Demethylated hopanoids in ‘Ca. Methylomirabilis oxyfera’ as biomarkers for environmental nitrite-dependent methane oxidation

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1. Introduction

Hopanoids are lipids that are widespread in the bacterial domain and well established molecular biomarkers in modern and paleo environments. In particular, the occurrence of 13C-depleted 3-methylated hopanoids are characteristic of aerobic bacteria involved in methane oxidation. Previously the intra-aerobic methanotroph ‘Candidatus Methylomirabilis oxyfera’ (‘Ca. M. oxyfera’), which performs nitrite-dependent methane oxidation in anoxic environments, has been shown to synthesize bacterio-hopanepolyols (BHPs) and their 3-methylated counterparts. However, since ‘Ca. M. oxyfera’ does not utilize methane as a carbon source, its biomass and lipids do not show the characteristic 13C-depletion. Therefore, the detection of ‘Ca. M. oxyfera’ in various environments is challenging, and still underexplored. Here, we re-investigated the hopanoid content of ‘Ca. M. oxyfera’ bacteria using enrichment cultures. We found the GC-amenable hopanoids of ‘Ca. M. oxyfera’ to be dominated by four demethylated hopanoids of which only one, 22,29,30-trisnorhopan-21-one, had been identified previously. The three novel hopanoids were tentatively identified as 22,29,30-trisnorhopan-21-ol, 3-methyl-22,29,30-trisnorhopan-21-one and 3-methyl-22,29,30-trisnorhopan-21-ol. These unique demethylated hopanoids are most likely biosynthesized directly by ‘Ca. M. oxyfera’ bacteria. Bioinformatical analysis of the ‘Ca. M. oxyfera’ genome revealed potential candidate genes responsible for the demethylation of hopanoids. For the sensitive detection of the four trisnorhopanoid biomarkers in environmental samples, a multiple reaction monitoring (MRM) method was developed and used successfully to detect the trisnorhopanoids in a peatland where the presence of ‘Ca. M. oxyfera’ had been confirmed previously by DNA-based analyses. These new biomarkers may be a novel tool to trace nitrite-dependent methane oxidation in various (past) environments. © 2019 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
functional groups. Both BHPs and derived diagenetic products are extensively used as lipid biomarkers for (specific) microbial communities and their metabolic role in modern and ancient ecosystems (e.g., Brocks and Pearson, 2005; Talbot et al., 2007).

One important example is the use of hopanoids to trace microbes involved in methane oxidation. BHPs with penta- and hexa-functionalized side chains containing an amine group at the C-35 position are characteristic of methane-oxidizing bacteria (MOB) from the alpha- and gammaproteobacterial classes (Talbot and Farrimond, 2007; van Winden et al., 2012). Another important structural feature in some hopanoids is the methylation at the C-3 position of the A-ring. This methylation was first shown in acetic acid bacteria (Rohmer and Ourisson, 1976) and later in MOBs (Zundel and Rohmer, 1985). Subsequently, C-3 methylated hopanoids were used as biomarkers for the presence of aerobic methanotrophy in ancient sediments (Summons and Jahnke, 1992). However, Welander and Summons (2012) identified the hpnR gene responsible for 3-methylation in hopanoids and showed that it is widely distributed among various bacterial taxa, including those not involved in the methane cycle. Nevertheless, the origin of hopanoids from MOB can be inferred from their depletion in $^{13}C$ from methane (Kool et al., 2012). However, the use of fatty acids deficiency as an indicator for methanotrophy if accompanied by additional evidence of $^{13}C$-depleted carbon isotope values.

An apparent exception to this $^{13}C$ depletion is the intra-aerobic methanotroph, Candidatus Methyloirribilis oxyfera’ (‘Ca. M. oxyfera’), which belongs to the NC10 phylum and occurs in clusters with ‘Ca. Methanoperedens nitroreducens’ archaea (ANME-2d) (Raghoebarsing et al., 2006; Ettwig et al., 2010; Vaksmaa et al., 2017). ‘Ca. M. oxyfera’ lives under anoxic conditions and reduces nitrite to nitric oxide, and subsequently is believed to produce its own intracellular oxygen from nitric oxide for the intra-aerobic oxidation of methane (Ettwig et al., 2010). Thus, ‘Ca. M. oxyfera’ directly connects the methane and nitrogen cycle (Ettwig et al., 2010). In contrast to most other MOB lipids, ‘Ca. M. oxyfera’ lipids were shown not to be depleted in $^{13}C$ from methane (Kool et al. 2012, 2014), as they autotrophically fix carbon dioxide via the Calvin cycle (Rasigraf et al., 2014). These lipids include 3-methyl hopanoids, first recognized through genomic analysis (Welander and Summons, 2006), such as the rare BHP-hexol and a novel hopanoid from MOB can be inferred from their depletion in $^{13}C$ from methane (Kool et al., 2012). However, the use of fatty acids deficiency as an indicator for methanotrophy if accompanied by additional evidence of $^{13}C$-depleted carbon isotope values.

