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Research

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Novel bacterial chloromethane degraders of a living tree fern evidenced by ¹³C-chloromethane incubations

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Abstract (181 words)

Background: Chloromethane (CH_3Cl) is the most abundant chlorinated volatile organic compound in the atmosphere and contributes to stratospheric ozone depletion. CH_3Cl has mainly natural sources such as emissions from vegetation. In particular, ferns have been recognized as strong emitters. Mitigation of CH_3Cl to the atmosphere by methylotrophic bacteria, a global sink for this compound, is likely underestimated and remains poorly characterized.

Results and Conclusions: We investigated chloromethane-degrading taxa associated with intact and living tree fern plants of the species *Cyathea australis* by stable isotope probing (SIP) with ^{13}C -labelled CH_3Cl combined with metagenomic DNA sequencing. Metagenome assembled genomes (MAGs) related to *Methylobacterium* and *Friedmanniella* were identified as being involved in the degradation of CH_3Cl in the phyllosphere, i.e., the aerial parts of the tree fern, while a MAG related to *Sorangium* was linked to CH_3Cl degradation in the fern rhizosphere. The only known metabolic pathway for CH_3Cl degradation, via a methyltransferase system including the gene *cmuA*, was not detected in metagenomes or MAGs identified by SIP. Hence, a yet uncharacterised methylotrophic *cmuA*-independent pathway likely drives CH_3Cl degradation in the investigated tree ferns.

Keywords: Chloromethane, Phyllosphere, Rhizosphere, Stable Isotope Probing (DNA-SIP), Metagenome-assembled genomes (MAGs), tree fern, one-carbon metabolism

Background (678 words)

With an average global mixing ratio of $\sim 553 \pm 5$ pptv, an atmospheric lifetime of 0.9 years and an estimated global emission rate of 4 to 5 Tg (1 Tg = 10^{12} g) per year [1, 2], chloromethane (CH_3Cl , methyl chloride) is one of the most abundant chlorinated volatile organic compounds (VOCs) in the Earth's atmosphere. Natural emissions of CH_3Cl have been reported from grasslands [3-5], plants [6-11], salt marshes [12-14], peatlands [15], ventilated and waterlogged soil [16-18] and oceans [19]. Ferns are especially potent natural emitters of CH_3Cl [6, 8, 10].

The main sink for atmospheric CH_3Cl is abiotic degradation by photochemically formed hydroxyl radicals [20], estimated at about 2.5 to 3.4 Tg per year [21]. Microbial degradation in soils is another possible significant global sink of CH_3Cl [22-25]. However, current estimates are highly uncertain, ranging from 0.1 to 1.6 Tg per year [21, 23, 26]. Methylotrophic bacteria able to use CH_3Cl as sole carbon and energy source for growth have been isolated from diverse environments, including soils [22, 24, 27], wastewater sludge [28-30], seawater [31, 32], and the phyllosphere (aerial parts of the plants) [33, 34]. The ubiquitous presence of such bacteria in soil and the phyllosphere implies that they play a more important role in mitigating the emission of CH_3Cl to the atmosphere than previously thought [14, 35].

The bacterial chloromethane utilisation (*cmu*) pathway was characterised in detail in *Methylobacterium extorquens* (previously *Methylobacterium extorquens*) strain CM4 [36, 37]. A corrinoid- and tetrahydrofolate (H_4F)-dependent methyltransferase system, encoded by genes *cmuA* and *cmuB* [38, 39], respectively, effect dehalogenation of chloromethane. Since the *cmuA* gene is strongly conserved in CH_3Cl -degrading bacteria [33], it has been used as a metabolic marker gene to study the biodiversity of CH_3Cl -degrading microorganisms [22, 24, 40-42]. Phylogenetically diverse *cmu*-dependent chloromethane degraders have been isolated and belong to the genera *Acetobacterium*, *Aminobacter*, *Hyphomicrobium*, *Leisingera*, *Pseudomonas*, *Methylobacterium*/*Methylobacterium*, and *Roseovarius* [33]. However, the *cmuA* biomarker is seldom found in metagenomes [10, 25, 35]. In addition, *cmu* genes are absent in the genomes of some sequenced CH_3Cl utilizers [35, 43] such as marine

strains (*Leisingera methylohalidivorans* MB2) [32], which points towards the existence of yet uncharacterized pathways for CH₃Cl degradation. All these microorganisms have in common that they have to adapt to several stresses in order to grow on CH₃Cl, for instance increased intracellular levels of protons and chloride, higher needs for the production of cofactors (H₄F and cobalamin-related compounds) and the regulation of their metabolism for efficient CH₃Cl utilisation [35, 37, 44, 45].

Chloromethane degradation associated with plants has been studied e.g. in *Arabidopsis thaliana* [11] and ferns such as *Osmunda regalis*, *Cyathea cooperi* and *Dryopteris filix-mas* [10]. The *cmuA* gene was found to be associated with living *Arabidopsis thaliana* plantlets [11], but CH₃Cl degradation and microbial biodiversity were not assessed. In a subsequent study, both production and consumption of CH₃Cl associated with three tropical fern species was investigated on cut-off plant material [10]. This may represent a stressful and potentially unphysiological condition, especially knowing that plants release VOCs after physical damage [46-48]. Therefore, cut-off plant material might release increased amounts of CH₃Cl compared to intact plants. Microbial 16S rRNA and *cmuA* gene diversity analysis did not allow to correlate CH₃Cl degradation with the microorganisms detected, nor could the corresponding metabolic pathways be identified [10].

In this study, we used an integrative experimental approach to identify the active CH₃Cl-degrading members of microbiota associated with rhizospheric soil and the phyllosphere of intact tree ferns known to produce CH₃Cl [10], by applying a combination of DNA stable isotope probing (DNA-SIP) and metagenomics [49, 50] under conditions closely matching the native environment.

Material and Methods

Tree ferns, gas-tight plant-growth chambers and labelling of transplanted plants

Tree ferns (*Cyathea australis*) with a shoot height of about 25 cm were obtained from a commercial supplier (A L'ombre Des Figuiers, Combrit, France). Plants were grown in Müncheberg Germany, and adapted to the local climate for three months before starting the

DNA-SIP experiment on August 17th 2017. Incubations were carried out in gas-tight plant-growth acrylic-glass chambers (height of 120 cm, volume of ~85 L) specifically manufactured for the experiments (Reli Kunststoffe, Erkner, Germany). Chambers were fitted with ports capped with air-tight butyl rubber stoppers (Merck Millipore Corporation, Darmstadt, Germany) for injection of CH₃Cl (Supplementary Figure 1A). In addition, ventilators were installed inside the chambers to ensure an even distribution of gas throughout the chamber. Gas-tightness was tested by injecting CO₂ into each chamber and measuring the concentration over time with CO₂ loggers (data not shown). Since the aim of the experiment was to label CH₃Cl-degrading microorganisms associated with fern plants, the side walls of the chambers were covered with aluminium foil (Supplementary Figure 1B) to reduce photosynthesis and thus microbial CO₂ consumption during the labelling period. Nonetheless, some light still entered the chambers through the transparent lid.

At the start of the experiment, a tree fern plant was placed in each of the six plant growth chambers (Supplementary Figure 1B). The tree fern plants were incubated with either ¹³C-labelled CH₃Cl (Campro Scientific GmbH, Berlin, Germany; named hereafter [¹³C]-CH₃Cl) or with CH₃Cl at natural abundance (99%, Linde GmbH, Pullach, Germany; named hereafter [¹²C]-CH₃Cl). Each labelling period lasted for about 6 hours during daylight, with plant growth chambers opened and ventilated overnight until the next round of labelling took place at the next day. At the start of the labelling experiment (August 17th), 20 mL CH₃Cl ([¹³C]-CH₃Cl or [¹²C]-CH₃Cl) was added to the chambers, so that the initial mixing ratio in the chamber was ~200 ppm. Chambers were closed and ventilation was turned on. For the following 18 days, 10 mL of CH₃Cl ([¹³C]-CH₃Cl or [¹²C]-CH₃Cl) was added every day, providing an initial mixing ratio in the chambers of ~100 ppm. Gas samples were taken at the start of the incubation period, after 2 and 4 hours and at the end of the incubation period with gas-tight syringes and stored in 3 mL, pre-evacuated Exetainers (Labco Limited, England) for further analysis by gas chromatography combined with single quadrupole mass spectrometry (GC MS). ¹³C-labelling [¹³C]-CH₃Cl and [¹²C]-CH₃Cl was carried out for 19 days. Soil humidity and salinity in the fern soil was monitored throughout the experiment (data not shown).

Gas chromatography

Chloromethane was measured in headspace gas by injecting 100 µL of a headspace gas sample into a ISQ™ Quadrupole GC MS System using a TRACE™ Ultra gas chromatograph (Thermo Fisher Scientific, USA) fitted with a 60 m, 0.32 mm GS-GasPro capillary column (Agilent Technologies, California, USA) with helium as the carrier gas (constant column flow rate, 1.5 mL min⁻¹). Headspace gas samples (100 µL) were injected into the column with a temperature ramp from 40°C to 200°C (15°C increase min⁻¹). High-throughput measurements were carried out with a MultiPurpose autosampler MPS (Gerstel Inc., USA). Chromatograms were analysed with Openchrome® (Lablicate GmbH, Germany). CH₃Cl concentrations were calculated by regression analysis based on a five-point calibration curve.

Analysis of carbon mass fraction (C %) and differential stable carbon isotope ratio (δ¹³C value)

From each pot, an aliquot of the plant biomass was sampled at the start of the experiment, after 4 days and at the end of the incubation period (after 19 days). Samples were dried for 24h at 60°C and finely ground using a vibrating disc mill (RS200, Retsch, Germany). Stable isotope ratios (¹³C/¹²C) were determined using an Elemental Analyzer (EA) Flash 2000 HT (Thermo Fisher Scientific, Bremen, Germany), coupled with a Delta V isotope ratio mass spectrometer (IRMS) via a ConFlo IV interface (Thermo Fisher Scientific, Bremen, Germany). Stable carbon isotope ratios (δ¹³C) are expressed in permil (‰) relative to the international standard, as defined by the equation:

$$\delta^{13}\text{C} = \frac{R_{\text{Sample}} - R_{\text{Reference}}}{R_{\text{Sample}}}$$

where R_{Sample} is the isotopic ratio (¹³C/¹²C) of the sample and $R_{\text{Reference}}$ is the known isotopic ratio of the standard. δ¹³C values were normalized to the international scale Vienna Pee Dee Belemnite (VPDB) by analyses of the international standards USGS40 and USGS41 (L-glutamic acid) within the sequence [51]. Precision, defined as the standard deviation (±1σ) of the laboratory control standard along the run was better than ±0.2‰ for C.

For calculation of the element mass fraction C (percentage, %) of plant biomass, different amounts of the laboratory standard (apple leaves, $\delta^{13}\text{C} = -27.11\text{‰}$, C = 48.12 %) were analysed and the linear regression between peak area and sample weight was used to calculate the C (%). Precision, defined as the standard deviation ($\pm 1\sigma$) of the laboratory control standard along the runs was better than $\pm 0.5\%$ for C.

Sample collection, DNA extraction and processing of DNA-SIP samples

After labelling, phyllosphere and rhizosphere soil samples of *Cyathea australis* plants were collected as follows. For the phyllosphere, leaf wash (LW) and leaf (L) samples were collected. Leaf wash samples were prepared as previously described [52], using 5 g of leaves and collecting the leaf surface communities on a 0.22 μm Durapore® membrane filter (Merck Millipore Corporation, Darmstadt, Germany), and therefore comprised mainly epiphytes. Leaf samples were cut off from the plants without washing using sterile razor blades, and hence included epi- and endophytes. For the rhizosphere, ~20 g of soil (S) samples were collected near the roots (ectorrhizosphere).

DNA extraction was carried out using the FastDNA™ Spin Kit for Soil (MP Bio Science Ltd., Derby, UK) following the manufacturer's instructions. ^{13}C -labelled heavy DNA was separated from unlabelled light ^{12}C -DNA using cesium chloride density gradient ultracentrifugation, as described previously [53]. Density gradient formation across 12 fractions (250 μL each) was confirmed by measuring refractive indexes using a digital refractometer (Reichert AR2000). To identify ^{13}C -DNA and ^{12}C -DNA 'heavy' and 'light' fractions, respectively, and hence labelled microorganisms, DNAs of all SIP fractions were first PCR-amplified. Primers 799F labelled at the 5'-end with 6-carboxyfluorescein (6-FAM) and 1193r were used to amplify 16S rRNA gene DNA fragments of 500 bp [54]. For profiling of the bacterial communities present in the different SIP fractions, terminal restriction fragment length polymorphism (T-RFLP) was used as described [55], with normalization of T-RFLP profiles [56].

