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Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms

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Phylogeny based on ribosomal RNA sequences alone is rarely a reliable indicator of microbial function. To circumvent this problem, nucleic acid based techniques have been developed that exploit the physical properties of stable isotopes to study microbially mediated processes within complex environmental samples. Investigations using labelled substrates, or which detect variations in the natural abundance of isotopes, have thus revealed the metabolic function of microorganisms without the need to isolate them in culture.

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Abbreviations

FISH	fluorescent *in situ* hybridisation
IRMS	isotope ratio mass spectrometry
PCR	polymerase chain reaction
PLFA	phospholipid-derived fatty acids
SIMS	secondary ion mass spectrometry
SIP	stable-isotope probing
SSU rRNA	small subunit ribosomal RNA

Introduction

Targeting of the small subunit ribosomal RNA (SSU rRNA; i.e. 16S and 18S rRNA) or the corresponding genes, has become an established and robust means to describe the phylogenetic diversity of microbial communities [1,2]. However, the rRNA sequences that have revealed a remarkably vast microbial diversity, generally provide few direct clues regarding the interactions and metabolic capabilities of the microorganisms that these sequences represent. Thus, one of the fundamental questions in microbiology is as pertinent as ever: which functions are attributable to which microorganisms in the natural environment?

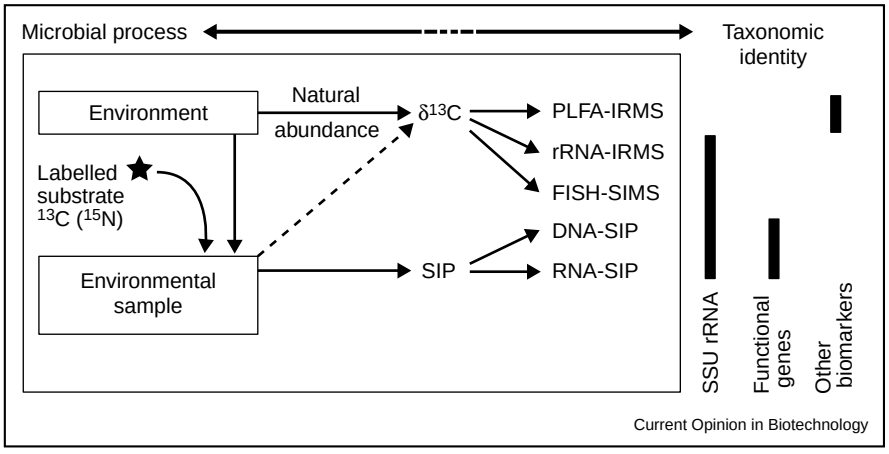
To date, the most successful way to address this question has first involved isolation of microbial strains into laboratory culture, after which they can be identified and characterised at the physiological, biochemical and genetic levels. Such metabolic properties and cellular interactions can be used to infer potential functions of these microbes and their close relatives in a natural environment. However, it is recognised that the microbial phyla that are isolated most readily into culture only represent a fraction of the phylogenetic groups that are widely distributed in the environment [3,4].

Several recently published methods have adopted the converse approach to address this question, by first establishing the biological process and then identifying the microorganisms that are involved. These methods exploit physical properties of the atoms that constitute all cellular components, in particular the isotopes of carbon. An example is the technique of stable-isotope probing (SIP); a term coined to describe the use of ^{13}C-labelled growth substrates to link microbial function with identity via selective recovery of 'heavy' ^{13}C-labelled DNA [5]. In a wider context, however, SIP is not exclusively a DNA-based technique (Figure 1). Analysis of labelled biomarkers including RNA and phospholipid-derived fatty acids (PLFAs) can also be used to identify the microorganisms involved in a particular function within a complex environmental sample. It has also been possible to use natural abundance levels of stable isotopes in cells or nucleic acids as a signature that a microbial population is involved in specific processes. Here, we review this broader definition of SIP, outlining current applications, advantages, limitations and potential future applications.

