DNA buoyant density shifts during $^{15}$N-DNA stable isotope probing

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Summary

DNA-based stable isotope probing (SIP) is a novel technique for the identification of organisms actively assimilating isotopically labeled compounds. Herein, we define the limitations to using $^{15}$N-labeled substrates for SIP and propose modifications to compensate for these shortcomings. Changes in DNA buoyant density (BD) resulting from $^{15}$N incorporation were determined using cultures of disparate GC content ($E. coli$ and $Micrococcus luteus$). Incorporation of $^{15}$N into DNA increased BD by $0.015 \pm 0.002 \, g \, ml^{-1}$ for $E. coli$ and $0.013 \pm 0.002 \, g \, ml^{-1}$ for $M. luteus$. The DNA BD shift was greatly increased ($0.045 \, g \, ml^{-1}$) when dual isotope ($^{13}$C plus $^{15}$N) labeling was employed. Despite the limited DNA BD shift following $^{15}$N enrichment, we found the use of gradient fractionation, followed by a comparison of T-RFLP profiles from fractions of labeled and control treatments, facilitated detection of enrichment in DNA samples from either cultures or soil.

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Introduction

Nucleic-acid-based stable isotope probing (SIP) is a novel method that facilitates the examination of links between metabolic function and taxonomic identity, and precludes the need for culture isolation. SIP entails the exposure of microbes to labeled target compounds, and the recovered pool of cellular nucleic acid is separated by density gradient centrifugation. A resulting increase in the buoyant density (BD) of isotopically enriched nucleic acids corresponds to cells that assimilated the target compound. The recovered nucleic acid fractions can then be characterized by a number of available...
molecular-based methods. Though SIP might be possible using substrates containing isotopically labeled C, H, O or N, to date, the technique has been applied almost exclusively to C-labeled compounds (Radajewski et al., 2003). Clearly our investigative opportunities could be greatly expanded with the use of other isotope labels (15N, 2H, 18O).

The use of 15N-DNA-based SIP is particularly attractive because of its potential for investigating microbial processes such as environmental nitrogen cycling, or the biodegradation of environmentally important nitrogen-containing compounds. For example, 15N-DNA SIP could be utilized to investigate the biodegradation of the nitrogen-based pollutants 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Keith and Telliard, 1979; Van Aken et al., 2004) or to expand on previous research addressing the fate of nitrogen from agrochemicals (Bichat et al., 1999). The primary concern for using 15N-DNA SIP, compared to the already established method of 13C-DNA SIP, is the nitrogen content of DNA, which limits the potential BD increase following 15N incorporation into target DNA. Owing to differences in GC content among bacterial taxa, the range in BD of unlabeled DNA is significant (Lueders et al., 2004a) compared to the change in BD expected from 15N enrichment (Meselson and Stahl, 1958). Based partly on theoretical values, one group of researchers (Cadisch et al., 2005) concluded that it should be possible for 15N SIP to resolve labeled from unlabeled DNA among organisms of differing guanine–cytosine (% GC) contents, though this remains to be proven empirically.

In this study, we quantify the DNA BD shifts associated with 15N-DNA SIP using two organisms (Escherichia coli and Micrococcus luteus) of disparate (medium and high) GC content (~50% and ~72% for E. coli and M. luteus, respectively (Orskov, 1984; Stackebrandt et al., 1995)) using a simple substrate (15NH4Cl) to maximize enrichment. Additionally, we report for the first time the effect of dual labels (15N and 13C) on the separation of light and heavy DNA. We also examine the effectiveness mixed DNA separation (14N-and 15N-DNA from E. coli and M. luteus) using terminal restriction fragment length polymorphism (T-RFLP) and quantitative real-time PCR. Finally, we investigate the feasibility of 15N-DNA SIP with mixed soil communities.

