10 Stable Isotope Probing: A Critique of Its Role in Linking Phylogeny and Function

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10.1 Introduction

Microbes are responsible for an enormous array of the biochemical transformations in the Earth's biogeochemical cycles and are capable of mineralising almost all chemicals derived from human industry. Whilst these assertions are in no doubt, microbial ecologists have had limited success in attributing biogeochemical and biodegradative transformations to specific microbial taxa in the environment (Gray and Head 2001). This can be attributed to the difficulties associated with observing microscopic processes and our inability to reconstruct all but the simplest of microbial communities in the laboratory. However, in recent times, novel methodological approaches promise to shed light on the relationship between microbial phylogeny and certain community functions in situ (Murrel and Radajewski 2000; Boschker and Middelburg 2002; Radajewski et al. 2003; Wellington et al. 2003). This chapter summarises the lead role a novel methodological approach known as stable isotope probing (SIP) is playing in attributing the assimilation of specific elements from certain compounds to particular microorganisms within complex microbial communities.

Many of the important biogeochemical and biodegradative processes mediated by microbes take place in soil environments (Conrad 1996; Kozdroj and Van Elsas 2001). Owing to their complex and heterogeneous nature, soil environments support the highest microbial species richness per unit of space of any environment known (Torsvik et al. 1990). It follows that the diversity of chemical transformation occurring in soils is also rich; therefore, attributing processes of interest to specific microbial taxa in soil environments is never likely to be a menial task. Despite this, it is clear that through the rigorous implementation of SIP protocols, microbial ecologists are making headway in this endeavour.

SIP involves tracking stable isotope atoms from particular substrates into components of microbial cells that provide phylogenetic information, often

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referred to as biomarkers. The most common biomarkers are nucleic and fatty acids. By aligning the incorporation of an isotopic label with a specific phylogenetic group it is possible to attribute the assimilation of a substrate to certain microbes. Because SIP is founded on assimilatory processes, its utility is restricted to chemical transformations that microbes perform, at least in part, for anabolic purposes. Non-assimilatory chemical transformations, of which there are many relevant to ecology, biogeochemistry and biodegradation, fall outside the applicability of SIP. SIP approaches can theoretically be applied to trace the assimilation of any element that has a stable isotope. Almost all of them do, including most of the abundant elements in biological materials (C, N, O, H, and S). In practice the application of SIP has almost exclusively been restricted to the use of ^{13}C.

SIP methodologies vary in the use of biomarker and the means by which biomarkers are analysed both for isotopic and phylogenetic content (summarised in Fig. 10.1). Whilst not all of the published methodologies have been tried and tested in soil environments they each have potential relevance and are therefore worthy of review here. The remainder of this

