PERSPECTIVES

INNOVATION

Stable isotope probing — linking microbial identity to function

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Abstract | Stable isotope probing (SIP) is a technique that is used to identify the microorganisms in environmental samples that use a particular growth substrate. The method relies on the incorporation of a substrate that is highly enriched in a stable isotope, such as ^{13}C, and the identification of active microorganisms by the selective recovery and analysis of isotope-enriched cellular components. DNA and rRNA are the most informative taxonomic biomarkers and ^{13}C-labelled molecules can be purified from unlabelled nucleic acid by density-gradient centrifugation. The future holds great promise for SIP, particularly when combined with other emerging technologies such as microarrays and metagenomics.

One of the biggest challenges that microbial ecologists face is to identify which microorganisms are carrying out a specific set of metabolic processes in the natural environment; that is, who is doing what? Until recently, the best way to address this question was to cultivate microbial strains in the laboratory, using growth media that contained a specific substrate, and then to identify the cultivated bacteria at the physiological, biochemical and, more recently, molecular level. The metabolic properties of these bacterial isolates could then be used to infer the potential roles of these and related microorganisms *in situ* in the environment.

An important limitation to this approach has been that most microorganisms that are present in the natural environment are not easily cultivated in the laboratory[1]. However, it is the duty of microbiologists to use new molecular biological data to assist in enriching and cultivating so-called 'unculturable' microorganisms in the laboratory, perhaps not in pure culture as has been the tradition, but in defined mixed cultures or CONSORTIA[2] (see Glossary). In the absence of methods to cultivate many environmental organisms, several techniques have been developed that enable microbial ecologists to identify the activities of microorganisms.

DNA sequencing efforts have yielded over 120,000 bacterial 16S rRNA gene sequences, which are available through the Ribosomal Database Project[3] and nucleotide sequence databases such as GenBank and EMBL. Cultivation-independent methods to study microbial community composition were pioneered in the 1980s and have provided insight into the enormous bacterial diversity[1,4]. Although these methods, such as the polymerase chain reaction (PCR), dot-blot and fluorescence *in situ* hybridization (FISH), offer microbiologists the means to detect and identify novel prokaryotes in the environment, they do not necessarily enable us to infer their functions.

In the last decade, techniques have been developed that combine cultivation-independent identification of microorganisms with metabolic analyses. One such method is FISH–microautoradiography[5,6]. The method involves short-term incubations of environmental samples with specific radiolabelled substrates, fixation of thin sections of these samples to glass slides and subsequent analysis by FISH and inverse confocal laser scanning microscopy (FIG. 1a). This powerful technique allows the detection of bacteria that are biologically active in taking up a specific radiolabelled substrate using pre-designed, specific 16S rRNA-targeted FISH probes. Good examples of the use of this technique involved the analysis of nitrifiers and denitrifiers in complex microbial communities[7].

The isotope array is a second approach that makes it possible to study the function and activity of microorganisms in their natural environment. This technique involves incubating an environmental sample with a ^{14}C-labelled substrate, after which the RNA is extracted from the sample, labelled with a fluorophore and analysed with an oligonucleotide array that targets 16S rRNA of the bacteria of interest. The array is then scanned for fluorescence and incorporation of the radioactive isotope to determine which community members have incorporated the ^{14}C isotope into their RNA[8]. In this manner, Adamczyk and co-workers showed the incorporation of ^{14}C-bicarbonate by autotrophic ammonia oxidizers in activated sludge (FIG. 1b).

FISH–microautoradiography and the isotope array both use radioactive tracers to monitor the incorporation of substrate. An alternative approach is to use stable isotopes to identify functionally active members of a microbial community. Using FISH combined with secondary ion mass spectrometry, Orphan and colleagues[9] exploited the extremely low natural abundance of ^{13}C in methane compared with other carbon compounds to demonstrate that methane is oxidized in anoxic cold seep sediments by a group of Archaea that are associated with the Methanosarcinales. These Archaea mediate the anaerobic oxidation of methane in a consortium with sulphate-reducing bacteria[10].

