Microbial Ecology

Methodological Considerations for the Use of Stable Isotope Probing in Microbial Ecology

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Abstract

Stable isotope probing (SIP) is a method used for labeling uncultivated microorganisms in environmental samples or directly in field studies using substrate enriched with stable isotope (e.g., ^{13}C). After consumption of the substrate, the cells of microorganisms that consumed the substrate become enriched in the isotope. Labeled biomarkers, such as phospholipid-derived fatty acid (PLFA), ribosomal RNA, and DNA can be analyzed with a range of molecular and analytical techniques, and used to identify and characterize the organisms that incorporated the substrate. The advantages and disadvantages of PLFA-SIP, RNA-SIP, and DNA-SIP are presented. Using examples from our laboratory and from the literature, we discuss important methodological considerations for a successful SIP experiment.

Introduction

Stable isotope probing (SIP) has become a focal method in microbiology since its adoption by microbial ecologists who seek to link the phylogeny and function of uncultivated microorganisms in the natural environment. A commercially prepared, labeled substrate (typically >99.5% stable isotope) is added to an environmental sample, and biomarkers are purified and analyzed following the consumption of the substrate. Variations of SIP focus on different biomarker molecules that become labeled by growth on ^{13}C-substrate. These SIP variations include labeling of membrane lipids, such as phospholipid-derived fatty acid (PLFA-SIP), deoxyribonucleic acid (DNA-SIP), and ribonucleic acid (RNA-SIP). PLFA molecules were first analyzed by SIP [4] and the technique was later extended to nucleic acids [20, 26]. RNA-SIP has enabled the analysis of 16S ribosomal RNA (rRNA) genes and DNA-SIP has enabled analysis of both 16S rRNA and physiological genes from organisms that grow on specific carbon substrates. Figure 1 summarizes experimental designs and data analyses that are commonly used for SIP experiments.

The history and application of SIP methodology has been reviewed extensively in recent years [3, 9, 19, 21, 27, 32, 34, 35] and may be second only to metagenomics in its ratio of reviews to primary publications. This attests to the ability of SIP to stimulate the imagination and enthusiasm of microbial ecologists, by enabling a link between organisms and their function in the natural environment; however, a lack of primary publications may also reflect challenges in applying the technique to address specific ecological questions. Here, we discuss methodological issues that should be considered when planning SIP experiments, providing examples from the literature and results from our own laboratory.

Sensitivity of the SIP Technique

The most appropriate approach for a study using SIP depends on the environment, the substrate being consumed, and the duration of the incubation. A problem arises, particularly for nucleic acids, when substrate incorporation and incubation time are insufficient, generating poorly labeled biomarker molecules that are not distinguishable above a background of relatively abundant unlabeled molecules. As discussed in subsequent sections, too much substrate and excessive incubation times may be problematic as well, leading to (1) enrichment bias that does not reflect the natural process of substrate metabolism in the environment and (2) the potential for enhanced cross-feeding of the substrate. Identifying the appropriate substrate concentration and incubation time are critical for a successful SIP, as well as monitoring the appropriate biomarker for a particular experiment (Table 1). In general, PLFA-SIP provides the highest sensitivity. DNA-SIP is the least sensitive SIP

