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^{13}C-chloromethane incubations provide evidence for novel bacterial chloromethane degraders in a living tree fern

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Summary

Chloromethane (CH_3Cl) is the most abundant halogenated 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. We identified and characterized CH_3Cl-degrading bacteria 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 metagenomics. 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 uncharacterized methylotrophic *cmuA*-independent pathway may drive CH_3Cl degradation in the investigated tree ferns.

Introduction

Chloromethane (CH_3Cl, methyl chloride) is the most abundant chlorinated volatile organic compound (VOC) in the Earth's atmosphere. It occurs with an average global mixing ratio of ~ 553 ± 5 pptv, and exhibits 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 (Keppler *et al*., 2005b; Xiao *et al*., 2010). Natural emissions of CH_3Cl have been reported from grasslands (Rhew and Abel, 2007; Teh *et al*., 2008; Rhew, 2011), plants (Yokouchi *et al*., 2002; Hamilton *et al*., 2003; Blei *et al*., 2010; Yokouchi *et al*., 2015; Ul Haque *et al*., 2017; Jaeger *et al*., 2018b), salt marshes (Rhew *et al*., 2000; Valtanen *et al*., 2009; Keppler *et al*., 2020), peatlands (Dimmer *et al*., 2001), ventilated and waterlogged soils (Keppler *et al*., 2000; 2001; Redeker *et al*., 2000) and oceans (Moore *et al*., 1996). Ferns are especially potent natural emitters of CH_3Cl (Yokouchi *et al*., 2002; 2015; Jaeger *et al*., 2018b). Whereas the physiological function of enzyme-produced chloromethane *in planta* remains to be demonstrated, CH_3Cl emissions in *Arabidopsis* involve the tissue damage increased expression of the gene encoding *S*-adenosylmethionine-dependent methyltransferase harmless to the ozone layer (Nagatoshi and Nakamura, 2009).

The main sink for atmospheric CH_3Cl is abiotic degradation by photochemically formed hydroxyl radicals (Khalil and Rasmussen, 1999) with an estimated global annual rate of approximately 2.5 to 3.4 Tg (Carpenter *et al*., 2014). Microbial degradation in soils is another significant global sink of CH_3Cl (McAnulla *et al*., 2001; Harper and Hamilton, 2003; Miller *et al*., 2004; Jaeger

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et al., 2018a). Current estimates are highly uncertain, ranging from 0.1 to 1.6 Tg per year (Harper and Hamilton, 2003; Keppler *et al*., 2005a; Carpenter *et al*., 2014). Methylotrophic bacteria that are able to use CH_3Cl as the sole carbon and energy source for growth have been isolated from diverse environments, including soils (Doronina *et al*., 1996; McAnulla *et al*., 2001; Miller *et al*., 2004), wastewater sludge (Hartmans *et al*., 1986; Traunecker *et al*., 1991; Freedman *et al*., 2004), seawater (Schäfer *et al*., 2005; Nadalig *et al*., 2014), and the phyllosphere (aerial parts of the plants) (Doronina *et al*., 2004; Nadalig *et al*., 2011). The ubiquitous presence of such bacteria in soil and the phyllosphere implies that they play a more important role in the mitigation of CH_3Cl emissions than previously thought (Bringel *et al*., 2019; Keppler *et al*., 2020).

The bacterial CH_3Cl utilization (*cmu*) pathway was characterized in detail in *Methylorubrum extorquens* (previously *Methylobacterium extorquens*) strain CM4 (Vannelli *et al*., 1999; Roselli *et al*., 2013). A corrinoid- and tetrahydrofolate (H_4F)-dependent methyltransferase system, encoded by the genes *cmuA* and *cmuB* (Studer *et al*., 1999; 2001), respectively, facilitates the dehalogenation of CH_3Cl. Since the *cmuA* gene is strongly conserved in known CH_3Cl-degrading bacteria (Nadalig *et al*., 2011), it has been used as a metabolic marker gene to study the biodiversity of CH_3Cl-degrading microorganisms in soils and plants (McAnulla *et al*., 2001; Miller *et al*., 2004; Schäfer *et al*., 2007; Cox *et al*., 2012; Chaignaud *et al*., 2018a). Phylogenetically diverse *cmu*-dependent CH_3Cl degraders have been isolated and belong to the genera *Acetobacterium*, *Aminobacter*, *Hyphomicrobium*, *Leisingera*, *Pseudomonas*, *Methylobacterium*/*Methylorubrum* and *Roseovarius* (Nadalig *et al*., 2011). However, the *cmuA* biomarker is seldom found in metagenomes (Jaeger *et al*., 2018a,b; Bringel *et al*., 2019). In addition, *cmu* genes are absent in the genomes of some sequenced CH_3Cl utilizers (Buddruhs *et al*., 2013; Bringel *et al*., 2019) such as marine bacterial strains (*Leisingera methylohalidivorans* MB2) (Nadalig *et al*., 2014), which points towards the existence of yet uncharacterized pathways for CH_3Cl degradation. These microorganisms have to adapt to several stresses enabling growth on CH_3Cl, for instance increased intracellular levels of protons and chloride, higher needs for the production of cofactors (H_4F and cobalamin-related compounds) and the regulation of their metabolism for efficient CH_3Cl utilization (Roselli *et al*., 2013; Michener *et al*., 2016; Chaignaud *et al*., 2017; Bringel *et al*., 2019).

Chloromethane degradation of plant microbiomes has been studied in *Arabidopsis thaliana* (Ul Haque *et al*., 2017) and ferns such as *Osmunda regalis*, *Cyathea cooperi* and *Dryopteris filix-mas* (Jaeger *et al*., 2018b). The *cmuA* gene was found to be associated with living *A. thaliana* plants in a previous study (Ul Haque *et al*., 2017). However, the CH_3Cl degradation and its microbial biodiversity were not assessed. In a subsequent study, both the production and consumption of CH_3Cl associated with three tropical fern species were investigated by cutting off plant material (Jaeger *et al*., 2018b). This may represent a stressful and potentially unnatural physiological condition, especially given that plants release such VOCs after physical damage (Piesik *et al*., 2006; 2010; Wang *et al*., 2009). Therefore, cut-off plant material might release increased amounts of CH_3Cl compared to intact plants. Microbial 16S rRNA and *cmuA* gene diversity analysis did not allow us to correlate CH_3Cl degradation with the microorganisms detected, nor could the corresponding metabolic pathways be identified (Jaeger *et al*., 2018b).

In the current study, we used an integrative experimental approach to identify the active CH_3Cl-degrading members of the microbiome associated with the rhizospheric soil and the phyllosphere of intact tree ferns known to produce CH_3Cl (Jaeger *et al*., 2018b), by applying a combination of DNA stable isotope probing (DNA-SIP) and metagenomics (Chen and Murrell, 2010; Kröber and Eyice, 2019) under conditions closely matching the native environment.

