A REPORT FROM THE AMERICAN ACADEMY OF MICROBIOLOGY

THE UNCHARTED MICROBIAL WORLD:

microbes and their activities in the environment

AMERICAN SOCIETY FOR MICROBIOLOGY

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EXECUTIVE SUMMARY

Microbes are the foundation for all of life. From the air we breathe to the soil we rely on for farming to the water we drink, everything humans need to survive is intimately coupled with the activities of microbes. Major advances have been made in the understanding of disease and the use of microorganisms in the industrial production of drugs, food products and wastewater treatment. However, our understanding of many complicated microbial environments (the gut and teeth), soil fertility, and biogeochemical cycles of the elements is lagging behind due to their enormous complexity. Inadequate technology and limited resources have stymied many lines of investigation. Today, most environmental microorganisms have yet to be isolated and identified, let alone rigorously studied.

The American Academy of Microbiology convened a colloquium in Seattle, Washington, in February 2007, to deliberate the way forward in the study of microorganisms and microbial activities in the environment. Researchers in microbiology, marine science, pathobiology, evolutionary biology, medicine, engineering, and other fields discussed ways to build on and extend recent successes in microbiology. The participants made specific recommendations for targeting future research, improving methodologies and techniques, and enhancing training and collaboration in the field.

Microbiology has made a great deal of progress in the past 100 years, and the useful applications for these new discoveries are numerous. Microorganisms and microbial products are now used in industrial capacities ranging from bioremediation of toxic chemicals to probiotic therapies for humans and livestock. On the medical front, studies of microbial communities have revealed, among other things, new ways for controlling human pathogens. The immediate future for research in this field is extremely promising. In order to optimize the effectiveness of community research efforts in the future, scientists should include manageable systems with features like clear physical boundaries, limited microbial diversity, and manipulability with the goal of understanding fundamental principles that may apply to more complex systems. A great deal of microbial genetic and phenotypic diversity remains to be explored, and the commercial and medical potential locked up in these unknowns should compel the field to move forward.

Future microbiology research will build on the successes of the past using new techniques and approaches. Uncultivated microbes hold great promise for industry, medicine, and the recycling of precious resources, and research and technology must make inroads in overcoming the barriers that prevent their study. In many cases, we will no longer be able to rely on isolated, pure cultures of microorganisms, but must use communities of microorganisms, which presently are poorly understood. Indeed, community-level studies can benefit from deconstructing microbial communities and analyzing the component members separately, but this is not feasible in every system. The effects of perturbation on microbial communities also require study. Humans rely on the services of microbes in innumerable

ways, but we have little or no predictive understanding of how microbial communities respond to disturbance.

Research must address current limitations in detecting microscale interactions among microbes by enhancing current technologies and fostering new microscopic tools, biosensors, and gas sensors for appropriate small scales. Genomics, which has enabled great progress in microbiology research of individual species, must be applied to communities of microorganisms. This will require improved methods of DNA extraction and amplification from environmental samples and improved strategies for DNA sequence assembly. In the future, genome sequencing efforts should continue the exploration of evolutionarily diverse microbes, as well as help reveal the mechanisms by which closely related microbes evolve.

Technological advances have spurred every great leap in microbial biology, and in order to move forward, new methods for revealing the activities of microorganisms must be continually developed. Today, researchers need access to better techniques for enriching and isolating novel microorganisms, particularly approaches that enable them to mimic the low nutrient conditions to which many environmental microbes are adapted. Other outstanding needs include methods for performing *in situ* work and bioinformatics tools.

Finally, there are several ways that training and education in microbiology are failing to adequately prepare the next generation of scientists for the challenges ahead. Training in some of the long-established disciplines, including enrichment and isolation, physiology, enzymology, and biochemistry, needs to be revitalized.

INTRODUCTION

Humans live in the midst of a seething, breathing microbial world. Microorganisms populate every conceivable habitat, both familiar and exotic, from the surface of human skin to rainforest soils to hydrothermal vents in the ocean floor. All around us, microbes are exploiting locally available, favorable chemical reactions and living off the energy released by these transformations. In doing so, microbes are the engine behind global biogeochemical cycles that release and absorb oxygen and greenhouse gases in the atmosphere, fix nitrogen for plant growth, and recycle dead material into useful nutrients for new life. In short, microorganisms are the keystone of global health, and make life as we know it possible.

Despite their undeniable roles in the processes and conditions that sustain life, science is only beginning to understand microorganisms. Many microbes and microbial systems have been isolated and studied in the laboratory, and new techniques are constantly being devised for studying microbes without cultivation, but the vast majority of microbial life has resisted or escaped these efforts. This part of the microbial world largely remains a mystery to science. Microbes are of fundamental importance to every form of life on earth, but in spite of major recent advances we lack the technological tools and resources to explore them fully.

Ignorance of the microbial world and of the specific roles microorganisms play in the biogeochemical cycles that sustain our planet is a massive waste of the enormous potential for microorganisms to benefit humankind. Through fossil fuel burning, pollution, and resource exploitation, humans have impacted the very ecosystems that provide the essential elements for our continued existence. Researchers currently lack an understanding of the normal, baseline state of many microbial systems, and they know even less about how microorganisms will be affected by drastic environmental changes, whether microbes will amplify or dampen these effects in their habitats and what those effects signify for human beings. For example, it is not known how climate change will impact carbon cycling by microorganisms, an important factor in predicting the future concentrations of carbon dioxide in the atmosphere. Also, clearing forested land for farming increases the emission of nitrous oxide (a significant greenhouse gas) from soil and diminishes the soil's ability to absorb methane (another important greenhouse gas), but it is not known why this happens or whether it can be prevented. Microbial systems are robust but can be pushed to the limit, and science needs to find ways microbial consortia may eventually fail in their roles in maintaining ecosystem integrity. These scientific challenges must be met with a resolve befitting the real urgency of the environmental problems we face.

In addition to their roles in the global biogeochemical cycles, microorganisms perform innumerable other functions related to ecosystem health and human health. The influence of microorganisms can be felt in the structure of food webs, bioenergy production, waste management and treatment, food production, and symbiotic nitrogen fixation for plants, to name a few examples. As human popula-

tions climb, reliance on microorganisms to perform these functions and others for maintaining human and ecological health will grow too, and the stakes could not be higher.

Research must continue to explore the microbial world in order to enable accurate predictions and effective manipulations of environmental systems. This report suggests avenues to explore for accelerating the pace of discovery in microbial biology.

ESTIMATES OF THE NUMBER OF MICROORGANISMS ON THE PLANET

On a global scale, one estimate places the total number of microbial cells at 10^{30} and the number of viral particles at 10^{31} (Whitman, 1998), but these large numbers are associated with uncertainty, particularly with respect to inaccessible environments like deep sediments, which are little explored and poorly characterized as to their microbial inhabitants. Moreover, estimates of the total number of microbes on the planet are less instructive than estimates of total microbial biomass or biomass turnover, which are not available.

In individual microbial systems, it can be useful to derive estimates of the number of participating microbial cells, since fluctuations in cell density can be an indication of perturbation. However, for many health applications and most environmental systems, baseline shifts can go undetected because information about cell density in the undisturbed state is lacking.

ESTIMATING THE TAXONOMIC DIVERSITY OF MICROORGANISMS

Estimates of the total number of distinct microbial types (also called taxonomic diversity) vary widely, but, judging from the known microbial species, this diversity is extremely large. To estimate the total number of prokaryotic species on Earth requires speculation on the average volume of material that would have to be sampled to encounter a unique species. A simple calculation illustrates this:

In the example presented here, we limit ourselves to estimating the prokaryotic species present in the top 1 km of the Earth's crust. Based on the known dimensions of the Earth, this is estimated to be 5.1×10^{17} m^3.

If each 1 m^3 of soil (one million grams) contains only one unique species (by the 70% DNA-DNA hybridization measure defining a species), there are ~10^{17} prokaryotic species in the top 1 km of Earth's crust.

A more conservative estimate assumes there is only 1 unique species in each km^3 (one billion m^3 or one quadrillion, 10^{15}, grams), in which case there are "only" ~10^8 prokaryotic species on Earth.

Incredibly, this conservative estimate yields a total number of species that is only two orders of magnitude larger than some estimates of the species diversity found in a single gram of soil (Gans et. al., 2002), but still an order of magnitude more than the estimated number of insect species (30 million; Erwin, 1982), he most diverse class of macroorganisms known.

The small toy boat represents the Known Biological Diversity, while the great, complex ship represents the number of Unknown Microbial Species.