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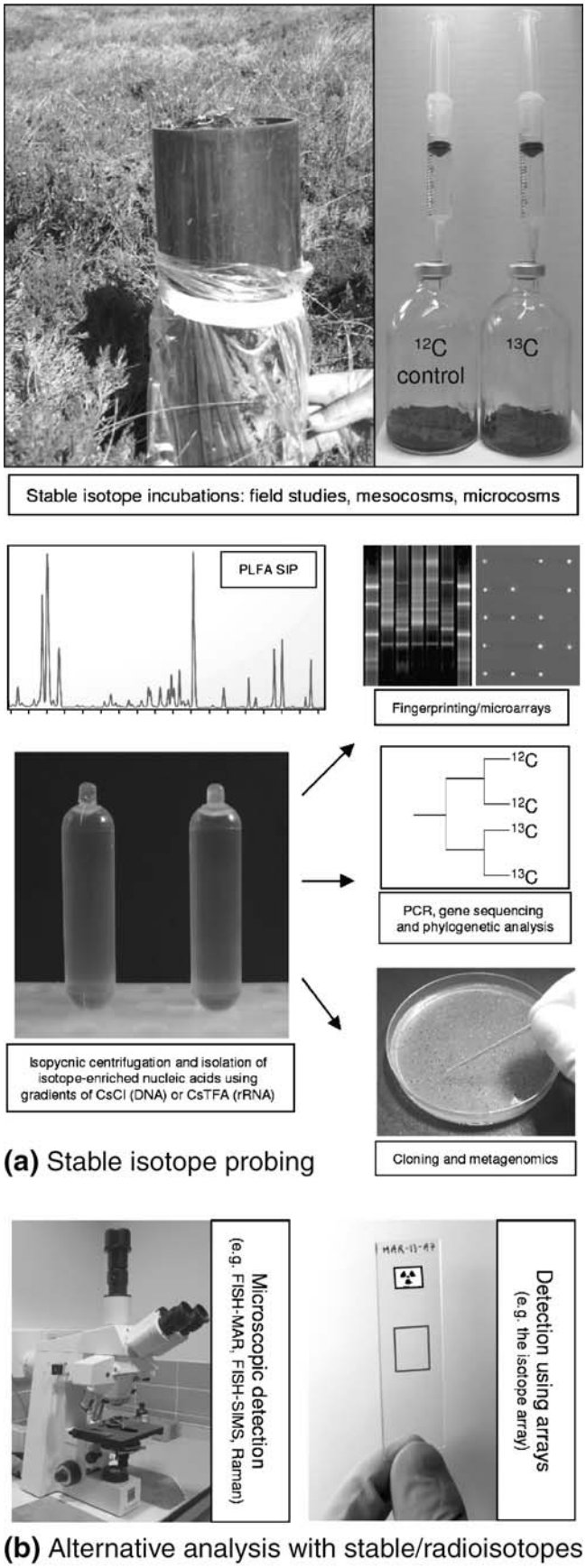

approach, because unlike RNA and PLFA regeneration, DNA replication normally requires cell division. Therefore, successful DNA-SIP experiments require cell division in the presence of labeled substrate to achieve sufficient incorporation for separation of labeled DNA.

Phospholipid-Derived Fatty Acid Stable Isotope Probing. In some instances, the sensitivity provided by the analysis of stable-isotope-labeled PLFA is required. PLFAs are analyzed by a combination of gas chromatography and isotope ratio mass spectrometry (GC-c-IRMS) and, unlike with RNA-SIP and DNA-SIP, do not need to be first purified from unlabeled molecules. Therefore, small amounts of label and only partial incorporation of ^{13}C isotope into PLFA molecules is sufficient for the analysis.

Phospholipid-derived fatty acid SIP is the method of choice when probing a population composed of relatively low cell numbers or growth rates and therefore incorporating minimal amounts of labeled substrate. An example of this scenario is that of the microbial community responsible for the consumption of atmospheric CH_4 in upland soils. CH_4 is present in the atmosphere at 1.75 ppmv and soil microbial communities with relative high affinity compared with that of extant methane-oxidizing bacteria are capable of oxidizing this CH_4. Because the organisms involved in atmospheric CH_4 oxidation are not abundant in soil and concentrations of available labeled substrate are low, PLFA-SIP is an appropriate method for labeling the organisms responsible. In addition, the organisms that oxidize methane at atmospheric concentrations may supplement their carbon intake with other substrates, such as methanol [2], which would decrease label incorporation and make RNA-SIP and DNA-SIP more difficult or unfeasible. Bull *et al.* [5] incubated soil for 6 months at low CH_4 concentrations (<3.6 ppmv) before repeatedly pulsing the microcosms with 1.9 ppmv $^{13}CH_4$ for an additional 3 weeks to label sufficient PLFA. The resulting patterns contained individual PLFAs that suggested the involvement of an unknown methanotroph, possibly related to the alpha *Proteobacteria Methylosinus/Methylocystis* genera. Knief *et al.* [15] conducted a similar PLFA-SIP and found additional PLFA patterns associated with $^{13}CH_4$ uptake, including patterns most closely resembling species of methanotrophs from both the gamma *Proteobacteria* (type I methanotrophs) and the alpha *Proteobacteria* (type II methanotrophs), indicating that atmospheric CH_4 oxidation may be a widespread ability

Figure 1. Stable isotope probing (SIP) experimental design. (A) SIP of PLFA and nucleic acids enable downstream investigations including fingerprint, phylogenetic, and metagenomic (for DNA-SIP) analyses. (B) Additional methods use stable isotopes and radioisotopes to provide sensitive confirmation of SIP-derived phylogenetic data.