Results and discussion

The tree fern Cyathea australis *and its associated microbiome are capable of CH_3Cl degradation*

Cut-off leaves from the tree fern *C. australis* are capable of CH_3Cl degradation (Jaeger *et al*., 2018b). However, it has not been investigated whether intact plants behave in the same way and degrade CH_3Cl. In addition, the active members of the tree fern-associated microbiome involved in CH_3Cl degradation have not been resolved. Therefore, six tree ferns were incubated with either [^{13}C]-CH_3Cl or [^{12}C]-CH_3Cl in plant growth chambers (Supplementary Fig. S1) and CH_3Cl concentrations were monitored over a time period of 19 days. On the first day of incubation, 200 ppm of [^{13}C]-CH_3Cl or [^{12}C]-CH_3Cl was added to the incubation chambers (Fig. 1). Rapid consumption was evident, and most likely due to adsorption to the soil–plant interface. However, in the following 18 days, steady degradation of CH_3Cl was observed following daily CH_3Cl addition, especially from day 12 onwards, with an average CH_3Cl consumption of 7 to 15 ppm d^{-1} (Fig. 1). To

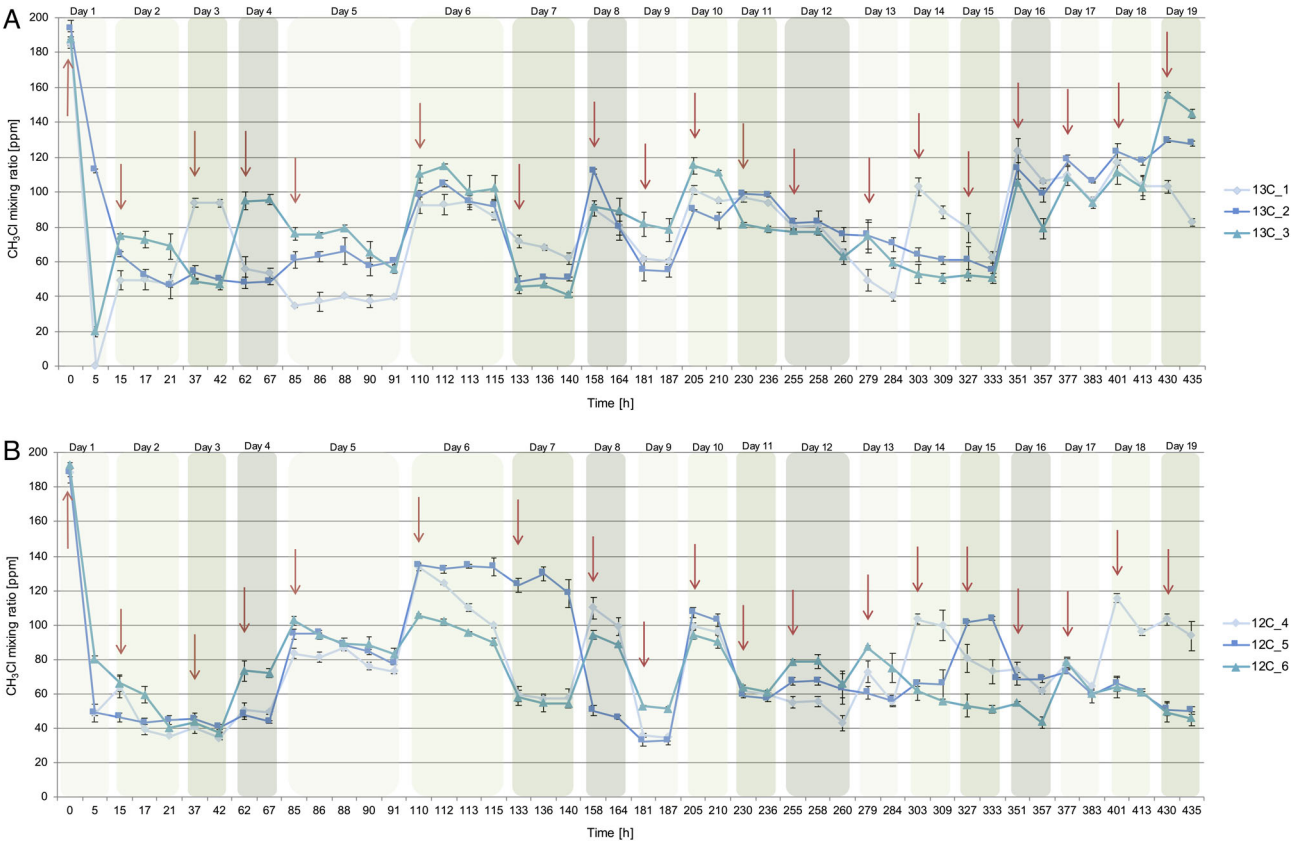

Fig. 1. Monitoring of the SIP-incubation of ferns. Incubations were carried out over 19 days with three biological replicates of intact fern plants in gas-tight incubation chambers per tested condition. At the start of the SIP-incubation, 20 ml CH_3Cl was added to the chambers (initial mixing ratio of ~ 200 ppm) 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 CH_3Cl was added to the chambers (initial mixing ratio of ~ 100 ppm). Red arrows indicate the point of CH_3Cl addition.
A. ^{13}C-labelled CH_3Cl.
B. ^{12}C-labelled CH_3Cl. [Color figure can be viewed at wileyonlinelibrary.com]

further prove whether the ^{13}C was incorporated by the plant or by epiphytic microorganisms, $\delta^{13}C$ values of bulk leaves were determined using EA/IRMS. Stable carbon isotope values (Supplementary Fig. S2) revealed an increase in the [^{13}C]-CH_3Cl incubated samples from the start of the experiment (^{13}C T_0, approximately −29‰) to the end of the experiment (T_{end}, approximately −7.5‰) in the incubated ferns 1 and 3 (Supplementary Fig. S1), demonstrating the incorporation of ^{13}C. Washed leaves (^{13}C T_{end} washed) exhibited much lower deviations of $\delta^{13}C$ values (Supplementary Fig. S2; ferns 1 and 3) indicating incorporation of ^{13}C mainly by the microorganisms living on leaf surfaces. Thus, our results proved that intact living tree fern plants of the species *C. australis* and their associated microbiota are capable of CH_3Cl degradation and that the microorganisms play an important role in the degradation of this chlorinated VOC.

Methylobacterium is an abundant genus with CH_3Cl-degrading bacteria of the fern phyllosphere, and active CH_3Cl-utilizing bacteria differ between the fern phyllosphere and rhizosphere

The identification of ^{13}C-labelled microorganisms and hence those microorganisms that incorporated CH_3Cl-derived carbon was achieved by SIP of six fern plants, collecting DNA from ‘heavy’ and ‘light’ fractions from phyllosphere and rhizosphere samples and T-RFLP (data not shown). DNA fractions with labelled bacteria were used for subsequent 16S rRNA gene amplicon sequencing (Supplementary Fig. S3).