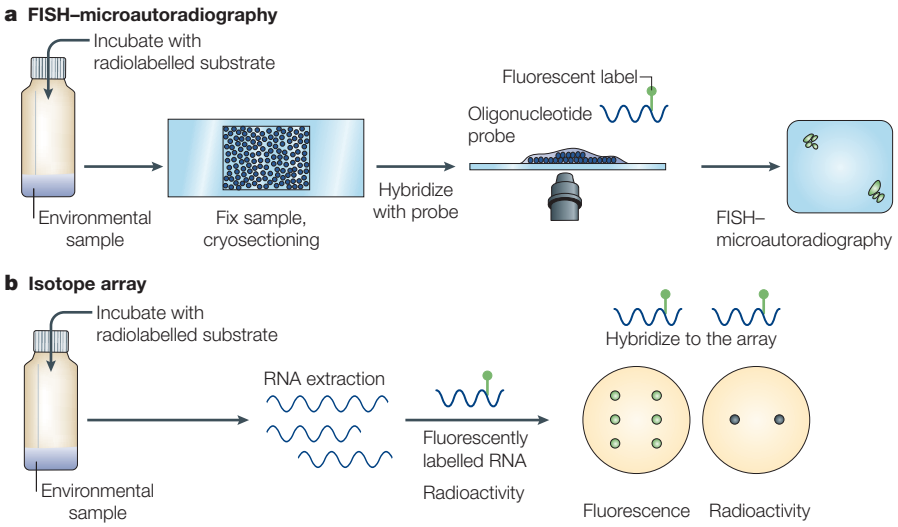

Figure 1 | **Cultivation-independent identification of microorganisms using radioisotopes.** **a** | FISH (fluorescence *in situ* hybridization)–microautoradiography. An environmental sample is incubated with labelled substrates such as ^{3}H-acetate, ^{14}C-pyruvate, ^{14}C-butyrate or ^{14}C-bicarbonate, and then fixed onto a glass slide. Samples are analysed by FISH using fluorescently labelled oligonucleotide probes specific for various 16S rRNA sequences chosen by the researcher. The slides are treated with an autoradiographic emulsion, and silver grains associated with radioactive cells are visualized by inverse confocal laser scanning microscopy. FISH together with detection of radioactivity can identify those microorganisms that are present and metabolizing the specific radiolabelled substrate under the conditions tested. **b** | Isotope array. The environmental sample is incubated with a ^{14}C-labelled substrate, after which the RNA is extracted from the sample. RNA is then labelled with a fluorescent dye and hybridized to an oligonucleotide array containing DNA probe sequences specific for the 16S rRNA genes of the bacteria of interest. Hybridization to the array (fluorescence) can show microorganisms are present in the sample, and radioactivity indicates which of the microorganisms have metabolized the labelled substrate and incorporated the isotope into their RNA.

Stable isotope probing

Stable isotope probing (SIP) is now used to describe a suite of techniques that involve exposing an environmental sample to commercially manufactured, stable-isotope-enriched substrates (which do not radioactively decay) and subsequently analysing the labelled biomarkers. SIP has only been carried out using ^{13}C, but there is potential for some applications to use other stable isotopes, such as ^{15}N. SIP was first applied in the analysis of phospholipid fatty acids (PLFA) that can be extracted from an environmental sample and analysed by isotope-ratio mass spectrometry (IRMS). Groups of microorganisms often have signature PLFA molecules and therefore, in some instances, it is possible to identify the microorganisms that have incorporated the ^{13}C-substrate. A good example of how this technique can be used to link microbial populations to specific biogeochemical processes is the use of ^{13}C-acetate and ^{13}C-methane to study active sulphate reducers and methanotrophs, respectively[11]. The disadvantage of this technique is that nothing is known about the PLFA patterns of microorganisms for which there are no cultivated representatives. Although PLFA analysis offers great sensitivity, the use of labelled nucleic acids as biomarkers has the potential to identify a wider range of bacteria with a greater degree of confidence.

