Stable Isotope Probing Links Taxonomy with Function in Microbial Communities

Microbial ecologists have a culture-independent means for analyzing sources of metabolic activities among complex mixtures of microbes

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Stable isotope probing (SIP) is proving to be a powerful tool for molecular microbial ecologists, providing a culture-independent means of investigating populations that conduct particular metabolic processes in specific environments. Moreover, this alternative means for linking taxonomic identity with function may also assist microbiologists in developing strategies for isolating elusive microorganisms that are involved in biogeochemical processes.

One approach relies on density gradient centrifugation to separate organisms on the basis of having different guanidine-cytosine (GC) contents. Furthermore, with steadily increasing availability of ^{13}C-enriched substrates and the potential of using other isotopes such as ^{15}N, microbial ecologists can now address challenging questions surrounding natural processes.

Several New Approaches to Studying Microbes in Ecological Settings

Microbial ecologists face ongoing challenges when trying to identify members of microbial communities that are directly involved in particular metabolic processes in specific natural environments. Traditionally, microbiologists were trained to isolate and culture microorganisms from samples. However, relying on this approach can bias results in favor of rapidly growing microorganisms, which may not be responsible for the specific metabolic processes of interest.

Advances in molecular techniques are providing alternative strategies for microbial ecologists to characterize organisms within particular habitats. In some instances, ecosystem function can be inferred by relating the microbial groups to the physical, chemical, and biological characteristics of the particular environment in which they are growing. This strategy can work well in simple, structured environments where local microbial communities are dominated by only a few morphologically distinct organisms. In more complex communities where the microbial populations are heterogeneous, it remains difficult to unravel specific ecological roles played by particular organisms within a group.

Several recently developed techniques relate phylogeny to ecosystem functions, providing insights into ecological questions. One example is microautoradiography, which when combined with fluorescent in situ hybridization (FISH) of rRNA signatures, determines the uptake of specific radiochemicals by individual cells. However, FISH requires large numbers of probes to provide taxonomic resolution equivalent to that of rRNA-based cloning or fingerprint techniques.

Another approach relies on using lipid biomarkers to analyze functionally active components within a microbial community. This method uses stable carbon isotopes to label specific lipid biomarkers, such as phospholipid fatty acids (PLFA). Samples are incubated with a ^{13}C-enriched substrate, and the biomarkers are extracted and analyzed by isotope ratio mass spectrometry. An advantage of PLFA analysis is that it provides information on biomass of different groups. There is also the possibility of obtaining composite PLFA profiles from mixtures of uncultivated microbes. Despite an increasing knowledge of signature lipids that can

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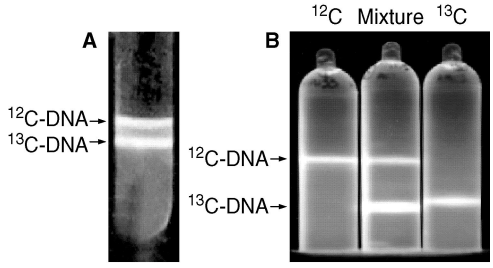
FIGURE 1



(a) Density gradient of isotopically labelled DNA from a sediment enrichment culture incubated with [13C]-CO2 (Whitby et al., 2001.) Copyright permission pending from Blackwell Publishing Ltd. (b) Density gradient of DNA (pure fractions and a mixture of DNA) from *Methylobacterium extorquens* grown on either -[^{12}C]- or -[^{13}C]-methanol (Radajewski et al., 2000. Copyright permission obtained from Nature Publishing Group http://www.nature.com/)

be used to identify specific microbial groups, however, this method is limited in its resolution, and hence, applicability.

Michael Friedrich and colleagues at the Max Planck Institute for Terrestrial Ecology, Marburg, Germany, recently developed techniques for enhancing the sensitivity of DNA- and RNA-based SIP, including quantitative analysis of isopycnic centrifugation gradients. Moreover, they are combining RNA- and DNA-SIP to monitor the dynamics of methylotroph communities and their trophic interactions with fungi and protozoa in rice field soils. Their experiments also illustrate how SIP-based techniques can be used to analyze microbial food webs and interactions.

Stable Isotope Probing Encompasses a Broad Range of Taxa

Labeled nucleic acids are biomarkers with the potential to identify a very wide range of microbial taxa. For example, Colin Murrell and his collaborators at the University of Warwick, United Kingdom, began using SIP as a method for linking metabolic processes in situ directly with specific members of particular microbial communities. Their analytical approach involves adding to environmental samples a substrate labeled with a stable isotope. To date, their efforts have involved ^{13}C-labeled substrates, but other stable isotopes could also be used.

During incubations, such labels are incorporated into the biomass of organisms actively assimilating that labeled substrate. Once nucleic acids become labeled, they can be used as biomarkers. SIP therefore provides a targeted approach to the analysis of in situ ecosystem functions. A typical analysis entails separating the labeled and unlabeled nucleic acids by means of isopycnic density gradient centrifugation (Fig. 1a and b).