THE CURRENT LANDSCAPE

Technological advances and creative, collaborative research have enabled a great wave of discoveries in microbiology in the past 100 years, including novel organisms and communities for use by industry and agriculture, as well as treatments and preventatives for human infectious diseases. There are several characteristics to target when selecting microbial communities for future research, including clearly delineated physical characteristics, reproducibility, and replicability.

METHODOLOGY FOR DISCOVERING NOVEL MICROBES

The methods used for discovering new microorganisms can be roughly divided into two broad categories: **molecular techniques** and **cultivation- or enrichment-dependent** approaches. (In this context "molecular" connotes the use of genetic material for analysis.) There is a degree of overlap between these two approaches, and they do not capture all of the methods available to researchers in microbial biology. Also, molecular methods are often combined with cultivation-dependent approaches in a single investigation. Most microbiological research tools fit nicely into either the "molecular" or "cultivation" categories, however, and they summarize a fundamental decision that must be made when planning an investigation into microbial systems: to cultivate or not to cultivate?

Molecular techniques

There are two types of information that can be recovered by analyzing the gene content of environmental DNA: phylogenetic or functional. Phylogentically informative genes (e.g., 16S rRNA) have been used to survey diversity, whereas genes encoding specific functions (nitrogen fixation, nitrification, denitrification, etc.) have been used to evaluate potential processes. In one molecular approach that is common today, researchers extract DNA from environmental samples, copy the ribosomal RNA (rRNA) genes in the sample using the polymerase chain reaction (PCR), clone the rRNA genes in a fast-growing organism, sequence the genes, and analyze the phylogenetic relationship of those genes to each other and to genes from known organisms. In another approach, referred to as metagenomics, researchers eliminate the PCR and clone and sequence large segments of DNA directly from environmental samples. This eliminates the restrictions and biases associated with PCR.

DNA hybridization approaches, including microarray technology, are used to detect organisms for which the target genes are already known. This type of molecular approach can provide "barcodes" or "fingerprints" of individual microbes or communities of microbes. These identification techniques enable researchers to track changes in community composition and relative abundance over time or between various experimental treatments. Researchers may also use these

approaches to predict or identify which barcodes represent microbes that should be targeted for cultivation.

A drawback to applying molecular techniques is that it is not always possible to predict the physiology of a microorganism from its phylogenetic relationship to other organisms. Also, similarity at the genetic level does not always translate into similarity at the functional level, since extremely subtle variations in gene sequence can have drastic effects on the function of a gene. Most molecular methods only examine a single gene or a subset of genes and, therefore, neglect the vast majority of the genetic information that dictates function, and many novel organisms bear little genetic resemblance to known organisms in any case. Members of the bacterial genus *Dehalococcoides*, for example, catalyze anaerobic dehalogenation of several very toxic chemicals, and they constitute a novel phylum level group of organisms. Phylogenetic analysis of the organism placed it far from any reference organisms, so the physiology of *Dehalococcoides* was only understood after it was cultivated in the laboratory. However, recent methodological advances now enable microbiologists to investigate with molecular tools important aspects of the physiology of uncultured microorganisms in their natural habitat on a single cell level. For example, these approaches, which include the combination of FISH (fluorescence *in situ* hybridization) and microautoradiography, FISH and Raman spectroscopy, and FISH and secondary ion mass spectrometry, provide insights into which substrates are assimilated by uncultured microorganisms under different environmental conditions and thus also provide important guidance for subsequent cultivation efforts (Wagner et al, 2006).

The development of molecular techniques has contributed to:

- Our current perception of microbial diversity. For example, the number of recognized bacterial phyla increased from 12 in 1887 to more than 80 in 2004. Of these 80 bacterial phyla, more than 50 have no cultured representative.

- An appreciation that many uncultivated groups of bacteria are present in high numbers in some environments, e.g., the discovery that crenarchaeota represent one of the ocean's most abundant microbial cell types.

- The use of metagenomics to harvest genes from uncultured microorganisms and use them (via heterologous expression) for production of biotechnologically important products, such as antibiotics and enzymes.

- The identification of the microorganisms important for chemical processes, e.g., the discovery of microbes that catalyze nitrogen and phosphorous removal in wastewater treatment plants and which are thus essential to prevent eutrophication. These key players in the world's largest biotechnological process are mostly uncultured (*Nitrospira, Accumulibacter*), and since we can now detect them by molecular tools, the conditions in treatment plants can be optimized for their activities.

Cultivation and Enrichment

The alternative to molecular methods—cultivation and enrichment—has limitations. Researchers have estimated that less than 1% of the microorganisms observed by microscopy in soil yield to conventional cultivation efforts (Torsvik and Øvreås, 2002), and once isolated, a researcher can never be certain that an organism's behavior under controlled conditions mirrors its behavior in the environment. Furthermore, cultivation-based approaches are generally not suitable to determine microbial community structure and dynamics over time and often lead to the isolation of microbial weeds, which are well adapted to the conditions offered in the laboratory but not necessarily important in the environment under investigation. Despite these drawbacks, the value of cultivation-dependent approaches for making discoveries in microbiology is undeniable. They have formed the historical foundation of all microbiology, and today studying microbes in the laboratory is still pivotal to making many important discoveries. Even the interpretation of data acquired independently of cultivation, like metagenomics data in which the genomes of a community of organisms are pooled and studied as a unit, relies on information gathered from cultured and characterized organisms.

The possible inspirations for initiating an enrichment experiment include the need to acquire a disease-causing organism for study, biogeochemical observations that provoke inquiries into microbial activities, a need to acquire specific metabolisms for commercial purposes, or a need to acquire microbes with specific properties for use in agriculture.

Researchers usually use one or more "knowns" to tailor enrichments for an organism of interest. Using an understanding of the environmental conditions favored by an organism, together with molecular (genetic clues about the microbe's abilities) or physical information (e.g., whether the organism is more likely to thrive on surfaces or in liquid) as a guide for designing culture conditions, is a powerful approach. For example, if researchers know that a certain microbial transformation is thermodynamically possible, they can recreate those conditions in the laboratory to single out the organisms that carry out that process. Enrichment and cultivation approaches like these are increasingly being supplemented with environmental observations made using techniques that do not rely on cultivation, leading to ever more targeted isolation strategies. Sulfate-reducing bacteria, for example, were first isolated years ago, but new molecular techniques have helped identify novel, numerically-dominant types of sulfate reducers that have subsequently been targeted by enrichment experiments.

In some cases, biogeochemical observations have led to very targeted cultivation of previously unknown organisms, including iron-reducing bacteria and a defined anaerobic methane-oxidizing consortium of microbes. Some types of bacteria were predicted by thermodynamic calculations to exist, and then the respective conditions in laboratory bioreactors were established that led to their subsequent isolation.

Cultivation has allowed researchers to discern some of the fundamental principles that govern biology and enabled basic discoveries about microorganisms themselves. Discoveries using cultivation and enrichment have contributed to:

- **Discerning the unifying biological principles of life.** Studies of isolated microbes have revealed a great deal about biochemistry, genetics, bioenergetics, evolution, ecology, and population biology that can be applied to the whole of biology. Also, molecular biological reagents derived from cultivated bacteria (Taq polymerase, restriction enzymes, etc.) have enabled genetic studies of animals and plants.

- **Identifying sources and mechanisms of biogeochemical processes.** Selective enrichment of microorganisms has enabled researchers to understand the processes that make life on earth possible—the biogeochemical cycles of the elements. This is especially true of the nitrogen cycle, which tends to be driven by specialized groups of microbes that mediate each different step in the transformation of ammonia to nitrogen gas back to ammonia.

- **Defining the limits and nature of life.** Work with microbial extremophiles (which live in habitats in which most higher organisms cannot) and bacteria with small genomes has illuminated the limits of life—the chemical, physical, and genetic constraints beyond which life (as we know it) is not possible.

- **Understanding the mechanisms of disease and pathogenesis.** Thanks to work with cultured organisms, we now know that microbes and microbial communities are not only the cause of infectious disease, they are also essential for normal immune development and defense against pathogens. Other suspected links between certain diseases (possibly including heart disease, inflammatory bowel syndrome, and others) and bacteria and bacterial communities have yet to be confirmed. Enrichment and isolation will be important in these investigations.