### Materials and methods

#### Strains and culture conditions

E. coli (ATCC 15524) was grown overnight (shaking at 37°C) on LB (Fisher Chemicals) and transferred (4% v/v) twice successively to a phosphate-buffered (pH 7) minimal medium (Fries et al., 1994) modified to contain glucose (1 g L−1) as the sole carbon source, and 15NH4Cl (98 atom %, Isotec, Miamisburg, OH) or unlabeled NH4Cl (0.35 g L−1) as the sole source of assimilable nitrogen. M. luteus (ATCC 49442) was grown (shaking at 25°C) on ATCC medium 1780 modified by substituting pyridine with glucose (1 g L−1) and NH4Cl (0.35 g L−1) and transferred (10% v/v) twice successively to minimal medium containing glucose and unlabeled NH4Cl or 15NH4Cl (as described above) but supplemented with thiamine (2 μM (Sims et al., 1986)). To investigate SIP with dual label, E. coli, following growth on LB, was transferred (4% v/v) twice successively on minimal medium modified to contain 13C-lactate (1.3 g L−1) as the sole carbon source and NH4Cl (0.35 g L−1) as the sole nitrogen source as follows: unlabeled lactate (Sigma) with unlabeled NH4Cl (0.35 g L−1); unlabeled lactate with 15NH4Cl (0.35 g L−1); unlabeled lactate (13C3, 98 atom %, Cambridge Isotope Laboratories, Inc., Andover, MA) with unlabeled NH4Cl; or 13C-lactate with 15NH4Cl. Following growth on minimal media, DNA was extracted from cultures as described below. Additionally, washed cell pellets were subject to isotopic analyses by the 15N Analysis Service at the University of Illinois, as previously described (Khan et al., 1997).

#### Soil experiment

Glucose (Sigma) (12.5 mg g soil−1), (15NH4)2SO4 (15N, 99.4 atom %, Isotec) or unlabeled (NH4)2SO4 (Fisher Scientific) (0.68 mg g soil−1) and water (50% water holding capacity) were added to samples (0.5 g, dry weight) of a Westola soil (coarse-loamy, mixed, superactive, calcareous, thermic Typic Ustifluvents), and following incubation (30°C) for 1 week, DNA was extracted with the UltraClean Soil DNA Kit (MoBio Laboratories, Inc.), following the manufacturer's instructions.

#### DNA extraction and CsCl density gradient ultracentrifugation

Cells were harvested during late exponential growth and DNA was extracted from the pure cultures with a DNeasy tissue system (Qiagen, Inc, Valencia, CA), following the manufacturer’s instructions (Gram-positive protocol for M. luteus). Throughout this study, DNA was quantified by fluorometry with the PicoGreen nucleic acid quantification dye (Molecular Probes),...
analyses of E. coli DNA by quantitative real-time PCR

Quantification of the E. coli-specific 16S rDNA was performed by real-time PCR amplification (Opticon 2 Real Time Thermal Cycler) using previously described primers (Hu and Edenberg, 2002) and QuantiTect SYBR® Green PCR Master Mix (Qiagen), following the manufacturer’s instructions. PCR conditions were: 95°C (15 min); 94°C (10 s), 60°C (20 s), 72°C (30 s) (26 cycles); then 72°C (7 min). Melting curves were performed from 50°C to 95°C (read every 1°C). Standard curves were generated using serial dilutions of genomic E. coli DNA.

Results and discussion

14N- and 15N-DNA separation when centrifuged individually

The distributions of E. coli and M. luteus DNA following ultracentrifugation over a range of BD values, both with and without 15N incorporation, are illustrated in Fig. 1. As expected, based on the GC contents of E. coli (~50%) and M. luteus (~72%) (Orskov, 1984; Stackebrandt et al., 1995), unlabeled E. coli DNA was found at lower BD values (1.701 ± 0.001 g mL⁻¹, average peak DNA mass, n = 3) than unlabeled M. luteus DNA (1.715 ± 0.001 g mL⁻¹, n = 3). Similarly, 15N-labeled E. coli DNA was present in fractions with lower BD values (1.715 ± 0.002 g mL⁻¹, n = 3) compared to 15N-labeled M. luteus DNA (1.727 ± 0.002 g mL⁻¹, n = 3). The DNA BD increase following 15N incorporation was 0.015 ± 0.002 g mL⁻¹ (n = 3) for E. coli and 0.013 ± 0.002 g mL⁻¹ (n = 3) for M. luteus. Isotopic analyses indicated M. luteus and E. coli cells contained 87.7 and 88.0 at% 15N, respectively.

To date, only one report has addressed the use of 15N for DNA SIP. These investigators (Cadisch et al., 2005) quantified the width (mm) of separation of unlabeled and 15N-DNA bands from Pseudomonas putida by ultracentrifugation using ethidium bromide staining to visualize DNA bands. However, without quantitative data on the increase in DNA BD caused by 15N incorporation, these researchers were unable to compare their results to the range of DNA BD values found in natural communities (difference between highest and lowest DNA BD of ~0.03 g mL⁻¹ (Lueders et al., 2004a)). Our results suggest the limited increase in DNA BD caused by 15N incorporation could result in unlabeled, heavy DNA (high GC content) co-eluting with lower BD...
15N-DNA regions. This concern is illustrated in Fig. 1, where unlabeled M. luteus DNA (high GC content) is found at a similar BD as 15N-labeled E. coli DNA. Therefore, previously reported methods for 13C-DNA SIP involving removal of the well separated, heavy band may not be appropriate for 15N-DNA SIP.

