

Stable Isotope Probing: Linking Functional Activity to Specific Members of Microbial Communities

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Linking organisms or groups of organisms to specific functions within natural environments is a fundamental challenge in microbial ecology. Advances in technology for manipulating and analyzing nucleic acids have made it possible to characterize the members of microbial communities without the intervention of laboratory culturing. Results from such studies have shown that the vast majority of soil organisms have never been cultured, highlighting the risks of culture-based approaches in community analysis. The development of culture-independent techniques for following the flow of substrates through microbial communities therefore represents an important advance. These techniques, collectively known as stable isotope probing (SIP), involve introducing a stable isotope-labeled substrate into a microbial community and following the fate of the substrate by extracting diagnostic molecular species such as fatty acids and nucleic acids from the community and determining which specific molecules have incorporated the isotope. The molecules in which the isotope label appears provide identifying information about the organism that incorporated the substrate. Stable isotope probing allows direct observations of substrate assimilation in minimally disturbed communities, and thus represents an exciting new tool for linking microbial identity and function. The use of lipids or nucleic acids as the diagnostic molecule brings different strengths and weaknesses to the experimental approach, and necessitates the use of significantly different instrumentation and analytical techniques. This short review provides an overview of the lipid and nucleic acid approaches, discusses their strengths and weaknesses, gives examples of applications in various settings, and looks at prospects for the future of SIP technology.

Abbreviations: DGGE, denaturing gradient gel electrophoresis; FISH, fluorescent in situ hybridization; GC, gas chromatography; GC-C-IRMS, gas chromatography–combustion–isotope ratio mass spectrometry; GC-MS, gas chromatography–mass spectrometry; GC-Py-IRMS, gas chromatography–pyrolysis–isotope ratio mass spectrometry; IRMS, isotope ratio mass spectrometry or isotope ratio mass spectrometer; NA-SIP, stable isotope probing of nucleic acids; PCR, polymerase chain reaction; PLFA, polar lipid-derived fatty acids; rDNA, DNA encoding ribosomal RNA; rRNA, ribosomal RNA; RT-PCR, reverse transcription polymerase chain reaction; SIMS, secondary ion mass spectrometry; SIP, stable isotope probing; SSU, small subunit.

An ongoing challenge for microbial ecologists is to identify which organisms are carrying out various metabolic activities in their natural environments. Until recently, the only way to approach this question was to inoculate enrichment cultures with natural samples, determine what bacteria grew in the cultures, and make inferences from that data about activity in the environment. The development of culture-independent molecular biological tools for characterizing the components of microbial communities revealed the inherent weakness of this approach: only a small percentage of the organisms found in soil commu-

nities had ever been cultured (Giovannoni et al., 1990; Ward et al., 1990; Amann et al., 1991, 1995; Fuhrman et al., 1992; Rappe and Giovannoni, 2003). If culturing soil organisms in the laboratory with standard enrichment techniques automatically eliminates most of the species present, the data garnered from enrichment cultures may not be very relevant to natural settings. An excellent discussion of the problem of ecological significance in laboratory culture experiments was presented in a recent perspective (Madsen, 2005). An ideal experimental approach to dissecting community function would be one that allowed investigators to trace substrate usage to specific organisms without disturbing community structure.

Ecologists have used stable isotope ratios to answer questions about resource use for several decades. It has long been established that the stable isotope ratios of tissues and molecules of organisms are a function of the isotope ratios of the substrates they assimilate (DeNiro and Epstein, 1978, 1981; Estep and Dabrowski, 1980). This relationship has been used to decipher food webs, reconstruct ancient and modern diets (Schoeninger et al., 1983; Macko et al., 1999; Kelly, 2000), and even to link cultured microorganisms to laboratory water and media for forensic attribution (Kreuzer-Martin et al., 2003, 2004).

The past few years have seen the development of techniques for using light element stable isotopes to follow the flow of substrates through microbiological communities and identify which members of the community are taking up the substrate without laboratory

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culturing. The general approach has been to introduce a stable isotope-labeled substrate into a microbial community and follow the fate of the substrate by following the appearance of the isotope in diagnostic molecules. The types of molecules and the approaches used to analyze them vary, but the techniques as a whole have come to be known as stable isotope probing. Stable isotope probing allows direct observations of substrate assimilation in minimally disturbed communities, and thus represents an exciting new tool for linking microbial identity and function in a natural setting.

Stable isotope probing has become possible because of various combinations of advances in stable isotope ratio measurement technology and molecular biology. The molecular biology advances in microbial identification techniques and bioinformatics have been described elsewhere. This short review presents a brief introduction to the relevant stable isotope ratio measurement techniques, reviews approaches to stable isotope probing and the application of the approaches in various settings, and looks at prospects for the future of the technology.