2. Material and methods

2.1. ‘Ca. M. oxyfera’ enrichment cultures

An enrichment culture of ‘Ca. M. oxyfera’ bacteria was obtained from a bioreactor operated under the conditions described previously by Ettwig et al. (2009). The reactor contained bright flocculent biomass which originated from active ‘Ca. M. oxyfera’ bacteria, and black material from the bottom of the bioreactor that was most likely dead ‘Ca. M. oxyfera’ biomass. The bacteria in the bioreactor contained ca. 67% of ‘Ca. M. oxyfera’ while the rest was composed of a mix of ANME-2d archaea and different minor bacteria phyla (e.g., Anaerolinea thermophila, Caldithrix abyss or Melioribacter roseus) at abundances less than 7% for each phylum. The mineral medium containing CaCl2·2H2O (0.24 g/L), MgSO4·7H2O (0.16 g/L), KHCO3 (0.1 g/L), NaNO3 (1.39 g/L), KH2PO4 (0.01 g/L), 1 mL of trace element solution and 3 mL of iron stock solution was sparged with Ar/CO2 mixture (95:5, v/v) and supplied to the bioreactor at a rate of 1.5–2 L per day. The trace element solution contained ZnSO4·7H2O (1.44 g/L), CoCl2·6H2O (0.6 g/L), CuSO4 (4 g/L), NiCl2·6H2O (0.95 g/L), H2BO3 (0.07 g/L), MnCl2·4H2O (1 g/L), Na2WO4·2H2O (0.1 g/L), Na2MoO4·2H2O (0.48 g/L), SeO2·0.13 g/L, CeCl3 (0.12 g/L) and nitritotrificic acid (NTA, 30 g/L) as chelating agent. The iron stock solution contained FeSO4·7H2O (5 g/L) and NTA (10.31 g/L) as chelating agent. The steady state concentration of nitrite in the bioreactor was kept at 0–5 mg/L. Biomass from a second reactor enriched from Italian paddy field soils consisted of less ‘Ca. M. oxyfera’ cell material (30%), more ‘Ca. Methanoperedens nitroreducens’ (ANME-2d) archaea (30%) and other microbial communities (40%) as described previously (Vaksmaa et al., 2017).

2.2. Peatland core

A peatland core from the Brunsummerheide peatland, Netherlands (BRH) was sampled and divided into nine sections from 51 to 102 cm depth (1e 51–60 cm, from 60 to 95 cm, 95–102 cm) (Kool et al., 2012; Kool et al., 2014). Previous quantitative polymerase chain reaction (qPCR) analysis of ‘Ca. M. oxyfera’ specific 16S rRNA genes revealed the peak of their abundance at 70–90 cm depth. In this zone both methane and nitrate showed depletion in pore water concentrations (Zhu et al., 2012).

2.3. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) of ‘Ca. M. oxyfera’ enrichments from the two bioreactors was performed for the estimation of bacterial and archaeal abundance. Liquid samples of 1.5 mL from an active enrichment culture were centrifuged for 10 min at 7000 g (Eppendorf® 5424, Hamburg) and biomass pellets were washed 2 times with 1 mL phosphate-buffered saline (PBS: 130 mM NaCl and 10 mM phosphate buffer at pH 7.4). After washing, the samples were fixed with 4% paraformaldehyde at a 3:1 (v/v) ratio to the sample at 4 °C overnight. FISH was performed as previously described (Ettwig et al., 2008). Formamide concentration in the hybridization buffer was set to 35%. The following oligonucleotide probes were used: DAMOBACT-0193 (CCG TCG CCC CCT TGG TGT GGC) specific for ‘Ca. M. oxyfera’-like bacteria; DAMOARCH-0641 (GTT CCC AAG CCT ACC AGT) specific for ‘Ca. M. nitroreducens’-like archaea; EUB 338 (S-D-Bact-0338-a-A-18) (Amann et al., 1990), EUB 338 II (S-D-Bact_0338-b-A-18) and EUB 338 III (S-D-Bact-0338-c-A-18) (Daims et al., 1999) for most bacteria; S-D-Arch-0915-a-A-20 for most archaea. Images were collected with a Zeiss Axiosplan 2 epifluorescence microscope equipped with a CCD camera and processed with the Axiosvision software package (Zeiss, Germany).