Table 1. Summary of SIP techniques, relative sensitivities, separation methods, and examples of possible downstream analyses for SIP experiments

Method	*Sensitivity*	*Separation*	*Analyses*
DNA-SIP	Low	Needle extraction, CsCl fractionation	Fingerprinting, clone libraries, metagenomics, microarrays
RNA-SIP	Medium	CsTFA gradient fractionation	Fingerprinting, clone libraries, microarrays
PLFA-SIP	High	None	GC-c-IRMS profile analysis

of many diverse species. More recently, Knief *et al.* [14] continued to exploit the high sensitivity of PLFA to demonstrate that in some hydromorphic upland soils, type II methanotrophs are primarily responsible for the oxidation of low CH_4 mixing ratios (30 ppmv) and fatty acids typical of type I methanotrophs become labeled with higher $^{13}CH_4$ concentrations (500 ppmv).

Aquatic environments (ocean, lakes, and rivers) are characterized by microbial communities with relatively low cell numbers that result in low absolute carbon incorporation rates. Such environments may be best suited to PLFA-SIP to achieve sufficient resolution of labeled biomarkers. PLFA-SIP has been used to study primary producers involved in estuarine [^{13}C]bicarbonate incorporation. Under light and dark conditions, PLFA profiles corresponded to algal phytoplankton and chemoautotrophic nitrifying bacteria, respectively [3].

Although the sensitivity of PLFA-SIP is high, general taxonomic identifications of associated organisms are tentative and difficult to confirm. In some instances, as with methanotrophs, it may be possible to identify uncultivated representatives based on the comparison of their PLFA profiles with those of extant strains. However, PLFA profiles provide no phylogenetic information for most uncultivated microorganisms. An advantage of nucleic-acid-based SIP experiments is that the phylogenetic resolution of the labeled biomarkers is high, with sequence databases providing an ever-increasing resource for robust taxonomic and functional assignments.

DNA Stable Isotope Probing. The appeal of DNA-SIP has been the potential to retrieve labeled genomic DNA from the environment, which offers the opportunity to analyze the purified DNA with a range of molecular techniques (Table 1). DNA-SIP is unparalleled in its ability to link metabolic functions in the environment with ecologically relevant phylogenetic and metabolic "functional" genes. Recently, the potential to combine DNA-SIP with metagenomic analysis has been recognized [34] and applied to identify a BAC clone containing an operon involved in one-carbon metabolism [9, 10].

An important limitation of DNA-SIP is the prerequisite for DNA synthesis and cell division to obtain incorporation of sufficient label into DNA for gradient separation. In the presence of 100% ^{13}C-labeled compound, with each cell division, the "heavy" carbon fraction of DNA increases by 50%, as one parent chromosome strand is retained by each progeny. As a result, increasing the number of cell divisions increases the successful isolation of labeled nucleic acid, but may increase the enrichment bias of the SIP experiment.

An ideal DNA-SIP experiment is one in which the amount of substrate provided to the sample is representative of expected *in situ* concentrations and the experiment proceeds for no longer than necessary for the detection of labeled DNA above the background. As an example of enrichment bias, we recently exposed 4 L of seawater to 5 mmol of $^{13}CH_3OH$ for 6 days. At the end of the incubation, the sample became turbid, indicating