STABLE ISOTOPE RATIO ANALYSIS

The stable isotope ratios of light elements (e.g., C, N, O, H, and S) are measured on specialized mass spectrometers (isotope ratio mass spectrometers, or IRMSs) with fixed magnets and Faraday cups positioned to detect a small number of masses. This configuration generates very high sensitivity and precision, enabling the accurate calculation of isotope ratios, but gives no molecular structure information. Isotope ratios can be determined with quadrupole mass spectrometers, but these determinations require a much higher concentration of the rare isotope than when an IRMS is used. The IRMSs measure isotope ratios in comparison to reference materials, which are analyzed in parallel with the samples. In natural-abundance studies, isotope ratios are expressed in relation to international standards as delta (δ) values in parts per thousand. In enrichment studies and SIP literature, isotope ratios are often expressed as atom percent, which expresses the number of atoms of the isotope in question for every one hundred atoms of that element in the sample.

Samples are introduced into an IRMS as gases, and therefore solids and liquid samples must be converted into gases before analysis. Offline methods have been developed for producing gases suitable for injection into an IRMS from numerous materials, but the development of a variety of sample introduction interfaces has obviated the need for many of them. The interfaces for solids and liquids are furnaces that convert samples to gases at high temperatures in the presence (combustion) or absence (pyrolysis) of O₂. Combustion is typically used for the analysis of C (as CO₂), N (as N₂, combustion gases are passed over a reduction column to convert nitrogen oxides to N₂), and S (as SO₂). Pyrolysis in the presence of glassy C is used for the analysis of O (as CO) and H (as H₂) from organic materials. Analytical methods for IRMS have been well described (DeGroot, 2004).

Combustion and pyrolysis furnaces require that the sample be in the desired state of purity when it is introduced into the interface, and these systems typically require at least tens of micrograms of the element to be analyzed. If the isotope ratio of an individual compound is desired, that compound must first be purified in sufficient quantity before introduction. These requirements put severe constraints on the use of stable isotope ratio measurements in the context of environmental microbiol-

ogy, although laboratory-produced material can be readily analyzed (MacGregor et al., 2002; Kreuzer-Martin et al., 2004).

Compound-Specific Isotope Ratio Analysis

A key development in isotope ratio analysis came in 1990 with the linking of gas chromatography (GC) and IRMS. A gas chromatograph–combustion furnace (GC-C) interface was developed in which the effluent stream from a GC column was fed into a combustion furnace (Hayes et al., 1990). As the separated compounds flowed into the furnace, the C in each compound was converted into peaks of CO₂ that then flowed sequentially into the IRMS, permitting the measurement of the C isotope ratio of each eluted compound. This GC-C-IRMS technology makes it possible to determine the C isotope ratios of individual volatile compounds in mixtures. The analysis of C stable isotope ratios of individual lipids isolated from environmental samples has become one of the two fundamental approaches in SIP (Zhang, 2002; Pancost and Damste, 2003). An interface that feeds a GC effluent stream into a pyrolysis reactor, enabling H isotope ratio determinations of individual eluted compounds (GC-Py-IRMS), has also been developed (Burgoyne and Hayes, 1998). The investigation of the relationship of H isotope ratios of individual compounds to growth conditions is beginning and is finding potential applications in the reconstruction of past environmental conditions (Sessions et al., 1999, 2002; Sauer et al., 2001).

A recent exciting innovation in compound-specific IRMS technology is a moving-wire interface for liquid chromatography–IRMS for the analysis of small (roughly 10 nmol) amounts of organic C from nonvolatile compounds such as sugars, nucleic acids, and proteins (Sessions et al., 2005). This device promises to streamline the analysis of many individual compounds of biological interest and will undoubtedly find myriad applications in fields from environmental science to medicine.

STABLE ISOTOPE PROBING

Stable isotope probing involves exposing a microbial community to an isotopically labeled substrate of interest, and using the subsequent appearance of the heavy isotope in biomarker molecules as an indication that the source of those biomarkers was active in the assimilation of the substrate. To date, lipids and nucleic acids have been used as the biomarker molecules. The first published example of SIP (Boschker et al., 1998) used polar lipid-derived fatty acids (PLFA) as the indicator molecular species, and this approach to SIP has been reviewed (Boschker and Middelburg, 2002; Zhang, 2002; Pancost and Damste, 2003). The term SIP did not come into use to describe the approach until 2000, with the publication of the first demonstration of SIP using DNA (Radajewski et al., 2000). Since then, the term has sometimes been used to describe only nucleic acid techniques (Radajewski et al., 2003; Wellington et al., 2003), and sometimes to denote both nucleic acid and lipid techniques, as in a recent excellent overview (Dumont and Murrell, 2005). Here the term is used inclusively.

Although the logic behind SIP of lipids and nucleic acids is similar, the techniques and instrumentation requirements are quite different. Lipid-SIP requires the separation of lipids isolated as mixtures and the measurement of stable isotope ratios of individual lipids, necessitating the use of chromatography and mass spectrometry. In contrast, nucleic acid SIP (NA-SIP) does not require that isotope ratio measurements be made. Instead, NA-SIP exploits the greater

buoyant density conferred on nucleic acid molecules when they incorporate a heavy isotope instead of a light one. Following exposure of the community to a labeled substrate, the nucleic acids of organisms that assimilated the labeled substrate are separated from the nucleic acids of those that did not by isopycnic centrifugation. The nucleic acids are subsequently recovered from the density gradients and analyzed. No stable isotope ratio measurement is required, although they are sometimes made as adjuncts to the experiments, for example of respired CO₂. Both the relative specificities of lipids and nucleic acids as biomarkers and the technology used to analyze them in SIP affect the utility of the two approaches.