2.4. Genomic analysis

Identification of potential genes involved in hopanoid biosynthesis in ‘Ca. M. oxyfera’ (NCBI: taxid671143) was carried out on the NCBI (National Center for Biotechnology Information) server with the PSI-BLAST algorithm (Schäfer et al., 2001) using characterized proteins from different organisms as queries (Supplementary Table S1). Operon analysis and visualization was performed...
by using the Gene Context Tool NG (http://biocomputo2.ibt.unam.mx/gctng/) and protein analysis was conducted with Uniprot (UniProt Consortium, 2018).

2.5. Hopanoid analyses

2.5.1. Extraction of hopanoids

Freeze dried biomass of the ‘Ca. M. oxyfera’ enrichments as well as freeze dried BRH peat core material (Kool et al., 2012) were extracted using a modified Bligh and Dyer technique (Schouten et al., 2008; Bale et al., 2013). The samples were ultrasonically extracted with a solvent mixture containing methanol (MeOH), dichloromethane (DCM) and phosphate buffer (2:1:0.8, v/v/v). After sonication (10 min) and centrifugation, the solvent layer was collected and the residue re-extracted twice. The combined solvent layers were separated from the aqueous layer by adding additional DCM and phosphate buffer to achieve a ratio of MeOH, DCM and phosphate buffer (1:1:0.9, v/v/v). The separated organic bottom layer was removed and collected while the aqueous layer was washed two more times with DCM. The combined DCM layers were dried under a continuous flow of N₂.

Aliquots of the total lipid extracts (TLEs) (ca. 0.5–3 mg) were base hydrolyzed (saponified), with 2 mL of 1 N KOH in MeOH solution, refluxed for 1 h at 130 °C, the pH adjusted to 5 with a 2 N HCL in MeOH solution, separated with 2 mL of distilled water and 2 mL DCM and washed with DCM two more times. The combined DCM layers were dried over a Na₂SO₄ column. Afterwards the saponified fraction was methylated with diazomethane in diethyl ether, filtered over a small silica column with ethyl acetate and silylated with pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 60 °C for 20 min. These total lipid extracts (TLEs) were analyzed using GC and GC–mass spectrometry (MS).

2.5.2. Instrumental analysis

GC–MS analyses of the TLEs of different ‘Ca. M. oxyfera’ biomass and BRH peat core material were performed on an Agilent Technologies GC–MS Triple Quad 7000C in full scan and multiple reaction monitoring (MRM) mode. The chromatography was carried out using a CP-SIL5 CB column (25 m × 0.32 mm with a film thickness of 0.12 μm; Agilent Technologies) with helium as carrier gas (constant flow 2 mL/min). For each sample, 1 μL was injected on-column at 70 °C, the temperature was increased by 20 °C/min to 130 °C, raised further by 4 °C/min to 320 °C, which was held for 10 min for full scan mode and for the MRM analysis. GC–MS full scan analysis was conducted over a mass range of m/z 50–850, the gain was set to 3 and with a scan rate of 700 ms. Compounds were identified by comparison with previously published data (Zundel and Rohmer, 1985; Sinninghe Damsté et al., 2004; Kool et al., 2012).

A new GC–MS–MS MRM method was developed to detect hopanoid ketones and alcohols in environmental samples using transitions and collision energies shown in Table 1. The MS1 and MS2 resolution was set on wide, the gain on 10 and the dwell time on 10 ms for all scans. As collision gases in the MRM scan mode, He was admitted at a flow of 1.5 mL/min and N₂ at a flow of 1 mL/min.

