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Validation of Heavy-Water Stable Isotope Probing for the Characterization of Rapidly Responding Soil Bacteria▽†

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Rapid responses of bacteria to sudden changes in their environment can have important implications for the structure and function of microbial communities. In this study, we used heavy-water stable isotope probing ($H_2{}^{18}O$-SIP) to identify bacteria that respond to soil rewetting. First, we conducted experiments to address uncertainties regarding the $H_2{}^{18}O$-SIP method. Using liquid chromatography-mass spectroscopy (LC-MS), we determined that oxygen from $H_2{}^{18}O$ was incorporated into all structural components of DNA. Although this incorporation was uneven, we could effectively separate ^{18}O-labeled and unlabeled DNAs derived from laboratory cultures and environmental samples that were incubated with $H_2{}^{18}O$. We found no evidence for *ex vivo* exchange of oxygen atoms between DNA and extracellular H_2O, suggesting that ^{18}O incorporation into DNA is relatively stable. Furthermore, the rate of ^{18}O incorporation into bacterial DNA was high (within 48 to 72 h), coinciding with pulses of CO_2 generated from soil rewetting. Second, we examined shifts in the bacterial composition of grassland soils following rewetting, using $H_2{}^{18}O$-SIP and bar-coded pyrosequencing of 16S rRNA genes. For some groups of soil bacteria, we observed coherent responses at a relatively course taxonomic resolution. Following rewetting, the relative recovery of *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* increased, while the relative recovery of *Chloroflexi* and *Deltaproteobacteria* decreased. Together, our results suggest that $H_2{}^{18}O$-SIP is effective at identifying metabolically active bacteria that influence soil carbon dynamics. Our results contribute to the ecological classification of soil bacteria while providing insight into some of the functional traits that influence the structure and function of microbial communities under dynamic soil moisture regimes.

One of the overarching goals of microbial ecology is to understand the contributions of different taxa to ecosystem processes. This seemingly straightforward task is often hindered by the tremendous complexity of microbial communities. For example, environmental samples typically contain thousands of taxa (44), but a large fraction of this diversity is comprised of dormant or inactive individuals (10, 24, 30). Furthermore, microbial communities are dominated by rare taxa (49), and it is unclear whether the activity of these microorganisms is important for understanding the biogeochemical fluxes of material and energy in different ecosystems.

One approach that has been used successfully for assigning function to microbial taxa is stable isotope probing (SIP). This technique involves the addition of an isotopically labeled substrate (e.g., $^{13}CH_4$) to an environmental sample (39). Following a sufficient incubation period, isotopically enriched macromolecules (e.g., lipids or nucleic acids) can be extracted and linked to a targeted microbial process, such as methane oxidation (31), contaminant degradation (27), or nitrogen fixation (3).

Recently, heavy water ($H_2{}^{18}O$) was introduced as a novel substrate to be used with SIP (48). Two features make $H_2{}^{18}O$-SIP attractive for linking microbial structure and function. First, it has the potential to be more sensitive than some nucleic acid-based SIP techniques that employ other labeled substrates. For example, most SIP studies use substrates that have only one additional neutron compared to the most abundant naturally occurring isotope (e.g., ^2H versus ^1H, ^{13}C versus ^{12}C, or ^{15}N versus ^{14}N). In contrast, ^{18}O has two more neutrons than the most abundant naturally occurring oxygen isotope (^{16}O). Therefore, the incorporation of a single ^{18}O atom compared to a single ^{13}C or ^{15}N atom should increase the degree of physical separation between labeled and unlabeled fractions during the isopycnic centrifugation step of SIP. Second, most SIP studies to date have used isotopically labeled substrates that target somewhat phylogenetically constrained metabolic processes (e.g., methane oxidation). In contrast, $H_2{}^{18}O$-SIP has the potential to label all metabolically active (RNA-SIP) or growing (DNA-SIP) microorganisms due to the universal requirements of water for cellular maintenance and biosynthesis (48).

Although $H_2{}^{18}O$-SIP holds promise for linking the identity and activity of microorganisms, there are some important assumptions and unknowns that remain to be addressed. The location and evenness of ^{18}O incorporation into nucleic acids are not well established. For example, ^{18}O has the potential to be located in the phosphate group, in deoxyribose/ribose sugars, and in nucleobases (i.e., cytosine, guanine, and thymine), since all of these molecular components contain oxygen. There are three specific pathways by which ^{18}O can be incorporated into the phosphate group of nucleic acids. First, ^{18}O can be exchanged between H_2O and inorganic phosphate species of

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ATP, which is facilitated by pyrophosphatases (6). Second, the F1 component of ATPase can promote isotopic exchange so that the γ-phosphate group of ATP contains ^{18}O (25). The ^{18}O-labeled ATP may then catalyze the formation of glucose-6-phosphate, which serves as a precursor of ribose-5-phosphate and, eventually, nucleic acid biosynthesis. Third, $H_2{}^{18}O$ can be used as a substrate to convert nucleoside diphosphates to deoxyribonucleoside triphosphates (33). Furthermore, ^{18}O may be incorporated into pyrimidine nucleobases during hydrolysis reactions with citric acid cycle intermediates (e.g., aspartate and carbamoyl phosphate), into deoxyribose via gluconeogenesis, and into pentose phosphate pathways that use small carbon and oxygen precursors (34). Variation in the evenness of ^{18}O incorporation in all of these pathways may lead to differences in the degree of separation between labeled and unlabeled DNAs during SIP, which can have important implications for drawing conclusions about the metabolic activity of a microbial population (3). Another concern is the potential exchange between ^{18}O and ^{16}O in nucleic acids following DNA replication or RNA transcription. For example, it is fairly well documented that oxygen isotope exchange can occur between water and a variety of substances, including metal complexes (36, 53), organic matter (55), and monosaccharides such as ribose and deoxyribose (7), but the extent of this phenomenon for nucleic acids remains unclear. If a significant amount of isotopic exchange occurs between nucleic acids and cytoplasmic or extracellular water, then it could be problematic to associate microbial taxa with metabolic processes by using $H_2{}^{18}O$-SIP.

