms, can also be used to study the genetic reservoir of entire communities, i.e., the metagenome (Vergin et al., 1998; Rondon et al., 2000; Béjà et al., 2000b). Several metagenomics studies have recovered large fragments of DNA from an environment, cloned them into vectors, and sequenced them to produce either end-sequence fragments or completely sequenced regions (Béjà et al., 2000a; Gillespie et al., 2002; Brady, Chao, and Clardy, 2002). Thus, genetic material from a natural community can be analyzed without first culturing the organisms. Although technical challenges and funding limitations are important concerns, environmental metagenomics has the potential to provide new biological insights, recover and detect novel functional genes in the environment, and determine physiological diversity of environmental samples. This methodology is currently being pioneered in several MO projects, and has enormous potential for future microbial life research in all types of environments.

"16S rRNA work alone has identified the presence of more than 13,000 new prokaryotes."

Before metagenomics can take a leading position in microbial-life research, there are many challenges that must be met. For example, access to microorganisms in extreme or inaccessible environments can be difficult, and obtaining sufficient DNA to build genomic libraries (e.g., from insect guts or the human mouth) will be a true challenge. One multicellular organism can be host to untold numbers of microbes, but can we build metagenomic libraries from a single host?

Metagenomics at Microbial Observatories

Jo Handelsman and colleagues at the University of Wisconsin discovered hemolytic clones in a screen of a soil metagenomics library. Analysis of pigmented compounds produced by one of the clones indicated that the products inhibited bacterial growth, and led to the discovery of the novel antibiotics turbomycin A and turbomycin B (Gillespie et al., 2002). This same metagenomic approach is being used by Handelsman at the Alaskan Soil Microbial Observatory (MCB-0132085) to identify new microbial functions for coping with phosphorous limitation. Metagenomic libraries of DNA from uncultured soil microbes are being screened for genes involved in bacterial utilization of reduced forms of phosphorus and solubilization of mineral P in the forest soil.

A gene that encodes a novel bacterial rhodopsin was discovered by Oded Béjà, Ed DeLong, and colleagues in a metagenomic library of DNA from the Monterey Bay Coastal Ocean MO (MCB-0084211) (Béjà et al., 2000a). The presence of this gene, previously known only from extreme halophytic Archaea, indicates that marine Bacteria have a previously unsuspected capability for a light-driven mode of energy acquisition. Metagenomic analysis of this uncultured marine microbial community has changed scientists' views of carbon cycling in the surface ocean.

Even the midgut of lepidopteran larvae is fertile ground for metagenomics. Robert M. Goodman of theUniversity of Wisconsin and Anna-Louise Reysenbach of Portland State University are using metagenomics to examine phylogenetic and functional diversity of microbes in the gut of tropical caterpillars (MCB-0084222 and MCB-0084224). The goal of this project, which is being carried out in the Area de Conservacion Guanacaste in northwestern Costa Rica, is to better understand the role of internal microbes in caterpillar ecology and determine whether the diversity of the gut microbiota parallels that of animal and plant diversity in this endangered ecosystem.

Understanding the heterogeneity in microbial communities over time and space, the hallmark of most natural environments, must also be captured in future metagenomic approaches. For example, in the marine environment, changes in microbial community structure, functional gene reservoirs, and activities can be extremely rapid, sometimes occurring over the course of minutes rather than days, months, or years. Developing high-throughput metagenomic analysis that can adequately represent the dynamics of natural microbial communities is a significant challenge. The difficulty of obtaining high-quality DNA from certain microbes or microbial habitats is another potential hurdle. Some metagenomics studies require large (ideally, ~100 kb) fragments for library construction; yet Bacteria and Archaea with hard-to-break cell walls necessitate extraction procedures that yield fragmented DNA. Many environments, particularly soils and sediments, have contaminants that are co-extracted with DNA and, thus, limit its quality and susceptibility to cloning.

Once isolated, how will the DNA be cloned and maintained for metagenomic analysis? Present methods use *E. coli* as the primary vector for cloned environmental DNA; but there is concern that sequences harboring genes that produce proteins toxic to *E. coli* will never be retrieved (Béjà et al., 2000b). Alternate vectors, new extraction methods, and smaller sample requirements will all be important technical challenges as

environmental metagenomics develops as a tool for microbial-life research. Finally, how and for how long will metagenomic libraries be maintained?