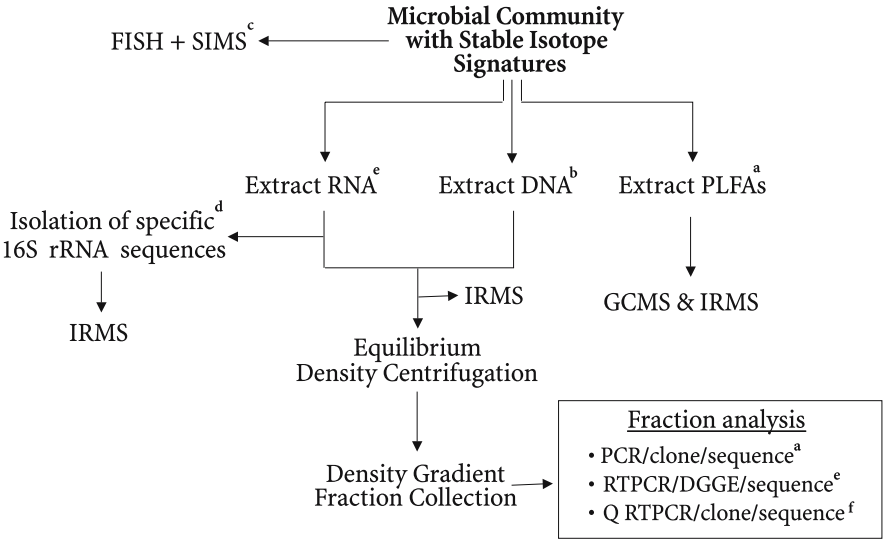

Fig. 10.1. Summary of stable isotope probing (SIP) approaches to link function with phylogeny. Approaches pioneered by *a* Boschker et al. 1998; *b* Radajewski et al. 2000; *c* Orphan et al. 2001; *d* MacGregor et al. 2002; *e* Manefield et al. 2002b; and *f* Lueders et al. 2004a. Equilibrium density centrifugation based SIP methods require pulse enrichments of stable isotope signatures. Refer to text for details. *GCMS* Gas chromatography/mass spectrometry; *IRMS* isotope ratio mass spectrometry; *Q RTPCR* quantitative reverse transcription polymerase chain reaction; *FISH* fluorescence in situ hybridization; *SIMS* secondary ion mass spectrometry; *PLFA* polar lipid derived fatty acid

chapter discusses each in turn with emphasis on practical limitations and the relative contributions each has made to date in linking microbial community functions with phylogeny in the soil environment.

10.2 Polar Lipid Derived Fatty Acid Based Stable Isotope Probing (PLFA-SIP)

Boschker et al. (1998) published the first stable isotope probing investigation, which identified microorganisms responsible for the assimilation of C from the greenhouse gas methane in a freshwater sediment environment. Stable isotope labelled methane ($^{13}CH_4$) was pulsed into the microbial community occupying the sediment resulting in the labelling of polar lipid derived fatty acids (PLFAs) from methane-assimilating organisms. Subsequently, PLFAs were extracted, separated and analysed for ^{13}C enrichment by isotope ratio mass spectrometry (IRMS). Because specific phylogenetic groups produce signature PLFA profiles the stable isotope enrichment of certain PLFAs revealed which organisms were dominating the use of methane as a C source. The labelled PLFAs generated throughout the duration of the pulse were only produced by species belonging to the *Methylobacter* and *Methylomicrobium* genera, thereby unambiguously linking these organisms with methane oxidation in this environment. In the same study, Boschker et al. (1998) identified the sulphate reducing microorganisms responsible for acetate oxidation in estuarine and brackish sediments. PLFA-SIP has also been used to identify the microorganisms responsible for methane oxidation in temperate forest soils (Bull et al. 2000) and marine sediments (Hinrichs et al. 1999) and propionate consumption in anoxic brackish sediments (Boschker et al. 2001). Finally, a number of authors have traced complex substrates such as ^{13}C-labelled plant litter or microalgae into PLFAs in order to investigate the fate of decomposing photosynthetic organisms (Sun 2000; Malosso et al. 2004).

Hanson et al. (1999) were the first to use PLFA-SIP to link the degradation of a xenobiotic to specific microbial community members. Yolo silt loam was pulsed with ^{13}C-labelled toluene and PLFAs were analysed for ^{13}C enrichment. This study attributed toluene degradation to genera within the order *Actinomycetales*. The majority of research attention for toluene degradation has been absorbed by genera within the order *Pseudomonadales*. In this case SIP offered a clear rationale to turn some of this attention to the *Actinomycetales*, and may eventually result in more successful attempts to augment natural toluene remediation. Pelz et al. (2001) performed a similar investigation to identify sulfate-reducing, toluene-degrading microorganisms in the sediment of a petroleum hydrocarbon contaminated aquifer.

Johnsen et al. (2002) traced ^{13}C-labelled phenanthrene into the PLFAs of various phenanthrene-degrading taxa in soils unpolluted, lightly polluted and heavily polluted with polyaromatic hydrocarbons. Alexandrino et al. (2001) used PLFA-SIP to identify styrene-degrading organisms from biofilters used for the treatment of styrene-containing waste gases. Uniquely, they applied a ^{2}H- rather than ^{13}C-labelled substrate in their investigation. To date ^{2}H has not been widely used in SIP applications, and there are no published examples of its application in the soil environment.

The most striking advantages of using PLFAs as biomarkers in SIP is the rapidity with which PLFAs become detectably labelled and the quantitative information generated from the analysis of PLFAs with IRMS. The primary limitations of PLFA-based SIP are that the phylogenetic resolution offered by this biomarker is inferior to that offered by nucleic acid (NA)-based biomarkers and that it requires signature PLFAs to have been identified from close culturable relatives. A number of researchers also regard the extraction of PLFAs from soils as a labour-intensive process. Despite these limitations PLFA-based SIP has implicated more soil microbes in environmental processes of interest than any other form of SIP.