Early applications of SIP involved characterizing organisms that metabolize one-carbon (C-1) compounds in soils. In those experiments, ^{13}C-labeled methanol was added to soil microcosms, which were incubated aerobically. Then the ^{13}C-enriched DNA was stained with ethidium bromide and separated from the ^{12}C-DNA in a cesium chloride gradient. Both labeled and unlabeled DNA fractions can be retrieved using a needle and syringe under UV illumination.

As little as 20% incorporation of a ^{13}C-labeled substrate into DNA is sufficient for resolving ^{13}C-DNA from ^{12}C-DNA using this approach. In this case, approximately 15 μg of ethidium-bromide-stained DNA are required to visualize distinct bands under UV illumination, and this quantity of DNA sometimes proves difficult to recover from natural environments such as oligotrophic freshwater lakes where biomass is low. However, Murrell and his colleagues have shown that small amounts of ^{13}C-DNA can be removed from gradients on a "blind" basis, when relying on PCR amplification to recover those labeled but otherwise invisible DNA sequences.

The sensitivity of the SIP method was greatly enhanced by an rRNA-based SIP approach developed at the Centre of Ecology and Hydrology (CEH), Oxford, by Mike Manefield and coworkers. They can analyze as little as about 500 ng of rRNA on cesium trifluoroacetate gradients (CsTFA), using equilibrium density centrifugation to separate labeled and unlabeled RNA molecules. In such CsTFA gradients, labeled and unlabeled RNA molecules are distributed over several fractions, and typically are detected using RT-PCR and DGGE analysis.

There is an advantage to targeting 16S rRNA molecules rather than 16S rRNA genes. The molecules in the ribosomes are present in higher copy numbers, show higher turnover rates, and are produced independently of cellular replica-

tion. Since RNA molecules offer the same sequence-based resolution for identifying an organism as DNA analysis, this approach provides a useful biomarker for linking metabolic activity with the organisms responsible for producing it.

SIP Is Being Applied in a Variety of Ecological Settings

SIP unambiguously links taxonomic identity to metabolic function across a wide range of ecosystems. It is applicable to microorganisms involved in driving natural biogeochemical processes such as the cycling of carbon and nitrogen and also to those organisms involved in degrading xenobiotics. Moreover, it provides a culture-independent means of investigating how complex microbial communities respond to changes in temperature, pH, and substrate concentrations.

Specifically, Stefan Radajewski and colleagues at Warwick have shown that SIP is useful for investigating methanol-consuming microorganisms in soil. They used ^{13}C-labeled DNA to analyze microorganisms in an oak forest soil that used methanol as a metabolic substrate for growth. Phylogenetic analysis of the 16S rRNA bacterial gene sequences amplified from the ^{13}C-containing DNA indicated that these soil-dwelling organisms belonged to two phylogenetically distinct groups of eubacteria, the α-Proteobacteria and *Acidobacterium* lineages.

In addition to that soil ecosystem, Elena Hutchens and colleagues at the University of Warwick used DNA-SIP to characterize active aerobic methanotrophic bacteria in a closed environment. Organisms in a natural groundwater system in Movile Cave, whose atmosphere contains 1–2% ambient methane plus higher concentrations in gas bubbles that support the growth of microbial mats, were studied. To identify the active methanotrophs present in the water and mat material, microcosms were incubated with 10% ^{13}C-labeled methane in the headspace. Subsequently, the ^{13}C-DNA was analyzed by looking at both 16S rRNA genes and functional genes by using primer sets that target methane monooxygenase genes. The analysis indicates that strains of *Methylomonas*, *Methylococcus,* and *Methylocystis/Methylosinus* assimilate the labeled methane. These researchers surmised that aerobic methanotrophs were actively converting methane into complex organic compounds in the cave that are used by a diverse microbial community in this closed ecosystem.

DNA-SIP has also been successfully applied by Ju-Ling Lin and coworkers at Warwick to study methanotrophs in soda lake sediments. Methanotrophs in these highly alkaline environments appear to be dominated by Type I methanotrophs of the genera *Methylomicrobium* and *Methylobacter*.

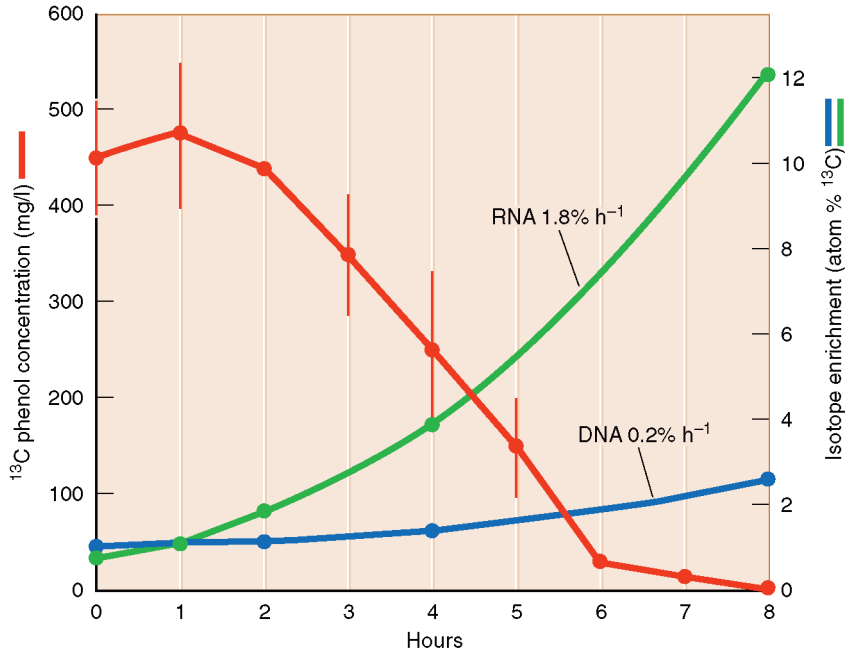
SIP also Provide Insights into Bioremediation