Stable Isotope Probing of Lipids

Fatty acid and lipid profiling are well-established techniques for the identification of microorganisms and characterization of microbial communities (White et al., 1994; Cavigelli et al., 1995; Zelles, 1999). In SIP of lipids, the approach is to expose a community of interest to an isotopically labeled substrate (usually ¹³C), then isolate lipids from the community and trace the label to specific molecules. The lipids are extracted from soil in a mixture, the components of which can be characterized by conventional gas chromatography–mass spectrometry (GC–MS). Most published studies have focused on PLFA, but simple and complex hopanoids have also been analyzed (Crossman et al., 2001). Examples of chromatograms, data analysis, and a tabulated list of current publications in this field can be found in a recent review of PLFA-SIP of microbial communities (Evershed et al., 2006).

In an alternative approach, Alexandrino et al. (2001) used deuterium as the isotopic label to follow biodegradation of styrene. Because the degree of deuterium enrichment was very large, they were able to monitor the appearance of deuterium in lipids by conventional GC–MS rather than by IRMS. For this type of analysis, GC–Py–IRMS could also be used.

A distinct advantage of lipid-SIP is that the identification of labeled lipids does not depend on extensive incorporation of a heavy isotope, in contrast to NA-SIP (discussed below). All the lipids in a sample, labeled or not, are extracted together and separated by GC. Isotope ratio measurements of each individual eluting peak are made, permitting even small differences in ¹³C content to be detected. This advantage is particularly important when experimental conditions work against heavy labeling. Such conditions could be a requirement for a very brief labeling period, the desire to follow a label through more than one trophic level, or circumstances where alternative C (or other label) sources may be available to the community. Any of these conditions could work against the heavy labeling required for NA-SIP and make lipid-SIP a more productive approach. In addition, biosynthesis of lipids, like that of RNA, does not require cell replication.

Lipid-SIP has two major disadvantages. The first is that with the exception of a few biomarker lipids, lipids are not highly phylogenetically specific molecules. Many of the same fatty acids are made by different organisms, and fatty acid profiles of individual organisms can change with environmental conditions (see reviews cited above). In addition, since the lipids of all organisms in a sample are extracted together, the enriched fatty acid profile probably represents a group of an unspecified number of unknown organisms. Within those limitations, however, it is possible to extract information as to general groups of organisms, and some fatty acids are synthesized by quite restricted clades. Lipid-SIP has

been very useful in situations where a restricted number of organisms are likely to be metabolically active or when the organisms of interest produce biomarker fatty acids (Pelz et al., 2001; Elvert et al., 2003; Tillmann et al., 2005).

The second disadvantage of lipid-SIP is that if an organism has not been cultured, its lipid profile is unknown. Unlike a previously unencountered DNA or RNA molecule, which can be amplified, cloned, and sequenced, a previously unknown lipid profile could be embedded within the composite enriched lipid profile and would be difficult to specify unambiguously. If the isotopically enriched lipid profile contained an unusual pattern, it would not be clear whether it was entirely novel or the sum of a known profile plus an unknown one. When researchers encounter novel lipid profiles, they can compare them to known profiles but at this point no further progress can be made to identify the relevant organism(s) without application of additional techniques. The presence of novel lipids does, however, indicate that a previously uncharacterized organism is playing an important role in the process being studied (Knief et al., 2003).

Stable Isotope Probing of Nucleic Acids

In NA-SIP, the community, either in the field or in microcosm, is exposed to a labeled substrate (again usually ¹³C). After labeling, either DNA or RNA is isolated and subjected to buoyant density gradient centrifugation. The nucleic acids of organisms that have assimilated the ¹³C have a higher buoyant density than the nucleic acids of organisms that have not. The ¹³C-containing nucleic acid will be separated from unlabeled nucleic acid during the centrifugation process, and labeled nucleic acid will be concentrated in heavier gradient fractions.

Following centrifugation, the nucleic acid at various positions in the gradient is collected and typically amplified by the polymerase chain reaction (PCR) or reverse transcription (RT) PCR using primers complementary to 16S rDNA, or rRNA. Specific functional genes are also occasionally used as amplification targets. Amplified products can be separated by denaturing gradient gel electrophoresis (DGGE), cloned directly, or subjected to terminal restriction fragment length polymorphism analysis for phylogenetic characterization. The efficacy of NA-SIP is clearly dependent on the efficiency and biases of each step in this process, from label assimilation to nucleic acid extraction and purification to PCR.