DNA-SIP

The elegant rationale underlying DNA-based SIP (DNA-SIP) is not recent. In 1958, Meselson and Stahl [6] anticipated that a labelled atom which increases the density of DNA might help to resolve the distribution of parent and progeny molecules following DNA replication. Their well-known results were achieved with *Escherichia coli* grown on a nitrogen source (NH_4Cl) that was labelled with the ^{15}N or ^{14}N stable isotope, coupled with the technique of caesium chloride (CsCl) density-gradient centrifugation. The nucleotide units that comprise DNA also contain a large proportion of carbon and hydrogen, due in part to the deoxyribose backbone (Table 1), and it was subsequently found that DNA could also be labelled with the heavy stable isotopes ^{13}C and ^{2}H (reviewed in [7]). Although the buoyant density of DNA varies with its guanine-cytosine (G+C) content [8], the incorporation of a high proportion of a naturally rare stable isotope into DNA greatly enhances the density

Figure 1



Schematic diagram illustrating the use of stable isotopes and nucleic acids to obtain a link between a microbial process and microbial identity. Current approaches have incubated environmental samples with a ^{13}C-labelled substrate to define the process and analysed SSU rRNA or functional gene sequences in labelled DNA or RNA fractions to establish taxonomic identity by SIP. The ratio of stable carbon isotopes ($\delta^{13}C$) can also be measured at natural abundance, using IRMS or SIMS, to reveal signatures for certain processes occurring in the environment. When combined with rRNA capture, FISH or analysis of non-nucleic acid biomarkers (e.g. PLFAs), microbial communities can be identified. Stable isotope ratios can also be determined following incubation with labelled substrates. The biomarkers that are used by each technique to obtain taxonomic identity are indicated by the vertical bars.

difference between labelled and unlabelled fractions (Figure 2).

Separation of heavy (labelled) and light DNA using density-gradient centrifugation can ultimately be attributed to the large difference between the natural abundance of the heavy and light stable isotopes (Table 1). Consequently, DNA-SIP is dependent upon the availability of compounds that are highly enriched with a rare stable isotope. To date, the substrates $^{13}CH_3OH$, $^{13}CH_4$ or $^{13}CO_2$ (99 atom% ^{13}C; i.e. 99% of the carbon atoms are the ^{13}C isotope) have been added to soil samples or enrichment cultures for DNA-SIP. These studies have focussed on the process of methylotrophy (i.e. metabolism of reduced one-carbon compounds) [5,9••,10••] or on aerobic autotrophic ammonia oxidation [11•].

The most important feature of DNA-SIP is that the heaviest DNA (^{13}C-DNA) fraction collected following density-gradient centrifugation contains the combined genomes of a microbial population that is able to incorporate the labelled substrate into their nucleic acids. Therefore, 'universal' polymerase chain reaction (PCR) primers that amplify the SSU rRNA genes of Bacteria, Archaea or Eukarya are integral for the identification, *a priori*, of microorganisms involved in the process of interest. When a specific metabolic function has been previously established for cultivated microorganisms, it has also been possible to use PCR primers to amplify functional genes that encode key enzymes in specific metabolic pathways (e.g. *mxaF*, which encodes the active-site subunit of methanol dehydrogenase). These principles were illustrated in the first application of DNA-SIP,

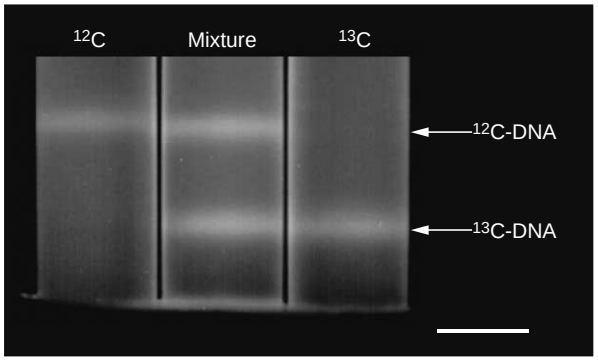
Table 1

Elements that constitute nucleic acids and some properties that are important for SIP.

