Chapter 14

Analysis of Active Methylotrophic Communities: When DNA-SIP Meets High-Throughput Technologies

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Abstract

Methylotrophs are microorganisms ubiquitous in the environment that can metabolize one-carbon (C1) compounds as carbon and/or energy sources. The activity of these prokaryotes impacts biogeochemical cycles within their respective habitats and can determine whether these habitats act as sources or sinks of C1 compounds. Due to the high importance of C1 compounds, not only in biogeochemical cycles, but also for climatic processes, it is vital to understand the contributions of these microorganisms to carbon cycling in different environments. One of the most challenging questions when investigating methylotrophs, but also in environmental microbiology in general, is which species contribute to the environmental processes of interest, or "who does what, where and when?" Metabolic labeling with C1 compounds substituted with ^{13}C, a technique called stable isotope probing, is a key method to trace carbon fluxes within methylotrophic communities. The incorporation of ^{13}C into the biomass of active methylotrophs leads to an increase in the molecular mass of their biomolecules. For DNA-based stable isotope probing (DNA-SIP), labeled and unlabeled DNA is separated by isopycnic ultracentrifugation. The ability to specifically analyze DNA of active methylotrophs from a complex background community by high-throughput sequencing techniques, i.e. targeted metagenomics, is the hallmark strength of DNA-SIP for elucidating ecosystem functioning, and a protocol is detailed in this chapter.

Key words Carbon-13, DNA stable isotope probing, DNA-SIP, High-throughput sequencing, Isotopic labeling, Methylotrophy, Metagenomics, One-carbon compounds

1 Introduction

One carbon (C1) compounds, as well as compounds with multiple carbons but no carbon–carbon bonds, such as methylated amines, are diverse and widespread in the environment. These compounds play key roles in the biogeochemical cycles of carbon, and also nitrogen, sulfur, and phosphorus [1–3]. Some of these compounds have an influence on climatic processes through their release to the atmosphere [1], and thus a direct relevance for global ecology. Microorganisms that can metabolize these compounds, called

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methylotrophs, are ubiquitous in the environment. Next to physicochemical reactions, microbial activities often are the only major processes involved in C1 compound conversion [3–5]. Thus, the composition and activity of the microbial community in a specific habitat is a major factor that modulates the release or uptake of C1 compounds into and from the atmosphere. Consequently, investigation of these microorganisms in different habitats, as well as assessment of their activity and contribution to biogeochemical cycles, is essential for understanding and modeling the environmental processes that shape and sustain our planet.

Most knowledge of C1 compound metabolism was obtained from isolation and characterization of pure cultures of methylotrophs [6]. However, insights deduced by these cultivation-dependent approaches are difficult to transfer directly to environmental systems, where microorganisms are tightly integrated in metabolic networks and potentially dissimilar to those readily cultivated microorganisms. Actual microbial communities that catalyze processes of interest often remain "black boxes" for the environmental microbiologist, making it difficult to answer the key question of "who is doing what, where and when?" [7] in a particular environment.

Classical approaches for environmental studies of methylotrophs rely on the analysis of specific biomarkers. The detection of 16S rRNA genes similar to those of known and characterized methylotrophs in environmental samples is often used to infer a corresponding function to these detected organisms. In addition, structural genes can be used to identify environmental distribution of key enzymes for the conversion of C1 compounds, including a range of dehydrogenases, monooxygenases, and methyl transferases [8]. Various PCR primer sets have been introduced to target these genes in environmental surveys [9–15]. For example, *pmoA* and *mmoX*, encoding subunits of the particulate and soluble methane monooxygenase, have been used to target methanotrophs, and *mxaF*, encoding the large subunit of methanol dehydrogenase, to target methylotrophs [9, 10, 13]. High-throughput sequencing technologies have improved rapidly over the past decade, allowing much deeper sequencing of environmental samples. Pyrosequencing, reversible dye terminator sequencing, or ion semiconductor sequencing [16] are often used in combination with biomarker approaches. Selection for biomarkers of interest can either be done prior to sequencing, for example by using PCR amplicon pyrosequencing [17, 18], or by screening of shotgun metagenomic datasets [19, 20]. However, these approaches do not provide information on the real metabolic activities of the microbial communities being investigated.

In order to unravel the functional contributions of methylotrophs in microbial communities, cultivation-independent approaches are needed that can establish a direct link between phylogeny and function. Stable isotope probing (SIP), a metabolic labeling approach with

substrates enriched with heavy, nonradioactive isotopes, can fulfill these requirements. In a SIP experiment targeting methylotrophs, environmental material (e.g. water, soil or sediment) is incubated with a ^{13}C-labeled C1 compound. Active methylotrophs that use this compound as a carbon source incorporate the heavy carbon atoms into their biomass, including and notably their DNA. Detection of ^{13}C enrichment in biomarkers of specific organisms is therefore evidence for methylotrophic activity resulting in substrate assimilation. This approach was first described in combination with the investigation of microbial polar lipid derived fatty acids (PLFA), using isotope ratio mass spectrometry to detect the heavy isotopes [21]. The combination with metagenomics (DNA-SIP) followed 2 years later, and allowed the implementation of SIP with the classical approaches described above to detect active methylotrophs in the environment [22–26]. Compared to a PLFA-based approach, DNA-SIP offers better phylogenetic resolution and provides substantial functional information from the labeled DNA sequences (*see* e.g. [14, 15, 27]). Even the retrieval of whole genomes of the active methylotrophs is possible [28].

A SIP experiment employing ^{13}C-labeled C1 compounds, followed by DNA extraction, typically results in a mix of heavy (^{13}C-labeled) DNA from active methylotrophs and unlabeled light (^{12}C) DNA from other organisms, including inactive methylotrophs. In this chapter, we outline requirements for a DNA-SIP experiment, describe the methods necessary for isolation and identification of the labeled DNA and give advice for troubleshooting and interpretation of subsequent results. In addition, we highlight strategies for the analysis of metagenomics DNA by high-throughput sequencing.