DNA-based SIP

DNA-based SIP[12] (DNA-SIP) is increasingly being used in attempts to link the identity of microorganisms to their functions. The rationale behind the SIP technique is not new; in 1958, Meselson and Stahl showed that DNA labelled with the stable isotope ^{15}N could be separated from DNA containing ^{14}N by density-gradient centrifugation in an experiment that proved the semi-conservative mechanism of DNA replication in *Escherichia coli*[13]. Subsequently, it was found that DNA could also be labelled with the heavy stable isotopes ^{13}C and ^{2}H (REF. 14). To date, only SIP experiments with ^{13}C-enriched compounds have been described. Although the buoyant density of DNA varies with its GC (guanine and cytosine) content, the incorporation of a high proportion of ^{13}C isotope into DNA greatly enhances the density of labelled DNA compared with unlabelled (^{12}C) DNA (FIG. 2).

DNA-SIP is dependent on the commercial availability of compounds that are highly enriched in ^{13}C. The earliest substrates that were used in DNA-SIP were $^{13}CH_3OH$ and $^{13}CH_4$, which contained >99 atom% ^{13}C. These one-carbon compounds are substrates for a group of bacteria known as methylotrophs. The $^{13}CH_3OH$ and $^{13}CH_4$ substrates were added to soil samples or enrichment cultures and, after an incubation period, the DNA was isolated and subjected to caesium chloride (CsCl) buoyant density-gradient centrifugation with ethidium bromide. The ^{13}C-DNA ('heavy') separates with remarkable efficiency from the ^{12}C-DNA ('light'), forming a second band in the gradient that is visible under ultraviolet illumination. The two DNA fractions can be retrieved separately from the gradient with either a needle and syringe or by fractionation. The heavy ^{13}C-DNA can be purified away from the light ^{12}C-DNA by a further round of centrifugation if necessary[15]. The fraction that contains the ^{13}C-DNA harbours the combined genomes of the microorganisms in the environmental sample that are active and have incorporated the labelled substrate into their nucleic acids (FIG. 2).

Once ^{13}C-DNA has been isolated, it can be used as a template in PCR, with general primer sets that amplify rRNA genes of most known Bacteria, Archaea or Eukarya. The analysis of the rRNA gene PCR products enables the identification of microorganisms that have assimilated the ^{13}C-substrate. In DNA-SIP studies with $^{13}CH_3OH$ and $^{13}CH_4$, it was possible to target 'functional' genes in addition to the 16S rRNA gene, as methylotrophic bacteria have been relatively well-studied and the genes that encode enzymes involved in methanol and methane oxidation are known. The genes amplified by PCR can also be analysed using microarrays, as described for the *pmoA* gene[16], which encodes a subunit of the particulate methane monooxygenase enzyme. Although PCR is the easiest method by which to obtain information from a DNA-SIP experiment, it is also possible to directly clone ^{13}C-DNA and then sequence the cloned DNA, which might reveal genes of known function that have been found in cultivated microorganisms.

DNA-SIP and methylotrophs

SIP is ideal for studying methylotrophs, as these organisms are known to use one-carbon compounds as their sole carbon source. The first DNA-SIP experiment involved the incubation of forest soil with $^{13}CH_3OH$, and the monitoring of $^{13}CH_3OH$ consumption by gas chromatography[12]. When approximately 2 mmol of substrate had been consumed, total DNA was isolated from the soil and subjected to density-gradient