- **Basic discoveries about microorganisms themselves.** Microbiological discovery has traditionally been driven by the identification of new capabilities in organisms that are newly cultivated. For example, the isolation of bacteria that can completely degrade environmental pollutants made untenable the widely held view that many of these substances were completely recalcitrant to biodegradation. Also, our understanding of mechanisms of antibiotic resistance derives from studies of antibiotic resistant isolates of bacteria and fungi. The novel and striking features of microorganisms that have been discovered by studying isolates can be divided among morphology, physiology, abundance, and phylogeny.

Specific discoveries that have arisen from cultivation and enrichment include, but are not limited to:

- **Proteorhodopsin-containing bacteria.** Proteorhodopsin, discovered as late as 2000 and found in numerous species of marine bacteria, probably plays a significant role in energy cycling the biosphere.

- **Methods to genetically engineer crops** come from basic studies of the Ti plasmid from the plant pathogen *Agrobacterium tumefaciens.*

- **The nanoarchaea.**

- **Autotrophic ammonia oxidizing crenarchaea.**

- **Aerobic anoxygenic phototrophs.**

- **Anaerobic methane-oxidizing denitrifiers.**

- **Probiotic organisms.**

- **Interactions in biofilms.** Communities of microbes that form biofilms have been approached through an understanding of model organisms, which allow discovery of the mechanisms of intercellular communication and cooperation.

- **Genome sequencing.** Pure cultures have enabled the first genomes to be sequenced, leading to the discovery of evolutionary mechanisms in microbes.

- **Modern day analogues of ancient metabolisms.** Researchers have been able to reconstruct ancient biogeochemical cycles and interpret Earth's history using microbes that mirror early life forms.

- **New pathogens.** The discovery of novel pathogens has led to new treatments and new thinking about the epidemiology of certain diseases, such as AIDS, *Legionella pneumophila* (the causative agent of Legionnaires' disease), and *Helicobacter*-induced stomach ulcers.

COMMERCIAL PROGRESS ENABLED BY STUDY OF MICROORGANISMS

Microorganisms have great commercial significance, and the wealth of bacteria, viruses, archaea, and microscopic eukaryotes that have yet to be cultivated and understood pose a tantalizing untapped resource for industry. There are two general approaches for adapting uncultivated microbes and microbial communities for commercial purposes: using an understanding of community function to identify useful community components, and bioprospecting.

An understanding of community functions and the chemical language of microorganisms can identify new targets for commercial applications. Quorum sensing, for example, was discovered by studies carried out at the community level. Discoveries in this area may lead to more refined methods of controlling biofilms that contribute to biofouling or antibiotic resistance.

Bioprospecting (also sometimes referred to as "biodiversity prospecting") seeks to capture useful aspects of the world's biological diversity in order to apply them in industrial endeavors, medicine, or other areas. It is most reasonable to carry out bioprospecting efforts in communities where the desired activities have been demonstrated or are probably occurring. For example, to find compounds involved in microbe-microbe interactions, research should focus on biofilm communities and other settings where multiple species of microbes live in close proximity. Bioprospecting for DNA from microbial communities in specific environments with desirable properties (e.g., high or low temperatures, or a certain level of acidity) has proven valuable for selectively plucking out genes that encode useful enzymes. Certain antimicrobials and anticancer drugs (including bryostatin) have been isolated through bioprospecting in the microbial communities in and around marine invertebrates.

Commercial applications for microbes and microbial products include:

- **Bioremediation and bioaugmentation.** Microbes have been put to use degrading organic chemicals through direct metabolism (in which the microbe uses the material for food or energy) and through co-metabolism (through which the microbe apparently gains nothing). They have also been used to carry out chemical transformations of inorganic materials in order to make those products less mobile or bioavailable in the environment. Applications include both *in situ* treatment (at the site of contamination) and treatment of waste streams in manufacturing settings.

- **Aids in mining operations.** Bacteria are used in microbial enriched oil recovery and to extract precious materials from ore.

- **Probiotics.** Numerous probiotics products (consumables containing microorganisms that are thought to offer health benefits) are available to consumers today. Probiotics are occasionally used in medical settings as well; patients are sometimes administered a collection of probiotic microorganisms to head off colonization by *Clostridium difficile* after a dose of broad-spectrum antimicrobials.

- **Manufacture of biofuel and other energy products.** Bacteria are used to digest corn and sugarcane in the manufacture of ethanol, and researchers are exploring their use in transforming chemical energy into electrical energy in microbial fuel cells.

- **Agricultural applications.** Bacteria are used to digest grasses and other fodder to make silage, a feed material that can be stored for use during winter months when pastures are not available. Also, legume seeds, such as beans and peas, are often coated with nitrogen-fixing bacteria prior to planting to ensure the plants develop the proper nitrogen-fixing communities. A gene that encodes the insecticidal delta-endotoxin of *Bacillus thuringiensis* (a bacterium commonly called Bt) has been inserted into certain crops to improve insect resistance, and the bacteria themselves are sometimes sprinkled on crops to limit infestations. Although the neurotoxins produced by *Clostridium botulinum* have been a persistent problem in the food canning industry, the botulinum toxin is used in the medical and cosmetic (Botox) industries. Finally, the bacterial compound monensin is used to increase digestion efficiency in dairy cattle.

- **Food manufacture.** Microorganisms are put to work in food manufacture in many different capacities, including fermentation processes and flavor enhancement. Microbes are also significant in terms of food spoilage and food safety. There have been enormous and frequent food recalls due to microbial contamination.

- **Industrial applications.** Heat stable enzymes isolated from thermophilic bacteria, like Taq, lipase, esterases and others, have proven extremely useful in biotechnology.

- **Wastewater treatment.** This exploits the natural capability of microorganisms to degrade and recycle the essential elements on Earth. Millions of tons of organic and inorganic waste are treated annually, and more and more of the energy contained in this waste is recovered as biogas (methane). Important advances have also been made in recycling of sulfur and heavy metals.

- **Others.** Microbes are used in the manufacture of biodegradable plastics, green chemistry applications, and bacterial ice nucleation proteins are used in snow manufacture.

PROGRESS IN UNDERSTANDING PATHOGENESIS AND DISEASE ENABLED BY STUDY OF COMMUNITIES OF MICROORGANISMS

Through studying human-associated microorganisms in sick and healthy individuals, scientists have discovered that microbial communities can define not only human disease, but also human health. Subtle perturbations in the microbial communities that exist on or in our bodies can give rise to illness or even death. Oral health, in particular, relies on microbial community interactions; fluctuations in the numbers of various microorganisms in the human mouth can bring on conditions ranging from bad breath to periodontal disease and has also been associated with systemic problems, such as arthritis and cardiovascular disease. In the gut, micro-

bial communities are now known to be necessary for the production and absorption of certain nutrients, and their development is intricately linked with the development of proper immune response and gut blood vessels. Studying microbial communities has also illuminated the mechanisms by which opportunistic human pathogens take hold. Scientists now know that vaginosis, for example, is not solely due to invasion by a foreign pathogen, but often results from an imbalance in the various members of the steady state ("normal") vaginal microbial community that allows an opportunist to grow unchecked. The use (and overuse) of antibiotics has precipitated many of these insights into human health by triggering a change or collapse in the steady state communities that exist in and on the human body.

Research into microbial communities in the environment has yielded insights and tools for managing human health and disease. Scientists are continually discovering new antibiotics, anti-inflammatories, and other bioactive compounds in the natural communities associated with marine invertebrates and other niches. Community-level studies have also revealed the dynamics of antibiotic resistance genes in human-associated and environmental communities and how antibiotic resistance genes and infections are spread. Studies of *Vibrio cholerae* in its natural surroundings have revealed that the pathogen exists in communities on the surface of comparatively large copepods. These creatures, along with their bacterial passengers, are effectively removed by filtering contaminated water through several layers of cloth, rendering the water safe to drink (Colwell et al., 2003).

Current work with human-associated microbial communities could lead to even greater advancements in medicine. Researchers have identified a tentative association between the composition of gut microbial communities and obesity. Further studies may illuminate the interactions that bring about this correlation, possibly allowing doctors to prescribe community-altering therapies for overweight patients. Other experimental work has successfully fashioned a strain of the cavity-causing bacterium *Streptococcus mutans* that does not produce acid, a deficiency that makes the strain unable to degrade teeth. Researchers are currently investigating whether inoculating the mouth with acid deficient *S. mutans* can force native, acid-producing strains to extinction and lower the incidence of dental caries.

Scientists have learned that the health of animals and plants is also linked to the state of their respective microbial communities. In cattle, rapid changes in diet from grass to grain unbalance the microbial community of the rumen, leading to poor production efficiency, rumen acidosis, and potentially death. A slow transition allows the microbiota to adapt and preserves the health of the animal. Black band disease in corals is caused by a consortium of microorganisms and may require the contributions of polymer degraders, fermentative organisms, and sulfidogens. Conditions like rumen acidosis and black band disease can only be understood by studying entire communities of organisms.

