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Editor's note: With this article, BioScience *inaugurates a series of occasional articles that will explore some of the wide-ranging impacts of modern molecular methods on biology. The articles were invited from noted researchers on the basis of recommendations by AIBS member societies. Douglas J. Futuyma, distinguished professor of ecology and evolution at Stony Brook University and president of AIBS in 2007, proposed the series.*

Progress in the Ecological Genetics and Biodiversity of Freshwater Bacteria

JÜRG B. LOGUE, HELMUT BÜRGMANN, AND CHRISTOPHER T. ROBINSON

The field of microbial ecology has grown tremendously with the advent of novel molecular techniques, allowing the study of uncultured microbes in the environment, and producing a paradigm shift: now, rather than using bacteria cultures for evaluating cell-specific questions, researchers use RNA and DNA techniques to examine more broad-based ecological and evolutionary constructs such as biogeography and the long-debated biological species concept. Recent work has begun to relate bacteria functional genes to ecosystem processes and functioning, thereby enabling a better understanding of the interactive role of bacteria in different and often-changing environments. The field continues to mature and will most likely make substantial contributions in the future with additional efforts that include metagenomics and genomics. Here we review progress in the application of molecular techniques to study microbial communities in freshwater environments.

Keywords: molecular methods, metagenomics, microbial ecology, microbial function, bacterioplankton

Since Robert Koch first grew cells in pure cultures and identified *Bacillus anthracis* (1876) and *Mycobacterium tuberculosis* (1882), the world of microbes has been divided into two groups: those that can be cultured and those that remain uncultured. The most prominent example of this dichotomy is the often observed discrepancy between numbers of viable plate counts and total microscopic counts of natural microbial cells; this phenomenon, termed the "great plate count anomaly" by Staley and Konopka (1985), was first observed in oligotrophic and mesotrophic aquatic environments. This imparity between culturable and *in situ* microbial diversity and the realization that most environmental microorganisms are refractory to cultivation stimulated the application of a series of seminal methodological breakthroughs in environmental microbial ecology (figure 1). The first was the discovery made by Zuckerkandl and Pauling (1965) that macromolecules carrying the information of genes (or transcripts thereof) were most suitable for unraveling evolutionary history. This realization that evolutionary relationships can be inferred from sequence differences found between homologous macromolecules revitalized the study of molecular phylogeny.

One of the first to take advantage of this new knowledge was Carl Woese, who attempted to establish a sequence-based framework of evolutionary diversity among prokaryotes. By using 16S ribosomal ribonucleic acid (rRNA) and comparing rRNA sequences from cultivated microorganisms, Woese and Fox (1977) developed a phylogenetic tree of the three major domains: Eukarya, Bacteria, and Archaea. The assumption behind the use of the 16S rRNA gene to study diversity and phylogeny is that the gene's sequences reflect

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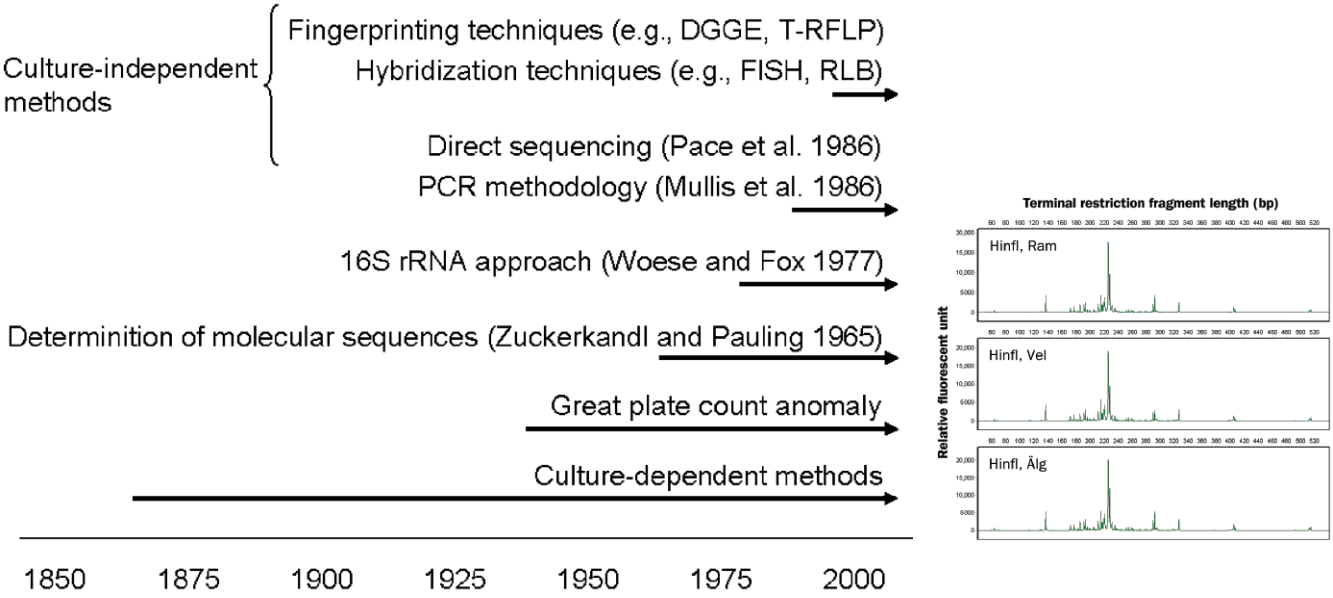

Figure 1. Timeline of significant breakthroughs characterizing the ongoing revolution within molecular microbiology, 1850 to the present. The T-RFLP peak electropherogram (inset at right) was provided by Jürg B. Logue. Abbreviations: DGGE, denaturing-gradient gel electrophoresis; FISH, fluorescent* in situ *hybridization; RLB, reverse line blot; T-RFLP, terminal-restriction fragment-length polymorphism.

evolutionary relatedness and, hence, act as a molecular clock (figure 2). On the basis of this work, Pace and colleagues (1986) led the way to a paradigm shift from cultivation-dependent to cultivation-independent molecular methods. They developed an approach using rRNA gene sequence information retrieved directly from environmental microbial populations, without requiring cultivation. In this approach, the total deoxyribonucleic acid (DNA) extracted from natural microbes is analyzed by having a phage or plasmid (clone) replicate an inserted DNA fragment, which is subsequently sequenced. This molecular approach marked the advent of cultivation-independent techniques to examine bacteria diversity, thus circumventing the great plate count anomaly.