Despite some methodological uncertainties, $H_2{}^{18}O$-SIP is a promising technique for linking the activity of microorganisms to changes in their environment. In particular, $H_2{}^{18}O$-SIP may help to assign ecological significance to groups of bacteria that respond to sudden changes in soil moisture. In nature, rainfall patterns can create rewetting events that elicit dramatic changes in soil microbial dynamics and ecosystem activity (18, 20, 58). For example, dry soils generate large pulses of CO_2 following episodic precipitation events that are important for balancing terrestrial carbon budgets (47). It is unclear, however, which bacteria are responsible for moisture-mediated pulses of ecosystem activity. Are there coherent taxonomic responses to rewetting (38)? Do these taxa possess functional traits that select for rapid responses to rewetting? Arguably, these questions are likely to become more important given the predictions of altered rainfall patterns under future climate change scenarios (56, 59).

In this study, we conducted a series of experiments to address some of the methodological questions surrounding the $H_2{}^{18}O$-SIP technique. First, we used liquid chromatography-mass spectrometry (LC-MS) to determine the location and evenness of ^{18}O incorporation within bacterial DNA. Second, we conducted $H_2{}^{18}O$-SIP experiments with DNA from *Escherichia coli* to test for the exchange of ^{18}O-^{16}O between DNA and extracellular H_2O. Third, we tracked the incorporation of ^{18}O into bacterial DNA to assess whether $H_2{}^{18}O$-SIP can be used to capture the rapid metabolic responses generated from soil rewetting. We then used bar-coded pyrosequencing of 16S rRNA genes to identify bacterial taxa that responded to a rewetting event in grassland soils and thus contributed to measured pulses of CO_2.

MATERIALS AND METHODS

Location of ^{18}O in DNA. To evaluate the location and evenness of ^{18}O incorporation into bacterial DNA, we measured the incorporation of oxygen atoms from $H_2{}^{18}O$ into the nucleotide dTMP in a population of *Escherichia coli* cells by using LC-MS. Because the mass of dTMP does not overlap with those of any of the other nucleoside monophosphates (i.e., guanosine, adenosine, and cytidine), this nucleotide offered the best mass spectrum for identifying ^{18}O incorporation. In triplicate, we obtained labeled (^{18}O) and unlabeled (^{16}O) DNAs by inoculating 10 μl of log-phase *E. coli* (K-12 strain MG1655) into 140 μl of 10× Luria-Bertani (LB) liquid medium containing 1.5 ml of $H_2{}^{18}O$ (97 atom% ^{18}O; Isotech, Sigma-Aldrich, St. Louis, MO) and unlabeled nuclease-free water ($H_2{}^{16}O$), respectively. Cells were incubated for 5 h at 37°C on a shaker table (150 rpm). Genomic DNA was extracted from *E. coli* by using an UltraClean microbial DNA isolation kit (MoBio, Carlsbad, CA) and was eluted in 150 μl of nuclease-free H_2O. Genomic DNA (≈3 μg) was then digested with DNase I (Invitrogen Corporation, Carlsbad, CA) for 48 h at 37°C to create dTMP. Each 300-μl digestion mixture contained 150 μl of DNA, 30 μl of 10× DNase reaction buffer, 30 μl of DNase I (amplification grade; 1 unit μl^{-1}), and 90 μl of nuclease-free H_2O. We terminated the reaction by placing the digestion mixtures at −20°C. The resulting dTMP was purified with a Novagen SpinPrep PCR cleanup kit (EMD Chemicals, Gibbstown, NJ) to remove DNA polymerases and then stored at −20°C until being run on the LC-MS. We used an LC-MS system consisting of a Q-ToF Ultima API LC-MS, a Waters 2795 separation module, and an Acquity UPLC BEH C_{18} column (2.1 mm by 100 mm by 1.7 μm; Waters Corporation, Milford, MA) with electrospray ionization in negative-ion mode. The resulting data were analyzed with MassLynx 4.0 software (Waters Corporation, Milford, MA).

***Ex vivo* exchange of ^{18}O in DNA.** We tested for the *ex vivo* exchange of ^{18}O in bacterial DNA by using $H_2{}^{18}O$-SIP. Specifically, we tracked changes in the location of ^{18}O-labeled DNA in cesium trifluoroacetate (CsTFA; GE Healthcare, Salt Lake City, UT) gradients following isopycnic centrifugation before and after the DNA was incubated in $H_2{}^{16}O$. First, ^{18}O-labeled and unlabeled DNAs from *E. coli* were obtained following the methods described above. We then incubated ^{18}O-labeled and unlabeled DNAs (≈1 μg) separately in $H_2{}^{16}O$ (500 μl) for 7 days at 4°C. Next, $H_2{}^{18}O$-SIP was performed in triplicate, using the procedures described above. Finally, we quantified the amounts of DNA in the different density fractions by using quantitative PCR (qPCR) with primers targeting a fragment of the 16S rRNA gene (see "qPCR detection of ^{18}O-labeled DNA"). The resulting quantities of 16 rRNA gene copies were standardized by expressing the number of gene copies in each buoyant density fraction relative to the number of gene copies in the buoyant density fraction that contained the maximum number of 16S rRNA gene copies. This standardization of copy numbers within the gradient for each replicate helped to determine the locations of ^{18}O-labeled and unlabeled DNAs. For a given replicate, the entire gradient was analyzed in the same qPCR assay to minimize the potential for qPCR bias between runs. We tested for the effects of isotopic exchange on ^{18}O-labeled DNA by comparing the ratios of maximum quantities of ^{18}O-rRNA genes within the different density fractions (i.e., 11 fractions, with densities ranging from 1.531 to 1.649 g ml^{-1}) before and after the incubation, using two-way analysis of variance (ANOVA). Assumptions of ANOVA were tested using the Shapiro-Wilk test for normality and Levene's test for homogeneity of variance (46).