WELL-DEFINED MICROBIAL SYSTEMS AND COMMUNITIES FOR STUDY

The work of "defining" certain microbial communities has been ongoing for several decades, but progress toward this goal can be measured in different ways. There are two levels in defining a community: achieving a process level understanding (knowing what the community does) and achieving a compositional understanding (knowing which species make up the community).

The oral microbiota is somewhat defined, in terms of both its processes and its composition. The successional processes of these communities, whereby different species of microorganisms come and go in the wake of disturbance, are well characterized and reasonably well understood. Relationships between community composition in the mouth and the oral health of the patient are also well established, but gum disease does not appear to fit the paradigm established by Koch, who introduced a set of postulates meant to test the causative relationship between a microorganism and a disease. Rather than following a one-microbe-one-disease trajectory, research shows that gum disease is the result of complex multispecies community interactions (Jenkinson and Lamont, 2005). Some organisms are associated with health, while others are associated with disease.

KOCH'S POSTULATES

Three rules for experimental proof of the pathogenicity of an organism were presented in 1883 by the German bacteriologist, Robert Koch. A fourth was appended by E.F. Smith in 1905. Briefly, these rules state:

1. The suspected causal organism must be constantly associated with the disease.
2. The suspected causal organism must be isolated from an infected plant and grown in pure culture.
3. When a healthy susceptible host is inoculated with the pathogen from pure culture, symptoms of the original disease must develop.
4. The same pathogen must be re-isolated from plants infected under experimental conditions.

These rules of proof are often referred to as "Koch's Postulates."

Other microbial communities that could be labeled "well-defined," in terms of their composition and the processes they carry out, include the communities that inhabit the rumen of cattle, anaerobic sludge granules and other waste water treatment communities, deep subsurface sediment communities, the symbiotic community of medicinal leeches, and the communities in certain extreme environments, such as acid mine drainage pools at Iron Mountain in California (Tyson et al., 2004). The communities important for certain food products (including yogurt, pickles, sauerkraut, and cheese) are well-defined, but the role of microbial communities in permitting pathogens to survive or coexist with natural communities in food is not understood. Although the processes and compositions of each of these communities have been studied extensively, less is known about the effects of perturbation (including the addition of antibiotics and other micropollutants, for example) on these communities.

Obtaining a robust, precise, high-resolution community profile remains a challenge even in the simplest of communities. An understanding of the processes—and which organisms are carrying them out—is more meaningful and important than an exhaustive census of each and every organism in a community.

Phages (viruses that infect bacteria or archaea) probably play a profound role in the organization and functions of microbial communities, but their diversity and host range are seldom evaluated in community-level studies, and science knows relatively little about them.

Selecting "easy" communities for study

No single microbial community will meet all of the criteria for an "easy" system to study, but the following is a list of guidelines for targeting manageable communities for these investigations. (Not all criteria are equally important for different types of studies, and discovery-driven studies, which should not necessarily aim to meet these criteria, maintain an important place in research.) Communities targeted for complete characterization should be:

- **Clearly delineated by physical boundaries.** The boundaries of a microbial community should be easily delineated so that researchers can determine with precision which microorganisms are part of the community and which are not. The human digestive tract is one example of a clearly bounded community.

- **Reproducible.** Research data on a particular microbial community should be reproducible among different laboratories.

- **Replicable.** Microbial systems for which replicates can be obtained (including termite guts or the oral cavity) are preferable to those which cannot (including unique geological features, like Lake Michigan, for example).

- **Manipulable.** For research purposes, the ideal microbial community is capable of being altered for experimental purposes.

- **Accessible.** Microbial communities targeted for study should be physically accessible for sampling purposes and technically accessible for measuring inputs and outputs of the system.

- **Scaled appropriately.** The physiochemical that a particular community of microbes interacts with can vary in size, depending on the type of community. It is helpful if this environment is sufficiently large to be amenable to measurement and characterization.

- **Growing and active.** The activities and fluxes of active microbial communities are, in general, easier to measure than they are in less active communities.

- **The key fluxes into and out of the system should be known.** Substantive and credible background information is helpful.

- **Manageably diverse.** This is technology-dependent.

- **Stable over reasonably long time frames.**

- **Genetically tractable.**

WHISTLEBLOWERS: MICROBES AS SIGNALS OF ENVIRONMENTAL DEGRADATION

Microbes and microbial communities are intimately linked with their environments, and in many cases, ecological disturbances are reflected in changes in the abundance or behavior of these organisms before detection of other outward signs. Toxic algal blooms, for example, can result from nutrient pollution in coastal marine environments. Under the right conditions, these blooms may also signify the presence of *V. cholerae* (the bacterium that causes cholera) in coastal waters. Pathogen-related illnesses in wildlife can also signify ecological disturbance; black band disease and bleaching (by Vibrio species) in coral, for example, has been linked to increases in water temperature resulting from global climate change. Malodorous products from sulfur reducing bacteria can signify tainted water or even spoiled food products and generally serve as a good indicator of "things and places to avoid." The presence of genes for degrading human-made chemicals could potentially be used to identify areas of chemical contamination. Fecal coliform bacteria and other organisms found in the human gut are routinely used to identify sewage contamination in water. Lichens, which are extremely sensitive to atmospheric sulfur dioxide, can be used to detect elevated levels of this substance in areas of concern. The pollution-sensitive bacterium *Thioploca* gradually disappeared from Lake Erie waters as that lake became more and more eutrophied (Larkin and Strohl, 1983), serving as an indicator of adverse conditions.

UNEXPLORED ACTIVITIES ENCODED BY MICROBIAL GENES

Despite the extensive amount of work done to characterize microbes and their communities, many unknowns remain at the genetic level. Today, as much as a third of the genetic content of even the most thoroughly studied organisms remains to be annotated, so although the sequences of many genes (in many organisms) are known, the functions and significance of these genes remain unresolved.

Judging from what is already known of the functional diversity of the microbial world, the potential locked up in poorly understood genes is considerable. These "unknown" genes could reveal useful processes for industry, bring to light processes that science has not yet conceived of, fill gaps in our understanding of metabolic pathways, be responsible for disease processes not yet understood, and unearth previously unknown functions of microorganisms in the environment. Since the functions of many of the genes in well-known microbial species are not known, many of the current predictions about microbial metabolism may be incomplete, and serious limits are placed on the ability to carry out genomic and metagenomic studies. Every new gene that is characterized has multiplicative value in science's ability to identify similar, related genes in other organisms.

Functional studies of genes in the past 20 years show that, although general categories of function can often be assigned, specific functions of genes are often not predictable from their sequences, so care must be taken in extrapolating the functions of unknown genes from the gene sequences of known proteins.

RESEARCH ISSUES

The future of research in microbial biology is inspiring. Research concerns include the large fraction of microbial life that remains uncultivated, accurate construction and deconstruction of microbial communities for study in the laboratory, predicting the effects and outcomes of disturbance in microbial communities, finding the functions of unknown microbial genes, spatial scale in community interactions, co-evolution of microbial species, the role of genomics in microbiology research, and the ways in which the principles of population biology and evolution can be applied in microbiology.

UNCULTIVATED MICROORGANISMS: FUTURE BENEFITS FOR THE PLANET AND COMMERCIAL PURPOSES

Microbiologists estimate that over 90% of bacteria are not captured by the current collection of cultivation techniques, a deficit that leaves the vast majority of the microbial world largely hidden from the eyes of science (Staley and Konopka, 1985). These uncultivated microorganisms could potentially be useful for increasing the sustainability of human activities on this planet and for a number of commercial purposes.

There are great hopes for microbes to repair the damage of human activities on the health of ecosystems. Microbes may eventually be isolated for degrading and detoxifying recalcitrant wastes that cannot currently be treated, for example, or the powers of uncultivated nitrifiers in the soil could be directed to produce more fertile farmland without the use of chemical fertilizers or other nitrogen-rich amendments. Other uncultivated microorganisms may eventually be induced to impact the atmosphere and climate in some beneficial way. Oxygenic phototrophs in the oceans, for example, could be encouraged to generate more oxygen, or aerobic methane oxidizers in soils could be induced to consume a greater share of the atmospheric methane than they already bear. In all probability, science is still not aware of all the ways in which microorganisms could be useful for managing greenhouse gases.