Separation of heavy and light DNA is achieved in a density gradient because the substitution of ^{12}C with ^{13}C proportionally increases the density of DNA. Ultracentrifugation of the extracted DNA mix in a cesium chloride solution results in the migration of DNA according to its density within the gradient, forming bands of increasingly labeled DNA down the gradient. The density gradient is partitioned into a number of fractions, and the 16S rRNA gene profiles of the DNA recovered from each of these fractions are investigated via denaturing gradient gel electrophoresis (DGGE) [29]. This fingerprinting technique represents a straightforward method that separates PCR amplicons based on their GC content and sequence [14, 15].

The rRNA gene fingerprints are important to rapidly identify the fractions containing ^{13}C-labeled DNA by comparison with corresponding fraction profiles from an unlabeled (^{12}C) control incubation. These fractions containing DNA enriched with genetic material of the active methylotrophs can subsequently be used for sequence analysis, starting with amplicon sequencing targeting 16S rRNA genes and functional genes (e.g. *pmoA*, *mxaF*), to obtain phylogenetic and functional information. If necessary,

labeled DNA can be amplified by multiple displacement amplification (MDA) to obtain sufficient material prior to shotgun metagenomics [30], enabling a more in-depth functional investigation of active methylotrophs, including the potential for genome assembly even with very low quantities of labeled material.

2 Materials

Use analytical grade reagents and ultrapure water for the preparation of all solutions. For suspending DNA, use nuclease-free water. All solutions should be prepared and stored at room temperature, unless otherwise indicated.

2.1 Density Gradient Centrifugation Components

1. EDTA solution: 0.5 M EDTA, pH 8.0. Dissolve 186.1 g of disodium ethylenediamine tetraacetate dihydrate (EDTA) in 900 mL of water. Add 2 M NaOH to adjust the pH to 8.0 (*see* **Note 1**) and make up to 1 L with water. Sterilize in an autoclave.
2. Tris-EDTA (TE) buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 8.0. Dissolve 60.6 mg of Tris in 40 mL of water. Add 100 μL of a 0.5 M EDTA solution, mix and adjust pH to 8.0 with 0.5 M HCl. Make up to 50 mL with water. Filter sterilize (0.22 μm) or autoclave.
3. DNA from a metabolic labeling experiment using the ^{13}C-labeled C1 compound of interest and DNA from a control treatment with the same ^{12}C compound, in TE buffer or water (*see* **Note 2**), with known DNA concentrations.
4. CsCl solution: Dissolve 603.0 g of CsCl in water to a final volume of 500 mL, resulting in a 7.163 M CsCl solution (*see* **Note 3**). Adjust the density to a final value between 1.88 and 1.89 g/mL at 20 °C (*see* **Note 4**).
5. Gradient buffer (GB): 0.1 M Tris–HCl, 0.1 M KCl, 1 mM EDTA, pH 8.0. Dissolve 12.11 g of Tris and 7.46 g of KCl in 900 mL of water. Add 2 mL of a 0.5 M EDTA solution, mix and adjust pH to 8.0 with HCl. Make up to 1 L with water. Sterilize in an autoclave.
6. Ultracentrifuge tubes: 5.1 mL, 13 mm × 51 mm Polyallomer Quick-Seal Centrifuge Tubes (Beckman Coulter Ltd., High Wycombe, UK).
7. Ultracentrifuge rotor capable of withstanding 177,087 × *g* average: e.g. VTi 65.2 Beckman Coulter Vertical (Beckman Coulter Ltd., High Wycombe, UK).
8. Pump for fractionation: Syringe pump or peristaltic pump able to deliver a constant flow of 425 μL/min.
9. Digital refractometer: e.g. AR200 Digital Handheld Refractometer (Reichert Technologies, Depew, NY, USA).

10. APS solution: 10 % ammonium persulfate (w/v). Dissolve 1 g of ammonium persulfate (APS) in 10 mL of water. Aliquot in 1 mL portions and store at −20 °C. Frozen APS solution can be used for several months.
11. Linear Polyacrylamide (LPA): Mix (in order) 250 mg of acrylamide, 4.25 mL of water, 200 μL of 1 M Tris–HCl pH 8.0, 33 μL of 3 M sodium acetate pH 7.5, 10 μL of 0.5 M EDTA solution, 50 μL of 10 % ammonium persulfate solution and 5 μL of tetramethylethylenediamine (TEMED) in a 50-mL tube, leave at room temperature for 30 min. Add 12.5 mL of 95 % ethanol to precipitate for 5 min. Remove liquid (squeeze pellet), wash with 70 % ethanol and remove liquid again. Air dry for 10 min. Suspend pellet overnight in 50 mL of water, aliquot and store at −20 °C.
12. Polyethylene glycol-NaCl (PEG-NaCl) solution: 30 % PEG 6000, 1.6 M NaCl. Dissolve 150 g of PEG 6000 and 46.8 g of NaCl into a final volume of 500 mL. Sterilize in an autoclave. Two phases may form after autoclaving or prolonged storage, so mix well before each use.