$H_2{}^{18}O$-SIP procedure. We followed the DNA-SIP procedure outlined by Neufeld et al. (35), with a few modifications to improve the recovery of the ^{18}O-labeled DNA from unlabeled DNA. Approximately 1 μg of genomic DNA (ranging from 0.85 to 1.3 μg) was loaded into 4.7-ml OptiSeal polyallomer tubes (Beckman Coulter Inc., Brea, CA) containing CsTFA with a buoyant density of 1.61 g ml^{-1} (27). The CsTFA mixture was prepared in bulk before centrifugation, with each 4.7-ml tube receiving approximately 2.9 ml of CsTFA and 1.75 ml of nuclease-free H_2O. The DNA was added to each tube in 45 μl of nuclease-free H_2O to help maintain similar starting densities between tubes. Heavy and light DNAs were separated in each tube through isopycnic ultracentrifugation at a speed of 65,135 rpm (177,000 × g_{av}) for 72 h. Tubes were balanced within ±0.005 × g, loaded into a TLA 110 rotor (Beckman Coulter Inc., Brea, CA), and centrifuged on an Optima Max-XP benchtop ultracentrifuge (Beckman Coulter Inc., Brea, CA) at 20°C. We retrieved DNA in 20 fractions (235 μl each) from the resulting gradient, using a fraction recovery system (Beckman Coulter Inc., Brea, CA) connected to a single-syringe infusion pump (Cole-Parmer, Vernon Hills, IL) set at a rate of 0.47 ml min^{-1}, which displaced the CsTFA mixture with nuclease-free water. We incorporated two blanks without DNA in each ultracentrifugation step to calculate the densities of the 20 fractions and create a standard CsTFA buoyant density gradient (n = 12) for our specific SIP conditions. Buoyant densities of all fractions were weighed on a microbalance (Sartorius, Goettingen,

Germany). DNA was precipitated from all fractions through two isopropanol (molecular biology grade) washes. Each fraction received 600 μl of ice-cold isopropanol and was incubated overnight at −20°C. These fractions were then centrifuged at 14,000 × g_{av} for 40 min, followed by a 10-min centrifugation in 200 μl ice-cold isopropanol. DNAs in all fractions were resuspended in 30 μl of nuclease-free H_2O.

qPCR detection of ^{18}O-labeled DNA. We performed qPCR assays to determine the distribution of DNA within the CsTFA gradient. Following centrifugation and fractionation, qPCR was used on all 20 fractions from each SIP run, using a Mastercycler EP Realplex qPCR machine (Eppendorf, Hamburg, Germany) and SYBR green to quantify bacterial DNA obtained with primers that targeted regions of the 16S rRNA gene. Each 30-μl reaction mixture contained the following: 13.5 μl of 2.5× SYBR Rox Real master mix (5 Prime, Inc., Gaithersburg, MD); 0.5 μl of forward primer Eub338 and 0.5 μl of reverse primer Eub518, which make up a universal bacterial primer set (17); 1 μl of DNA template; and 14.5 μl of nuclease-free H_2O. The qPCR standard was generated from a soil bacterium (*Micrococcus* sp.) by use of a Topo TA cloning kit (Invitrogen). We extracted plasmids from transformed cells (45) and used M13 forward and reverse primers from the cloning kit to generate PCR products that we used for our standard curve (10^1 to 10^8 copies/μl). The coefficients of determination (r^2) for our assays ranged from 0.96 to 0.99, while amplification efficiencies fell between 0.93 and 0.99. Based on melting curve analyses, we found no evidence for primer dimers. The resulting quantities of rRNA gene copies were normalized by expressing gene copies as ratios to the maximum quantity of copies per grassland replicate, similar to the *E. coli* qPCR methods described above (see "*Ex vivo* exchange of ^{18}O in DNA").

Grassland soil sampling. We collected soils from the successional grassland treatment (T7) located at the W. K. Kellogg Biological Station (KBS) Long-Term Ecological Research (LTER) site in southwestern Michigan. This replicated land-use treatment consists of 1-ha plots that comprise a mixture of perennial grasses and forbs that became established after the field was abandoned from agricultural activity in 1989. The dominant plant species in the T7 treatment include *Solidago canadensis* (L.), *Apocynum cannabinum* (L.), and *Phleum pratense* (L.) (43). Our sampling was conducted on 2 September 2008 following summer dry-down of the soils. We collected and pooled 10 random soil cores (10 cm by 2 cm) from three randomly selected grassland treatments. On average, annual precipitation at the KBS LTER site is 890 mm, with half falling as snow, and the mean annual temperature is 9.7°C (http://www.lternet.edu/sites/kbs). All soils are fine-loamy, mixed, mesic typic Hapludalfs with a total soil C content of 1.3% and N content of 0.13%, pH of 5.5, and cation exchange capacity of 5.5 cmol kg^{-1}. Soil samples were collected from the Ap horizon, sieved to 2 mm, and used in $H_2{}^{18}O$-SIP microcosms within 5 h of removal.

Soil rewetting and $H_2{}^{18}O$-SIP. We performed $H_2{}^{18}O$-SIP experiments to estimate the rate of ^{18}O incorporation into the DNA of soil bacteria. This information was then used to link bacterial taxa to the metabolic activity associated with soil rewetting (see "Soil CO_2 production"). For both of these objectives, we simulated rewetting events with $H_2{}^{18}O$ in microcosms containing grassland soil. Microcosms consisted of 40-ml borosilicate glass vials with polypropylene, silicone-septum screw caps. Each microcosm contained 3 g of grassland soil. Soils were incubated with $H_2{}^{18}O$ for different durations (0, 48, and 72 h). The experiment was initiated by evenly applying 600 μl of $H_2{}^{18}O$ (97 atom% ^{18}O; Isotech, Sigma-Aldrich, St. Louis, MO) to the surface of each grassland soil microcosm (3 grassland replicates × 3 sampling times = 9 microcosms). The rewetting increased gravimetric soil moisture 5-fold, from ≈0.05 to 0.25 g H_2O g $soil^{-1}$. Prior to rewetting, gravimetric water content was estimated as the difference in the masses of soil before and after drying at 105°C for 48 h. The microcosms were incubated at 25°C in a temperature-controlled incubator. Total genomic DNA was extracted from the microcosm soil by using a PowerSoil DNA isolation kit (MoBio, Carlsbad, CA). We performed $H_2{}^{18}O$-SIP on the resulting DNA by using the methods described above.