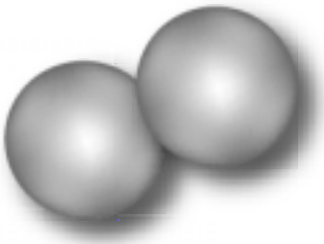
"Alternate vectors, new extraction methods, and smaller sample requirements will all be important technical challenges as environmental metagenomics develops as a tool for microbial-life research."

Environmental Microarrays

Environmental microarrays are genes or gene fragments of ecological interest that are arrayed on microscopic grids. The arrayed genes may be of taxonomic (Cho and Tiedje, 2001; Loy et al., 2002) or functional (Wu et al., 2001; Cho and Tiedje, 2002) interest, and may be used to assess presence (Koizumi et al., 2002) or expression of microbial genes in nature. DNA or RNA extracted from environments is hybridized with the gene array, and the strength of hybridization is measured as an indication of the abundance and/or expression of each gene.

Use of microarray technology represents another potentially valuable, yet challenging, prospect for microbial life research. Chief among the challenges of adapting microarray approaches to environmental applications is sensitivity. Current detection sensitivity is between 10-100 pg of nucleic acid, which means that large DNA samples are required. For example, given current methods, ~4 x 10^6 copies of a 500-base molecule would be required for successful detection (Murray, pers. comm.). In some environmental samples, these amounts of DNA or RNA can be nearly impossible to obtain. The complexity of the community and the size of the genomes within the community also have a direct impact on the sensitivity of microarray methods: How many different genomes are present? How many genes are in each genome? What is the frequency of each genome within the environment? Do genome types exhibit dominance or evenness within the environment? Is gene expression high or low? All of these factors affect the number of any single gene or gene transcript within an environmental sample, and, therefore, the sensitivity of microarray analysis.

Another important issue in developing environmental microarrays is the specificity required to identify a specific gene sequence. Is "noise" problematic because it represents imperfect matches to the target sequence, or is "cross hybridization" desirable because it identifies sequences related to the target sequence? Microarrays can be constructed with oligonucleotides, gene fragments, whole genes, or multiple genes, and each provides differences in specificity. One particular advantage of microarray technology for environmental research is that lower specificity may allow study of functional sequences that are similar, but not necessarily identical, to those previously characterized in cultured organisms.



A reasonably complete set of functional genes collected from an environment or collection of environments can serve as a useful database for microbial biologists and biogeochemists. Functional gene arrays developed from this database would provide information on the expression of key genes of microbially mediated processes (Wu et al., 2001). Biogeochemical gene arrays can give shorthand information on the processes occurring over time and space in a given habitat (Taroncher-Oldenburg et al., 2003).

Other Molecular Approaches and Issues

Other molecular approaches hold promise for revealing the workings of microbes in natural environments. For example, proteomics is likely to play an important future role in understanding microbial activities. Protein arrays, which are similar to gene arrays but target the proteome, are currently under development, although they are more technically challenging than DNA microarrays. Environmental protein arrays, developed for use with mixed communities in natural environments, will be even more challenging. Two-dimensional (2-D) gel electrophoresis techniques are well known and often used, but are limited in that they often detect only the most abundant proteins. In natural environments, less abundant proteins that play key roles in regulating microbial activities may be missed. Nonetheless, environmental proteomics holds great promise for bridging the gap between genes and function, and allowing the study of changes in microbial function in response to environmental changes.

Faster, more affordable genetic fingerprinting methods for microbial communities may also play a key role in future research. Unless or until metagenomics and related sequencing methods become more accessible, better tools are needed for examining natural communities from large numbers of samples over broad spatial and temporal scales. The heterogeneity inherent in most ecological communities and most environmental processes requires methodological tools that can capture the complexity of diverse systems that are changing through time.