DNA STABLE ISOTOPE PROBING

In the first published example of DNA-SIP, Radajewski et al. (2000) produced pure cultures on ¹³C methanol and ¹³C methane and demonstrated that DNA extracted from these cultures could be separated from DNA produced on ¹²C substrates by isopycnic centrifugation in CsCl. The ¹²C- and ¹³C-DNA bands in the CsCl gradient were separated by about 1 cm and could be easily isolated individually and analyzed. A modified centrifugation later gave twice the separation of ¹³C- and ¹²C-DNA (Hutchens et al., 2004).

Radajewski et al. (2000) isolated DNA from their gradient by removing it with a syringe while visualizing it directly via ethidium bromide staining, which requires about 15 µg of DNA (Lueders et al., 2004a). This approach is quite effective if there is sufficient sample to see, but it is not necessary to see the DNA to isolate it. Padmanabhan et al. (2003) used a control labeled culture to visualize where the ¹³C-DNA band should be. They then harvested that area of a gradient containing experimentally labeled DNA from soil field tests even though no stained DNA was visible at that position.

They successfully recovered clones of rDNA following PCR amplification (Padmanabhan et al., 2003). In controls to which they added no ^{13}C substrate, they did not recover clones from the ^{13}C -DNA position in the gradient. This approach has been used successfully in other studies to isolate smaller quantities of labeled DNA.

A question that arises frequently in such studies is whether the " ^{13}C position" in the gradient also contains unlabeled ^{12}C -DNA. Padmanabhan et al. (2003) reported detecting no cross-contamination, but other investigators have reported finding ^{12}C -DNA in the ^{13}C -DNA gradient position and vice versa. In careful studies of the separation of ^{12}C - and ^{13}C -nucleic acids by isopycnic centrifugation, Lueders et al. (2004b) prepared both ^{12}C - and ^{13}C -DNA from pure cultures of several organisms. They measured the buoyant density of individual DNA preparations and found a difference of about 0.03 g/mL between species, while the difference between ^{12}C and ^{13}C of a single species was 0.04, suggesting that the separation of light and heavy DNA of mixed populations might not be as clear-cut as previously thought. Lueders et al. (2004b) additionally centrifuged mixtures of ^{12}C - and ^{13}C nucleic acids and analyzed fractions collected from the gradient with a highly sensitive real-time PCR assay. They detected nonspecific nucleic acid in all gradient fractions. Other investigators have since reported detecting a small amount of contaminating ^{12}C -DNA in the ^{13}C -DNA fractions they isolated from labeled environmental samples (Singleton et al., 2005). When short-term pulses of label are applied to complex samples, as is typical for many environmental analyses, the products are likely to form a smear of DNA molecules of varying buoyant density. Under these circumstances, harvesting DNA gradients in fractions and analyzing the content of each separately (as in RNA-SIP below) would probably yield the most complete data set.

A study published in 2005 demonstrated that the addition of ^{13}C -labeled archaeal DNA to a CsCl gradient as a carrier could increase the sensitivity of ^{13}C -labeled eubacterial DNA detection in the gradient (Gallagher et al., 2005). Gallagher et al. (2005) reported being able to detect labeled eubacterial DNA by PCR of rDNA and of a functional gene only 1 h after the addition of labeled substrate to a microcosm. Their assay detected no DNA in the ^{13}C region of the gradient containing DNA from a microcosm fed ^{12}C substrate. With time, they saw a shift in the composition of the labeled population, an indication of either cross-feeding (see below) or the assimilation of substrate by slower metabolizing members of the consortium.

The use of elements other than C for labeling DNA has been briefly explored but not developed. In their original SIP study, Radajewski et al. (2000) produced a culture in CD_4 . Following isopycnic centrifugation, D-nucleic acid was separated from nucleic acid of organisms fed CH_4 by only about 0.5 cm, probably because organisms incorporate H atoms from both nutrients and culture water into their organic molecules and so the nucleic acid was incompletely labeled (Sessions et al., 1999; Kreuzer-Martin et al., 2004). The use of deuterium as a label in DNA-SIP has received little further attention to date. In a recent study, investigators explored the use of ^{15}N to label DNA for SIP and reported that a minimum of 40% enrichment would be required to produce separate bands of DNA (Cadisch et al., 2005). In labeling experiments with soil samples, they observed a smear of DNA in the gradient from which they collected fractions having approximately 80, 60, 30, 20, and 15% enrichment in the nucleic acid

subsequently isolated from the fractions, suggesting that ^{15}N labeling could be successful at lower levels of incorporation.

The advantage of DNA-SIP is that it directly links functionality to phylogeny without the intervention of laboratory isolation and culturing of organisms. Insights into the function of uncultivated microorganisms gained through the application of DNA-SIP have been discussed in a recent review (Friedrich, 2006). The nucleic acid sequences of rDNA detected in the ^{13}C -labeled products can be plugged into bioinformatics resources for identification and for comparison with results from other laboratories. In addition, the amplified nucleic acids are typically cloned, meaning they are available to be developed into probes for techniques such as fluorescent in situ hybridization (FISH). Nucleic acid SIP indeed provides a bridge between organism function and the power of molecular biology and bioinformatics.