2.2 DGGE Components

1. PCR primers for DGGE: Primer set 341f_GC (CGCCCG CCGC GCGCGGCGGG CGGGGCGGGG GCACGGGG GG CCTACGGGAG GCAGCAG) and 518r (ATTACCGCGG CTGCTGG) targeting bacterial 16S rRNA genes [31].
2. 50× Tris-Acetate-EDTA (TAE) buffer: 2 M Tris–HCl, 1 M Acetic acid, 0.05 M EDTA. Dissolve 242 g of Tris in 800 mL of water. Add 57.1 mL of 100 % acetic acid and 100 mL of 0.5 M EDTA solution. Make up to 1 L with water.
3. 30 % DGGE solution: 1× TAE, 10 % acrylamide/bis-acrylamide, 12 % formamide (v/v), 12.6 % urea (w/v). Dissolve 6.3 g of urea in 10 mL of water. Add 6 mL of formamide, 1 mL of 50× TAE buffer and 12.5 mL of 40 % acrylamide/bis (37.5:1). Make up to 50 mL with water while the remaining urea dissolves.
4. 70 % DGGE solution: 1× TAE, 10 % acrylamide/bis-acrylamide, 28 % formamide (v/v), 29.4 % urea (w/v). Dissolve 14.7 g of urea in 10 mL of water. Add 14 mL of formamide, 1 mL of 50× TAE buffer and 12.5 mL of 40 % acrylamide/bis (37.5:1). Make up to 50 mL with water while the remaining urea dissolves. 5 mg of bromophenol blue can be added for visual differentiation from the 30 % DGGE solution.
5. 5× DGGE loading dye: 50 % glycerol (v/v), 0.2 M EDTA, 0.05 % bromophenol blue (w/v). Mix 2.5 mL of glycerol, 2 mL of 0.5 M EDTA solution and 2.5 mg of bromophenol blue. Make up to 5 mL with water.
6. DGGE system: e.g. DCode Universal Mutation Detection System (Bio Rad, Hemel Hempstead, UK) or DGGEK-2001-110 (C.B.S. Scientific, San Diego, CA, USA).

Table 1
PCR primer sets for functional genes involved in methylotrophy

Gene	Name	Sequence	Reference
pmoA/amoA	pmoA189F	GGNGACTGGGACTTCTGG	Holmes et al. [9]
pmoA/amoA	pmoA682R	GAASGCNGAGAAGAASGC	
pmoA	mb661R[a]	CCGGMGCAACGTCYTTACC	Costello and Lidstrom [11]
mxaF	1003F	GCGGCACCAACTGGGGCTGGT	McDonald and Murrell [10]
mxaF	1561R	GGGCAGCATGAAGGGCTCCC	
mxaF	1555R	CATGAABGGCTCCCARTCCAT	Neufeld et al. [14]
mmoX	206F	ATCGCBAARGAATAYGCSCG	Hutchens et al. [13]
mmoX	886R	ACCCANGGCTCGACYTTGAA	
mmoX	mmoX166F	ACCAAGGARCARTTCAAG	Auman et al. [12]
mmoX	mmoX1401R	TGGCACTCRTARCGCTC	
mauA	mauAf1	ARKCYTGYGABTAYTGGCG	Neufeld et al. [14]
mauA	mauAr1	GARAYVGTGCARTGRTARGTC	
gmaS	557F	GARGAYGCSAACGGYCAGTT	Wischer et al. [15]
gmaS	1332R[b]	GTAMTCSAYCCAYTCCATG	
gmaS	970R[c]	TGGGTSCGRTTRTTGCCSG	

[a]For nested PCR
[b]Beta- and Gammaproteobacteria
[c]Alphaproteobacteria

2.3 DNA Amplification Components and Bioinformatics Tools

1. PCR primer sets targeting functional genes (Table 1).
2. Multiple displacement amplification (MDA) kit: e.g. REPLI-g Mini Kit (QIAGEN Ltd., Manchester, UK).
3. Software package mothur: www.mothur.org [32].
4. Software package USEARCH: www.drive5.com/usearch/ [33].

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Metabolic Labeling with ^{13}C-Labeled C1 Compounds

Setup conditions for metabolic labeling experiments are complex and depend on many factors, including the composition of the microbial community, type of heavy isotope substrate used, metabolic activity of the target population, conversion efficiency and biochemical processes of interest. Thus, no comprehensive protocol can be given for this part of the experiment (*see* **Note 5**).

The following section gives a basic guideline highlighting key steps and crucial points of a metabolic labeling experiment.

1. Obtain environmental material containing the microbial community of interest, e.g. soil, sediment, sludge, biofilm, or aquatic material. Ensure enough material to obtain sufficient DNA after incubation: 5 μg of genomic DNA are required. Furthermore, process the environmental material as soon as possible after sampling. Excessive transport or storage times might influence the microbial community and bias the experimental outcome.
2. Mix the environmental sample to avoid experimental inconsistencies due to sample heterogeneity. Split into individual batches (e.g. bottles, microcosms) for incubation. Prepare all incubations in duplicates or triplicates. In addition to incubations with the ^{13}C-labeled C1 compound, incubations with the corresponding ^{12}C compound are also required. This is critical to identify ^{13}C-labeled DNA later on. Also prepare controls without substrate and sterile controls as necessary.
3. Select the incubation time(s) for your experiment. This depends largely on the metabolic activity of the microbial community of interest. Based on that, an incubation time that is too short will result in insufficient labeling; an incubation time that is too long results in unspecific labeling (i.e. cross-feeding). A preliminary experiment to assess the microbial activity can be useful. Furthermore, performing a time series experiment can give additional information about the carbon flux through the microbial community.
4. Choose the substrate concentration and incubation conditions. The concentration of the added C1 compound should be as close as possible to the concentration present in the environment. Too low a substrate concentration can result in insufficient labeling. Aim for incorporation of 5–500 μmol of ^{13}C per gram of soil or sediment and 1–100 μmol of ^{13}C per liter of water. Incubation conditions (i.e. temperature, light level, nutrient and oxygen concentration) should be as close to natural conditions as possible to reduce biases on the active microbial community detected [34].
5. Monitor substrate consumption. This will allow quantification of incorporation and facilitate selection of the most suitable sampling times. If no reliable method for determination of substrate concentrations is available, consider monitoring $^{13}CO_2$ production or enrichment in biomass (e.g. using isotope ratio mass spectrometry) to have a proxy for microbial activity.

3.2 Preparation and Setup

1. Prepare a calibration curve for calculation of the density of mixtures of the CsCl solution and GB from refractive indices. Mix 450 μL of CsCl solution with 0, 10, 20, 35, 50, 65, 80,

100, 120, and 140 μL GB. Measure the density of the mixtures (*see* **Note 4**). Measure refractive indices with a digital refractometer with a resolution of at least 0.0001 and temperature correction (nD-TC) to 20 °C. Plot density versus nD-TC and calculate a linear regression. The calibration curve is required to convert nD-TC readings to density to set up samples of the correct density for density gradient centrifugation and to verify gradient formation afterward (*see* **Note 6**).