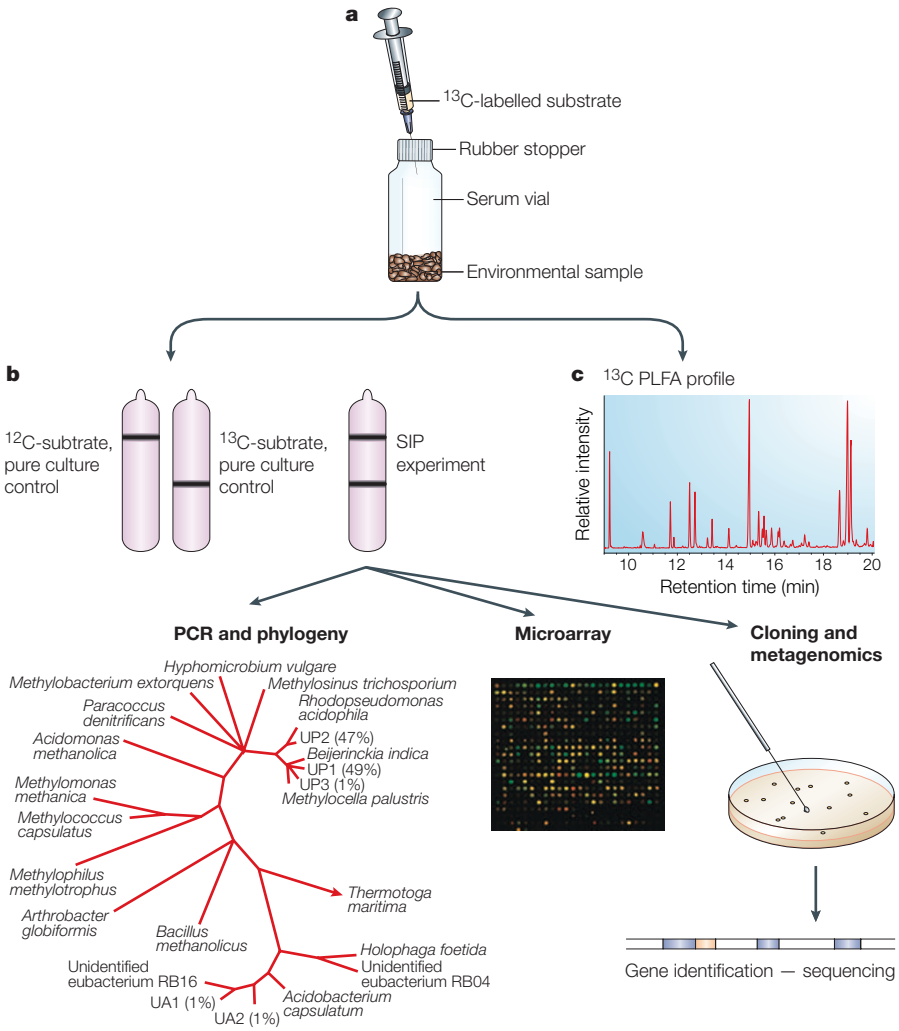

Figure 2 | **DNA-based stable isotope probing (SIP) and ^{13}C-phospholipid fatty acids (PLFA) analyses. a** | A ^{13}C-labelled substrate is added to an environmental sample, such as soil, water or plant material, either *in situ* in the field, or in a serum vial (as depicted). The sample is incubated so that the labelled carbon from the substrate can be incorporated into the biomass of the active microorganisms in the sample. **b** | Total DNA that has been purified from the incubated sample should represent those microorganisms that grew using the ^{13}C-labelled substrate. This genomic DNA — enriched with the ^{13}C isotope — can be separated from the community DNA (^{12}C-DNA) by CsCl gradient centrifugation. Phylogenetic analyses of sequence data produced by PCR amplification of the isolated ^{13}C-labelled DNA using selected primers sets (chosen by the researcher based on their knowledge of probable community members) such as 16S rRNA, *pmoA* (particulate methane monooxygenase), *mmoX* (soluble methane monooxygenase), *cmuA* (chloromethane utilization) and *mxaF* (methanol dehydrogenase) can help to identify organisms that are active in the soil sample (phylogenetic tree reproduced, with permission, from *Nature* REF. 12 © (2000) Macmillan Magazines Ltd; UA1–2 and UP1–3 are cloned 16S rRNA gene PCR products). Microarrays can also be used to identify which of the amplified genes are the most numerous. **c** | PLFA can also be purified, and PLFA profiles can reveal which microorganisms incorporated the ^{13}C isotope. Image reproduced, with permission, from *Nature* REF. 45 © (2000) Macmillan Magazines Ltd.

centrifugation. The ^{13}C-DNA extracted from the gradient was then used as a template in PCR, with primers targeting 16S rRNA genes of Bacteria and the functional gene *mxaF* (encoding a subunit of the methanol dehydrogenase enzyme). Gene sequences retrieved by SIP were similar to those of α-proteobacterial species already known to grow on methanol in the laboratory. In addition, 16S rRNA gene sequences similar to acidophilic methane-oxidizing bacteria that had recently been isolated from northern peat wetlands[17] were obtained, showing for the first time that these bacteria were present in forest soil. More surprisingly, a small number of 16S rRNA gene sequences related to *Acidobacterium* and *Beijerinckia* species were also recovered from the ^{13}C-DNA. It now seems that some *Beijerinckia* species can grow on methanol in the laboratory (P.H. Janssen, personal communication), therefore illustrating the utility of the DNA-SIP technique for identifying new metabolic capabilities in bacteria present in the environment that had previously been considered to be non-methylotrophic.