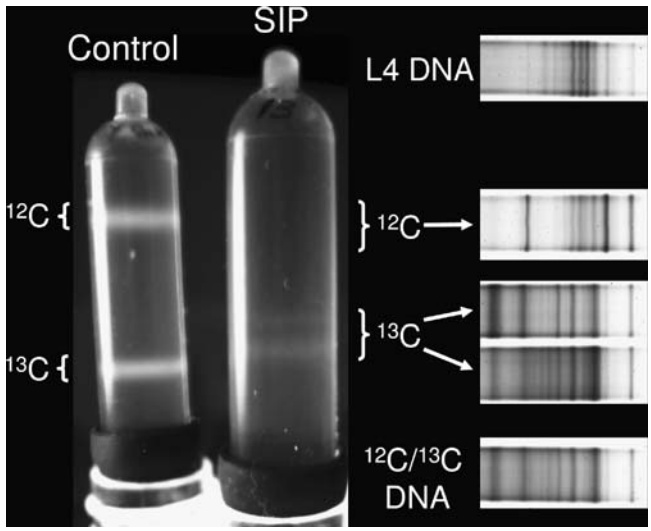

Figure 2. Summary of a seawater SIP experiment in which 4 L of seawater (supplemented with dilute minimal salts medium) was exposed to 5 mmol of $^{13}CH_3OH$ for 6 days. The control gradient contained ^{12}C-labeled DNA from *Methylomonas methanica* S1 and ^{13}C-labeled DNA from *Methylococcus capsulatus* (Bath). The SIP gradient contained 5 μg of seawater DNA. Both gradients were centrifuged at 220,000 × *g* for 48 h at 20°C. Approximately 500 μL of gradient solution was retrieved by syringe from both the "heavy" and "light" portions of the gradient and the DNA was purified by 1-butanol extraction, precipitated, and suspended in H_2O. PCR and DGGE analysis of 16S rRNA of the original seawater DNA sample (Station L4 DNA; Plymouth, Devon, UK), raw SIP DNA (^{12}C/^{13}C DNA), and light and heavy DNA was performed as previously described [16]. Note that the heavy DNA appeared as a dual band. Based on the DGGE fingerprints, it appeared as if two phylotypes, possibly differing significantly in their G + C contents, were predominant in each of the ^{13}C bands that were separately extracted with two needles.