Direct sequencing is rather laborious, however, and its beginnings were not simple. The next breakthrough occurred that same year with the development of polymerase chain reaction (PCR) technology, which facilitated and invigorated cultivation-independent approaches (Mullis et al. 1986). Employing purpose-designed oligonucleotide primers, PCR methodology can be used to copy and amplify specific regions of DNA (Mullis et al. 1986). PCR amplification, cloning, and sequencing of rRNA and DNA from environmental samples led to the discovery of numerous new taxa, and provided sound sequence information for the study of phylogenetic comparisons. Yet this approach for sequencing clone libraries is labor-intensive, time consuming, and, above all, quite costly. To improve efficacy, mainly from larger sample numbers, microbiologists have more recently turned to DNA fingerprinting and to hybridization techniques.

Fingerprinting methods take advantage of different properties of the amplified environmental sequences (e.g., sequence length, presence or absence of restriction sites, melting behavior) to obtain a qualitative representation of the presence and abundance of different phylotypes in a sample. Frequently used fingerprinting methods are ribosomal intergenic spacer analysis (RISA), denaturing-gradient gel electrophoresis (DGGE), single-strand conformation polymorphism, temperature-gradient gel electrophoresis, and amplified ribosomal DNA restriction analysis (ARDRA), or the newer variant, terminal-restriction fragment-length polymorphism (T-RFLP) (for a recent review on fingerprinting methods, see Nocker et al. [2007]). All allow a rapid, inexpensive, and reproducible assessment of environmental microbial communities. By profiling the genetic diversity, composition, and structure of microbial communities, these techniques are valuable for tracking genotypic community changes over time, as well as for comparative analysis of microbial communities inhabiting different environments. These community-profiling techniques are at most semiquantitative in nature, as the community fingerprints they generate are subject to potential PCR bias, do not directly translate into taxonomic information, and provide only an overview of the most abundant taxa.

At present, T-RFLP is the most extensively used fingerprinting method, although DGGE and, more recently, RISA are also commonly applied. T-RFLP and RISA are potentially more applicable than DGGE for comparative community analysis because they are standardized between different runs and laboratories, they can be automated (to an extent), and their data can be cross-referenced with organized sequence databases (e.g., the Ribosomal Database Project). However, obtaining consistent restriction digestion can be a challenge for