Nucleotide unit	H	C	N	O*	P
Number of atoms per nucleotide unit in DNA					
β-D-2-Deoxyribose backbone	7	5	0	5	1
(A) Deoxyadenylate	11	10	5	5	1
(G) Deoxyguanylate	11	10	5	6	1
(T) Deoxythymidylate	12	10	2	7	1
(C) Deoxycytidylate	11	9	3	6	1
Isotope natural abundance (atom %)†					
Light stable isotope	(^{1}H) 99.99	(^{12}C) 98.93	(^{14}N) 99.63	(^{16}O) 99.76	(^{31}P) 100
Heavy stable isotope(s)	(^{2}H) 0.01	(^{13}C) 1.07	(^{15}N) 0.37	(^{17}O) 0.04 (^{18}O) 0.20	

*In RNA there is one additional oxygen atom present in each ribonucleotide. †Data taken from www.webelements.com

Figure 2



Equilibrium centrifugation of isotopically labelled DNA in CsCl/ethidium bromide gradients. Individual and combined samples of DNA extracted from *Methylosinus trichosporium* OB3b grown on ^{12}C- or ^{13}C-labelled methane as the sole carbon source. Bar, 1 cm.

in which ^{13}C-labelled methanol was added to a 10 g soil sample [5]. Following density-gradient centrifugation, the separation observed between the discrete DNA bands was used to estimate that the ^{13}C-DNA fraction was approximately 65 atom% ^{13}C, and therefore represented a methanol-assimilating population. Indeed, it was easy to reconcile that many of the 16S rRNA and *mxaF* gene sequences retrieved by PCR from the ^{13}C-DNA were very similar to those of α-*Proteobacteria*, which can grow on methanol in culture in the laboratory. However, the presence of a smaller number of 16S rRNA gene sequences in the labelled DNA suggested that methylotrophy may be a function of certain members of the *Acidobacterium* division. The acidobacteria are a diverse and widely distributed group of microorganisms that has few cultivated representatives, none of which has been reported to grow on methanol.

A valuable secondary aspect of DNA-SIP is that incorporation of a labelled substrate into DNA implies that the microorganisms represented by the ^{13}C-DNA fraction were active under the conditions tested. Whitby *et al.* [11•] were thus able to identify the metabolically active $^{13}CO_2$-assimilating autotrophic ammonia-oxidising bacteria in an enrichment culture inoculated with a freshwater sediment. As ammonia oxidisers form a coherent rRNA group, ^{13}C- and ^{12}C-labelled DNA was amplified with group-specific 16S rRNA PCR primers. Analysis of the amplification products indicated that nitrosomonads had out-competed nitrosospiras in laboratory culture. DNA-SIP and analysis of 16S rRNA gene sequences has also been used to identify the active aerobic methane-oxidising bacteria (methanotrophs) in soil microcosms exposed to $^{13}CH_4$ [9••,10••]. The activity of one or both of two broad methanotroph groups was further supported by analysis of the functional genes *mmoX* and/or *pmoA*, which encode the active-site subunits of the soluble and particulate methane monooxygenase, respectively. Morris *et al.* [9••] also observed that many 16S rRNA gene sequences amplified from the ^{13}C-DNA fraction were not related to those of known methanotrophs. Certain 16S rRNA and *mxaF* gene sequences suggested that some bacteria had grown on methanol (an oxidation product of CH_4), whereas others may have predated ^{13}C-labelled biomass. Although these observations demonstrate the limitations of using DNA-SIP to link microbial function with taxonomic identity, they also provide a rational basis for further investigations into the microbial community that may be actively involved in the cycling of a specific substrate.