10.3 DNA- and RNA-Based Stable Isotope Probing (NA-SIP)

In comparison to PLFAs as a biomarker, polymerase chain reaction (PCR)-based sequence analyses of 16S rDNA genes or rRNA itself offers high-resolution, culture-independent phylogenetic information (Olsen et al. 1986). Radajewski et al. (2000) enhanced the phylogenetic resolution of SIP by demonstrating that stable isotope labelled DNA could be isolated from mixed microbial communities based on the increase in buoyant density associated with isotopic enrichment. Equilibrium density centrifugation in CsCl gradients was used to separate 'heavy' (labelled) from 'natural' (unlabelled) DNA and 16S rRNA gene clone libraries constructed from 'heavy' DNA were sequenced to obtain the identity of organisms assimilating the ^{13}C-labelled substrate used in the study (Fig. 10.2).

This technique was used to attribute methane and methanol utilisation in oak forest soil microcosms to moderately acidophilic methylotroph populations (Radajewski et al. 2000, 2002). Radajewski et al. (2002) went on to amplify functional genes involved in the oxidation of one-carbon compounds from high density gradient positions. Along with implicating methanol dehydrogenase and methane monooxygenase genes in methanol and methane assimilation, this study found that species encoding ammonia monooxygenase had assimilated ^{13}C. It was proposed that ammonia-oxidising species had assimilated ^{13}C second hand through the $^{13}CO_2$ generated by the

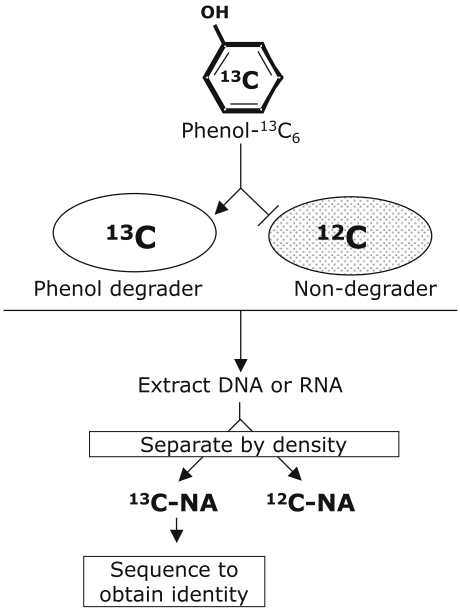

Fig. 10.2. An example of nucleic acid (NA)-based stable isotope probing. A ^{13}C-labelled substrate (e.g. phenol) is pulsed into a mixed microbial community composed of phenol-degrading and non-phenol-degrading organisms. After the pulse is consumed, total community DNA or RNA is extracted and separated by equilibrium density centrifugation. NA sequences are then obtained from the ^{13}C-labelled fraction thereby revealing the identity of organisms acquiring carbon from the chosen substrate (phenol)

methylotrophs. This raises the interesting challenge posed by metabolic crosstalk. Whilst SIP has the potential to dissect microbial food webs, to date the possibility of secondary feeding on breakdown products of the primary substrate has emerged as a frustrating caveat researchers cannot ignore.

SIP with DNA has also been used to identify active methanotrophs in peat soil (Morris et al. 2002) and ammonia-oxidising microbes in freshwater sediments (Whitby et al. 2001). One of the primary limitations of NA-SIP is the requirement for a large isotopic enrichment in the biomarker. This, in conjunction with concerns over the presence of unlabelled substrates native to the system that will compete for assimilation, has led researchers to apply artificially high concentrations of labelled substrates into soil microcosms for extended periods of time. The in situ relevance of doing so has been questioned (Jeon et al. 2003; Padmanabhan et al. 2003). Jeon et al. (2003) pulsed ^{13}C-labelled naphthalene at realistic concentrations into a contaminated sediment to identify naphthalene degraders in situ. Further, Jeon et al. (2003) were able to isolate a β-proteobacterium, implicated in

naphthalene mineralisation by DNA-SIP, and demonstrate that it encodes a naphthalene dioxygenase present at the field site. It is pertinent to mention at this point that, owing to the heterogeneous nature of soil environments, the diversity between different soil environments (including the ability to adsorb different compounds) and the diversity in solubility and volatility of the many substrates relevant to SIP in soil environments, that careful planning of the delivery of a pulse is crucial to the generation of meaningful results.