Eukaryotic microbes need to be fully integrated into molecular biology methods. Although researchers recognize the tremendous diversity—both discovered and as-yet undiscovered—of Archaea and Bacteria, there may still be a perception in the scientific community that eukaryotic microbial diversity is not high (Finlay, 2002). Currently, some molecular techniques used for prokaryotes are difficult to employ in studying eukaryotes. For example, the large size of many dinoflagellate genomes makes environmental genomic approaches considerably more difficult. Nonetheless, understanding the relative roles of prokaryotic and eukaryotic microbes and their ecological interactions is a key challenge for future microbial life research.

Sampling strategies and design are critical issues that all microbial life researchers need to address. For example, it is important to maintain sample integrity to preserve relevant gene expression patterns,

Working in the Antarctic

In the Antarctic, extremes in solar irradiance are mirrored in the adaptations of the biological community. LExEn researcher Alison Murray from the Desert Research Institute is interested in ecophysiological and strategic processes that facilitate microbial survival in this extreme environment, including metabolic plasticity, temperature compensation, and specialized macromolecules that determine cold response (OPP-0085435). A DNA microarray is being constructed to identify genes that are expressed in the sub-zero temperatures of Antarctic marine waters and to understand how these genes are regulated. The arrays will provide information on organisms that remain to be cultivated, including groups such as the marine Crenarchaeota. Microarray technologies developed for the Antarctic should have widespread application in other natural microbial habitats.

protein profiles, and spatial organization of community members. Advances in technology may also permit researchers to consider developing techniques that assess "transient products" produced during distinct time frames or in times of rapid changes.

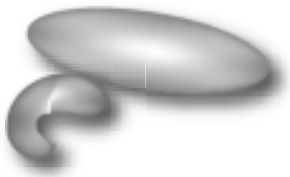
Long-term storage of environmental samples and culture collections, including mechanisms for distribution to other researchers, is a frequent requirement of microbial research. A non-trivial impediment to this requirement is the cost of sample storage and sample dissemination to the scientific community. Special funding needs include monies for maintenance of collections within individual laboratories that are establishing archives of novel organisms or novel environmental samples. Established collections, such as the American Type Culture Collection, archive characterized strains as frozen stocks, but have no capacity to store environmental samples. These samples will only survive if individual investigators can make long-term commitments to their maintenance despite a typically short-term funding environment.

"Environmental proteomics holds great promise for bridging the gap between genes and function, and allowing the study of changes in microbial function in response to environmental changes."

Recommendations for Future Research

To help understand microbial diversity and function, the following future research directions should be considered as priorities.

- The continued development of environmental genomics with particular attention to capturing ecological heterogeneity;
- The continued development of environmental microarray technology with particular emphasis on understanding sensitivity and complexity;
- The development of environmental proteomics;
- The expansion of molecular environmental methods to include eukaryotic organisms;
- The encouragement of bioinformatics and modeling as research tools for solving critical problems (e.g., reconstructing genomic information from mixed samples and understanding the ecological significance of genomic complexity and evolution); and
- The development of cost-effective mechanisms for environmental sample and culture collection archiving and for dissemination to microbial-life researchers.

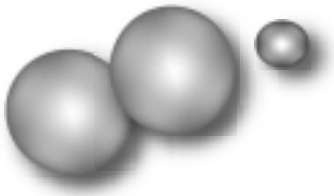


Novel Approaches for Culturing and Isolating Microorganisms

LExEn and MO projects have shown that microbial life can be found in every terrestrial environment where there are sources of carbon, water, and energy. The application of molecular techniques over the past several decades has revealed the enormous biodiversity of microbes in these previously unexplored ecosystems. Interestingly, this emphasis on molecular characterization of microbial communities is leading us back to a renewed interest in cultivating representatives of the diverse microbes that are revealed through nucleic acid-based studies, but have proven refractory to culture in the laboratory using traditional enrichment techniques. The availability of pure cultures of strains that are known largely by their presence in clone libraries can aid in "ground truthing" DNA microarray libraries and bacterial artificial chromosome (BAC) libraries of genes isolated directly from the environment. Of equal importance, pure cultures still remain the best and most cost-

effective means for understanding an organism's physiology, and for natural product discovery.