Soil CO_2 production. We quantified the effects of rewetting on soil microbial metabolism by measuring the evolution of CO_2 in the headspace of the microcosms over the duration of the experiment. To accurately measure transient CO_2 pulse dynamics and the rate of CO_2 production, microcosms were destructively harvested at 12-h increments over a 96-h time span, resulting in a total of 27 experimental units (3 grasslands × 9 sampling times from 0 to 96 h = 27 microcosms). For the 0-, 48-, and 72-h sampling times, we measured soil respiration after rewetting with $H_2{}^{18}O$. For the remaining time points (12, 24, 36, 60, 84, and 96 h), rewetting was performed with nuclease-free unlabeled water. We evaluated the rate of soil respiration by removing 1 ml of gas from the headspace of each microcosm with a 1-ml syringe, measuring the CO_2 concentration (ppm) in the gas on a CO_2 gas analyzer (LI-820; Li-Cor Biosciences, Lincoln, NE), and regressing the total amount of CO_2 respired (μg C-CO_2 g $soil^{-1}$) by the harvest time of each microcosm. We stored microcosms at −20°C until further processing.

Taxonomic responses to rewetting. To link bacterial taxa to moisture-induced pulses of CO_2, we pyrosequenced the 16S rRNA genes from bacteria in the 0-h and 72-h $H_2{}^{18}O$ soil incubations. Specifically, we selected, pooled, and sequenced two fractions within the 0-h gradients (fractions 12 and 13, with buoyant densities in CsTFA of 1.531 to 1.548 g/ml) to identify bacterial taxa immediately following the rewetting event. We then selected, pooled, and sequenced two fractions within the 72-h gradients containing ^{18}O-labeled DNA (fractions 9 and 10, with buoyant densities in CsTFA of 1.574 to 1.585 g/ml). This combined SIP procedure was performed on soil from three replicate grassland plots.

We used bar-coded pyrosequencing of the 16S rRNA gene to characterize shifts of bacterial taxa following rewetting. The pyrosequencing procedures are described in greater detail elsewhere (16, 23). Briefly, 16S rRNA genes were amplified using a 27F and 338R primer set with unique 12-nucleotide (nt) error-correcting Golay bar codes under the following thermal cycle conditions: an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 30 s, and extension at 72°C for 90 s. PCR amplicons from each sample were pooled at approximately equimolar concentrations prior to pyrosequencing. Samples were sequenced at the Environmental Genomics Core Facility at the University of South Carolina in a 454 Life Sciences FLX genome sequence (Roche, Branford, CT) instrument. Sequences were analyzed according to the methods outlined by Fierer et al. (16). To ensure the accuracy and quality of pyrosequencing, only sequences of >200 bp were included in the analysis (22). Phylotypes were identified using Megablast at a minimum coverage of 99% and a minimum pairwise identity of 97%. The phylogenetic identities of sequences were determined with BLAST searches against the Greengenes database (http://greengenes.lbl.gov/) (12), using an E value cutoff of $1e^{-10}$ and the Hugenholtz taxonomy.

To assess compositional responses to rewetting, we first calculated the relative recoveries of taxonomic groups as the total number of phylotypes in a given group divided by the total number of phylotypes present in each sample. We restricted our analyses to taxa that comprised more than 1% of all phylotypes and demonstrated ≥0.5% differences in relative recovery. Next, shifts in bacterial composition in response to rewetting were assessed for each grassland replicate (n = 3) by quantifying the change in relative recovery of taxa between 0 and 72 h of $H_2{}^{18}O$ application. We tested for the significance of these shifts in bacterial composition following rewetting by constructing 95% confidence intervals (sample mean ± [1.96 × standard error]) around the mean change for each taxonomic group. If the confidence intervals overlapped with zero, we concluded that there was no significant change in the relative recovery of a taxonomic group following rewetting. Furthermore, we quantified changes in bacterial taxon richness over the rewetting periods by using Chao1 diversity estimation (4) for each of the $H_2{}^{18}O$-SIP runs.

RESULTS

Location of ^{18}O in DNA. Results from LC-MS revealed that oxygen from $H_2{}^{18}O$ was incorporated into all structural components of dTMP of *E. coli*, including the phosphate group, deoxyribose, and thymine. Naturally occurring dTMP has a molar mass of 322 and contains a total of eight oxygen atoms; four of these are in the phosphate group, two in the pentose sugar, and two in the thymidine nucleobase. If any of the ^{16}O atoms were replaced by ^{18}O, we would expect the molar mass of dTMP to increase by 2, for a total possible mass shift of 16 in a fully ^{18}O-labeled dTMP molecule. After incubating unlabeled *E. coli* DNA with $H_2{}^{16}O$, the resulting dTMP peaked predominantly at a mass-to-charge ratio (m/z) of 321 in the LC-MS spectrum (Fig. 1A). After incubating *E. coli* with $H_2{}^{18}O$, however, the resulting dTMP exhibited substantial peaks at 321, 325, 327, 331, 333, and 335 m/z (Fig. 1A). These multiple peaks suggest that the number of ^{18}O atoms incorporated into dTMP is variable, ranging from approximately 4 to 14 extra neutrons (or 2 to 7 ^{18}O atoms). The largest of these peaks occurred at 333 and 335 m/z, corresponding to the addition of six and seven ^{18}O atoms, respectively. Because dTMP contains only eight O atoms and no structural component of