DNA barcoding for taxonomical identification and definition of the ^{13}C -labelled OTUs

Bacterial composition in [^{13}C]-CH₃Cl and [^{12}C]-CH₃Cl 'heavy' and 'light' fractions was determined by amplicon sequencing using the primer set 5'-AACMGGATTAGATACCCCKG-3', 5'-ACGCATCCCCACCTTCCTC-3' targeting the V5 and V7 hypervariable regions of the bacterial 16S rRNA gene. Amplicon sequencing was performed on an Illumina Miseq platform by GenoScreen (Lille, France). Amplicon reads were analysed using the QIIME pipeline [57] and singletons and chimeras were removed using USEARCH v7 [58] and UCHIME [59]. Labelled OTUs were defined according to four criteria (adapted from [41]): 1) the relative abundance of the OTU in the 'heavy' DNA fraction of the ^{13}C -labelled microcosm was higher than in the 'heavy' DNA fraction of the corresponding ^{12}C -labelled microcosm; (2) the relative abundance of the OTU was higher in the 'heavy' DNA fraction than in the 'light' fraction of the ^{13}C -labelled microcosm; (3) the relative abundance of the OTU in the 'heavy' DNA fraction of the ^{13}C -labelled microcosm was $\geq 0.015\%$, and (4) the relative abundance difference of the OTU between 'heavy' and 'light' DNA fractions was $\geq 0.005\%$ and higher in the ^{13}C -labelled treatment than in the corresponding unlabelled control treatment.

Metagenomics and bioinformatics

Metagenomic sequencing was carried out using 'heavy' DNA fractions of [^{13}C]-CH₃Cl-amended fern samples combined from three biological replicates. The ^{13}C -labelled heavy fraction of leaf (L), leaf wash (LW) and soil (S) samples were sequenced on a HighSeq platform and data was filtered and quality trimmed by GenoScreen (Lille, France). Processed reads were assembled with MEGAHIT v.1.2.9 [60] with default options and additional parameter "*--presets meta-large*", that sets a *k-mer* list from 27 to 127 in steps of 10. An additional co-assembly of leaf and leaf wash metagenomes was performed with the same parameters, to improve recovery of low abundant species.

Binning was carried out using a combination of CONCOCT v1.1.0 [61], MetaBAT2 v2.12.1 [62] and MaxBin2 [63] under default parameters. For binning with CONCOCT and MetaBAT2 reads were first mapped to the assemblies using bowtie2 v2.3.5.1 [64] with the parameters "*-sensitive-local --threads 80 --seed 100 --maxins 1000*". Mapping results were used to

generate depth files. For binning with MaxBin2 the binning module of MetaWRAP v1.2.1 [65] was used. The MetaWRAP *bin_refinement* module was used to consolidate binning results from MetaBAT2, MaxBin2 and CONCOCT into a final set of bins. Quality of the bins was estimated using CheckM v1.1.2 [66]. Bins with a reported completeness >70% and contamination <10% were selected as MAGs (Metagenome-Assembled Genomes) for further analysis (Supplementary Table S1). MAGs were annotated with RAST-tk [67] and the closest taxon was used for initial taxonomic classification. Identified proteins for MAGs were downloaded as 'faa-files' from RAST and used to place MAGs in a tree with 2850 references using the CVTree3 Web Server [68]. The deduced tree was visualized at K=6 using the Interactive Tree of Life v 5.5.1 [69], and used to select reference genomes for genome comparisons. The closest references around each MAG cluster were included in a pruned tree.

Closely related reference genomes were chosen for selected MAGs. PhyloPhlAn 3.0 [70] was run with "*--diversity low*" to identify common marker genes and construct a concatenated multiple sequence alignment (MSA) between MAGs and references. Maximum-likelihood trees were constructed with MEGAX [71] from MSAs using the JTT+G+I model with 500 bootstrap replications. ANI values between MAGs and reference genomes were calculated with fastANI [72] v1.3 with "*--fragLen 1500*". AAI values were calculated with the CompareM v1.1.2 AAI workflow (*aai_wf*) (<https://github.com/dparks1134/CompareM>), where homology was defined as > 30% identity and > 70% alignment length. MAGs and metagenomes were screened for the presence of the chloromethane utilisation gene (*cmuA*) using BLAST [73], and prokka [74] under default settings, respectively. ShortBRED (version 0.9.3) [75] was used to identify *cmuA* in unassembled metagenomic reads. MAGs were submitted to the MicroScope platform [76, 77] for annotation and analysis of genomic content and features using the Magnifying Genome (MaGe) platform Web interface (<https://mage.genoscope.cns.fr/microscope/mage/>). In addition, BLAST [73] and RAST [78] were used to further compare MAG genetic content and assess potentials for CH₃Cl utilization by screening for functional marker genes involved in CH₃Cl utilization, one-carbon

metabolism, central metabolism, co-factor synthesis, transport systems, and stress response. Enzymes that use or produce halides were detected using the BRENDA database available at www.brenda-enzymes.org, and adapted from [79]. This more in-depth MAG genetic content analysis was performed for six MAGs from the phyllosphere (LEAF and LEASFWASH) and rhizosphere (SOIL) named hereafter: 'Fern CH₃Cl SIP LEAF bin s3', 'Fern CH₃Cl SIP LEAF bin co1', 'Fern CH₃Cl SIP LEAF bin co3', 'Fern CH₃Cl SIP LEAFWASH bin co7', 'Fern CH₃Cl SIP SOIL bin s1', and 'Fern CH₃Cl SIP SOIL bin s7'.

Comparative genome analyses

Genome comparisons within the genus *Methylobacterium* and the closely related clade *Methylobacterium* to determine pan, core and dispensable genes and singletons (unique genes) was carried out using EDGAR v2.0 [80] as described previously [81]. The MAG Fern CH₃Cl SIP LEAF bin s3 was used as a reference and strains of *Methylobacterium* (including *Methylobacterium*) used for this analysis are listed in Supplementary Table S2.

Accession numbers for datasets

Read data of the tree fern metagenomes have been submitted to the National Center for Biotechnology Information (NCBI) under the BioProject number PRJNA659413. The metagenome assembled genomes for 'Fern CH₃Cl SIP LEAF bin s3', 'Fern CH₃Cl SIP LEAF bin co1', 'Fern CH₃Cl SIP LEAF bin co3', 'Fern CH₃Cl SIP LEAFWASH bin co7', 'Fern CH₃Cl SIP SOIL bin s1', and 'Fern CH₃Cl SIP SOIL bin s7', can be found in MaGe (<https://mage.genoscope.cns.fr/microscope/home/index.php>) and under the same BioProject with BioSample Accession numbers SAMN17394138, SAMN17394139, SAMN17394140, SAMN17394141, SAMN17394142, SAMN17394143, respectively.

Results and discussion

The tree fern Cyathea australis and its associated microbiome is capable of CH₃Cl degradation

As previously shown, cut-off leaves from the tree fern *Cyathea australis* are capable of CH₃Cl degradation [10]. However, it has not been investigated if intact plants behave in the same way and degrade CH₃Cl. In addition, the active members of the tree fern-associated microbiota possibly involved in CH₃Cl degradation has not been studied. Therefore, tree ferns were incubated with either [¹³C]-CH₃Cl or [¹²C]-CH₃Cl and CH₃Cl concentrations were monitored over a time period of 19 days. On the first day of incubation, 200 ppm [¹³C]-CH₃Cl or [¹²C]-CH₃Cl was added to the incubation chambers (Supplementary Figure 2). Rapid consumption was evident, and most likely due to adsorption to the soil-plant-interface. However, in the following 18 days, steady degradation of CH₃Cl was observed following daily CH₃Cl addition, especially from day 12 onwards, with an average CH₃Cl consumption of 7-15 ppm d⁻¹ (Supplementary Figure 2). In order to investigate if the ¹³C was incorporated by the plant or by microorganisms living on the surface of plant leaves (epiphytes), δ¹³C values of bulk leaves were determined using EA/IRMS. Stable carbon isotope values (Supplementary Figure 3) showed an increase in the [¹³C]-CH₃Cl incubated samples from the start of the experiment (¹³C T₀, about -29 ‰) to the end of the experiment (T_{end}, about -7.5 ‰) in the incubated ferns 1 and 3, demonstrating an incorporation of ¹³C. Washed leaves (¹³C T_{end} washed) showed much lower deviations of δ¹³C values (Supplementary Figure 3; fern 1 and 3, about -24 ‰), indicating incorporation of ¹³C mainly by the microorganisms living on leaf surfaces. Therefore, we have shown here that intact living tree fern plants of the species *Cyathea australis* and their associated microbiota are capable of CH₃Cl degradation and that the microorganisms most likely play a more important role in the degradation of this chlorinated volatile organic compound than previously anticipated.

Methylobacterium is an abundant genus with CH₃Cl-degrading representatives in the fern phyllosphere, and active chloromethane-utilizing bacteria differ in the fern phyllosphere and rhizosphere

In order to identify ¹³C-labelled microorganisms and hence, the microorganisms most likely responsible for CH₃Cl degradation in the fern phyllosphere, 'heavy' and 'light' DNA fractions

were identified using T-RFLP (showing an enrichment of certain TRFs in the ^{13}C -‘heavy’ fraction compared to the ^{12}C -‘heavy’ and ^{13}C -‘light’ fractions; data not shown), and the corresponding fractions were used for 16S rRNA amplicon sequencing.

Methylobacterium was identified as the most abundant ^{13}C -labelled genus in the phyllosphere (leaf) of *Cyathea australis* (Figure 1). It is well-established that *Methylobacterium* are abundant in the phyllosphere of a variety of plants [83] including *Arabidopsis thaliana* [11, 84-86], soybean [86-88], clover [86], cotton [87], maize [87], sunflower [87] and rice [89], and that representatives of the genus *Methylobacterium* can utilize CH_3Cl via the chloromethane utilization pathway (*cmu*) [33]. Whereas *Methylobacterium* has been identified previously in the phyllosphere of three different CH_3Cl -producing or CH_3Cl -degrading ferns, *Osmunda regalis*, *Cyathea cooperi* and *Dryopteris filix-mas* [10], a correlation between in planta CH_3Cl degradation and the microorganisms detected could not be demonstrated in these previous studies.

In the fern phyllosphere (leaf and leaf wash samples), other ^{13}C -labelled microorganisms than *Methylobacterium*-related, included members of families *Pseudomonadaceae* (leaf), *Propionibacteriaceae*, and *Solirubrobacteraceae* (leaf wash), among others (Figure 1). *Pseudomonadaceae* were detected in a CH_3Cl -degrading mixed culture [33], but their role in CH_3Cl degradation was not assessed. Among those, members of the family *Pseudomonadaceae* are well known colonizers of the phyllosphere of a variety of plants, such as soybean, clover, rice, *A. thaliana* [90, 91], grapevine [92], maize [93] and lettuce [94], to name only a few. *Pseudomonadaceae* have been associated with ferns involved in CH_3Cl production and consumption [10], and some isolates of the genus *Pseudomonas* capable of CH_3Cl degradation have been reported [10, 30, 33]. In contrast, representatives of *Propionibacteriaceae* and *Solirubrobacteraceae* capable of CH_3Cl degradation have not been described so far. *Propionibacteriaceae* closely related *Friedmaniella* members have been found in the phyllosphere of magnolia trees [95], hemlock plants [96], in grasses such as *Arrhenatherum elatius* [97], in *Baccharis dracunculifolia* [98] and in hardwood forest trees such as maple, oak and lime [99]. Bacteria of the family *Solirubrobacteraceae* are strictly aerobic

and chemoorganotrophic [100], and have been identified in the phyllosphere of *Baccharis dracunculifolia* [98], in grasses such as *Arrhenatherum elatius* [97], and in olive trees [101]. Compared to the phyllosphere, different taxa were enriched in the ¹³C-‘heavy’ DNA of rhizosphere samples (Figure 1), e.g. from *Xanthobacteraceae* and *Myxococcales*, but a potential association of these families with degradation of CH₃Cl has not yet been reported. *Xanthobacteraceae* were enriched in a CH₃Cl-incubated forest soil in a previous study [25], but also detected in the phyllosphere of grassland plants [97], tomatoes [102], and hay [103]. Worthy of note in the context of chloromethane utilisation, *Xanthobacter autotrophicus*, a member of family *Xanthobacteraceae*, is a methylotrophic organism capable of growing with methanol [104, 105]. *Myxococcales* were detected in e.g. the phyllosphere of magnolia trees [95], apple trees [106] and rocket [107], and also present in the core microbiome of the phyllosphere of five different trees species in a temperate forest in Quebec [108]. Thus, the living fern tree is host to a variety of potential CH₃Cl-utilisers, of which most differ from previously characterized representatives.