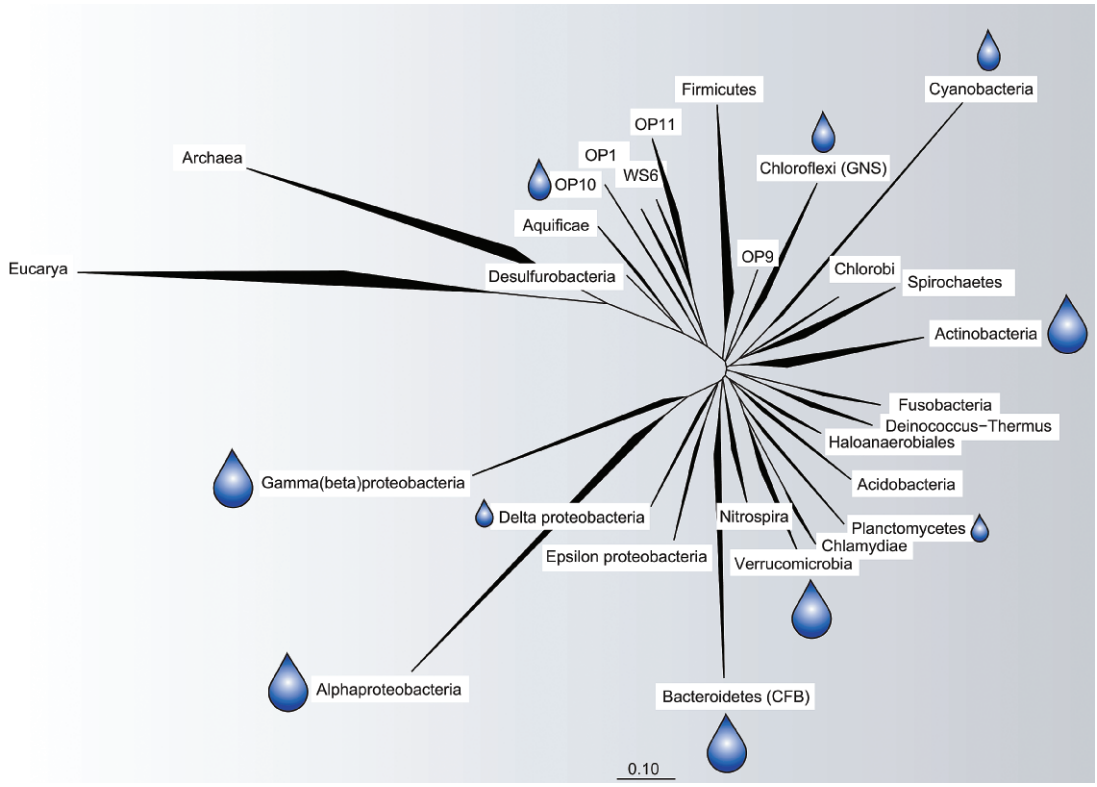

Figure 2. Phylogenetic inference tree based on small subunit ribosomal RNA sequences, showing major bacterial lineages. Large drops indicate typical and frequently dominant groups of freshwater bacteria, intermediate size drops indicate groups that contain clusters of typical freshwater bacteria that are not usually dominant, and small drops indicate other groups frequently observed in freshwater, but neither dominant nor exclusive to freshwater. Freshwater lineages are from data in Zwart and colleagues (2002). Abbreviations: CFB, cytophaga-flavobacteria-bacteroidetes group; GNS, green nonsulfur; OP and WS, candidate phylogenetic divisions. The scale bar indicates 0.10 change per nucleotide.

T-RFLP (as well as for ARDRA), and selecting the correct restriction enzyme is essential for successful application. Phylogenetic interpretation of the terminal fragments in T-RFLP, or the spacer length in RISA, can also lead to misinterpretation, because more than one species or phylogenetic group can share the same fragment length, or one organism may contain multiple deviating copies of the target sequence, resulting in more than one phylotype per strain. RISA may suffer from additional bias introduced by the different lengths of the products. DGGE is less amenable to automation, but it offers a relatively straightforward way to compare a limited set of samples, and phylogenetic information about interesting bands can be obtained by cutting bands from the gel for reamplification and sequencing. As with other fingerprinting methods, identity of phylotype and species is generally assumed, but exceptions occur.

Hybridization techniques such as fluorescent *in situ* hybridization (FISH) (DeLong et al. 1989) and reverse line blot hybridization (Kaufhold et al. 1994) use labeled oligonucleotides to detect a target sequence. For FISH, the target is ribosomal RNA in the intact cell, allowing the localization of individual cells under the microscope (figure 3). These techniques enable the analysis of bacteria communities in natural environments through visualization, taxonomic identification, and cell enumeration without potentially biased PCR amplification (Amann et al. 1995)—a large advantage over fingerprinting methods—and facilitate community analysis of species richness and diversity. One major constraint of these techniques, however, is specificity: to design oligonucleotide probes, sufficient knowledge of the community is needed in advance. If the hybridization target is ribosomal RNA (e.g., with FISH), the resulting information is limited to the phylogenetic viewpoint specific to the marker, and little can be learned about organism function.

Even though each of the methods discussed above has advantages and disadvantages, when they are used in combination, this "rRNA approach" (Amann et al. 1995) for phylogenetic discovery and community study has been highly successful. The difficulties facing researchers today relate less to the application of a specific method than to the need to understand what the methods tell us about the system and the ecological roles of the observed organisms. It is difficult to grasp the nature of freshwater environments as a microbial habitat, and even more so to determine how the measured genetic diversity relates to the actual ecological diversity and functional role of microbes (e.g., their metabolic

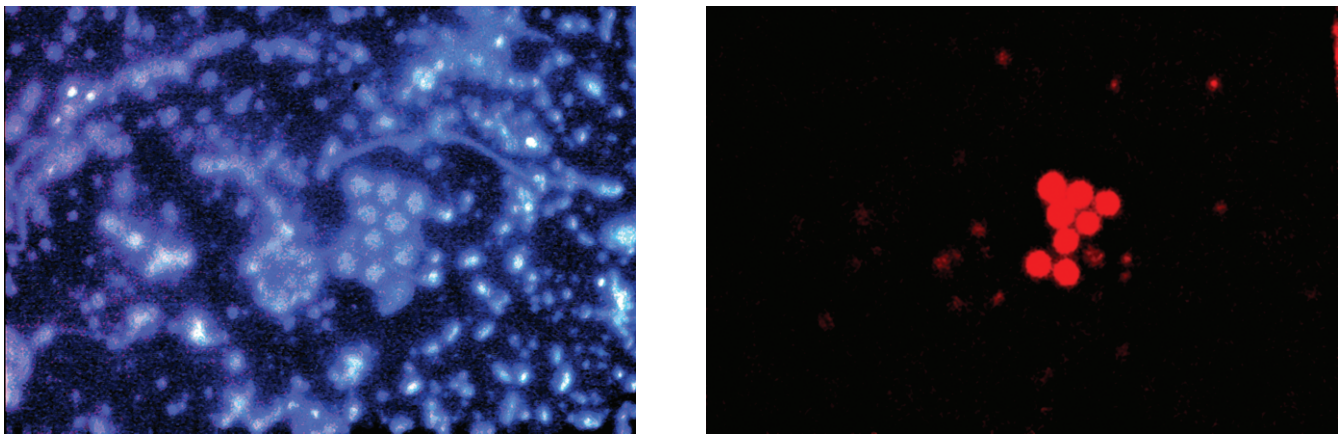

Figure 3. Photographs of a lake microbial community stained using 4', 6-diamidino-2-phenylindole fluorescent stain (left) and the same community with bacteria stained specifically by fluorescent **in situ** *hybridization (right).*

activities). This challenge leads us to a discussion of freshwater microbial species and community concepts, and thence to a view of advanced and emerging methods that may help bridge the gap between observing genetic diversity and gaining a better understanding of microbial ecology.

