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$^{15}N_2$–DNA–stable isotope probing of diazotrophic methanotrophs in soil

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Abstract

Nucleic acid stable isotope probing (SIP) is a powerful tool that can identify and characterize the microorganisms that mediate specific soil processes and explore the flow of C and N through functional groups in the soil food web. While ^{13}C–SIP has been used successfully in a range of applications, methodological constraints have limited the applicability of ^{15}N-labelled compounds in nucleic acid SIP. However, ^{15}N–DNA–SIP can now be achieved and this method when used with $^{15}N_2$ provides a powerful new tool for characterizing free-living diazotrophs in natural ecosystems. A diverse array of non-cultivated diazotrophs have been observed in soil and yet the characteristics of these organisms and their environmental significance remain almost completely unknown. $^{15}N_2$–DNA–SIP can identify those diazotrophs that are active in situ while providing access to gene sequences and genome fragments that can yield insights on their evolutionary history and functional capacities. Further insights on the ecology of free-living diazotrophs in soil can be provided by performing $^{15}N_2$–DNA–SIP on microcosms in which the response of the diazotrophic community is determined in relation to experimental manipulation. We describe the use of $^{15}N_2$–DNA–SIP to explore linkages between different C sources and N-fixation by specific diazotroph populations in soil. Methane addition to soil was observed to stimulate N-fixation and the organisms that were found to be responsible for this activity were Type II methanotrophs most closely related to the genus *Methylocystis*. This report provides insights on the use of nucleic acid SIP to identify and characterize microorganisms that mediate specific soil processes and represents the first time that a specific group of methanotrophs has been shown to mediate N-fixation while in the soil environment.

Keywords: SIP; Diazotroph; Non-cultivated; *nifH*; Methanotroph; $^{15}N_2$; Nitrogen fixation; Microorganism; Bacteria; Microbial community; DNA

1. Introduction

In their metabolic diversity and their sheer numbers, microbes dominate the soil processes that sustain agricultural productivity and underlie ecosystem processes in terrestrial environments. Yet, the magnitude of their contribution lies in stark contrast to the little we know about soil microorganisms and the principles that govern their function. The vast majority of microorganisms continue to resist cultivation in the laboratory and even when cultivation can be achieved the traits expressed by a microorganism in culture may not be representative of those expressed in its natural habitat. Stable isotope probing (SIP) of nucleic acids offers a means to study the microorganisms that facilitate soil processes as they occur in soil, to characterize novel organisms that have escaped detection previously, and to make significant advances in our understanding of the biological principles that drive soil processes.

In nucleic acid, SIP an isotopically labeled substrate is added to soil and microorganisms are given a chance to incorporate the labeled substrate into biomass. DNA or RNA is then extracted from the soil and the labeled 'heavy' nucleic acids are separated from their unlabeled counterparts on the basis of buoyant density. The labeled nucleic acids can then be analyzed by a variety of methods to identify and characterize the organisms that were active in situ. Nucleic acid SIP was initially used to characterize soil methylotrophs that metabolize $^{13}CH_3OH$ (Radajewski et al., 2000) but this technique has subsequently proved useful in a wide range of applications (for review, see Dumont

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and Murrell, 2005; Friedrich, 2006; Madsen, 2006; McDonald et al., 2005; Radajewski et al., 2003; Whiteley et al., 2006) including the recent demonstration of $^{15}N_2$–SIP of DNA from diazotrophs in soil (Buckley et al., 2007b). One of the advantages of nucleic acid SIP experiments is the ability to track the movement of isotopically labeled compounds into microbial food webs over time (DeRito et al., 2005; Hutchens et al., 2004; Lueders et al., 2004b; Mahmood et al., 2005; McDonald et al., 2005; Morris et al., 2002; Ziegler et al., 2005). Another advantage is that DNA–SIP can be used to access genomic DNA from microorganisms as a function of their environmental activity and this DNA can be used to construct genome fragment libraries which can be used to further characterize microorganisms (Friedrich, 2006). As a result it is now possible to conduct hypothesis-driven studies in environmental genomics (Dumont et al., 2006). A conceptual diagram of this approach is depicted in Fig. 1. Initial nucleic acid SIP experiments can be used to identify, through analysis of gene sequences or genome fragments, organisms that assimilate an isotopically labeled compound under defined conditions. The gene and genome sequences that are obtained can be used to generate hypotheses about factors that govern the in situ activity of these organisms, and then subsequent SIP experiments can be used to test these hypotheses without the need for cultivation of microorganisms.