Despite the successful application of DNA-SIP to naphthalene degradation in the field, the requirement for cell replication during pulse labelling, which is notoriously slow in soil environments, will always be a limitation to the isotopic enrichment of DNA. Whilst extending the duration of a pulse can alleviate this limitation it also exacerbates the issue of metabolic crosstalk. To resolve this dilemma, 16S rRNA-based SIP methodologies have been developed (Manefield et al. 2002a,b). Because RNA is turned over in bacteria independently of replication, rates of RNA synthesis are always higher than for DNA. For this reason RNA will be labelled more rapidly, making it a more responsive biomarker with a high degree of phylogenetic resolution.

The applicability of RNA-SIP was demonstrated with the identification of bacterial species responsible for phenol degradation in the activated sludge community of an industrial waste water treatment plant (Manefield et al. 2002b). Manefield et al. (2002b) have additionally highlighted the importance of fractionating equilibrium density gradients and monitoring increases in NA density over time as a substrate pulse is assimilated. The utility of the technique in the soil environment has been demonstrated by Lueders et al. (2004b) who identified methylotrophs responsible for methanol assimilation in a rice field soil and implicated certain fungi and flagellates in grazing on these methylotrophs. They have gone on to use RNA-SIP to identify the bacteria and archaea responsible for syntrophic propionate degradation in flooded soil, including representatives of uncultured phylogenetic groups (Lueders et al. 2004c). Lueders et al. (2004a) have introduced the quantification of transcripts or genes in gradient fractions with real-time PCR. Even though the use of RNA as a biomarker and the rigorous quantitative examination of gradient profiles have enhanced the sensitivity of NA-SIP, there remain certain limitations to its application in soil environments. An obvious hurdle is the ability to extract clean and intact DNA or RNA from the soil environment under investigation. A less intuitive limitation is exemplified by the following contemplation of unpublished data from our laboratory.

Given that the soil microbiota plays a large role in regulating the concentrations of greenhouse gases in the atmosphere, many researchers have recognised the value of attributing particular processes in soil to specific

taxonomic groups present therein. For example, there is a need to identify the microbial taxa dominating the acquisition of carbon from plant root exudates. To address this question, we pulsed labelled grassland turfs with $^{13}CO_2$ to generate and deliver ^{13}C-labelled root exudates to the soil community (Ostle et al. 2003), and then analysed the 16S rRNA from root-associated soil by IRMS and equilibrium density centrifugation (Griffiths 2003). Whilst the IRMS analysis revealed that the RNA was enriched with ^{13}C, the degree of labelling was too low to allow conclusive results to be drawn from density centrifugation experiments. A PLFA-SIP analysis of soil samples derived from a $^{13}CO_2$ plant pulse was able to attribute assimilation of root exudates at low phylogenetic resolution to Gram-negative bacteria and fungi (Treonis et al. 2004).

This raises an important issue related to any SIP methodology that is dependent on the degree of labelling. If the labelled substrate of choice will ultimately be consumed by a rich diversity of organisms then the degree of labelling in any given taxa will be low, making separation by density problematic. Conversely, if the substrate of choice is consumed by a small number of taxa, then the degree of labelling in those taxa will be high, facilitating isolation by density. The use of $^{13}CO_2$ to label soil microbes via root exudates is particularly problematic both because the initial substrate is translated into a large range of organic compounds and the microbial diversity in the soil is extremely rich. Further, the physiological state of genetically identical cells in the soil environment can be very different at any one time. Ultimately, attributing the assimilation of root exudates to specific taxa by NA-based SIP will involve pulsing labelled exudates directly into root-associated soil. There are experimental systems available to mimic the delivery of root exudates into soil (Falchini et al. 2003).

10.4 Alternative Stable Isotope Based Approaches

Two additional and distinct stable isotope based approaches have been developed to unveil the relationship between microbial community functions and diversity. Orphan et al. (2001, 2002) have published investigations in which individual cells or mixed aggregates of cells in methane-consuming communities were identified by fluorescence in situ hybridisation (FISH) and subsequently analysed for ^{13}C content by secondary ion mass spectrometry (SIMS). The natural abundance of ^{13}C in methane is conveniently low, enabling Orphan et al. (2001) to associate cells harbouring depleted ^{13}C signatures with methane consumption. In this way, the involvement of specific Archael groups in the consumption of oceanic methane reservoirs in marine sediments has been demonstrated.