Tree fern MAGs from [¹³C]-CH₃Cl DNA are closely related to Methylobacterium, Friedmanniella and Sorangium

Metagenomics of [¹³C]-CH₃Cl labelled fern phyllosphere and rhizosphere samples was carried out to complement and expand the 16S rRNA amplicon sequencing analysis. Of the 34 MAGs, three carried 16S rRNA-encoding genes (Supplemental Table S1). To further elucidate the phylogeny of the [¹³C]-CH₃Cl labelled microorganisms, phylogenetic trees were calculated using the MAGs ‘Fern CH₃Cl SIP LEAF bin co1’, ‘Fern CH₃Cl SIP LEAF bin s3’, ‘Fern CH₃Cl SIP LEAF bin co3’, ‘Fern CH₃Cl SIP LEAFWASH bin co7’, ‘Fern CH₃Cl SIP SOIL bin s1’ and ‘Fern CH₃Cl SIP SOIL bin s7’ (Figure 2). MAG’s ‘Fern CH₃Cl SIP LEAF bin co1’ and ‘Fern CH₃Cl SIP LEAF bin s3’ clustered within the genus *Methylobacterium* (Figure 2A). The MAG ‘Fern CH₃Cl SIP LEAF bin s3’ was more closely related to *Methylobacterium phyllosphaerae* CBMB27, *M. phyllostachyos* BL47, *M. pseudosasicola* BL36 and *M. oryzae* CBMB20, which were all isolated from phyllospheric samples (Supplementary Table S2). Bins

'Fern CH₃Cl SIP LEAF bin co3' and 'Fern CH₃Cl SIP LEAFWASH bin co7' clustered more closely to the genus *Friedmaniella* of the family *Propionibacteriaceae* (Figure 2B). Bins 'Fern CH₃Cl SIP SOIL bin s1' and 'Fern CH₃Cl SIP SOIL bin s7' showed closest but more distant similarity with *Sorangium* (Figure 2C), possibly representing a new genus within the order *Myxococcales*. Taken together, the phylogeny based on MAGs supported the findings on ¹³C-labelled OTUs based on the results from 16S rRNA amplicon sequencing.

cmuA genes are not present in metagenomes and MAGs

The *cmu* pathway is the only characterized pathway for utilisation of CH₃Cl [35]. Of the *cmuA*- and *cmuB*-encoded methyltransferases of chloromethane dehalogenase, the *cmuA* gene is the most conserved and has been frequently used as the *cmu* pathway gene marker [109]. The MAGs and metagenomes were screened for the presence of *cmuA*. On the basis of BLAST searches and prokka annotations, no *cmuA* homologs could be identified in metagenome-assembled genomes. Using shortBRED, unassembled short metagenomic reads were screened for the presence of *cmuA*. Unlike reference genes for methanol oxidation (*xoxF* and *mxoF*) which were detected in leaf, leafwash and soil samples (data not shown), the *cmuA* gene was not detectable in leafwash and leaf samples. In soil samples, only seldom and very short sequences (between 8 and 15 amino acids) matched to a small fragment of CmuA (data not shown) when using shortBRED. This lack of evidence for the presence of *cmuA* suggests that CH₃Cl degradation in tree fern samples occur by other routes.

CH₃Cl in tree ferns is degraded by yet unidentified chloromethane utilisation pathways

The genome sequences retrieved from ¹³C-labelled MAGs offered an opportunity to investigate the metabolic traits of these bacteria. Metabolic reconstruction was performed with the two MAGs related to *Methylobacterium* ('Fern CH₃Cl SIP LEAF bin co1' and 'Fern CH₃Cl SIP LEAF bin s3'), one of the bins related to *Friedmaniella* ('Fern CH₃Cl SIP LEAF bin co3') and one of the bins related to *Sorangium* ('Fern CH₃Cl SIP SOIL bin s1').

Beyond the already noted lack of *cmuA*, analysis of these MAGs also revealed the absence of the other essential genes (*cmuB* [36] and *metF2* [45, 110]) of the *cmu* pathway (Figure 3), confirming that CH₃Cl-degrading bacteria on tree ferns may use other pathways for CH₃Cl utilization. In contrast, genes *folD* encoding the bifunctional enzyme methylene-H₄F dehydrogenase/methenyl-H₄F-cyclohydrolase and *purU* encoding the formyl-H₄F deformylase which allow downstream processing of one-carbon units derived from CH₃Cl [36] were detected in three and two of the four MAGs, respectively (Figure 3). Similarly, other genes associated with one-carbon metabolism including *mtdA* for methylene-H₄F dehydrogenase, *fch* for methenyl-H₄F cyclohydrolase, and *ftfL* for formate-H₄F ligase were also detected in *Methylobacterium*-related MAGs, as expected [111-113] (Figure 3, Supplementary Table S3). In many methylotrophs, methylene-H₄F is the entry point for carbon assimilation through the ethylmalonyl-CoA pathway (EMCP) cycle, and most genes were detected in two out of four MAGs, and/or the glycine cleavage system pathway (GCP) with genes detected in the two MAGs lacking the EMCP cycle (Figure 3, Supplementary Table S3). Furthermore, the last step in one-carbon compound reduction/oxidation is the conversion of formate to CO₂ that involves the *fdh*-encoded formate dehydrogenase found in the two MAGs related to *Methylobacterium* and the MAG related to *Friedmaniella*. Thus, analysis of the MAGs obtained from heavy DNA confirmed that they possess the genetic potential for one-carbon compound reduction/oxidation, and also suggest that H₄F is a one-carbon-group carrier in all four MAGs (Figure 3, Supplementary Table S3). While these data clearly suggest that methylene-H₄F is also the entry point to central metabolism in tree fern MAGs from CH₃Cl degradation, how this is achieved by the corresponding strains remains unknown. Moreover, it is puzzling that MAGs related to species not previously described as methylotrophs (e.g. *Friedmaniella* and *Sorangium*) and which are lacking reference genes for primary oxidation of one-carbon compounds (Supplemental Table S3) were [¹³C]-chloromethane labelled. One hypothesis is that genes encoding methylotrophic genes were excluded from the assembly, for instance if plasmid-borne, associated to mobile elements or if present on genomic islands. Another

possibility is that these MAGs correspond to microorganisms that harbour so far uncharacterized genes for methyltrophic growth.

The MAG more closely related to *Methylobacterium* ('Fern CH₃Cl SIP LEAF bin s3') harbours in addition the genes for the one-carbon compound oxidation pathway via tetrahydromethanopterin (H₄MPT). One explanation for this phenomenon might be the incompleteness of MAGs. The last step of the one-carbon compound oxidation pathway, the oxidation of formate to carbon dioxide, is mediated by the formate dehydrogenase (*fdh*), which was detected in three out of the four MAGs. The gene encoding for the formaldehyde-activating enzyme (*fae*), which catalyses the reduction of formaldehyde with H₄MPT was only detected in the MAG more closely related to *Methylobacterium* ('Fern CH₃Cl SIP LEAF bin s3') suggesting that the other three MAGs lack the H₄MPT pathway for formaldehyde oxidation (Figure 3, Supplementary Table S3).

Gene content for halide metabolism in MAGs

Halide ions such as Cl⁻, Br⁻, I⁻ are substrates, products or both for dehalogenases. Dehalogenases can hydrolyse a broad range of haloalkanes to e.g. the corresponding alcohols, and as a rule this will be accompanied by the release of protons and halide ions. Searches using the BRENDA database led to the detection of putative genes encoding halogenases (3) and dehalogenases (40) in MAGs obtained from ¹³C-labelled DNA (Supplemental Table S4), which may potentially be associated with dehalogenation of chloromethane. Further experiments are needed to investigate this point.

Comparative genomics of Methylobacterium-related MAGs

Since *Methylobacterium* represents the best-investigated bacterial genus for utilisation of one-carbon compounds, including CH₃Cl, an in depth-analysis of the two MAGs most closely related to *Methylobacterium* was performed. Thus, Fern CH₃Cl SIP LEAF bin c1 and Fern CH₃Cl SIP LEAF bin s3 were compared with representative complete genomes of other members of the genus *Methylobacterium* including *Methylobacterium* (Figure 4, Supplementary

Table S2) to identify genes present in all strains (core genes), two or more strains (accessory or dispensable genes), and only in one strain (singleton genes). According to pan-genome analysis of 15 *Methylobacterium* genomes (including three *Methylobacterium* strains and two MAGs from this study), a total of 25,534 genes were identified, of which 52.4% were singletons (13,375 CDS), and only 2.5% were shared by all 15 *Methylobacterium* genomes (core genome, 626 CDS; Figure 4a). An UpSet plot [114] shows the number of CDS in the core genome, the singletons but also the number of CDS shared by the different *Methylobacterium* genomes (including *Methylobacterium*, Figure 4b). These analyses confirmed the phylogeny of the phylogenetic tree (Figure 2A), i.e. it revealed a high similarity between *Methylobacterium phyllosphaerae* and *Methylobacterium oryzae* (392 uniquely shared CDS) and between *Methylobacterium populi* and *Methylobacterium extorquens* (233 uniquely shared CDS). Genes encoding for enzymes that use or produce halides (Supplementary Table S4) and are present in the two MAGs obtained in this work do not belong to the core genome shared by the 15 *Methylobacterium* strains (including *Methylobacterium*).

Conclusions

Chloromethane is massively produced by some tree ferns [8, 10], and these plants have therefore been suggested to be important emitters of this compound to the atmosphere [6]. However, microorganisms living in close association with these plants might substantially mitigate the emission of CH₃Cl into the atmosphere. In this context, recent isotopic mass balances of atmospheric CH₃Cl including isotopic analysis of carbon [115], hydrogen [116] and chlorine [117] point to large unidentified terrestrial sinks and sources of CH₃Cl. The present study reveals the complexity of CH₃Cl associated processes occurring in tree ferns. In addition, the observed lag in the onset of CH₃Cl degradation associated with tree ferns could also point to microbial adaptation. As such, we demonstrated that ¹³C-labelled CH₃Cl is incorporated into DNA of microorganisms of the leaf surface of tree ferns, and not to plant biomass, since insignificant ¹³C incorporation was found in washed leaves. Moreover, we identified key microorganisms involved in CH₃Cl degradation in the phyllosphere and

rhizosphere. These were closely related to *Methylobacterium*, *Friedmanniella* and *Sorangium*, all known to be capable of inhabiting the phyllosphere. Of these genera however, only *Methylobacterium* (including *Methylobacterium*, e.g. *Methylobacterium extorquens* strain CM4) strains were previously described to be capable of CH₃Cl degradation. Moreover, the canonical *cmu*-dependent CH₃Cl degradation pathway could not be detected in the heavy DNA fraction obtained in ¹³C-CH₃Cl incubations of tree ferns. These findings suggest that biodegradation pathways other than the *cmu* pathway were predominantly involved in degradation of CH₃Cl in the investigated tree fern species *Cyathea australis*. Our study thus demonstrates that CH₃Cl emissions from living plants is modified by microbial consumption by other bacteria than from the well-known genus *Methylobacterium*. For future studies, incubations under lower, environmentally observed concentrations (~600 pptv), coupled with targeted proteomics and/or transcriptomics approaches, may help to identify as yet unidentified CH₃Cl degradation pathways. Finally, the application of triple element isotope analysis [117] might provide clues on the global relevance of vegetation and their associated microbes in mitigating emissions of CH₃Cl to the atmosphere.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Read data of the tree fern metagenomes have been submitted to the National Center for Biotechnology Information (NCBI) under the BioProject number PRJNA659413. The

metagenome assembled genomes for 'Fern CH₃Cl SIP LEAF bin s3', 'Fern CH₃Cl SIP LEAF bin co1', 'Fern CH₃Cl SIP LEAF bin co3', 'Fern CH₃Cl SIP LEAFWASH bin co7', 'Fern CH₃Cl SIP SOIL bin s1', and 'Fern CH₃Cl SIP SOIL bin s7', can be found under the same BioProject with BioSample Accession numbers SAMN17394138, SAMN17394139, SAMN17394140, SAMN17394141, SAMN17394142, SAMN17394143, respectively.

Competing interests

Not applicable

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Authors' contributions

SK and FB conceived the research. EK, SW, FB analyzed the data. EK carried out the experimental research. EK, FB, CBT, SK, SV, FK discussed experiments and interpreted the results. The manuscript was written under the lead of EK and FB with contributions of all authors.

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Supplementary Materials:

Supplementary Figure S1. Plant growth chamber setup.

Supplementary Figure S2. Monitoring of the SIP-incubation of ferns.

Supplementary Figure S3. Analysis of carbon mass fraction (C %) and differential stable carbon isotope ratio ($\delta^{13}\text{C}$ value) of fern leaves and washed leaf samples (T_{end} washed) via EA/IRMS.

Supplementary Table S1. List of metagenome-assembled genomes (MAGs).

Supplementary Table S2. List of *Methylobacterium* (including *Methylobacterium*) genomes used for pan-genomics.

Supplementary Table S3. Methylophilic pathway genes in labelled MAGs.

Supplementary Table S4. Enzymes potentially using or producing halides in labelled ^{13}C -MAGs.

Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

1. Keppler F, Harper D, Röckmann T, Moore R, Hamilton J: **New insight into the atmospheric chloromethane budget gained using gained using.** *Atmospheric Chemistry and Physics* 2005, **5**.
2. Xiao X, Prinn RG, Fraser PJ, Simmonds PG, Weiss RF, O'Doherty S, Miller BR, Salameh PK, Harth CM, Krummel PB, et al: **Optimal estimation of the surface fluxes of methyl chloride using a 3-D global chemical transport model.** *Atmospheric Chemistry and Physics* 2010, **10**.
3. Rhew RC: **Sources and sinks of methyl bromide and methyl chloride in the tallgrass prairie: applying a stable isotope tracer technique over highly variable gross fluxes.** *Journal of Geophysical Research: Biogeosciences* 2011, **116**.
4. Rhew RC, Abel T: **Measuring simultaneous production and consumption fluxes of methyl chloride and methyl bromide in annual temperate grasslands.** *Environmental science & technology* 2007, **41**:7837-7843.
5. Teh YA, Rhew RC, Atwood A, Abel T: **Water, temperature, and vegetation regulation of methyl chloride and methyl bromide fluxes from a shortgrass steppe ecosystem.** *Global change biology* 2008, **14**:77-91.
6. Yokouchi Y, Ikeda M, Inuzuka Y, Yukawa T: **Strong emission of methyl chloride from tropical plants.** *Nature* 2002, **416**:163-165.
7. Hamilton JT, McRoberts WC, Keppler F, Kalin RM, Harper DB: **Chloride methylation by plant pectin: an efficient environmentally significant process.** *Science* 2003, **301**:206-209.
8. Yokouchi Y, Takenaka A, Miyazaki Y, Kawamura K, Hiura T: **Emission of methyl chloride from a fern growing in subtropical, temperate, and cool temperate climate zones.** *Journal of Geophysical Research: Biogeosciences* 2015, **120**:1142-1149.
9. Blei E, Hardacre CJ, Mills GP, Heal KV, Heal MR: **Identification and quantification of methyl halide sources in a lowland tropical rainforest.** *Atmospheric Environment* 2010, **44**:1005-1010.
10. Jaeger N, Besaury L, Röhlings AN, Koch F, Delort A-M, Gasc C, Greule M, Kolb S, Nadalig T, Peyret P, et al: **Chloromethane formation and degradation in the fern phyllosphere.** *Science of the Total Environment* 2018, **634**:1278-1287.
11. Ul-Haque MF, Besaury L, Nadalig T, Bringel F, Mutterer J, Schaller H, Vuilleumier S: **Correlated production and consumption of chloromethane in the *Arabidopsis thaliana* phyllosphere.** *Scientific reports* 2017, **7**:1-10.
12. Rhew RC, Miller BR, Weiss RF: **Natural methyl bromide and methyl chloride emissions from coastal salt marshes.** *Nature* 2000, **403**:292-295.
13. Valtanen A, Solloch S, Hartikainen H, Michaelis W: **Emissions of volatile halogenated compounds from a meadow in a coastal area of the Baltic Sea.** *Boreal Environment Research* 2009, **14**:915-931.
14. Keppler F, Röhlings AN, Jaeger N, Schroll M, Hartmann SC, Greule M: **Sources and sinks of chloromethane in a salt marsh ecosystem: constraints from concentration and stable isotope measurements of laboratory incubation experiments.** *Environmental Science: Processes & Impacts* 2020, **22**:627-641.
15. Dimmer CH, Simmonds PG, Nickless G, Bassford MR: **Biogenic fluxes of halomethanes from Irish peatland ecosystems.** *Atmospheric Environment* 2001, **35**:321-330.

16. Keppler F, Eiden R, Niedan V, Pracht J, Schöler H: **Halocarbons produced by natural oxidation processes during degradation of organic matter.** *Nature* 2000, **403**:298-301.
17. Keppler F, Eiden R, Niedan V, Pracht J, Schöler H: **Correction: Halocarbons produced by natural oxidation processes during degradation of organic matter.** *Nature* 2001, **409**:382-382.
18. Redeker K, Wang N-Y, Low J, McMillan A, Tyler S, Cicerone R: **Emissions of methyl halides and methane from rice paddies.** *Science* 2000, **290**:966-969.
19. Moore RM, Groszko W, Niven SJ: **Ocean atmosphere exchange of methyl chloride: Results from NW Atlantic and Pacific Ocean studies.** *Journal of Geophysical Research: Oceans* 1996, **101**:28529-28538.
20. Khalil M, Rasmussen R: **Atmospheric methyl chloride.** *Atmospheric Environment* 1999, **33**:1305-1321.
21. Carpenter LJ, Reimann S, Burkholder JB, Clerbaux C, Hall BD, Hossaini R, Laube JC, Yvon-Lewis SA, Engel A, Montzka S: **Update on ozone-depleting substances (ODSs) and other gases of interest to the Montreal protocol.** *Scientific Assessment of Ozone Depletion* 2014:1.1-1.101.
22. McAnulla C, McDonald IR, Murrell JC: **Methyl chloride utilising bacteria are ubiquitous in the natural environment.** *FEMS Microbiology Letters* 2001, **201**:151-155.
23. Harper DB, Hamilton JT: **The global cycles of the naturally-occurring monohalomethanes.** In *Natural Production of Organohalogen Compounds*. Springer; 2003: 17-41
24. Miller LG, Warner KL, Baesman SM, Oremland RS, McDonald IR, Radajewski S, Murrell JC: **Degradation of methyl bromide and methyl chloride in soil microcosms: use of stable C isotope fractionation and stable isotope probing to identify reactions and the responsible microorganisms.** *Geochimica et Cosmochimica Acta* 2004, **68**:3271-3283.
25. Jaeger N, Besaury L, Kröber E, Delort AM, Greule M, Lenhart K, Nadalig T, Vuilleumier S, Amato P, Kolb S, et al: **Chloromethane degradation in soils: A combined microbial and two dimensional stable isotope approach.** *Journal of Environmental Quality* 2018, **47**:254-262.
26. Keppler F, Harper DB, Röckmann T, Moore RM, Hamilton JTG: **New insight into the atmospheric chloromethane budget gained using stable carbon isotope ratios.** *Atmospheric Chemistry and Physics* 2005, **5**:2403-2411.
27. Doronina N, Sokolov A, Trotsenko YA: **Isolation and initial characterization of aerobic chloromethane-utilizing bacteria.** *FEMS Microbiology Letters* 1996, **142**:179-183.
28. Hartmans S, Schmucke A, Cook AM, Leisinger T: **Methyl chloride: naturally occurring toxicant and C-1 growth substrate.** *Microbiology* 1986, **132**:1139-1142.
29. Traunecker J, Preuß A, Diekert G: **Isolation and characterization of a methyl chloride utilizing, strictly anaerobic bacterium.** *Archives of Microbiology* 1991, **156**:416-421.
30. Freedman DL, Swamy M, Bell NC, Vercé MF: **Biodegradation of chloromethane by *Pseudomonas aeruginosa* strain NB1 under nitrate-reducing and aerobic conditions.** *Applied and Environmental Microbiology* 2004, **70**:4629-4634.
31. Schäfer H, McDonald IR, Nightingale PD, Murrell JC: **Evidence for the presence of a CmuA methyltransferase pathway in novel marine methyl halide-oxidizing bacteria.** *Environmental Microbiology* 2005, **7**:839-852.

32. Nadalig T, Greule M, Bringel F, Keppler F, Vuilleumier S: **Probing the diversity of chloromethane-degrading bacteria by comparative genomics and isotopic fractionation.** *Frontiers in Microbiology* 2014, **5**:523.
33. Nadalig T, Ul-Haque MF, Roselli S, Schaller H, Bringel F, Vuilleumier S: **Detection and isolation of chloromethane-degrading bacteria from the *Arabidopsis thaliana* phyllosphere, and characterization of chloromethane utilization genes.** *FEMS Microbiology Ecology* 2011, **77**:438-448.
34. Doronina N, Ivanova E, Suzina N, Trotsenko YA: **Methanotrophs and methylotrophs are found in woody plant tissues within the winter period.** *Microbiology* 2004, **73**:702-709.
35. Bringel F, Besaury L, Amato P, Kröber E, Kolb S, Keppler F, Vuilleumier S, Nadalig T: **Methylotrophs and methylotroph populations for chloromethane degradation.** *Current Issues in Molecular Biology* 2019, **33**:149-172.
36. Vannelli T, Messmer M, Studer A, Vuilleumier S, Leisinger T: **A corrinoid-dependent catabolic pathway for growth of a *Methylobacterium* strain with chloromethane.** *PNAS USA* 1999, **96**:4615-4620.
37. Roselli S, Nadalig T, Vuilleumier S, Bringel F: **The 380 kb pCMU01 plasmid encodes chloromethane utilization genes and redundant genes for vitamin B₁₂- and tetrahydrofolate-dependent chloromethane metabolism in *Methylobacterium extorquens* CM4: a proteomic and bioinformatics study.** *PLoS One* 2013, **8**:e56598.
38. Studer A, Vuilleumier S, Leisinger T: **Properties of the methylcobalamin: H₄folate methyltransferase involved in chloromethane utilization by *Methylobacterium* sp. strain CM4.** *European Journal of Biochemistry* 1999, **264**:242-249.
39. Studer A, Stupperich E, Vuilleumier S, Leisinger T: **Chloromethane: tetrahydrofolate methyl transfer by two proteins from *Methylobacterium chloromethanicum* strain CM4.** *European Journal of Biochemistry* 2001, **268**:2931-2938.
40. Schäfer H, Miller LG, Oremland RS, Murrell JC: **Bacterial cycling of methyl halides.** *Advances in Applied Microbiology* 2007, **61**:307-346.
41. Chaignaud P, Morawe M, Besaury L, Kröber E, Vuilleumier S, Bringel F, Kolb S: **Methanol consumption drives the bacterial chloromethane sink in a forest soil.** *ISME Journal* 2018, **12**:2681-2693.
42. Cox MJ, Schäfer H, Nightingale PD, McDonald IR, Murrell JC: **Diversity of methyl halide-degrading microorganisms in oceanic and coastal waters.** *FEMS Microbiology Letters* 2012, **334**:111-118.
43. Buddhuhs N, Chertkov O, Petersen J, Fiebig A, Chen A, Pati A, Ivanova N, Lapidus A, Goodwin LA, Chain P: **Complete genome sequence of the marine methyl-halide oxidizing *Leisingera methylohalidivorans* type strain (DSM 14336T), a representative of the *Roseobacter* clade.** *Standards in Genomic Sciences* 2013, **9**:128-141.
44. Chaignaud P, Maucourt B, Weiman M, Alberti A, Kolb S, Cruveiller S, Vuilleumier S, Bringel F: **Genomic and transcriptomic analysis of growth-supporting dehalogenation of chlorinated methanes in *Methylobacterium*.** *Frontiers in Microbiology* 2017, **8**:1600.
45. Michener JK, Vuilleumier S, Bringel F, Marx CJ: **Transfer of a catabolic pathway for chloromethane in *Methylobacterium* strains highlights different limitations for growth with chloromethane or with dichloromethane.** *Frontiers in Microbiology* 2016, **7**:1116.

46. Piesik D, Weaver DK, Peck GE, Morrill WL: **Diel patterns in volatiles released by mechanically-damaged wheat plants.** *Electronic Journal of Polish Agricultural Universities, Agronomy* 2006, **9**:1-12.
47. Piesik D, Łyszczarz A, Tabaka P, Lamparski R, Bocianowski J, Delaney K: **Volatile induction of three cereals: influence of mechanical injury and insect herbivory on injured plants and neighbouring uninjured plants.** *Annals of Applied Biology* 2010, **157**:425-434.
48. Wang Z-P, Gullledge J, Zheng J-Q, Liu W, Li L-H, Han X-G: **Physical injury stimulates aerobic methane emissions from terrestrial plants.** *Biogeosciences* 2009, **6**:615-621.
49. Chen Y, Murrell JC: **When metagenomics meets stable-isotope probing: progress and perspectives.** *Trends in microbiology* 2010, **18**:157-163.
50. Kröber E, Eyice Ö: **Profiling of active microorganisms by stable isotope probing metagenomics.** In *Stable Isotope Probing*. Springer; 2019: 151-161
51. Coplen TB: **Guidelines and recommended terms for expression of stable isotope ratio and gas ratio measurement results.** *Rapid Communications in Mass Spectrometry* 2011, **25**:2538-2560.
52. Atamna-Ismaeel N, Finkel OM, Glaser F, Sharon I, Schneider R, Post AF, Spudich JL, von Mering C, Vorholt JA, Iluz D, et al: **Microbial rhodopsins on leaf surfaces of terrestrial plants.** *Environmental Microbiology* 2011, **14**:140-146.
53. Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, Friedrich MW, Murrell JC: **DNA stable-isotope probing.** *Nature Protocols* 2007, **2**:860-866.
54. Liu W-T, Marsh TL, Cheng H, Forney LJ: **Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA.** *Applied and Environmental Microbiology* 1997, **63**:4516-4522.
55. Ulrich A, Becker R: **Soil parent material is a key determinant of the bacterial community structure in arable soils.** *FEMS Microbiology Ecology* 2006, **56**:430-443.
56. Dunbar J, Ticknor LO, Kuske CR: **Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities.** *Applied and Environmental Microbiology* 2001, **67**:190-197.
57. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, et al: **QIIME allows analysis of high-throughput community sequencing data.** *Nature Methods* 2010, **7**:335-336.
58. Edgar RC: **Search and clustering orders of magnitude faster than BLAST.** *Bioinformatics* 2010, **26**:2460-2461.
59. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R: **UCHIME improves sensitivity and speed of chimera detection.** *Bioinformatics* 2011, **27**:2194-2200.
60. Li D, Liu C-M, Luo R, Sadakane K, Lam T-W: **MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph.** *Bioinformatics* 2015, **31**:1674-1676.
61. Alneberg J, Bjarnason BS, de Bruijn I, Schirmer M, Quick J, Ijaz UZ, Lahti L, Loman NJ, Andersson AF, Quince C: **Binning metagenomic contigs by coverage and composition.** *Nature Methods* 2014, **11**:1144-1146.
62. Kang D, Li F, Kirton ES, Thomas A, Egan RS, An H, Wang Z: **MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies.** *Journal of Life & Environmental Sciences (PeerJ Preprints)* 2019, **7**:e27522v27521.