2. Calculate the required amount of GB to get to the desired starting density for density gradient centrifugation of 1.725 g/mL. This can be done using the formula:

$$\text{Required volume} = \left(\text{CsCl stock density} - 1.725\ \text{g/mL}\right) \times \text{volume of CsCl stock added} \times 1.52\ \text{mL/g}\ \left(\textit{see}\ \textbf{Note 7}\right).$$

3. Based on the DNA concentrations of each sample, calculate the volume required to obtain 5 μg of DNA per sample. The amount of GB for each sample needs to be corrected by this volume.
4. Prepare a 15 mL tube for each DNA sample with 4.8 mL of CsCl stock solution. Add 5 μg of DNA for each sample. Add the calculated volume of GB that is reduced by the volume of DNA solution you added for each sample. Calculate the targeted refractive index based on the calibration curve prepared in step 1 for a desired final density of 1.725 g/mL. This typically will be around an nD-TC of 1.4040, but can vary slightly for different stock solutions. Add small amounts of GB and CsCl stock solution to reach the desired refractive index, mix well after each addition (*see* **Note 8**). Samples should be within +/− 0.0002 of the targeted refractive index.
5. Fill ultracentrifuge tubes with the prepared CsCl/GB/DNA mixtures. Use disposable Pasteur pipettes for convenience. To remove air bubbles that stick to the tube walls, fill the tubes up to 1 cm below the top, then gently tilt and rotate the tube, allowing the remaining air to run over the tube walls to gather any smaller air bubbles. Carefully top up the tubes to the tube stem.
6. Balance pairs of ultracentrifuge tubes using an analytical balance. Weight differences below 2 mg are essential for each pair. Heat seal the tubes according to the manufacturer's instruction. Squeeze tubes firmly to make sure that they are properly sealed. Reweigh the paired tubes to ensure that they remain balanced (*see* **Note 9**).
7. Load tubes into the ultracentrifuge rotor, taking care to position balanced tube pairs opposite each other. Note sample

names and rotor positions; tube labels can come off during ultracentrifugation. Prepare the rotor according to the manufacturer's instructions.

3.3 Ultra centrifugation and Fractionation

1. Ultracentrifugation should be carried out for at least 40 h to ensure proper gradient formation and focused migration of DNA to the corresponding densities. Extended run times of 60–72 h, i.e. over weekends, can also be used. Set the centrifuge to a speed according to 177,087 × *g* average (e.g., 44,100 rpm for the VTi 65.2 Beckman Coultier Vertical rotor; *see* **Note 10**) and a temperature of 20 °C. Note that temperature influences density directly. Set the centrifuge to maximum acceleration and select the "no brake" option for deceleration. Calculate between 1.5 and 2 h of additional run time until the centrifuge has stopped. Follow the manufacturer's instructions when operating the ultracentrifuge.
2. Collect all tubes carefully from the rotor, keeping each tube vertical at all times. A pump with adjustable speed and uniform flow rate is needed to fractionate each SIP gradient. The required flow rate is 425 μL/min. A syringe pump should be used for best results, or a peristaltic pump instead. Adjust the speed of the pump by running it with water for 10 min and measuring the volume of the flow-through to get to the desired flow rate. Make sure that the tubing connected to the pump is fitted with a male Luer fitting. Before fractionating the first tube, rinse and fill tubing with water (*see* **Note 11**).
3. After ultracentrifugation, fit the ultracentrifuge tube in a stand with a suitable clamp for fractionation. Handle the tube carefully to prevent disturbing the density gradient. The clamp should be only tight enough to hold the tube securely, without squeezing it. Connect a 23-gauge (0.6 × 25 mm) needle to the tubing of the pump. Run the pump momentarily to remove all air from the needle. Carefully pierce the top of the ultracentrifuge tube with the needle, adjacent to the tube stem (*see* **Note 12**). Ensure that the needle and tubing are secured and cannot slip away during fractionation. Use a second needle to pierce the tube at the bottom, then remove this needle again (*see* **Note 13**).
4. Prepare a series of 12 tubes (1.5 mL) to capture all sample fractions. Activate the prepared pump to fill the ultracentrifuge tube with water, replacing the CsCl solution, together with a timer (*see* Fig. 1). Collect the CsCl solution at the bottom of the tube in the prepared tubes. Collect 425 μL per fraction (i.e. 1 min per fraction). An automated fraction collector might be used, but is not necessary. A full ultracentrifuge tube will result in 12 fractions with a flow rate of 425 μL/min, but keep additional 1.5 mL tubes ready for a potential 13th fraction.