DNA-SIP techniques have been used to study the functionally active populations of methane-oxidizing bacteria (methanotrophs) in peat soil[18] and acidic forest soil[19]. Initial studies involved relatively long incubation times (~40 days), which had the drawback that continued exposure of soil organisms to a specific ^{13}C-labelled substrate might result in detection of organisms that do not directly assimilate this substrate but instead use ^{13}C-labelled intermediates or by-products generated by the primary consumer organisms. However, more recent studies using $^{13}CH_4$ with samples from unusual environments, such as the enclosed ecosystem at the Movile Cave in Romania[20] and Russian soda lake sediments[21], have used shorter incubation times and have yielded heavy DNA. When analysed by PCR with rRNA and functional gene probes for methanotrophs, this heavy DNA resulted in a high proportion of DNA sequences recognizable as those from extant methanotrophs.

The effectiveness of working with ^{13}C-labelled one-carbon compounds has also been demonstrated in studies using $^{13}CO_2$ to identify nitrifiers in freshwater sediments[22], and $^{13}CH_3Br$ and $^{13}CH_3Cl$ to investigate the active populations of methyl-halide-degrading bacteria in soils[23] (E. Borodina and J.C.M., unpublished observations). In the latter studies, PCR primers were used that target the *cmuA* gene, which encodes a methyltransferase that is essential for growth on methyl halides, thereby linking global cycling of methyl halides to a poorly characterized group of bacteria[24].

Wagner and colleagues[25] demonstrated the usefulness and power of combining SIP with other techniques in their study of denitrification by microbial communities in activated sludge. Again, a methylotrophic substrate, $^{13}CH_3OH$, was used in DNA-SIP, and DNA that was enriched with ^{13}C was used as a template to amplify 16S rRNA genes by PCR. 16S rRNA gene sequences that were closely related to those of the methylotroph genera *Methylophilus* and *Methylobacillus* were abundant in the heavy DNA. These sequence data were then used to design FISH probes, and after uptake of $^{14}CH_3OH$ under denitrifying conditions, FISH–microautoradiography was used to prove that the bacteria identified in SIP experiments were important denitrifiers in the activated sludge under study.

DNA-SIP/multi-carbon compounds

DNA-SIP has been used with multi-carbon compounds, particularly in the context of biodegradation of organic pollutants. Padmanabhan and colleagues[26] added ^{13}C-labelled glucose, caffeine, naphthalene and phenol to soil in field experiments, estimated the rates of substrate degradation by monitoring soil respiration rates and recovered ^{13}C-DNA and PCR-amplified 16S rRNA genes using general bacterial primers. Jeon and co-workers[27] extended this study by further field-based work using ^{13}C-naphthalene in a contaminated site to monitor the flow of carbon from pollutants into the microbial community in that environment. PCR and 16S rRNA gene analysis revealed a cluster of β-proteobacteria involved in the metabolism of the added ^{13}C-naphthalene. The strength of carrying out traditional enrichment and isolation methods alongside the DNA-SIP technique was demonstrated by the isolation of a strain from this environment with a 16S rRNA gene that matched the dominant sequence in the clone library made from heavy DNA. This strain was closely related to *Polaromonas vacuolata* and harboured a naphthalene dioxygenase sequence that was prevalent in the sediment from the test site and had only previously been detected in environmental DNA samples. The potential for DNA-SIP in biodegradation research has recently been reviewed[28,29].

Another exciting application of DNA-SIP has been in investigating SYNTROPHIC ASSOCIATIONS of microorganisms in anoxic environments. Because of the difficulties in isolation and cultivation of often highly fastidious syntrophic bacteria and the lack of suitable molecular markers for detecting these bacteria, the ecology and diversity of syntrophic propionate-oxidizing acetogens is poorly understood. Lueders and colleagues[30] have used ^{13}C-labelled propionate to identify, by DNA-SIP and subsequent retrieval of 16S rRNA gene sequences from ^{13}C-DNA, active anaerobic microorganisms that are involved in the thermodynamically unfavourable syntrophic oxidation of propionate under methanogenic conditions.