Methylobacterium was identified as the most abundant ^{13}C-labelled genus in the phyllosphere (leaf) of *C. australis* (Fig. 2). It is well-established that *Methylobacterium* strains are abundant in the phyllosphere of a variety of plants (Bringel and Couée, 2015), including

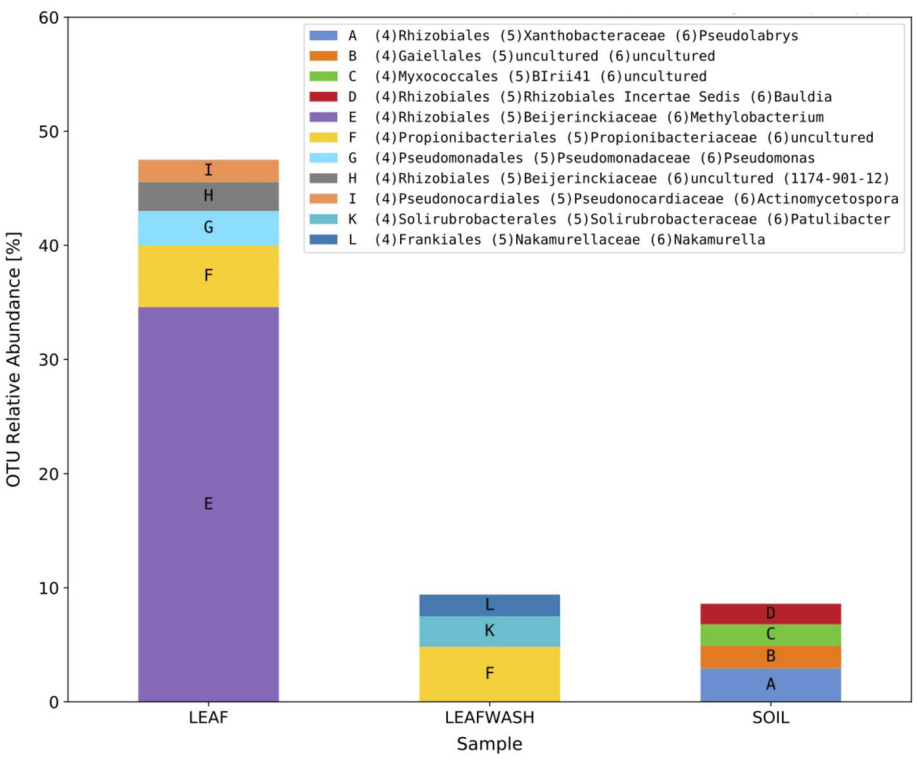

Fig. 2. Relative abundance of ^{13}C-labelled bacterial OTUs at the genus level from fraction samples LEAF, LEAFWASH and SOIL. OTUs were classified as 'labelled' by the following criteria: (i) ^{13}C-heavy > ^{12}C-heavy; (ii) ^{13}C-heavy > ^{13}C-light; (iii) ^{13}C-[heavy-light] > ^{12}C-[heavy-light]; and (iv) ^{13}C[heavy-light] > threshold, with threshold = 0.005%. The taxonomic affiliation of each labelled OTU is indicated at the family, order and genus levels (4–6 respectively), with a specific colour and capital letter (see legend box). [Color figure can be viewed at wileyonlinelibrary.com]

A. thaliana (Delmotte *et al*., 2009; Knief *et al*., 2010a,b; Ul Haque *et al*., 2017), soybean (Raja *et al*., 2008; Delmotte *et al*., 2009; Minami *et al*., 2016), clover (Delmotte *et al*., 2009), cotton (Raja *et al*., 2008), maize (Raja *et al*., 2008), sunflower (Raja *et al*., 2008) and rice (Madhaiyan *et al*., 2009), and that representatives of the genus *Methylobacterium* can metabolize CH_3Cl via the CH_3Cl utilization pathway (*cmu*) (Nadalig *et al*., 2011). Whereas *Methylobacterium* has been identified previously in the phyllosphere of three different CH_3Cl-producing or CH_3Cl-degrading ferns, *O. regalis*, *C. cooperi* and *D. filix-mas* (Jaeger *et al*., 2018b), 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), ^{13}C-labelled microorganisms other than *Methylobacterium*-related microorganisms included members of the families *Pseudomonadaceae* (leaf), *Propionibacteriaceae* and *Solirubrobacteraceae* (leaf wash), among others (Fig. 2). *Pseudomonadaceae* were detected in a CH_3Cl-degrading mixed culture (Nadalig *et al*., 2011), but their role in CH_3Cl degradation was not resolved. 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* (Vorholt, 2012; Karasov *et al*., 2020), grapevine (Perazzolli *et al*., 2020), maize (Chen *et al*., 2018) and lettuce (Zwielehner *et al*., 2008). *Pseudomonadaceae* have been associated with ferns involved in CH_3Cl production and consumption (Jaeger *et al*., 2018b), and some isolates of the genus *Pseudomonas* capable of CH_3Cl degradation have been reported (Freedman *et al*., 2004; Nadalig *et al*., 2011; Jaeger *et al*., 2018b). In contrast, representatives of *Propionibacteriaceae* and *Solirubrobacteraceae* capable of CH_3Cl degradation have not been described thus far. *Friedmaniella* members closely related to *Propionibacteriaceae* have been detected in the phyllosphere of magnolia trees (Jackson and Denney, 2011), hemlock plants (Rogers *et al*., 2018), in grasses (Aydogan *et al*., 2020), shrubs (Santana *et al*., 2016) and hardwood forest trees (Herrmann *et al*., 2020). Bacteria of the family *Solirubrobacteraceae* can be strictly aerobic and chemoorganotrophic (Albuquerque and da Costa, 2014), and have been identified in the phyllosphere of grasses (Aydogan *et al*., 2020), shrubs (Santana *et al*., 2016) and in olive trees (Fausto *et al*., 2018).

Compared to the phyllosphere, divergent taxa were enriched in the ^{13}C-'heavy' DNA of rhizosphere samples (Fig. 2), e.g., from *Xanthobacteraceae* and *Myxococcales*, but a potential association of these families with degradation of CH_3Cl has not yet been reported. *Xanthobacteraceae* were enriched in a CH_3Cl-incubated forest soil in a previous study (Jaeger *et al*., 2018a), but they were also detected in the phyllosphere of diverse plants (Moore-Colyer *et al*., 2018; Ottesen *et al*., 2019; Aydogan *et al*., 2020). Notably, in the context of chloromethane utilization, *Xanthobacter autotrophicus*, a member of the family *Xanthobacteraceae*,

is a methylotrophic organism capable of growing on methanol (Wiegel *et al*., 1978; Beck *et al*., 2015). *Myxococcales* were also detected in the phyllosphere of several tree species (Jackson and Denney, 2011; Yashiro *et al*., 2011; Laforest-Lapointe *et al*., 2016). Thus, the living fern tree is host to a variety of potential CH_3Cl utilizers, most of which differ from previously characterized representatives.