In the future, the most significant commercial feature of uncultivated microbes may be their novel metabolites, including antimicrobials and other bioactive compounds. Genome studies of cultivated bacteria hint at the wealth of compounds waiting to be discovered. *Myxococcus xanthus* alone appears to have 50-60 pathways for the synthesis of small molecules, most of which have yet to be characterized in any way. *Streptomyces avermitilis* makes several useful compounds that we know about, but it is suspected of making 30 other small molecules that may be valuable.

As demand for novel compounds grows, it will become increasingly important to develop innovative techniques for accessing them. Isolating DNA from environmental samples and then inducing another microorganism to express those genes has

been a successful approach in the past, but it remains impossible in most cases to isolate and express entire pathways, due to a lack of suitable expression hosts. It may be important to develop more and better hosts for expression of uncultivated microbial genes in the future.

Targeting uncultivated microorganisms and their animal hosts in the search for bioactive compounds is often rewarding, since symbionts and hosts often develop chemical defenses to prevent other microbes from establishing spurious infections. It is important to note that useful compounds that may eventually come from uncultivated microorganisms are not limited to antibiotics; microorganisms produce an incredibly diverse array of bioactive compounds. The availability of appropriate screening methods to test the activities of these substances currently limits the ability to find and use them.

Novel enzymes, which may eventually be identified and from DNA extracted from uncultivated microorganisms in the environment, also present a great potential resource for commercial use. The symbionts of termite guts, for example, are thought to be species-specific, and each symbiotic community produces a vast number of biocatalysts for wood degradation—a process that is critical for any number of industrial processes, including ethanol manufacturing. Considering that there are over 2,600 different species of termites, the commercial resource these communities represent is impressive. A number of enzymes from other uncultivated microorganisms are already on the market, including enzymes that can tolerate detergents and heat.

An understanding of microbial ecology/diversity should enable more effective development of both probiotics and prebiotics. (Prebiotics are defined as nondigestible food ingredients that may beneficially affect the host by selectively stimulating the growth and/or the activity of a limited number of bacteria in the colon.)

Barriers to cultivation

The ability to cultivate the majority of microorganisms found in the environment is likely impeded by difficulties in replicating the conditions the microorganisms prefer, genetic and ecological barriers, overgrowth by nontarget organisms, and basic human error, including researcher impatience. Many bacteria, archaea, viruses, and microscopic eukaryotes prosper only in exceedingly narrow sets of conditions, and replicating these microbial environments in controlled laboratory settings is very difficult. The presence of trace contaminants, incorrect concentrations of nutrients, the absence of signal molecules, failure to recreate microbe-microbe or microbe-host interactions, and failure to provide the right nutrients can all work against the researcher trying to isolate a particular strain. Growing microorganisms may also change the chemical conditions of the growth media, thereby inhibiting their own growth. The unknown presence of lysogenic phage, which selectively destroys certain species of bacteria and archaea, may also impede cultivation.

Genetic and ecological barriers to cultivation include the dependence of microorganisms on symbiotic or mutualistic settings that cannot realistically be recreated in the laboratory. Cell density-dependent microbial growth can also prevent researchers from collecting sufficient organisms for study. Batch culture methods, which dominate the established methods for cultivation, can impair cultivation by preventing the isolation of organisms that are not adapted to grow to high densities. Still other organisms require a minimum inoculum size that cannot be achieved with today's methods. It is also possible that some organisms have complex life strategies that make growth in simple laboratory-devised habitats impossible. In these microbes, reproduction may be triggered by seasonal or environmental cues that researchers do not (or cannot) replicate.

Oftentimes, the organism of interest does not have time to establish itself under the culture conditions before the flask is taken over by other, faster growing organisms. Mutations can also arise that change the strain of interest in favor of faster growth and greater fitness in the culture environment. For example, under the selective pressures of cultivation, dellovibrios that are largely host dependent can acquire the ability to grow independently or other talents that are completely different from the *in situ* organism.

Human error, including failure to wait a sufficient length of time for cultures to grow and a lack of imagination and creativity in designing cultivation conditions, probably prevents successful cultivation in some cases. Moreover, although the ability to study cultivated microorganisms has enabled many of the most important advances in the field, a bias in funding agencies in favor of molecular, culture-independent techniques may ultimately discourage cultivation efforts.

It may not be possible to separate microorganisms that have co-evolved to fit one another's functions. Symbionts and pathogens, for example, have developed smaller, sparer genomes as their relationships with other organisms allowed them to do so, and they may not be able to survive outside of these carefully balanced arrangements. Microbiology needs to move beyond its dependence on pure cultures of organisms and appreciate the value of defined but mixed communities.

IS THE WHOLE A SUM OF ITS PARTS? DECONSTRUCTING AND MODIFYING MICROBIAL COMMUNITIES

In general, it will be difficult to isolate and identify every individual component of a natural microbial system. However, research on an intact microbial community can get extremely complicated if the community or the analysis is not distilled to target the process or organisms of greatest interest. Community deconstruction, including studies of waste water systems, pulp mill systems, and artificial rumens, has successfully illuminated community processes and functions of a number of systems. There is unsurpassed clarity in the interpretation of results from studies of pure cultures; using a co-culture of two organisms squares the complexity of a

pure culture, and a culture of three organisms cubes the complexity. Results from pure culture studies can lead back to the intact community by generating hypotheses that are testable on *in situ* processes.

Although there are significant technical difficulties in dismantling communities, it is possible, and there are far more tools available for these projects now than ever before, including single cell optical tweezers, improved FISH and flow cytometry techniques, and other single cell technologies.

The availability of long-term microbiological study sites is important for studies that seek to deconstruct natural microbial communities. Model systems in which a researcher can start simply and add additional complexity one step at a time are also helpful. Suitable model systems that have been or could be used include:

- waste water treatment communities,
- rumen communities,
- some chemostat communities,
- winogradsky columns, and
- phytoplankton communities.

Ideally, microbiologists would develop a set of rules (or "Koch's postulates for communities") for testing success in deconstructing communities. Possible tenets of this system could include:

1. Gain an understanding of the environment, including chemical processes and members,
2. Acquire the isolates that carry out these processes, and
3. Return to the environment and demonstrate that the selected organisms are responsible for the processes of interest.

A deconstructed community that is subsequently reconstructed from constituent key players should retain the key characteristics and process rates of the original, and there is probably redundancy in the microorganisms that can be targeted to accomplish this. Success in deconstructing a microbial community can often be measured by reproducibility and observation of the predicted results.

Although deconstruction can offer valuable insights into microbial communities, researchers need to bear in mind that, even in laboratory cultures, populations of microorganisms in the environment are never genetically identical. Even single nucleotide polymorphisms, in which one base out of thousands differs from that of

the most closely related organism, can have a huge impact on the characteristics of a microbe, so subtle genetic differences among members of a microbial population can be meaningful to community function. When isolating an organism from a community, researchers generally whittle a population down to a single ribotype (genetic type), but it is not known how well this organism represents the rest of the population. Moreover, there is a chance that the process of dissecting a community will introduce genetic changes to a given member (see **Barriers to Cultivation**, above) by spontaneous mutation or gene/plasmid loss.

There are alternatives to community deconstruction for pursuing an understanding of microbial communities. In the "encapsulation approach," individual cells can be encapsulated within small agarose beads, one cell per bead, then a meta-community can be assembled by combining certain beads and incubating under conditions like those found *in situ*. After clonal replication within the beads, individual beads may be removed for characterization. This approach permits cross-talk among the different members of the community during incubation and allows the isolation of single cells, but alters the physical and spatial interactions of the community.

Another alternative to deconstruction is to selectively remove (or "knock out") an individual member of a community using antibiotics, phage, antibodies, filtration by size, or another tool, and then observe community function to determine the role of that member. Metagenomics, proteomics, or transcriptomics approaches may be used to reveal genetic or protein components of the community. Flow cytometry can also be used in these efforts as an analytical tool or as a cell sorting technique to physically separate individual components.

A microbial community does not necessarily need to be disassembled in order to learn about it. Microbiologists could take a cue from ecology research by manipulating individual parameters in a system and observing the effect on community structure.

Constructing artificial communities

Constructing artificial microbial communities can be revealing. In general, the basic properties and individual processes of a community can be recompiled relatively easily by drawing representatives from different categories of metabolisms. Phenomena and processes that can be studied in artificially constructed systems include:

- Host-symbiont and host-pathogen interactions,
- Metabolic interactions (including syntrophy),
- Predator-prey interactions,
- Evolution (either experimentally or *in silico* using computer models),
- Quorum sensing,

- Dehalogenation,
- Gene exchange, and
- Biofilm formation.