Still other groups of soil-dwelling methylotrophs, specifically those that degrade methyl bromide and methyl chloride, can be identified by using SIP, according to Larry Miller and Ron Oremland at the U.S. Geological Survey in Menlo Park, Calif., working in collaboration with Colin Murrell and colleagues at Warwick. Their approach entailed adding ^{13}C-labeled methyl bromide and chloride to microcosms and then amplifying with PCR primers for 16S rRNA genes and also for *cmuA*, which encodes a specific methyltransferase involved in methyl halide metabolism. Their results suggest that the diversity of methyl halide degraders in soils is considerably greater than one would suppose based on those few strains that have been isolated. *Hyphomicrobium* spp. seem to be the dominant MeCl-utilizers in woodland soils.

Corinne Whitby and colleagues at Liverpool University applied SIP to analyze one component of the nitrogen cycle, namely nitrification in freshwater sediments. In this study, ^{13}C carbon dioxide was added to sediment enrichment cultures to identify active members within the autotrophic-ammonia oxidizing communities in a hypereutrophic freshwater lake. Sequence analysis of the labeled fraction indicates 97% homology with *Nitrosomonas europaea-eutropha*. Maneesha Ginige, at the University of Queensland, Australia, looked at denitrification at a wastewater treatment plant. SIP combined with FISH/microautoradiography identify a microbial community at a wastewater treatment plant that can use methanol as a carbon substrate during denitrification.

Researchers seeking to understand particular bioremediation processes often extensively characterize genes and proteins involved in the relevant degradative biochemical pathways. Typically, this information is obtained by study-

FIGURE 2



Degradation of phenol-$^{13}C_6$ concentration (▲) and subsequent increase in ^{13}C atom percent of RNA (●) and DNA (■) in the microbial community from an industrial bioreactor.

ing culturable isolates that degrade the compounds of interest.

However, more recently the RNA-SIP approach is being applied to identify key organisms involved in bioremediation processes. For example, Mike Manefield and colleagues at the Centre for Ecology and Hydrology, Oxford, are using RNA-SIP to analyze how microbial communities deal with phenol, a recalcitrant compound that is widely distributed, highly toxic, and difficult to biodegrade. In their bioreactor study (Fig. 2), RNA-SIP analysis indicates that the percentage of ^{13}C in RNA increases more than 10-fold as the cells use labeled phenol, while that of DNA increases 2-fold. Subsequently, they learned that a member of the *Thauera* genus is critical for degrading phenol and controlling this process. This type of information could not have been obtained without the use of the SIP-based method.

Recognizing Several Limitations of SIP

Although SIP represents an important advance in microbial ecology, using this method has some disadvantages. For instance, researchers assume that microbial populations do not discriminate between labeled and unlabeled substrates but, instead, will use either one equally. However, small differences in the natural abundance of ^{13}C and ^{12}C isotopes may lead to preferential assimilation of compounds containing the latter isotope in many biochemical processes. This, variation in $^{13}C/^{12}C$ ratios occurs due to differences in substrate, metabolic pathways, or environmental factors.

One problem in trying to label DNA with a stable isotope is that the organism being observed has to be actively replicating when the isotope is added, which may not always be the case. However, this problem can be circumvented by doing RNA-SIP instead.

Another consideration is that indigenous unlabeled substrates may dilute the ^{13}C-labeled substrate being added to a particular environment, thereby reducing the proportion of label incorporated into the biomass. To produce detectable levels of material, it may be necessary to add excess labeled substrate for extended incubations. As with culture-based methods, these excesses could well bias the results—for example, by promoting growth of particular organisms and promoting substrate cross-feeding. Long incubation times can also lead to ^{13}C-labeled metabolites being assimilated by secondary users within a microbial community.

Production of corresponding ^{12}C-labeled compounds and other trophic interactions would dilute labeled substrates in complex systems such as soils. This problem also comes up when studying pathways in which a microbial consortium generates many intermediates while degrading compounds, making it impossible to unravel the role each organism plays in the overall process. However, this may be overcome by labeling the intermediate substrates and tracking the flux of ^{13}C uptake through different members of the consortium. Another approach would entail introducing compounds containing different isotopes, such as ^{15}N, ^{2}H, and ^{3}H.

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