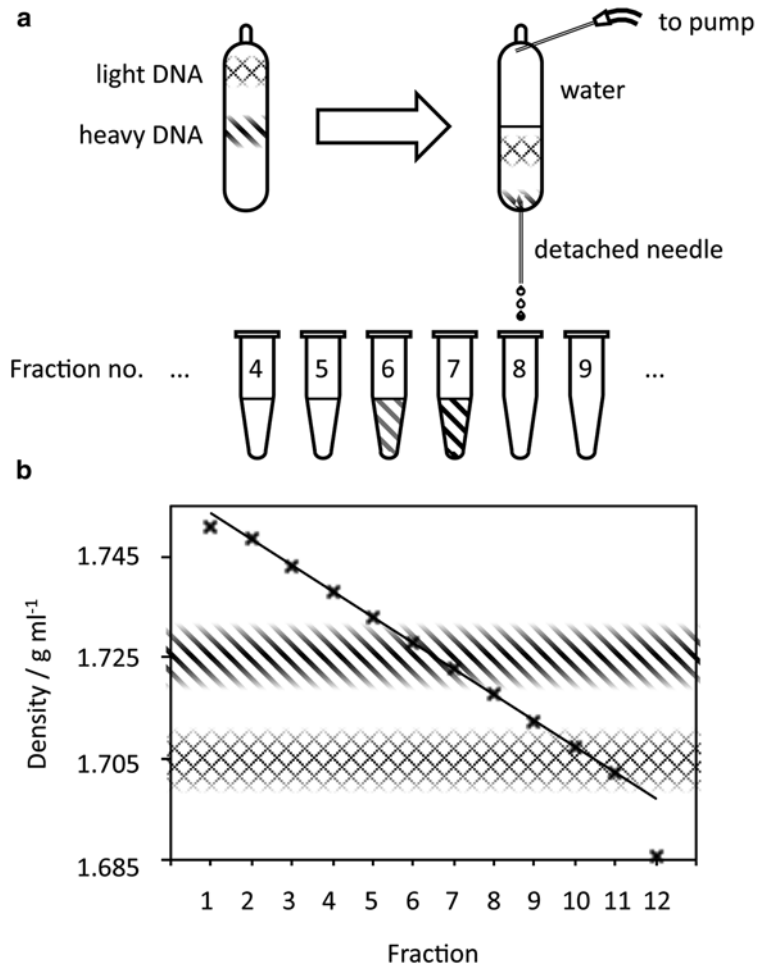

Fig. 1 Illustration of the density gradient fractionation process after separation of labeled and unlabeled DNA by ultracentrifugation. (a) The ultracentrifuge tube is pierced at the top and bottom and the CsCl solution is replaced by water and collected in 1.5 mL tubes. The refractive indices of individual fractions are determined for calculation of densities. ^{13}C-labeled DNA is typically found in fractions 6–8, at a density of around 1.725 g/mL. (b) The density curve typically shows a deviation from linearity for the first fraction (due to diffusion) and the last fraction (due to mixing with water). Labeled DNA is indicated by diagonal line pattern, unlabeled DNA by a checked pattern

Label the fractions in the order of collection, 1–12. Repeat the fractionation process with the next sample (*see* **Note 14**).

5. Measure the refractive indices of all individual fractions to ensure proper gradient formation (*see* **Note 15**). The refractive indices typically are +/− 0.0025 around the refractive index measured before ultracentrifugation, with the first fractions having the highest refractive index and the last fractions the lowest. With the refractive indices, the densities of the fractions

can be calculated based on the calibration curve prepared in Subheading 3.1, **step 1**. On average, ^{13}C-labeled DNA has a density of 1.725 g/mL; unlabeled DNA has an average density of 1.705 g/mL.

6. In order to purify DNA from the CsCl solution, precipitate DNA by adding 5 μL LPA (5 mg/mL) per fraction and mix well (*see* **Note 16**). Add 850 μL of PEG-NaCl solution and mix well. Leave at room temperature for at least 2 h to allow precipitation. Incubation overnight is also possible. Centrifuge at 13,000 × *g* for 30 min and withdraw supernatant with a 1 mL pipette. A transparent pellet should be visible after the supernatant is removed. Wash with 400 μL of 70 % ethanol, centrifuging at 13,000 × *g* for 10 min. Discard supernatant as before. A white pellet should be visible now, which can easily become detached from the tube wall. Air-dry for 15 min, then suspend in 50 μL TE buffer for 30 min on ice, tapping the tube every 5–10 min to ensure that DNA dissolves fully.

3.4 Identification of Labeled DNA by DGGE Fingerprinting

1. Check retrieval and quality of DNA obtained from individual fractions by applying 5 μL to 1 % (w/v) agarose gel electrophoresis following standard laboratory procedures. Quantification of DNA is possible by fluorometric assays. Do not use photometric DNA quantification based on absorbance in the UV range, because this is usually not sensitive enough to detect the low amounts of DNA that might be present. High molecular mass DNA bands should be visible under UV light after staining with ethidium bromide (0.5 μg/mL gel) in at least some of the fractions, typically between fractions 6 and 12. For trouble-shooting on DNA retrieval, *see* Table 2.
2. Perform a PCR with primers targeting rRNA genes of the organisms of interest, including a GC clamp. To target bacterial 16S rRNA genes, we typically use the primer set 341f_GC/518r which amplifies a ~230 bp portion of the gene, spanning the V3 hypervariable region. The PCR conditions are: 95 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by a final extension of 72 °C for 5 min [31]. The final reaction volume is 50 μL. Check the PCR products by applying 5 μL to 1 % (w/v) agarose gel electrophoresis. Prepare samples for DGGE by mixing 4–40 μL of the PCR product, according to band intensity on the agarose gel, with DGGE loading dye to achieve a 1× final concentration.
3. Prepare a gel for denaturing gradient gel electrophoresis. The following volumes are given for DGGE equipment supporting 20 × 20 cm glass plates in a 6.5 L tank. Transfer 12.5 mL of the 30 % and 70 % DGGE solution to two 15 mL falcon tubes and keep them on ice. Add 12.6 μL of TEMED and 126 μL of APS solution to each tube and transfer to a gradient mixer. Cast

Table 2
Potential sources of problems in fractionation of DNA from SIP experiments and recommended solutions

Problem	Potential reason	Solution
No gradient formation	Problems with ultracentrifugation	Repeat ultracentrifugation, ensure no brakes are applied for deceleration and no errors during run
No DNA recovery	Wrong density (DNA sticking on side of tube)	Check correct starting density
	DNA amount too low	Use >5 µg of DNA as starting material
	Loss during DNA precipitation	Make sure to use carrier. Make sure to visualize pellet
DNA at unexpected densities	Incorrect calibration density/nD-TC	Repeat density calibration
	Temperature deviation during ultracentrifugation	Do ultracentrifugation at 20 °C
No difference between ^{12}C and ^{13}C experiment *or* Low amount of ^{13}C DNA	Insufficient labeling	Increase substrate concentrations and/or incubation times[a]
		Try to amplify by MDA
DNA at intermediate densities (partially labeled)	Insufficient labeling (partially and unlabeled DNA)	Increase incubation time
	Crossfeeding (partially and fully labeled DNA)	Reduce incubation time
	Organisms use alternative carbon source (e.g. CO_2)	Perform additional metabolic labeling experiment with ^{13}C-CO_2 for confirmation
Only labeled DNA in ^{13}C experiment	High enrichment of active organisms	Reduce substrate concentrations and/or incubation times
	Crossfeeding	Reduce incubation time
Same genotypes in all fractions	Contamination during/after fractionation	Change solutions, repeat ultracentrifugation with fresh DNA

[a]Organisms that metabolize a C1 compound without using it as carbon source cannot be detected

gradient gel according to standard laboratory protocols, with the 70 % solution at the bottom and the 30 % solution on top. Overlay the gel with 0.5 mL of isopropanol to achieve an even surface. Wait 45 min for the gel to polymerize.