A disadvantage of DNA-SIP is that uptake of ^{13}C into DNA requires DNA replication, which can require longer incubation times. This could present a problem if the rate of C turnover into forms usable by other community members is significant when compared with the rate of DNA replication (Radajewski et al., 2000). So-called cross-feeding, the conversion of ^{13}C substrate into metabolites that are taken up by organisms other than the primary assimilator, is a potential problem for any SIP approach, but most particularly in DNA-SIP, the approach that potentially requires the most time for labeling of the biomarker. It has been shown that, with time, the appearance of heavy C in community nucleic acids becomes less and less specific (Manefield et al., 2002a; Gallagher et al., 2005; Rangel-Castro et al., 2005). In a deliberate study of cross-feeding, DeRito et al. (2005) showed that autoclaved ^{13}C microbial biomass could serve as a substrate for soil respiration. They further showed that repeated pulsing of a soil community with ^{13}C phenol during a 2-wk period labeled cross-feeding microorganisms that were not labeled by a single substrate pulse (DeRito et al., 2005).

RNA STABLE ISOTOPE PROBING

To circumvent the requirement for replication and to ease the temporal requirement for labeling, RNA-SIP was developed (Manefield et al., 2002a), and this approach has been recently reviewed (Whiteley et al., 2006). In the first demonstration of RNA-SIP, Manefield et al. (2002a) isolated DNA and RNA from industrial bioreactor sludge fed a pulse of ^{13}C phenol. They showed that the appearance of ^{13}C -RNA was more rapid than the appearance of ^{13}C -DNA and correlated better with the disappearance of the substrate. They isolated labeled 16S rRNA, amplified it by RT-PCR, and were able to identify the phenol assimilator in the bioreactor sludge from its 16S rRNA sequence.

There are technical differences between the approaches for DNA-SIP and RNA-SIP. Because of its greater buoyant density, isopycnic centrifugation of RNA is performed in cesium trifluoroacetate (CsTFA) rather than CsCl. The amount of RNA loaded into a gradient is also crucial, as too much sample can lead to aggregation and incomplete separation (Manefield et al., 2002a; Lueders et al., 2004a). Centrifugation of RNA in these gradients appears to be less able to focus populations of rRNA into tight bands, and nonspecific RNA is typically detected throughout the gradient (Manefield et al., 2002a, 2002b; Lueders et al., 2004a; Rangel-Castro et al., 2005).

In RNA-SIP, the gradient is harvested in a series of fractions, and RT-PCR is performed on each fraction using primers specific

to rRNA. The products from each fraction are separated by DGGE and the rRNA content of each fraction is compared. Specific metabolism of the labeled substrate is evidenced by increasing presence of a particular rRNA in the heavier fractions.

Although RNA was more quickly labeled than was DNA in the original RNA-SIP study, use of RNA as the probed population does not entirely circumvent the issue of cross-feeding. If communities are incubated long enough, labeled C can potentially flow from the initial assimilators to other community members, as was seen in mixed cultures of a phenol metabolizer and nonmetabolizer grown in culture in the presence of ^{13}C phenol (Manfield et al., 2002a). With increasing incubation time, the ^{13}C label began to appear in the RNA of the nonassimilator. The design of the pulsing regime and the time at which samples are taken for analysis in any SIP experiment are crucial.

Cross-feeding has been seen in RNA-SIP studies of rice (*Oryza sativa* L.) field soils in oxic cultures (Lueders et al., 2004c). Incorporation of ^{13}C from labeled methanol into bacterial rRNA was detected by real-time PCR analysis of CsTFA gradient fractions after 6 d of labeling. By Day 13, fully half of the bacterial RNA had shifted toward heavier buoyant densities, as had some fungal 18S RNA and 18S RNA subsequently determined to come from the *Cercozoa* group of soil flagellates. The appearance of label in fungi and protozoa suggests that either they were assimilating the methanol directly or, more probably, they were cross-feeding on the primary methylotrophs or their metabolic products. Thus the potential problem of cross-feeding is simultaneously a potential opportunity for studying the flow of elements through communities and trophic interactions.

An important limitation for both DNA- and RNA-SIP is the requirement for extensive labeling. Nucleic acid SIP is predicated on the assumption that sufficient heavy isotope will be incorporated into either DNA or RNA to permit separation by isopycnic centrifugation. This can be an issue particularly if there are alternative, unlabeled C sources available to the community.

APPLICATIONS OF STABLE ISOTOPE PROBING

All applications of SIP concern themselves with detecting the assimilation of specific substrates. For purposes of this discussion, three focus areas will be mentioned. The first area is comprised of studies that follow the direct uptake of naturally occurring substrates by microorganisms. The second focus area consists of experiments to detect the flow of C from plants into soil microorganisms. The third focus area is bioremediation, in which investigators have designed experiments to follow the assimilation of compounds of anthropogenic origin. This discussion is not meant to be a complete review of the literature, but rather to give an overview of current applications of SIP and to consider which approaches have been fruitful to date.