Most limitations associated with DNA-SIP stem from the need to add a substrate and wait for the synthesis of labelled DNA by the microorganisms that are involved in the process under investigation. Long incubation periods (>40 days) have been used before labelled DNA extracted from small soil samples (less than 10 g) can be observed in a CsCl gradient [5,9••]. Larger volumes (200 ml) used to enrich ammonia-oxidising bacteria only required a shorter incubation period (5 days) [11•]. Any such incubation will result in the formation of labelled products and intermediates of substrate metabolism, which could then be assimilated by non-target microorganisms (cross-feeding). It has been suggested that cross-feeding effects might be detected as a successional change occurring during a DNA-SIP time-course experiment, thereby permitting a more sensitive resolution of the link between function and taxonomic identity [10••]. Further studies are still required to quantify these limiting aspects of DNA-SIP.

RNA-SIP

The principles of density-gradient centrifugation have also been used to link function with taxonomic identity using a very promising RNA-based stable-isotope probing approach (RNA-SIP). Manefield *et al.* [12••] reasoned that RNA could serve as a more responsive biomarker than DNA for use in SIP, because in active cells, RNA synthesis occurs at a high rate and labelling can also occur without the need for DNA synthesis or replication of the organism. The sequence-based resolution offered by SSU rRNA should permit identification of the microorganisms involved in a biological process, without any prior knowledge of their identity. These authors used RNA-SIP in this capacity for identification of the microorganism(s) that are responsible for phenol metabolism in an aerobic industrial bioreactor. A pulse of phenol-$^{13}C_6$ was added to a bioreactor sludge sample, and an RNA fraction enriched with ^{13}C was collected 8 h later. Contrary to expectations based on previous conventional (cultivation) and molecular microbiological studies, reverse transcriptase (RT)-PCR amplification of ^{13}C-labelled 16S rRNA identified a

Thauera species as the bacterium that dominated the acquisition of carbon from phenol.

A fundamental difference between RNA-SIP and its DNA counterpart is that it relies on the 'transcriptome' of the active target microorganism(s). Therefore, one key advantage of the RNA-SIP approach is the natural amplification of the phylogenetic signature molecule (rRNA) in active cells. Furthermore, it was found that RNA became ^{13}C-labelled much more rapidly than DNA in a densely populated bioreactor sample [12••]. This suggests that RNA-SIP may have greater sensitivity than DNA-SIP, because it should be possible to reduce the amount and/or concentration of substrate or the length of an incubation that is required to obtain a link between metabolic function and taxonomic identity. It remains to be determined whether microbial communities in other environmental samples will respond in the same manner.

Several important considerations are critical for the successful application of RNA-SIP [13•]. Native RNA has a higher buoyant density than DNA and of several density gradient media that were evaluated, caesium trifluoroacetate (CsTFA)/formamide performed optimally. As the direct observation of RNA was problematic, because this medium absorbed UV light, RNA-containing gradients were fractionated (up to 20 fractions) using a syringe pump to displace CsTFA with water. Agarose-gel electrophoresis revealed that nearly all the RNA of a given buoyant density focussed over three or four gradient fractions; however, it was noted that RT–PCR amplification detected RNA in fractions that spanned the entire length of a density gradient. To circumvent this problem it was essential to analyse each gradient fraction by RT–PCR and a community profiling technique (e.g. denaturing gradient gel electrophoresis), and monitor the changes in band intensity over the duration of the labelled substrate pulse. In this manner it was possible to identify cross-feeding effects within a defined, mixed bacterial culture and to identify phenol-assimilating organisms that were important for bioreactor function [12••].