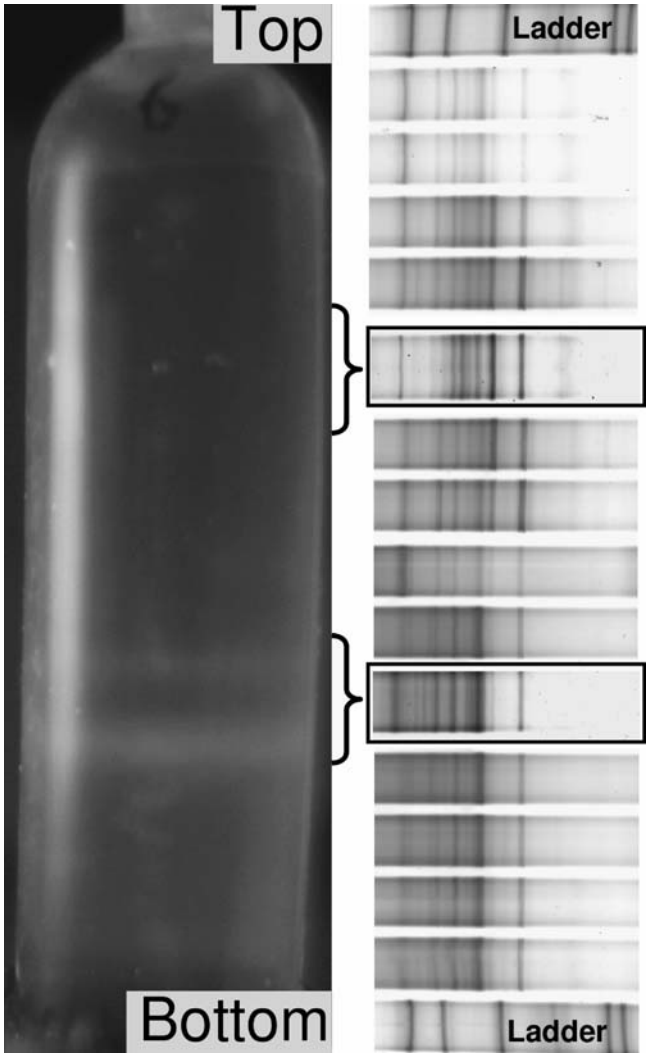

Figure 3. A comparison of needle extraction and gradient fractionation of the same SIP DNA using 16S rRNA gene fingerprints. Seawater SIP DNA (as described in Fig. 1) was centrifuged in a CsCl gradient similarly to a previously described protocol [17] and the 5.1-mL gradient was fractionated in 400-μL aliquots. DNA was precipitated with polyethylene glycol and then suspended in H_2O. PCR and DGGE was performed on gradient fractions as previously described [16] and fingerprints are arranged top to bottom from low density to high density, respectively. The boxed DGGE fingerprints were generated from DNA retrieved by needle extraction, both light and heavy, and are similar to fingerprints generated from the respective DNA fractions.

microbial growth. Figure 2 demonstrates that DNA extracted from this SIP experiment was almost entirely composed of [^{13}C]DNA. Not only was the [^{13}C]DNA band the only visible DNA in the gradient, but the denaturing gradient gel electrophoresis (DGGE) fingerprint of the raw DNA extract was essentially the same fingerprint as the purified [^{13}C]DNA. The [^{12}C]DNA retrieved from the gradient generated a similar DGGE fingerprint to that of the original seawater. The results were the inverse of those occasionally obtained with several grams of soil that have incorporated a similar amount of carbon [22] and represented a significant enrichment scenario that necessitated revisiting the experimental design. To minimize bias for DNA-SIP experiments, the amount of DNA in the [^{13}C]DNA band should be substantially less than that in the corresponding [^{12}C]DNA band.

RNA Stable Isotope Probing. Stable isotope probing of RNA offers higher sensitivity than DNA-SIP. In the first demonstration of the RNA-SIP method, a phenol-degrading community in an industrial bioreactor was fed [$^{13}C_6$]phenol, and the incorporation of isotopic label was measured for RNA and DNA [20]. Over 8 hours, labeled carbon accumulated in RNA almost 10-fold more quickly than in DNA [35]. After separation of the [^{13}C]rRNA and [^{12}C]rRNA molecules by cesium trifluoracetate (CsTFA) gradient centrifugation and analysis by reverse transcriptase polymerase chain reaction (RT-PCR) of the 16S rRNA molecule, a *Thauera* species was implicated in phenol degradation [20].

RNA-SIP has since been applied to the more technically challenging soil environment and has been used to explore plant–microorganism interactions. The first application of RNA-SIP in a soil environment was performed using [^{13}C]methanol added at low concentrations to microcosms of oxic rice field soil [18]. Using a combination of RNA-SIP and DNA-SIP, it was possible to monitor the flow of carbon from methylotrophs to fungi and predatory soil flagellates. Rangel-Castro *et al.* [29] determined that the lower limit for detection of RNA-SIP was between 10^5 and 10^6 labeled bacterial cells per gram of soil. Above this number, it was possible to clearly distinguish labeled 16S rRNA over unlabeled background RNA using RT-PCR and DGGE. After pulse labeling grassland soil with $^{13}CO_2$, they were able to detect rhizosphere microorganisms that incorporated plant exudates and investigate the effect of liming. RNA-SIP is well suited to studies of plant-associated microorganisms and can detect utilization by microbes of plant exudates, providing sensitivity and phylogenetic resolution not readily accessible by other methodologies.

Despite the sensitivity of RNA-SIP being higher than for DNA-SIP, downstream applications have been limited to the analysis of rRNA (Table 1), which has received criticism for not being tightly linked to the physiological role of organisms in the environment. Theoretically, isotopically enriched mRNA could also be extracted and fractionated to retrieve genes from organisms actively incorporating labeled substrate. This has yet to be demonstrated and has thus far been limited by low yields of mRNA retrieved from environmental samples, the difficulty of cloning mRNA independent of background rRNA

and potentially by technical difficulties in the separation of mRNA species by isotopic density centrifugation.