RNA-based SIP

The recovery and analysis of RNA molecules after SIP has recently been developed and has some advantages over DNA-SIP[31,32]. One of the drawbacks of DNA-SIP is the relatively long incubation times that are required for DNA replication and incorporation of the ^{13}C-label into newly synthesized DNA. Because RNA synthesis occurs at a faster rate than DNA synthesis, it is possible to obtain ^{13}C-RNA more quickly than ^{13}C-DNA. RNA-SIP was used to identify bacteria that degrade phenol in an aerobic industrial bioreactor. A pulse of ^{13}C-phenol was added to a bioreactor sludge sample and RNA was collected for analysis 8 hours later. The ^{13}C-labelled RNA was separated from ^{12}C-RNA by caesium trifluoroacetate density-gradient centrifugation. Reverse transcription (RT-) PCR amplification of ^{13}C-labelled 16S rRNA revealed that a *Thauera* species was a key player in phenol degradation in this bioreactor.

Unlike DNA-SIP, ^{13}C-labelled RNA with a specific buoyant density is distributed in several fractions in density gradients, so it is necessary in RNA-SIP to analyse each gradient fraction by RT-PCR and denaturing gradient gel electrophoresis (DGGE). Analysis of the shifts in band intensities that occurred during the pulse of ^{13}C-phenol in the bioreactor made it possible to determine which bacteria were being labelled. The first use of RNA-SIP in soil has recently been shown using ^{13}C-pentachlorophenol (REF. 33). RNA-SIP is a powerful technique and its usefulness will increase as methods to purify and separate light and heavy nucleic acids are refined[34].

Studies combining RNA-SIP and DNA-SIP are particularly powerful. A good example of the combined use of RNA-SIP and DNA-SIP was a study by Lueders and colleagues[35], in which they simultaneously analysed the incorporation of the carbon isotope in $^{13}CH_3OH$ in microcosms containing oxic rice-field cover soil for 42 days. RNA-SIP, owing to its increased sensitivity, allowed the identification of the population of methylotrophs that was active first in the microcosm when only small amounts of $^{13}CH_3OH$ had been metabolized, whereas DNA-SIP gave insights into the primary methanol-using communities, their enrichment during the incubation period and their interaction with fungi in the microbial food web. The detection of fungal and protozoal sequences in ^{13}C-enriched nucleic acid fractions indicated their involvement in either direct assimilation of $^{13}CH_3OH$, ^{13}C-labelled intermediates or by-products of methylotrophic metabolism or possibly predation of labelled methylotrophs. These types of SIP-based time-course experiments with environmental samples will probably yield valuable information on microbial interactions and microbial food webs in the environment.

Glossary

CONSORTIUM
Physical association between cells of two or more types of microorganisms. Such an association might be advantageous to at least one of the microorganisms.

SYNTROPHIC ASSOCIATION
An association in which the growth of one organism depends on, or is improved by, growth factors or substrates provided (or in some cases removed) by another organism growing nearby.

SIP/plant–microorganism interactions

Another area of microbial ecology in which SIP might provide valuable information is in rhizosphere–microorganism interactions. The rhizosphere, the biologically active zone in the soil around plant roots, is the site for important and complex plant–microorganism interactions, many of which are poorly understood. For example, root exudation is an important process in carbon transfer and cycling in soil, and these exudates can influence the dynamics of microbial populations surrounding plant roots[36]. One approach to study these interactions is to incubate plants with $^{13}CO_2$ and subsequently extract nucleic acids from the rhizosphere soil. Isolation of ^{13}C-DNA (or ^{13}C-RNA) from this soil yields DNA directly (or indirectly through RT-PCR) for use as a template in PCR to analyse 16S rRNA genes or functional genes by clone library, DGGE or microarray analysis. This would yield important information about rhizosphere bacterial populations that are sequestering carbon through plant exudates. A potential problem with this approach is that the labelling of bacterial nucleic acids might be inefficient, making it difficult to track the flow of ^{13}C from the plant to rhizosphere microorganisms[37]. Prosser and colleagues[38] have examined the effects of liming on the structure of microbial communities that metabolize root exudates in the rhizosphere using RNA-SIP. A $^{13}CO_2$-pulse-labelling field experiment was successfully carried out on upland grassland soil and ^{13}C-RNA was recovered. The lower limit of detection for soil samples inoculated with *Pseudomonas fluorescens* was 10^5–10^6 cells per gram of soil. Nevertheless, 16S rRNA-DGGE analysis showed that there were significant differences in microbial communities using root exudates in limed versus unlimed grassland soils, with most of the more complex communities being in limed soils. This method offers great potential for the future.