It is generally argued that less than one percent of all microorganisms are known and culturable. But are any microorganisms truly "unculturable," or have our attempts simply failed to provide the environmental conditions essential for growth? Traditional culturing approaches have largely relied on growth media that attempt to mimic the bulk chemistry of the natural environment. For example, saline media at pH 7 and containing abundant yeast extract are commonly used in attempts to culture neutrophilic, marine heterotrophs. However, workshop participants recognized the value and challenges of developing new culture techniques that incorporate an understanding of the localized physical structure and chemical environment of an organism's habitat (e.g., speciation of dissolved organic matter, minor and trace element compositions, and mineral surfaces).



Furthermore, many environmentally important microbes may grow slowly and prefer nutrient-limited conditions. This is especially true of microbes from the open ocean or the deep subsurface. In traditional media, the amount of energy available from targeted metabolic reactions is at least several orders of magnitude higher than in most natural ecosystems; but the adage, "If one is good, two are better," may not necessarily apply to culturing poorly understood or completely unknown microorganisms. Attempts to provide an energy environment that connects more closely to the natural environment may bring to the forefront the abundant, but often slow-growing, representatives of these groups. Finally, most culturing is done on liquid or gelatinous media in batch or, less commonly, in stirred vessels. Porous and permeable solid substrates (to mimic soils, sediments, and particle aggregates) combined with flowing or percolating aqueous solutions (to mimic fluid transport) are rarely included in attempts to culture novel microbes. New culturing protocols emerging from molecular biology have gained favor in the environmental microbiology community, but often at the expense of those based on analytical, experimental, and theoretical geochemistry.

"Are any microorganisms truly 'unculturable,' or have our attempts simply failed to provide the environmental conditions essential for growth?"

Workshop participants also noted the growing realization that an individual microbe is often dependent upon its associations with other microbes. Recognition of these associations may be crucial in our ability to culture either individuals or simple consortia in the laboratory. In future, emphasizing purified consortia may be as important as the historical microbiological emphasis on pure cultures.

Recent advances in the culture of novel microbes include high-throughput cultivation and screening techniques for isolating oligotrophic, open-ocean microbes (Connon and Giovannoni, 2002), and a unique micro-drop gel technique (Zengler et al., 2002) for isolation of taxonomically diverse bacteria from soil and aquatic environments. Other studies have shown the efficacy of using very dilute media for cultivating soil microbes of the genera *Acidobacteria* and *Verrucomicrobia* (Janssen et al., 2002), as well as including signaling factors, such as acyl homoserine lactones in media for culturing marine or-

Making Culturing Better

Using fluorescent in situ hybridization (FISH), high-throughput dilution culturing techniques, and robotic approaches, PI Steven Giovannoni of the Oceanic Microbial Observatory has developed a method to screen large numbers of miniature cultures in search of elusive microorganisms (Connon and Giovannoni, 2002; MCB-9977930). The method has paid off in the isolation of members of the SAR11 bacterial clade. This clade has been shown to be abundant and widespread throughout the world's oceans, but it has long resisted traditional methods used for culturing. SAR11 is extremely small and slow growing. In contrast to many microbes that grow better when associated with surfaces, SAR11 appears to grow best in suspension.

New techniques that enhance the isolation of microbes in extreme thermophilic environments grew out of the combination of theoretical geochemical constraints and traditional culturing approaches. LExEn researcher Jan Amend has been characterizing the inorganic and organic fluid geochemistry of extreme ecosystems, such as Yellowstone hot springs and hydrothermal systems of the Aeolian Islands, Italy. By coupling his measurements with calculations of the energetics of biochemical pathways, Amend has successfully designed specialized media to culture organisms that have previously been considered unculturable.

ganisms (Bruns, Cypionka, and Overmann, 2002). The challenges now lie in overcoming the limitations of traditional culturing techniques-those of selectivity-and improving culturing techniques to isolate novel organisms known only from 16S rRNA sequences.

"The challenges now lie in overcoming the limitations of traditional culturing techniques."

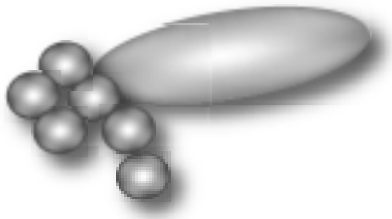
Recommendations for Future Research

To further efforts to culture more microorganisms, leading to increased understanding of the genetic potential and biogeochemical function of microbial life, the following should be considered as priorities:

- The continued development of culture-based technologies that take advantage of recent advances in substratum types, microfluidics, and other micro- and nanotechnologies;
- Development of improved microsensor techniques to identify and quantify important organic and inorganic metabolites in situ, as well as follow reactant sources and products in real time; and
- Building of new data schema for rapidly identifying novel microbes that can be coupled with advances in molecular genotyping and phenotyping methods.