Gnotobiotic animals, which are born and raised in a sterile environment, and gnotobiotic mice in particular, area good platform on which to build complex microbial systems one step at a time. *Bacteroides*' metabolic interaction, for example, has been studied in the mouse gut, where they stimulate complex carbohydrate production and then metabolize these sugars.

Identifying communities that impact human health and environmental safety that should be studied at the community level

There are some questions about microbial communities that currently cannot be answered using a reductionist approach that treats the whole as a sum of its parts. Studies of *V. cholerae* within the context of its microbial community, for example, led to the discovery that the bacterium tends to adhere to the surface of much larger zooplankton, making the bacterium easy to filter out using a folded piece of fabric. This simple treatment, which has saved countless lives since it was recommended, may not have been considered without information gathered in community-level studies.

Among the microbial communities that impact environmental integrity and human health, it is important to identify those that are most in need of study as intact entities. Communities can be identified and prioritized by evaluating them according to ecological criteria (Is it widespread? Does it perform a keystone function? Does it perform a quantitatively important function?) or according to the degree to which they impact human health and well being. Epidemiologists and experts in exposure assessment can also play an important role here.

Almost every agent of infectious disease needs to be studied at the community level, since pathogens either arise from or pass through environmental communities during the infection process. Virulence (the degree to which a pathogen is able to cause disease) is partly determined by community interactions, and in most cases it is not known which factors enhance or inhibit virulence. It is particularly critical to understand community dynamics in cases where natural communities serve as reservoirs of disease, thereby maintaining a baseline level of infection in humans despite treatment and eradication efforts. Communities that expose humans to toxic microbial products also require study. Some examples of pathogen-laden communities that should be studied more thoroughly include communities in coastal environments (which have become breeding grounds for pathogens), the communities of the human gut and mouth, and the communities that thrive in moist air conditioning ducts and water lines in homes.

The impact of microbial communities on chemical contaminants in the environment is another area where study is needed. Some compounds are degraded or detoxified only by communities of organisms working together, while other compounds resist degradation or are made more toxic by microbial communities. Research needs to address these issues and determine which compounds are broken down and which are made more toxic and how microbial communities accomplish these transformations.

Researchers need to study drug effectiveness on pathogens within the context of their communities in the human body. Chemicals that are effective on pathogens in a Petri plate in the laboratory are not always effective when used to treat an infection. It is also important to consider microbial communities present in agricultural animals as these are often the source of food contamination and reservoirs of human disease.

Examples of directing and modifying natural communities

A microbial community may be designed and constructed piece by piece to carry out a specific process, but it is also possible to alter or direct an intact community to achieve the same goal. There are a number of examples in which this type of management has been accomplished successfully. Very good examples are various civil and industrial wastewater treatment plants where a complex but selected microbial community is doing its daily or seasonal job of sewage or industrial waste degradation. It is possible, for instance, to stimulate methanotrophic activity in a community in order to boost the co-metabolism of trichloroethylene, and *Dehalococcoides* bacteria can be stimulated or added to a community to boost anaerobic dehalogenation of tetrachloroethylene. Other examples of microbial community alteration include:

- The addition of **probiotic microorganisms** to the microbial consortia of the gut in humans and animals, which are commonly administered to aid digestion or fight pathogens,

- The addition of **Geobacter species** to stimulate microbial uranium precipitation in ground water systems,

- Coating legume seeds with ***Rhizobium*** **bacteria** to prompt colonization of the plant and improve plant productivity, and

- Use of ***Bacillus cereus*** for biocontrol of fungal infections in plants.

In the future, microbial community manipulation may successfully replace acid-forming species of *Streptococcus mutans* in the mouth with strains that are less prone to forming dental caries. Phage control of bacteria is another promising area of research. The FDA recently approved the use of a Listeria phage as a food safety additive (Bren, 2007).

MICROBIAL COMMUNITY STABILITY

The stability of a microbial community, that is, its ability to maintain species composition and processes within certain bounds, mostly relies on three basic features: the variability of the environment, the diversity of the community, and the interactions among community members.

Environmental variation has a profound influence on gene expression, species composition, and the relative abundance of members within microbial communities. High microbial community diversity, specifically functional diversity and redundancy, is critical to overcoming perturbation. There are many settings, including sewage treatment communities, the human microflora (including the gut, mouth, and vagina), bioremediation, and agriculture, in which a better knowledge of stability-diversity connections is needed. The presence of cells in resting phase may also boost a community's stability. Since macromolecule synthesis, transport, and metabolism, in general, are greatly slowed in resting microbes, slow-growing and non-growing organisms may not have to copy with chemical contaminants to the degree actively-growing members do. Systematic studies of the connection between diversity and stability are few and far between, and much of what is known on this topic comes from anecdotal evidence. It is not known whether keystone groups (without which the community could not recover) exist, for example. This situation may change as new high throughput methods for monitoring community composition, like DNA microarrays, become available.

Predicting the outcome of disturbance

Very little is currently known about the specific outcomes of disturbance in microbial communities, even in communities that are directly related to human health and well being, like wastewater treatment and the human gut. In Los Angeles, for example, a seemingly innocuous ban on metals dumping in municipal sewers changed the microbial system drastically, encouraging the unpredicted growth of *Thiobacillus denitrificans*, which in turn promoted widespread corrosion of sewer pipes. Mathematical models for predicting the results of community-level disturbance are very limited in number and scope.

Research needs to address this deficit in the predictability of microbial systems by evaluating model systems at "baseline" or preperturbation state and then directly measuring the effects of perturbation in these systems.

PREDICTING RESPONSES FROM AN UNCHARACTERIZED COMMUNITY: WHAT COMES OUT OF THE BLACK BOX?

In certain settings, the composition and processes of a particular community do not need to be understood in detail in order to predict community response to change, since experience and the laws of thermodynamics are sufficiently instructive. Wastewater treatment and many food fermentation processes are examples of longstanding successes in this capacity. However, there is now concern that existing wastewater treatment systems are not effectively removing pharmaceuticals (e.g., birth control, antibiotics) and food additives. These are seriously impacting natural systems to which the treated water is released. Thus, it is essential that established microbially-based treatment processes be revisited to ensure that they retain function as the composition of waste streams change with changes materials discharged by both households and industry. In most cases, however, it is not acceptable to treat microbial communities as black boxes with unknown contents and predictable outputs. A novel synthetic chemical, for example, may be introduced to soil or water, but without understanding soil and water microbial communities, their metabolic resources, and the processes they carry out is not possible. Research should continue to investigate the composition and activities of microbial communities in order to predict the reactions of communities to disturbance.

UNLOCKING THE FUNCTIONS OF UNKNOWN MICROBIAL GENES

A great deal of uncertainty remains in drawing the link between a nucleotide sequence in the genome of a microorganism and the processes that organism carries out in its environment. The difficulty of this problem can be illustrated by past experience with a thoroughly studied model organism; the genome of *Escherichia coli* K12, for which mutants have been created for many open reading frames, maintains a large fraction of unknown genes. Apparently, all the standard tools have been applied to studying this organism, but little is currently understood.

There has obviously been some progress in identifying gene functions, however, and several different approaches to the problem that have met with success can provide guidance for making future discoveries. These approaches include:

- Relate gene expression patterns to environmental (chemical/physical/biotic) parameters like nutrient limitation. Other, more qualitative factors, like where the gene is expressed within the cell or microcolony or consortium, can also point to a gene's function.

- Construct a strain with a knockout mutation for a known gene, then examine changes in the relative amounts of transcribed genes to infer which of the unknown genes are associated with the known, interrupted gene.

- Study adaptive radiation within a clade to understand what functions have been selected.

- Create a metabolic model of an organism, then create targeted knockouts of unknown genes and reexamine metabolic fluxes within the cell.

- Use transposon mutagenesis to create libraries of mutants, then expose the mutants to selective regimes to determine via microarray hybridization which mutants are selected against.

- Use protein structure modeling to predict functions.

SPATIAL SCALE OF MICROBIAL COMMUNITY FUNCTIONS AND INTERACTIONS

When designing an investigation of a microbial community, it is important to determine the spatial scales on which the community and the system operates, since sampling and other factors rely directly on the scaling of the system. For example, in biofilms and sediments the use of microelectrodes has revealed that steep gradients of oxygen or sulfide can exist over a few millimeters or less. Unfortunately, there is no stock set of rules to follow for determining the scales relevant in all community-oriented research, but there are some general guidelines that can be tailored on a case-by-case basis. They include:

- **Identify the spatial constraints** on the community by making chemical and physical measurements. Do multiscale sampling and determine the scale at which the desired property or process is relevant. However, due to the small scales involved and the limitations of the current generation of technology, researchers often have limited ability to determine the relevant parameters at the right scales.