Current ecological perspectives on freshwater bacteria

Assimilation of the aforementioned methodological breakthroughs in freshwater microbial ecology has had notable impacts on the field. Glöckner and colleagues (2000), for instance, used clone libraries and FISH to identify globally distributed freshwater bacteria. The comparative 16S rRNA sequence analysis of bacterioplankton from three lakes in Austria, Germany, and Russia revealed that the majority of sequences had their origin in freshwater or soil. They infer the existence of a globally distributed set of freshwater bacterioplankon, and show that Actinobacteria are a bacterial cluster highly abundant in freshwater bacterioplankton communities. FISH results from Lake Gossenköllesee (Austria) showed that 49% of all 4', 6-diamidino-2-phenylindole stained cells belonged to this cluster, constituting 63% of the bacterioplankon biomass. Zwart and colleagues (2002), combining a meta-analysis of the 16S rRNA gene sequence database of freshwater bacterioplankton with clone libraries and DGGE, showed that the sequences were affiliated with 34 freshwater bacterioplankton clusters, and thereby inferred a numerically confined set and worldwide distribution of "typical freshwater bacterioplankton."

To date, freshwater bacterial communities have been shown to be characterized by Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia, and Planctomycetes (figure 2; Methe et al. 1998, Glöckner et al. 2000, Zwart et al. 2002). The ß-Proteobacteria are particularly abundant in freshwaters and are essentially absent in marine systems, although individual members have been found in coastal environments (Methe et al. 1998). There is a growing consensus that bacteria communities in different habitats are distinct, and that aquatic ecosystems support fewer taxa than do terrestrial soils (Curtis et al. 2002, Torsvik et al. 2002); there also appears to be a distinct difference in the taxonomic composition of oceanic and freshwater bacterial communities. Temporal changes in bacterial communities appear particularly pronounced in the freshwater environment. Yannarell and Triplett (2004) used an automated ribosomal intergenic spacer analysis (ARISA) to show seasonal changes in diversity and community composition in three different lakes, at a temporal resolution of two weeks.

The shift from cultivation-dependent to cultivation-independent methods constitutes a quantum leap in microbiology, one that has fundamentally changed our knowledge and perception of the microbial world in a wide range of environmental systems (e.g., terrestrial, marine, and freshwater). Despite the prodigious impacts of cultivation-independent approaches on microbiology, however, identifying, isolating, and characterizing microorganisms with respect to phylogeny as well as physiology remains a challenge for microbiologists. The phenomenon of the great-plate count anomaly is still an inherent element of microbial ecology. Drawbacks, biases, and other difficulties of molecular methodologies in use today make the choice of an appropriate method a crucial issue. We may not yet be able to overcome the discrepancy between what does exist in nature and what we manage to see, but by selecting the right technique to analyze microbial communities, we may be able to minimize the discrepancies.

Although the methodologies discussed above are used for microbial ecology studies regardless of the environmental system under scrutiny, and although they pose no fundamentally different methodological problems when applied to various habitats, studies of freshwater ecosystems are presented with unique conceptual challenges. High seasonal (e.g., flooding or mixing) and structural (i.e., hyporheic zone, sediment-water interface) variability and the inherent interrelated variability of microbial communities make freshwater systems exciting fields of study. In addition, interactions of food-web

components in freshwater systems, such as predation on bacterioplankton communities, are unparalleled.

Bacteria species concept. Despite remarkable methodological progress in assessing microbial communities, there is still strong controversy over what constitutes a bacterial species (Rossello-Mora and Amann 2001, Cohan 2002, Gevers et al. 2005). Microbiologists have yet to agree on whether bacterial species should be viewed as a cluster of phenotypically and genetically similar organisms, or whether a species should have distinct genetic, phylogenetic, evolutionary, or ecological traits. Indeed, bacterial species demarcation is more arbitrary compared with delineating species for higher organisms. Genetic diversity within bacterial species is not constrained by the cohesive force of genetic exchange as it is within highly sexual animals and plants. Lateral gene transfer, for instance, results in highly dynamic bacterial genomes, thereby convoluting bacterial phylogenies and creating major difficulties for defining a bacterial species.

A bacterial species is currently described as "a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions" (Rossello-Mora and Amann 2001). In practice, a bacterial species is often defined simply as a group of strains exhibiting more than 70% DNA-DNA-hybridization (DDH) similarity (or < 5% difference in their melting temperature, Δ Tm) and more than 97% of 16S rRNA gene sequence identity (subspecies: 75% to 80% DDH similarity and < 3% difference in Δ Tm). Furthermore, phenotypic consistency within species and differences among species are required to facilitate demarcation of genomic limits (Rossello-Mora and Amann 2001, Gevers et al. 2005). Yet this definition may critically underestimate bacterial diversity by orders of magnitude, and the usefulness of biogeographic assessments strictly on the basis of 16S rRNA sequences may be limited.

Bacterial species should be characterized by integrating phenotypic (biochemical data), genotypic (DNA fingerprinting data), and phylogenetic information (rRNA gene sequences), an approach generally known as polyphasic. But in this method, ecological species properties are entirely omitted. To address this, Cohan (2002) suggested analyzing bacterial species by smaller units that integrate the concept of the ecotype; he defines an ecotype as "a set of strains using the same or very similar ecological niches, such that an adaptive mutant from within the ecotype out-competes to extinction all other strains of the same ecotype; an adaptive mutant does not, however, drive to extinction strains from other ecotypes." Hence, ecotypes are genetically cohesive and ecologically distinct populations. We need a theoretical concept that considers biological processes affecting genetic cohesion within species and divergence among them (Curtis and Sloan 2004). A clear view of microbial diversity, as well as spatial and temporal patterns, relies on a consensus about the species concept for microbes, especially when comparing scaling relationships of macroorganisms and the generality of spatial scaling rules.