3. Results and discussion

3.1. Lipid analysis of ‘Ca. M. oxyfera’ reveals novel demethylated hopanoids

The microbial community composition and microstructure of the ‘Ca. M. oxyfera’ enrichment culture was assessed by FISH. Based on the average fluorescence signal, we estimated that approximately two-thirds of the biomass granules were composed of bacteria related to ‘Ca. M. oxyfera’ while the rest was composed of a mix of ANME-2a archaea and different minor bacteria phyla (e.g., Anaerolinea thermophila, Caldithrix abyssi or Meliborientoraceae) (Supplementary Fig. S1). The lipid composition of this biomass was dominated by a series of fatty acids with high relative abundances of C16:0 (22%), 10MeC16:0 (5%) and 10MeC16:0 (37%) fatty acids as described previously (Kool et al., 2012). We also detected a series of hopanoids using mass chromatograms for m/z 191 and m/z 205 revealing diploptene, diplopteron, C₂₅ 17[(21p)-hopenoic acid and some of their 3-methylated homologues (Fig. 1). These hopanoids, especially diploptene and diplopteron, are found in a diverse range of aerobic and anaerobic bacteria (Rohmer et al., 1984; Summons et al., 1994; Sinninghe Damsté et al., 2004; Blumenberg et al., 2006). Furthermore, these hopanoids have been found in various environments like oxygen minimum zones (Wakeham et al., 2007), marine hydrocarbon seeps (Thiel et al., 2003; Niemann et al., 2006), lacustrine sediments (Innes et al., 1997), peatlands (van Winden et al., 2010) or soils (Crossman et al., 2005).

The GC–MS analysis also revealed the presence of four more minor peaks which we tentatively identified as hopanoids with relative abundances each about 5–36% compared to the most abundant hopanoid diplopteron, and 0.1% compared to the most abundant fatty acids, the 10MeC 16:0 and C 16:0 (Figs. 1 and 2). Of these four, 22,29,30-trisnorhopan-21-one (compound 1–TNHop in Fig. 1) was tentatively identified based on a published mass spectrum (Simoneit, 1977). This hopanoid was first identified in sediments from the Black Sea (Simoneit, 1977) and has since been found in various marine sediment and water column samples, where it is believed to be formed by aerobic diagenesis of BHPs (Conte et al., 1998; Botz et al., 2007).

Directly eluting after 22,29,30-trisnorhopan-21-one is a compound (labelled 2 in Fig. 1) with a diagnostic fragment of m/z 191 suggesting a non-A-ring methylated hopanoid (Fig. 2B). The difference of 74 Da between the molecular ions of 22,29,30-trisnorhopan-21-one and unknown compound 2, as well as ions at m/z 73 and 75, suggests the presence of a TMSI-derivatized alcohol. This is confirmed by the second diagnostic fragment of m/z 237, compared to the m/z 163 in the 22,29,30-trisnorhopan-21-one, suggesting that the alcohol is located at the D- or E-ring position. The fragment at m/z 367 indicates that the alcohol is likely at the E-ring position (Fig. 2B). This hopanoid was therefore tentatively identified as 22,29,30-trisnorhopan-21-ol (TNHol).

A similar doublet of peaks (compounds 1a and 2a in Fig. 1) eluted after the 22,29,30-trisnorhopan-21-one and 22,29,30-trisnorhopan-21-ol, respectively. Both mass spectra (Fig. 2C and D) show the diagnostic fragment of m/z 205, which indicates a methylation of the A-ring. Furthermore, both have molecular ions which are 14 Da heavier (m/z 398 and m/z 472) than that of 22,29,30-trisnorhopan-21-one and 22,29,30-trisnorhopan-21-ol, respectively (Fig. 2A and B, respectively). The first eluting

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target transition (m/z)</th>
<th>Qualitative transition (m/z)</th>
<th>Collision energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>trisnorhopan-21-one</td>
<td>384.3 → 191.1</td>
<td>384.3 → 369.3</td>
<td>10</td>
</tr>
<tr>
<td>trisnorhopan-21-ol</td>
<td>458.3 → 182.1</td>
<td>458.3 → 237.2</td>
<td>15</td>
</tr>
<tr>
<td>3-methyl-trisnorhopan-21-one</td>
<td>398.4 → 205.2</td>
<td>398.4 → 383.3</td>
<td>10</td>
</tr>
<tr>
<td>3-methyl-trisnorhopan-21-ol</td>
<td>472.4 → 182.1</td>
<td>472.4 → 237.2</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 1 Specifications of the MRM method for the four ‘Ca. M. oxyfera’ trisnorhopanoids, showing the target transition (m/z), qualitative transition (m/z) and optimized collision energy (V).
HpnR gene responsible for the 3-methylation of hopanoids in the (3-MeTNHone) and 22,29,30-trisnorhopan-21-ol (3-MeTNHol), as the methylated counterparts of 22,29,30-trisnorhopan-21-one. Therefore, we tentatively identify these two compounds as 3-methyl-22,29,30-trisnorhopan-21-one (3-MeTNHone; Fig. 2C) and 3-methyl-22,29,30-trisnorhopan-21-ol (3-MeTNHol; Fig. 2D).