Assimilation of Naturally Occurring Substrates

A number of studies using both lipid- and NA-SIP have been designed to follow the assimilation of methane in various settings (e.g., Boschker et al., 1998; Nold et al., 1999; Bull et al., 2000; Elvert et al., 2003; Crossman et al., 2004, 2005; Hutchens et al., 2004) and to identify the organisms responsible. The application of NA-SIP to methanotrophy and methylotrophy has been recently reviewed (Crossman et al., 2005;

McDonald et al., 2005). Stable isotope probing of both lipids and nucleic acids has been successfully used to identify organisms that assimilate various other naturally occurring substrates in soils and sediments, such as methanol (Radajewski et al., 2000, 2002; Lueders et al., 2004c; Nercessian et al., 2005), formate (Nercessian et al., 2005), acetate and propionate (Boschker et al., 1998, 2001), and bicarbonate (Knief et al., 2003). The oxidation of methanol under denitrifying conditions (Ginige et al., 2004) and of propionate under methanogenic (Lueders et al., 2004b) conditions have been investigated with RNA-SIP. It is clear that SIP is an effective approach for identifying community members that assimilate labeled elements from specific substrates.

In a particularly elegant series of experiments that demonstrate the power of combining several approaches, Orphan et al. (2001a, 2001b, 2002) first used lipid analyses of organisms from anoxic methane seeps to determine that the isotopically depleted methane was assimilated into archaeal lipids and into biomarker lipids of sulfate-reducing bacteria. Analysis of archaeal small-subunit (SSU) rRNA from the samples revealed two candidate archaeal lineages, ANME-1 and ANME-2, and whole-cell FISH analysis revealed aggregations containing ANME-2 archaea and sulfate-reducing bacteria (Orphan et al., 2001a, 2002). The group used secondary ion mass spectrometry (SIMS) to analyze the C isotope ratios within individual microbial aggregates and showed that the presence of ^{13}C -depleted C in the aggregates coincided with hybridization to the sulfate reducer FISH probes on the outside of the granules and ANME-2 FISH probes in the granule cores (Orphan et al., 2001b). This study did not require the application of labeled substrate, as the naturally occurring methane at the study sites was highly depleted in ^{13}C and therefore isotopically distinct. Identification of the naturally isotopically labeled lipids guided the design of nucleic acid probes that both confirmed the SIP results and revealed three-dimensional community organization.

Tracing Carbon Flow from Plants to Soil Microbiota

Stable isotope probing offers a means to identify microorganisms that take up C from plant root exudates (recently discussed by Prosser et al., 2006) or from decomposition of plant material, processes that have been difficult to dissect. Experiments using PLFA-SIP to follow C from plants into microorganisms have been successful, within the inherent specificity limitations of PLFA analysis. In a field study, a ^{13}C pulse provided to algae in the intertidal zone at low tide could be rapidly detected in the PLFA of bacteria as well as in hand-picked nematodes and macrofauna (Middelburg et al., 2000). Pulse labeling of grass with $^{13}\text{CO}_2$ has been used to trace photosynthate into rhizosphere microorganisms, showing uptake into the lipids of fungi and Gram-negative bacteria and, to a lesser extent, Gram-positive bacteria (Butler et al., 2003; Treonis et al., 2004). Lu et al. (2004) performed a pulse-chase labeling experiment in a wetland rice system and observed incorporation of ^{13}C into rhizosphere PLFA immediately after the plant assimilation. They, too, found more incorporation into PLFAs characteristic of fungi and Gram-negative bacteria rather than Gram-positive organisms (Lu et al., 2004).

The assimilation of C from the decomposition of ^{13}C -labeled plant material has also been traced using PLFA-SIP. McMahon

et al. (2005) followed the incorporation of ^{13}C from ryegrass (*Lolium perenne* L.) into phospholipids isolated from the rhizosphere. Williams et al. (2006) followed the uptake of ^{13}C from labeled crimson clover (*Trifolium incarnatum* L.) and ryegrass root and straw residues into PLFA isolated from the rhizosphere at varying times following application (Williams et al., 2006). They observed that the distribution of ^{13}C among individual PLFA differed from the relative contributions of the individual PLFA (in mol%) to total PLFA-C, suggesting that only a subset of the soil microbiota was responsible for assimilating residue-derived C. In addition, the distribution of ^{13}C differed between sampling times, indicating that different community members were more actively assimilating ^{13}C at different time points.

Studies using NA-SIP to follow photosynthate uptake have had mixed success. In a nonspecific proof-of-principle demonstration, C from $^{13}\text{CO}_2$ pulsed at ambient concentration to grass and soil explants growing in chambers could be detected in total DNA and RNA purified from soil samples taken at intervals after the pulse (Ostle et al., 2003). In these experiments, the total nucleic acid C was converted to CO_2 offline, and the C isotope ratio of the CO_2 was determined. Their results showed that ^{13}C was detectably incorporated into soil nucleic acids, with the peak of ^{13}C enrichment of soil-derived RNA occurring 4 to 8 d after pulse labeling. The RNA incorporated more ^{13}C and measurements displayed less variation than did those of soil-derived DNA, which was significantly enriched above natural variation in only one post-labeling sample.