- **Measure the inputs and outputs** of the system and determine the scaling. Technology also limits the ability to get this done in some settings.

- **Identify how rates and fluxes change with scale** to see if change with scale is predictable or if relationships are scale-dependent. For example, in a small, well-stirred fermentor, the mixing time of a growth limiting nutrient may be ignored relative to its rate of biological consumption, whereas in a large fermentor, the mixing time may become the rate-limiting factor and/or lead to strong fluctuations of this nutrient in the growth vessel. In such cases, the proper engineering approach is to perform mathematical modeling

and experimental verification to identify the critical time constants (bottle necks) of the process.

- **Use mathematical modeling** to predict scales when possible.

Microscale interactions among species are critical in almost all microbial systems, and they play roles in many different phenomena related to microbial communities, including competition for nutrients, symbiotic relationships, quorum sensing, gene transfer (especially by conjugation), interspecies hydrogen transfer, viral infection, predation, cross feeding, antibiotic production, and density-dependent growth. Bulk measurements will often miss microscale activities and underestimate the rates of many microbial transformations. For example, clustering around particulate nutrient sources in the oceans, where nutrient concentrations are relatively high, can lead to a greater growth rate than would be estimated from measurements of the bulk solution. Modeling suggests that if these interactions are taken into account, the overall carbon turnover of the oceans would have to be scaled up by a factor of two or three.

In systems in which the black box approach (which treats a microbial system as a simple unit with inputs and outputs) works well at answering the relevant questions, as it does with issues of substrate conversion rates in a bioreactor; for example, microscale interactions may be neglected.

Tools for studying microscale interactions include reporter genes (which are extremely useful for studying biofilms), fluorescence *in situ* hybridization (FISH) coupled with digital image analysis, FISH-microautoradiography and FISH-Raman spectroscopy to reveal cross feeding, fluorescent quenching techniques (which can be used to study physical interactions), microelectrodes, microoptodes, single cell reporter tools (e.g., green fluorescent protein system and carbon source reporters), chemical-specific dyes, and secondary ion mass spectroscopy. More work is needed to enhance these technologies. Further work is also needed to miniaturize scanning electron microscopy and other microscopic tools, develop biosensors, microsensors, and gas sensors, and to generally improve the ability to make *in situ* environmental measurements at appropriate scales.

CO-EVOLUTION IN INTERACTING MICROBIAL SPECIES

Microorganisms are constantly co-evolving to improve their standing in the context of competitive and symbiotic relationships with other microbes. Arms races with antibiotics can drive competing microbes to create new weapons and, in turn, create new defenses. Another form of antagonism—phage-host interactions—determines the success or failure of certain genotypes by influencing host population size and other factors. Succession in oral biofilms is partly determined by ligand-receptor binding that determines physical assembly of different species.

Evolved changes in microorganisms can reverberate through a microbial community and cause the adaptive landscape to shift for other organisms. There may be synergistic interactions in which an organism has reached a fitness peak and any changes the organism makes will decrease the fitness of the interacting partners. Involvement in an interaction like this may also decrease the ability of an organism to act as a generalist within the community, since any new adaptation moves it out of sync with a partner that it needs to survive.

MICROBIAL COMMUNITIES IN AGRICULTURAL SYSTEMS—MANAGING HUMAN IMPACTS

Intensive agricultural practices are known to have serious ecological impacts, and microbial communities are usually the first to feel these effects. The concern here lies not necessarily in the impacts to the microorganisms themselves, but in the implications these effects hold for livestock health, human health, and the environment. Examples of the impacts of agriculture on microbial communities include the fostering of antibiotic resistant bacteria in livestock-associated consortia, which can eventually lead to untreatable infections in animals as well as humans. Pathogen loads, including human pathogens, can be drastically elevated in lakes and soils near industrial farms. Nutrient pollution in water bodies and groundwater down gradient of farms can cause eutrophication—blooms of toxic dinoflagellates and toxic cyanobacteria.

By developing more microbial sentinels to display specific stress responses indicative of system disturbance, monitoring strategies could be devised to make use of impacts to microbial communities and head off consequent impacts to animals, humans, and the environment.

GENOMICS AND ENVIRONMENTAL MICROORGANISMS

The ability to sequence and interrogate entire microbial genomes has revolutionized microbiology. Genome sequencing, on its own, is important for examining the evolution of strains over relatively short periods; researchers can use sequencing to follow genetic adaptations over time in a single strain. The tools of genomics, which study an organism's genome for information regarding the organism's activities, are superb hypothesis generators. **Comparative genomics**, for example, allows estimation of the mechanisms and rates of genome diversification, adaptation, and speciation. Metagenomics, in which groups of genomes from diverse organisms are studied as a whole, has revealed many new details, including the importance of transposons in bacteriophages. **Environmental genomics** can expose the differential distribution of genes and metabolic types in different types of environments.

Although the cost of sequencing genomes is coming down, in the future, researchers must continue to be discerning about which microorganisms they select

for sequencing. Among the genome sequences currently available in the public domain, there is a marked overrepresentation by human pathogens, and there are many underrepresented groups that remain to be characterized. It is important to sequence clusters of closely related organisms to reveal the eco-physiological properties that maintain diversity within a family or species, as well as more divergent organisms. Viruses are agents of gene transfer in microbial communities and also are important targets for sequencing. It is important to ground predictions arising from genomics with experiments in the laboratory wherever possible.

The pace of genome sequencing is brisk, but the current lack of resources in bioinformatics and data analysis (which are used to make sense of raw sequence data) pose a serious limitation on progress in this field. More work is particularly needed in functional genomics, which is used to link sequences with specific functions.

APPLYING THE PRINCIPLES OF POPULATION BIOLOGY AND EVOLUTION TO THE STUDY OF MICROBIAL COMMUNITIES

Although microbes share many characteristics with larger organisms, science has historically dealt with them very differently, excluding microbes and their communities from the scrutiny afforded in the endeavors of population biology, ecology, and evolution studies. Scientists now recognize that it is useful and appropriate to apply the principles learned in these fields to the study of microorganisms, since a deep understanding of ecology, natural history, and physiology is perhaps the most important foundation for advancing understanding of gene function. Some of the fundamental themes of microbiology that should be addressed using these principles include:

- **The definition of a microbial species.** Questions about the nature of the species concept as it applies to microbes, how species are formed, and how to define diversity should all be dealt with.

- **The origins of novel phenotypes and how they are maintained.** Research should address the how genes are carried into a community, the nature of genetic drift in microorganisms, the environmental parameters that control the selection of phenotypes, and whether or not there are measurable rates of gene transfer in nature.

- **The biogeography of microorganisms.** It is clear that as far as principle types of metabolism is concerned the old axiom "everything is everywhere" is undoubtedly true, but it is unclear whether any given microbial type/(sub)species can be found wherever one looks. When it comes to the fine detail of niche differentiation it may well be that specific variants of species/families operate on a more localized basis (Foti et al., FEMS Microbial Ecology 56, 95-101 (2006)).

- **Improving the predictive capacity with respect to the activities of microbial communities.** Ecology, which focuses on developing predictive hypotheses, has a great deal to offer in the study of microorganisms.

- **Resolution and precision in community analysis.** The practice of blind testing in data collection and in data analysis is standard in ecology research, but it has eluded microbiology, a shortfall that probably has an impact on standardization of methods.

SPECIFIC TOPICS IN MICROBIOLOGY TO ADDRESS USING PRINCIPLES FROM POPULATION BIOLOGY, ECOLOGY, AND EVOLUTION

More specific topics that could be addressed using principles from population biology, ecology, and evolution include:

- The mechanisms of community assembly and interaction,
- How predation dictates structure in microbial communities,
- Host switching by bacteria and viruses (e.g., SARS),
- Antibiotic resistance, including gain and loss of resistance from populations,
- Emerging infectious diseases,
- Co-evolution of hosts and parasites,
- The effect of different kinds of selection on microbes,
- Development of new vaccines (the phenomenon of herd immunity needs to be understood), and
- The environmental manifestations of organismal interactions.