Tree fern metagenome-assembled genomes (MAGs) from [^{13}C]-CH_3Cl DNA are closely related to Methylobacterium*,* Friedmanniella *and* Sorangium

Metagenomics of three [^{13}C]-CH_3Cl-labelled fern phyllosphere and rhizosphere samples were carried out to complement and expand the 16S rRNA amplicon sequencing analysis. Of the 34 MAGs, 3 carried 16S rRNA-encoding genes (Supplemental Table S1). To further elucidate the phylogeny of the [^{13}C]-CH_3Cl-labelled microorganisms, phylogenetic trees were calculated using the MAGs 'Fern CH_3Cl SIP LEAF bin co1', 'Fern CH_3Cl SIP LEAF bin s3', 'Fern CH_3Cl SIP LEAF bin co3', 'Fern CH_3Cl SIP LEAFWASH bin co7', 'Fern CH_3Cl SIP SOIL bin s1' and 'Fern CH_3Cl SIP SOIL bin s7' (Fig. 3). Metagenome-assembled genomes 'Fern CH_3Cl SIP LEAF bin co1' and 'Fern CH_3Cl SIP LEAF bin s3' clustered within the genus *Methylobacterium* (Fig. 3A).

The MAG 'Fern CH_3Cl SIP LEAF bin s3' was more closely related to various strains of *Methylobacterium* that were isolated from phyllosphere samples (Supplementary Table S2). Bins 'Fern CH_3Cl SIP LEAF bin co3' and 'Fern CH_3Cl SIP LEAFWASH bin co7' clustered more closely to the genus *Friedmaniella* of the family *Propionibacteriaceae* (Fig. 3B). Bins 'Fern CH_3Cl SIP SOIL bin s1' and 'Fern CH_3Cl SIP SOIL bin s7' had the closest but more distant similarity with *Sorangium* (Fig. 3C), possibly representing a new genus within the order *Myxococcales*. Taken together, the phylogeny based on MAGs supported the findings of ^{13}C-labelled OTUs based on the results from 16S rRNA gene amplicon sequencing.

cmuA genes are not present in metagenomes and MAGs

The *cmu* pathway is the only characterized pathway for the utilization of CH_3Cl (Bringel *et al*., 2019). Of the *cmuA*- and *cmuB*-encoded methyltransferases of CH_3Cl dehalogenase, the *cmuA* gene is the most conserved and has been frequently used as the *cmu* pathway gene marker in environmental studies (Chaignaud *et al*., 2018b). The MAGs and metagenomes were screened for the presence of *cmuA*. On the basis of BLAST searches and prokka annotations, no *cmuA* homologues were identified in the MAGs. Unassembled short metagenomic reads were also screened for the presence of *cmuA* using shortBRED and GraftM. Unlike reference genes for methanol oxidation (*xoxF* and *mxaF*) which were detected in leaf, leafwash and soil samples (data not shown), the *cmuA* gene was not detectable in leafwash and leaf samples using either ShortBRED or GraftM. 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. Only 0.0001% of the metagenomic reads from soil samples could be aligned to *cmuA* reference sequences using GraftM. However, these sequences did not cover full-length *cmuA* reference sequences. This lack of evidence for the presence of *cmuA* suggests that CH_3Cl degradation in tree fern samples occurs by alternative and still unresolved enzymes.

CH_3Cl from tree ferns is degraded by yet unidentified enzymes

The genome sequences retrieved from ^{13}C-labelled MAGs offered an opportunity to investigate the metabolic traits of these bacteria. Metabolic reconstruction was conducted with the two MAGs related to *Methylobacterium* ('Fern CH_3Cl SIP LEAF bin co1' and 'Fern CH_3Cl SIP LEAF bin s3'), one of the bins related to *Friedmaniella* ('Fern CH_3Cl SIP LEAF bin co3') and one of the bins related to *Sorangium* ('Fern CH_3Cl SIP SOIL bin s1').

Beyond the already noted lack of *cmuA*, analysis of these MAGs also revealed the absence of the other essential genes of the *cmu* pathway *cmuB* (Vannelli *et al*., 1999) and *metF2* (Studer *et al*., 2002; Michener *et al*., 2016) (Fig. 4), i.e., confirming that CH_3Cl-degrading bacteria on tree ferns may use other pathways for CH_3Cl degradation. In contrast, *folD* genes encoding the bifunctional enzyme methylene-H_4F dehydrogenase/methenyl-H_4F-cyclohydrolase and *purU* encoding the formyl-H_4F deformylase which allow downstream processing of one-carbon units derived from CH_3Cl (Vannelli *et al*., 1999) were detected in three and two of the four MAGs respectively (Fig. 4). Similarly, other genes associated with one-carbon metabolism including *mtdA* for methylene-H_4F dehydrogenase, *fch* for methenyl-H_4F cyclohydrolase and *ftfL* for formate-H_4F ligase were also detected in *Methylobacterium*-related MAGs as expected (Vuilleumier *et al*., 2009; Marx *et al*., 2012; Kwak *et al*., 2014) (Fig. 4; Supplementary Table S3). In many methylotrophs, methylene-H_4F 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