63. Wu Y-W, Simmons BA, Singer SW: **MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets.** *Bioinformatics* 2015, **32**:605-607.
64. Langmead B, Salzberg SL: **Fast gapped-read alignment with Bowtie 2.** *Nature Methods* 2012, **9**:357-359.
65. Uritskiy GV, DiRuggiero J, Taylor J: **MetaWRAP—a flexible pipeline for genome-resolved metagenomic data analysis.** *Microbiome* 2018, **6**:158.
66. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW: **CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes.** *Genome Research* 2015, **25**:1043-1055.
67. Brettin T, Davis JJ, Disz T, Edwards RA, Gerdes S, Olsen GJ, Olson R, Overbeek R, Parrello B, Pusch GD, et al: **RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes.** *Scientific Reports* 2015, **5**:8365-8365.
68. Zuo G, Hao B: **CVTree3 Web Server for whole-genome-based and alignment-free prokaryotic phylogeny and taxonomy.** *Genomics Proteomics Bioinformatics* 2015, **13**:321-331.
69. Letunic I, Bork P: **Interactive Tree Of Life (iTOL) v4: recent updates and new developments.** *Nucleic Acids Research* 2019, **47**:W256-W259.
70. Asnicar F, Thomas AM, Beghini F, Mengoni C, Manara S, Manghi P, Zhu Q, Bolzan M, Cumbo F, May U, et al: **Precise phylogenetic analysis of microbial isolates and genomes from metagenomes using PhyloPhlAn 3.0.** *Nature Communications* 2020, **11**:2500.
71. Kumar S, Stecher G, Li M, Knyaz C, Tamura K: **MEGA X: Molecular evolutionary genetics analysis across computing platforms.** *Molecular Biology and Evolution* 2018, **35**:1547-1549.
72. Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S: **High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries.** *Nature Communications* 2018, **9**:5114.
73. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *Journal of Molecular Biology* 1990, **215**:403-410.
74. Seemann T: **Prokka: rapid prokaryotic genome annotation.** *Bioinformatics* 2014, **30**:2068-2069.
75. Kaminski J, Gibson MK, Franzosa EA, Segata N, Dantas G, Huttenhower C: **High-specificity targeted functional profiling in microbial communities with ShortBRED.** *PLoS computational biology* 2015, **11**:e1004557.
76. Vallenet D, Calteau A, Dubois M, Amours P, Bazin A, Beuvin M, Burlot L, Bussell X, Fouteau S, Gautreau G, et al: **MicroScope: an integrated platform for the annotation and exploration of microbial gene functions through genomic, pangenomic and metabolic comparative analysis.** *Nucleic Acids Research* 2019, **48**:D579-D589.
77. Vallenet D, Calteau A, Dubois M, Amours P, Bazin A, Beuvin M, Burlot L, Bussell X, Fouteau S, Gautreau G, et al: **MicroScope: an integrated platform for the annotation and exploration of microbial gene functions through genomic, pangenomic and metabolic comparative analysis.** *Nucleic Acids Research* 2020, **48**:D579-D589.
78. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M: **The RAST Server: rapid annotations using subsystems technology.** *BMC Genomics* 2008, **9**:1-15.

- 798 79. Aslan-Üzel AS, Beier A, Kovář D, Cziegler C, Padhi SK, Schuiten ED, Dörr M,
799 Böttcher D, Hollmann F, Rudroff F: **An ultrasensitive fluorescence assay for the**
800 **detection of halides and enzymatic dehalogenation.** *The European Society Journal*
801 *for Catalysis* 2020, **12**:2032-2039.
- 802 80. Blom J, Albaum SP, Doppmeier D, Pühler A, Vorhölter F-J, Zakrzewski M,
803 Goesmann A: **EDGAR: a software framework for the comparative analysis of**
804 **prokaryotic genomes.** *BMC Bioinformatics* 2009, **10**:154.
- 805 81. Kröber E, Schäfer H: **Identification of proteins and genes expressed by**
806 ***Methylophaga thiooxydans* during growth on dimethylsulfide and their presence**
807 **in other members of the genus.** *Frontiers in Microbiology* 2019, **10**:1132.
- 808 82. Madhaiyan M, Poonguzhali S, Senthilkumar M, Lee J-S, Lee K-C: ***Methylobacterium***
809 ***gossipiicola* sp. nov., a pink-pigmented, facultatively methylotrophic bacterium**
810 **isolated from the cotton phyllosphere.** *International Journal of Systematic and*
811 *Evolutionary Microbiology* 2012, **62**:162-167.
- 812 83. Bringel F, Couée I: **Pivotal roles of phyllosphere microorganisms at the interface**
813 **between plant functioning and atmospheric trace gas dynamics.** *Frontiers in*
814 *Microbiology* 2015, **6**:486.
- 815 84. Knief C, Frances L, Vorholt JA: **Competitiveness of diverse *Methylobacterium***
816 **strains in the phyllosphere of *Arabidopsis thaliana* and identification of**
817 **representative models, including *M. extorquens* PA1.** *Microbial Ecology* 2010,
818 **60**:440-452.
- 819 85. Knief C, Ramette A, Frances L, Alonso-Blanco C, Vorholt JA: **Site and plant species**
820 **are important determinants of the *Methylobacterium* community composition in**
821 **the plant phyllosphere.** *The ISME Journal* 2010, **4**:719-728.
- 822 86. Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, von
823 Mering C, Vorholt JA: **Community proteogenomics reveals insights into the**
824 **physiology of phyllosphere bacteria.** *PNAS USA* 2009, **106**:16428-16433.
- 825 87. Raja P, Balachandar D, Sundaram S: **Genetic diversity and phylogeny of pink-**
826 **pigmented facultative methylotrophic bacteria isolated from the phyllosphere of**
827 **tropical crop plants.** *Biology and Fertility of Soils* 2008, **45**:45-53.
- 828 88. Minami T, Anda M, Mitsui H, Sugawara M, Kaneko T, Sato S, Ikeda S, Okubo T,
829 Tsurumaru H, Minamisawa K: **Metagenomic analysis revealed methylamine and**
830 **ureide utilization of soybean-associated *Methylobacterium*.** *Microbes and*
831 *Environments* 2016:ME16035.
- 832 89. Madhaiyan M, Poonguzhali S, Kwon S-W, Sa T-M: ***Methylobacterium***
833 ***phyllosphaerae* sp. nov., a pink-pigmented, facultative methylotroph from the**
834 **phyllosphere of rice.** *International Journal of Systematic and Evolutionary*
835 *Microbiology* 2009, **59**:22-27.
- 836 90. Vorholt JA: **Microbial life in the phyllosphere.** *Nature Reviews Microbiology* 2012,
837 **10**:828-840.
- 838 91. Karasov TL, Neumann M, Duque-Jaramillo A, Kersten S, Bezrukov I, Schröppel B,
839 Symeonidi E, Lundberg DS, Regalado J, Shirsekar G: **The relationship between**
840 **microbial population size and disease in the *Arabidopsis thaliana* phyllosphere.**
841 *BioRxiv* 2020:828814.
- 842 92. Perazzolli M, Nesler A, Giovannini O, Antonielli L, Puopolo G, Pertot I: **Ecological**
843 **impact of a rare sugar on grapevine phyllosphere microbial communities.**
844 *Microbiological Research* 2020, **232**:126387.
- 845 93. Chen Q-L, An X-L, Zheng B-X, Ma Y-B, Su J-Q: **Long-term organic fertilization**
846 **increased antibiotic resistome in phyllosphere of maize.** *Science of the Total*
847 *Environment* 2018, **645**:1230-1237.

- 848 94. Zwielehner J, Handschur M, Michaelsen A, Irez S, Demel M, Denner EB, Haslberger
849 AG: **DGGE and real time PCR analysis of lactic acid bacteria in bacterial**
850 **communities of the phyllosphere of lettuce.** *Molecular Nutrition & Food Research*
851 2008, **52**:614-623.
- 852 95. Jackson CR, Denney WC: **Annual and seasonal variation in the phyllosphere**
853 **bacterial community associated with leaves of the southern magnolia (*Magnolia***
854 ***grandiflora*).** *Microbial Ecology* 2011, **61**:113-122.
- 855 96. Rogers TJ, Leppanen C, Brown V, Fordyce JA, LeBude A, Ranney T, Simberloff D,
856 Cregger MA: **Exploring variation in phyllosphere microbial communities across**
857 **four hemlock species.** *Ecosphere* 2018, **9**:e02524.
- 858 97. Aydogan EL, Budich O, Hardt M, Choi YH, Jansen-Willems AB, Moser G, Müller C,
859 Kämpfer P, Glaeser SP: **Global warming shifts the composition of the abundant**
860 **bacterial phyllosphere microbiota as indicated by a cultivation dependent and**
861 **independent study of the grassland phyllosphere of a long-term warming field-**
862 **experiment.** *FEMS Microbiology Ecology* 2020, **96**.
- 863 98. Santana RS, Fernandes G, Ávila MP, Reis MP, de Araújo FM, Salim A, Oliveira G,
864 Chartone-Souza E, Nascimento A: **Endophytic microbiota associated with the root**
865 **tips and leaves of *Baccharis dracunculifolia*.** *Brazilian Archives of Biology and*
866 *Technology* 2016, **59**.
- 867 99. Herrmann M, Geesink P, Richter R, Küsel K: **Canopy position has a stronger effect**
868 **than tree species identity on phyllosphere bacterial diversity in a floodplain**
869 **hardwood forest.** *Plant Microbe Interactions* 2020, **81**:157-168.
- 870 100. Albuquerque L, da Costa M: **The Families *Conexibacteraceae*, *Patulibacteraceae***
871 **and *Solirubrobacteraceae*.** *The Prokaryotes Springer Berlin Heidelberg* 2014:185-
872 200.
- 873 101. Fausto C, Mininni AN, Sofo A, Crecchio C, Scagliola M, Dichio B, Xiloyannis C:
874 **Olive orchard microbiome: characterisation of bacterial communities in soil-**
875 **plant compartments and their comparison between sustainable and conventional**
876 **soil management systems.** *Plant Ecology & Diversity* 2018, **11**:597-610.
- 877 102. Ottesen A, Ramachandran P, Reed E, Gu G, Gorham S, Ducharme D, Newell M,
878 Rideout S, Turini T, Hill T: **Metagenome tracking biogeographic agroecology:**
879 **phytobiota of tomatoes from Virginia, Maryland, North Carolina and California.**
880 *Food Microbiology* 2019, **79**:132-136.
- 881 103. Moore-Colyer MJ, Longland A, Harris P, Crosthwaite S: **Mapping the bacterial**
882 **ecology on the phyllosphere of grass hay and the potential hazards of soaking**
883 **fodder for horse gut health.** *BioRxiv* 2018:494799.
- 884 104. Beck DA, McTaggart TL, Setboonsarng U, Vorobev A, Goodwin L, Shapiro N,
885 Woyke T, Kalyuzhnaya MG, Lidstrom ME, Chistoserdova L: **Multiphyletic origins**
886 **of methylotrophy in Alphaproteobacteria, exemplified by comparative genomics**
887 **of Lake Washington isolates.** *Environmental microbiology* 2015, **17**:547-554.
- 888 105. Wiegel J, Wilke D, Baumgarten J, Opitz R, Schlegel HG: **Transfer of the nitrogen-**
889 **fixing hydrogen bacterium *Corynebacterium autotrophicum* Baumgarten *et al.* to**
890 ***Xanthobacter* gen. nov.** *International Journal of Systematic and Evolutionary*
891 *Microbiology* 1978, **28**:573-581.
- 892 106. Yashiro E, Spear R, McManus P: **Culture dependent and culture-independent**
893 **assessment of bacteria in the apple phyllosphere.** *Journal of Applied Microbiology*
894 2011, **110**:1284-1296.
- 895 107. Darlison J, Mogren L, Rosberg AK, Grudén M, Minet A, Liné C, Mieli M, Bengtsson
896 T, Håkansson Å, Uhlig E: **Leaf mineral content govern microbial community**

