# Techniques
# Conversations with the silent majority
## Editorial overview
Jo Handelsman and Kornelia Smalla

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**Jo Handelsman**

Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin 53706, USA
e-mail: joh@plantpath.wisc.edu

The Handelsman lab dissects the structure and function of microbial communities by describing their molecular diversity and by decoding the language by which the members communicate with each other and with plant and animal hosts.

**Kornelia Smalla**

Institute for Plant Virology, Microbiology and Biosafety, Federal Biological Research Centre for Agriculture and Forestry, Messeweg 11/12, D-38104 Braunschweig, Germany
e-mail: k.smalla@bba.de

For more than a decade the Smalla lab has focused on the development of cultivation-independent methods to study the diversity of microbial communities and, in particular, their horizontal gene pool. Molecular fingerprinting techniques have been developed and are applied to find out how bacterial and fungal communities change in response to environmental stimuli. Novel experimental approaches are also taken to elucidate the contribution of mobile genetic elements to bacterial adaptability and diversity.

If only bacteria would howl, cheep, squawk, trill, purr, or rustle, how easy would be the job of the microbial ecologist! The challenges of microbial ecology spring from the difficulty in finding the subjects of study and in interpreting their behavior once we find them. Tracking bacteria in their native environments has traditionally involved light microscopy and culturing on agar medium, but these methods have substantive limitations. Microscopy is limited by the relatively low diversity of microscopic forms that distinguish microorganisms, the opacity of many of the matrices in which bacteria are found, and the insufficiency of methods for efficient quantification. Likewise, the utility of culturing is limited by the current technology, which typically accesses 0.1 to 10% of the diversity in natural environments.

Recent advances in molecular microbial methods have let us hear from microorganisms that were silent before. The use of PCR to amplify 16S rRNA genes directly from environmental samples, circumventing culturing, led to the discovery of thousands of new sequences and some entirely new divisions of bacterial life. The current challenge is to take the bold step beyond simply cataloguing sequences to elucidate the functions and ecophysiology of these newly described members of the microbial world.

In this issue of *Current Opinion in Microbiology*, we explore the emerging technology that is the platform for modern microbial ecology. The articles describe methods that assess who is there and others that dissect the functions of organisms or communities in environmental settings. The field is far from providing a complete description of the diversity in any environment, but new methods attempt reasonable estimates of the extent of species-level diversity and abundance of individuals in a community. Functional analysis, which is intended to understand the role of individual species in communities, is advancing with techniques that facilitate measurements of gene activity, protein structure and cellular morphology at levels of resolution unimagined until recently.

The advent of PCR-based detection of microbial diversity called for improved strategies for culturing that might bring organisms known only by their molecular signatures into the captivity of the Petri dish. Toward this end, Leadbetter reviews recent advances in cultivating the hard-to-culture majority of the microorganisms on earth. He argues that cultivation is far from a dying sport, and is, perhaps, entering its golden age as discoveries made with culture-independent tools impel microbiologists to develop new cultivation techniques. Some successes in cultivating microorganisms known only by their molecular signatures have been supported by dramatic intellectual steps that require rejecting old paradigms, some by technological advances, and some by tweaking and patience. For example, the cultivation of a marine sulfate reducer that oxidizes phosphite required the investigators to reject microbiological dogma and recognize that some bacteria oxidize reduced forms of phosphorous. Technical innovation propelled the cultivation of deep lineages of Planctomyces and Bacteroides from seawater. Giovannoni's group devised a simple, yet sophisticated gel microdroplet that encapsulates bacteria and can then be packed in a column, which is bathed in medium. In this case, sterilized seawater stimulated growth of diverse phylotypes. In contrast, success in the cultivation of the elusive SAR11, a bacterial division that represents a scandalous 50% of the microorganisms in seawater, and members of the *Verrucomicrobia*, which represent massive populations in soil ($10^8$–$10^9$ per gram dry weight), depended on innovations as simple as changing the solidifying agent in the medium, diluting the medium and waiting. And waiting. And waiting. An emerging leitmotif is that cultivation of recalcitrant microorganisms requires patience — they grow on a biological schedule that is their own and is not necessarily compatible with the acceleration of modern science.

The rapid accumulation of 16S rRNA gene sequences stimulated the need for mathematical approaches to recognize patterns and derive organization from the morass of data. Bohannan and Hughes compare and contrast parametric and nonparametric approaches to estimate microbial diversity from an assemblage of 16S rRNA gene sequences that is not a complete census of a community. Statistical analysis adds rigor to comparisons of diversity by demonstrating the lack of significance where a visual scan of the data suggests a difference between two communities, while also enhancing the resolution of our scrutiny, detecting differences that are not apparent to the eye. Broader application of these tools is essential for meaningful conclusions about the factors that influence species richness and abundance in microbial habitats.