3.2. Origin of (3-methyl) 22,29,30-trisnorhopan-21-one and 22,29,30-trisnorhopan-21-ol

To the best of our knowledge, the three novel hopanoids (22,29,30-trisnorhopan-21-ol, 3-methyl-22,29,30-trisnorhopan-21-one and 3-methyl-22,29,30-trisnorhopan-21-ol) have not been identified before in any other bacterial cultures nor have they previously been detected in environmental samples. Since 'Ca. M. oxyfera' biomass derives from highly enriched, but not pure cultures, it cannot be fully excluded that other bacteria in the bioreactor produce these hopanoids. However, this possibility seems rather unlikely due to the low relative abundances of the other bacterial species present in the enrichment culture: only ten species had abundances >1% (and always less than 7%) in the enrichment culture. The most abundant bacteria, besides 'Ca. M. oxyfera', were Anaerolinea thermophila (6.5%), Calditrichia abyssi (5.5%), Gemmatimonas aurantiaca (3.2%) and Meloribacter roseus (1.7%). These species were previously investigated for their fatty acid inventory but not for hopanoids ( Sekiguchi et al., 2003; Zhang et al., 2003; Podosokorskaya et al., 2013), although none of them has been shown to contain the gene responsible for hopanoid synthesis (e.g., Sinninghe Damsté et al., 2017) or hopanoid methylation at the C-3 position (Welander and Summons, 2012). Furthermore, we investigated another enrichment culture from a methanotrophic bioreactor fed with nitrite and methane which contained a lower proportion of 'Ca. M. oxyfera' (30%) and higher abundance of other species, mainly ANME-2D archaea (30%) (Vaksmaa et al., 2017). This showed a much lower abundance of the trisnorhopanoids (Fig. 3), suggesting that 'Ca. M. oxyfera' is the likely source of these hopanoids.

Of the four trisnorhopanoids, only the 22,29,30-trisnorhopan-21-one has been reported previously, i.e. in anaerobic enrichment cultures of planctomycetes by Sinninghe Damsté et al., (2004) and in various environmental samples such as the anoxic brine-filled basins in the Mediterranean (ten Haven et al., 1987), anoxic sediments (Pancost and Sinninghe Damsté, 2003), in coals and shales from Indonesia (Hoffmann et al., 1984) and Green River oil shale kerogen (Barakat and Yen, 1990). These studies have suggested that 22,29,30-trisnorhopan-21-one is a diagenetic or microbial alteration product of BHPS such as bacteriohopanetetrol (BHT) and diplopterol formed under oxic conditions (Santos et al., 1994; Conte et al., 2003). Moreover, 22,29,30-trisnorhopan-21-one is believed to be an indicator for high bio-productivity when found in the water column or sediments (Simoneit, 1977; Botz et al., 2007). However, since 'Ca. M. oxyfera' biomass was incubated under strictly anaerobic conditions, aerobic degradation processes can be excluded. We also analyzed black cell material from the bottom of the 'Ca. M. oxyfera' enrichment bioreactor, which most likely represents dead cell material from 'Ca. M. oxyfera'. This material had no elevated relative abundances of the novel trisnorhopanoids compared to the more readily degradable fatty acids (Fig. 3), suggesting that the trisnorhopanoids are not formed after cell death. Together, these results suggest that the trisnorhopanoids in 'Ca. M. oxyfera' bacteria are more likely formed via a direct biosynthesis rather than by degradation processes in the bioreactor itself.