A related approach involves isolating the small-subunit (SSU) rRNA of particular phylogenetic groups from total community RNA and determining stable isotope levels therein by IRMS. Specific rRNA molecules can be captured by the hybridisation of biotin-labelled probes to target sequences followed by retrieval of hybridised targets using magnetic beads. MacGregor et al. (2002) demonstrated the potential utility of this approach using laboratory grown cultures. Again, natural differences in the stable isotope composition of substrates could be exploited to trace their assimilation into phylogenetic groups of interest. The primary technical challenge associated with this method is the isolation of sufficient quantities of rRNA for isotopic characterisation. This approach awaits application in the field.

The primary advantage of the Orphan and the Macgregor approaches is that there is no requirement for high degrees of labelling for the method to be informative. As demonstrated, these methods can be applied to scrutinise natural stable isotope signatures. Further, the method can also be used in conjunction with an enrichment pulse. The disadvantage of these approaches lies in the fact that it is dependent on sets of predetermined oligonucleotides that must be hybridised to sequences of interest. This necessarily transforms the question being asked from 'which taxa assimilate the substrate?' to 'does a specified taxa assimilate the substrate?', thereby pre-determining the analysis to some degree.

10.5 Radioactive Isotope Based Approaches

The use of stable isotopes to trace the assimilation of elements from substrates of interest by microbial taxa in soil environments has, in some ways, overshadowed exciting developments of radioactive isotope based methods aimed at achieving the same alignment of function with phylogeny. By combining FISH with microautoradiography (MAR), Lee et al. (1999) demonstrated that the assimilation of ^{14}C-labelled acetate, butyrate and bicarbonate could be attributed to various taxa in activated sludge samples. Whilst the number of published studies making use of MAR-FISH continues to accrue, it remains to be applied to soil environments. Technical aspects of the methodology in the context of soil matrices may ultimately make it too difficult to do so.

An approach that has more potential in its application to soil environments makes use of 16S rRNA microarrays (Adamczyk et al. 2003). After pulse labelling activated sludge samples with ^{14}C-labelled bicarbonate, Adamczyk et al. (2003) hybridised community 16S rRNA samples to a microarray which was then scrutinised for the location of the radioactive isotope in order to identify the autotrophic members of the community.

Despite the obvious utility of these radioactive isotope based techniques, their potential to link community structure to function in field experiments is nullified by the associated and valid health and safety concerns. As evidenced by the rapid development and application of alternative stable isotope based approaches, many researchers prefer to avoid working with radioactivity.

10.6 Notes on Isotopic Enrichments

Most of the studies described above have involved enriching isotopic signatures in biomarkers of microbes performing assimilatory functions. A small number of them have exploited natural isotopic signatures derived from processes that fractionate against rare isotopes. The presence of natural isotope signatures are a fortuitous phenomenon inherent to a limited number of biochemical transformations, not only because there is a ready-made signature to observe, but also because it avoids two important considerations that researchers performing enrichment pulses must take into account. The first is that the presence of a label may cause a substrate to be discriminated against. Our experience with phenol and SIP suggests fractionation does not occur on a discernable level in the face of an enrichment pulse, but this does not rule out the possibility that fractionation may manifest itself in other situations. The use of natural isotopic signatures also avoids the expense of acquiring labelled substrates. In many cases the cost of having a particular fully labelled substrate custom-synthesised is too high to justify the use of a stable isotope probing approach.

10.7 Conclusions

Herein we have described the benefits being derived from the development of SIP alongside related caveats in the context of linking microbial phylogeny and function in soil environments. It is clear from the increasing frequency of publications in which SIP plays a role that it has great utility in its various guises, even in soil environments. We believe that with considered application SIP has the potential to confirm or discover the identity of microbes responsible for a large range of processes of interest to humans both in fundamental ecology and bioremediation. Despite this, we would still argue that there is room for methodological improvement. Ideally, we require a SIP methodology that allows the quantification of label incorporation in conjunction with sequencing based phylogenetic

resolution. Further, it is important to emphasise that SIP can only penetrate the relationship between microbial community composition and function when the function of interest involves anabolic activity. There are countless important activities partaken by microbes that do not involve substrate assimilation. Despite the promises of SIP, microbial ecologists have at hand the challenge of developing even more ingenious means of unravelling the relationship between specific taxa and non-assimilatory functions.

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