- structure in the phyllosphere of spinach (*Spinacia oleracea*) and rocket (*Diplotaxis tenuifolia*). *Science of The Total Environment* 2019, **675**:501-512.
108. Laforest-Lapointe I, Messier C, Kembel SW: **Tree phyllosphere bacterial communities: exploring the magnitude of intra-and inter-individual variation among host species.** *Journal of Life & Environmental Sciences (PeerJ)* 2016, **4**:e2367.
 109. Chaignaud P, Morawe M, Besaury L, Kröber E, Vuilleumier S, Bringel F, Kolb S: **Methanol consumption drives the bacterial chloromethane sink in a forest soil.** *The ISME journal* 2018, **12**:2681-2693.
 110. Studer A, McAnulla C, Büchele R, Leisinger T, Vuilleumier S: **Chloromethane-induced genes define a third C1 utilization pathway in *Methylobacterium chloromethanicum* CM4.** *Journal of Bacteriology* 2002, **184**:3476-3484.
 111. Vuilleumier S, Chistoserdova L, Lee M-C, Bringel F, Lajus A, Zhou Y, Gourion B, Barbe V, Chang J, Cruveiller S: ***Methylobacterium* genome sequences: a reference blueprint to investigate microbial metabolism of C1 compounds from natural and industrial sources.** *PLoS One* 2009, **4**:e5584.
 112. Marx CJ, Bringel F, Chistoserdova L, Moulin L, Ul Haque MF, Fleischman DE, Gruffaz C, Jourand P, Knief C, Lee M-C: **Complete genome sequences of six strains of the genus *Methylobacterium*.** *Journal of Bacteriology* 2012, **194**:4746-4748.
 113. Kwak M-J, Jeong H, Madhaiyan M, Lee Y, Sa T-M, Oh TK, Kim JF: **Genome information of *Methylobacterium oryzae*, a plant-probiotic methylotroph in the phyllosphere.** *PLoS One* 2014, **9**:e106704.
 114. Lex A, Gehlenborg N, Strobel H, Vuilleumier R, Pfister H: **UpSet: visualization of intersecting sets.** *IEEE Transactions on Visualization and Computer Graphics* 2014, **20**:1983-1992.
 115. Bahlmann E, Keppler F, Wittmer J, Greule M, Schöler HF, Seifert R, Zetzsch C: **Evidence for a major missing source in the global chloromethane budget from stable carbon isotopes.** *Atmospheric Chemistry and Physics* 2019, **19**:1703-1719.
 116. Keppler F, Bahlmann E, Greule M, Schöler HF, Wittmer J, Zetzsch C: **Mass spectrometric measurement of hydrogen isotope fractionation for the reactions of chloromethane with OH and Cl.** *Atmospheric Chemistry and Physics* 2018, **18**:6625-6635.
 117. Keppler F, Barnes JD, Horst A, Bahlmann E, Luo J, Nadalig T, Greule M, Hartmann SC, Vuilleumier S: **Chlorine isotope fractionation of the major chloromethane degradation processes in the environment.** *Environmental Science & Technology* 2019, **54**:1634-1645.
 118. Tani A, Ogura Y, Hayashi T, Kimbara K: **Complete genome sequence of *Methylobacterium aquaticum* strain 22A, isolated from *Racomitrium japonicum* moss.** *Genome Announcements* 2015, **3**.
 119. Madhaiyan M, Poonguzhali S: ***Methylobacterium pseudosasicola* sp. nov. and *Methylobacterium phyllostachyos* sp. nov., isolated from bamboo leaf surfaces.** *International Journal of Systematic and Evolutionary Microbiology* 2014, **64**:2376-2384.
 120. Kang Y-S, Kim J, Shin H-D, Nam Y-D, Bae J-W, Jeon CO, Park W: ***Methylobacterium platani* sp. nov., isolated from a leaf of the tree *Platanus orientalis*.** *International Journal of Systematic and Evolutionary Microbiology* 2007, **57**:2849-2853.
 121. Chaudhry V, Baidara P, Pal VK, Chawla N, Patil PB, Korpole S: ***Methylobacterium indicum* sp. nov., a facultative methylotrophic bacterium isolated from rice seed.** *Systematic and Applied Microbiology* 2016, **39**:25-32.

- 947 122. Ardley J, Tian R, Howieson J, Yates R, Bräu L, Han J, Lobos E, Huntemann M, Chen
948 A, Mavromatis K: **Genome sequence of the dark pink pigmented *Listia bainesii***
949 **microsymbiont *Methylobacterium* sp. WSM2598.** *Standards in Genomic Sciences*
950 2014, **9**:1-9.
- 951 123. Gallego V, García MT, Ventosa A: ***Methylobacterium variabile* sp. nov., a**
952 **methylophilic bacterium isolated from an aquatic environment.** *International*
953 *Journal of Systematic and Evolutionary Microbiology* 2005, **55**:1429-1433.
- 954 124. Belkhelfa S, Labadie K, Cruaud C, Aury J-M, Roche D, Bouzon M, Salanoubat M,
955 Döring V: **Complete genome sequence of the facultative methylophilic**
956 ***Methylobacterium extorquens* TK 0001 isolated from soil in Poland.** *Genome*
957 *Announcements* 2018, **6**.
- 958 125. Van Aken B, Tehrani R, Schnoor JL: **Endophyte-assisted phytoremediation of**
959 **explosives in poplar trees by *Methylobacterium populi* BJ001(T).** In *Endophytes of*
960 *Forest Trees*. Springer; 2011: 217-234
- 961 126. Wang X, Sahr F, Xue T, Sun B: ***Methylobacterium salsuginis* sp. nov., isolated from**
962 **seawater.** *International Journal of Systematic and Evolutionary Microbiology* 2007,
963 **57**:1699-1703.
- 964

Figure and Table legends

Figure 1. Relative abundance of ^{13}C -labelled bacterial OTUs on genus level from fraction samples LEAF, LEAFWASH and SOIL. OTUs are classified as 'labelled' by the following criteria: 1) ^{13}C -heavy > ^{12}C -heavy; 2) ^{13}C -heavy > ^{13}C -light; 3) ^{13}C -[heavy-light] > ^{12}C -[heavy-light]; 4) ^{13}C [Heavy-Light] > threshold, with threshold = 0.005%. The taxonomic affiliation of each labeled OTU is indicated at the family, order and genus levels (4-6, respectively), with a specific color and capital letter (see legend box).

Figure 2. Maximum-likelihood phylogenetic trees showing the position of selected MAGs within a tree of closely related reference genomes. Multiple sequence alignments of concatenated marker genes were constructed using PhyloPhlAn 3.0. Phylogenetic analysis was performed based on a matrix of pairwise distances estimated using the LG+F+G+I model with 500 bootstrap replications. Numbers at branch nodes refer to bootstrap values. **(A) Phylogenetic tree of *Methylobacterium* species.** Two metagenomic bins isolated from the LEAF fraction were classified as *Methylobacterium* (marked in pink and purple) and were placed within 19 selected reference genomes of Alphaproteobacteria. Tree was annotated with ANI and AAI values in relation to 'Fern CH_3Cl SIP LEAF bin s3'. **(B) Phylogenetic tree of *Propionibacterium* species.** Two bins classified as related to *Propionibacterium* (marked in yellow) from fractions LEAF and LEAFWASH were placed in a tree with 7 selected reference genomes from *Microlunatus* and *Friedmaniella*. Annotated ANI and AAI values are in relation to 'Fern CH_3Cl SIP LEAF bin co3'. **(C) Phylogenetic tree of *Myxococcales* species.** Two bins classified as related to *Sorangium* (marked in green) from fraction SOIL were placed in a tree with 16 reference genomes of Deltaproteobacteria. Tree was annotated with ANI and AAI values in relation to 'Fern CH_3Cl SIP SOIL bin s1'; AAI values significantly under 50 and ANI values significantly under 70 were not computed and are annotated with NA.

Figure 3. Metabolic reconstruction of [¹³C]-CH₃Cl labelled metagenome assembled genomes. A coloured dot next to a gene or pathway shows presence of this pathway or gene in the corresponding genome. 'Fern CH₃Cl SIP LEAF bin s3' is represented by a pink dot, 'Fern CH₃Cl SIP LEAF bin co1' is highlighted in purple, 'Fern CH₃Cl SIP LEAF bin co3' is represented with a yellow dot and 'Fern CH₃Cl SIP SOIL bin s1' is highlighted in green.

Figure 4. Pan-genome analysis of genomes from 10 *Methylobacterium* isolated strains, 3 representatives of the closely related clade *Methylobacterium* and two [¹³C]-CH₃Cl-enriched fern leaf MAGs. (A) Fractional pan genome representation. (B) UpSet plot of the pan-genome of the 15 *Methylobacterium* genomes. The metagenomic bin 'Fern CH₃Cl SIP LEAF bin s3' was chosen as reference genome. The analysis was carried out using the "Efficient Database framework for comparative Genome Analyses using BLAST score Ratios" (EDGAR) platform.

Supplementary Figure S1. Plant growth chambers setup. A) Details of gas-tight plant chamber. Gas-tight plant chambers were constructed with acryl glass (thickness: 5 mm). Butyl rubber stoppers at the top and the lower part of the chamber were used as ports for injecting CH₃Cl and extracting gas samples. A small ventilator in the lower part of the chamber ensured an even distribution of the gases in the chamber. **B) Gas-tight plant chambers with fern plants inside at the end of the incubation period.** Aluminium foil was wrapped around the chambers during incubation with CH₃Cl to reduce photosynthesis and microbial CO₂ consumption during the labelling experiment. Sunlight entered the chambers through the clear top of the chamber.

Supplementary Figure S2. Monitoring of the SIP-incubation of ferns. Incubations were carried out over 19 days with three biological replicated intact fern plants in gas-tight incubation chambers per tested condition. At the start of the SIP-incubation, 20 mL CH₃Cl was added to the chambers (initial mixing ratio of ~ 200ppm) and degradation was

monitored with GC-MS. At the end of each day, incubation chambers were opened and ventilated overnight. From day 2 of the incubation, 10 mL CH₃Cl was added to the chambers (initial mixing ratio of ~ 100ppm). (A) ¹³C-labelled CH₃Cl, and (B) ¹²C-labelled CH₃Cl. Red arrows indicate point of CH₃Cl addition.

Supplementary Figure S3. Analysis of carbon mass fraction (C %) and differential stable carbon isotope ratio ($\delta^{13}\text{C}$ value) of fern leaves and washed leaf samples (T_{end} washed) via EA/IRMS. Stable isotope ratios ($\delta^{13}\text{C}$) are expressed in permil (‰) relative to the international standard.

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B

C

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Supplementary Table S1. List of Metagenome Assembled Genomes (MAGs). Only MAGs with > 70% completeness and < 10% contamination are shown. MAGs highlighted in pink, yellow and green were used for further analysis, such as taxonomic classification, metabolic reconstruction and/or pan-genomics. Single and co-assembly refer to processed reads assembled with MEGAHIT v.1.2.9 [60] once, and with an additional co-assembly of leaf and leaf wash metagenomes, respectively.