In experiments in which $^{13}\text{CO}_2$ was pulsed to grass at ambient CO_2 concentrations, one group of investigators could detect ^{13}C enrichment in IRMS analyses of total RNA, but no ^{13}C enrichment could be detected following isopycnic centrifugation, PCR amplification, and DGGE of gradient RNA (Griffiths et al., 2004). Another group was able to detect enrichment of bacterial, archaeal, and fungal rRNA in limed soils, but no archaeal assimilation could be detected in unlimed soil (Rangel-Castro et al., 2005). Dilution of the ^{13}C label because of availability of alternative C sources or insufficient numbers of active organisms in the sample may be issues for NA-SIP in these experiments.

Stable isotope probing of RNA was successfully applied to identify populations of methanogens growing on rice roots (Lu and Conrad, 2005) using plant-transmitted C as a substrate. They isolated the aboveground portion of rice plants and exposed them to brief, high-concentration (3000 ppm) pulses of $^{13}\text{CO}_2$ seven times per day for 7 d, a much more intensive labeling regime than that used in the grass labeling experiments of Griffiths et al. (2004) and Rangel-Castro et al. (2005). They detected specific labeling of the rRNA of archaea from the uncultured Rice Cluster I group of organisms. Cross-feeding was not an issue in this specialized community.

Bioremediation

Stable isotope probing has obvious potential in the field of bioremediation, for demonstrating biological processing of contaminants, for determining the identity of assimilators, and for comparing consortia involved in biodegradation of anthropogenic chemicals (Manefield et al., 2004; Wackett, 2004; Madsen, 2006). Stable isotope probing of PLFA has been used to characterize groups of organisms responsible for the degradation of toluene in soil (Hanson et al., 1999; Pelz et al., 2001) and in sediment from

a petroleum-contaminated aquifer (Pelz et al., 2001), styrene in a bioreactor film (Alexandrino et al., 2001), phenanthrene in soil (Johnsen et al., 2002), and polychlorinated biphenyls (PCBs) in a biofilm (Tillmann et al., 2005). Stable isotope probing of PLFA may be more useful in biodegradation studies than in general metabolic studies such as uptake of photosynthate, because often only a limited number of community members degrade the anthropogenic compounds, leading to more specific enriched fatty acid profiles (e.g., see Hanson et al. [1999], who found that only 16 of a total of 50 PLFAs isolated from their samples were enriched by ^{13}C from toluene), or because groups of organisms with distinctive fatty acids are often involved in biodegradation (see Pelz et al., 2001; Tillmann et al., 2005).

Stable isotope probing of nucleic acids has also been quite useful in identifying organisms that assimilate C from a variety of pollutants. The first example of RNA-SIP was performed using phenol as a substrate and an industrial bioreactor as a source of the microbial community (Manefield et al., 2002a). Small-scale DNA-SIP field studies have since traced the uptake of phenol by organisms in soil (Padmanabhan et al., 2003) and followed the spread of ^{13}C from phenol into the community via cross-feeding (DeRito et al., 2005). Similar experiments by the same research group also traced the degradation of caffeine and naphthalene in soil (Padmanabhan et al., 2003), resulting in the discovery of a new naphthalene assimilator (Jeon et al., 2003). Singleton et al. (2005) used DNA-SIP to investigate the degradation of salicylate, naphthalene, and phenanthrene by a community from a bioreactor used to treat contaminated soil. They observed that very similar consortia of organisms assimilated C from salicylate and naphthalene, but that the phenanthrene degraders were a separate group. In their tests, there was little change in the labeled profiles with time, suggesting that very little cross-feeding took place.

Investigators have used DNA-SIP with the analysis of both 16S rRNA genes and functional genes to study degradation of methyl chloride and methyl bromide, and found that a greater diversity of organisms were active than previously recognized by laboratory enrichment techniques, including potentially novel organisms (Miller et al., 2004; Borodina et al., 2005). Mahmood et al. (2005) performed side-by-side RNA-SIP and community analysis by PCR amplification of 16S rRNA and rDNA in a study of pentachlorophenol degradation in soil. The results of the two studies correlated: community analysis showed that the composition of the community changed with time, and the organisms that became dominant were the same ones whose rRNA was enriched by ^{13}C in the SIP experiments.

Manefield et al. (2005) recently used community analysis and RNA-SIP to characterize two sludge reactors used to treat coking effluent, one of which was performing more efficiently than the other. Both communities were established at the same time, and had been used to degrade the same split effluent stream under the same operating conditions. The design of the treatment plant was such that the communities did not exchange with one another and so had evolved independently. Community analysis showed the same level of diversity but different dominant species in each reactor stream. Stable isotope probing of RNA using labeled phenol as a substrate revealed that phenol was being assimilated by a single *Acidovorax* lineage in the efficient reactor, while in the less efficient reactor, phenol was being assimilated by it and by a second *Acidovorax* lineage, con-

tradicting the hypothesis that the poor performance of the second reactor was the result of lower diversity among the phenol degraders (Manefield et al., 2005).