4. Prepare the DGGE tank with 6.4 L of water and add 130 mL of 50× TAE buffer to a final concentration of 1× TAE, heat up to 60 °C. Remove the isopropanol from the polymerized gel and rinse the surface with water three times. Cast top-up gel with 5 mL of 0 % DGGE solution, 5 µL of TEMED and 50 µL

of 10 % APS solution. Insert a 16-well comb without introducing air bubbles. Wait 30 min for the top-up gel to polymerize.

5. Submerge the gels in the DGGE tank and rinse the wells with buffer. Load the samples prepared in **step 3**. Load DGGE ladder if available and load empty wells with 1× DGGE loading dye. Run the DGGE at 75 V for 16 h overnight. Ideally, run all fractions of a ^{13}C sample and the corresponding ^{12}C sample on two gels in parallel. After electrophoresis, stain the gels according to standard laboratory protocols (e.g. with SYBR Gold) and image the gel for evaluating fractionation results.
6. Check band patterns to identify fractions of the ^{13}C sample containing labeled DNA by careful comparison with the gel of the corresponding ^{12}C sample. Unlabeled DNA typically is found in fractions 10–12, fully labeled DNA in fractions 6–8. Ignore bands that are present in all fractions, as these are not likely to have originated from ^{13}C-DNA alone. Look for bands that are consistently present in the light fractions of both the ^{12}C and ^{13}C sample to identify unlabeled DNA. Then look for bands that change their position in relation to the unlabeled DNA to identify the labeled DNA (*see* **Note 17**). Select the appropriate fractions for further experimentation (*see* Fig. 2). *See* also Table 2 for troubleshooting advice.

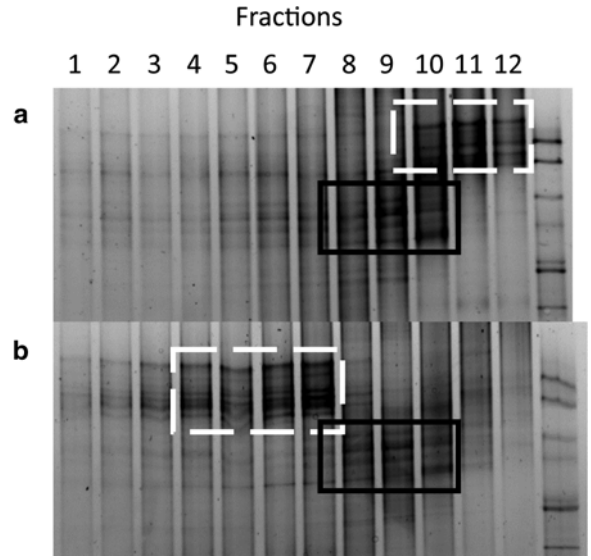

Fig. 2 DGGE gels obtained from fractionated DNA of (a) ^{12}C and (b) ^{13}C incubations on ^{13}C-labeled methanol after electrophoresis for 16 h at 75 V. *Black box*: bands occurring in the same fractions in ^{12}C and ^{13}C incubations representing unlabeled DNA. *White box*: bands enriched in the heavy fractions of the ^{13}C incubation due to labeling of DNA by methylotrophic activity

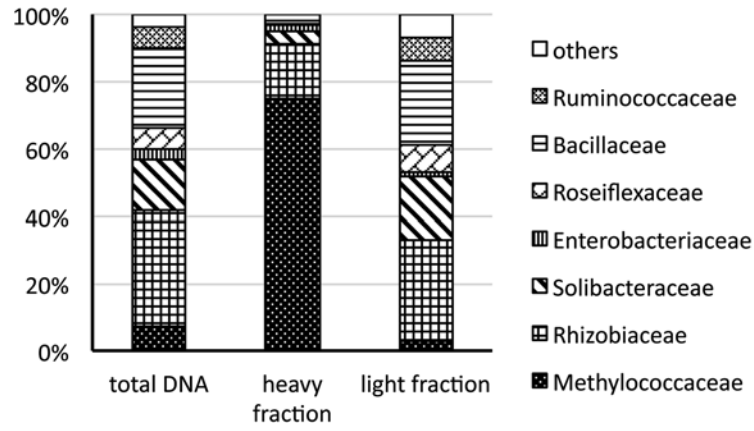

Fig. 3 Theoretical expected results of 454 amplicon pyrosequencing data targeting 16S rRNA genes in unfractionated DNA, DNA from heavy fractions and from light fractions. The heavy fractions show a strong enrichment, compared to unfractionated DNA, of the putative active methylotroph of the family Methylococcaceae, while the light fractions show sequences of the remaining, non-methylotrophic/inactive bacteria also detected in the total DNA