Potential limitations to SIP using labelled substrates

The application of DNA-SIP and RNA-SIP is still in its infancy and many technical aspects have not been fully evaluated. The principal consideration for determining whether SIP will be suitable for investigating a specific process is whether the nucleic acids of the target organisms will contain a sufficient proportion of ^{13}C-labelled atoms to permit collection of a heavy nucleic acid fraction. Isotope ratio mass spectrometry (IRMS) has confirmed extensive enrichment of ^{13}C in heavy DNA and RNA fractions [11•,12••]. A series of experiments using a pure culture grown on increasing proportions of phenol-$^{13}C_6$ (between 0–100%) established empirically that RNA fractions with a minimum ^{13}C content of 20 atom% could be resolved from unlabelled RNA [13•]. This value is equivalent to the lower limit that was previously estimated for DNA-SIP, while still maintaining a link between metabolic function and taxonomic identity [5]. However, microbial DNA (^{12}C) with a G+C content of 35–70% ranges in density from approximately 1.69–1.73 g cm^{-3} [8], whereas the calculated buoyant density for the same DNA with a ^{13}C content of 100 atom% is approximately 1.75–1.79 g cm^{-3}. Although not yet verified experimentally, labelled nucleic acids extracted from environmental samples might therefore need a ^{13}C content greater than 50 atom% to avoid the isolation of high G+C content ^{12}C-DNA in the ^{13}C-DNA fraction.

At present, relatively high concentrations (e.g. 10% CH_4 or 500 μg/ml phenol) and large amounts of labelled substrates have been used to obtain labelled nucleic acid fractions. The sensitivity of PCR may permit the use of less substrate or shorter incubations, provided that a microbial population can still be associated with substrate assimilation. Nevertheless, SIP will be vulnerable to the problems associated with microbial enrichment, albeit within a physically, chemically and biologically complex environment such as a bioreactor or soil. CsCl density gradients observed in DNA-SIP studies have reported a distinct ^{13}C-DNA band [5,11•], a 'smear' of heavy and intermediate density DNA species [9••], or even no observed ^{13}C-DNA fraction [10••]. It has been argued that the most heavily labelled fraction of ^{13}C-DNA will contain sequences of the target population and may also include organisms in very tight nutritional relationships (syntrophy). DNA of intermediate buoyant density may occur due to cross-feeding effects or the growth of target organisms on both labelled and naturally occurring (i.e. ^{12}C-labelled) substrates. As the results of further applications of SIP are interpreted, it will become important to establish the significance of minor components of the labelled nucleic acid pool. These may reflect slow growing microorganisms that are out-competed during enrichment or biases introduced by PCR amplification. Thus, it is important to stress that if a microorganism is not detected in a ^{13}C-labelled nucleic acid fraction, it is not necessarily precluded from involvement in a metabolic process.

SIP requires the uniform labelling of atoms in the compound of interest. One-carbon substrates are thus particularly amenable to this technique. The use of $^{13}C_6$-phenol demonstrated the potential to investigate the processing of larger molecules, although it is likely that very complex substrates and/or processes will be less suitable for SIP techniques that require the labelling of nucleic acids. Alternative techniques involving IRMS analysis of non-nucleic acid biomarkers have been reviewed recently [14•,15] and have been especially informative when combined with molecular biological or cultivation-based analyses [16,17•,18]. However, techniques that amend

samples with labelled substrates cannot be used to identify definitively which microorganisms are active *in situ*, because inactive populations may be stimulated by the substrate addition. Results of labelling studies should rather be used to provide a rational basis for the application of molecular biological techniques to study the role of specific organisms that are likely to be involved in defined processes.

Natural abundance studies

Two recent approaches that can detect extremely small differences in the isotopic ratio ($\delta^{13}C$) between the heavy and light stable carbon isotopes, such as those occurring at natural abundance, hold considerable potential for linking microbial function with phylogeny. The process of anaerobic methane oxidation has been studied intensively for several interesting reasons including its global importance to the methane cycle and the poor understanding of the microorganisms that are involved. This process is particularly suitable for IRMS or secondary ion mass spectrometry (SIMS), because the natural abundance of ^{13}C in methane is especially low. In methane-rich anoxic sediments, rRNA-targeted oligonucleotide probes and fluorescence *in situ* hybridisation (FISH) have identified the microbial population within cell aggregates [16,19,20••]. Using SIMS, Orphan and colleagues [19,20••] subsequently obtained an isotopic section through phylogenetically identified microbial aggregates and individual cells. As some very low $\delta^{13}C$ values were observed, it was inferred that the major portion of this biomass must have been derived from the only plausible ^{13}C-depleted carbon source, methane. Thus, the combined technique of FISH-SIMS has identified microorganisms that are likely to be involved in methane cycling within anaerobic environments.