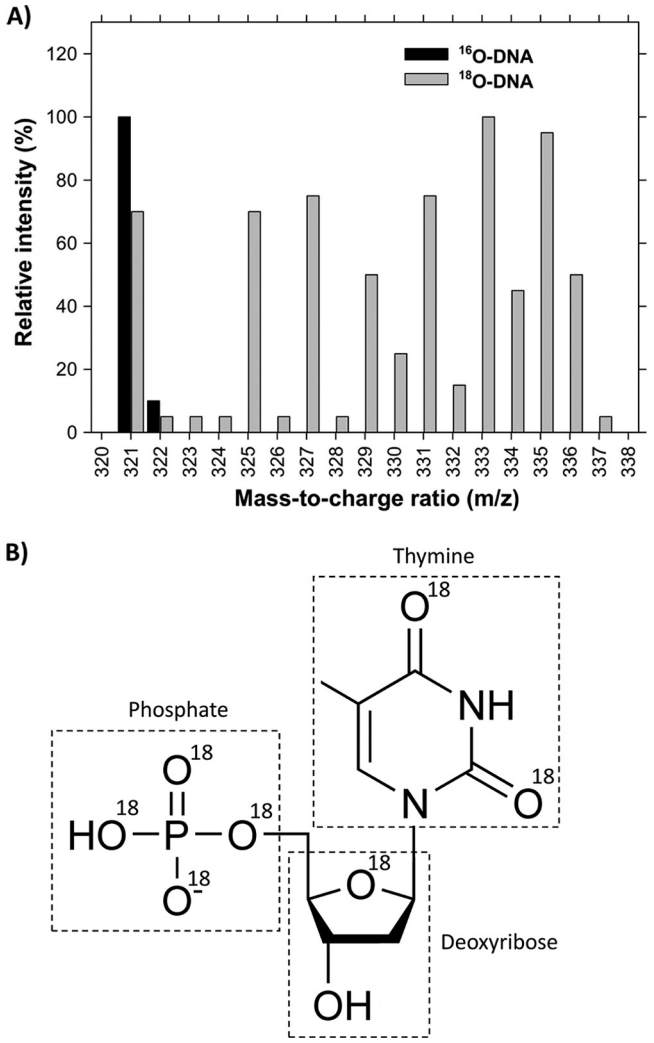

FIG. 1. Mass spectra of unlabeled (^{16}O) and labeled (^{18}O) dTMP in actively growing *E. coli*. Bars represent the relative intensities of dTMP ions with various mass-to-charge (*m/z*) ratios from LC-MS. Relative intensity was based on the number of ions in each *m/z* ratio relative to the ratio with the largest number of ions detected for unlabeled ($6.31e^3$) or ^{18}O-labeled ($1.23e^3$) dTMP ions. (B) Diagram of dTMP with an *m/z* of 335, highlighting the possible locations of ^{18}O.

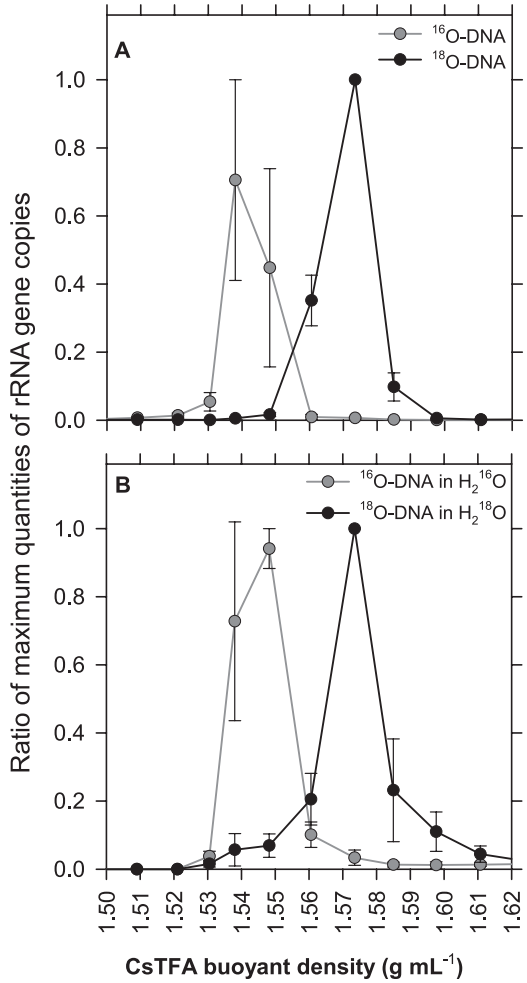

FIG. 2. ^{18}O-DNA SIP of unlabeled (^{16}O) and labeled (^{18}O) *E. coli* rRNA genes before (A) and after (B) a 7-day incubation in $H_2{}^{16}O$. Values are means ± SEM for the ratio of maximum quantities of rRNA gene copies ($n = 3$) within CsTFA gradients. Ratios are based on the number of gene copies in each buoyant density fraction relative to the buoyant density fraction that contained the maximum number of 16S rRNA gene copies.

DNA contains only one O atom, we can infer that ^{18}O incorporation from $H_2{}^{18}O$ occurred in all three components of DNA (Fig 1B). The relatively smaller peaks throughout the spectrum, at 322 *m/z* in the unlabeled DNA and at 324, 326, and 328 *m/z* in the ^{18}O-labeled DNA, most likely occurred from the addition of an extra neutron into DNA from ^{13}C. This phenomenon is common and can most likely be attributed to the natural abundance of $^{13}C/^{12}C$ (≈1%) in the *E. coli* inoculum and the medium used in the $H_2{}^{18}O$ incubations.

***Ex vivo* exchange of ^{18}O in DNA.** Our results suggest that it is unlikely that $H_2{}^{18}O$-SIP is strongly affected by *ex vivo* isotopic exchange. ^{18}O in the ^{18}O-labeled DNA from *E. coli* did not undergo *ex vivo* exchange with oxygen from H_2O. If significant isotopic exchange had occurred, we would have expected there to be a decrease in the buoyant density of ^{18}O-labeled DNA following incubation. In contrast, there was no difference in the buoyant density for the fraction with the maximum quantity of ^{18}O-rRNA genes within 11 fractions of the gradient (with densities ranging from 1.531 to 1.649 g ml^{-1}) before and after the 7-day incubation in $H_2{}^{16}O$ ($F_{1,10} = 1.02$; $P = 0.32$) (Fig. 2). Therefore, ^{18}O-labeled DNA possessed the same buoyant density and retained its extra neutrons from ^{18}O following incubation in unlabeled H_2O. ^{18}O-labeled DNA resided in fractions with densities of 1.561 to 1.585 g/ml, while unlabeled DNA resided in fractions with densities of 1.531 to 1.548 g/ml.