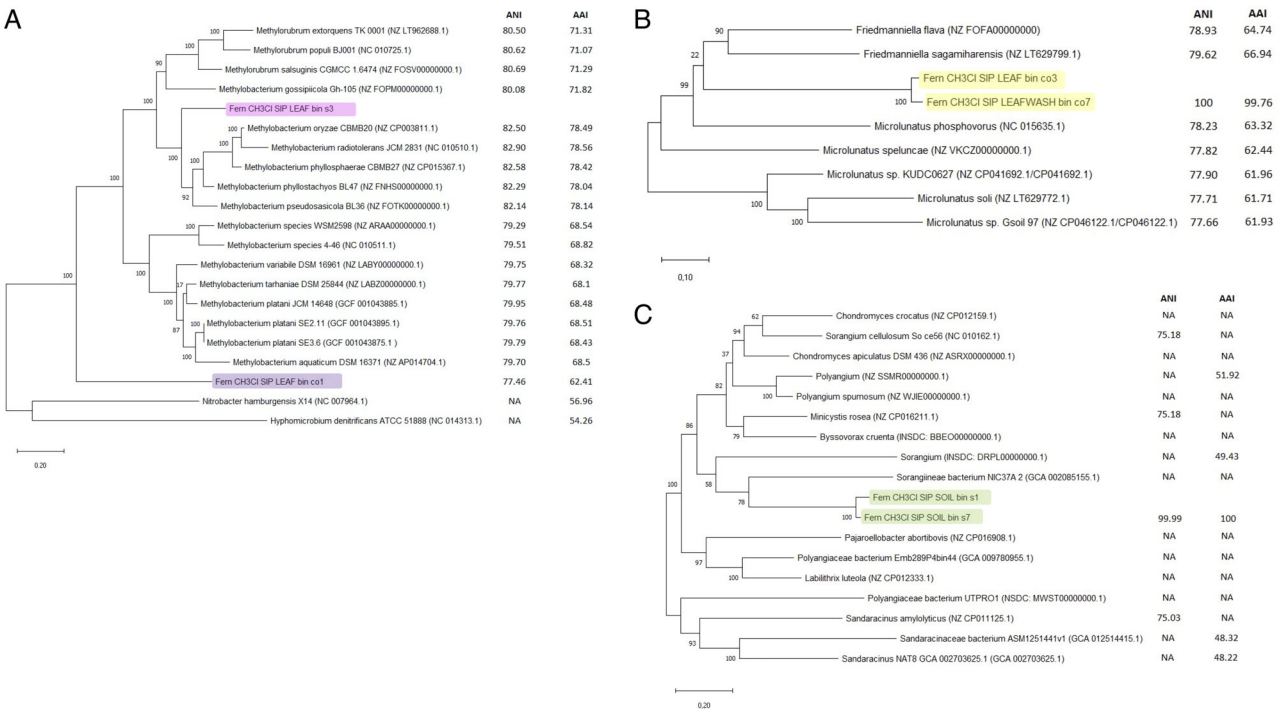

Fig. 3. Maximum-likelihood phylogenetic trees showing the position of selected MAGs within a tree of closely related reference genomes. Multiple sequence alignments of concatenated marker genes were constructed using PhyloPhlAn 3.0. Phylogenetic analysis was performed based on a matrix of pairwise distances 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. The tree was annotated with ANI and AAI values in relation to 'Fern CH_3Cl SIP LEAF bin s3'.

B. Phylogenetic tree of *Propionibacteriaceae* species. Two bins classified as related to *Propionobacteriaceae* (marked in yellow) from fractions LEAF and LEAFWASH were placed in a tree with seven 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*. The 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. [Color figure can be viewed at wileyonlinelibrary.com]

(Fig. 4; Supplementary Table S3). Furthermore, the last step in one-carbon compound oxidation is the conversion of formate to CO_2, which involves the *fdh*-encoded formate dehydrogenase found in the two MAGs related to *Methylobacterium* and the MAG related to *Friedmaniella*. Thus, the analyses of the MAGs obtained from heavy DNA confirmed that they possess the genetic potential for one-carbon compound oxidation and suggested that H_4F is a one-carbon-group carrier in all four MAGs (Fig. 4; Supplementary Table S3). While these data clearly suggest that methylene-H_4F is also the entry point to the central metabolism in tree fern MAGs, how CH_3Cl is dehalogenated by the corresponding strains remains unknown. Moreover, it is puzzling that MAGs related to species not previously described as methylotrophs (e.g., *Friedmaniellea* and *Sorangium*) and that lack reference genes for primary oxidation of one-carbon compounds (Supplemental Table S3) were [^{13}C]-chloromethane labelled. One hypothesis is that genes encoding methylotrophy 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 uncharacterized genes for methylotrophic growth to date. A third option could be that these microorganisms cross-feed on the by-products of CH_3Cl degradation, e.g., *Methylobacterium* or other CH_3Cl degraders living in the fern phyllosphere.

The MAG more closely related to *Methylobacterium* ('Fern CH_3Cl SIP LEAF bin s3') additionally harboured the genes for the tetrahydromethanopterin (H_4MPT)-dependent one-carbon compound oxidation pathway. The last step of the one-carbon compound oxidation pathway, the oxidation of formate to CO_2, is mediated by formate dehydrogenase (*fdh*), which was detected in three out of the four MAGs. The gene encoding for the formaldehyde-activating enzyme (*fae*), which catalyzes the reduction of formaldehyde with H_4MPT, was only detected in the MAG more closely related to