16S rRNA gene sequence analysis provides detailed information about the phylotypes in an environment, but is too laborious to be used to attain a snapshot of diversity or monitor changes in gene expression induced by environmental change. Nucleic acid array technology provides an efficient means to assess the presence of organisms or the expression of genes in communities. Zhou reviews diverse applications and limitations of microarrays in microbial ecology. He cautions that the performance of microarray hybridization in environmental microbiology studies has to be carefully evaluated, and a number of technological challenges need to be solved before this technique can reliably inventory nucleic acids from complex environmental samples. In particular, when environmental DNA is used without prior PCR amplification, specificity, sensitivity and quantification of microarray hybridization are critical. As the technical challenges are overcome, microarrays are likely to emerge as one of the central technologies for analysis of the structure and dynamics of microbial communities.

A critical advance in nucleic acid technology is the direct cloning of DNA from the environment, circumventing the limitations of both culturing and PCR. This approach, known as metagenomics, environmental genomics, or community genomics, is reviewed by Wellington *et al.*, who provide insight into the power of this method for discovering new functions in microbial communities. Recent discoveries have included genes encoding novel light harvesting proteins, new antibiotic biosynthetic pathways and new enzymes. Wellington *et al.* discuss methods that can be coupled with metagenomics to study a subset of a microbial community with a particular function. Among these, stable isotope probing is emerging as a powerful device to amplify the voices of some members of the community, while dampening the rest by selectively isolating and cloning DNA from organisms that metabolize a labeled substrate. Metagenomics technology is young and offers growth potential for understanding microbial ecology in complex communities.

Just as improvements in culturing and 16S rRNA gene analysis give these methods new applications in microbial ecology, modern advances in microscopy have revitalized the oldest tool of the microbiologist. An important technique to study the silent majority is fluorescence *in situ* hybridization (FISH) with rRNA-targeted probes in combination with microscopy or flow cytometry. The review by Wagner *et al.* describes the methodological improvements of this method, which provide new ways to link structure and function in microbial ecology studies. FISH has unique potential to study the composition and spatial organization of bacterial communities *in situ*. Moreover, it can be used to augment or complement culturing or diversity analysis. Wagner *et al.* discuss the limitations that remain to be addressed as well as the opportunities for coupling FISH to other technologies, such as modern imaging systems and microautoradiography to acquire a better understanding of spatial organization of microbial communities with certain metabolic activities.

Marker gene technology has emerged as a powerful tool to study the spatial distribution of marked bacteria, to assess their metabolic activity and to follow transfer of their genes in a community. Recent applications of this technology are reviewed by Jansson. The rapid establishment of this technique as a central and versatile member of the microbial ecologist's arsenal of devices designed to amplify the signal of a single bacterium against background noise is reflected by the numerous recent publications in which marker or reporter genes are used as biosensors for environmental monitoring or for monitoring horizontal gene transfer *in situ*. Reporter genes have contributed to understanding bacterial colonization patterns of surfaces as diverse as minerals and hexadecane droplets, they have facilitated tracking bacteria as they move among habitats and they provide visual evidence for the complex structure of biofilms. Jansson discusses the ramifications of the dependence of the signal intensity on the physiological status of the cell, necessitating better infusion of microbial physiology into ecological studies.

Microbiologists, more than any other group of biologists, are confronted and often stumped by the challenge to listen to the world at a scale appropriate to the subject of study. Just as dilution plating estimates an average population across an environment, most biochemical techniques blur the subtleties at the cellular level by studying populations of molecules isolated from many individuals. Recent developments have begun to address this challenge by providing images obtained at the molecular scale. Dufrêne reviews the application of atomic force microscopy (AFM) in microbiology, one of the most dramatic advances in technology designed for a molecule-by-molecule dissection of bacteria. AFM employs a tiny probe that skims a surface, and the force of its deflection across the surface is integrated to construct an image of the relief of the surface. AFM has been used to study the topology of protein crystals, cells attached to surfaces and biofilms. Study of a bacterial-mineral interface revealed pitting of the mineral surface due to dissolution caused by bacterial activity. In one study, an AFM probe was used as a tiny tool to scrape mucilage from a diatom surface, revealing the silicaceous material underneath. At the subcellular level, AFM can measure variations in elasticity of cell walls across the surface of a cell and unfold individual protein molecules. Dufrêne discusses future challenges and applications of this powerful technology to home in on molecular events that underpin microbial lifestyles.

Bacteria do not openly reveal their secrets. But they have signals that can be amplified, reconfigured and translated through the medium of technology, giving voice to what is hidden. The microbiologist's task is to listen.