Current concepts of bacteria biodiversity. A long-standing notion among microbiologists is that "everything is everywhere and the environment selects" (Baas-Becking 1934)—that is, bacteria species will occur anywhere throughout the globe, assuming that specific habitat requirements are met. Extreme abundance, rapid proliferation, ready dispersal, and improbable extinction of bacterial species are the arguments propounded in favor of the concept of cosmopolitanism (Finlay and Clarke 1999, Fenchel and Finlay 2004). As a consequence of the absence of geographical barriers and local extinctions, every habitat will contain a majority of globally occurring bacterial species in the form of a seed bank (Finlay and Clarke 1999). The most frequently cited argument in favor of cosmopolitanism is large population size (Fenchel and Finlay 2004), which implies that dispersal is more likely and extinction is less likely (Curtis et al. 2002, Torsvik et al. 2002). On the one hand, dispersal is indeed facilitated by the small size of bacteria (Fenchel and Finlay 2004); on the other hand, the likelihood of extinction is minimized by resting or inactive stages.

Another explanation for the ubiquity of bacteria is that low rates of extinction and speciation limit local diversification. For example, Finlay and Clarke (1999) recorded 32 *Paraphysomonas* species during a study of a freshwater pond in the United Kingdom, representing 78% of the globally identified species within the flagellate genus *Paraphysomonas* at the time. Therefore, diversity would be expected to be high at the local level but low at the global level. Local environmental features are the prominent regulating factors of bacterial community assemblages within the cosmopolitan concept of biogeography (Hughes Martiny et al. 2006). Most researchers also agree that aquatic bacteria assemblages are controlled by local physico-chemical factors such as water chemistry, temperature, ultraviolet radiation, organic matter, and nutrients. One question of concern is whether bacteria assemblages exhibit biogeographical constraints and properties similar to those of higher-order organisms, and how this might relate to ecosystem function.

The metacommunity concept has evolved as a counterpart to the cosmopolitan view and is now being introduced into microbial community ecology step by step (see the review by Leibold et al. [2004] and Logue and Lindström [2008]). A metacommunity can be defined as a set of local communities that are connected by dispersal. The metacommunity concept focuses on structural and biotic processes that emerge at local and regional scales of organization. For instance, Papke and coworkers (2003) studied island-like hot-spring Cyanobacteria communities in globally distant regions (United States, Japan, New Zealand, and Italy). They observed distinct patterns of phylogeny and a distribution of genotypes consistent with geographical isolation at both global and local scales. The concept of metacommunity comprises four different perspectives that attempt to explain various aspects of

biogeographical community dynamics (figure 4; Leibold et al. 2004).

The *species-sorting perspective* assumes the existence of multiple heterogeneous patches, and emphasizes that community dynamics depend on spatially differing aspects of environmental conditions that are independent of spatial gradients because dispersal is global (figure 4a). For instance, Eiler and colleagues (2003) observed a gradual change in bacteria diversity along a dissolved organic carbon (DOC) gradient. The ß-Proteobacteria and Bacteroidetes were represented at all DOC concentrations, whereas the α-Proteobacteria subclass was present only at the lowest DOC concentration. In streams, bacteria assemblage differences have been strongly associated with differences in water source, such as between glacial and nonglacial streams (Logue et al. 2004). This view appears to support both the Baas-Becking hypothesis and the environmental variation hypothesis of Hughes Martiny and colleagues (2006).

The *patch-dynamics perspective* proceeds from the assumption that a multitude of identical patches exist (figure 4b). The stochastic and deterministic extinctions that take place in some patches are counteracted by dispersal, which provides a source of colonization into empty patches. Furthermore, the patch-dynamics perspective predicts that species composition does not vary with environmental conditions. For instance, Yannarell and Triplett (2004) demonstrated that the spatial distribution of lakes substantially affected the composition of bacteria communities. More closely situated lakes had higher similarities in bacteria diversity than more distant lakes. The patch-dynamics perspective clearly contrasts with the Baas-Becking hypothesis that everything is everywhere, instead inferring that historical events are responsible for most of the spatial variation in community assembly.

The *mass-effects perspective* anticipates environmental heterogeneity and focuses on immigration and emigration in local community dynamics (figure 4c). Asymmetric dispersal influences both immigration and emigration, and spatial community patterns may be affected by local population changes following source-sink dynamics. Depending on whether dispersal is global, the mass-effects approach predicts community changes along environmental or spatial gradients. For instance, Lindström and colleagues (2006) showed that a continuous supply of bacteria cells from the surrounding catchment strongly influenced local patterns in bacteria diversity in lakes.

Last, the *neutral perspective* assumes that all species display similar competitive abilities, mobility, and fitness. The drivers of community dynamics are stochastic probabilities of species loss (extinction, emigration) and gain (speciation, immigration; Sloan et al. 2006). Consequently, changes in community composition are a result of geographical distance, not environmental conditions. This perspective is similar to that described by Hughes Martiny and colleagues (2006), in which deterministic forces are lacking and bacteria are randomly distributed throughout space. It is most likely that both stochastic and deterministic forces act to determine global, regional, and local diversity patterns in freshwater bacteria (Loreau et al. 2003), as inferred by the metacommunity concept and related perspectives (Loreau et al. 2003, Leibold et al. 2004).