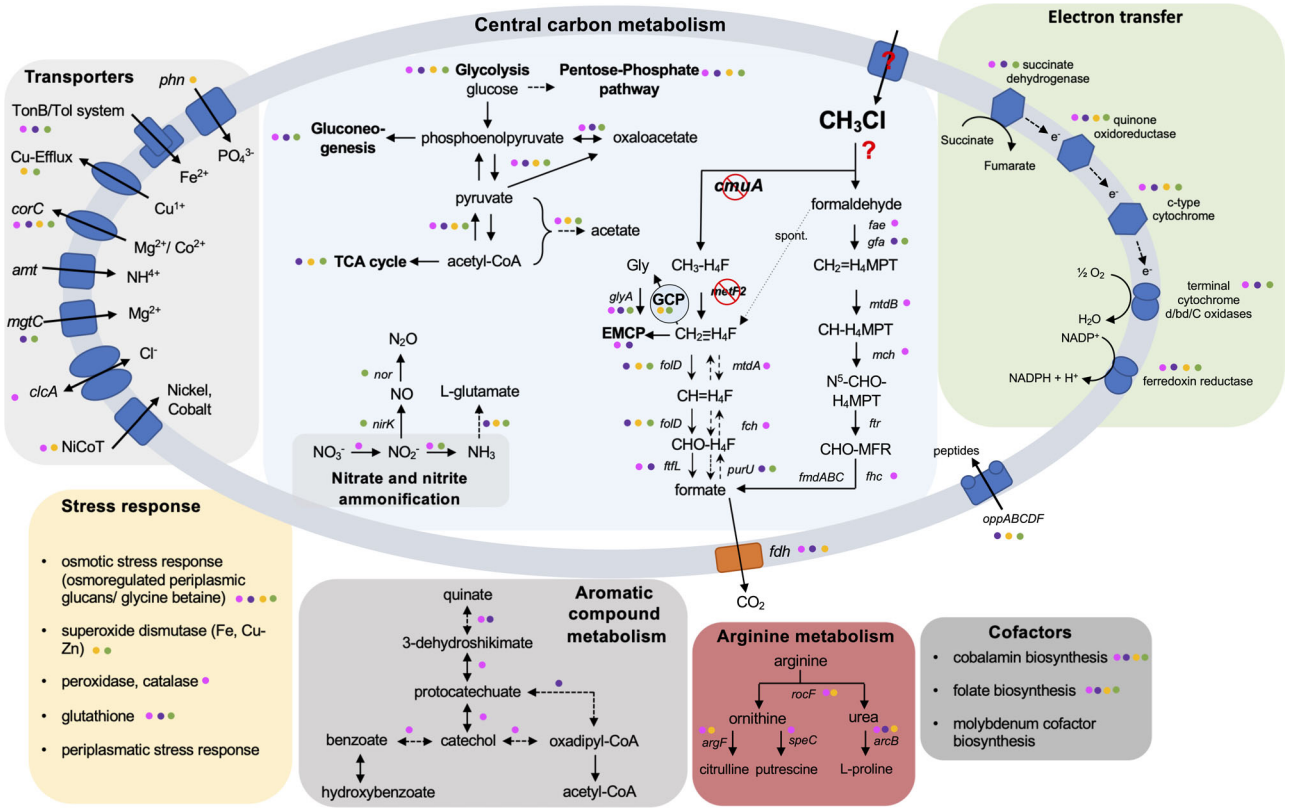

Fig. 4. Metabolic reconstruction of [^{13}C]-CH_3Cl-labelled metagenome-assembled genomes. A coloured dot next to a gene or pathway shows the presence of this pathway or gene in the corresponding genome. 'Fern CH_3Cl SIP LEAF bin s3' (MAG related to *Methylobacterium*) is represented by a pink dot, 'Fern CH_3Cl SIP LEAF bin co1' (MAG related to *Methylobacterium*) is highlighted in purple, 'Fern CH_3Cl SIP LEAF bin co3' (MAG related to *Friedmaniella*) is represented with a yellow dot and 'Fern CH_3Cl SIP SOIL bin s1' (MAG related to *Sorangium*) is highlighted in green. Pathways such as glycolysis or the TCA cycle are shown in bold. The chloromethane degradation pathway would require the cleavage of the C-Cl bond involving a yet uncharacterized dehalogenases, different from CmuAB. This essential chloromethane degradation step would dehalogenate chloromethane directly or indirectly if the substrate is a metabolite derived from a previous activating reaction step. Reactions of one-carbon compounds involve C_1-carriers (common ones include biotin, tetrahydrofolate, corrinoids, S-adenosyl methionine, coenzyme M, methanofuran and tetrahydromethanopterin) (Ujungdahl, 1987), and a dedicated C_1-carrier associated with chloromethane assimilation of this yet uncharacterized pathway would also be needed. Analysis of the Fern-associated *Methylobacterium* MAGs, like other *Methylobacterium* genomes (Vuilleumier *et al*. 2009), harbours the genetic repertoire to use methylene tetrahydrofolate (H_4F) as the entry point of reduced one-carbon compounds into the serine cycle for carbon assimilation. In contrast, few genes related to tetrahydromethanopterin (H_4MPT) have been detected in the Fern-associated *Methylobacterium* MAGs, suggesting that H_4F would be a more likely cofactor of chloromethane oxidation towards formate production. [Color figure can be viewed at wileyonlinelibrary.com]

Methylobacterium ('Fern CH_3Cl SIP LEAF bin s3') suggesting that the other three MAGs lack the H_4MPT pathway for formaldehyde oxidation (Fig. 4; Supplementary Table S3).

Gene content for halide metabolism in MAGs

Halide ions such as Cl^-, Br^- and I^- are substrates, products or both for dehalogenases. Dehalogenases can hydrolyze a broad range of haloalkanes to the corresponding alcohols accompanied by the release of protons and halide anions. Searches using the BRENDA database led to the detection of putative genes encoding halogenases (3) and dehalogenases (40) in MAGs obtained from ^{13}C-labelled DNA (Supplemental Table S4), which may potentially be associated with dehalogenation of CH_3Cl. Further experiments are required to resolve this open issue.

Comparative genomics of Methylobacterium*-related MAGs*

Methylobacterium represents a well-characterized bacterial genus with regard to the utilization of one-carbon compounds, including CH_3Cl. Thus, an in-depth analysis of the two MAGs most closely related to *Methylobacterium* was conducted. Fern CH_3Cl SIP LEAF bin c1 and Fern CH_3Cl SIP LEAF bin s3 were compared with representative complete genomes of other members of the genus *Methylobacterium* including *Methylorubrum* (Fig. 5; Supplementary Table S2) to identify genes present in all strains (core genes), two or more strains

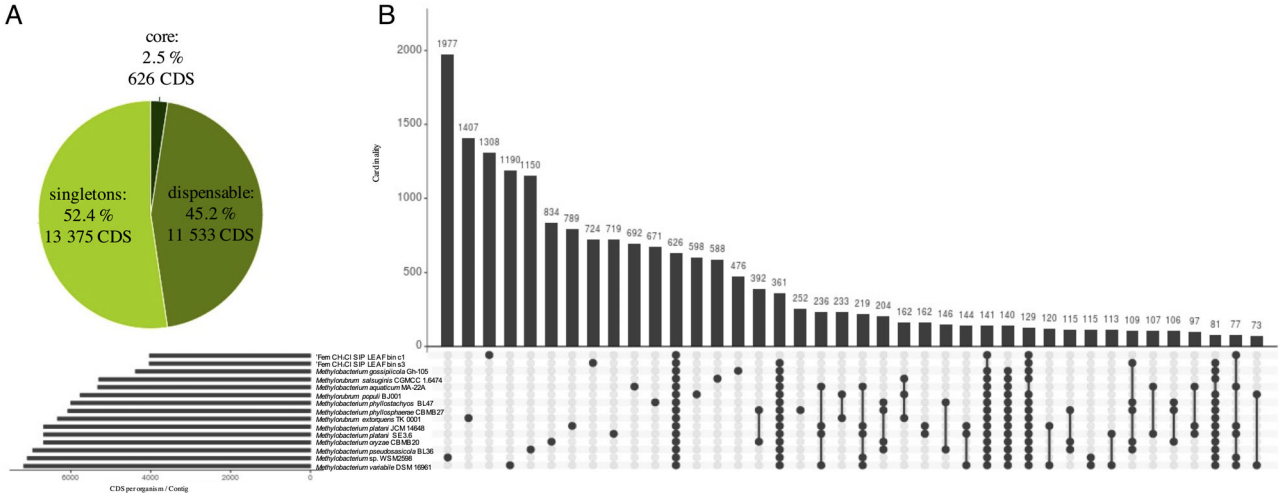

Fig. 5. Pangenome analysis of genomes from 10 *Methylobacterium*-isolated strains, 3 representatives of the closely related clade *Methylorubrum* and two [^{13}C]-CH_3Cl-enriched fern leaf MAGs.
A. Fractional pan genome representation.
B. UpSet plot of the pangenome of the 15 *Methylobacterium* genomes. The metagenomic bin 'Fern CH_3Cl SIP LEAF bin s3' was chosen as the reference genome. The analysis was carried out using the 'Efficient Database framework for comparative Genome Analyses using BLAST score Ratios' (EDGAR) platform. [Color figure can be viewed at wileyonlinelibrary.com]

(accessory or dispensable genes), and only in one strain (singleton genes). According to pangenome analysis of 15 *Methylobacterium* genomes (including three *Methylorubrum* 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; Fig. 5A). An UpSet plot (Lex *et al*., 2014) shows the number of CDSs in the core genome, the singletons and the number of CDSs shared by the different *Methylobacterium* genomes (Fig. 5B). These analyses confirmed the phylogeny of the phylogenetic tree (Fig. 3A), i.e., it revealed a high similarity between *Methylobacterium phyllosphaerae* and *Methylobacterium oryzae* (392 uniquely shared CDSs) and between *Methylorubrum populi* and *M. extorquens* (233 uniquely shared CDSs). Genes encoding the chloromethane utilization pathway (*cmuA* and *cmuB*) and enzymes that use or form halides (Supplementary Table S4) and were present in the two MAGs obtained in this work do not belong to the core genome shared by the 15 *Methylobacterium* strains (including *Methylorubrum*).