3.3. Potential biosynthesis of demethylated hopanoids in 'Ca. M. oxyfera'

To further investigate the potential direct biosynthesis of the trisnorhopanoids, we explored biosynthetic genes that could be
involved in the demethylation of the hopanoids (i.e., 22,29,30-trisnorhopan-21-one, 22,29,30-trisnorhopan-21-ol and their 3-methyl homologues) in ‘Ca. M. oxyfera’. This demethylation of the C22, C29 and C30 carbon atoms can either take place before the cyclization of squalene to a hopane or thereafter. Squalene biosynthesis in bacteria involves a three-step reaction encoded by the HpnD, HpnC and HpnE genes and are usually found in one operon (Pan et al., 2015). We found the gene that potentially codes for HpnD (DAMO_1512) in the genome of ‘Ca. M. oxyfera’, but we did not find any homologous sequence for HpnC nor HpnE using BLAST searches (Supplementary Table S1). Previous studies suggested that these steps can alternatively be performed by farnesyl diphosphate farnesyl transferase (FdfT) activities as found in for example acidobacteria, gammaproteobacteria or cyanobacteria (Lee and Poulter, 2008; Ohtake et al., 2014; Sinninghe Damsté et al., 2017). The gene DAMO_0933 is part of the predicted operon mox-DAMO_0930 which is composed of genes that codes for a protein of unknown function (DAMO_0930), a predicted glycosyltransferase (DAMO_0931), a potential methyltransferase (DAMO_0932), the NAD-dependent epimerase/dehydratase (DAMO_0933), a short-chain alcohol dehydrogenase (DAMO_0934) and a potential oxidoreductase (DAMO_0935). This combination of enzymes suggests that the trisnorhopanoids can be formed by a sequential oxidative process of diplopterol catalyzed by the genes in the DAMO_0930 operon (Supplementary Fig. S2). We propose that the oxidoreductase activity coded by the DAMO_0935 gene in combination with the dehydrogenase activity coded by the DAMO_0934 gene and the potential dehydratase coded by DAMO_0933 could be involved in a sequential demethylation process (Supplementary Fig. S2). Future work should involve the verification of the proposed enzymatic processes for the demethylation of hopanoids to better understand the mechanism.

![Mass spectra with fragmentation and molecular structures of trisnorhopanoids in ‘Ca. M. oxyfera’ biomass. The mass spectra of 22,29,30-trisnorhopan-21-one was described previously by Simonet (1977).](image-url)
involved in the synthesis of trisnorhopanoids in ‘Ca. M. oxyfera’ and potentially other demethylated hopanoids in bacterial phyla.

3.4. Environmental occurrence of novel ‘Ca. M. oxyfera’ trisnorhopanoids

To investigate whether the novel hopanoids detected in ‘Ca. M. oxyfera’ are also present in the environment, we developed an MRM method to sensitively detect the trisnorhopanoids in environmental samples. For this, product ion scans of the molecular ions for 22,29,30-trisnorhopan-21-one, 22,29,30-trisnorhopan-21-ol and their 3-methylated counterparts were conducted using GC–MS–MS. The selection of the target ions was based on the most selective and abundant ions in these product scans (Fig. 2). The collision energies were then optimized to provide an optimal abundance for the different transitions within the MRM method (Table 1). The resulting MRM chromatogram shows substantially enhanced signal to noise ratio compared to the full scan chromatograms (Figs. 1 and 3) and extracted ion chromatograms of the typical archaeal lipids archaeol and OH-archaeol (sn2-hydroxy-archaeol) as annotated in the chromatograms.

Application of this MRM method to a peatland core from the Brunsummerheide, which previously revealed the presence of demethylated hopanoids (ten Haven et al., 1987; Botz et al., 2007), in contrast to its 3-methylated version. A peak of the 3-Me trisnorhopanone/(3-Me trisnorhopanone + trisnorhopanone) ratio occurs at 70–90 cm depth, and corresponds to the peak in ‘Ca. M. oxyfera’ cell abundance data based on qPCR 16S rRNA gene analysis as well as the specific 10MeC16:0 and 10MeC16:1 fatty acids (Fig. 5B and C; Kool et al., 2012). The 3-methyl-trisnorhopan-21-one is present in low abundance in the upper section of the core (50–70 cm depth), which might be related to the low abundance of NC10 cells in this section (Kool et al., 2012; Zhu et al., 2012). Furthermore, temporal variations of the water level in the peatland could have shifted the depth of the oxic–anoxic interface, and therefore the ‘Ca. M. oxyfera’ niche. This remnants of past NC10 abundance may have been picked up by our more sensitive MRM method for trisnorhopanoids compared to that of the specific fatty acids reported by Kool et al. (2012). Thus, this ratio of trisnorhopanoids may be a complimentary biomarker tool for the detection of ‘Ca. M. oxyfera’ in the environment. Moreover, these trisnorhopanoids may be better preserved over geological timescales than fatty acids and thus be a tool for the detection of past intra-aerobic methanotrophy in paleo-environments, in contrast to δ^{13}C analysis of hopanoids (Kool et al., 2014; Rasigraf et al., 2014).