Environmental Sequence Databases

Existing public sequence databases are insufficient for ecological purposes. Designed for scientific fields in which single organisms are studied in laboratory situations, current databases archive sequences of genes and proteins primarily as a collection of nucleotides and amino acids. For microbial ecologists, these sequences have much less value, both now and in the future, than do sequences archived along with information on environmental context, e.g.,



where the sample was collected, the conditions at the time of sampling, and the other sequences collected at the same time. Furthermore, sequences obtained from Polymerase Chain Reaction (PCR) amplification or genomic analysis of environmental samples can be difficult to shoehorn into existing database structures. For example, end sequences of bacterial artificial chromosome (BAC) libraries produced during genomic analyses of environmental samples (e.g., Béjà et al., 2000a; Gillespie et al., 2002) may contain only partial gene sequences lacking phylogenetic context.

Sequence databases with an environmental slant build knowledge of where, when, and under what conditions microbial sequences were retrieved. Such databases provide a mechanism for data exchange within the research community, enhance the value of sequences obtained in single laboratories, and provide a data catalog to be mined (at different times and with different questions) by microbial diversity and microbial biogeography researchers. Ecological sequence databases could, conceivably, contain more information than any single research group will ever be able to analyze. Efforts facilitating the development of such databases, available in a format that is usable by the entire research community, are highly desirable (Sheldon, Moran, and Hollibaugh, 2002).

Important issues concerning environmental microbial databases revolve around whether the database(s) should be centralized and permanent, or whether they should be independent and potentially ephemeral at different project sites. The former allows for maximum accessibility and uniformity of structure, but is expensive to build and maintain. The latter is relatively inexpensive and tailored to individual projects, but may not be widely accessible to the community, permanent, nor available in a format useful for comparative analyses. Furthermore, database design and construction efforts may be duplicated among many different projects. The “distributed database” model is the current template for MO researchers, with several projects producing their own data management systems that meld se-

Sequence Databases at Microbial Observatories

How will new information on the identity and distribution of genes in microbial communities be organized and disseminated? One way is through the Comprehensive Environmental BAC Resource being assembled by the Monterey Bay MO (MCB-0084211). John Heidelberg and colleagues are creating an environmental genomics sequence database in which microbial genes plucked directly from natural environments are stored and annotated using genomic tools, and are available for searching and retrieval through a web interface. Researchers can search for the wild version of their favorite gene at www.tigr.org/tdb/MBMO/BAC-ends.shtml.

The sequence database of the Sapelo Island MO (MCB-0084164) has a somewhat different focus: to establish a framework for linking individual microbes with their environmental contexts. A 16S rRNA sequence database of coastal bacteria, complete with information on when, where, and under what ecological conditions each sequence was retrieved, is available at simo.marsci.uga.edu.

quence data and environmental information. However, these individual databases are not standardized, and cross correlations are often cumbersome and time consuming.

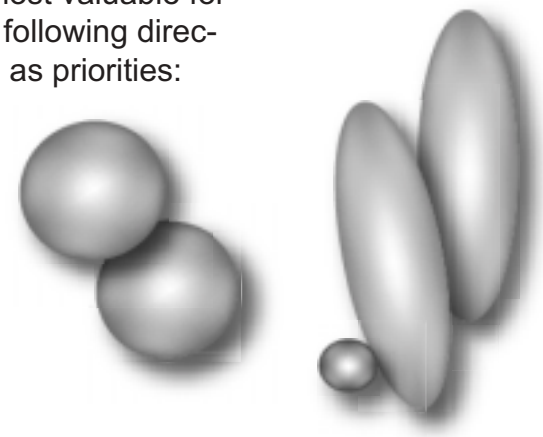
"Sequence databases with an environmental slant build knowledge of where, when, and under what conditions microbial sequences were retrieved."