Conclusions

Chloromethane is produced in significant quantities by certain tree ferns (Yokouchi *et al*., 2015; Jaeger *et al*., 2018b), and these plants have therefore been suggested to be important emitters of this compound into the atmosphere (Yokouchi *et al*., 2002). However, microorganisms living in close association with these plant hosts might substantially mitigate fluxes of CH_3Cl into the atmosphere. In this context, recent isotopic mass balances of atmospheric CH_3Cl, including isotopic analysis of carbon (Bahlmann *et al*., 2019), hydrogen (Keppler *et al*., 2018) and chlorine (Keppler *et al*., 2019), point to large unidentified terrestrial sinks and sources of CH_3Cl. The present study reveals the complexity of CH_3Cl budget-associated processes. In addition, the observed lag in the onset of CH_3Cl degradation in our experiments may point to physiologic adaptation. Hence, we demonstrated that carbon of [^{13}C]-CH_3Cl was incorporated into the DNA of bacteria colonizing the leaf surface of tree ferns, and not into plant biomass or microbial endophytes, since insignificant ^{13}C incorporation was found in washed leaves. Moreover, we identified key microorganisms involved in CH_3Cl degradation in the phyllosphere and rhizosphere. These were closely related to *Methylobacterium*, *Friedmanniella* and *Sorangium*, all known to be inhabitants of the phyllosphere. Of these genera however, only *Methylobacterium* (including *Methylorubrum*, e.g., *M. extorquens* strain CM4) strains were previously described to be capable of CH_3Cl degradation. The canonical *cmu*-dependent CH_3Cl degradation pathway could not be detected in the heavy MAGs and metagenomes of ^{13}C-labelled DNA. Our findings suggest that metabolic degradation pathways other than the *cmu* pathway were involved in the degradation of CH_3Cl in the microbiome of the tree fern species *C. australis*. Hence, our study very likely suggests that CH_3Cl emissions from living plants are 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 CH_3Cl degradation pathways. Nonetheless, a combination of isotope labelling with meta-omics is a promising approach to describe and characterize *in planta* active metabolic traits of microbiota members without their separation from or the killing of the plant host. Finally, the application of triple element isotope analysis (Keppler *et al*., 2019) might provide further clues on the global relevance of certain plant species and their associated microbiomes for the regulation of CH_3Cl emissions into the atmosphere.

Experimental procedures

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

Tree ferns (*C. australis*) with a shoot height of approximately 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 3 months before starting the DNA-SIP experiment on August 17, 2017. Incubations were carried out in gas-tight acryl-glass plant-growth 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 airtight butyl rubber stoppers (Merck Millipore Corporation, Darmstadt, Germany) for injection of CH_3Cl (Supplementary Fig. S1A). In addition, ventilators were installed inside the chambers to ensure an even distribution of gas throughout the chamber. Gas tightness was tested by injecting CO_2 into each chamber and measuring the concentration over time with CO_2 loggers (data not shown). Since the aim of the experiment was to label CH_3Cl-degrading microorganisms associated with fern plants, the sidewalls of the chambers were covered with aluminium foil (Supplementary Fig. S1B) to reduce photosynthesis and thus microbial CO_2 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 Fig. S1B). The tree fern plants were incubated with either ^{13}C-labelled CH_3Cl (Campro Scientific GmbH, Berlin, Germany; hereafter [^{13}C]-CH_3Cl) or with CH_3Cl at natural abundance (99%, Linde GmbH, Pullach, Germany; hereafter [^{12}C]-CH_3Cl). Each labelling period lasted for approximately 6 h 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 CH_3Cl ([^{13}C]-CH_3Cl or [^{12}C]-CH_3Cl) 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 CH_3Cl ([^{13}C]-CH_3Cl or [^{12}C]-CH_3Cl) 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 h 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). ^{13}C-labelling [^{13}C]-CH_3Cl and [^{12}C]-CH_3Cl was carried out for 19 days. Soil humidity and salinity of the fern soil were monitored throughout the experiment (data not shown).

Gas chromatography

Chloromethane was measured in the headspace gas by injecting 100 µl of a headspace gas sample into an 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 analyzed with Openchrome® (Lablicate GmbH, Germany). CH_3Cl concentrations were calculated by regression analysis based on a 5-point calibration curve.

Analysis of the carbon mass fraction (C %) and differential stable carbon isotope ratio ($\delta^{13}C$ 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 24 h at 60°C and finely ground using a vibrating disc mill (RS200, Retsch, Germany). Stable isotope ratios ($^{13}C/^{12}C$) 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). Stable carbon isotope ratios ($\delta^{13}C$) are expressed in permil

(‰) relative to the international standard, as defined by the equation:

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

where R_{Sample} is the isotopic ratio ($^{13}C/^{12}C$) of the sample and $R_{Reference}$ is the known isotopic ratio of the standard. $\delta\ ^{13}C$ values were normalized to the international Vienna Pee Dee Belemnite scale by analyses of the international standards USGS40 and USGS41 (L-glutamic acid) within the sequence (Coplen, 2011). Precision, defined as the standard deviation ($\pm\ 1\sigma$) of the laboratory control standard along the run was lower than $\pm$ 0.2‰ for C.

For calculation of the element mass fraction C (percentage, %) of plant biomass, different amounts of the laboratory standard (apple leaves, $\delta\ ^{13}C = -27.11$‰, C = 48.12%) were analyzed 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 lower than $\pm$ 0.5% for C.

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

After labelling, phyllosphere and rhizosphere soil samples of all six *C. 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 (Atamna-Ismaeel *et al*., 2011), using 5 g of leaves and collecting the leaf surface communities on a 0.22 μm Durapore® membrane filter (Merck Millipore Corporation), 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 soil (S) samples were collected near the roots (ectorhizosphere) of the plants.

DNA extraction was carried out from these samples 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 caesium chloride density gradient ultracentrifugation, as described previously (Neufeld *et al*., 2007). 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 (Liu *et al*., 1997). For profiling of the bacterial communities present in the different SIP fractions, terminal restriction fragment length polymorphism (T-RFLP) was used as previously described (Ulrich and Becker, 2006), with normalization of T-RFLP profiles (Dunbar *et al*., 2001).