Confirming Isotopic Enrichment

When designing a SIP experiment, it is critical that adequate measures are taken to ensure that retrieved sequences are derived from [^{13}C]DNA or [^{13}C]RNA molecules and not from a background of ^{12}C-nucleic acid. Even pure culture nucleic acid distributes across the length of density gradient such that it contaminates the more dense fractions [17, 31]. In our hands, we have found by PCR and RT-PCR that DNA and RNA can distribute in trace, but detectable, amounts along the length of respective gradients, as shown in Fig. 3 and discussed by Manefield *et al.* [20]. This could lead to the mistaken analysis of "light" nucleic acids that occur lower in a density gradient. Another concern is that high-GC content can increase the buoyant density approaching that of lower-GC content ^{13}C molecules [17]. This is particularly a concern for SIP studies that incorporate ^{15}N as a stable isotope [6, 7] because the proportional amount of nitrogen in nucleic acid is less than that of carbon.

There are several approaches available to help confirm isotopic enrichment for nucleic-acid based SIP approaches. (1) Obtaining a δ^{13}C value by IRMS provides a useful confirmation that the retrieved nucleic acids are enriched in ^{13}C and to what extent. This was first demonstrated in the initial publication of RNA-SIP [20]. (2) Performing SIP experiments in a time series offers one of the most robust indications of both isotopic enrichment of particular phylotypes, but also verification of the initial community members that consume a particular substrate and possible subsequent consumers of metabolic by-products (cross-feeding). In this way, Lueders *et al.* [18] monitored the bacterial methylotrophic communities in soil that consumed labeled methanol and reported that the enriched community changed with time. This population shift would have been overlooked if only the last time point (42 days) had been analyzed. Their study was particularly "holistic" in that they also monitored the isotopic enrichment of eukaryotic nucleic acid, which may have been a result of direct methanol assimilation or indirect incorporation through a microbial food web. (3) An unlabeled control is always a useful (or necessary) comparison. This involves incubating a parallel sample with ^{12}C-labeled substrate alongside the samples treated with ^{13}C-labeled substrate. With a successful SIP experiment, the control gradient should contain less nucleic acid in the more dense fractions than is present in the sample incubated with ^{13}C-substrate. The first example of this control was demonstrated with gradients derived from soils exposed to $^{12}CH_4$ and $^{13}CH_4$ [22]. The sequences obtained from lower fractions in the ^{12}C control, which may require more PCR cycles to amplify, can be shown (e.g., by DGGE analysis) to be different from the genuine ^{13}C-enriched sequences. (4) The identification of identical sequences by both RNA-SIP and DNA-SIP (and alternatively with PLFA-SIP) analyses can confirm that the organisms first to respond to the presence of a labeled substrate are the same organisms that grow on that substrate. (5) If one-carbon compounds are used for SIP experiments, additional validation may be obtained through 16S rRNA gene sequence analysis. A predominance of sequences affiliated with methylotrophic organisms provides reasonable evidence that the extracted DNA used as template was isotopically enriched. Unfortunately, for experiments in which the general target population is not known *a priori*, and more complex substrates are used, as exemplified by Padmanabhan *et al.* [25], this approach for validating isotopic enrichment is not feasible.

Finally, a recent approach for validation of isotopic enrichment was provided by Singleton *et al.* [31]. They used unlabeled DNA from *Escherichia coli* K12 as an indicator of unlabeled DNA in the high-density fractions. The *E. coli* DNA was added to the sample to a level equivalent to that of a well-represented indigenous organism. *E. coli* K12 was specifically targeted by PCR to determine the extent to which unlabeled DNA contaminated the high-density fractions. The *E. coli* DNA could be detected in the high-density fraction of the gradient when 40 PCR cycles were used, but in most instances was detectable only in the low-density fractions when 25 PCR cycles were performed. This provided an indication of DNA separation efficiency. The presence of *E. coli* K12 genes in clone libraries served as an additional indicator of [^{12}C]DNA contamination.