Soil rewetting and $H_2{}^{18}O$-SIP. As expected, bacteria in the dry grassland soil rapidly responded to rewetting. CO_2 accumulated in microcosms at a rate of 1.34 μg C-CO_2 g^{-1} soil h^{-1} (linear regression $r^2 = 0.76$; $P < 0.0001$). By 72 h, CO_2 concentrations had increased from 3.04 ± 0.35 (mean ± standard error of the mean [SEM]) to 113 ± 9.11 μg C-CO_2 g^{-1} soil. At the same time, ^{18}O from $H_2{}^{18}O$ was rapidly incorporated into the DNA of metabolically active soil bacteria. Based on $H_2{}^{18}O$-SIP time series, the ^{18}O incorporation into DNA

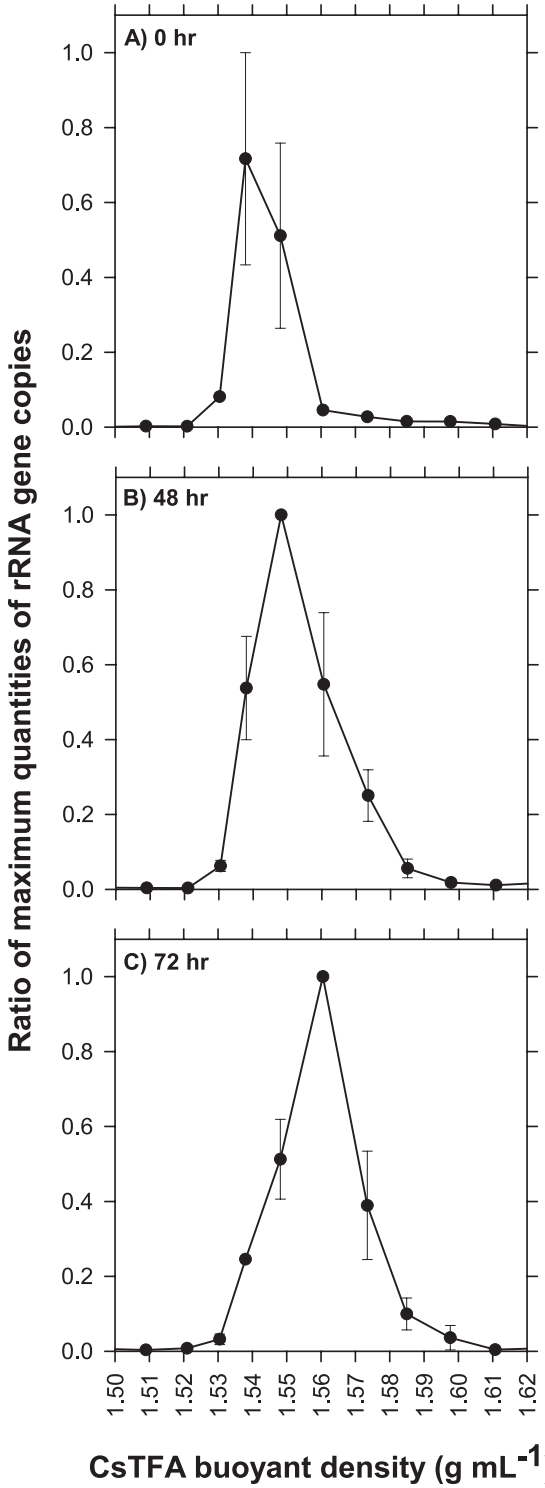

FIG. 3. Rapid incorporation of ^{18}O from $H_2{}^{18}O$ into bacterial DNA following rewetting of dried soils. Panels show the resulting distributions of 16S rRNA genes from replicate ($n = 3$) grassland soils in CsTFA gradients. Soils were incubated with $H_2{}^{18}O$ for 0 h (A), 48 h (B), and 72 h (C). Values are mean ratios of maximum quantities of rRNA genes ($n = 3$) ± SEM from qPCRs.

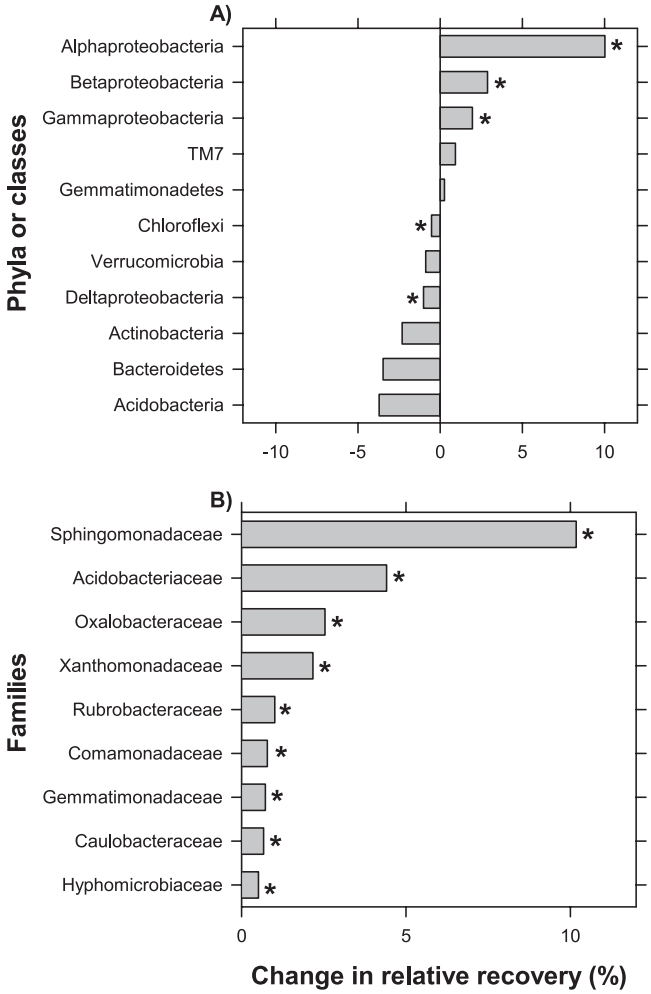

FIG. 4. Taxonomic responses to soil rewetting. Values are mean changes ($n = 3$) in relative recoveries of taxa after rewetting (72 h) and before rewetting (0 h) with $H_2{}^{18}O$ for phyla or classes (A) and families (B). Asterisks denote taxonomic groups that responded significantly to rewetting based on the overlap of 95% confidence intervals with zero change in relative recovery.

caused the buoyant density of DNA to increase by 0.013 to 0.054 g/ml within the first 48 h (Fig. 3). We observed an increase in the denser CsTFA fractions (i.e., fractions 8 to 11, with buoyant densities of 1.561 to 1.598 g/ml) in the 72-h incubation, suggesting that bacteria continued to produce ^{18}O-labeled DNA over the duration of the experiment. However, at both 48 and 72 h, there was always a substantial quantity of unlabeled rRNA genes present in the less dense CsTFA fractions (i.e., fractions 12 to 14, with buoyant densities of 1.531 to 1.548 g/ml).