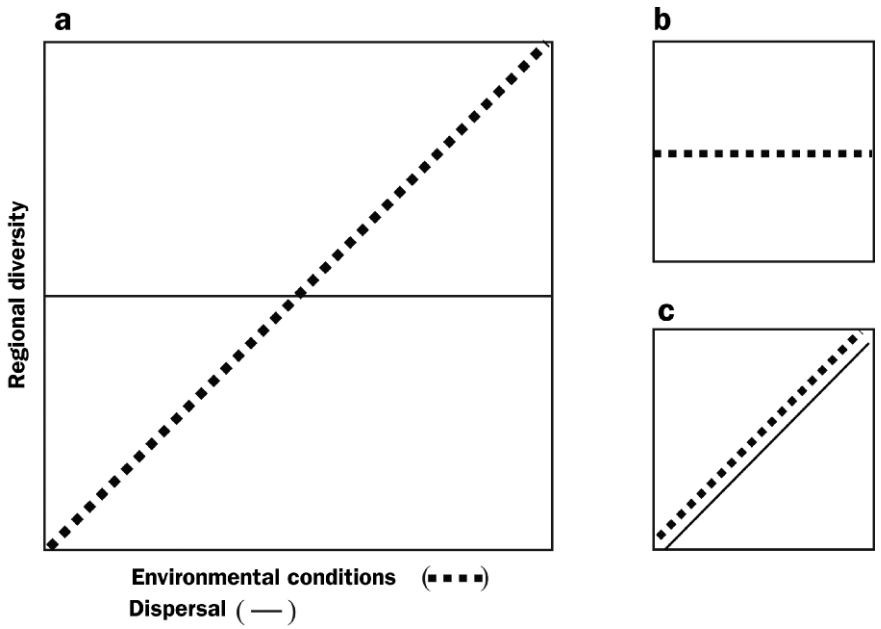

Figure 4. Schematic depicting the predictive effects of environmental conditions and dispersal on the regional diversity of bacterial communities. (a) The cosmopolitan concept, or species-sorting perspective, assumes that regional diversity varies with environmental conditions, but shows no effect of dispersal. (b) The patch-dynamics perspective and the neutral model hypothesize that diversity will show no effects with changing environmental conditions. Predictions on the relationship between regional diversity and dispersal have not been clarified to date. (c) The mass-effects perspective assumes that regional bacterial diversity varies with environmental conditions as well as with dispersal.

Advanced methods in microbial molecular ecology

Although molecular methods have revolutionized microbial ecology, the field continues to profit from novel methodological developments occurring at breakneck speed. Techniques introduced in recent years, often derived from the medical field, are important new tools for studying microbial communities in greater detail. A key issue that many of these innovative techniques address concerns better linking observed microbial diversity with ecological functions and biological strategies.

Incorporating functional information. The importance of bacterioplankton and sediment bacteria in biogeochemical cycles of freshwater ecosystems is well known (Peterson et al. 2001). Only recently have microbial ecologists begun in earnest to link microbial community structure to microbial functioning in different environments, such as in soil (reviewed by Torsvik et al. [2002]), marine (Fuhrman 2002), and freshwater (Gutknecht et al. 2006) habitats. In the context of the limitations of the current bacterial species concept, it is difficult to relate bacterial species defined by, for example, rRNA similarity to their respective functional roles because bacteria physiology can vary significantly even within so-defined "species" (Jaspers and Overmann 2004). Furthermore, some microbial functions, such as nitrogen fixation, denitrification, and the use of certain carbon substrates, are not restricted to distinct phylogenetic groups but are widespread throughout the bacterial and archaeal domains. Although new cultivation strategies, such as low nutrient concentrations, nontraditional nutrient sources, incorporation of signaling molecules, and long-term incubation, or incubation within the inoculum's original environment (Stevenson et al. 2004), are increasingly overcoming barriers to culturing many "unculturable" bacteria, culturing many microbial species remains difficult (Zengler et al. 2002), and culturing the entire natural diversity of an environment is still out of reach. To date, the use of molecular markers as proxies of functional activity has proved highly successful for studying microbial phenotypes in the environment.

Using the rRNA-based molecular toolbox for assessing microbial diversity, microbiologists have turned to functional genes as markers of functional groups. The use of functional genes as markers for studying genetic diversity requires that the gene sequence be reasonably conserved (to allow group-specific probes or primer design) and, ideally, contain phylogenetic information to facilitate interpretation of environmental sequence data. A large number of functional genes have proven amenable for studying functional processes, frequently in freshwater environments. Some examples include nitrogen fixation (*nifH*; Affourtit et al. 2001, Steward et al. 2004), denitrification (*nirS, nirK, nosZ, narG*; Smith et al. 2007), nitrification (*amoA*; Cebron et al. 2004), methanogenesis (*pmoA, mmoX*; Banning et al. 2005), and methanotrophy (Kalyuzhnaya et al. 2005). For example, this approach was used to study *nifH* along a salinity gradient of the Neuse River estuary in North Carolina (Affourtit et al. 2001), where changes in the nitrogen-fixing community were related to changes in salinity and nitrate concentration. Steward and colleagues (2004) studied the vertical distribution of *nifH* in hypersaline Mono Lake using PCR amplification, cloning, and sequencing. They found a diversity of *nifH* sequences but few changes in community composition with depth, despite strong gradients in oxygen and ammonium; however, nitrogen fixation rate measurements were below detection. This latter result emphasizes the fact that relating functional gene diversity to bacteria functioning is problematic, as bacteria may not express a particular marker gene under specific environmental conditions—that is, what is observed is a potential community, not necessarily an active one. Other kinds of data such as biochemical or isotopic measurements can be used to better understand bacterial functioning. For example, MacGregor and colleagues (2001) used a combination of nitrogen isotope measurements, microscopic techniques, and amplification and sequencing of *nifH* to show the presence of nitrogen-fixing microbes. Despite apparent phosphate limitation, active nitrogen fixation appeared to contribute significantly to the nitrogen budget of Lake Michigan.