SIP and metagenomics

Metagenomics, or the culture-independent genomic analysis of microbial communities, is a growing field that promises to provide great insights into the genetic diversity of uncultivated microorganisms[39,40]. It should be possible to clone ^{13}C-labelled DNA from a DNA-SIP experiment to generate a metagenomic library of the microorganisms in an environment that have incorporated a specific substrate, and therefore function in a particular environmental process.

There have been several notable successes in the construction and analysis of metagenomic libraries from soil[41], the marine environment[42] and more extreme environments such as acid mine drainage[43]. Metagenomic libraries from such complex communities require the screening or sequencing of many thousands of clones to identify specific genes of interest. An important advantage of the use of ^{13}C-DNA derived from a carefully designed SIP experiment is that it will be enriched in the genomes of organisms that are involved in a specific metabolic process; for example, methane oxidation. This considerably reduces the number of clones that need to be screened for a particular set of genes, and aids the reconstruction of a smaller number of targeted genomes in a microbial population[39].

One of the potential problems in creating a metagenomic library using ^{13}C-DNA is the recovery of sufficiently intact, high quality DNA from a SIP experiment, given that it must be subjected to CsCl density-gradient centrifugation and other manipulations. In a proof-of-concept experiment, we generated a small metagenomic library, using ^{13}C-DNA from a SIP experiment with a forest soil sample and $^{13}CH_4$ as the substrate. To clone ^{13}C-DNA from the SIP enrichment, the total DNA from the $^{13}CH_4$-labelled soil slurry was gently extracted and subjected to CsCl density-gradient centrifugation. Purified ^{13}C-DNA was partially cut with a restriction enzyme and ligated into a BAC (bacterial artificial chromosome) cloning vector. The clones were found to contain 10–30 kb inserts and a modest library of 2,300 clones was generated. The library was screened by hybridization with the *pmoA* gene.

A BAC clone that hybridized with the probe was sequenced and found to contain 15.2 kb of sequence and the complete *pMMO* gene operon and several flanking genes, including some encoding enzymes known to be involved in growth on one-carbon compounds. This experiment demonstrated that it is possible to directly clone ^{13}C-DNA from a SIP experiment and obtain target genes in a small library (M.G.D. and J.C.M., unpublished observations). Judging by the size of the DNA fragments obtained from this SIP experiment, it is probably feasible to clone ^{13}C-DNA into a cosmid or fosmid vector.

RNA-SIP has the advantage of greater sensitivity than DNA-SIP, and extension of RNA-SIP techniques to examine mRNA could in theory access the entire transcriptomes of bacteria involved in a specific metabolic process in the environment. However, the inherent instability of mRNA might preclude large-scale transcriptome analyses of environmental samples in the near future. On the other hand, DNA-SIP technology is currently poised for applications in metagenomic studies.

Caveats and future perspectives

SIP is a powerful technique and has provided valuable insights into the diversity and activity of methylotrophic bacteria in particular. These organisms are ideally suited to the technique, as their growth substrate can be fully labelled with ^{13}C and it is used as the sole source of carbon. Organisms that incorporate other forms of carbon that are naturally present in the environment will dilute the amount of ^{13}C incorporated into the cell. It is not yet clear how much ^{13}C must be incorporated to successfully resolve the ^{13}C-DNA from the ^{12}C-DNA in a sample containing a complex community. As described in this review, SIP has more recently been used with several complex carbon substrates and therefore shows great promise for studying the activity of various microbial communities. In addition, SIP has only been carried out using ^{13}C-substrates and there is still scope to use heavy stable isotopes of N and O (REF. 44). In the future, SIP might also prove useful for investigating microbial communities that colonize humans, as stable isotope labelling of substrates would offer important advantages over radioactive compounds.