Taxonomic responses to rewetting. Based on pyrosequencing of the 16S rRNA genes from $H_2{}^{18}O$-SIP, we found that soil bacteria exhibited different responses to rewetting (Fig. 4A). Bacterial taxa were considered positive responders if they produced new cells labeled with ^{18}O and increased in relative recovery following rewetting. For example, the *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* all increased in relative recovery following rewetting. The most dra-

matic change occurred for the *Alphaproteobacteria*, which increased in their relative recovery by 10%. Together, these positively responding taxa accounted for 30% ± 1.7% of all phylotypes prior to the rewetting event. In contrast, the relative recovery of the *Chloroflexi* and *Deltaproteobacteria*, which accounted for 3.2% ± 0.4% of the initial phylotypes, decreased after the rewetting event. The response of some bacterial taxa to rewetting was variable. For example, the mean changes in relative recovery of *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, TM7, and *Verrucomicrobia* ranged from −3.7 to 0.9%; the 95% confidence intervals around the average shifts in relative recoveries overlapped with zero, indicating that these taxonomic groups did not respond significantly to rewetting. This variability could be explained in part by examining the bacterial responses at a finer taxonomic resolution. At the family level, we found that nine families increased in relative recovery by at least 0.5% following the rewetting event, and three of these nine came from phyla with variable responses (Fig. 4B). For example, we found that the *Rubrobacteraceae* (*Actinobacteria* phylum), *Acidobacteriaceae* (*Acidobacteria* phylum), and *Gemmatimonadaceae* (*Gemmatimonadetes* phylum) all responded positively and significantly to rewetting. The remaining six families belonged to phyla that demonstrated coherent positive responses to rewetting. Within the *Alphaproteobacteria*, we observed significant positive responses by the *Caulobacteraceae*, *Hyphomicrobiaceae*, and *Sphingomonadaceae*. In particular, the relative recovery of the *Sphingomonadaceae* increased by 10% following rewetting. In addition, we saw positive responses by the *Comamonadaceae* (*Betaproteobacteria*) and the *Xanthomonadaceae* (*Gammaproteobacteria*). Based on Chao1 estimates, there was no difference between the numbers of phylotypes in the initial (0 h) and final (72 h) SIP samples (5,963 ± 587.0 versus 6,679 ± 204.1 [mean ± SEM], respectively; paired *t* test $t_2 = 1.15$ [$P = 0.42$]).

DISCUSSION

Over the past decade, SIP has emerged as an effective tool for linking the structure and function of microbial communities. In this study, we evaluated some of the mechanisms and underlying assumptions of $H_2{}^{18}O$-SIP (48). In laboratory experiments with *E. coli*, we found that ^{18}O was incorporated into all of the structural components of DNA and that these labeled materials did not exchange with extracellular water. With environmental samples, we measured rapid (48 to 72 h) incorporation of ^{18}O into the DNA of soil bacteria, which coincided with the production of soil CO_2. Consistent with previous studies, we found that many taxa belonging to the *Proteobacteria* were able to respond rapidly to changes in soil environmental conditions. We discuss some of the functional traits that may be involved in the tolerance of fluctuating soil moisture and the contribution of these organisms to ecosystem activity.

Validation of $H_2{}^{18}O$-SIP. LC-MS data retrieved from labeled dTMP indicated that ^{18}O from $H_2{}^{18}O$ could be incorporated into all structural components of DNA. However, this incorporation was variable, consisting of as few as two ^{18}O atoms to as many as seven ^{18}O atoms per nucleotide. There is additional evidence that ^{18}O incorporation into microbial DNA can be variable. For example, after 2-week incubations of soils with 95 atom% $H_2{}^{18}O$, three bands of DNA (instead of two) were observed in cesium chloride gradients stained with SYBR green 1 (48). It was hypothesized that the additional band of DNA belonged to "cross-feeding" taxa, which over time preyed upon or decomposed ^{18}O-labeled organisms and their cellular constituents. Alternatively, an additional band could reflect semiconservative DNA replication (48). Although our incubations with *E. coli* were much shorter than those in previous work with environmental samples (48), the preferential labeling of different DNA components may also give rise to variability in the buoyant densities in $H_2{}^{18}O$-SIP. Specific pathways for DNA synthesis directly incorporate ^{18}O from $H_2{}^{18}O$ (i.e., formation of phosphate), while others derive ^{18}O from both water and oxygen-containing organic compounds. For example, it was shown that oxygen associated with C-3 and C-5 of deoxyribose was derived predominantly from glucose, not from the surrounding cytoplasmic water (41). Thus, any unlabeled C substrate from growth media or the surrounding environment (e.g., soil) has the potential to be incorporated into deoxyribose over ^{18}O from $H_2{}^{18}O$, but this interaction may change as more carbon becomes labeled with ^{18}O. Regardless of the uneven labeling during $H_2{}^{18}O$-SIP, ^{18}O incorporation was sufficient to clearly separate fractions of ^{18}O-labeled and unlabeled DNAs from both cultures and environmental samples (Fig. 2 and 3). In addition, we found that the ^{18}O in ^{18}O-labeled DNA from *E. coli* did not undergo any detectable *ex vivo* exchange with ^{16}O from unlabeled H_2O. These results suggest that ^{18}O incorporation into DNA is relatively stable and that $H_2{}^{18}O$-SIP is appropriate for making inferences about the metabolic activity of microbial taxa. Future studies, however, should evaluate whether *in vivo* isotopic exchange associated with processes such as DNA repair (54) facilitates ^{18}O-^{16}O substitutions.