Gradient Considerations

In a DNA-SIP experiment, the [^{13}C]DNA is separated from the community [^{12}C]DNA by CsCl gradient centrifugation. Historically, CsCl gradients with ethidium bromide (EtBr) have been widely used for the isolation of supercoiled plasmid DNA from uncoiled molecules [30]. This approach is effective because linear DNA binds more EtBr than supercoiled DNA. At saturating EtBr concentrations, the dye alters the conformation of the DNA helix and decreases the density of the DNA molecule such that the supercoiled plasmid DNA forms a band lower in the gradient than chromosomal DNA. When developing the DNA-SIP technique, Radajewski *et al.* [26] used the CsCl and EtBr concentrations and centrifugation conditions optimized for plasmid isolation. The [^{13}C]DNA and [^{12}C]DNA separated based on atomic density and not a differential effect of the EtBr, but the dye enabled convenient and reassuring visualization of distinct DNA bands. DNA-SIP is the only SIP approach suited to the use of CsCl–EtBr centrifugation, which

enables bands to be visualized by UV irradiation. Note that centrifugation speeds and times should be chosen carefully as these factors affect the magnitude of ^{12}C-band and ^{13}C-band separation and resolution [28]. If both bands can be visualized under UV irradiation, then it is a simple matter of piercing the tube with a needle and syringe and retrieving the bands. If only the [^{12}C]DNA is visible, it is possible to insert a needle at multiple positions (e.g., 1 and 2 cm) below the band to collect fractions that might contain [^{13}C]DNA.

The complete fractionation of gradients is essential for RNA-SIP and Lueders *et al.* [17] optimized the conditions for this method. Low relative RNA concentrations in gradients (around 500 ng to prevent aggregation) prevent visualization with nucleic acid stains. Lueders *et al.* [17] also optimized the conditions necessary for separation of [^{12}C]DNA and [^{13}C]DNA in CsCl gradients without EtBr (i.e., a denser solution) and the DNA was recovered by fractionation of the gradient and analysis of the fractions.

For gradient fractionation, water is typically pumped into the top of the ultracentrifuge tube and fractions are collected dropwise from the bottom of the tube. High-performance liquid chromatography or syringe pumps are ideal because they provide smooth delivery of displacement liquid, which minimizes disruption of the gradient and ensures a consistent volume in the fractions collected. We have found that light mineral oil may be used instead of water for fractionating gradients and, unlike water, is not miscible with CsCl and CsTFA gradients, thus avoiding the convective mixing that occurs at the water-gradient interface.

Gradient fractionation has the advantage of reducing the effort associated with purification of DNA from EtBr, removing the necessity to detect DNA in the gradient visually and eliminating the potential hazard of handling relatively concentrated EtBr solutions. Furthermore, fractionation of DNA gradients avoids exposure of DNA to UV irradiation as is common for needle extraction. Even low-level exposure to UV light damages DNA and can frustrate downstream applications such as the preparation of metagenomic libraries. Alternatives to UV are also available to enable needle extraction from gradients, such as the Dark Reader (Clare Chemical Research).

We compared needle extraction and gradient fractionation for the same DNA sample extracted from a SIP experiment (Fig. 3). DGGE fingerprints of 16S rRNA genes confirmed that the community fingerprints from the needle-extracted [^{12}C]DNA and [^{13}C]DNA matched the fingerprints obtained from the corresponding regions of the fractionated gradient (Fig. 3). These data provide an indication that results are not heavily biased by the method chosen to retrieve labeled and unlabeled DNA from SIP experiments. On the other hand, using labeled DNA from one organism and unlabeled DNA from a different organism, needle extraction resulted in less mixing between the [^{12}C]DNA and [^{13}C]DNA than observed with complete fractionation of the gradient (J.V., unpublished data). Needle extraction may be preferred for applications in which maximum separation of [^{12}C]DNA and [^{13}C]DNA is critical.

^{13}C-Carrier Nucleic Acid in Gradients

In some SIP experiments, the amount of isotope incorporation and total extracted nucleic acid can be relatively low. Consequently, the retrieval and detection of labeled biomarkers may be difficult. The benefit of adding ^{13}C-tracer or carrier molecules to gradients is now starting to be explored. A carrier molecule could be nucleic acid obtained from archaeal or eukaryotic origin (if bacteria and bacterial genes are being targeted in the study) or synthetic [^{13}C]DNA. The obvious advantage with DNA-SIP is that it could enable visualization of a band in a CsCl–EtBr gradient that would not normally be visible. The band could be easily removed from the gradient without requiring the analysis of multiple fractions. The benefit of a tracer may be even more significant than this because, as demonstrated by Gallagher *et al.* [11], ^{13}C-carrier DNA may increase the sensitivity of the method. In their study, they prepared [^{13}C]DNA from *Halobacterium salinarium* grown in a ^{13}C-labeled ISOGRO powder growth medium (Isotec, Inc.), and added an equal quantity (300 ng) of this archaeal DNA to the [^{13}C]benzoate SIP-labeled environmental DNA. With the addition of the carrier, the detection of *NosZ* genes from denitrifying microorganisms that utilized the [^{13}C]benzoate as a carbon source decreased from days to hours.