MAG Name	Sample	Assembly Type	Completeness	Contamination	GC	GC std	Genome size	# contigs	Longest contig	N50 (contigs)	Mean contig length	Coding density	# predicted genes	16S rRNA in MAG (RNAmmer)	RAST Taxonomy
Fern CH3CI SI LEAF		Co-Assembly	74.1	6.9	0.69	0.01	2,589,893	422	26327	6979	6137	0.92	2859	no	<i>Brevundimonas</i> sp BAL3
Fern CH3CI SI LEAF		Co-Assembly	74.7	1.6	0.62	0.02	3,566,997	945	26720	4942	3775	0.89	3645	no	<i>Burkholderia mallei</i> ATCC 23344
Fern CH3CI SI LEAF		Co-Assembly	76.2	2.1	0.62	0.02	3,727,886	1008	31300	4829	3698	0.89	3858	no	<i>Burkholderia mallei</i> ATCC 23344
Fern CH3CI SI LEAF		Single	86.2	4.1	0.70	0.01	3,121,099	234	72936	16915	13338	0.91	3124	no	<i>Clavibacter michiganensis</i>
Fern CH3CI SI LEAF		Single	87.5	3.0	0.74	0.02	2,895,226	400	49341	10204	7238	0.91	3124	no	<i>Clavibacter michiganensis</i>
Fern CH3CI SI LEAF		Co-Assembly	78.4	2.0	0.73	0.01	3,206,454	302	54365	12926	10617	0.93	3148	no	<i>Janibacter</i> sp HTCC2649
Fern CH3CI SI LEAF		Single	80.7	2.9	0.73	0.01	3,298,717	320	49896	11919	10308	0.93	3230	no	<i>Janibacter</i> sp HTCC2649
Fern CH3CI SI LEAF		Single	85.7	4.5	0.73	0.01	3,667,181	375	49896	11466	9779	0.92	3592	no	<i>Janibacter</i> sp HTCC2649
Fern CH3CI SI LEAF		Co-Assembly	76.4	6.4	0.74	0.02	5,175,864	963	34258	6859	5375	0.92	5489	no	<i>Kineococcus radiotolerans</i> SRS30216
Fern CH3CI SI LEAF		Co-Assembly	81.6	4.8	0.74	0.01	2,633,679	435	32366	8104	6054	0.91	2911	no	marine actinobacterium PHSC20C1
Fern CH3CI SI LEAF		Co-Assembly	78.1	7.3	0.70	0.01	3,454,154	251	66164	18012	13762	0.91	3403	no	marine actinobacterium PHSC20C1
Fern CH3CI SI LEAF		Co-Assembly	79.4	1.6	0.72	0.02	3,437,766	460	39543	9299	7473	0.92	3693	no	<i>Methylobacterium extorquens</i> PA1
Fern CH3CI SI LEAF		Co-Assembly	73.9	7.1	0.70	0.01	3,599,730	301	42665	13382	11959	0.87	3627	no	<i>Methylobacterium extorquens</i> PA1
Fern CH3CI SI LEAF		Single	81.5	3.7	0.71	0.02	3,823,638	420	71582	12689	9104	0.88	3909	no	<i>Methylobacterium radiotolerans</i> JCM 2831
Fern CH3CI SI LEAF		Co-Assembly	71.6	7.3	0.74	0.02	3,066,080	562	27103	6693	5456	0.93	3233	no	<i>Nocardioides</i> sp JS614
Fern CH3CI SI LEAF		Co-Assembly	80.8	10.0	0.72	0.01	3,692,543	345	51678	11824	10703	0.92	3711	yes	<i>Propionibacterium acnes</i> KPA171202
Fern CH3CI SI LEAF		Co-Assembly	85.0	2.2	0.65	0.01	3,978,621	509	49156	9622	7817	0.93	4150	no	<i>Rhodococcus jostii</i> RHA1
Fern CH3CI SI LEAF		Single	80.4	6.9	0.65	0.01	3,912,515	992	24370	4591	3944	0.93	4414	no	<i>Rhodococcus jostii</i> RHA1
Fern CH3CI SI LEAFWASH		Co-Assembly	76.2	2.1	0.62	0.02	3,727,886	1008	31300	4829	3698	0.89	3858	no	<i>Burkholderia mallei</i> ATCC 23344
Fern CH3CI SI LEAFWASH		Co-Assembly	81.7	9.9	0.62	0.02	4,717,577	1629	26720	3751	2896	0.88	5196	no	<i>Burkholderia mallei</i> ATCC 23344
Fern CH3CI SI LEAFWASH		Single	73.3	1.3	0.62	0.02	3,273,165	804	27709	5203	4071	0.89	3278	no	<i>Burkholderia mallei</i> ATCC 23344
Fern CH3CI SI LEAFWASH		Co-Assembly	76.2	4.3	0.74	0.02	2,212,892	342	32366	9008	6470	0.91	2428	no	<i>Clavibacter michiganensis</i>
Fern CH3CI SI LEAFWASH		Co-Assembly	86.8	8.1	0.73	0.01	3,544,565	356	54365	12722	9957	0.92	3518	no	<i>Janibacter</i> sp HTCC2649
Fern CH3CI SI LEAFWASH		Co-Assembly	70.2	8.5	0.70	0.01	3,366,239	256	66164	18000	13149	0.92	3318	no	marine actinobacterium PHSC20C1
Fern CH3CI SI LEAFWASH		Co-Assembly	79.6	1.2	0.72	0.02	3,413,711	456	39543	9410	7486	0.92	3656	no	<i>Methylobacterium extorquens</i> PA1
Fern CH3CI SI LEAFWASH		Single	82.8	6.6	0.74	0.02	3,222,653	632	24538	6100	5099	0.92	3450	no	<i>Nocardioides</i> sp JS614
Fern CH3CI SI LEAFWASH		Co-Assembly	74.2	5.4	0.72	0.01	3,118,906	275	54326	12680	11341	0.92	3111	yes	<i>Propionibacterium acnes</i> KPA171202
Fern CH3CI SI LEAFWASH		Single	74.9	1.3	0.62	0.02	3,393,326	849	31300	5079	3997	0.89	3424	no	<i>Ralstonia solanacearum</i> GM1000
Fern CH3CI SI LEAFWASH		Co-Assembly	86.6	2.6	0.65	0.01	4,120,025	543	49156	9324	7588	0.93	4319	no	<i>Rhodococcus jostii</i> RHA1
Fern CH3CI SI SOIL		Single	72.9	3.8	0.71	0.02	2,231,874	545	23284	4891	4095	0.92	2647	no	<i>Ammonifex degensii</i> KC4
Fern CH3CI SI SOIL		Single	74.3	2.2	0.67	0.02	3,755,866	949	17944	4419	3958	0.92	4334	no	<i>Mycobacterium tuberculosis</i> H37Rv
Fern CH3CI SI SOIL		Single	73.4	1.4	0.65	0.02	2,344,994	487	23872	5961	4815	0.93	2607	yes	<i>Rhodoferrax ferrireducens</i> DSM 15236
Fern CH3CI SI SOIL		Single	81.2	4.4	0.65	0.01	7,667,279	945	67204	9821	8114	0.91	7236	no	<i>Sorangium cellulosum</i> So ce 56
Fern CH3CI SI SOIL		Single	83.8	9.2	0.65	0.01	8,685,556	1130	69346	9351	7686	0.91	8550	no	<i>Sorangium cellulosum</i> So ce 56

Supplementary Table S2. List of *Methylobacterium* / *Methylobacterium* genomes used for pan-genomics.

Genus	Source	INSDC No./ Accession No.	Genome size [bp]	No. of predicted genes	Reference
<i>Methylobacterium aquaticum</i> MA-22A	Moss	AP014704	7,557,960	6,993	[118]
<i>Methylobacterium gossypiicola</i> Gh-105	Cotton phyllosphere	FOPM01000046	4,524,655	4,224	[82]
<i>Methylobacterium oryzae</i> CBMB20	Rice phyllosphere	CP003811	6,286,629	5,795	[113]
<i>Methylobacterium phyllosphaerae</i> CBMB27	Rice phyllosphere	CP015367	6,203,749	5,820	[89]
<i>Methylobacterium phyllostachyos</i> BL47	Bamboo phyllosphere	FNHS01000044	6,015,046	5,790	[119]
<i>Methylobacterium platani</i> JCM 14648	<i>Platanus orientalis</i> phyl.	JXOD01000099	6,770,243	6,358	[120]
<i>Methylobacterium platani</i> SE3.6	Rice seed	JTHG01000099	6,930,824	6,635	[121]
<i>Methylobacterium pseudosasicola</i> BL36	Bamboo phyllosphere	FOTK01000093	6,845,277	6,795	[119]
<i>Methylobacterium</i> sp. WSM2598	<i>Listia bainesii</i> nodule	KB900613	8,130,841	7,551	[122]
<i>Methylobacterium variabile</i> DSM 16961	Aquatic environment	LABY01000099	7,432,100	7,183	[123]
<i>Methylobacterium extorquens</i> TK 0001	Soil	LT962688	5,715,512	5,435	[124]
<i>Methylobacterium populi</i> BJ001	Poplar tree tissue	NC_010725	5,848,997	5,464	[125]
<i>Methylobacterium salsuginis</i> CGMCC 1.6474	Seawater	FOSV01000046	5,323,773	5,012	[126]
Metagenomic bin Fern CH ₃ Cl SIP LEAF bin s3	<i>Cyathea australis</i> phyllosphere	SAMN17394138	3,823,638	3,909	This study
Metagenomic bin Fern CH ₃ Cl SIP LEAF bin co1	<i>Cyathea australis</i> phyllosphere	SAMN17394139	3,437,766	3,693	This study

Supplementary Table S3. Gene presence in labeled MAGs.

Pathway, gene and gene product		MAGs related [a]					
		<i>Methylobacterium</i>		<i>Friedmanniella</i>		<i>Sorangium</i>	
		Fern CH3Cl SIP LEAF bin co1 (METHYLO1CO_v1_#)	Fern CH3Cl SIP LEAF bin s3 (METHYLO3_v1_#)	Fern CH3Cl SIP LEAF bin co3 (PROPION13_v1_#)	Fern CH3Cl SIP LEAFWASH bin co7 (PROPION17_v1_#)	Fern CH3Cl SIP SOIL bin s1 (MYXOCOCC1_v1_#)	Fern CH3Cl SIP SOIL bin s7 (MYXO7SOR_v1_#)
methanol oxidation							
<i>mxoFI</i>	methanol dehydrogenase (cytochrome c) subunits [EC:1.1.2.7]	nd [b]	510001 210010-like	nd	nd	nd	nd
<i>xoxF</i>	lanthanide-dependent methanol dehydrogenase [EC:1.1.2.10]	nd	2580029	nd	nd	nd	nd
<i>exaF(A)</i> (<i>mdh2</i>)	PQQ-dependent methanol/ethanol dehydrogenase precursor, lanthanide-containing [EC:1.1.1.244, EC:1.1.99.-]	nd	210010-like 1870009	nd	nd	nd	nd
methane oxidation to methanol							
<i>mmoXYZ</i>	methane monooxygenase component A chains [EC:1.14.13.25]	nd	nd	nd	nd	nd	nd
<i>pmoABC</i>	methane/ammonia monooxygenase subunits	nd	nd	nd		nd	
methylamine oxidation							
<i>mauAB</i>	methylamine dehydrogenase [EC:1.4.99.3] subunits	nd	nd	nd	nd	nd	nd
<i>gmaS</i>	γ -glutamylmethylamide synthase [EC:6.3.4.12]	nd	nd	nd	nd	nd	nd
<i>mgdABCD</i>	<i>N</i> -methylglutamate dehydrogenase [EC:1.5.99.5] subunits	1080002-3 1080006	nd	nd	nd	nd	nd
<i>mgsABC</i>	<i>N</i> -methylglutamate synthase [EC:2.4.2.-] subunits	nd	nd	3030003-5	2420003-5	nd	nd
C1 transfer reactions: primary oxidation							
<i>fae</i>	5,6,7,8-tetrahydromethanopterin hydro-lyase [EC:4.2.1.147]	nd	3420072 1110002 1540002	nd	nd	nd	nd
<i>mtdB</i> (<i>mdtA</i>)	methylene H4MPT-dehydrogenase [EC:1.5.1.-]	nd	2750003	nd	nd	nd	nd
<i>mch</i>	methenyltetrahydromethanopterin cyclohydrolase [EC:3.5.4.27]	nd	2750004	nd	nd	nd	nd
<i>fhcABCD</i>	formyltransferase/hydrolase complex Fhc	nd	3210016_19	nd	nd	nd	nd
<i>fch</i>	methenyl H4F-cyclohydrolase [EC:3.5.4.9]	nd	3410009	nd	nd	nd	nd

<i>mtdA</i>	methylene H4MPT-dehydrogenase/methylene H4F-dehydrogenase [EC:1.5.1.5, 1.5.1.-]	nd	3410008	nd	nd	nd	nd
<i>glyA</i>	glycine hydroxymethyltransferase [EC:2.1.2.1] (Ser cycle)	4570002	2340008	nd	nd	6950008	8320007
C1 transfer reactions: primary demethylation H4F-linked							
<i>metF2</i>	methylenetetrahydrofolate reductase [EC:1.5.1.20]	nd	nd	nd	nd	nd	nd
<i>folD</i>	methylene H4F-dehydrogenase/methenyl H4F-cyclohydrolase [EC:1.5.1.5 3.5.4.9]	3800010	nd	990020	850020	7530008	8980008
<i>purU</i>	formyltetrahydrofolate deformylase [EC:3.5.1.10]	4300009	nd	nd	nd	6950006	8320005
<i>fdh</i>	S-(hydroxymethyl)glutathione dehydrogenase [EC:1.1.1.284]	4420002	3990004	10005 2770014	10005 2260014	nd	nd
<i>fhs (ftfL)</i>	formate--tetrahydrofolate ligase [EC:6.3.4.3]	910006	1050006	nd	nd	nd	nd
ethylmalonyl-CoA pathway (EMCP)							
<i>sgaA</i>	serine--glyoxylate aminotransferase [EC:2.6.1.45] (Ser cycle)	1410013	3410006	nd	nd	nd	nd
<i>hprA</i>	glycerate dehydrogenase [EC:1.1.1.29] (Ser cycle)	nd	3410007	nd	nd	2430007	2800007
<i>gck</i>	glycerate kinase [EC:1.1.1.81] (Ser cycle)	4010001-like	800010	nd	nd	8150004	nd
<i>eno</i>	enolase [4.2.1.11] (Ser cycle)	1900007	nd	2600009	2100009	3610005	4190005
<i>ppc</i>	phosphoenolpyruvate carboxylase [EC:4.1.1.31] (Ser cycle)	1510001-like	1700001	nd	nd	4820006	5610006
<i>mdh</i>	malate dehydrogenase [EC:1.1.1.37]	2680002	nd	nd	nd	nd	nd
<i>mtkAB</i>	malate thiokinase [EC:6.2.1.9], large and small subunits (Ser cycle)	2680003-like 2680004-like	3410010 3410011	990013-like 990014-like	850013-like 850014-like	nd	nd
<i>mcl</i>	L-malyl-CoA/beta-methylmalyl-CoA lyase [EC:4.1.3.24]	680002	1700002	nd	nd	nd	nd
<i>phaA</i>	beta-ketothiolase [EC:2.3.1.16]	2150009	1590015	1410005	1180005	3350025	3900027
<i>phaB</i>	acetoacetyl-CoA reductase [EC:1.1.1.36]	2150007	1590014	nd	nd	nd	nd
<i>croR</i>	crotonase [EC:4.2.1.55]	nd	1430007	nd	nd	nd	nd
<i>ccr</i>	crotonyl-CoA reductase [EC:1.3.1.85]	nd	660006	nd	nd	nd	nd
<i>ecm (meaA)</i>	ethylmalonyl-CoA mutase [EC:5.4.99.63]	2740001	3140013	nd	nd	nd	nd
<i>msd</i>	methylsuccinyl-CoA dehydrogenase [EC:1.3.8.4]	4310001	3350010	nd	nd	nd	nd
<i>mcd</i>	methylsuccinyl-CoA dehydrogenase [EC: 1.3.8.4]	4310001	3350010	nd	nd	nd	nd
<i>pccAB</i>	propionyl-CoA carboxylase [EC:6.4.1.3] alpha and beta subunits	3140006 3770008	2280001 100012	1360011	1160011	4470013	5200012
<i>meaB</i>	ethylmalonyl-CoA mutase accessory protein	nd	nd	1410004	2140009	3520009 4590001	4090009
<i>mcmAB</i>	methylmalonyl-CoA mutase [EC:5.4.99.2] alpha subunit	1190003 2730001	2530011 2880016	2840002	2140010 2290002	4180008	4880008 8690001
<i>sdhABCD</i>	succinate dehydrogenase [EC:1.-.-.-], flavoprotein subunit	2770004-6 2970003	160015	nd	nd	5810004-5 like	6080002-3 6900005-6
<i>fumC</i>	fumarate hydratase [EC:4.2.1.2]	2860002	2910008	3430003	2740003	950003	1080003
formaldehyde oxidation (glutathione-dependent)							
<i>gfa</i>	glutathione-dependent formaldehyde-activating enzyme [EC:4.4.1.22]	4510002	nd	nd	nd	2640010 1400003	3050009 1560003
<i>frmA</i>	alcohol dehydrogenase class III/glutathione-dependent formaldehyde dehydrogenase [1.1.1.1, 1.1.1.284]	470005	770022-like	nd	nd	2580006	2980006