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

The bacterial composition in [^{13}C]-CH_3Cl and [^{12}C]-CH_3Cl 'heavy' and 'light' fractions from the phyllosphere and rhizosphere samples of all six fern plants was determined by amplicon sequencing using the primer set 5′-AACMGGATTAGATACCCKG-3′ and 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 analyzed using the QIIME pipeline (Caporaso *et al*., 2010) and singletons and chimeras were removed using USEARCH v7 (Edgar, 2010) and UCHIME (Edgar *et al*., 2011). Labelled OTUs were defined according to four criteria (adapted from Chaignaud *et al*., 2018a): (i) 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; (ii) the relative abundance of the OTU was higher in the 'heavy' DNA fraction than in the 'light' fraction of the ^{13}C-labelled microcosm; (iii) the relative abundance of the OTU in the 'heavy' DNA fraction of the ^{13}C-labelled microcosm was ≥ 0.015%; and (iv) the relative abundance difference of the OTU between 'heavy' and 'light' DNA fractions was ≥ 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]-CH_3Cl-amended fern samples combined from three biological replicates. The ^{13}C-labelled heavy fraction of L, LW and S samples were sequenced on a HighSeq platform, and data were filtered and quality trimmed by GenoScreen. Processed reads were assembled with MEGAHIT v.1.2.9 (Li *et al*., 2015) with default options and the additional parameter '–presets meta-large', which sets a k-*mer* list from 27 to 127 in steps of 10. An additional co-assembly of L and LW metagenomes was performed with the same parameters, to improve the recovery of low-abundant species.

Binning was carried out using a combination of CONCOCT v1.1.0 (Alneberg *et al*., 2014), MetaBAT2 v2.12.1 (Kang *et al*., 2019) and MaxBin2 (Wu *et al*., 2015) under

default parameters. For binning with CONCOCT and MetaBAT2, reads were first mapped to the assemblies using bowtie2 v2.3.5.1 (Langmead and Salzberg, 2012) 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 (Uritskiy *et al*., 2018) was used. The MetaWRAP *bin_refinement* module was used to consolidate the binning results from MetaBAT2, MaxBin2 and CONCOCT into a final set of bins. The quality of the bins was estimated using CheckM v1.1.2 (Parks *et al*., 2015). Bins with a reported completeness > 70% and contamination < 10% were selected as MAGs for further analysis (Supplementary Table S1). Metagenome-assembled genomes were annotated with RAST-tk (Brettin *et al*., 2015), 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 (Zuo and Hao, 2015). The deduced tree was visualized at K = 6 using the Interactive Tree of Life v 5.5.1 (Letunic and Bork, 2019) 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 (Asnicar *et al*., 2020) 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 (Kumar *et al*., 2018) from MSAs using the JTT+G+I model with 500 bootstrap replications. ANI values between MAGs and reference genomes were calculated with fastANI (Jain *et al*., 2018) 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. Metagenome-assembled genomes and metagenomes were screened for the presence of the chloromethane utilization gene (*cmuA*) using BLAST (Altschul *et al*., 1990) and prokka (Seemann, 2014) under default settings respectively. ShortBRED (version 0.9.3) (Kaminski *et al*., 2015) and GraftM (version 0.13.0) (Boyd *et al*. 2018) were used to identify *cmuA* in unassembled metagenomic reads. Metagenome-assembled genomes were submitted to the MicroScope platform (Vallenet *et al*., 2019; 2020) 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 (Altschul *et al*., 1990) and RAST (Aziz *et al*., 2008) were used to further compare MAG genetic content and assess the potential for CH_3Cl utilization by screening for functional marker genes involved in CH_3Cl utilization, one-carbon metabolism, central metabolism, cofactor 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 (Aslan-Üzel *et al*., 2020). This more in-depth MAG genetic content analysis was performed for six MAGs from the phyllosphere (LEAF and LEASFWASH) and rhizosphere (SOIL); hereafter: 'Fern CH_3Cl SIP LEAF bin s3', 'Fern CH_3Cl SIP LEAF bin co1', 'Fern CH_3Cl SIP LEAF bin co3', 'Fern CH_3Cl SIP LEAFWASH bin co7', 'Fern CH_3Cl SIP SOIL bin s1' and 'Fern CH_3Cl SIP SOIL bin s7'.

Comparative genome analyses

Genome comparisons within the genus *Methylobacterium* and the closely related clade *Methylorubrum* to determine pan, core and dispensable genes and singletons (unique genes) were carried out using EDGAR v2.0 (Blom *et al*., 2009) as described previously (Kröber and Schäfer, 2019). The MAG Fern CH_3Cl SIP LEAF bin s3 was used as a reference and strains of *Methylobacterium* (including *Methylorubrum*) 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 MAGs for 'Fern CH_3Cl SIP LEAF bin s3', 'Fern CH_3Cl SIP LEAF bin co1', 'Fern CH_3Cl SIP LEAF bin co3', 'Fern CH_3Cl SIP LEAFWASH bin co7', 'Fern CH_3Cl SIP SOIL bin s1' and 'Fern CH_3Cl 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 and SAMN17394143 respectively.

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Author contributions

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

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Figure S1. Plant growth chambers setup. A) Details of gas-tight plant growth chambers. 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 CH_3Cl 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 growth chambers with fern plants inside at the end of the incubation period. Aluminium foil was wrapped around the chambers during incubation with CH_3Cl to reduce photosynthesis and microbial CO_2 consumption during the labelling experiment. Sunlight entered the chambers through the clear top of the chamber.

Supplementary Figure S2. Analysis of carbon mass fraction (C %) and differential stable carbon isotope ratios ($\delta^{13}C$ value) of fern leaves and washed leaf samples (T_{end} washed) via EA/IRMS. Stable isotope ratios ($\delta^{13}C$) are expressed in permil (‰) relative to the international standard.

Supplementary Figure S3. Relative distribution and phylogenetic affiliation of OTUs detected by bacterial 16S rRNA high-throughput amplicon sequencing of fractionated DNA from triplicate [^{13}C]-CH_3Cl and [^{12}C]-CH_3Cl SIP incubations of phyllosphere (leaf washings – LW and leaves - L) and rhizosphere samples (soil – S) of *Cyathea australis* at the order-level after incorporation of an approximately 100 μmol C g^{-1} sample. Each column represents the relative abundance of microbial taxa detected in a sample. Columns marked with a black frame are ^{13}C-'heavy' fractions that should represent the enriched community under [^{13}C]-DMS treatment and therefore the active DMS-degrading community. For detailed enrichment analysis and results see Fig. 2.

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

Supplementary Table S2. List of *Methylobacterium* (including *Methylorubrum*) genomes used for pangenomics.

Supplementary Table S3. Methylotrophic pathway genes in labelled MAGs.

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