While SIP provides a useful tool for characterizing microbial activity under in situ conditions the method has notable limitations (DeRito et al., 2005; Manefield et al., 2002b; Radajewski et al., 2000; Radajewski et al., 2003). One limitation is the need to add labeled substrates at concentrations that are substantially higher than those typically experienced by cells in situ. Elevated substrate addition is required because cells will assimilate substrates from both native and labeled sources resulting in the dilution of an isotopic label in the receiving community (Radajewski et al., 2000). Another problem encountered when performing nucleic acid SIP experiments is that low growth rates in soil may require prolonged incubations to permit sufficient labeling of nucleic acids. During prolonged incubations cross-feeding and trophic cascades can result in the movement of an isotopic label into nucleic acids from non-target functional groups (DeRito et al., 2005; Hutchens et al., 2004; Lueders et al., 2004b; Mahmood et al., 2005; McDonald et al., 2005; Morris et al., 2002; Ziegler et al., 2005). As a result, DNA from isotopically enriched environmental samples can contain a range of isotopic signatures from 0% to 100% label incorporation.

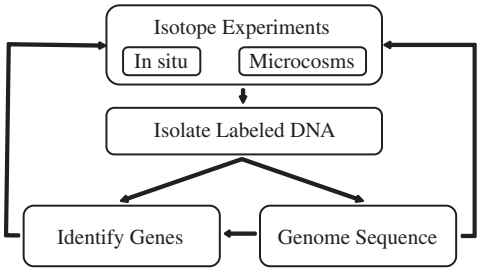

Fig. 1. This flow diagram demonstrates how stable isotope probing can be used to engender a hypothesis-driven approach to environmental genomics.

Several strategies have been developed to deal with these issues and each requires the collection and analysis of density gradient fractions in order to determine the degree of isotope incorporation into nucleic acids (Lueders et al., 2004a; Manefield et al., 2002a, b). Examination of nucleic acids fingerprints (i.e.: T-RFLP or DGGE analysis of 16S rRNA genes) as a function of gradient density can be used to identify templates from organisms that have incorporated the isotopic label by comparing the buoyant density of given templates in isotopically enriched samples relative to unlabeled controls (Manefield et al., 2002b). In addition, since the change of buoyant density for a given DNA molecule is proportional to the degree of isotope incorporation (Birnie and Rickwood, 1978; Buckley et al., 2007a) it can be possible to infer the degree of isotopic labeling for given templates in this manner. Thus, by following the incorporation of the isotopic label into the community over time and in comparison to control treatments that receive unlabeled substrates it is possible to track the movement of the label from a substrate into particular functional groups and then into other components of the soil food web (DeRito et al., 2005; Lueders et al., 2004a; Lueders et al., 2006).

A final consideration that can complicate the interpretation of DNA–SIP experiments is the effect that genome G+C content has on DNA buoyant density (Rolfe and Meselson, 1959). The native buoyant density of DNA in a CsCl gradient can differ by as much as 0.05 g ml^{-1} over the range of genome G+C contents that can occur in soil (Birnie and Rickwood, 1978; Holben and Harris, 1995; Nusslein and Tiedje, 1998). While the G+C problem is a potential concern in any DNA–SIP experiment it represents a fundamental obstacle to the successful application of ^{15}N–DNA–SIP. The change in DNA buoyant density for fully ^{15}N-labeled DNA (0.016 g ml^{-1}; Birnie and Rickwood, 1978) is less than half of the change in density that occurs naturally as a result of variation in genome G+C content (Rolfe and Meselson, 1959). While this concern is largely absent from experiments that use ^{15}N-labeled DNA obtained from pure cultures (Meselson and Stahl, 1958), significant overlap in the buoyant densities of ^{15}N-labeled and unlabeled DNA can occur in experiments performed with complex communities (Cadisch et al., 2005; Cupples et al., 2007). It is now possible to disentangle the effects of isotope incorporation and genome G+C content on DNA buoyant density in DNA–SIP experiments by exploiting the ability of DNA intercalating agents such as bis-benzimide to change the buoyant density of DNA as a function of G+C content (Buckley et al., 2007a), and this method has made it possible to use effectively ^{15}N-labeled substrates, such as $^{15}N_2$, for DNA–SIP of soil communities (Buckley et al., 2007b).