Another approach is to obtain information on gene transcription from the environment. As transcription is often tightly regulated, and mRNA is relatively short-lived, the presence of gene transcripts in environmental samples is a good indicator of activity (Saleh-Lakha et al. 2005). Recently improved methods for RNA extraction—purification and reverse transcription—have increasingly allowed the detection of gene transcripts from sediment and water samples (Saleh-Lakha et al. 2005). Zani and colleagues (2000) were among the first to detect functional gene transcripts in a natural environment. They demonstrated that Cyanobacteria and α-Proteobacteria were actively transcribing the nitrogen fixation marker gene *nifH* in Lake George, New York. Other genes that have been studied with respect to functional gene transcription in surface-water ecosystems include chitinases (LeCleir et al. 2007), methanotrophy (Kalyuzhnaya et al. 2005), and denitrification (Smith et al. 2007). The study by Smith and coworkers (2007) is notable for its use of quantitative reverse transcription PCR to quantify copies of the environmental mRNA of various denitrification genes. They found a general trend of decreasing transcript numbers from head to mouth of the studied estuary, indicating a decreasing importance of denitrification along the salinity gradient.

Lately, a number of studies have added such a quantitative element to the study of ribosomal or functional genes by using real-time quantitative PCR (QPCR). This technology, based on the optical detection of product formation during a PCR reaction, provides accurate quantification of target sequence numbers with high specificity and sensitivity. In comparison with FISH, the advantages of QPCR lie in the lower limit of detection and the versatility afforded by being able to choose any piece of genetic information as the reaction target, such as rRNA, ribosomal spacers, functional genes, or reverse-transcribed mRNA. QPCR has been used to study, for example, functional genes like *rbcL,* a gene of the rubisco carbon fixation pathway in diatoms (Wawrik et al. 2002); phylogenetic groups for community analysis in methanogenic lake sediments (Schwarz et al. 2007); and *nirS* genes of *Pseudomonas stutzeri* (denitrification) in lake and marine samples (Gruntzig et al. 2001). Additional quantitative data on the distribution of microbial species (microbial, phylogenetic, and functional groups) or specific genes and their transcripts over time and space are important to obtain to improve our understanding of microbial dynamics in response to environmental change.

There also have been improvements in the FISH technique that now make it capable of detecting genes or low-copy RNA sequences in cells. Advanced versions of FISH (RING-FISH, CARD-FISH) may allow the possible cross-application of functional genes and microscopic techniques, thereby providing both quantitative and spatial or organizational information. RING-FISH employs multilabeled polynucleotide probes that organize themselves into large networks around the target (Zwirglmaier 2005). CARD-FISH uses horseradish peroxidase-labeled oligonucleotides to perform a signal amplification based on formation of fluorescently labeled tyramide that deposits on cellular proteins (Pernthaler and Amann 2004). Microautoradiography in combination with FISH has proven to be a valuable tool for identifying microbes active in the uptake of specific radioisotope-labeled substrates, such as methanotrophs and methylotrophs in freshwater and freshwater sediments (Schwarz et al. 2007). Microautoradiography has also been used to study microbes degrading carbon sources, such as propionate in a freshwater marsh in the everglades (Chauhan and Ogram 2006), and the microorganisms degrading chitin and amino sugars in marine systems (Cottrell and Kirchman 2000).

An ongoing development in the study of functional genes in environmental microbial communities is the examination of multiple genes in parallel, to study how different genes work together or dominate different habitats. A powerful technology designed for this purpose and recently introduced into microbial ecology is microarray hybridization (Zhou and Thompson 2002). In microarrays, multiple probes (e.g., oligonucleotides or PCR products) are immobilized on a surface such as a glass slide, and a fluorescently labeled nucleic acid sample is then hybridized to the probes. Analysis of the fluorescent signals after hybridization gives information on the abundance of each target in the sample. Arrays targeting rRNA sequences (so-called phylochips) can be used to assess community composition of a sample in a single assay, and also seem attractive for studying multiple functional genes in parallel. The high density of probes on microarrays allows the high-resolution study of genetic diversity and gene expression in communities, not only comparing different genes but also resolving homologs that correspond to different phylogenetic groups. The method was first developed for the marine environment, overcoming significant challenges (Zhou and Thompson 2002). Targeting important genes in the nitrogen cycle, Taroncher-Oldenburg and colleagues (2003) successfully applied microarrays to study a river estuary ecosystem. To date, in comparison with marine environments, for example, the distribution of functional microbial groups and functional genes and their transcripts remains relatively poorly studied in freshwater ecosystems.

Genome sequencing and metagenomics. Prominent emerging fields in environmental microbiology are genomics (Pedros-Alio 2006) and metagenomics. Metagenomics is the study of the genomic features of entire bacterial communities (Handelsman 2004). The approach usually involves DNA extraction from an environmental sample and creation of large construct libraries (bacterial artificial chromosomes or fosmid clones), followed by physiological or genetic screening and sequencing (figure 5). A considerable challenge for this approach is the enormous diversity found in many natural environments. Assembling even partial genomes of the most abundant organisms requires enormous sequencing efforts, and the assembly process is anything but simple (Venter et al. 2004, Handelsman 2004). Furthermore, there is a real danger of assembling nonexistent "chimeric" genomes. Because certain sequences may be conserved among different species, genome fragments of these species could be erroneously assembled into a continuous sequence (Venter et al. 2004). Nevertheless, this kind of study offers unique views of the entire genetic diversity in a system. As high-throughput sequencing facilities become increasingly available, this now rather expensive approach will become more feasible for a growing number of researchers, and thus our understanding of freshwater microbes will be enhanced.

Metagenomic techniques combined with different enrichment techniques are particularly promising and have already been highly successful at understanding microbial genomics and metabolic pathways in uncultured microorganisms. For instance, such studies have revealed the genes most likely involved in anaerobic methane oxidation "reverse methanogenesis" (Hallam et al. 2004) and anaerobic ammonia oxidation (Strous et al. 2006). Thus, metagenomics, as well as standard genomics of newly cultured environmental microbes, will be instrumental in understanding the genetic capabilities of microbes. Similar approaches for transcribed genes (Poretsky et al. 2005) and proteins (Wilmes and Bond 2006) will most likely provide new insights on the active genes within entire communities.