Linking $H_2{}^{18}O$-SIP to soil CO_2 production. In a previous study, separation of ^{18}O-labeled and unlabeled DNAs was achieved after incubating soil for 6 to 21 days (48). In this study, we measured consistent shifts in the buoyant density of 16S rRNA genes from soil bacteria after only 48 h of incubation with $H_2{}^{18}O$ (Fig. 2 and 3). This shorter incubation time may reduce the occurrence of artifacts associated with exposure of environmental samples to laboratory conditions for prolonged periods. In addition, a 3-day $H_2{}^{18}O$ incubation is a time frame that captures rapid pulses of ecosystem activity generated from rewetting of dry soils (19). Therefore, it is likely that many of the ^{18}O-labeled bacterial taxa contributed, at least in part, to the observed pulses of CO_2 that followed rewetting. There are a number of important caveats, however, that must be considered in using $H_2{}^{18}O$-SIP to make inferences about the contributions of soil microorganisms to CO_2 pulses. First, not all bacteria generate CO_2. For example, ammonia-oxidizing bacteria use $NH_4{}^+$ as an electron donor and O_2 as an electron acceptor; CO_2 is not generated as a byproduct of their metabolism. Second, it is possible that some heterotrophic bacteria contributed to soil CO_2 production but did not grow and become labeled with ^{18}O. For example, soil bacteria may have responded to rewetting but used $H_2{}^{18}O$ predominantly for catabolism to meet maintenance energy requirements (e.g., motility, energized cell membrane, DNA repair, etc.) in the absence of new cell growth (52). These active but nonreplicating bacteria would presumably be detected if

RNA-SIP were used instead of DNA-SIP (57). Lastly, there were nonbacterial taxa (e.g., archaea, fungi, and nematodes) in our grassland soil samples that were not targeted by our PCR primers but almost certainly produced CO_2 in response to rewetting. Therefore, it is important to acknowledge that our $H_2{}^{18}O$-SIP assay captured some microorganisms that did not contribute to CO_2 pulses but also missed some microorganisms that did contribute to CO_2 pulses.

Coherent taxonomic responses to rewetting. Given the tremendous diversity of microorganisms, recent attention has focused on whether the ecological responses of bacteria can be predicted at relatively high levels of taxonomic resolution (38). In other words, can we make generalizations about how phyla respond to environmental changes, or must we understand bacterial behaviors on a strain-by-strain basis? In general, our results provide evidence that bacterial responses to rewetting are coherent at fairly coarse taxonomic levels of resolution. Specifically, the *Betaproteobacteria* and *Gammaproteobacteria* exhibited moderate increases in relative recovery, and *Alphaproteobacteria* increased by 10% following soil rewetting. This positive response represents the rapid growth of ~500 individual phylotypes within the *Alphaproteobacteria* alone. In particular, we found that the *Sphingomonadaceae*—a group of Gram-negative, aerobic heterotrophs that are common in soils (26, 40)—were chiefly responsible for the increase in relative recovery of the *Alphaproteobacteria* following rewetting. Conversely, the relative recovery of *Chloroflexi* and *Deltaproteobacteria* declined after rewetting. This reduction in relative recovery may reflect the fact that some bacteria experience stress by sudden changes in osmotic pressure and are unable to rapidly grow following rewetting (47). Alternatively, a decrease in relative recovery could represent a dilution effect whereby neutrally responding taxa were less likely to be detected in our pyrosequencing efforts owing to the increased probability of recovering positively responding taxa. Furthermore, it is possible that we missed the responses of other bacterial taxa since we evaluated only two ^{18}O-labeled fractions within the $H_2{}^{18}O$-SIP study; other taxa may have been recovered in fractions immediately adjacent to the fractions evaluated in our study.

The response of bacterial taxa to rewetting is likely to be influenced by the distribution of functional traits. For example, fast-growing (*r*-selected), copiotrophic microorganisms have high rates of resource uptake (i.e., V_{max}) that would allow them to take advantage of sudden increases in substrate availability that accompany soil rewetting events (47). In contrast, slow-growing (*k*-selected), oligotrophic microorganisms benefit from having a higher affinity (i.e., lower half-saturation constant [K_m]) for growth-limiting resources, which may allow them to persist under less productive conditions (e.g., prolonged periods of drought). Using this framework, our data are consistent with the view that the *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* exhibit copiotrophic behavior, while some of the *Chloroflexi*, *Deltaproteobacteria*, and other nonresponsive taxa may include oligotrophic bacteria. Specifically, the sphingomonads are recognized as a copiotrophic group of soil bacteria. These microorganisms exhibit extreme metabolic versatility, as demonstrated by their ability to consume a wide range of organic carbon substrates, including glucose, polysaccharides, and aromatic hydrocarbons (1, 28, 29). The taxonomic responses from our rewetting experiment are consistent with other studies that have attempted to classify the ecology of soil bacteria. For example, the relative recovery of *Betaproteobacteria* increased along a carbon mineralization gradient (15), while the relative recoveries of *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* from different land-use types responded positively to the addition of simple carbon substrates such as glucose, glycine, and citric acid (13).

It seems likely, however, that functional traits other than those associated with resource acquisition may also contribute to bacterial rewetting responses. For example, many *Proteobacteria*, including the *Sphingomonadaceae*, secrete a diverse compilation of exopolymeric substances (11, 14). These substances can retain moisture in dry soils (37, 42) and allow bacteria to capitalize on resources when they become available. Also, the presence of aquaporins, channel proteins that enhance the permeability of cell membranes, should allow bacteria to rapidly expel osmolytes following rewetting and thus maintain equilibrium with their environment (8, 47). Of the sequenced Gram-negative bacteria with aquaporins, approximately 80% belong to phyla that responded positively to rewetting (i.e., *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*) (50). Other traits that may be important for soil microbes that experience drying-rewetting cycles are motility (9), tolerance to low oxygen (32), and the ability to enter and exit a dormant state (24).

Summary and future applications. Our findings demonstrate that $H_2{}^{18}O$-SIP is a viable method for identifying bacteria that rapidly respond to soil rewetting and potentially contribute to pulses of ecosystem activity (e.g., CO_2 flux). A number of studies have documented the effects of rewetting stress on individual strains of soil bacteria (2, 21, 42, 51), but this information comes largely from a phylogenetically restricted group of cultured bacteria. In examining an intact soil bacterial community, we found that there were coherent positive responses of the *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* to rewetting. Additional studies are needed to elucidate the mechanisms behind these patterns, especially given the predicted changes in precipitation regimens under future climate change scenarios (56, 59). Besides understanding bacterial responses to fluctuations in moisture, $H_2{}^{18}O$-SIP has many other potential applications in microbial ecology. For example, this form of SIP may help to identify temperature-sensitive microorganisms involved in decomposition, characterize the activity of functional genes, or serve as an isotopic backdrop to identify bacteria that are capable of remediating contaminated ecosystems. Last, $H_2{}^{18}O$-SIP could also be used in combination with metagenomic approaches (5) to characterize genes that are regulated under conditions causing fluctuations in microbial growth and activity.

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