Many of the DNA-SIP experiments described were done in microcosms and therefore do not represent the conditions that the organisms experience *in situ*. A current limitation of the technology is that a relatively large amount of ^{13}C-substrate must be consumed to visualize a ^{13}C-DNA band in a CsCl gradient. For example, incubations with $^{13}CH_4$ require approximately 0.2 mmol of ^{13}C to be assimilated to produce a visible band. For this reason, artificially high substrate concentrations are often used and the incubations are carried out over several weeks. The high substrate concentration could cause a bias and the long incubation time increases the risk of cross-feeding of ^{13}C from the primary consumers to the rest of the community. Of course, the amount of ^{13}C-DNA that is required to be visible in a gradient far exceeds the amount that is required for PCR detection of genes. It is possible to use less ^{13}C-substrate and extract an 'invisible' ^{13}C-DNA band from CsCl gradients by reference to a ^{13}C-DNA marker in a control gradient, or by simply fractionating the gradient and examining the ^{12}C/^{13}C ratios of the DNA by isotope-ratio mass spectrometry. Care must be taken when using less ^{13}C, to ensure that the DNA is indeed ^{13}C-labelled and not simply GC rich (which also increases the density of DNA) or the result of contamination. In other words, the noise to signal ratio will be greater.

It should be possible to optimize the technique so that the incubation conditions resemble those *in situ* more closely. The cross-feeding of ^{13}C metabolites produced by the primary consumers of the ^{13}C substrate or by subsequent lysis or predation of the target population will always remain a potential problem. On the other hand, this could be turned to an advantage because ultimately, by sampling at various times during the incubations and careful examination of DNA (and/or RNA), it should be possible to follow the flow of ^{13}C through microorganisms in the environment and to examine microbial food chains and succession of microorganisms under conditions approaching those *in situ*.

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doi:10.1038/nrmicro1162
Published online 10 May 2005

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Acknowledgements

M.G.D. received financial support during his Ph.D. from the Fonds de Recherche sur la Nature et les Technologies (Quebec, Canada). J.C.M. gratefully acknowledges support from the Natural Environment Research Council, the Biotechnology and Biological Sciences Research Council and the European Union for funding work in his laboratory.

Competing interests statement

The authors declare no competing financial interests.

Online links

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OPINION

Viral metagenomics

Robert A. Edwards and Forest Rohwer

Abstract | Viruses, most of which infect microorganisms, are the most abundant biological entities on the planet. Identifying and measuring the community dynamics of viruses in the environment is complicated because less than one percent of microbial hosts have been cultivated. Also, there is no single gene that is common to all viral genomes, so total uncultured viral diversity cannot be monitored using approaches analogous to ribosomal DNA profiling. Metagenomic analyses of uncultured viral communities circumvent these limitations and can provide insights into the composition and structure of environmental viral communities.

The genomic age began in 1977 when ΦX174, a virus that infects *Escherichia coli*, was sequenced[1]. The metagenomics of viruses began in 2002 with the publication of two uncultured marine viral communities[2]. In both cases, the small size of viral genomes — approximately 50 kb on average[3,4] — was an advantage because less sequencing was required. However, several unique challenges are encountered when sequencing viruses that are not associated with the sequencing of cellular organisms. These challenges include the abundance of free DNA in the environment[5,6], viral genes that kill the cloning host cells[7] and unclonable, modified viral DNA[8]. These problems have now been overcome (BOX 1) and viral metagenomic libraries are starting to provide information about the types of viruses that are present in environmental samples.

Diversity of environmental viruses

Viral metagenomes mostly comprise novel sequences. There are currently five published viral metagenomic libraries, all of which contain sequences from double-stranded DNA viruses only (see BOX 1): two from near-shore marine water samples[2], a marine sediment sample[9], a human faecal sample[10] and an equine faecal sample[11]. When the marine sequences were first published, approximately 65% of them had no significant similarity (E-VALUE (see Glossary) >0.001) to any sequence in the GenBank non-redundant database (FIG. 1). Analyses 2 years later revealed that most of the viral sequences are still unique, despite the fact that the GenBank database has since more than doubled in size. Likewise, 68% of