The results from metagenomic studies will very likely have a great influence on the debate regarding the microbial species concept, as they will help reveal the extent of genetic and physiological diversity at different levels of rRNA similarity and provide data on the evolution or resilience of microbial genomes under different environmental conditions. Since environmental genomics and metagenomics offer access to both functional traits and neutral genetic elements as markers of evolution, they may eventually provide a more suitable grammar for microbial diversity and evolution than the operational species concepts used today.

The road ahead

Developments in molecular techniques will keep advancing and invigorating the field of environmental microbiology. With improvements in the molecular toolkit, and with the ever-growing throughput of these methods, we are increasingly able to test ecological theory as it applies to bacterial populations. A considerable challenge is understanding microbial interactions with each other, with predators, and with the environment. What heterogeneity in time and space do microbes really experience, and how do these effects shape microbial activity and microbial reactions at different scales?

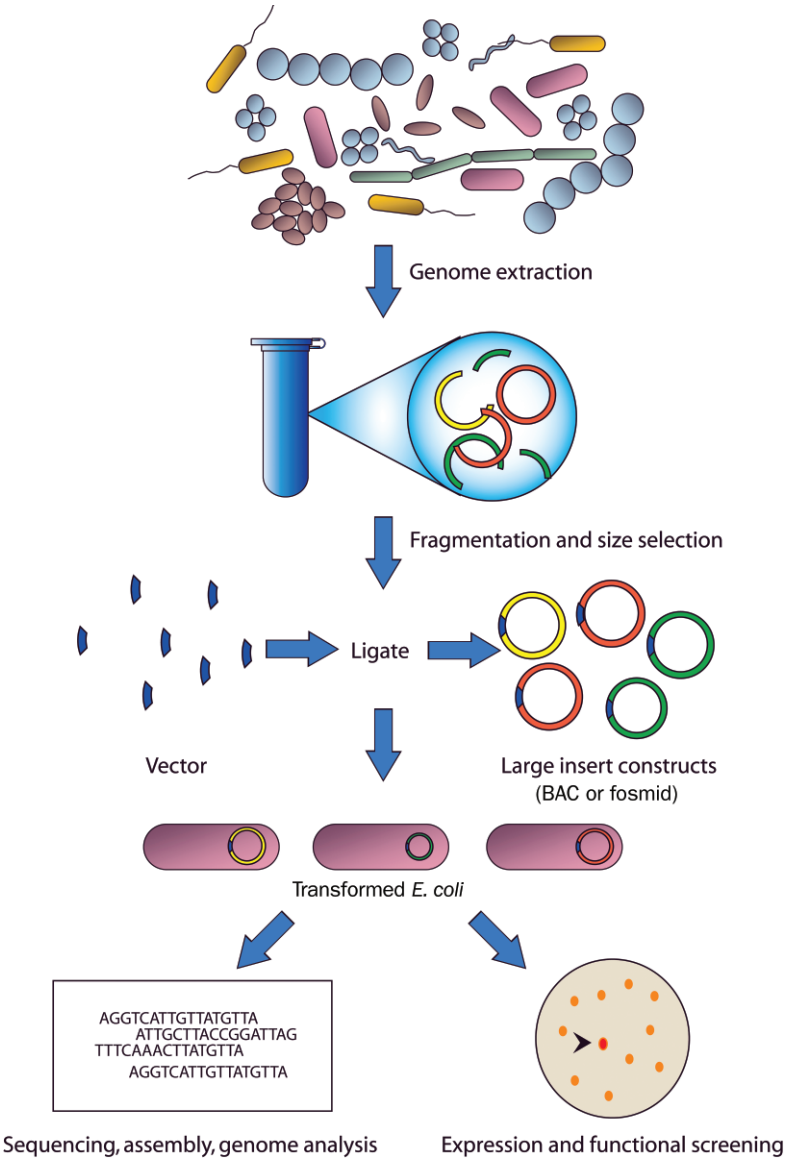

Figure 5. Schematic of the metagenomics approach. Genomes of environmental microbial communities are extracted and cloned into large construct vectors. After transforming a host bacterium with the vector, the clones can be further subcloned and sequenced, or screened for the expression of specific properties. Abbreviation: BAC, bacterial artificial chromosome.

This is a question not only for microbiologists but also for biogeochemists. Emerging methods to characterize environments at the microscopic level—for example, raman microspectroscopy (which provides chemical information at the micrometer scale) or micro-SIMS (secondary ion mass spectrometry; allows isotopic measurements at the microscopic scale)—may give new insights into the true shape and nature of the microbial environment. Automated sampling and analysis systems for monitoring microbial communities are already in development (Chandler and Jarrell 2004) and will produce data on microbial community dynamics with much improved temporal and spatial resolution, a development that will be especially significant for the study of the highly dynamic freshwater bacterioplankton.

The knowledge to be gained with these tools will help us better understand the microbial component in nutrient cycles and their involvement in feedback loops in a changing global environment. A better understanding of the spatial and temporal niches of microbial populations will also lead to an improved understanding of the differences in diversity and species composition between different habitats.

As molecular methods increase in throughput and information content, dealing with these data will become a challenge of its own for the field of bioinformatics. Maintaining databases for sequence and other molecular data is already a massive undertaking and will remain a challenge as the generation of sequencing data skyrockets. Likewise, efficient data mining and linking to environmental metadata is an important issue. Molecular methods will certainly continue to advance our understanding of the ecology of freshwater bacteria. Considering that freshwater is one of the most important resources for humans and that it sustains very diverse ecosystems, our knowledge of freshwater microbial ecology is still poor compared with, for example, marine systems. With a powerful and expanding toolbox at hand, exciting decades of discovery lie ahead for scientists in this field.

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