3.5 Analysis of Labeled DNA

1. Perform PCR assays with primers targeting bacterial 16S rRNA genes on the labeled DNA. Purify PCR products obtained using PEG-NaCl precipitation as described in Subheading 3.2, **step 6**. Perform sequencing of 16S rRNA gene PCR amplicons to acquire an overview of the phylogenetic composition of the labeled DNA and to identify putative methylotrophs (*see* **Note 18**). This may also be done with the unlabeled (light) DNA of the ^{13}C sample for comparison, to illustrate the relative enrichment of genes from methylotrophic organisms in the labeled DNA (*see* Fig. 3).
2. Screen for functional genes encoding key enzymes for methylotrophy by PCR. Depending on the investigated processes and applied substrates, different genes can be of interest. Commonly targeted are *mxaF*, encoding the large subunit of methanol dehydrogenase, *pmoA* and *mmoX*, encoding subunits of the particulate and soluble methane monooxygenase, as well as *mauA* and *gmaS*, encoding genes for alternative pathways of methylamine degradation (*see* Table 1 for PCR primers and references). Purify obtained PCR products using PEG-NaCl precipitation as described in Subheading 3.2, **step 6**.
3. Sequence functional gene PCR amplicons by 454 pyrosequencing. We commonly use the software packages mothur and USEARCH when analyzing data from a GS FLX Titanium system (*see* **Note 19**). Use Mothur to extract flowgrams from raw *.sff data files with the sffinfo() command. Discard flowgrams with less than 450 usable flows and cut remaining flowgrams to 720 flows with trim.flows(). Denoise flowgrams and translate to

nucleic acid sequences using shhh.flows(). Use the trim.seqs() command to demultiplex sequences and remove barcode and primer sequences, to discard sequences with errors in the barcode or primer region, with ambiguous bases or homopolymer runs >6 bp and to filter sequences by length, depending on the expected product size. The count.seqs() command can be used to obtain quantitative information. Use USEARCH to sort sequences by abundance (-sortbysize) and for binning of operational taxonomic units (OUT), chimera removal and singleton removal (-cluster.otus). Use a 90 % identity threshold for this step (*see* **Note 20**).

4. The obtained OTUs can be analyzed either by approaches based on the basic local alignment search tool (BLAST, [35]) or by generating phylogenetic trees after aligning with reference sequences (*see* **Note 21**). The resulting phylogenetic affiliation of the functional genes of interest can be compared to the data obtained by 16S rRNA gene sequencing to confirm the presence of putative methylotrophs.
5. For a more comprehensive analysis of the enriched DNA, shotgun metagenomic sequencing can be used. Due to the low DNA amounts typically present in the fractions, multiple displacement amplification (MDA) can be used to obtain sufficient material for sequencing. Use a commercially available MDA kit and follow the manufacturer's instructions. We commonly use the REPLI-g Mini Kit (QIAGEN) with 1–10 ng of DNA as template, incubating for 16 h overnight at 30 °C, followed by heat inactivation for 3 min at 65 °C. Perform amplification in replicates and check fidelity of the amplified DNA by DGGE (*see* Subheading 3.3, **steps 2–5**; *see* **Note 22**). Merge and purify amplified DNA (*see* Subheading 3.2, **step 6**) before shotgun metagenomic sequencing.
6. Perform shotgun metagenomic sequencing using in-house protocols or a commercially available service (also *see* **Note 19**). First analysis of the sequences can be done by using the metagenomics Rapid Annotation using Subsystem Technology (MG-RAST) analysis server (metagenomics.anl.gov, [36]). This platform is designed to call and annotate the genes in a large set of short DNA sequence reads by comparison with DNA and protein databases. This allows an in-depth phylogenetic and functional analysis of the reads, as well as screening for functional genes of interest. *See* Chapter 4 "MG-RAST" for more information. If one or a few species are specifically enriched, assembly of the reads can be used to obtain larger DNA sequence fragments or even nearly complete genomes of the investigated methylotrophs, leading to additional information about organization of gene clusters and allowing reconstruction of bacterial metabolism.

4 Notes

1. EDTA will slowly dissolve as the pH gets near 8.0. When using solid NaOH pellets, around 18–20 g are required. Use a 2 M NaOH solution for more precise adjustment of the pH.
2. DNA extraction protocols will differ based on the source material (e.g. soil, sediment, sludge, biofilm, or aquatic samples) and, consequently, no specific instructions can be given. Do test extractions from source material obtained directly from the environment to establish a suitable DNA extraction method before starting a metabolic labeling experiment.
3. The high amount of CsCl leads to an increase in volume when dissolving. Make sure not to add too much water initially. Stirring and gently warming in a water bath will help to dissolve the CsCl more quickly.
4. For measuring density, use a digital density meter or carefully weigh 1-mL aliquots in triplicate. Make sure the solution is at 20 °C before beginning this process. If the density is too low, add more CsCl. Adding 5–10 g of CsCl increases the density by ~0.01 g/mL. A density above 1.89 g/mL can still be used if adjustments are done when setting up samples for ultracentrifugation (*see* Subheading 3.1, **step 4**).
5. This is discussed in more detail elsewhere [24, 25].
6. Density measurement using an analytical balance is tedious and much less accurate than refractive index measurement, and also provides a higher chance for sample loss or DNA contamination.
7. For example, when using 4.8 mL of a stock solution with a density of 1.890 g/mL, this equates to:

   $$\text{Required volume} = (1.890\text{–}1.725\ \text{g/mL}) \times 4.8\ \text{mL} \times 1.52\ \text{mL/g}$$

   $$\text{Required volume} = 1.20\ \text{mL of GB}$$

8. Before starting with your samples, prepare a sterile 15 mL tube with 4.8 mL of CsCl stock solution and the calculated volume of GB; mix well by inversion. Measure the refractive index and adjust as described. Addition of 10 µL of GB will decrease the refractive index by ~0.0001, and addition of 40 µL of CsCl solution will increase it by ~0.0001. Keep track of the additions to correct the required volume of GB calculated in **step 2**. The prepared solution can later be used to top up ultracentrifuge tubes in case there is too little solution for a sample, or for balancing tubes.
9. Sometimes the sealing process leads to a change in tube weight. If this occurs, or if you are in doubt about the sealing on a tube, it is best to prepare a completely new ultracentrifuge tube. For recovery of the sample, cut off the top of the suspicious tube

and empty it into the 15 mL tube used to prepare that sample by holding the ultracentrifuge tube upside down and squeezing repeatedly.