METHODOLOGIES AND TECHNIQUES

Microbiology is both enabled and limited by technology. Although the technology and methodology support for environmental microbiology has progressed quickly in recent years, the challenges that lie ahead cannot be met without further progress in cultivation methods, functional genomics, and other techniques.

NEEDED METHODS FOR ISOLATION AND ENRICHMENT

Researchers need access to better cultivation technologies in order to adequately explore the world of uncultivated organisms that hold promise for medicine, industry, and the environment. Microbiology requires more methods that allow researchers to mimic the conditions microorganisms encounter in their natural habitats, particularly the conditions in low nutrient environments and in nutrient

and oxygen gradients that form at interfaces and surfaces. In the environment, microorganisms probably rely heavily on the activities of other species, but these biotic services are often neglected in laboratory culture conditions. Cultivation techniques that provide these partners for the organisms of interest are needed. *In situ* culture technologies, like nutrient-permeable bags, encourage the replication of the organism of interest in a growth chamber inserted into a microbial system. More and better refined technologies like this are needed.

Flow cytometry, which enables researchers to separate cells either on the basis of their intrinsic properties, like cell shape, or on the basis of fluorescent tags, can be extremely useful in selective cultivation, but continued improvements are needed. For example, it would be extremely helpful if cells could be sorted after FISH identification and if this FISH staining would not kill the cells (as current protocols do) and, thus, the sorted cells could be used for cultivation. There is a single paper which postulates that FISH staining of living cells might be possible (Nucleic Acids Res. 2005 33:4978-86). Laser capture microscopy with optical tweezers is a promising technique for aiding cultivation, too, but here again, improvements are needed. In an ideal situation, scientists would be able to use lasers to capture organisms sharing the same general habitat, sequence those organisms, perform the bioinformatics research to reconstruct their metabolisms, then sample environmental parameters to ultimately decide how to cultivate the organisms in the laboratory.

Finally, research has long neglected the value of cultivating consortia of microorganisms, partly because of a lack of suitable techniques. New methods are needed that make it easier to exploit meta-interactions and grow microbial consortia in the laboratory.

NEEDS FOR *IN SITU* WORK

Oftentimes in microbiology, it is best to study the organisms of interest within the context of their habitats, *in situ*. This type of work can be difficult, and researchers often find their investigations are limited by technology. More sensitive methods for making *in situ* measurements are needed, as are improvements in the ability to make real time measurements of environmental conditions. Research would benefit from miniaturized instrumentation for making real-time, long-term *in situ* measurements of cellular responses. Researchers also need better tools for monitoring substrate consumption and compound excretion patterns, gene expression, and the proteome in the environment.

BIOINFORMATICS NEEDS

Bioinformatics, which applies mathematics and computer science to problems in biology, has opened up grand new possibilities for exploring the microbial world, but more bioinformatics tools are needed to facilitate further progress and

there are a number of specific areas where improvements can be made. Research needs include:

- Methods for rapidly identifying nonfunctional genes ("junk DNA"),
- New algorithms for assembling genomes from short sequence reads and from metagenomic data,
- Comparative bioinformatics approaches to identifying metabolic modules that might allow inference of novel function of specific genes that are part of that module.

OTHER NEEDED TECHNOLOGIES AND METHODS

Other key developments in technology and methodology to enable future research on microorganisms and their activities in the environment include:

- Improved **high throughput molecular and proteomic techniques** for monitoring community compositions.
- Better **single cell genome replication techniques** that give higher coverage and fewer errors. Current rolling circle genome replication technologies operate at low temperatures, a situation that encourages non-specific binding and imperfect replication. Thermophilic phages should be mined for more suitable polymerases.
- **"Live" stains** that can identify uncultured cells without killing them.
- Improvements in **autonomous underwater vehicles** to enable aseptic water sampling.
- New **genetic systems**, particularly environmentally important but so far understudied groups.
- Better **models for protein prediction** from gene sequences.
- Development of **ordered mutant libraries** of more organisms.
- Better **protein crystallization methods** and methods of predicting and solving crystal structures so that the functional details of more proteins can be elucidated.
- More **remote sensing techniques** for monitoring community structure.
- Refined **techniques** for measuring the function of uncultured microbial cells at the single cell level.

EDUCATION, TRAINING, AND COMMUNICATIONS ISSUES

Although the challenges that lie ahead in microbiology are compelling, it is important to remember that discovery relies on the work of individuals. Emphasis should be placed on effective training and fostering productive collaborations.

CURRENT GAPS IN TRAINING

The future of microbiology relies on the training and education going on today, but there are a number of identifiable gaps in training that must be addressed. Current training programs in microbial science do not place enough emphasis on critical thinking and hypothesis or question building, and observational skills are being lost. This sort of preparation may have to begin in K-12 education, where students could be more effectively introduced to the excitement of natural discovery.

Although microbiology training seems to be keeping up with advances in technology, it is falling short when it comes to some of the more long-established disciplines, including physiology, enzymology, and biochemistry, and long-established techniques like culturing. Other weak areas in training include bioinformatics, small molecule structural identification, biophysics, and chemistry. Quantitative analysis, including mathematical modeling and complex statistics is also not taught to a sufficient extent.

The failure of graduate programs to provide sufficient training in physiology is particularly troubling, since a detailed knowledge of physiology is needed to interpret the vast amounts of genomics data that are currently being generated by high throughput techniques. Fellowships that emphasize physiology and improved microbial physiology textbooks are sorely needed.

Some specific recommended changes for education and training that would help to keep microbial science vital include:

- Fellowships and travel grants for encouraging cross disciplinary interactions,
- Web-based courses and reading lists for students and other scientists,
- Intensive short courses in microbiology (including microbial diversity courses) which offer students good exposure to the field and networking possibilities,
- Revitalize devoted microbial science departments in colleges and universities as they have often of late succumbed to consolidation with genetics and molecular biology departments, a development that has weakened collaborative ties between microbiologists. Re-equip many outdated college and university laboratories with new tools for microscopy and molecular analyses,

- Make evolution and ecology standard components of microbiology training, since almost all questions in microbiology must be viewed in these contexts, and

- Develop new and better textbooks for microbial physiology, microbial diversity and systematics, bioinformatics, and microbial ecology.

RECOMMENDED COLLABORATIONS

Collaborations among professionals from diverse areas of expertise are a hallmark of successful microbiology research. Collaborations provide fresh perspectives on study topics and help inspire dialog and creativity. There are a number of ways to improve the state of collaborative interaction in microbial science. Microbiologists should make connections with experts on particular environments in order to specifically tailor microbial cultivation conditions. Additional collaborations among environmental microbiologists and infectious disease specialists would be helpful. Engineers and applied physicists, too, should be involved in microbiology research to help design improved instrumentation. Long-term ecological research stations can provide good platforms for collaboration, provided they are associated with some sort of central institution that can foster these interactions.

In general, the field needs more nucleation points, more common ground where professionals can talk with each other. Proximity is essential for collaborative research; sharing space in a laboratory is one way to promote collaboration. Training courses should also involve students from diverse academic backgrounds.

Unfortunately, the administrative structure of academic departments sometimes inhibits interdisciplinary research. International collaboration is also difficult, since strict customs security measures have made moving microbiological samples across borders incredibly difficult. Exchange visits have also become more difficult for professionals from certain countries.

Collaborations with industry can be fruitful. Industry may also be interested in supporting graduate students conducting research in areas that interest the private sector but funding agencies avoid.

RECOMMENDATIONS

When selecting microorganisms for genome sequencing, it is important to sequence clusters of closely related organisms as well as more divergent organisms. Viruses, too, should be targeted by sequencing efforts, since they serve as important agents of gene transfer in microbial communities. Technical barriers to community sequencing projects should be lowered.

Microbiology also needs to move beyond its dependence on pure cultures of organisms and appreciate the value of defined but mixed communities of microbes. It may not always be possible to separate microorganisms that have coevolved to fit one another's functions and isolate them in pure cultures.

Research should test the responses of microbial communities to perturbation by evaluating model systems at "baseline" or preperturbation state, and then directly measuring the effects of perturbation in these systems. Very little is currently known about the specific outcomes of disturbance in microbial communities, even those that are directly related to human health and well-being.

Current technologies for making measurements at the microscale require enhancement. Work is also needed to miniaturize scanning electron microscopy and other microscopic tools, develop biosensors, and to generally improve the ability to make *in situ* environmental measurements.

Microbiology requires more methods that allow researchers to mimic the conditions microorganisms encounter in their natural habitats, particularly the conditions in low nutrient environments and in nutrient and oxygen gradients that form at surfaces.

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