<i>fdh</i>	S-(hydroxymethyl)glutathione dehydrogenase [EC:1.1.1.284]	4420002	3990004	10005 2770014	10005 2260014	nd	nd
glycine cleavage system (GCP)							
<i>gcvP</i>	PLP-dependent glycine dehydrogenase [EC:1.4.4.2]	nd	nd	2910006	2340006	1220003 9080023	10910023 1370003
<i>gcvH</i>	decarboxylating, aminomethyltransferase protein H [EC:1.4.4.2,2.1.2.10]	440029	nd	2910001	2340001	3880005	4390005
<i>gcvT</i>	aminomethyltransferase tetrahydrofolate-dependent protein T [EC:2.1.2.10]	nd	nd	320013	290013	3770004	4390004

[a] Annotation and analysis of genomic content and features as described in the MaGe platform [76, 77]

(<https://mage.genoscope.cns.fr/microscope/mage/>).

[b] Nd, not detected

Supplementary Table S4. List of enzymes that potentially use or produce halides in labeled ¹³C-MAGs.

Halogenase / dehalogenase [a]					MAGs related [b]						
					<i>Methylobacterium</i>		<i>Friedmanniella</i>		<i>Sorangium</i>		
					Ferm CH3Cl SIP LEAF bin co1 (METHYLO1CO_v1_#)	Ferm CH3Cl SIP LEAF bin s3 (METHYLO3_v1_#)	Ferm CH3Cl SIP LEAF bin co3 (PROPION13_v1_#)	Ferm CH3Cl SIP LEAFWASH bin co7 (PROPION17_v1_#)	Ferm CH3Cl SIP SOIL bin s1 (MYXOCOCC1_v1_#)	Ferm CH3Cl SIP SOIL bin s7 (MYXO7SOR_v1_#)	
EC #	Enzyme	Br-	Cl-	I-	Br-	Cl-	I-				
1.10.3.2	Laccase			x				nd	nd	nd	nd
1.11.1.10	Chloride peroxidase		x					nd	nd	nd	nd
1.11.1.11	L-ascorbate peroxidase			x				nd	nd	nd	nd
1.11.1.7	Peroxidase			x				nd	nd	370002 4030002	6050003
1.11.1.8	Iodide peroxidase			x				nd	nd	nd	nd
1.11.1.B2	Chloride peroxidase, vanadium-containing	x	x					nd	nd	nd	nd
1.11.2.1	Unspecific peroxygenase	x						nd	nd	nd	nd
1.11.2.2	Myeloperoxidase		x					nd	nd	nd	nd
1.13.11.49	Chlorite O2-lyase		x		x			nd	nd	1730007	1420007
1.13.11.64	5-nitrosalicylate dioxygenase				x			nd	nd	nd	nd
1.14.12.13	2-halobenzoate 1,2-dioxygenase				x	x	x	nd	nd	nd	0
1.14.13.1	Salicylate 1-monooxygenase						x	2490006	nd	nd	nd
1.14.13.22	Cyclohexanone monooxygenase			x				nd	nd	nd	nd
1.14.13.50	Pentachlorophenol monooxygenase				x	x		nd	nd	nd	nd
1.14.19.49	Tetracycline 7-halogenase		x					nd	nd	nd	nd
1.14.19.55	4-hydroxybenzoate brominase (decarboxylating)	x						nd	nd	nd	nd
1.14.19.56	1H-pyrrole-2-carbonyl-[peptidyl-carrier protein] chlorinase		x					nd	nd	nd	nd
1.14.19.57	1H-pyrrole-2-carbonyl-[peptidyl-carrier protein] brominase	x						nd	nd	nd	nd
1.14.19.58	Tryptophan 5-halogenase		x					nd	nd	nd	nd
1.14.19.59	Tryptophan 6-halogenase	x	x					nd	nd	nd	nd
1.14.19.9	Tryptophan 7-halogenase	x	x					nd	nd	nd	nd
1.14.20.14	Hapalindole-type alkaloid chlorinase	x	x					nd	nd	nd	nd
1.21.1.1	Iodotyrosine deiodinase	x	x	x			x	nd	nd	nd	nd
1.21.1.2	2,4-dichlorobenzoyl-CoA reductase				x			nd	nd	nd	nd
1.21.99.3	Thyroxine 5-deiodinase			x			x	nd	nd	nd	nd
1.21.99.4	Thyroxine 5'-deiodinase			x			x	nd	nd	nd	nd
1.21.99.5	Tetrachloroethene reductive dehalogenase		x		x	x		nd	nd	nd	nd
2.1.1.165	Methyl-halide transferase	x	x	x				nd	nd	nd	nd
2.1.1.9	thiol S-methyltransferase	x	x	x				nd	nd	nd	nd

2.5.1.18	Glutathione transferase		x x x	1240007 1510003 2100003 2390002 3140014 3450006 4000002 4040004	2170010 3990005	780005 3180005	650005	850017 2300010 2810007 4110004 6180010 6990006 7100006 7330012 8400001 8470003	960018 2660010 3260007 4790004 7330010 8370005 8510006 8770012 10090001 10160003
2.5.1.47	Cysteine synthase (<i>cysK</i> , <i>cysM</i>)		x	3510001 3240006 1210003	nd	nd	nd	1280022 7390005 8330003 8330007	1440022 1950002 8830005 9990003 9990007
2.5.1.63	Adenosyl-fluoride synthase	x		nd	nd	nd	nd	nd	nd
2.5.1.94	Adenosyl-chloride synthase	x x x	x	nd	nd	nd	nd	nd	nd
3.1.1.42	Chlorogenate hydrolase		x						
3.8.1.10	2-haloacid dehalogenase (configuration-inverting)		x x	nd	nd	nd	nd	nd	nd
3.8.1.2	(S)-2-haloacid dehalogenase		x x x	2750008	1720008	1200003	1020003	5030005 7330004 3900003	5860005 8770004 4560003
3.8.1.3	Haloacetate dehalogenase (<i>dehH</i>)		x	4400007	nd	nd	nd	nd	nd
3.8.1.5	Haloalkane dehalogenase (<i>dhmA</i>)		x x x	nd	nd	nd	nd	7120009	8540009
3.8.1.6	4-chlorobenzoate dehalogenase		x	nd	nd	nd	nd	nd	nd
3.8.1.7	4-chlorobenzoyl-CoA dehalogenase		x x x	nd	nd	nd	nd	nd	nd
3.8.1.8	Atrazine chlorohydrolase		x	nd	nd	nd	nd	nd	nd
3.8.1.9	(R)-2-haloacid dehalogenase		x x	nd	nd	nd	nd	nd	nd
4.4.1.16	Selenocysteine lyase		x	4160006	1060021	nd	nd	nd	nd
4.5.1.1	DDT-dehydrochlorinase		x	nd	nd	nd	nd	nd	nd
4.5.1.2	3-chloro-D-alanine dehydrochlorinase		x	nd	nd	nd	nd	nd	nd
4.5.1.3	Dichloromethane dehalogenase		x	nd	nd	nd	nd	nd	nd
4.5.1.4	L-2-amino-4-chloropent-4-enoate dehydrochlorinase		x	nd	nd	nd	nd	nd	nd

[a] According to the BRENDA database available at www.brenda-enzymes.org. Adapted from Table S1, a complete list of halogenases/dehalogenases [79]

[b] MAGs annotation protein label in MaGe platform. Names in bold when assigned gene names

[c] nd, not detected, MAGE automatic annotation

Figures

Figure 1

Relative abundance of ¹³C-labelled bacterial OTUs on genus level from fraction samples LEAF, LEAFWASH and SOIL. OTUs are classified as 'labelled' by the following criteria: 1) ¹³C-heavy > ¹²C-heavy; 2) ¹³C-heavy > ¹³C-light; 3) ¹³C-[heavy-light] > ¹²C-[heavy-light]; 4) ¹³C[Heavy-Light] > threshold, with threshold =0.005%. The taxonomic affiliation of each labeled OTU is indicated at the family, order and genus levels (4-6, respectively), with a specific color and capital letter (see legend box).

Figure 2

Maximum-likelihood phylogenetic trees showing the position of selected MAGs within tree of closely related reference genomes. Multiple sequence alignments of concatenated marker genes were constructed using PhyloPhlAn 3.0. Phylogenetic analysis was performed based on a matrix of pairwise distances estimated using the LG+F+G+I model with 500 bootstrap replications. Numbers at branch nodes refer to bootstrap values. (A) Phylogenetic tree of *Methylobacterium* species. Two metagenomic bins isolated from the LEAF fraction were classified as *Methylobacterium* (marked in pink and purple) and were placed within 19 selected reference genomes of Alphaproteobacteria. Tree was annotated with ANI and AAI values in relation to ‘Fern CH3Cl SIP LEAF bin s3’. (B) Phylogenetic tree of *Propionibacterium* species. Two bins classified as related to *Propionibacterium* (marked in yellow) from fractions LEAF and LEAFWASH were placed in a tree with 7 selected reference genomes from *Microlunatus* and *Friedmanniella*. Annotated ANI and AAI values are in relation to ‘Fern CH3Cl SIP LEAF bin co3’. (C) Phylogenetic tree of *Myxococcales* species. Two bins classified as related to *Sorangium* (marked in green) from fraction SOIL were placed in a tree with 16 reference genomes of Deltaproteobacteria. Tree was annotated with ANI and AAI values in relation to ‘Fern CH3Cl SIP SOIL bin s1’; AAI values significantly under 50 and ANI values significantly under 70 were not computed and are annotated with NA.

Figure 3. Overview of the metabolic network of *Shewanella* sp. strain ANAS-2. The diagram illustrates the central carbon metabolism, electron transfer, and various other metabolic pathways. Key components include:

- Central carbon metabolism:** Glycolysis, Pentose-Phosphate pathway, Gluconeogenesis, TCA cycle, and various intermediates like pyruvate, acetate, and acetyl-CoA.
- Electron transfer:** Fumarate, Succinate, succinate dehydrogenase, quinone oxidoreductase, and c-type cytochrome.
- Stress response:** osmotic stress response (osmoregulated periplasmic glucans/ glycine betaine), superoxide dismutase (Fe, Cu-Zn), peroxidase, catalase, glutathione, and periplasmic stress response.
- Aromatic compound metabolism:** quinate, 3-dehydroshikimate, protocatechuate, benzoate, catechol, oxadipyl-CoA, acetyl-CoA, and hydroxybenzoate.
- Arginine metabolism:** arginine, ornithine, urea, citrulline, putrescine, and L-proline.
- Co-factors:** cobalamin biosynthesis, folate biosynthesis, and molybdenum cofactor biosynthesis.

Due to technical limitations, the caption for figure 3 can be found in the manuscript file.

Pan-genome analysis of 13 *Methylobacterium* genomes of isolated strains and two $[^{13}\text{C}]$ -CH₃Cl-enriched fern leaf MAGs. (A) Fractional pan genome representation. (B) UpSet plot of the pan-genome of the 15 *Methylobacterium* genomes. The metagenomic bin 'Fern CH₃Cl SIP LEAF bin s3' was chosen as reference genome. The analysis was carried out using the "Efficient Database framework for comparative Genome Analyses using BLAST score Ratios" (EDGAR) platform.