10. Differences in centrifugation speed and thus centrifugal force will lead to differences in gradient formation. Higher centrifugal forces result in a steeper gradients and thus in poorer sample separation. Lower centrifugal forces result in shallower gradients. Although this can increase sample separation slightly, lower centrifugal forces also require highly extended run times. The proposed centrifugal force of 177,087 $\times g$ average is the best trade-off between sample separation and run time.
11. Previous protocols suggested the use of mineral oil for this purpose, but we found that water can be used to simplify the process of fractionation. Due to the high density difference between the CsCl solution in the tube and the water, only limited mixing will occur. For improved visualization, bromophenol blue or another dye can be added to the water.
12. Setting up the fractionation and piercing a tube can be difficult to do correctly for the first time. Prepare sealed ultracentrifuge tubes with water to test this process beforehand to ensure that it is working smoothly before processing the samples. Hold the tube with one hand to fix it securely in the clamp, otherwise it might slide down when you apply force with the needle. Put your thumb on top of the tube next to the tube stem and two other fingers under the tube. This ensures that you have the best control of the tube without having any fingers in line with the needle when piercing (potential danger of injury!). Make sure to apply controlled force to prevent the needle from entering too deep. Twisting the needle slightly can help to "drill" through the tube wall. The sharpened tip of the needle has to penetrate the tube wall completely to avoid spillage later on. This means that the first few millimeters of the needle will be inside the tube, but not more. Once through the tube wall, the needle will move much easier than before. To prevent deeper entry, you can wrap sticky tape around the needle or put a short piece of tubing over it beforehand, so the needle is blocked by the tube wall from going any deeper. If liquid from the tube is forced out once the tube is pierced, reduce the pressure from the clamp holding the tube.
13. Use the same precautions as when piercing the top of the tube. Under rare circumstances, when the needle at the top of the tube is not sealing properly, the tube can run out very suddenly at this step. Have a 15 mL tube ready to catch the CsCl solution in case this happens, so you can use the sample for a new ultracentrifugation. If a low amount of leaking occurs, a small drop of mineral oil applied to the top puncture hole can help prevent further sample loss. The following fractionation with

the pierced bottom of the tube will result in relatively large drops, and thus differences in fraction size. To create smaller drops and to allow easier fractionation, a detached 23-gauge needle can be fitted into the hole at the bottom of the tube. To do so, break a needle from the Luer slip (plastic part) by gently bending it left and right with tweezers, then carefully push it into the prepared hole.

14. The CsCl gradient is not stable over time and will mix again through diffusion. This will first be noticeable at the top and bottom of the gradient when measuring the refractive indices. Thus it is recommended to carry out the fractionation of all samples in a row as soon as the ultracentrifuge run has ended. Calculate roughly 20 min per sample (12 min of fractionation and 8 min of preparation). Fractionating eight samples in 3 h usually gives optimal results. If multiple pumps are available, fractionation in parallel is an option.
15. Although manufacturers of digital refractometers usually recommend covering the entire prism before measurement, a single drop in the centre of the prism is often sufficient for an accurate measurement. Depending on the model of refractometer used, accurate measurements can be obtained with volumes as small as 20 μL. This greatly reduces the loss of material at this step. Consistency of measurements should be checked before attempting to work with actual samples.
16. The addition of a carrier substance like LPA or glycogen is essential for the recovery of the small DNA amounts that might be present in the fractions (often <100 ng). Due to contamination issues with commercially available glycogen [37], we recommend LPA, which can be easily prepared in-house for a fraction of the cost of the commercially available product. UV treatment prior to use can ensure nucleic acid contamination will not affect downstream analysis.
17. DNA density is not only influenced by ^{13}C incorporation, but also by GC content: DNA with low GC content has a lower density than DNA with high GC content. This can lead to unlabeled genomic DNA spanning +/− 2 fractions in the described protocol. Hence it is essential to have fractions of a ^{12}C control experiment as reference for identifying labeled DNA bands; selecting fractions based only on density can be misleading. *See* [14, 15] for examples on identifying labeled DNA bands.
18. A variety of primer sets targeting different regions of bacterial 16S rRNA gene sequences have been described and can be used to acquire amplicons for sequencing. Likewise, different high-throughput sequencing methods are available for this purpose, also as commercial services that include bioinformatics analysis of the obtained sequences. If no in-house sequencing and analysis pipeline is available, use of such a service is recommended.

19. Alternative sequencing methods (Illumina dye sequencing, Ion semiconductor sequencing) can be used instead. Be aware that methods producing reads from a defined position of the gene of interest, i.e. the primer sequence, can be investigated in the way described and binned to OTUs. Methods producing random reads from the amplicons cannot be binned to OTUs with the tools described, but can be analyzed using an approach based on the basic local alignment search tool (BLAST) using Megan [38]. *See* [39] for an alternative approach employing Megan for 454 pyrosequencing data. Instead of mothur, also the software package QIIME can be used [40].
20. Higher identity thresholds can be used, but be aware that sequence diversity for functional genes can be rather different than for 16S rRNA genes when trying to assign OTUs to different phylogenetic levels. Furthermore, 454 pyrosequencing and ion semiconductor sequencing is prone to errors on homopolymer repeats, sometimes introducing up to 5 % sequencing errors. Thus, while an identity threshold of 90 % might lead to the loss of resolution on the highest taxonomic levels, it will effectively reduce artificial diversity introduced by sequencing errors.
21. Be aware that different algorithms can lead to different results, especially when only distantly related reference sequences are available. This is true for different clustering algorithms when constructing phylogenetic trees as well as for different BLAST algorithms. Also be aware that reference data for functional genes are usually much more limited than for 16S rRNA genes, and environmental samples can often yield sequences that can only be classified on lower taxonomic levels due to the lack of matching reference sequences.
22. MDA is highly prone to contamination and most available kits can introduce an amplification bias [41]. Thus, if somehow possible, it should be avoided. If very low DNA amounts are retrieved, and amplification before sequencing is essential, there are several possibilities to improve product quality. Split your sample into replicates (5–10) before amplification to reduce bias. Reduce the volume of each reaction as far as possible and reduce the incubation time to avoid contamination and unspecific amplification.

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