Lueders *et al.* [17] noted that [^{12}C]rRNA and [^{13}C]rRNA form more distinct and focused bands when centrifuged separately than when combined in the same gradient. They combined [^{12}C]RNA isolated from a pure culture *Archaea* and [^{13}C]RNA from a pure culture bacterium and found that when the RNA extracts were combined and centrifuged in a CsTFA gradient, the rRNA bands were closer together and showed increased overlap than when centrifuged individually. This is evidence that there is some interaction between rRNA of different buoyant densities. There might also be a benefit of adding a [^{13}C]RNA carrier to gradients for enhancing the recovery of small amounts of labeled RNA.

The addition of carrier DNA or RNA may be effective by improving the yield of nucleic acid recovered from ultracentrifuge gradients. We found that the addition of an inert carrier for DNA and RNA precipitation is critical for the quantitative recovery of low amounts of labeled nucleic acid commonly associated with SIP experiments. For example, almost complete recovery of low nanogram amounts of DNA from a CsCl

gradient using ethanolic or polyethylene glycol precipitations was only achieved by the addition of 20–60 μg of glycogen (Roche) as a carrier (J.D.N., unpublished data).

Combining SIP with Other Methods (Future Directions)

The retrieval of isotopically enriched biomarkers after pulsing a sample with a labeled substrate is a strong indication that those organisms are directly involved in the metabolism of that compound *in situ*. This is particularly true for experiments that use RNA-SIP and PLFA-SIP, as these approaches do not require cell division for generating labeled biomarkers. As previously discussed, DNA-SIP may be biased due to the multiple cell divisions that are required to detect labeled biomarker from target organisms. Although combining SIP approaches, such as RNA-SIP and DNA-SIP [18] or PLFA-SIP and DNA-SIP [33], can help to reinforce the results, additional techniques may also be used to help confirm the role of labeled organisms in degrading specific substrates (Fig. 1).

Ginige *et al.* [12] demonstrated a "full-cycle rRNA analysis" approach by conducting DNA-SIP on a denitrifying activated sludge bioreactor fed with [^{13}C]methanol. 16S rRNA gene clone libraries generated from the labeled DNA indicated that one phylotype represented half of the clones and clustered with *Methylobacillus* and *Methylophilus*, which are obligate methylotrophs. To confirm the role of these organisms in methanol utilization, an oligonucleotide probe targeting the 16S rRNA was designed for this phylotype. After exposing the sludge to [^{14}C]methanol, fluorescence *in situ* hybridization (FISH) was combined with microautoradiography. This confirmed that the radiolabeled cells were those that hybridized with the phylotype-specific probe.

Analogous to this approach, other available techniques take advantage of microscopy to analyze the environment of interest and confirm results from SIP. Two particularly powerful microscopic approaches in early development include Raman microscopy [13] and FISH coupled with secondary ion mass spectrometry [8, 24], both of which offer the potential to examine individual cells and aggregates for isotopic enrichment, complementing results obtained with SIP analysis. In addition, the isotope array [1] enables the combination of rapid and efficient radioactive labeling of nucleic acid in the environment with the phylogenetic resolution of microarrays.

Stable isotope probing is not a perfect technique and is subject to biases and limitations similar to other techniques used by microbial ecologists [23]. The successful application of SIP methodology will depend on careful experiment design, the required sensitivity, the approach selected for separating labeled nucleic acid biomarkers, and the downstream analysis intended for the labeled material. SIP has enabled a range of experimental approaches that provide community-based information for guiding cultivation attempts, relevant sequence information for improved design and application of specific hybridization probes, and ecological information about the functions of environmental organisms that have been largely unexplored. The utility of this method is best realized in combination with other techniques that help to confirm the data obtained and, as is the case with metagenomics, fully exploit the sequence data available from isotopically enriched genetic material.

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