Directed database building, in which projects systematically fill in gaps of important and useful functional genes, may also be desirable. For example, a collection of functional genes that catalyze key steps in biogeochemically important processes might be retrieved from a range of habitats. This functional gene database could include sequences of the genes encoding enzymes for CO_2 fixation, N_2 fixation, ammonium oxidization, denitrification, sulfate and iron reduction, and methanogenesis, among other reactions. Although development of such a database will be an iterative process, as current knowledge of microbial activities is incomplete, it could be used to develop microarray-based methods for detecting geochemically important activities in the environment. Similarly, underrepresented phylotypes might be targeted for increasing representation in environmental databases to more fully sample microbial diversity.

Recommendations for Future Research

To archive environmental microbial sequence data in a manner most valuable for future research efforts, the following directions should be considered as priorities:

- Determination of the feasibility and desirability of building a centralized, ecological sequence database that will serve as a community-wide resource, and
- Consideration of the relationship of an environmental database to existing gene archives, including the Ribosomal Database Project (RDP; rdp.cme.msu.edu/html) and NCBI/GenBank (www.ncbi.nlm.nih.gov).



Recommendations for Microbial-Life Research Funding at NSF

NSF maintains an intense interest in microbial sciences, with more than 50 programs supporting microbiological research in some way. These programs range from large programs in integrative biology and long-term grants for training taxonomists, to programs for the improvement of microbial collections, understanding geomicrobial processes, and supporting digital libraries of microbes. But how well will these programs support the critical elements of microbial life research in the coming decade? Are there better funding approaches or models that should be considered?

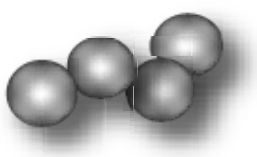
Currently, the LExEn program has run its five-year course. The MO program is an ongoing special competition, and could possibly be continued to support research on novel and poorly understood microbes, habitats, and environments in future years. Questions posed to workshop participants for discussion included: Does the MO program meet an important research need of the community? Should core funding for microbial research be established at NSF, analogous to programs previously established for plant and animal research? Are short-term, targeted research programs a better, more flexible approach for funding the rapidly evolving fields of microbial biogeochemistry and microbial ecology? Do significant funding

gaps exist despite the current NSF programs that support microbial life research?

Workshop participants judged the MO program to be overwhelmingly successful in addressing a critical research need in site-based microbial discovery and activity. Yet, despite the success of this and other NSF environmental microbiology funding opportunities, significant funding gaps were identified. Funding levels for microbial-life research at NSF have been increasing gradually, yet still are meager compared to what is needed to fulfill the promise that this field now holds for groundbreaking discoveries for science and society. These include understanding the history and distribution of life on Earth, realizing life's physical and chemical limits, simplifying understanding of the intricate workings of natural ecosystems, and discovering new antibiotics and enzymes for medicine and industry. Recent breakthroughs in analyzing and culturing microorganisms have poised microbial-life research at the beginning of its most important and exciting era.

Critical areas that currently fall outside existing programs and special competitions include:

- Microbial discovery that is not site-based;
- Microbe-microbe interactions;
- Microbial community interactions (physiological, biochemical, genetic);
- Natural patterns of microbial distribution;
- Environmental proteomics and functional genomics;
- Exploring extreme environments for biochemical and phylogenetic diversity;
- Specific programs to support eukaryotic microbial studies and soil microbial studies at a high level;
- Bioremediation; and
- Discovering natural products from microorganisms.



Recommendations:

1. The MO program has played a major role in advancing research on microbial life and should be continued in its current form, perhaps broadening its scope to include extreme environments and to cover habitat types, rather than single sites.
2. Nonetheless, significant and critical gaps exist in funding research on microbial life that can only be filled with an increased investment by NSF.
3. Consideration should be given to establishing long-term, renewable MO projects, perhaps analogous to the Long-Term Ecological Research (LTER) projects that focus on diversity and function, and are not necessarily restricted to a single locale.
4. Consideration should be given to establishing a core funding program for ecological microbiology.
5. Special short-term programs are a particularly valuable approach for funding research on microbial life, since they allow flexibility in responding to a rapidly changing field.
6. Continued support of multidisciplinary research that welcomes collaborations between microbiologists, geochemists, and molecular biologists should be encouraged.
7. New mechanisms for funding needs unique to research on microbial life should be considered. These needs include: maintenance of culture collections, sample archiving, establishment of environmental sequence databases, updating instrumentation, and increasing accessibility of molecular and in situ technologies to environmental microbiologists.