3.5. Implications

Our results may have consequences for how we view the sources of demethylated hopanoids detected in present and past environments. Up to now, hopanoid demethylation processes were mainly attributed to diagenetic alteration such as microbial reworking of organic matter for bisnor- and trisnorhopanones or heavy biodegradation of petroleum for the presence of 25-norhopanones (Moldowan et al., 1984; Noble et al., 1985; Moldowan and McCaffrey, 1995). For example 18x(H)-22,29,30-trisnorhopane (Ts) and the 17x(H)-22,29,30-trisnorhopane (Tm),

![Gas chromatograms of saponified and silylated total lipid extracts from (A) ‘Ca. M. oxyfera’ active biomass (bright flocculent cell material), (B) ‘Ca. M. oxyfera’ dead biomass (black cell material from bioreactor bottom) and (C) ‘Ca. M. oxyfera’ based on DNA and fatty acids (Kool et al., 2012, Kool et al., 2014; Rasigraf et al., 2014).](image)
are thought to derive from diplopterol by a simple side-chain cleavage followed by an acid-catalyzed methyl shift (Seifert and Moldowan, 1978) and are often used to determine the source and the thermal maturity of petroleum (Seifert and Moldowan, 1978; Peters et al., 2005). It has been suggested that the 25-demethylation of 28,30-bisnorhopane to 25,28,30-trisnorhopane, common biomarker lipids in petroleum and organic-rich sediments (e.g., Noble et al., 1985; Peters et al., 2005), occurs during advanced stages of petroleum biodegradation in which the 28,30-bisnorhopane probably derives from a hopene precursor having a 17(18) double bond (Rullkötter et al., 1982; Volkman et al., 1983). A tentative identification of 28,30-bisnorhop-17(18)-ene as a precursor has been made previously in sediments from the Gulf of California (Rullkötter et al., 1982). However, it was also suggested that 28,30-bisnorhopane and 28,30-trisnorhopane were biosynthesized directly since they are not found as sulfur-bound hydrocarbons (Schoell et al., 1992; Schouten et al., 2001a). The 13C-content of both compounds and their distribution strongly suggest that they are derived from anaerobic bacteria or those living under low oxygen conditions (Schoell et al., 1992; Schouten et al., 2001b). Our finding of demethylated hopanoids in 'Ca. M. oxyfera' as well as those reported in the anaerobic planctomycetes (Sinninghe Damsté et al., 2004) now suggest that demethylated hopanoids may not only be formed by diagenetic processes, but also biosynthesized directly.

4. Conclusions

The intra-aerobic methanotroph 'Ca. Methylomirabilis oxyfera' synthesizes a series of unique demethylated hopanoids identified as 22,29,30-trisnorhopan-21-one, 22,29,30-trisnorhopan-21-ol, 3-methyl-22,29,30-trisnorhopan-21-one and 3-methyl-22,29,30-trisnorhopan-21-ol. These unique hopanoids suggest a possible demethylation process of hopanoids by 'Ca. M. oxyfera'. This is further supported by the finding of potential candidate genes responsible for the demethylation of hopanoids in the 'Ca. M. oxyfera' genome. For the sensitive detection of these four hopanoids, an MRM method was developed and successfully applied to an environmental setting where 'Ca. M. oxyfera' was previously detected by specific fatty acids and genomic analysis. The novel trisnorhopanoids and the developed MRM method offer a new tool to investigate the presence of 'Ca. M. oxyfera' and nitrite-dependent methane oxidation in modern and past environments. Moreover, the identification of demethylated hopanoids in living bacterial biomass gives a new perspective on the origin and sources of demethylated hopanoids found in the geological record.

Author contributions

NTS, DR and SS planned research. MJM, OR and SCG provided biomass and FISH analysis data. NTS performed lipid analysis and NTS and SS interpreted the data. MV, SS and NTS developed MRM method. DXSC analyzed genome data for potential biosynthesis pathways of demethylated hopanoids. NTS wrote the paper with input from all authors.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

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