Stable isotope probing of PLFA has been demonstrated to work in large-scale field trials that combined the hydrogeological technique of push–pull testing with molecular analysis (Pombo et al., 2002, 2005; Kleikemper et al., 2005). In a push–pull test, a test well is injected with a solution containing a substrate of interest and a chemically and biologically inert tracer to control for dilution, and samples are subsequently removed for analysis to determine whether the test substance is being degraded. In the first test, Pombo et al. (2002) injected ^{13}C acetate along with NO_3^{2-} and a Br^- tracer into the denitrifying zone of a petroleum-contaminated aquifer. They detected assimilation of the ^{13}C label into both PLFA and dissolved inorganic carbon (DIC) as early as 4 h after introduction. The PLFA profiles suggested that the ^{13}C was being assimilated by denitrifiers, and FISH tests confirmed that they were present in high numbers. In the second series of experiments, Pombo et al. (2005) probed sulfate reducers by injecting a solution of ^{13}C acetate, sulfate, and Br^- into the transition zone of the aquifer, where sulfate-reducing and methanogenic conditions prevailed. They observed ^{13}C enrichment of PLFA and DIC. The enriched PLFA profile contained biomarkers for more than one sulfate-reducing genus, and FISH testing confirmed the presence of a mixed community. Thus it may be possible to use PLFA-SIP to characterize bioremediation at the field scale, especially if it is coupled to additional analyses such as FISH or SIMS.

CURRENT STATUS AND FUTURE PROSPECTS

The examples cited here illustrate the power of SIP to identify organisms that are assimilating specific substrates within complex communities. It requires no foreknowledge about the organisms in question, since no probes need be designed. It requires no classic laboratory enrichment culturing, and experiments can be performed with minimal disturbance to the community. Since it avoids radioactive labels, it can also be performed in field settings. In addition to identifying primary consumers, SIP can trace C through additional trophic levels. Stable isotope probing of nucleic acids is the most phylogenetically specific approach, but is not successful unless a sufficient degree of labeling can be achieved for the separation of labeled and unlabeled nucleic acids by buoyant density centrifugation. Successful experiments have used uniformly labeled substrates, mostly in microcosm, and have identified primary consumers, with exceptions primarily from the research group of E.L. Madsen (Padmanabhan et al., 2003; DeRito et al., 2005). With these caveats, nucleic acid SIP is the clearest method for linking biological function and microbial identity within communities.

Although SIP of lipids is phylogenetically less specific than NA-SIP, it is nonetheless the most effective approach in situations where label incorporation is limited. It can be very useful for identifying phylogenetic groups of varying breadth that are incorporating label from a particular substrate, particularly if the substrate is being incorporated by a limited group of organisms, or by organisms that synthesize biomarker lipids. In these cases, combining results from lipid SIP with community analysis can provide more specific information about the members of a community involved in assimilation. Information gleaned

from lipid-SIP can be used to guide the design of appropriate probes for other techniques such as FISH as well.

New Technology, New Possibilities

Technological advances in stable isotope ratio measurements make future prospects for SIP very interesting. The new interface for measuring C isotope ratios of very small nonvolatile samples (Sessions et al., 2005) will permit the use of a broad array of molecule types as biomarkers. With the new moving-wire technology, the isotope ratio of just 10 nmol of C can be analyzed—even less if the sample is spotted on the moving wire rather than introduced in a peak eluting from a liquid chromatograph. With this technology along with the existing GC interfaces, essentially any molecule that carries phylogenetic information and can be purified in sufficient quantity could be a target for SIP.

Perhaps the most exciting SIP prospect for the new technology is direct isotopic measurements of SSU rRNA. MacGregor et al. (2002) developed a technique for isolation of SSU rRNA from environmental samples for stable isotope ratio. They used hybridization with probes specific for varying phylogenetic levels of microorganisms to select SSU rRNA from groups of increasing specificity. The RNA was eluted from the probes and its isotope ratio measured by IRMS. MacGregor et al. (2002) used heavily labeled RNA so that they could dilute the samples with glucose to get sufficient C for analysis by conventional IRMS. The new moving-wire interface means that the analysis could be done with far less C. The requirement for 10 nmol of C translates into $<1 \mu\text{g}$ of nucleic acid, and less than that would be required for direct spotting. It may thus be possible to purify specific SSU rRNAs directly from environmental samples and measure their isotope ratios without the need for isopycnic centrifugation.

In such an application, one might start with information from other experiments, such as PLFA-SIP, that a particular group of microorganisms was involved in assimilation of a substrate. The SSU rRNA from that group could be selected from the sample and subcloned for community characterization. Individual probes to the cloned members of the community could then be synthesized and used to pull out specific SSU rRNA from the labeled sample, and the isotope ratios of those individual SSU rRNAs could be measured, indicating which community members were assimilating the substrate.

Such an approach would combine the advantage of the PLFA approach, in which far less label incorporation is needed because the separation of the biomarker is chromatographic and independent of label assimilation, with the phylogenetic power of NA-SIP. The next few years should be an exciting time for environmental microbiologists, offering new horizons for linking microbial identity to function.

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