SSU rRNA is an excellent candidate biomarker for linking function with taxonomic identity. MacGregor *et al.* [21••] recently described a method to isolate different classes of rRNA from mixtures of total RNA, which employed biotin-labelled oligonucleotide probes and streptavidin-coated magnetic beads. Universal and domain-level probes validated that the method could selectively recover SSU rRNAs from a soil sample, which in principle should permit rRNA capture at various phylogenetic levels. It was also shown that at natural abundance levels, the $\delta^{13}C$ value of SSU rRNA of pure cultures closely reflected that of the carbon source. As this relationship holds true for ^{13}C-labelled substrates [13•], IRMS analysis of captured rRNA may permit studies of carbon flow in natural microbial communities. In many natural samples the major limitation to this approach is likely to be the large amount (10–100 μg) of RNA required for reliable stable carbon isotope determination [21••]. One approach to increase the amount of carbon for analysis may involve the rRNA-based capture of entire cells [22]. However, as many biosynthetic and scavenging pathways result in more extreme isotopic fractionation than occurs for nucleic acids, studies involving natural isotope abundance will be restricted to processes where the carbon substrate has a particularly strong isotopic signature. Therefore, the use of rRNA-IRMS and FISH-SIMS techniques may be most suited to processes such as methane oxidation or those for which labelled substrates can be added.

Outlook

Interest in establishing the function and identity of microorganisms without the need for their cultivation has seen a recent rapid increase in the use of techniques that employ stable isotopes to dissect biological processes. Techniques using stable-isotope-labelled compounds have assumed that these have no effect on the microorganisms that assimilate the label. Although this appears to be the case for many substrates [23], it is apparent that some effects may need to be examined in more detail; for example, uniformly ^{13}C-labelled hexadecane retarded the growth of an enrichment culture more than a mixture of ^{13}C- and ^{12}C-labelled hexadecane [24]. An earlier study also found that replacement of isotopes of hydrogen, carbon, oxygen and nitrogen in an alga altered the quantity and distribution of cellular components, including nucleic acids [25]. There is scope for investigating certain processes in the nitrogen cycle with the techniques reviewed here, as ^{15}N-labelled compounds also increase the mass of nucleic acids in the same manner as ^{13}C. Although there are several hydrogen atoms in nucleic acids, the use of deuterated compounds may be more problematic because substantial exchange with ^{1}H is likely to occur during metabolism and the relative mass increase from an additional neutron could have a greater impact than with other elements [25]. Labelled substrates might yield additional information in the form of labelled metabolites, which may indicate metabolic pathways used by the target microorganisms under conditions that approach those occurring *in situ*. For example, the detection of ^{13}C-labelled benzenediols in bioreactor fluid suggested that a *Thauera* species degraded phenol aerobically [12••].

A feature of SIP that may become useful in the environmental genomics era is access to the entire nucleic acid pool of a microbial community that is involved in a specific function. In combination with the cloning of large DNA fragments [26,27] or PCR amplification of intact genes [28], it may thus be possible to devise strategies for the targeted recovery of genes that encode novel biotransformation pathways. Access to labelled mRNA also enables identification of the functional genes that are expressed by the active portion of a microbial community during substrate assimilation, although this avenue has yet to be explored.

Despite the enormous potential of techniques employing stable isotopes to investigate microbial community

function without the need for cultivation, it is clear that there are inherent limitations in each approach. Recent studies have demonstrated that careful observation, patience and subtle variations in widely used isolation methods can result in the cultivation of phylogenetic lineages that were previously only detected in cultivation-independent surveys [29•,30•,31•,32]. Therefore, a logical use of stable-isotope based techniques in combination with those of molecular and classical microbiology will offer the clearest view into the metabolic functions of microorganisms in the environment.

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