"Workshop participants judged the MO program to be overwhelmingly successful in addressing a critical research need in site-based microbial discovery and activity."

Conclusion

Despite the great successes of the LExEn and MO programs, there still is a need for expanded funding for research on microbial life: from identifying organisms in all environments (soil, ocean, air, and the myriad of extreme environments), to determining the role of the organisms in the ecosystem, to sequencing the organisms' genetic material for phylogenetic and evolutionary studies.

The meeting of LExEn and MO grantees lauded the programs' successes, but also pointed out some areas that remain unfunded and others that require additional funding. The grantees listed areas of critical concern, and also requested that programs such as these be continued and expanded. NSF is one of the few sources of research funds for microbial biology, thus, for such research to continue and expand, additional NSF programs remain essential.

References

Béjà, O. et al. Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. Science 289:1902-1906, 2000a.

Béjà, O. et al. Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. Environmental Microbiology 2:516-529, 2000b.

Brady, S.F., C.J. Chao, and J. Clardy. New natural product families from an environmental DNA (eDNA) gene cluster. J. Am. Chem. Soc. 124:9968-9969, 2002.

Bruns, A., H. Cypionka, and J. Overmann. Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central Baltic Sea. Appl. Environ. Microbiol. 68:3978-3987, 2002.

Cho, J.-C. and J.M. Tiedje. Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. Appl. Environ. Microbiol. 67:3677-3682, 2001.

Cho, J.-C. and J.M. Tiedje. Quantitative detection of microbial genes by using DNA microarrays. Appl. Environ. Microbiol. 68:1425-1430, 2002.

Connon, S.A. and S.J. Giovannoni. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl. Environ. Microbiol. 68:3878-3885, 2002.

Finlay, B.J. Global dispersal of free-living microbial eukaryote species. Science 296:1061-1063, 2002.

Gillespie, D.E. et al.. Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. Appl. Environ. Microbiol. 68:4301-4306, 2002.

Janssen, P.H. et al.. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Appl. Environ. Microbiol. 68:2391-2396, 2002.

Koizumi, Y. et al.. Parallel characterization of anaerobic toluene- and ethylbenzene-degrading microbial consortia by PCR-Denaturing Gradient Gel Electrophoresis, RNA-DNA membrane hybridization, and DNA microarray technology. Appl. Environ. Microbiol. 68:3215-3225, 2002.

Loy, A. et al.. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. 68:5064-5081, 2002.

Rondon, M.R. et al.. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl. Environ. Microbiol. 66:2541-2547, 2000.

Sheldon, W.M., M.A. Moran, and J.T. Hollibaugh. Efforts to link ecological metadata with bacterial gene sequences at the Sapelo Island Microbial Observatory. Proceedings of the 6th World Multiconference on Systemics, Cybernetics, and Informatics. Information Systems Development II 7:402-407, 2002.

Taroncher-Oldenburg, G., E.M. Griner, C.A. Francis, and B.B. Ward. Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. Appl. Environ. Microbiol. 69:1159-1171, 2003.

Vergin, K.L. et al. Screening of a fosmid library of marine environmental genomic DNA fragments reveals four clones related to members of the order Planctomycetales. Appl. Environ. Microbiol. 64:3075-3078, 1998.

Wu, L. et al. Development and evaluation of functional gene arrays for detection of selected genes in the environment. Appl. Environ. Microbiol. 67:5780-5790, 2001.

Zengler, K. et al. Cultivating the uncultured. Proc. Nat. Acad. Sci. (USA) 99:15681-15686, 2002.

Information on BLAST searches may be found at:
http://www.ncbi.nlm.nih.gov/BLAST/blast_overview.html

Additional Reading

Newman, D.K. and J.F. Banfield. Geomicrobiology: How molecular-scale interactions underpin biogeochemical systems. Science 296:1071-1077, 2002.

Reysenbach, A.-L. and E. Shock. Merging genomes with geochemistry in hydrothermal ecosystems. Science 296:1077-1082, 2002.