Separation of mixed DNA by T-RFLP and qPCR

For 13C-DNA SIP researchers have used community analyses methods such as T-RFLP or denaturing gradient gel electrophoresis (DGGE) to compare microbial communities in unexposed samples (no label added) with those from exposed samples (label added) in different BD fractions to identify organisms incorporating the labeled substrate (Manefield et al., 2002; Lueders et al., 2004b). Here, we examined the effectiveness of mixed DNA (14N- and 15N-DNA from E. coli and M. luteus) separation using T-RFLP. We expected T-RFLP analyses would demonstrate that the various DNA types could be separated and thus found in the BD fractions illustrated in Fig. 1. Although T-RFLP indicated DNA from both organisms was distributed throughout all fractions, the signals were concentrated in the appropriate fractions, i.e., E. coli and M. luteus signals were dominant in the lighter and heavier fractions, respectively (Fig. 2A). These results indicate that community analysis profiles must be carefully interpreted, as even small DNA residues when coupled with the sensitivity of PCR can mislead identification of target organisms. Quantitative PCR enabled a more precise target DNA BD determination (Fig. 2B), illustrating that E. coli DNA was proportionally dominant in fractions corresponding to E. coli DNA BD values illustrated in Fig. 1.

Mixed isotope DNA separation

15N-DNA SIP was compared to the more established method of 13C-DNA SIP by examining the BD increase in E. coli DNA resulting from the following: 15N incorporation; 13C incorporation; and 15N, 13C incorporation combined. The increase in E. coli DNA BD following 15N incorporation (0.012 g mL\(^{-1}\)) was less than that resulting from 13C incorporation (0.038 g mL\(^{-1}\)) or from both (0.045 g mL\(^{-1}\)) combined (Fig. 3). In agreement with our results, previous research with Methylobacterium extorquens (Lueders et al., 2004a) found the DNA BD increase following 13C incorporation was 0.04 g mL\(^{-1}\). To date, there are no reports of combining heavy isotopes for SIP; however, our results suggest this would provide a significant advantage (large increase in BD) to the method. Dual isotope SIP should be particularly useful for investigating compounds that could be used as both a nitrogen and carbon source, as this should guarantee a large increase in BD. Conversely, the approach may not be as beneficial if each compound contains only one heavy isotope, as the same microorganism may not be able to use both compounds.

15N-DNA SIP in soil communities

We applied 15N-DNA SIP to mixed community DNA (soil samples), comparing BD profiles from soils exposed to 15N-labeled or unlabeled (NH\(_4\))\(_2\)SO\(_4\), and found DNA BD increased in soils exposed to the label (Fig. 4A). This limited increase (0.008 g mL\(^{-1}\)) demonstrates the need to compare unlabeled controls for differentiating candidate taxa exhibiting BD increases due to 15N enrichment. The soil amendments (C, N and water) stimulated a flush in...
microbial activity, resulting in the emergence of 9 dominant operational taxonomic unit (OTU) fragments compared to the unamended profiles (Fig. 4B). These organisms exploited the relatively universal substrates, ammonium and glucose, and were dominant in both labeled and control populations, demonstrating the effect of $^{15}$N enrichment on DNA BD.

Figure 2. (A) TRFLP electropherograms of HaeIII digests of DNA from lightest and heaviest buoyant density fractions containing both unlabeled ($^{14}$N) and labeled ($^{15}$N) E. coli (31 bp) and M. luteus DNA (227 and 228 bp). All four DNA types were centrifuged together. (B) The proportional quantity of E. coli DNA measured by real-time quantitative PCR present in corresponding fraction.

Figure 3. Distribution of E. coli DNA over a range of BD values following no (closed diamonds), single ($^{15}$N open diamonds, $^{13}$C open triangles) or dual isotope (open circles) enrichment treatments. Each DNA type was centrifuged independently. DNA mass is normalized to highest value for each DNA type due to variability in DNA recovery.
In summary, for the first time, we quantified DNA BD increases from $^{15}$N enrichment, and from dual label enrichment. Our data illustrate dual labels will significantly increase BD shifts, enabling easier identification of enriched organisms. Although $^{15}$N incorporation resulted in a limited increase in DNA BD, gradient fractionation, followed by a comparison of T-RFLP from fractions of labeled and control treatments, facilitated detection of $^{15}$N enrichment with both pure cultures and soil DNA samples. Thus, despite its inherent limitations, $^{15}$N SIP is a promising technique that will enable researchers to better understand the ecology of organisms involved in transformation of nitrogenous compounds.

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